# UNIVERSITY OF SOUTHAMPTON

*THE AETIOLOGY & CELL BIOLOGY OF INFLAMMATION IN SEXUALLY TRANSMITTED BACTERIAL INFECTIONS*

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ABSTRACT

FACULTY OF MEDICINE, HEALTH, & BIOLOGICAL SCIENCES
SCHOOL OF MEDICINE (DIVISION OF CELL & MOLECULAR MEDICINE)

Doctor of Philosophy

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Genital infection with Neisseria gonorrhoeae (the gonococcus) can lead to different clinical outcomes between human hosts. While some individuals remain asymptomatic, others develop local symptoms of acute inflammation. In women, gonococci may ascend into the upper reproductive tract, eliciting an immunopathological reaction termed pelvic inflammatory disease (PID). In the presented study, clinical samples were obtained in order to investigate host responses (such as cytokine production) and pathogen factors (strain genotype, infectious load) that may determine the clinical manifestations of gonococcal infection. However, the prevalence of N. gonorrhoeae - as determined by polymerase chain reaction (PCR) assays - was 0% in two Thai study populations [480 asymptomatic male students and 90 mucopurulent cervicitis (MPC) patients], and 5.5 % (all porin genotype porB1b) in 348 Sri Lankan female sexually transmitted disease (STD) clinic attendees. This precluded a patient based study of the immunopathology of gonorrhoea. In contrast, Chlamydia trachomatis infection was present in 4.2% of the Thai students, 25.6% of the Sri Lankan women, and 26.7% of the Thai MPC patients.

Neither gonococcal nor chlamydial infection was significantly associated with cervical abnormalities in the Sri Lankan population, nor was the presence or infectious load of chlamydiae linked to the severity of MPC in Thai women. However, quantitative TaqMan® PCR assays revealed that in the latter group, women who shed large numbers of chlamydiae from the endocervix were more likely to have chlamydial urethritis or endometritis than were women who shed few or no organisms. Endocervical cytokine levels (measured by time-resolved fluorescence immunoassays) were not associated with the presence or load of chlamydiae; but secretion of interleukin (IL)-1β, IL-6, and IL-8 was increased in patients with overt cervical mucopus.

The inflammatory cytokine response to an STD pathogen was further explored in vitro, using N. gonorrhoeae strain P9 and human mononuclear phagocytes resembling 'tissue-resident' macrophages. The interaction between these phagocytes and viable gonococci resulted in the rapid, elevated release of IL-6, tumour necrosis factor-α, macrophage inflammatory protein-1α, RANTES, growth-related protein-α, and interferon-γ; but this was not dependent on the expression of opacity (Opa) proteins or pili by the inoculum. Purified gonococcal surface components [pili, lipooligosaccharide (LOS), and outer membranes (OM)] induced a similar cytokine profile to whole bacteria. However, when applied at equivalent concentrations, Opa-negative OM were more potent stimulators of cytokine production than was dissolved LOS. Relative to the Opa$^-$ phenotype, protein P9-Opa-b (whether present on the surface of isolated OM or viable organisms) tended to generate higher cytokine levels in macrophage cultures, whereas expression of P9-Opa-a was associated with a slightly diminished response. These findings suggest that macrophage-derived cytokines have important roles in the pathogenesis of both localised infections (acute symptoms) and PID (persistent inflammation).

*"Now faith is the assurance of things hoped for, the conviction of things not seen."*

Hebrews 11:1

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# DECLARATION OF CLINICAL COLLABORATION

Two components of the research project presented in this thesis (Chapters *3* & *4*) were dependent on the collection of genitourinary specimens by collaborating clinicians and scientists in Thailand and Sri Lanka. In addition, interpretation of the data I obtained during the course of this project occasionally required clinical information that was provided by our overseas collaborators.

The Molecular Microbiology Group at Southampton University has enjoyed a long-standing partnership with the Department of Obstetrics-Gynaecology at Prince of Songkla University, Hat Yai, Thailand. This has led to numerous exchange visits between the two research groups, organised by Professor Michael Ward (Southampton) and Dr. Verapol Chandeying (Chairperson of the Department of Obstetrics-Gynaecology at Prince of Songkla University). The ethical aspects of the studies herein were discussed and agreed between the collaborating departments, and Professor Ward and Dr. P.J. Rowe formulated protocols for gynaecological sampling. These were approved by the Steering Committee of the World Health Organisation Human Reproduction Programme. Dr. Chandeying was responsible for obtaining clearance from the local ethical committee of the University Hospital, Hat Yai.

The initial storage and transport of the Thai students' urine specimens (Section *3A*) was co-ordinated by Dr. Steven Skov of the Tri-State STD/HIV Project, Alice Springs (Australia). Similarly, Dr. Suphat Pecharatana was responsible for these aspects of the mucopurulent cervicitis study (Chapter *4*). Dr. Chandeying provided the data presented in Appendix *2* (clinical history and direct microscopic findings for the cervicitis study patients), except the results pertaining to chlamydial and gonococcal infection, which were obtained during my investigation. All further sample preparation, assay development, analysis of specimens, and statistical interpretation were my own work.

The cervical specimens that formed the basis of the investigations reported in Section *3B* were collected for a 4$^{th}$ year medical project conducted by Dr. Shamila Amarasekara, entitled "A Study To Determine The Prevalence Of Cervical Papillomavirus Infections & Cervical Precancer In Women Attending A Sri Lankan Sexually Transmitted Disease Clinic". Dr. Amarasekara's study was undertaken at the central STD clinic in the National Hospital at Colombo, and was supervised by Dr.

Iyanthimala Abeyewickereme of the National STD/AIDS Control Programme. Protocols used in this project were granted ethical approval by the clinic directorate, and all participants were volunteers who gave informed consent.

Dr. Stuart Lanham extracted DNA from the cervical specimens in Southampton, and supervised Dr. Amarasekara during assays for papillomavirus infection. These results form the basis of a published study [**Jordens *et al.* (2000)** *Journal of Virological Methods* (in press)]. As the remainder of the sample aliquots were available for further investigation, and classic STD have been implicated as cofactors in the development of cervical cancer, I analysed them for my thesis project. Clinical and diagnostic data for the Sri Lankan patients (contained in Appendix *1*) were provided by Dr. Amarasekara and Dr. Lanham, with the exception of the diagnoses for chlamydial and gonococcal infection, which are my own results.

# ABBREVIATIONS & ACRONYMS

| | |
|---|---|
| °C | degrees centigrade |
| µg | microgram(s) |
| µl | microlitre(s) |
| µm | micrometre(s) |
| µM | micromolar |
| µS | microsieverts |
| A | absorbance |
| AHS | autologous human serum |
| AIDS | acquired immune deficiency syndrome |
| ANAE | α-naphthyl acetate esterase |
| ANOVA | analysis of variance |
| Anti-Gal | anti-α1,3-galactosyl antibodies |
| BGMK | buffalo green monkey kidney |
| BGP | biliary glycoprotein |
| BIA | biomolecular interaction analysis |
| BSA | bovine serum albumin |
| CAP | cationic anti-microbial protein |
| CD | cluster of differentiation |
| cfu | colony-forming units |
| CI | confidence interval |
| CL | conserved loop |
| cm | centimetre(s) |
| (CMP-)NANA | (cytidine-5'-monophospho)-*N*-acetyl neuraminic acid |
| $CO_2$ | carbon dioxide |
| CrP | cysteine-rich protein |
| CSE | commercial sex establishment |
| CSW | commercial sex worker |
| $C_T$ | cycle threshold |
| CXC | cysteine-*(amino-acid)*-cysteine |
| Cy5 | cyanine-5 |
| d | day(s) |
| Da | dalton |
| DGI | disseminated gonococcal infection |
| $dH_2O$ | deionised water |
| DNA | deoxyribonucleic acid |
| dUTP | deoxyuridine triphosphate |
| EB | elementary body |
| EDTA | ethylenediaminetetraacetic acid |
| ELISA | enzyme-linked immunosorbent assay |
| ENA | epithelial-derived neutrophil attractant |
| F(ab') | fragment (antigen-binding) |
| FAM | 6-carboxy-fluorescein |

| | |
|---|---|
| Fc | fragment crystallisable |
| FCS | foetal calf serum |
| FITC | fluorescein isothiocyanate |
| Fmoc | 9-fluorenylmethyloxycarbonyl |
| g | gram(s), gravitational force |
| GC | gonococcal |
| GFP | green fluorescent protein |
| GM-CFC | granulocyte-macrophage colony-forming cell |
| GM-CSF | granulocyte-macrophage colony-forming factor |
| GRO | growth-related protein |
| HEPES | *N*– (2-hydroxyethyl)piperazine-*N*′-2-(ethanesulphonic acid) |
| HIV | human immunodeficiency virus |
| HPV | human papillomavirus |
| hr | hour(s) |
| HSD | honestly significant difference |
| HSPG | heparan sulphate-proteoglycans |
| HSV | herpes simplex virus |
| HV | hypervariable |
| IFN | interferon |
| ifu | inclusion-forming units |
| Ig | immunoglobulin |
| IL | interleukin |
| (k)bp | (kilo)base-pairs |
| kDa | kilodalton |
| l | litre(s) |
| LAMP | lysosome-associated membrane protein |
| LBP | lipopolysaccharide-binding protein |
| LOS | lipooligosaccharide |
| Lpb | lactoferrin-binding protein |
| LPS | lipopolysaccharide |
| M | molar |
| MΩ | megaohms |
| mAb | monoclonal antibody |
| mDa | megadalton |
| MEM | modified Eagle medium |
| mg | milligram(s) |
| min | minute(s) |
| MIP | macrophage inflammatory protein |
| ml | millilitre(s) |
| mm | millimetre(s) |
| mM | millimolar |
| MPC | mucopurulent cervicitis |
| mRNA | messenger ribonucleic acid |
| ng | nanogram(s) |
| NGU | non-gonococcal urethritis |
| NHS | normal human serum |
| nm | nanometre(s) |

| | |
|---|---|
| NTC | *no-template control* |
| OCP | oral contraceptive pill |
| OM | outer membrane(s) |
| OMP | outer membrane protein |
| Opa | opacity protein(s) |
| OR | odds ratio |
| PBMC | peripheral blood mononuclear cells |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| Pgp | phagocytic glycoprotein |
| PID | pelvic inflammatory disease |
| Pil | pilus |
| PMN | polymorphonuclear neutrophils |
| PorB | porin B |
| PPV | positive predictive value |
| psi | pounds per square-inch |
| PY | person-years |
| RANTES | regulated upon activation, normal T-cell expressed & secreted |
| RFU | relative fluorescence units |
| Rmp | reduction-modifiable protein |
| RNA | ribonucleic acid |
| ROX | carboxy-X-rhodamine |
| rRNA | ribosomal ribonucleic acid |
| RT°C | room temperature |
| RU | resonance units |
| SAI | sexually active individual(s) |
| SDS(-PAGE) | sodium dodecyl sulphate-(polyacrylamide gel electrophoresis) |
| SE | standard error of the mean |
| sec | second(s) |
| SFP | simulated fluorescence process |
| SPR | surface plasmon resonance |
| STD | sexually transmitted disease(s) |
| SV | semivariable |
| TAE | tris-acetate-ethylenediaminetetraacetic acid |
| TAMRA | 6-carboxy-tetramethyl rhodamine |
| *Taq* | *Thermus aquaticus* |
| Tbp | transferrin-binding protein |
| TET | tetrachloro-6-carboxy-fluorescein |
| TGF | transforming growth factor |
| $T_m$ | melting temperature |
| TNF | tumour necrosis factor |
| Tris | tris(hydroxymethyl)aminomethane |
| TRITC | tetramethyl rhodamine isothiocyanate |
| U | units (enzymatic activity) |
| UHQ | ultra high quality |
| UK | United Kingdom |
| UNG | uracil *N*-glycosylase |

| | |
|---|---|
| USA | United States of America |
| V | volts |
| v/v | volume-per-volume |
| w/v | weight-per-volume |
| WHO | World Health Organisation |

# CHAPTER 1

*SURVEY OF THE LITERATURE*

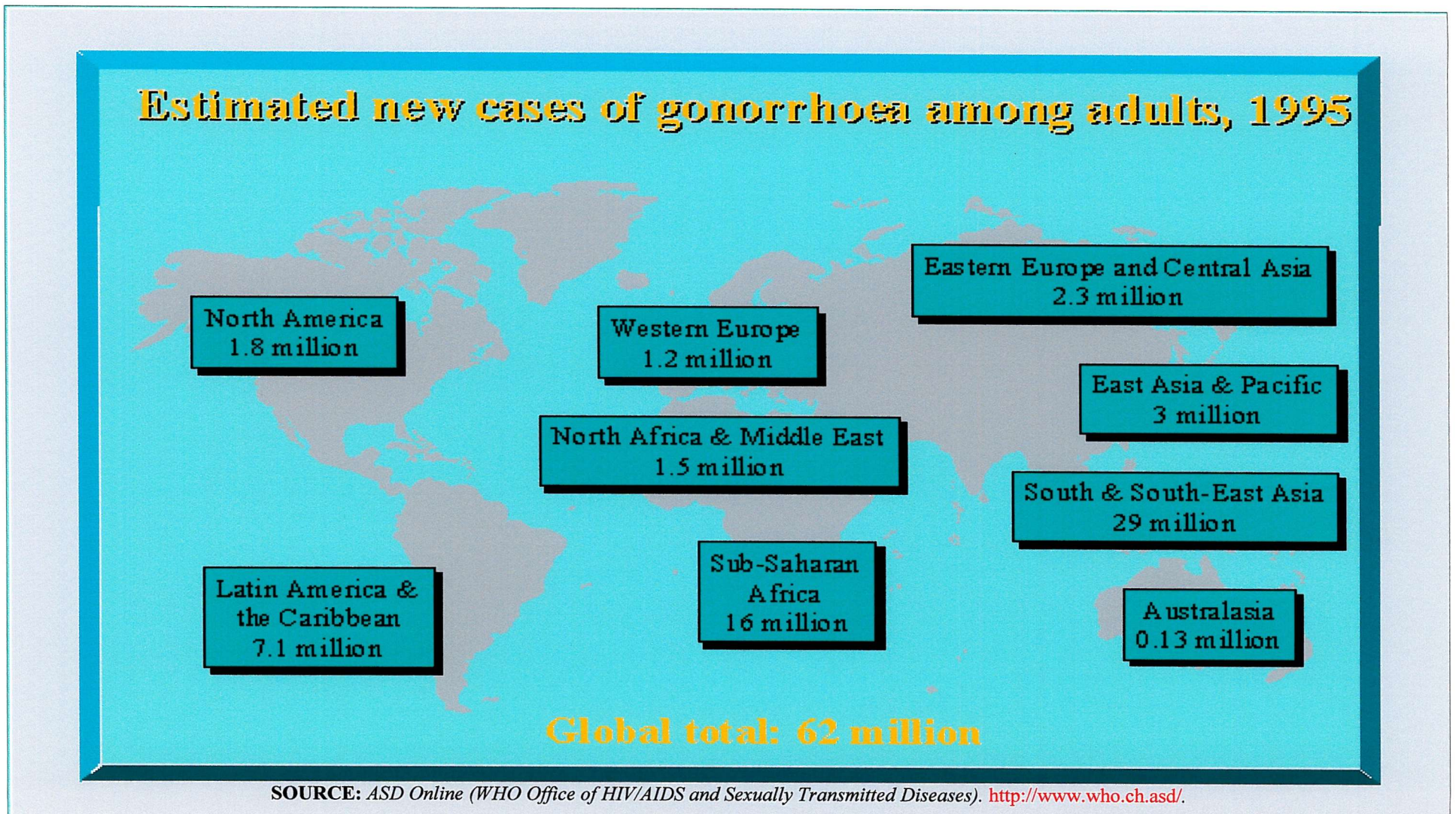
## *1A* - The Global Epidemiology Of Gonorrhoea

A uniquely human disease found throughout the world, gonorrhoea predominantly affects the reproductive health of young, sexually active individuals. Almost three-quarters of the estimated 62 million new cases of gonorrhoea each year occur in sub-Saharan Africa and south and south-east Asia (Gerbase *et al.*, 1998; see Figure *1·1*), and the prevalence in the former resembles that of Europe at the beginning of the 20$^{th}$ century (Schulz *et al.*, 1986; cited in Duncan *et al.*, 1991). In contrast, many western European countries (particularly those in Scandinavia) have experienced a sharp decline in reported cases of gonorrhoea since the early 1980s; and during the same period, the incidence rate in North America has also decreased, albeit more slowly (Sciarra, 1997).

The epidemiological patterns seen over large geographic areas mask very significant differences in gonorrhoea prevalence between sub-populations within a region. "Core groups" of individuals with high incidences of infection and rapid rates of partner exchange were originally predicted to exist by mathematical modelling, and were later identified in empirical studies (Rothenberg, 1983; cited in Lacey *et al.*, 1997). These groups maintain the transmission of gonorrhoea; and for Western populations, there are three principal risk factors that influence inclusion: young age, urban residence and black race (Curless & Kinghorn, 1997; Lacey *et al.*, 1997). An analysis of gonorrhoea statistics for Leeds, United Kingdom, revealed that differences in risk between ethnic groups occurred independently of socio-economic or residential factors, and may be due to cultural influences on beliefs and behaviour (Lacey *et al.*, 1997).

In common with most sexually transmitted diseases (STD), gonorrhoea has a markedly greater effect on the health of women than on that of men. Women are twice as likely to contract gonorrhoea from an infected male partner than vice-versa (Harlap *et al.*, 1991; cited in Sciarra, 1997), and STD (excluding human immunodeficiency virus) are the second most important cause of healthy life lost in women between the ages of 15 and 44 in the developing world (The World Bank, 1993; cited in Sciarra, 1997). Indeed, the median prevalence of gonorrhoea in women attending prenatal clinics in developing

**FIGURE *1·1*: Incidence Of Gonorrhoea By Region.**




**SOURCE:** *ASD Online (WHO Office of HIV/AIDS and Sexually Transmitted Diseases).* http://www.who.ch.asd/.

countries is 6%, whilst in prostitutes and STD clinic attendees it is 24% (Faúndes, 1994; cited in Sciarra, 1997).

The problems of controlling gonorrhoea and other STD - both in socially deprived areas of the West and in the developing world - are not simply restricted to inadequate health services or access to diagnosis and treatment. Ineffective and inappropriate health policies and education, stemming from an underestimation of the importance of socio-cultural factors, have hampered STD prevention (Gelmon & Moses, 1994). Targeting of the epidemiological "core-groups" with culturally appropriate interventions (Lacey *et al.*, 1997) may enable the success of Scandinavia to spread further afield.

## *1B* - The Nature & Structure Of The Pathogen

1B(i) *Taxonomy Of The Neisseriae*: In 1876, the first connection between infection with a microbe (*Bacillus anthracis*) and a specific disease (anthrax) was reported by Robert Koch (Kampmeier, 1978). Just three years later, Albert Neisser published his observations on a "micrococcus" that was exclusively found in gonorrhoeal pus (Neisser, 1879; cited in Kampmeier, 1978). His key findings [translated from the German by Kampmeier (1978)] were as follows:

> *"One prepares a thin film of gonorrhoeal pus on a slide and, after drying, floods it with an aqueous solution of methyl violet stain and permits it to dry. Under high magnification and with oil immersion, one immediately recognises the dark blue nuclei of various shapes in the pus cells whose cytoplasm, visible though weakly stained, contains a variable number of micrococci. ...Almost always they appear as two cocci lying closely together, so much so, in fact, as to suggest to the observer a figure of eight, a roll or biscuit shape."*

Originally designated *Micrococcus gonorrhoeae*, the etiological agent of gonorrhoea is now known as *Neisseria gonorrhoeae* – the gonococcus (Knapp, 1988). While several species within the genus *Neisseria* colonise humans, most are non-pathogenic commensals; and only *N. gonorrhoeae* and *N. meningitidis* (a major cause of Gram-negative bacterial meningitis) are frequently associated with human disease. As noted by Neisser, neisserial cells are diplococcoid structures approximately 0.8 μm in diameter; and all species produce positive oxidase reactions (Brooks *et al.*, 1998). However, three other genera within the Neisseriaceae (*Kingella, Moraxella, and*

*Acinetobacter*) also contain species that are found in humans (Knapp, 1988). Thus, differentiation between particular species within this family requires metabolic tests such as the detection of oxidase and catalase activity, acid production from carbohydrates, nitrite or nitrate reduction, and polysaccharide generation from sucrose (Knapp, 1988).

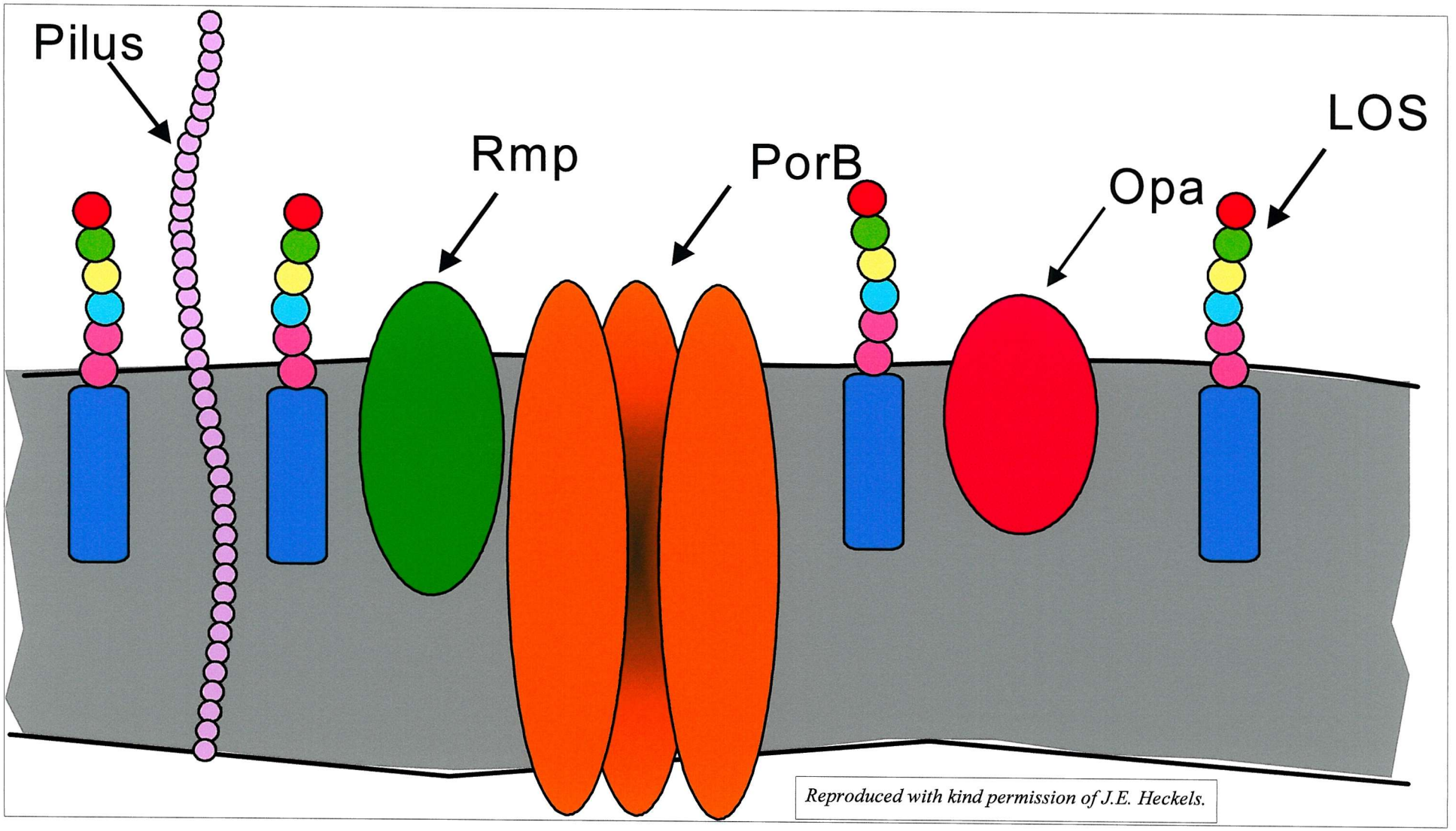
The co-evolution of the gonococcus with its single natural host has led to the development of a complex and sophisticated architecture for the bacterial outer membrane, since this represents the interface with a hostile environment. The major components of the gonococcal surface are displayed in Figure *1·2*, and these will be reviewed in turn in the following sections.

1B(ii) *Lipooligosaccharide (LOS)*: The term "lipopolysaccharide" (LPS) was coined by Shear [1941; cited in Apicella *et al.* (1994)] to describe a cytotoxic glycolipid component of the *Serratia marcescens* cell wall. This molecular family is now recognised as a principle distinguishing factor between Gram-negative and Gram-positive bacteria (Preston *et al.*, 1996), being found only in the former.

Lipopolysaccharides consist of a hydrophobic component, lipid A, which is imbedded in the bacterial outer membrane, and a carbohydrate portion, which extends into the surrounding environment (Apicella *et al.*, 1994). This latter moiety contains repetitive "O-antigen" side-chains in the Enterobacteriaceae (Apicella *et al.*, 1994); however, these structures are lacking in the equivalent surface glycolipids of mucosal-pathogenic genera such as *Neisseria*, *Haemophilus*, and *Bordetella* (Preston *et al.*, 1996). Therefore, these smaller LPS-like molecules are now generally referred to as "lipooligosaccharides" [(LOS) - Preston *et al.*, 1996; see Figure *1·2*].

Many early studies on the LOS of *Neisseria gonorrhoeae* relied principally on antigenic characterisation. Thus, it was revealed that LOS is stably associated with the protein PorB (Hitchcock, 1984), and that the common core of the molecule consists of D-galactosamine-*O*-D-galactopyranosyl-(1-4)-D-glucopyranose (Apicella *et al.*, 1981). It was soon realised that surface-exposed LOS epitopes differed markedly between *in vitro*- and *in vivo*-grown gonococci (Demarco de Hormaeche *et al.*, 1986), with modified LOS forming a capsule-like structure around the latter (Demarco de Hormaeche *et al.*, 1986; Rest & Frangipane, 1992; Smith *et al.*, 1995a). Furthermore, immunofluorescence experiments demonstrated that LOS was released from the gonococcal surface (Demarco de Hormaeche *et al.*, 1986), causing sloughing of ciliated epithelial cells from the fallopian tube mucosa (Cooper *et al.*, 1986).

**FIGURE *1·2*: The Major Surface-Exposed Components Of *Neisseria gonorrhoeae*.**



Pyocin typing methods divided gonococci into two LOS groups: those with "complex" LOS were serum-sensitive after subculture, whilst those with "simple" LOS were not (Winstanley *et al.*, 1984). It is the *N*-acetylglucosamine component of the former which binds natural IgM bactericidal antibodies (Rice, 1989). However, it was discovered that extracts from mammalian body fluids induced resistance in most "serum-sensitive" strains, and the causal component was identified as cytidine-5'-monophospho-*N*-acetyl neuraminic acid [(CMP-NANA) - Parsons *et al.*, 1988]. This compound donates sialyl groups to LOS (Parsons *et al.*, 1989) via a gonococcal sialyltransferase (Preston *et al.*, 1996), masking a lacto-*N*-neotetraose epitope (Smith *et al.*, 1995a). Expression of this modifiable LOS species has been found to increase under anaerobic conditions (Rest *et al.*, 1994) and during growth of gonococci in pyruvate (McGee & Rest, 1996), producing high-level serum resistance.

Ensuing experiments on the effects of LOS sialylation determined that it impairs adherence to neutrophils (Rest & Frangipane, 1992), and delays opsonophagocytosis of gonococci by these cells (Kim *et al.*, 1992). Moreover, binding of NANA blocks bactericidal epitopes on both the recipient molecule and PorB (Smith *et al.*, 1995a), although the most surface-exposed regions of the latter may be unaffected (de la Paz *et al.*, 1995). Human challenge experiments support the hypothesis that LOS species with acceptors for NANA are important in natural infection, and mimic human glycosphingolipids (Schneider *et al.*, 1991). Nevertheless, unsialylated gonococci are more efficient at initiating an infection (Schneider *et al.*, 1996), perhaps because sialylation inhibits Opa protein-mediated entry into epithelial cells (Smith *et al.*, 1995a; Gill *et al.*, 1996)

These observations suggest that phase variation of LOS must occur *in vivo*, allowing reversible switching between the invasive, unsialylated phenotype and the non-invasive, "disguised", sialylated state (van Putten, 1993). Recent studies have identified a locus of glycosyl transferase genes that contain polyguanosine tracts (Yang & Gotschlich, 1996): slipped strand mispairing of these tracts allows the production of multiple LOS species in a single gonococcus (Burch *et al.*, 1997).

1B(iii) *The Opacity Proteins (Opa)*: In contrast to the discovery of the relationship between gonococcal colony phenotype and pilus expression, which occurred within a few years of the seminal study of Kellogg and associates [Kellogg *et al.*, 1963; see Section *1B (iv)*], the cellular basis of colony coloration and opacity remained elusive. Swanson

(1977) noted that these colony characteristics were correlated with colony friability and granularity, as well as intercellular "zones of adherence" as determined by electron microscopy. Furthermore, protein profile differences in the 20-32 kDa region were observed between colony colour variants by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE), prompting Swanson to suggest that levels of protein expression determined the phenotype.

Subsequent investigations established that opaque colonies contained proteins that were entirely absent from their isogenic, transparent counterparts (Swanson, 1978), and that opacity was a discontinuous, qualitative phenomenon associated with outer membrane proteins that can vary within a strain (Lambden & Heckels, 1979). Swanson (1977) had claimed that these proteins "act as a sort of 'glue' to bind gonococci to one another"; this was confirmed by Blake *et al.* (1995) who reported that they form intercellular adhesions by binding to the non-reducing terminal portions of LOS molecules on adjacent cells. Further insight into their native structure was obtained by Achtman *et al.* (1988), who found evidence of a trimeric or tetrameric arrangement in the homologous proteins of *Neisseria meningitidis*.

Opacity proteins [(Opa) – see Figure *1·2*] - which were formerly designated Protein II - are now known to be encoded in gonococcal strain MS11 by a family of 11 genes contained in separate loci (Bhat *et al.*, 1991). This gene family has evolved by a combination of three diversifying mechanisms: gene duplication, gene replacement, and partial non-reciprocal recombination (Bhat *et al.*, 1991). However, during the time-frame of a single course of infection, control of Opa protein production at the level of translation - which is regulated by slipped strand mispairing in the pentameric-repeat leader sequences of individual *opa* genes (Stern *et al.*, 1986) - is of more importance. Such phase variation gives rise to clones expressing none, one, or several of the Opa proteins per cell; and thus colonies that are often visibly distinct.

In discussing his observations of *in vitro* interactions between gonococci and polymorphonuclear granulocytes, Swanson (1977) reported the existence of a "leukocyte-association factor" which appeared to be an outer membrane protein 28-29 kDa in mass. Indeed, non-opsonic interactions with these cells are mediated by several of the Opa proteins, which (in strain MS11) bind to granulocytic CD66-antigens such as carcinoembryonic antigen gene family member 1a (Chen & Gotschlich, 1996), non-specific cross-reacting antigen, and biliary glycoprotein [(BGP) - Gray-Owen *et al.*, 1997a]. Of these, BGP seems to have a unique role, as the ability of an Opa protein to

bind to this receptor correlates with the generation of a respiratory burst in granulocytes (Gray-Owen *et al.*, 1997b). The pattern of recognition observed in phagocytosis of $Opa^+$ gonococci by granulocytes is mirrored in monocytes, except that MS11-$Opa_{50}$ is only engulfed by the latter (Knepper *et al.*, 1997). Moreover, the monocyte receptors involved have yet to be identified.

The CD66-antigen family may act as important Opa receptors on other cell types, such as epithelial and endothelial cells. Gray-Owen *et al.* (1997b) reported that MS11 gonococci expressing BGP-specific Opa proteins adhere to primary endothelial cells, and this was increased by up-regulation of the receptor by the proinflammatory protein tumour necrosis factor (TNF)-α. However, MS11-$Opa_{50}$ attaches to and enters epithelial cells by an entirely independent mechanism involving the side chains of heparan sulphate-proteoglycans (van Putten & Paul, 1995; cited in Meyer, 1998). This interaction can be effectively inhibited by negatively-charged polysaccharides present in agar and serum, implying that structural homology and/or charge are important in the recognition of host molecules by Opa (van Putten *et al.*, 1997).

Although several lines of *in vitro* evidence support the hypothesis that Opa proteins determine tissue tropism during the course of infection (Kupsch *et al.*, 1993), this may not be their only (or even their major) role. Bos *et al.* (1997) discovered that several MS11 Opa proteins enhance gonococcal resistance to normal human serum by an unknown mechanism, independently of LOS phenotype and sialylation. This observation is undoubtedly relevant to the study of Jerse *et al.* (1994), who recorded the proportions of various Opa types in bacteria recovered from infected human volunteers [see Section *1C(ii)*].

1B(iv) *Pili*: The work of Kellogg *et al.* (1963) was instrumental in correlating colony phenotype with particular gonococcal characteristics, virulence being the most important of these [see Section *1C (ii)*]. Using their classification scheme comprising four distinct colony types, this group reported that 90% of primary isolates from acute gonorrhoea in men were type 1. Types 1 and 2 shared many properties in common, as did types 3 and 4: the former colonies were smaller than the latter, and exhibited a more pronounced convex morphology. In addition, subsequent investigations by Sparling (1966) established that the frequency of transformation with gonococcal DNA was much greater in types 1 and 2 than in types 3 and 4.

Jephcott *et al.* (1971) conducted an electron microscopic study of the colony types, with the rationale that any visible differences might be relevant to virulence. Indeed, they discovered that colony types 1 and 2 were associated with electron-dense bundles of fibrils that were not present in preparations of type 3 or 4 colonies. Speculation that these fibrils were related to the fimbriae or pili found on other Gram-negative bacteria was confirmed by subsequent research (Swanson, 1977).

The structure of the type-4, class I pilus fibres expressed by *Neisseria gonorrhoeae* has now been investigated by computational modelling based on X-ray crystallographic, biophysical, immunological, and genetic data. Pili are three-layered spiralling fibres, 6 nm in diameter and 3-4 μm in length, which are assembled by polymerisation of pilin, the predominant sub-unit protein (Forest & Tainer, 1997; see Figure *1·2*). The fully exposed surface of the fibre is comprised of a glycosylated, hypervariable layer that is under the genetic control of a RecA-dependent variation mechanism (Meyer *et al.*, 1994). As only one or two complete pilin gene sequences exist in the gonococcal genome, recombination between the expressed gene (*pilE*) and multiple, incomplete silent gene copies (*pilS*) gives rise to antigenic variants within a gonococcal population (Haas & Meyer, 1986; cited in Meyer *et al.*, 1994).

Several other genetic mechanisms are involved in pilin expression, some of which are responsible for phase variation between the pilus-positive ($Pil^+$) and pilus-negative ($Pil^-$) phenotype. Duplication of the 3' portion of the *pilE* gene can lead to the production of "L-pilin", which cannot be polymerised into pili; whilst some recombination events generate missense or nonsense *pilE* mutant sequences (Long *et al.*, 1998). Furthermore, some $Pil^-$ variants harbour a deletion of the *pilE* sequence, and both $Pil^+$ and $Pil^-$ cells may secrete a truncated form of pilin termed "S-pilin" (Long *et al.*, 1998). Because of the complexity of PilE regulation, colony morphology alone is a poor indication of true pilus phase variation (Long *et al.*, 1998).

An important pilus-associated protein in the neisseriae is PilC, which, in a similar fashion to the Opa proteins [see Section *1B(iii)*], undergoes phase variation via slipped strand mispairing of polyguanosine residues in the signal peptide DNA sequence (Jonsson *et al.*, 1991). In addition to its central role in pilus biogenesis (Jonsson *et al.*, 1991), PilC may act as an adhesin, possibly nestling within a hydrophobic pocket located on the pilus tip (Rudel *et al.*, 1995b, cited in Meyer, 1998). However, Rahman *et al.* (1997) demonstrated that PilC is exposed on the surface of gonococcal cells independently of pili, and in itself does not confer attachment to host cells.

The connection between piliated phenotype and an increased affinity for human cells was established during early investigations on gonococcal virulence. Swanson (1973) reported that type 2 gonococci were consistently isolated with the cell-associated fraction when incubated with amnion cells and subsequently centrifuged, whilst type 4 gonococci tended to remain in the supernatant. Microscopic studies confirmed that this interaction was mediated by pili. After many years of consequent research, insight into the molecular basis of Swanson's observations was obtained by Källström *et al.* (1997), who discovered that membrane cofactor protein (a ubiquitous human transmembrane glycoprotein) was a pilus receptor of epithelial cells.

In contrast to the described influence of piliation on gonococcal attachment to epithelial cells, the effect of pili on interactions with professional phagocytes appears to be ambiguous. Ofek *et al.* (1974) reported that colony variants 1 and 2 were more resistant to phagocytosis (by both human granulocyte/monocyte monolayers and murine peritoneal macrophages) than were types 3 and 4. These findings were supported in the discussion of Swanson (1977), who stated that his experiments revealed a negative influence of pili on the "leukocyte-association factor" (Opa) he had identified. Conversely, Knepper *et al.* (1997) found that defined pilus variants of strain MS11 (some of which carried a deletion in the *recA* gene) were phagocytosed by monocytes in much greater numbers than a Pil$^-$ control variant. Intriguingly, however, a respiratory burst was not generated in these cells unless the gonococcal challenge also expressed Opa proteins - a discovery which implicates complement receptor 3 in binding of pili by phagocytes (Knepper *et al.*, 1997).

Evidently, pili are associated with a variety of processes, including natural transformation, attachment to epithelial cells, and poorly understood interactions with phagocytic host cells. However, as the number of proposed roles for pili increases, questions about their functions *in vivo*, and in particular the purpose of their vast antigenic repertoire, remain to be answered. For instance, to what extent do pili contribute to cell tropism, interference with phagocyte-mediated killing, and evasion of specific immune responses during transmission between partners? These factors will be considered in the context of gonococcal pathogenesis in Section *1D(i)*.

1B(v) *Porin Protein (PorB)*: Johnson & Gotschlich (1974) performed the first isolation and analysis of the gonococcal outer membrane. Their central finding was that two-thirds of the radiolabelled protein of the membrane consisted of a single species with

a molecular weight of 34.5 kDa; hence this component became known as the "major" or "principal" outer membrane protein (OMP), and later as Protein I (Blake & Gotschlich, 1987). Subsequently, Newhall *et al.* (1980) used covalent cross-linking reagents to perform a nearest neighbour analysis of the intact membrane, which revealed that Protein I occurred in three *in situ* arrangements. Firstly, it formed a heterodimer with a 28-kDa protein (now known to be Rmp), secondly Protein I assembled into homotrimers (see Figure *1·2*), and thirdly tetramolecular complexes were found consisting of the homotrimer in association with a single Rmp molecule. In addition, Hitchcock (1984) determined by silver staining of SDS-polyacrylamide gels that Protein I was stably associated with lipooligosaccharide.

In a groundbreaking study by Douglas *et al.* (1981), the essential physiological role of Protein I was demonstrated when liposomes containing the protein were shown to swell when added to sugar solutions – evidence of aqueous channel-forming activity. Such molecules, which are termed "porins", are the predominant protein constituent of the outer membrane in Gram-negative bacteria - gonococcal porin exhibiting an anion-selective bias (Lynch *et al.*, 1984; cited in Blake & Gotschlich, 1987). The elucidation of the function of Protein I, together with comparative studies on the porin proteins and genes of gonococci and meningococci, have led Feavers & Maiden (1998) to propose "PorB" as an appropriate term for the gonococcal porin that is encoded at the *porB* locus.

Gonococcal PorB is antigenically stable within a strain, and in coagglutination tests forms the basis of a serogrouping system that was systematically investigated by Sandstrom *et al.* (1982). In a series of peptide mapping experiments, this group established that the WI serogroup shared a characteristic PorB now known as PorB1a (Feavers & Maiden, 1998), whilst the WII/WIII strains expressed PorB1b. These porins are encoded by separate alleles at a single locus; and analysis of *porB1a* sequences reveals positive selection for diversity indicated by a higher rate of non-synonymous than synonymous substitution between isolates (Smith *et al.*, 1995b). Furthermore, the non-synonymous differences are more frequent in regions of the sequence which code for surface-exposed loops of the functional protein, suggesting that the need to escape human immune surveillance - possibly in conjunction with selection for host cell-invasive phenotypes - maintains diversity in PorB1a (Smith *et al.*, 1995b).

Originally, the two PorB classes were thought to be mutually exclusive (Sandstrom *et al.*, 1982). However, small numbers of anomalous strains have been discovered which express unusual sequence variations in predicted surface-exposed

loops, and these isolates can be grouped into three distinct categories (Cooke *et al.*, 1998). "Intermediate" strains form a separate porin phenotype that is related to PorB1a but distinct from it, whilst "hybrid" strains appear to have arisen from a double crossover between *porB1a* and *porB1b* sequences (Cooke *et al.*, 1998). The third group is represented by the non-typeable strain M194, which is characterised by a deletion in the *porB1b* sequence arising from strand mispairing during replication (Cooke *et al.*, 1998).

Gonococcal PorB was implicated in important cellular interactions with the host by experiments in which it was shown to translocate into the plasma membrane of eukaryotic cells. Indeed, Blake & Gotschlich (1987) demonstrated that PorB was directly inserted into human red blood cell membranes when the latter were mixed with PorB1b-expressing gonococci, so that the surface-exposed loops of the protein protruded into the interior of the recipient cell. Subsequent experiments with Chang epithelial cells have revealed that the PorB1a confers invasive abilities to gonococci under particular conditions of low phosphate, independently of Opa phenotype (van Putten *et al.*, 1998). This finding is consistent with the report of Rudel *et al.* (1996), who found that binding of nucleoside triphosphates modulates the voltage-dependent gating of neisserial porins; hence, phosphorylated compounds may inhibit gonococcal invasion by closing PorB ion channels (van Putten *et al.*, 1998).

The role of PorB in gonococcal pathogenesis is unlikely to be limited to epithelial cell invasion. Purified neisserial porins have been found to have profound effects on the functioning of human neutrophils - inhibiting actin polymerisation, degranulation of both primary and secondary granules, phagocytosis of meningococci, and expression of opsonin receptors such as those for complement and the Fc region of immunoglobulin G (Bjerknes *et al.*, 1995). Although porins prime neutrophils to increase their oxidative burst, intracellular killing of gonococci may primarily depend on oxygen-independent mechanisms (Bjerknes *et al.*, 1995). Thus, the net effect of PorB on the antibacterial defences of neutrophils is probably detrimental, and may represent another element of the gonococcal strategy to establish and maintain an infection.

1B(vi) *Reduction-Modifiable Protein (Rmp)*: Utilising a variety of analytical techniques - including two-dimensional SDS-PAGE, *in situ* cross-linking, and immunoprecipitation - McDade & Johnston (1980) identified "Protein III", a novel outer membrane protein in gonococcal vesicles which was closely associated with PorB. They discovered that this protein exhibited decreased electrophoretic mobility when solubilised

in the presence of a reducing agent. Consequently, it was proposed that Protein III be known as the "reduction-modifiable protein" [(Rmp) - Hitchcock, 1989; see Figure *1·2*].

Since its initial description, a number of studies have reported that the structure of Rmp is highly conserved between gonococcal isolates – a unique characteristic among the major outer membrane proteins. Indeed, enzymatically-cleaved Rmp from several different sources yields peptides that appear identical by SDS-PAGE (Judd, 1982b; cited in Blake & Gotschlich, 1987), whilst monoclonal antibodies have been developed which react with Rmp from all tested gonococcal isolates (Swanson *et al.*, 1982; cited in Blake & Gotschlich, 1987).

Indubitably, the most significant attribute of Rmp discovered to date is its ability to bind IgG antibodies which block the action of bactericidal antibodies directed against other gonococcal components (Blake *et al.*, 1989). This phenomenon was originally observed in a series of experiments by Rice *et al.* (1986), who used both total IgG from normal human serum and immunopurified antibodies against Rmp to inhibit the killing of gonococci by convalescent immune serum. Furthermore, they discovered that the killing activity of an immune serum that lacked efficacy in bactericidal assays could be "unmasked" by depleting the serum of anti-Rmp antibody.

Several lines of evidence suggest that these blocking antibodies may act by more than one mechanism. As previously discussed [see Section *1B(v)*], Rmp exists as a hydrophobic complex with PorB in the gonococcal outer membrane; and in turn, PorB is associated with LOS. Therefore, anti-Rmp antibodies may be expected to obscure bactericidal epitopes on LOS and PorB by steric hindrance (Rice *et al.*, 1986). This is supported by the observation that $F(ab')_2$ fragments prepared from normal IgG exhibit equivalent blocking characteristics to intact molecules (Rice *et al.*, 1986). In contrast, Virji & Heckels (1989) reported that two monoclonal antibodies - with almost identical epitope specificities - differed markedly in blocking activity, suggesting a different mode of action in which one molecule diverted complement from bactericidal sites whilst the other did not.

The origin of natural anti-Rmp IgG antibodies is uncertain, although a potential source is suggested by the fact that Rmp displays significant sequence homology with the OmpA proteins of the Enterobacteriaceae (Blake *et al.*, 1989). Undoubtedly, however, the discovery that Rmp elicits extremely effective blocking antibodies, which have been isolated from patients with disseminated gonococcal infection (Rice *et al.*, 1986),

provides a compelling explanation for the otherwise incongruous homogeneity of Rmp between gonococcal strains (Blake & Gotschlich, 1987).

1B(vii) *Iron-Repressible Outer Membrane Proteins*: Bacteria that reside within the human body inhabit an environment in which the availability of iron, a critical micronutrient, is restricted by iron-sequestering host proteins such as lactoferrin (at mucosal surfaces) and transferrin [(in serum) - Alcorn & Cohen, 1994]. However, within the family Neisseriaceae, a unique mechanism has evolved which enables bacterial cells to bind human transferrin and lactoferrin in order to acquire iron for their own metabolism (Alcorn & Cohen, 1994). In addition to gonococci and meningococci, other human-specific species within the Neisseriaceae express this characteristic, which can be detected in iron-starved cells by dot-blot assays utilising enzyme-labelled transferrin and lactoferrin (Schryvers & Lee, 1988). Furthermore, when exposed to several mammalian iron-binding proteins in competition assays, all species tested have been found to exhibit binding activities specific for the human host (Schryvers & Lee, 1988).

Consequently, in SDS-PAGE analysis of affinity-isolated OMPs from Neisseriaceae family members, several transferrin-binding proteins were visualised (Schryvers & Lee, 1988). Indeed, in gonococci, two transferrin binding proteins are expressed on the cell surface, and deletion mutants expressing only one or neither proteins have been compared with the wild-type in liquid-phase transferrin-binding assays (Cornelissen & Sparling, 1996). Experiments were conducted in which receptors were quantified in mutants expressing a single protein only, revealing that there are fivefold fewer transferrin-binding protein A (TbpA) sites than TbpB sites on the outer membrane; however, the latter protein was found to have a threefold lower affinity for transferrin than the former (Cornelissen & Sparling, 1996). Both transferrin binding proteins exhibit limited molecular weight heterogeneity in gonococci, and the identification of the minimal domain for transferrin binding in the amino-terminal half of TbpB has led to suggestions for its use in immunoprophylaxis (Cornelissen *et al.*, 1997).

Cornelissen & Sparling (1996) have presented evidence suggesting that the two binding proteins interact on the gonococcal surface. Firstly, a high-affinity binding site for ferrated transferrin in the wild-type strain displays a lower dissociation constant than that of either TbpA in $TbpB^-$ mutants, or TbpB in $TbpA^-$ mutants. Secondly, the accessibility of the proteins to cleavage by trypsin is markedly different between the wild-type phenotype and these mutants. The simplest explanation for these data is that the

gonococcal transferrin-binding site consists of a complex of both proteins (Cornelissen & Sparling, 1996).

Subsequent to the detection of a 105 kDa lactoferrin-binding protein in several neisseriae and in *Moraxella catarrhalis* (Schryvers & Lee, 1988), cloning of the neisserial *lbpA* gene allowed a close relationship between the predicted protein and TbpA to be inferred (Schryvers *et al.*, 1998). Furthermore, since TbpA interacts with TbpB to form the gonococcal transferrin-binding site, the existence of a second lactoferrin-binding protein was expected. Consequently, a 95 kDa lactoferrin-binding protein (which is thought to be lipidated in a similar manner to TbpB) was identified in *M. catarrhalis*, and sequencing of the region upstream of the *lbpA* gene in meningococci has led to the characterisation of the *lbpB* gene within the *lbp* operon (Schryvers *et al.*, 1998).

It is becoming increasingly clear that within the Neisseriaceae, the molecules and mechanisms involved in binding lactoferrin parallel those described for transferrin. Both TbpA and LbpA, but neither TbpB nor LbpB, belong to a family of OMPs that are dependent on TonB, an inner membrane protein which is required to supply energy in nutrient-uptake processes (Schryvers *et al.*, 1998). Moreover, studies on the ability of neisserial isogenic mutants to grow *in vitro* with lactoferrin or transferrin as the sole source of iron have implied that TbpA and LbpA are essential for iron acquisition, whilst TbpB and LbpB have a subsidiary role (Schryvers *et al.*, 1998). Taken together, these observations support a model in which the former proteins act as gated pores for iron transport across the outer membrane (Schryvers *et al.*, 1998).

Accordingly, the subsidiary proteins may facilitate the initial recognition event by enabling the binding complex to switch between two different states (Schryvers *et al.*, 1998; Cornelissen & Sparling, 1996). The high-affinity conformation of the gonococcal transferrin-binding site has a lower dissociation constant than the mammalian transferrin receptor on host cells (Cornelissen & Sparling, 1996). However, in order to compete effectively for iron *in vivo*, the gonococcus must both preferentially bind ferrated transferrin and rapidly release the deferrated molecule (Cornelissen & Sparling, 1996). Indeed, Cornelissen & Sparling (1996) have reported that TbpB has a hundred-fold higher affinity for ferrated transferrin than for apotransferrin, thereby providing evidence for just such a mechanism.

1B(viii) *Immunoglobulin A1 Protease*: A diverse group of pathogenic bacteria that colonise mucosal surfaces, including gonococci, meningococci, various streptococci and

*Haemophilus influenzae,* secrete a protease which is specific for the hinge region of human IgA1 heavy chains (Brooks *et al.*, 1998). Cleavage by these enzymes inactivates secretory IgA1; and because they are not produced by commensal neisseriae, IgA1 proteases are assumed to be an important determinant of virulence (Brooks *et al.*, 1998).

Mulks *et al.* (1980), in an SDS-PAGE analysis of the cleavage products of IgA1 digested by the protease of *Neisseria meningitidis*, discovered that meningococcal strains varied in the antibody fragments produced. Two distinct profiles were identified, which were never generated simultaneously by the enzyme purified from a single isolate. Amino acid sequencing of the immunoglobulin fragments revealed "type 1" protease cleavage of a prolyl-seryl peptide bond within the IgA1 hinge, whilst "type 2" activity was specific for a prolyl-threonyl site nearby (Mulks *et al.*, 1980).

Sequencing of the *N. gonorrhoeae* MS11 IgA1 protease gene (*iga*) defined a protein with a predicted weight of 169 kDa – significantly heavier than the established weight (106 kDa) of the functional enzyme (Pohlner *et al.*, 1987). To resolve this discrepancy, Pohlner *et al.* (1987) conducted studies on the immunological cross-reactivity between purified IgA1 protease and fusion proteins expressed from the cloned *iga* gene in *Escherichia coli*. Consequently, three domains were identified in the *iga* sequence, which give rise to three regions of an IgA1 protease precursor protein: an amino-terminal signal peptide, the protease itself, and a carboxyl-terminal "helper" portion.

Further analysis involving a series of elegant immunoblotting experiments led to the presentation of a model to describe the processing and secretion of gonococcal IgA1 protease (Pohlner *et al.*, 1987). According to this proposal, the precursor protein enters the periplasmic space under the guidance of the leader peptide (which is subsequently cleaved off), and becomes associated with the outer membrane via the amphipathic helper region. The protease domain is extruded through a pore formed by the latter moiety, developing an active conformation in the process, and is then released into the medium surrounding the cell by an autoproteolytic event which leaves the "β-domain" of the helper portion behind in the membrane. Finally, the proform of IgA1 protease cuts within its own helper region a second time, yielding the mature enzyme and a small "α-protein" which can be detected in the supernatants of gonococcal broth cultures (Pohlner *et al.*, 1987).

Although IgA1 proteases have been credited with an important role in pathogenesis by many authors [for instance, see Brooks *et al.* (1998)], empirical evidence

supporting this assertion is scant. A particular puzzle is presented by the fact that these enzymes cannot digest IgA2, which lacks the hinge region targeted by the protease (Brooks *et al.*, 1998). Since ~40-55% of IgA-producing cells in the tissues of the female reproductive tract secrete this subclass (Kutteh *et al.*, 1988), it is unlikely that a colonising pathogen could neutralise a protective secretory antibody response. Hence, the discovery that gonococcal IgA1 protease exhibits autoproteolytic activity – and is therefore not functionally restricted to cleavage of the IgA1 hinge – has prompted further research into other candidate substrates for this enzyme.

Accordingly, Lin *et al.* (1997) have demonstrated that pathogenic neisseriae decrease levels of lysosome-associated membrane protein-1 (LAMP-1) in human epithelial cells during *in vitro* intracellular infection, whereas this phenomenon is not observed in cells challenged with *iga*$^-$ gonococci. Furthermore, LAMP-1 contains an IgA1-like hinge region that is cleaved *in vitro* by recombinant IgA1 protease (Lin *et al.*, 1997). Moreover, as LAMP-1 may be involved in protecting lysosomes and late endosomes from the activity of their own hydrolytic enzymes, digestion of this glycoprotein is liable to interfere with a key component of host defence. In support of this conjecture, the *iga*$^-$ gonococcal mutant GCM740$\Delta$4 has a much reduced intracellular growth rate in epithelial cells compared to its *iga*$^+$ parent strain (Lin *et al.*, 1997).

## *1C* - Characteristics & Clinical Aspects Of The Disease

1C(i) *Natural Infections*: Transmission of gonorrhoea requires direct contact, infection being acquired during sexual activity or by the perinatal route from an infected mother. Genital infection in men is characterised by acute urethritis, and the symptoms of urethral discharge and dysuria usually appear 2-5 days after infection, the range being 1 to 10 days or longer (Handsfield & Sparling, 1995). Within one to two days of onset, the discharge becomes distinctly purulent (Handsfield & Sparling, 1995); and is found to consist of serum, large numbers of neutrophils, and free and cell-associated Gram-negative diplococci (Harkness, 1948). In most cases, untreated infections resolve without complication over several weeks (Holmes, 1974).

The principal symptom of acute infection in women is mucopurulent cervicitis, sometimes in combination with any or all of the following: urethritis, vaginal discharge, dysuria, and intermenstrual bleeding (Curran *et al.*, 1975). The incubation period is more variable than that seen in men, but symptomatic cases are usually apparent within 10 days

of infection (Handsfield & Sparling, 1995). However, screening has revealed that up to 80% of women with a positive culture are asymptomatic (WHO, 1996).

It is not yet clear to what extent asymptomatic cases result from genuine unapparent infection, as opposed to episodes in which symptoms occurred initially, but were not treated effectively, if at all. In men, asymptomatic gonorrhoea was thought to be relatively uncommon [up to 10% of infections (WHO, 1996)]. However, a study in Tanzania by Grosskurth *et al.* (1996) revealed that of 158 men with urethritis (81% with gonorrhoea), 66% had neither symptoms nor signs at the time of examination, and 58% denied ever having a urethral discharge. Less controversially, rectal and pharyngeal infections, which are most often seen in women and homosexual men, are asymptomatic in the majority of cases (Handsfield & Sparling, 1995).

Complications due to gonorrhoea in men, such as acute epididymitis and urethral stricture, are now infrequently encountered (Handsfield & Sparling, 1995). In contrast, serious sequelae (such as endometritis, salpingitis, tubo-ovarian abscesses and pelvic peritonitis) are a common consequence of untreated infection in women (Handsfield & Sparling, 1995). Furthermore, gonorrhoea is also an important risk factor for female infertility. The World Health Organisation estimates that if 100 women (25% pregnant) were treated for gonorrhoea, on average, 25 cases of pelvic inflammatory disease (PID), 1 ectopic pregnancy and six cases of infertility would be averted (WHO, 1996). In addition, some cases of PID also result in acute perihepatitis (Handsfield & Sparling, 1995).

Disseminated gonococcal infection occurs in 0.5-3% of gonorrhoea cases and can affect either sex, but women may be at greater risk (Handsfield & Sparling, 1995). The most common manifestations are septic arthritis and a syndrome of polyarthritis and dermatitis; the latter characteristic symptom being present in 75% of cases (Handsfield & Sparling, 1995). Around 13% of patients have a complement deficiency that facilitates the initial bacteraemia, leading to dissemination of the organism to the skin and joints (Handsfield & Sparling, 1995).

An important component of the public health impact of gonorrhoea is the effect on new-borns. The risk of gonococcal conjunctivitis in babies born to infected mothers (ophthalmia neonatorum) can be up to 30%, and can lead to blindness if left untreated (WHO, 1996); however, administration of a 1% aqueous solution of silver nitrate to the conjunctivae soon after delivery is an excellent prophylactic. Systemic illness in new-borns can also occur, but is rare (Handsfield & Sparling, 1995). During the neonatal period, infections can be acquired non-sexually from an infected parent if hygiene is poor,

but most cases seen in children over 1 year of age reflect sexual abuse (Handsfield & Sparling, 1995).

Excluding neonatal infections, non-sexual transmission of gonorrhoea occurs very infrequently. However, gonococcal conjunctivitis, although often the result of autoinoculation in an individual with a primary infection elsewhere (Handsfield & Sparling, 1995), can be transmitted among young children or within close communities without sexual contact. Such outbreaks have been reported in the aborigines of central Australia, and have involved several hundred cases on more than one occasion (Matters *et al.*, 1998).

1C(ii) *Experimental Infections*: Since there is no adequate animal model of mucosal gonococcal infection (excluding chimpanzees), several studies have endeavoured to characterise aspects of the disease through the use of human volunteers. Women are not recruited for such research due to the substantial risk of chronic complications following infection (Cohen *et al.*, 1994). In modern experiments, subjects are infected by delivering a suspension of gonococcal cells into the urethra via a catheter; they are then requested not to urinate for about 2 hours (Cohen *et al.*, 1994). Antibiotics are administered to the volunteers immediately after the onset of urethral discharge (Cohen *et al.*, 1994).

In a series of trials by Cohen *et al.* (1994), in which 47 men were inoculated with different variants and quantities [$10^4$-$10^6$ colony-forming units (cfu)] of gonococcal strain FA1090, only 57% became infected, and 7% of these were asymptomatic. Most subjects had a purulent penile discharge, and dysuria was experienced only in the presence of a urethral exudate. The infection rate and the incubation period were clearly proportional to the size of the inoculum, with $10^6$ bacteria causing symptoms more rapidly (mean = 1.4 days, $n = 10$) than is seen in natural infections.

Interestingly, urine specimens collected 2 hours after the inoculation procedure contained very few viable organisms, although leukocyte numbers in the same samples were elevated until returning to a low level within 24 hours. Additionally, both leukocytes and gonococci increased in urine samples immediately before the onset of urethral exudate in affected subjects. The signs and symptoms of urethritis were indistinguishable from those presented in a clinical situation.

The location of gonococci during the incubation period remains unknown: most may die, or they may become attached or sequestered so as to be unaffected by the

passage of urine. However, Cohen *et al.* (1994) were unable to retrieve gonococci by swabbing the anterior urethra, and speculated that the bacteria may have reached a sub-mucosal site; if so, it is not clear whether the infection is transmissible prior to the onset of urethritis.

Insight into the development of gonorrhoea in men has been obtained by measuring the secretion of cytokines (soluble cell-signalling proteins) in urine during experimental infections with strain MS11mkA or MS11mkC. Ramsey *et al.* (1995) observed elevated levels of the proinflammatory cytokines interleukin (IL)-8, IL-6 and TNF-α in many symptomatic subjects within 2-14 hours of inoculation. Although IL-8 and IL-6 were also detected in the urine of some volunteers who were only transiently infected, the responses returned to baseline levels within 14 hours, whilst those in symptomatic cases continued to rise.

Interleukin-1β followed a different pattern: it did not increase at any time in transiently-infected subjects, but was elevated just prior to the onset of symptoms in the other volunteers. Ramsey *et al.* (1995) speculated that this cytokine might have therefore been derived from infiltrating inflammatory cells. The overall pattern suggested a cascade that followed the sequence IL-8/IL-6, TNF-α, IL-1β. Since several subjects secreted high levels of cytokines in urine in the absence of detectable amounts in their plasma, it was concluded that IL-8, IL-6 and TNF-α were derived from (alone or in combination) urethral epithelium cells, macrophages and mast cells (Ramsey *et al.*, 1995).

A more recent experimental challenge study utilising strain MS11mkC also found that IL-8 and IL-6 were secreted in the urine of infected volunteers, but time of onset was 24-48 hours post-inoculation (Warren *et al.*, 1996). In these subjects, peripheral blood antibody-secreting cell responses to gonococcal outer membrane complex (containing both pili and LOS) peaked at 10-12 days post-challenge, and serum IgG against LOS was also consistently detected (Warren *et al.*, 1996).

Human experimental infections provide a unique opportunity to explore the role of phase and antigenic variation of gonococcal surface components in pathogenesis. Using a piliated strain of gonococcus (MS11mkA) that expresses a single 3.6 kDa LOS, Schneider *et al.* (1991) tested the hypothesis that phase variation of LOS occurs *in vivo*. They discovered that recovery of defined LOS variants from urine sediments was coincident with certain clinical manifestations of the infection, with one variant appearing at the onset of inflammation and dysuria, and another at onset of urethritis and discharge. Furthermore, the transition from the non-sialylated inoculum that established the infection

to the disease-causing variants was associated with expression of potentially sialylated LOS species, the structures of which mimic human cell membrane glycosphingolipids (Schneider *et al.*, 1991). Gonococcal mutants have since been created which express a single truncated LOS and cannot undergo LOS variation; these organisms do not establish infections in volunteers (Cannon *et al.*, 1996).

Subsequent investigations involving one of the novel LOS variants (MS11mkC) reported by Schneider *et al.* (1991) revealed that it was more efficacious at establishing new infections than was MS11mkA. In fact, in one trial, 3 of 7 subjects inoculated with only ~250 MS11mkC gonococci developed urethritis between 65 and 89 hours later (Schneider *et al.*, 1995). When relatively small numbers ($<10^5$) of gonococci are used as inocula, very variable courses of infection are seen in terms of incubation time, shedding of organisms, and the relative timing of dysuria and discharge. This may reflect more accurately the situation in the natural disease (Schneider *et al.*, 1995).

As the LOS of variant MS11mkC can be sialylated in the presence of CMP-NANA, Schneider *et al.* (1996) investigated whether exogenous sialylation of gonococci affected the efficiency of infection. Contrary to expectations, sialylated bacteria infected fewer volunteers than did equivalent unsialylated inocula, and the incubation period was also extended. Therefore, sialylation as a mechanism of immune avoidance may not be utilised until after gonococci have initiated an infection. However, it is not known whether the CMP-NANA concentration in cervical mucous contributes to the relative low efficiency of female-to-male transmission (Schneider *et al.*, 1996).

To determine whether *in vivo* variations in opacity protein (Opa) expression are important in gonorrhoea, Jerse *et al.* (1994) infected volunteers with piliated, predominantly Opa-negative inocula of strain FA1090. They found that in all their infected subjects, the number of Opa-positive colonies cultured from urine samples increased substantially relative to the inoculum, and in six of nine of these volunteers, all cultured colonies were Opa-positive. Although strong selection for Opa expression was therefore evident [as also reported by Schneider *et al.* (1995) and Schneider *et al.* (1996)], the same Opa types were not recovered from all the infected subjects. Jerse *et al.* (1994) speculated that this could be due to chance survival of particular Opa variants present in the original inoculum. Indeed, when a variant expressing a single Opa type was compared with an Opa-negative variant in urethral challenge, there were no major differences between them in either the proportion of volunteers infected or in the incubation period;

but significantly, up to 10% of the "Opa-negative" inoculum was actually Opa-positive (Jerse *et al.*, 1994).

The major sub-unit of pili - pilin - is also capable of undergoing antigenic variation. In a study by Seifert *et al.* (1994) in which two volunteers were challenged with strain FA1090, the expressed pilin gene was sequenced in both the inoculum and in bacteria recovered during infection. Extensive pilin variation was discovered throughout the infection process both between and within subjects, and may have been stimulated by *in vivo* conditions independently of, or in conjunction with, intense selection of types present in the inoculum. One likely source of selective pressure is the non-specific immune response: indeed, one volunteer experienced a transitory inflammatory response immediately after urethral challenge, and the predominant inoculum pilin variant was not recovered again after the first urine sample (Seifert *et al.*, 1994).

Despite the findings of Kellogg *et al.* (1963), who reported that $Pil^+$ "type 1" gonococcal colonies infected test subjects after numerous *in vitro* passages whilst $Pil^-$ "type 4" colonies did not, pili may not be essential in establishing an infection. Hence, Cannon *et al.* (1996) developed a non-reverting, pilin-negative mutant of strain FA1090, and inoculated 6 volunteers with $10^6$ cfu. Although two subjects developed a watery discharge, the others remained asymptomatic, despite continual positive urine cultures over a 5-day period. Indubitably, pili are critical in producing virulent disease by directly or indirectly stimulating the acute inflammatory response which is characteristic of gonorrhoea in men.

## *ID* - Pathogenesis Of Gonococcal Infection At The Mucosal Interface

1D(i) *Invasion Of The Mucosal Epithelium*: Since gonorrhoea lacks a meaningful and practicable animal model, studies on the *in vivo* role of the gonococcal molecular armoury have been limited to the small number of human volunteer experiments described in the previous section. Therefore, investigators have turned to *ex vivo* methods - examining the interaction of gonococci with tissue sections, cultured human organs and cell lines from relevant sites, differentiated cell layers, and exudates from natural infections. These approaches have enabled a model of gonococcal pathogenesis at mucosal sites to be developed, which comprises a series of distinct but interactive events that lead to invasion of deeper tissues.

Ward *et al.* (1974) performed the first *ex vivo* studies of the gonococcal infection process using cultured human organs. Their experiments involving perfusion of live fallopian tubes with a gonococcal suspension were particularly important, since the bacteria were subject to the shearing forces of a flow of fluid, as is likely to be the case *in vivo*. Hence, the discovery that pili anchor gonococci to the epithelial surface under such conditions lends credence to the suggestion that these organelles mediate initial colonisation of the mucosa, perhaps further increasing resistance to the flow of urine or mucus by interconnecting adjacent bacteria into microcolonies (van Putten & Duensing, 1997). In addition, pili may enable gonococci to overcome the electrostatic repulsion that exists between the cell membranes of host and pathogen (Heckels *et al.*, 1976).

The infectious inoculum that encounters the mucosal epithelium of a sexual partner is likely to consist of a number of different phenotypes expressing distinct pilus species (Seifert *et al.*, 1994). Although phenotypic changes may contribute to evasion of both innate and specific immune responses, experiments conducted by Jonsson *et al.* (1994) reveal that pilus variation is also important in determining binding specificity to tissue sections. Therefore, their study provides a potential mechanism for the observed capability of gonococci to initiate infection at diverse anatomical sites, such as the cervix, urethra, rectum, pharynges and conjunctiva (Handsfield & Sparling, 1995).

Whilst pili undoubtedly facilitate anchorage of gonococci to host cell membranes exposed to fluid flows, most *in vitro* experiments allow bacteria to attach to epithelial cells by settling onto the monolayer under the influence of gravity. In such systems, pili are not required for gonococcal attachment; indeed, intimate binding of the gonococcal outer membrane to human cell surfaces is predominantly controlled by Opa protein expression (van Putten & Duensing, 1997). Furthermore, Opa is the principal gonococcal invasin, inducing uptake by epithelial cells via a process that resembles classical phagocytosis (Grassmé *et al.*, 1996). In Chang conjunctival epithelium cells, this mechanism involves Opa adherence to heparan sulphate-proteoglycans, accumulation of F-actin beneath the gonococcus, depolymerisation of the recruited actin, and internalisation of the organism by a microfilament-dependent process (Grassmé *et al.*, 1996).

Elucidation of the early stages involved in gonococcal interactions with mucosal surfaces raises the question of whether infected epithelial cells generate the proinflammatory cytokine responses recorded in challenged human volunteers (Ramsey *et al.*, 1995). *In vitro* stimulation of ME180 monolayers with both viable gonococci and

purified gonococcal components has revealed that whole bacteria, outer membrane complexes and pili, but not LOS, can induce production of IL-8 and IL-6 in these cervical carcinoma cells (van de Verg *et al.*, 1996). Dissection of the epithelial cell cytokine response at the molecular level has demonstrated increased synthesis of several mRNA transcripts (granulocyte-macrophage colony-stimulating factor, TNF-α, IL-8, monocyte chemotactic protein-1, transforming growth factor-β and IL-1β) after gonococcal challenge, in some cases within 15 minutes (Naumann *et al.*, 1997). Both $Pil^+/Opa^-$ and $Pil^-/Opa^+$ gonococci were effective in up-regulating transcription, which peaked at 2 hours after inoculation, but a $Pil^-/Opa^-$ variant did not significantly increase cytokine mRNA production (Naumann *et al.*, 1997).

Additional data presented by Naumann *et al.* (1997) support a mechanism in which gonococcal attachment - prior to invasion - activates nuclear factor-κB within 10 minutes, leading to expeditious transcription of cytokine genes and the development of inflammation. The spectrum of inflammatory proteins produced is consistent with two effects that are observed in most gonococcal infections – recruitment of neutrophils [see Section *1D(ii)*], and damage to cells of the mucosal surface. In fallopian tube explants, the latter phenomenon is dependent on TNF-α production, which is thought to be generated in response to gonococcal LOS and peptidoglycan released into the extracellular environment (McGee *et al.*, 1992; Cooper *et al.*, 1986). However, the cells responsible for TNF-α release in this system have not been identified, although they may be located in subepithelial tissues (McGee *et al.*, 1992).

Clearly, phagocytosis of gonococci by epithelial cells provides a route by which the infection can penetrate the mucosa, but it has taken many years to unravel the complexity of the invasive process, and some pieces of the jigsaw are still missing. Persuasive evidence that gonococci enter urethral epithelium cells *in vivo* during natural infections has been obtained by Apicella *et al.* (1996), who examined urethral exudates from men with confirmed gonorrhoea by both laser scanning confocal and immunoelectron microscopy. They determined that viable gonococci were present inside shed epithelial cells - some within vacuoles and others free in the cytoplasm. Detailed studies of the events leading to epithelial shedding were undertaken using excised human ureters by Mosleh *et al.* (1997). This work showed gonococcal invasion below the superficial epithelial layer, both by vacuolar transport and cell lysis. In the former process, ingested gonococci within a vacuolar membrane are exocytosed at the basal or lateral plasma membrane after translocation of the vacuole through the cytoplasm. Lysis

of infected cells occurs when the integrity of the vacuole is disrupted (probably by PorB and/or IgA1 protease), and results in extensive release of bacteria into intercellular locations. Consequently, damage to the intercellular connections leads to exfoliation of epithelial cells, penetration of gonococci into deeper cell layers, and thinning of the mucosa (Mosleh *et al.*, 1997).

Traversal of epithelial cell layers has also been investigated at the molecular level by Wang *et al.* (1998), who utilised a polarised colonic carcinoma cell line (T84) with an intact barrier function in their experiments. Gonococcal transmigration through this cell monolayer was dependent on initial binding of CD66-antigen family receptors by specific Opa proteins, and could not be mediated by attachment to heparan sulphate-proteoglycans. Furthermore, engulfed bacteria were contained within a phagosomal membrane, sometimes still in association with CD66 receptors, and began to appear beneath the basolateral surface of the intact monolayer as early as 12 hours post-infection.

As more becomes known regarding the details of gonococcal pathogenesis at the mucosal surface, many investigators have sought to explain the observation that in experimentally-infected volunteers, the first urine sample (collected 2 hours post-inoculation) contains very few viable gonococci (Cohen *et al.*, 1994). One possibility is that the majority of the inoculum is rapidly killed before the onset of overt symptoms (Cohen *et al.*, 1994), but there is no direct evidence that significant plasma exudation, bringing complement and antibodies to the mucosal surface, occurs early in infection of the genitourinary tract (Bos *et al.*, 1997). Certainly, normal human serum contains both complement-dependent bactericidal IgM antibodies directed against LOS epitopes (Rice, 1989), and antibodies elicited by enterobacteria that cross-react with neisserial porins (Henriksen *et al.*, 1998). However, the effectiveness of these serum factors may be compromised by inhibition of complement by urine (Bos *et al.*, 1997), and by gonococcal defences such as sialylation of LOS (Smith *et al.*, 1995a), expression of Opa proteins (Bos *et al.*, 1997), and adsorption of blocking antibodies (Blake *et al.*, 1989).

Alternatively, the "disappearance" of the gonococcal inoculum may relate to the natural history of the infection process, with recovery of bacteria from urine depending on the rupture or exfoliation of infected epithelial cells (Apicella *et al.*, 1996). Wang *et al.* (1998) noted that polarised epithelial cells display differential expression of surface receptors at their apical and basolateral interfaces. Accordingly, gonococci may initiate colonisation of the intact epithelium via CD66-antigens, and after acquiring extracellular factors such as vitronectin, invade other host cells from the basolateral surface by binding

to syndecan receptors. Thus, a cycle of infection may develop by which gonococci can re-enter the urethral lumen - from whence some may be released in urine, or in semen during sexual intercourse – in order to establish new foci of replication elsewhere on the mucosal surface (Wang *et al.*, 1998).

1D(ii) *Recruitment Of Neutrophils During Acute Inflammation*: The purulent discharge typical of acute gonorrhoea is characterised by a high concentration of polymorphonuclear leukocytes in a serous exudate, with many of the phagocytes containing intracellular gonococci (Harkness, 1948). This simple observation poses questions regarding how neutrophils are attracted to the site of infection, and what roles they play in the course of the disease.

Chemotaxis of leukocytes occurs during the inflammatory response to a wide variety of stimuli, and is mediated by several substances with a broad spectrum of activity, including complement-derived C5a, leukotriene $B_4$, and platelet-activating factor (Fantone & Ward, 1994; cited in Mukaida *et al.*, 1998). Interestingly, the ability of a gonococcal strain to induce C5a production correlates with *in vitro* susceptibility to normal human serum, and this in turn is linked to the propensity of the strain to cause inflammatory symptoms in natural infections (Rice, 1989). Thus, gonococci expressing a serum-susceptible phenotype are associated with symptomatic localised infections and acute salpingitis, whereas strains which generate C5a slowly exhibit stable serum resistance and are more likely to be recovered from asymptomatic localised infections and disseminated disease (Rice, 1989; Shafer & Rest, 1989). The sub-unit structure of LOS probably underpins this phenomenon, but the mechanism involved has yet to be determined (Shafer & Rest, 1989).

The chemokine superfamily of cytokines consists of many chemoattractant proteins, within which the "CXC" group tends to display granulocyte-specific activity, causing these cells to migrate along concentration gradients that emanate from the site of trauma (Mukaida *et al.*, 1998). Interleukin-8 is the most biologically dominant member of the CXC family, exhibiting a number of roles in addition to chemotaxis - including induction of shape change, lysosomal enzyme release, respiratory burst, free radical and lipid mediator generation, and adhesion molecule expression in neutrophils (Mukaida *et al.*, 1998). As discussed previously, IL-8 is secreted in the urine of men with experimental gonorrhoea (Ramsey *et al.*, 1995), as well as by epithelial cell lines stimulated by gonococci or gonococcal components (van de Verg *et al.*, 1996; Naumann *et al.*, 1997).

The study of Agace *et al.* (1993) demonstrated that urinary neutrophil numbers were strongly correlated with urinary IL-8 concentrations in women with experimental *Escherichia coli* urinary tract infections. Furthermore, the onset of IL-8 secretion occurred prior to neutrophil influx, and epithelial cells obtained from bladder irrigation fluid produced IL-8 in response to stimulation by *E. coli* – evidence that primary cells recovered directly from the site of infection generate this potent neutrophil chemoattractant. However, the roles of other CXC chemokines [such as epithelial-derived neutrophil attractant (ENA)-78 and the growth-related protein (GRO) subfamily] in neutrophil recruitment to the infected genitourinary tract have yet to be investigated. Rasmussen *et al.* (1997) have reported that 3 days post-infection, *Chlamydia trachomatis* causes an approximate hundred-fold increase in secretion of GRO-α by HeLa cervical epithelium cells, although IL-8 is simultaneously produced in much larger quantities. The expression of ENA-78 in genitourinary tissues infected with bacteria has not been determined, but in alveolar type II epithelial cells, *Mycoplasma hominis* is a potent stimulator of ENA-78 production, and is thought to contribute to granulocyte infiltration in bronchopulmonary dysplasia (Kruger & Baier, 1997).

Epithelial cells are not the only potential cytokine-generating cells present in the human genitourinary system. Immunohistochemistry techniques have revealed the presence of both T-lymphocytes and macrophages within the mucosa of the normal human ureter and urinary bladder (El-Demiry *et al.*, 1986). The latter cells are found mainly within the lamina propria but also within the urothelium (El-Demiry *et al.*, 1986), and as such may be important in interactions with gonococci early during infection. Indeed, blood-derived monocytes stimulated by LOS *in vitro* generate IL-8 by 1 hour after challenge, and production is greater in response to the gonococcal antigen than to equivalent amounts of *Salmonella typhimurium* LPS (Harper *et al.*, 1996). Moreover, stimulation of monocytes with gonococcal whole-cell lysates results in a two-fold increase in IL-8 production compared to LOS alone; an effect that cannot be attributed solely to the LOS component of the unfractionated material (Harper *et al.*, 1996).

1D(iii) *Interaction Of Gonococci With Neutrophils*: Neutrophils recruited to the site of mucosal infection in gonorrhoea clearly do not effectively control gonococci before acute symptoms and further transmission occur. Gonococci possess a number of virulence determinants that interact with professional phagocytes, but the contribution of each to ensuring the survival of an infectious inoculum is not fully understood. Early

studies on the function of pili suggested that they impeded engulfment of gonococci by attaching each bacterium to distal points on the phagocyte surface (Ofek *et al.*, 1974; Shafer & Rest, 1989). However, in the absence of serum opsonins, piliated MS11 organisms lacking Opa proteins display a modest increase in uptake by granulocytes when compared with the $Opa^-/Pil^-$ phenotype (Knepper *et al.*, 1997). Therefore, it is probable that variability of pilin and PilC expression in gonococci, perhaps in conjunction with the activation status of the phagocyte, results in different experimental outcomes during *in vitro* studies.

Potentially, gonococci will be opsonised *in vivo* via complement and natural antibodies present in inflammatory exudates (Shafer & Rest, 1989). A confounding factor is that the same secretions contain CMP-NANA which sialylates lacto-*N*-neotetraose residues on surface-exposed LOS, and as a consequence, organisms recovered from the infected urethra are usually sialylated (Smith *et al.*, 1995a). Hence, the interplay between complement and CMP-NANA during opsonophagocytosis of gonococci was investigated by Kim *et al.* (1992), who reported that *in vitro* sialylation caused a significant delay in neutrophil-mediated killing.

In the absence of serum, gonococci bind to neutrophils via the surface-exposed loops of Opa proteins; in particular, hypervariable region 2 is considered to be critical in this process (Naids *et al.*, 1991). As discussed in Section *1B(iii)*, the receptors on the neutrophil surface that recognise Opa proteins are glycoproteins of the CD66-antigen family, which share a non-glycosylated N-terminal domain responsible for ligand binding (Gray-Owen *et al.*, 1997a). Intriguingly, Opa-mediated adherence to neutrophils can be inhibited by incubation of gonococci in the presence of CMP-NANA, perhaps because sialylation increases electrostatic repulsion between the membranes of gonococcus and phagocyte (Rest *et al.*, 1994). Nevertheless, during assays conducted over 2-3 hours, sialylation does not prevent effective killing of gonococci by phagocytes, suggesting that turnover of LOS species on the gonococcal surface - or neutrophil sialidase activity – negates the inhibitory effect (Rest *et al.*, 1994). Furthermore, sialylated gonococci are not protected from the bactericidal action of neutrophil granule extracts *in vitro* (Rest *et al.*, 1994).

The specificity of the Opa/CD66-antigen interaction for phagocytosis of gonococci was investigated by Hauck *et al.* (1998). Binding of MS11 gonococci expressing $Opa_{52}$ triggered an activation cascade in neutrophils, which was transmitted from the CD66 receptors via Src-like protein tyrosine kinases and the G-protein Rac1 (a

mediator of actin reorganisation), to p21-activated kinase and Jun-N-terminal kinase. Whereas F(ab') fragments directed against CD66-antigens could initiate this signalling pathway, *E. coli*, *Neisseria cinerea*, and Opa$^-$ gonococci did not. Furthermore, the tyrosine kinase Syk was not involved in the non-opsonic uptake process, in contrast to its central role in mediating ingestion of antibody-coated particles via Fcγ receptors (Hauck *et al.*, 1998). Elucidation of the signals involved in Opa-dependent phagocytosis is important, as this phosphorylation pathway determines how internalised material is processed, and thus the eventually fate of intracellular gonococci.

Numerous *in vitro* experiments have been conducted to establish whether a sub-population of engulfed gonococci remains viable inside neutrophils. Indeed, a small proportion (~3%) of cell-associated gonococci appear to survive within phagocytes for over 2½ hours, independently of the metabolic conditions (aerobic or anaerobic) under which the assays proceed (Casey *et al.*, 1986). In addition, growth of organisms *in vivo* leads to the selection of a morphologically distinct population that displays enhanced resistance to phagocyte killing in subsequent experiments (Parsons *et al.*, 1985; cited in Smith, 1991). However, the issue of gonococcal survival inside exudate neutrophils during human infection has long been controversial (Shafer & Rest, 1989), and is yet to be settled unequivocally.

The finding that neutrophil-mediated killing of gonococci is oxygen-independent suggests that cationic granule proteins are the bactericidal factors employed (Shafer & Rest, 1989). Only a subset of these agents demonstrates anti-gonococcal activity *in vitro*: cathepsin G, cationic anti-microbial protein (CAP)-37 and CAP-57 are effective (Shafer & Rest, 1989), but *N. gonorrhoeae* appears to be uniformly resistant to neutrophil defensins (Qu *et al.*, 1996). Furthermore, gonococcal susceptibility to the action of cathepsin G is modulated by several phenotypic factors, including LOS structure, penicillin resistance, and phase of growth (Shafer & Rest, 1989).

Purified neisserial porins interfere with a number of neutrophil functions, including opsonophagocytosis and degranulation of both primary and secondary granules [Bjerknes *et al.*, 1995; see Section *1B(v)*]. Indeed, although gonococcal PorB causes an enhancement of the intracellular oxidative burst in neutrophils - as measured by hydrogen peroxide production (Bjerknes *et al.*, 1995) - impairment of myeloperoxidase release from secondary granules prevents the generation of a key bactericidal product, hypochlorite (Meyer, 1998). Additionally, insertion of PorB into the phagosomal membrane prevents fusion with lysosomal vesicles, thus circumventing degranulation

triggered by the CD66-antigen activation pathway (Meyer, 1998). The end result is that the secondary granules are depleted by discharging their contents at the plasma membrane rather than within the phagosome (Meyer, 1998).

Further analysis of the phagocytic oxidative burst elicited by interactions with gonococci expressing different phenotypes has demonstrated important roles for both Opa proteins and pili. Ingested piliated gonococci lacking Opa proteins do not stimulate a respiratory burst in neutrophils, but pilus expression does not suppress luminol-enhanced chemiluminescence following engulfment of $Opa^+$ variants (Knepper *et al.*, 1997). However, although opaque gonococci clearly do not prevent the triggering of neutrophil defences, the resultant oxidative burst is considerably weaker than that caused by phagocytosis of the commensal species *N. cinerea* and *N. sicca* in the JOSK-M myelomonocytic cell line (Hauck *et al.*, 1997). Because neutrophils use CD66 receptors to present sialylated Lewis-x antigen oligosaccharides to E-selectin on activated endothelial cells (Kuijpers *et al.*, 1992), Opa proteins may modify intracellular defences by mimicking a host mechanism of intercellular recognition (Hauck *et al.*, 1998).

Thus, a complex interplay occurs *in vivo* between pathogen-induced responses in the host and host-induced changes in the pathogen. Inflammatory cytokines secreted in response to stimulation by gonococcal antigens induce an influx of neutrophils and the expression of receptors for CD66-antigens on host cells, whilst the gonococcus undergoes variation in Opa phenotype, sialylation of LOS, and blebbing of excess $Opa^+$ outer membrane vesicles. The latter may interfere with phagocytosis by blocking binding of viable organisms (Shafer & Rest, 1989; Naids *et al.*, 1991).

Other important interactions between host cells may occur at the site of inflammation during gonorrhoea. Gregory & Wing (1998) have proposed a model describing clearance of systemic bacterial infections by the liver, involving co-operation between liver macrophages (Kupffer cells) and neutrophils. Kupffer cells are thought to trap blood-borne bacteria by lectin-like interactions, leading to increased surface expression of adhesion molecules and the release of cytokine signals. These products attract and stimulate neutrophils, which destroy the bound bacteria attached to the Kupffer cell by surface phagocytosis. As the genitourinary tract contains resident macrophages (El-Demiry *et al.*, 1986), it is plausible that such a mechanism could also operate during gonococcal infection.

# CHAPTER 2

*MATERIALS & METHODS*

## *2A* - Water & General Reagents

Deionised water ($dH_2O$) was produced by reverse osmosis on an Elgastat® Prima deioniser (Elga) to a conductivity of 3.6-7.0 µS/cm, whilst further purification to 18 MΩ-cm was achieved on an Elgastat® Ultra High Quality (UHQ) deioniser. Phosphate-buffered saline (PBS) was prepared by dissolution of tablets (Oxoid) in $dH_2O$ UHQ, generating a buffer of pH 7.3. The $dH_2O$ was not routinely sterilised, but UHQ water and PBS were autoclaved at 18-22 psi for 30 min at 120°C before use. Unless otherwise stated, all reagents were purchased from Merck as BDH AnalaR® grade products.

## *2B* - Growth Of Gonococci & Selection Of Phase-Variants

2B(i) *Gonococcal Variants*: Throughout this study, strain P9 of *Neisseria gonorrhoeae* [genotype *porB1b* (Ward *et al.*, 1992)] was used unless otherwise stated. This isolate was obtained from a male gonorrhoea patient with symptomatic urethritis, and is serum-sensitive in the absence of CMP-NANA (M.E. Ward, personal communication; Watt *et al.*, 1972). For stimulation experiments, five phase-variants of P9 - which exhibited the phenotypes $Opa^-/Pil^-$, $Opa^-/\alpha\text{-}Pil^+$, $Opa\text{-}a^+/Pil^-$, $Opa\text{-}b^+/Pil^-$, and $Opa\text{-}b^+/\alpha\text{-}Pil^+$ - were maintained.

Where indicated, a transformant of P9 expressing the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* (Chalfie *et al.*, 1994) was used for challenge of cell cultures. The full details of the transformation procedure have been reported by Christodoulides *et al.* (2000). Briefly, a mutant form of the *gfp* gene (*rs-gfp*), which encodes a protein with a red-shifted excitation peak [resulting in enhanced fluorescence (Heim *et al.*, 1995)], was incorporated into a hybrid shuttle vector containing the 4.2 kbp gonococcal cryptic plasmid. A promoter from the *porA* gene of *N. meningitidis* controlled expression of *rs-gfp*, whilst an ampicillin resistance cassette enabled selection of transformants. Initially, the construct was introduced into the $Opa^-/\alpha\text{-}Pil^+$ variant of strain

P9, but other phenotypes were subsequently isolated by colonial sub-culture [see Section *2B(iii)*].

2B(ii) *Culture Of Gonococci*: Gonococcal (GC) medium for growth and storage of gonococci contained 1% (w/v) proteose peptone ("Bacto® No. 3", Difco) and 0.1% (w/v) soluble starch in distilled water ($dH_2O$), with the following salts: sodium chloride (0.5% w/v), potassium dihydrogen orthophosphate (0.1% w/v), and di-potassium hydrogen orthophosphate 3-hydrate (0.524% w/v). For the preparation of solid plates, agar ("No. 1", Oxoid) was added to this mixture to 1% (w/v), and sterilisation was achieved by autoclave treatment at 120°C for 15 min at 15 psi. Heat-sensitive supplements, stored at -20°C, were added to the molten medium after cooling to 55°C. Consequently, the final concentrations of these compounds were: 1 g/l glucose, 0.1 g/l L-glutamine, 130 μg/l para-aminobenzoic acid (Sigma), 2.5 mg/l β-nicotinamide adenine dinucleotide (Sigma), 30 μg/l thiamine hydrochloride (Sigma), 1 mg/l cocarboxylase (Sigma), 0.1 mg/l cyanocobalamin (Sigma), 0.2 mg/l ferric nitrate, 260 mg/l L-cysteine hydrochloride (Sigma), 10 mg/l adenine (Sigma), 300 μg/l guanidine hydrochloride (Sigma), 8 mg/l uracil (Sigma), and 3.2 mg/l hypoxanthine (Sigma). Ampicillin (sodium salt, Sigma; 5 mg/l) was added at this stage if the plates were to be used with the fluorescent transformant.

The molten agar was poured into Petri dishes (90 mm diameter, 25 ml/plate), allowed to set, and dried in a laminar flow cabinet. Gonococci [stored in GC-broth/10% (w/v) glycerol at -135°C] were inoculated directly from the frozen state onto the GC-agar, and incubated for 22-24 hr at 37°C and 5% $CO_2$.

2B(iii) *Maintenance Of A Specific Phenotype*: With the use of a Carl Zeiss Jena dissecting microscope, 5-10 colonies of the required predominant phase-variant were selected on the basis of colour and morphology, and sub-cultured onto a new agar plate. These plates were incubated overnight as described, and were used for experiments only if growth was sufficient and colony homogeneity was ≥95%.

2B(iv) *Preparation & Quantification Of A Gonococcal Suspension*: Bacterial growth from a confluent plate was transferred into a tube containing 1-2 ml of balanced salt solution or cell culture medium, and gently suspended using a bacteriological loop.

The tube was spun at 50 g for 30 sec to remove any aggregates, and the top two-thirds of the suspension were transferred to a new tube.

A 200 µl aliquot of the gonococcal suspension was spun in a microcentrifuge at 660 g for 5 min, the supernatant discarded, and the pellet resuspended in 1 ml 1% (w/v) sodium dodecyl sulphate [(SDS) - "specially pure", BDH] before heating to 95°C for 5 min. In order to determine the original cell concentration, the absorbance (A) of the lysed material was measured in a quartz cuvette at 260 nm (ultraviolet) using a Hitachi U-1100 spectrophotometer, and the value interpolated into a previously generated standard curve. Thus, an $A_{260}$ value of 1.00 represented $2.73 \times 10^8$ colony-forming units (cfu) per ml for the wild-type strain or $1.16 \times 10^9$ cfu/ml for the fluorescent transformant.

## *2C* - Isolation, Cultivation, & Quantification Of Mononuclear Phagocytes

2C(i) *Source Of Monocytes*: Human buffy coat packs (prepared from blood donated the previous day) were obtained from the National Blood Service (Southampton Centre). All processing of the buffy coats and preparation of culture vessels took place in a laminar flow cabinet, using sterile media, UHQ water, glassware, and disposables.

2C(ii) *Preparation Of Culture Vessels For Monocyte Adherence*: One-millilitre aliquots of Matrigel® (Collaborative Biomedical Products) basement membrane matrix were thawed over several hours on ice, then diluted 1/10 with ice-cold serum-free medium, which consisted of a 1:1 mix of Dulbecco's Modified Eagle Medium [(MEM) – Gibco-BRL, Cat. No. 11880] and Nutrient Mixture Ham's F-12 (Gibco-BRL, Cat. No. 21765) in 20 mM HEPES [*N*—(2-hydroxyethyl)piperazine-*N*'-2-(ethanesulphonic acid)] buffer (1 M stock obtained from Sigma). Culture vessels were incubated with sufficient Matrigel® solution to form a thin, complete layer over the surface of the plastic or glass for 1 hr at room temperature (RT°C). For 96-well culture plates, one drop from a Pasteur pipette (~45 µl) was required per well; whilst 8-well glass chamber slides (Nunc Lab-Tek®, Gibco-BRL), 24-well plates (Costar), and individual cover slips ("No. 0", Chance Propper Ltd; 13 mm diameter) within these plates were coated with 200 µl/well. The Matrigel® solution was then removed from the culture vessels, which were rinsed once with PBS and either used the same day or stored at 4°C with a thin layer of diluent medium over the coated surfaces.

2C(iii) *Monitoring Of Cell Processing*: Monocyte yield and purity were determined using a Cell Dyn 3500 Coulter counter (Abbot Diagnostics). Recovery of suspended cells at each stage of the isolation procedure allowed comparisons to be made with cell populations present in the original buffy coat. Thus, the protocol was optimised to limit monocyte losses, whilst effectively removing red blood cells, granulocytes and platelets.

2C(iv) *Isolation Of Peripheral Blood Mononuclear Cells*: For each buffy coat (containing $2x10^7$-$1x10^8$ monocytes), 20 ml of HISTOPAQUE®-1077 cell separation medium (Sigma) was added to each of four centrifuge tubes and allowed to equilibrate to RT°C. With the centrifuge tubes held at a 30-45° angle, approximately 5 ml of undiluted buffy coat was carefully overlaid onto the HISTOPAQUE®-1077 in each tube. The tubes were returned to the vertical, and additional buffy coat was gradually added to a total of 20 ml per tube.

The tubes were spun at 700 g for 30 min in an IEC Centra MP4 centrifuge (all spins took place at RT°C in a swing-out rotor without use of the brake). The resultant gradients contained a thin interface of peripheral blood mononuclear cells (PBMC) between the HISTOPAQUE®-1077 layer and the uppermost plasma layer. The plasma layers in each tube were aspirated off and retained, whilst the PBMC bands (approximately 20 ml total) were transferred into two tubes and diluted 1/2 with ice-cold cell diluent comprising 2.5 mM ethylenediaminetetraacetic acid [(EDTA) - disodium salt, Eastman Kodak] and 10 mM glucose in PBS.

2C(v) *Washing Of PBMC Pellets And Reduction Of Platelet Contamination*: The diluted PBMC were spun at 360 g for 10 min, the cloudy supernatants (containing predominantly platelets and lymphocytes) discarded, and the pellets resuspended in 10 ml each of ice-cold cell diluent. The cell suspensions (concentration $1-4x10^7$ PBMC/ml) from each pellet were then carefully overlaid onto 30 ml of chilled foetal calf serum [(FCS) – Gibco-BRL] in centrifuge tubes, and spun at 200 g for 15 min to remove platelets (platelet reduction was 98.7-99.8%). After discarding the supernatants, this procedure was repeated once before resuspending the cell pellets in pre-warmed Dulbecco's MEM + Glutamax-I™ (Gibco-BRL, Cat. No. 61965) with 15% FCS and 25 μg/ml ampicillin. The cells (a yield of $2.5-7.4x10^8$ PBMC containing 4-19% monocytes) were pooled, then aliquoted to produce a density between $5x10^6$-$1x10^7$ PBMC/cm$^2$ in

Matrigel®-coated cell culture vessels. These were incubated overnight at 37°C with 5% $CO_2$.

2C(vi) *Removal Of Non-Adherent Cells & Preparation For Experiments*: Subsequent to the overnight incubation, the culture vessels were agitated by gentle shaking or rocking/tilting on a mixing platform at 37°C for 10 min. The medium containing non-adherent cells was discarded, and the remaining cells were washed twice using Dulbecco's MEM + Glutamax-I™ with 5% FCS and 25 μg/ml ampicillin; before replacement with Dulbecco's MEM + Glutamax-I™ containing 10% autologous human serum, 5% FCS, and 25 μg/ml ampicillin. The adherent cells were cultured for 8-10 d to allow macrophage morphology to develop, with the agitation and washing procedure being repeated twice or three times during this period. During infection experiments with viable gonococci, the standard culture medium was replaced with low glucose Dulbecco's MEM (Gibco-BRL, Cat. No. 31885; supplemented with 2% FCS and 300 mg/l L-glutamine) - hereafter referred to as "experimental medium" - to reduce bacterial growth.

2C(vii) *Preparation Of Human Serum From Buffy Coat Plasma*: Plasma retained from the first centrifugation step [50-60 ml; see Section *2C(iv)*] was transferred into a glass bottle containing 20 ml of autologous buffy coat, and calcium chloride solution was added to a final concentration of 50 mM. This mixture was incubated at 37°C for 1 hr to induce clotting before transferral to a refrigerator (4°C) overnight. The resultant serum was spun at 2000 g for 10 min to pellet any cell debris, then heat-inactivated at 56°C for 30 min prior to storage at -20°C.

2C(viii) *Quantification Of Macrophages*: A rapid method for the enumeration of matured macrophages was developed that utilised PicoGreen™ dsDNA Quantitation Reagent, a nucleic acid dye which is specific for double-stranded DNA (Haugland, 1996). Macrophage cultures in a 24-well plate were prepared for the assay by replacement of the culture medium with 250 μl/well $dH_2O$ UHQ, followed by freezing at -20°C. The plate was then thawed, and concentrated ammonium hydroxide solution ("DNA Synthesis Grade", PE Applied Biosystems) was added to a final concentration of ~1.5 M in each well. After a 30-min incubation at room temperature, cellular material was scraped off the plate surface (microscopy was used to confirm successful removal of cell remnants), and

200 µl of suspension from each well was transferred into screw-cap microcentrifuge tubes. A similar procedure was conducted in parallel for Matrigel®-coated wells lacking macrophages, and DNA was extracted from cells and control samples as described in Section *2H(i)*.

In order to determine cell numbers by their DNA content, a two-fold dilution series of PBMC (quantified on a Cell Dyn 3500 Coulter counter) was prepared in $dH_2O$ UHQ, and extraction was undertaken using the ammonium hydroxide technique [see Section *2H(i)*]. After dilution (1/400) of the PicoGreen™ reagent in Tris-EDTA buffer [10 mM tris(hydroxymethyl)aminomethane-hydrochloride (base supplied by Eastman Kodak), pH 7.5, with 1 mM EDTA], 50 µl was transferred to each well of a black microtitre strip (Labsystems). The PBMC standards ($2.6x10^3$ cell-equivalents/µl to $2.0x10^1$ cell-equivalents/µl), macrophage extracts, Matrigel® controls, and $dH_2O$ "blanks" were added in duplicate to the PicoGreen™ at 5 µl/well.

Following a 5-min incubation (RT°C) in the dark, the microtitre strip was placed in a Fluostar fluorometer (SLT), and the samples were excited at a wavelength of 485 nm. Fluorescence emission was measured at 538 nm, and the number of cell-equivalents (nuclei) for the macrophage extracts was derived from the standard curve by quadratic regression analysis. The DNA content of the Matrigel®-only controls was below the detection limit of this assay (~200 cell-equivalents/sample).

## *2D* - Gonococcal Surface Components Used As Macrophage Stimuli

Three types of purified gonococcal components were available for macrophage stimulation. These were generously donated by J.E. Heckels (LOS and outer membranes) and J.N. Robertson (pili). The LOS was obtained by extraction from a paste of bacterial cells ($Pil^+/Opa^-$) at 75°C using the phenol-water method (Lambden & Heckels, 1982). It was then solubilised by neutralisation of the free acid with triethylamine solution, and the concentration derived from weighing of the freeze-dried, pure compound.

Outer membrane vesicles from $Opa^-/Pil^-$, $Opa\text{-}a^+/Pil^-$, and $Opa\text{-}b^+/Pil^-$ variants of strain P9 were prepared by extraction of intact gonococcal cells with lithium acetate at 45°C, followed by differential centrifugation (Heckels, 1977). The vesicles were pilin-free as confirmed by SDS-PAGE. The concentration of outer membrane protein

(suspended in $dH_2O$ UHQ) was determined by the assay of Lowry *et al.* (1951), and these suspensions were stored at -20°C.

Pili (variant α) were sheared from the surface of intact $Pil^+/Opa^-$ gonococci in a cell homogeniser, separated from the cells by centrifugation in the presence of sucrose, and concentrated by ultracentrifugation in sodium chloride solution. Following further purification by density gradient centrifugation, the protein concentration of the cell-free pili suspension was determined by the method of Lowry *et al.* (1951). The absence of contaminating proteins and LOS was confirmed by deliberate overloading of an SDS-polyacrylamide gel (Lambden *et al.*, 1980) and sugar analysis (Robertson *et al.*, 1977), respectively. The pili were stored as a precipitate in 1 M sodium chloride solution at 4°C, with 0.05% (w/v) sodium azide added as a preservative.

## *2E* - Microscopic Analysis Of Mononuclear Phagocytes

2E(i) *Cytochemical Discrimination Between Mononuclear Cells & Determination Of Cellular Viability*: To distinguish mononuclear phagocytes from lymphocytes and other contaminating cells, the Sigma α-Naphthyl Acetate Esterase Kit was used to localise discriminatory enzyme activity [see Section *5B(i)*]. Cells cultured in 8-well glass chamber-slides were fixed, stained, and counterstained precisely as directed in the manufacturer's instructions. To inhibit enzymatic activity in monocytes, a parallel incubation was conducted with equivalent slides in staining solution containing sodium fluoride [final concentration = 0.08% (w/v)]. The slides were then examined on a Leitz DM-RB microscope (Leica).

To assess cellular viability, unfixed mature macrophages in an 8-well glass chamber-slide were stained using the Molecular Probes LIVE/DEAD® Reduced Biohazard Viability/Cytotoxicity Kit, exactly as instructed by the manufacturer. The glutaraldehyde post-fixative [4% (v/v)] was freshly prepared from an 8% aqueous stock ("Grade I", Sigma), which was stored at -20°C. The fixed slide was rinsed once with 0.5 ml/well PBS, dipped briefly in $dH_2O$ UHQ, and air-dried before application of a cover slip upon Vectashield® (Vector Laboratories) mountant (for retardation of photobleaching). Stained cells were immediately visualised through filter cube I3 [(Leica) – bandpass 450-490 nm] on a Leitz DM-RB fluorescence microscope.

2E(ii) *Preparation Of Uninfected Mononuclear Phagocytes For Confocal Microscopy*: Cells cultured in an 8-well glass chamber slide were washed twice with 0.5 ml/well PBS, and fixed with 0.5 ml/well pre-cooled absolute methanol (Riedel-de Haën® CHROMASOLV®, Sigma) at -20°C for 10 min. The cells were then washed once with PBS, and stored at 4°C under 0.05% (w/v) sodium azide in PBS for up to one week.

Immunological labelling was conducted at room temperature, and 0.5 ml/well PBS was used throughout for washing. To block non-specific binding, the fixed cells were incubated with 0.5 ml/well 10% normal goat serum (Serotec) in PBS for 30 min. Labelling of cell surface markers was undertaken using anti-mannose receptor (Pharmingen) or anti-phagocytic glycoprotein-1 [(Pgp-1) – Dako] primary monoclonal antibodies (mAbs), which were both murine isotype IgG1. As antibodies could potentially bind to cells via receptors for the Fc portion of IgG, an anti-human papillomavirus (HPV) type 16 early protein 7 mAb (Zymed Laboratories) served as an isotype-matched control. Following three washes, diluted mAbs (10 μg/ml for anti-mannose receptor and the control, or 7.1 μg/ml for anti-Pgp-1) were applied to separate chambers at 0.2 ml/well for 1 hr. The diluent was detergent-free assay buffer, composed of 150 mM sodium chloride, 0.023% (w/v) bovine γ-globulins ("Cohn fraction II", NBS Biologicals), 10 μM diethylenetriaminepentaacetic acid (Sigma), 0.025% (w/v) sodium azide, and 0.25% (w/v) bovine serum albumin [(BSA) - "RIA" grade, Sigma] in 25 mM Tris-hydrochloride, pH 7.8.

Unbound primary antibodies were removed by five washes, and a fluorescein isothiocyanate (FITC) conjugate [(Sigma) - fluorescently labelled $F(ab')_2$ fragments prepared from Fc-specific goat anti-mouse IgG] was diluted 1/250 in detergent-free assay buffer, and added to the cells at 0.2 ml/well. This incubation was conducted in the dark for 1 hr. After washing five times, cell nuclei were counterstained (10 min, protected from light) with 0.2 ml/well propidium iodide (Sigma) at 10 μg/ml in PBS. Finally, the slide was prepared for microscopy as previously explained [see Section *2E(i)*], and the cover slip was sealed with nail varnish prior to storage (protected from light) at 4°C.

2E(iii) *Preparation Of Infected Macrophage Cultures For Confocal Microscopy*: Unfixed macrophage cultures infected with fluorescent gonococci, prepared on cover slips in a 24-well culture plate, were stained with a single component (DEAD Red™) of the Molecular Probes LIVE/DEAD® kit. The green dye (SYTO® 10) was omitted from the staining step, but in all other respects the manufacturer's protocol was followed.

Glutaraldehyde fixation was conducted for 1 hr; and the cover slips were rinsed once with PBS, dipped briefly in $dH_2O$ UHQ, and mounted on glass slides using Vectashield®. The sealed cover slips were stored in the dark at 4°C, and scanned [see Section *2E(iv)*] within 24 hr of staining.

For the fixation of cultures reserved for immunological labelling, cover slips were removed from the 24-well plate, drained on edge using tissue paper, and transferred to a separate plate containing 0.5 ml/well 3% (w/v) paraformaldehyde ("GPR" grade, BDH) in PBS. After a 20-min incubation on ice, the fixative was removed and the residue neutralised with 50 mM ammonium hydrogen carbonate, which was applied at 1 ml/well for two incubations of 2 min each. The cover slips were then rinsed and stored as for methanol-fixed preparations [see Section *2E(ii)*].

The fixed cover slip cultures were blocked and labelled with primary mAbs (anti-mannose receptor or anti-HPV type 16 E7 protein) as previously described [see Section *2E(ii)*]. However, the FITC conjugate was replaced with either the exact equivalent secondary antibody with a tetramethyl rhodamine isothiocyanate (TRITC) label (Sigma), or a cyanine-5 (Cy5)-labelled goat anti-mouse IgG conjugate (Amersham) specific for heavy and light chains (both used at 1/250). Counterstaining with propidium iodide was omitted. At the blocking, primary antibody, and secondary antibody stages, saponin (Sigma) was present at 0.2% (w/v) to enable permeabilisation of the plasma membrane. The rinsed, mounted, and sealed cover slips were stored at 4°C in the dark until required.

2E(iv) *Confocal Microscopic Analysis Of Infected & Uninfected Mononuclear Phagocytes*: In order to generate images of the stained cells for subsequent analysis, slides were subjected to epifluorescent illumination using a Leica TCS 4D laser-scanning confocal microscopy system, which was controlled by SCANware 4.2 software (Microsoft). Simultaneous two-channel scanning was conducted in the *x-y* plane at excitation wavelengths of 488 nm for FITC and GFP, 568 nm for TRITC and propidium iodide, and 647 nm for Cy5. As DEAD Red™ exhibits intense fluorescence in response to excitation at both 488 and 568 nm, images of specimens stained with this dye were collected utilising the software's cross-talk correction facility.

The "optical sections" obtained by confocal scanning were examined as a series and/or combined as two-channel, three-dimensional images by computer processing. The "simulated fluorescence process" algorithm of the software, which mimics absorption and emission of light by a three-dimensional object, was applied to all images of infected

macrophages to highlight surface detail (particularly gonococci). In order to correct for inversion of cover slip cultures onto microscope slides, the series of sections was reversed during processing where appropriate. Images of control preparations were generated using similar parameters to the specimens of interest throughout the procedure.

## *2F* - Analysis Of Endocervical Secretions & Macrophage Culture Medium For Cytokines By Sandwich Immunoassay

2F(i) *Assay Principle & Source Of Reagents*: Cytokines present in the medium of stimulated macrophages were quantified using a sandwich immunosorbent assay technique, in which cytokine molecules are trapped in the solid phase between unlabelled capture antibodies and labelled detector antibodies. Capture antibodies, recombinant

**TABLE *2·1*: Cytokine Quantification - Concentrations Of Recombinant Standards & Suppliers Of Assay Reagents.**

| *CYTOKINE* | *SUPPLIER OF ASSAY REAGENTS* | *INITIAL CONCENTRATION OF CYTOKINE STANDARD IN DILUTION SERIES (ng/ml)* | |
|---|---|---|---|
| | | *A*[#] | *B*[~] |
| Granulocyte-Macrophage Colony-Stimulating Factor | Biosource International | - | 2 |
| Interferon-γ | R&D Systems | 1 | - |
| Interleukin-1β | R&D Systems | - | 0.5 |
| Interleukin-6 | R&D Systems | 5 | 1 |
| Interleukin-8 | R&D Systems | 2 | 2 |
| Interleukin-10 | Biosource International | - | 2 |
| Interleukin-12 | R&D Systems | - | 0.5 |
| Macrophage Inflammatory Protein-1α | R&D Systems | 10 | 2 |
| RANTES[*] | R&D Systems | 10 | 2 |
| Transforming Growth Factor-$\beta_1$ | R&D Systems | - | 2 |
| Tumour Necrosis Factor-α | R&D Systems | 5 | 1 |

[#]Assays on medium from macrophage cultures infected with viable gonococci for 6 hr. [~]All other assays. [*]*R*egulated upon *A*ctivation, *N*ormal *T*-cell *E*xpressed and *S*ecreted.

cytokine standards, and detector antibodies for quantification of the cytokines assayed were supplied from Biosource International or R&D Systems (see Table *2·1*).

For detection of the biotin-labelled antibodies, Delfia® europium (Eu)-labelled streptavidin (Wallac) was used instead of an enzyme/colorimetric substrate system. Lanthanide chelates, such as $Eu^{3+}$, enable the use of time-resolved fluorometry, which has several advantages over conventional colorimetric or fluorometric assays. Firstly, the decay time of the specific fluorescence signal greatly exceeds that of non-specific background fluorescence, which allows readings to be taken after the latter has expired. Secondly, the exceptionally large difference between the excitation and emission wavelengths of the chelates, coupled with a very narrow emission peak, increase the signal-to-noise ratio further. Finally, dissociation of the lanthanide label into the low pH enhancement solution - in which it forms a different, highly fluorescent chelate - generates a powerful signal and thus high sensitivity.

2F(ii) *Assay Protocol*: Unless otherwise stated, all incubations took place at 37°C in a humidified box, and reagents (100 µl/well) were added to plates at the concentration recommended by the manufacturer for each batch. Washes were conducted between each step with 250 µl/well 25 mM Tris-phosphate, pH 8.0, containing 100 mM sodium chloride and 0.05% (v/v) polyoxyethylene(20)sorbitan monolaurate ("Tween" 20, BDH Organics).

To coat the polystyrene surface of 96-well FluoroNunc™ Maxisorp plates, capture antibodies were applied in carbonate buffer, pH 9.6 [35 mM sodium hydrogen carbonate, 15 mM sodium carbonate, and 0.05% (w/v) sodium azide]. The plates were then sealed, incubated overnight at RT°C, and subsequently aspirated prior to the addition of 250 µl/well blocking solution comprising 1% (w/v) BSA and 5% (w/v) sucrose in PBS. Following one hour's incubation, the wells were washed three times immediately before transfer of the samples and standards to the plates.

Aliquots of macrophage medium or endocervical secretions stored in 96-well assay plates at -70°C were thawed and diluted with assay buffer [see Section *2E(ii)*] containing detergent [0.1% (v/v) polyoxyethylene ether W-1 (Sigma)], which was used as the diluent throughout the remainder of the protocol. A 2.5-fold dilution series of the recombinant cytokine standard was then prepared; the starting concentrations of which are given in Table *2·1* for each protein assayed. 'Blanks' consisted of assay buffer only.

The samples and standards (100 µl/well) were incubated for 2 hr on the plate, following which the wells were washed four times.

Subsequently, biotinylated detector antibodies were added to the wells, and the plate was again incubated for 2 hr. Four washes were conducted before the Eu-labelled streptavidin (100 ng/ml) was applied to the plate and incubated for 1 hr. Following a series of five washes, Delfia® Enhancement Solution (Wallac) was dispensed into the wells, after which the plate was agitated for 10 min on a Titertek® shaker (Flow Laboratories). Fluorescence was measured on a Wallac 1234 Delfia® fluorometer, and the resultant relative fluorescence units were converted into cytokine concentrations through the application of linear or quadratic regression analysis to the standard curves, taking into account the dilution factor employed during the immunoassay.

2F(iii) *Measurement Of Growth-Related Protein-α & Epithelial-Derived Neutrophil Attractant-78 Levels By Colorimetric Enzyme Immunoassay*: For the determination of growth-related protein (GRO)-α and epithelial-derived neutrophil attractant (ENA)-78 concentrations in macrophage medium, reagents supplied in the respective Quantikine™ (R&D Systems) enzyme-linked immunosorbent assay (ELISA) kits were utilised. Following dilution (1/5) of the samples with assay buffer [see Section *2E(ii)*] containing 0.1% (v/v) polyoxyethylene ether W-1, kit components were used exclusively throughout. However, some of the manufacturer's recommendations were modified to enable the two assays to be conducted in parallel.

Microtitre strips were supplied pre-coated with capture antibodies specific for the respective chemokines. Recombinant standards for each protein were diluted (duplicated two-fold series, beginning at 1 ng/ml) in the Calibrator Diluents that were indicated for use with cell culture supernatants. For the ENA-78 assay, 25 µl of each sample or standard was added to 200 µl/well of Assay Diluent; whilst for GRO-α, 200-µl aliquots were assayed directly. The plate was then incubated for 2 hr at 37°C in a humidified box.

After four washes (400 µl/well) with the kits' Wash Buffer, the appropriate horseradish peroxidase-conjugated antibodies were applied at 200 µl/well. The plate was incubated for 2 hr at 37°C, and five washes were conducted before the addition of 200 µl/well substrate solution [1:1 mix of Colour Reagent A (hydrogen peroxide) and Colour Reagent B (tetramethylbenzidine)]. The development of coloured product was allowed to proceed at room temperature until an optimum was reached for each assay, at which

points Stop Solution (2 N sulphuric acid) was administered at 50 µl/well. Optical densities were determined at 450 nm (reference filter 570 nm) on a HT II microplate reader (Anthos Labtec), and chemokine concentrations were derived from the standard curves by linear or quadratic regression analysis.

## *2G* - Studies On Gonococcal Opacity Protein B: Peptide Synthesis, Biomolecular Interaction Analysis, & Preparation Of Anti-Opa-b Monoclonal F(ab')$_2$ Fragments

2G(i) *Synthesis Of Biotinylated Opa-b Peptides Representing Putative Surface-Exposed Loops*: A series of fourteen overlapping peptides, encompassing regions of P9-Opa-b that were predicted to be surface-exposed (see Figure *5·12*), were synthesised by 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry on a Shimadzu PSSM-8 Peptide Synthesiser (scale = 6 µmol). The solid support was NovaSyn® TGR resin (Novabiochem), which did not require an initial deprotection step; and dimethylformamide (PerSeptive Biosystems) was used as the solvent. Coupling reagents [2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (Alexis Corporation) and *N*-hydroxybenzotriazole (PerSeptive Biosystems)] served to activate the amino-acid derivatives (Novabiochem) during synthesis of the nascent peptide chain, whilst Fmoc *N*-α-protecting groups were removed by piperidine (PerSeptive Biosystems). Finally, biotin (Sigma) was attached to the amino-terminus of each peptide via an aminohexanoic acid spacer.

Following retrieval from the machine, the resin was washed with methanol and dried under vacuum. Cleavage of the peptides from the solid support and removal of protecting groups from amino-acid side-chains were conducted using 10 ml of trifluoroacetic acid cleavage solution per 0.1-1.5 g of peptide-resin. To prevent the cleaved protecting groups from participating in secondary reactions, scavengers were present at the following concentrations: 7.5% (w/v) crystalline phenol (Sigma), 2.5% (v/v) 1,2-ethanedithiol (Sigma), 5% (v/v) thioanisole (Sigma), and 5% (v/v) $dH_2O$. The peptides were then precipitated with diethyl ether, and the resultant solids were dissolved in $dH_2O$.

Gel purification was conducted on Sephadex G-10 (Sigma) columns, and the recovered peptide solutions were shell-frozen in round-bottomed flasks held beneath liquid nitrogen. Following removal of water by sublimation under vacuum, the solids were stored desiccated at -20°C, and were dissolved in sodium carbonate solution

(peptide HV-1b only) or $dH_2O$ to 1 mg/ml as required. The amino-acid sequences of all the peptides are displayed in Table *2·2*; for additional information, see Section *5D(vii)*.

**TABLE *2·2*: Amino-Acid Sequences Of Fourteen Peptides Comprising The Putative Surface-Exposed Portions Of Gonococcal Opacity Protein B**

| *PEPTIDE DESIGNATION** | *AMINO-ACID SEQUENCE (N→C)*# |
|---|---|
| SV-a | Biotin-YAYEHITHDYPKPTGTGKN |
| SV-b | Biotin-YPKPTGTGKNKISTVSDYFR |
| SV-c | Biotin-KISTVSDYFRNIRTHSVHPR |
| HV-1a | Biotin-RYRKWNNNKYSVN |
| HV-1b | Biotin-NNKYSVNIEKGQEV |
| HV-1c | Biotin-IEKGQEVHKNRRGR |
| HV-1d | Biotin-HKNRRGRKTENQENG |
| HV-1e | Biotin-KTENQENGSFHAVSS |
| HV-2a | Biotin-HIRHGIDSTKKETTF |
| HV-2b | Biotin-TKKETTFLTSFYGVS |
| HV-2c | Biotin-LTSFYGVSPTVYTEKN |
| HV-2d | Biotin-PTVYTEKNTQNAHHQ |
| HV-2e | Biotin-TQNAHHQSDSIRCVGL |
| CL | Biotin-HNWGRLENTRFKTHE |

*SV = semivariable, HV = hypervariable, CL = conserved loop. #Aminohexanoic acid was coupled to the *N*-terminus prior to the addition of biotin.

2G(ii) *Principle Of Biomolecular Interaction Analysis*: Biosensors are analytical instruments that monitor real-time interactions between macromolecules via a physical detection mechanism. For instance, biomolecular interaction analysis (BIA) performed by the BIACORE® system (Biacore) exploits the optical phenomenon of surface plasmon resonance (SPR) to follow changes in analyte concentration. This effect occurs when monochromatic, p-polarised light is shone at a trilaminar plane consisting of a relatively dense, refractive medium on the illuminated surface; a thin layer of metal at the interface; and a basal medium of comparatively low refractive index. If a critical incident angle is exceeded, total internal inflection is observed. However, a component of the light energy is propagated through the medium of lower refractive index as an "evanescent wave", the transmission of which causes resonant oscillation in the electrons ("plasmons") of the metal film. This is associated with a reduction in the intensity of reflected light at a specific angle of incidence, termed the "SPR angle".

A key determinant of the SPR angle is the refractive index of the basal medium. In the BIACORE® instrument, a glass sensor chip coated with a thin film of gold forms two components of the trilaminar optical device, which is illuminated by a light-emitting diode. An integrated μ-fluidic cartridge delivers liquid containing the analyte to the chip

surface as a thin layer, thus providing the basal medium. As the solution flows over the sensor chip, ligands immobilised within a surface matrix on the gold film trap dissolved analytes, causing a change in surface concentration. This affects the refractive index of the medium, and concomitant shifts in the SPR angle are continually monitored by a fixed array of diode detectors. The data are collected by an integral microcomputer and processed by a separate computer connected to the BIACORE® instrument, which produces a sensorgram [resonance units (RU) plotted against time] as the run progresses. A signal of 1000 RU indicates a change in surface concentration of ~1 ng protein/mm$^2$ (protein binding typically generates a response of 100-20,000 RU).

The real-time information provided by BIA allows the derivation of kinetic rate constants, equilibrium constants, and analyte concentration. There is no intrinsic requirement for labelling of the reactants, which may otherwise necessitate lengthy purification procedures or even alter the nature of the interaction being studied. Furthermore, the ability of the sensor chip to be regenerated and re-used, coupled with computer-controlled continuous flow technology, promotes reproducibility between experimental runs. Finally, the BIACORE® system enables rapid and simultaneous definition of several parameters pertaining to macromolecular interactions on a self-contained instrument. For example, BIA can be used to characterise monoclonal antibodies in unfractionated hybridoma supernatants; determining antibody concentration, affinity of binding, and epitope specificity within a single investigation.

2G(iii) *Epitope Mapping Of Anti-Opacity Protein B Monoclonal Antibody SM40 By Biomolecular Interaction Analysis*: Six biotinylated peptides - designated HV-1a, HV-1b, HV-1c, HV-1d, HV-1e, and CL [see Section *2G(i)*] - were used in this experiment. On the BIACORE® 2000 instrument, each peptide was immobilised via a separate flow cell on the surface of a streptavidin-coated sensor chip ("SA5", Biacore), using 35 µl of solution (100 µg peptide/ml in $dH_2O$) at 5 µl/min. Two four-cell chips were utilised, providing a spare compartment on each chip for the no-peptide control.

Murine ascites containing a mAb [SM40, isotype IgG3 (Virji & Heckels, 1986)] that specifically binds P9-Opa-b was kindly provided by J.E. Heckels. This was diluted 1/100 and pumped through each cell for 7 min at a flow rate of 5 µl/min, after which the ascites was replaced with HEPES balanced salt buffer (Biacore) for a 15-min period of dissociation. To remove bound antibody, the surface of each chip was regenerated with 10 mM hydrochloric acid (a single 15-µl pulse per cell), and the SM40 binding assay was

repeated. Equivalent runs were also conducted with a control antibody [10Eii, murine isotype IgG2b (stored in 1:1 ascites/glycerol)] at a dilution of 1/100; this mAb was reactive against *Chlamydia trachomatis* major OMP (Conlan *et al.*, 1988). Data were collected at intervals of 1 sec, and sensorgrams were compared after adjustment for initial differences in baseline refractive index.

2G(iv) *Preparation Of Anti-Opacity Protein B F(ab')$_2$ Fragments From Purified Monoclonal Antibody SM40*: This procedure was conducted using materials supplied in the ImmunoPure® F(ab')$_2$ Preparation Kit (Pierce), but the recommended protocol was scaled-down to facilitate efficient processing of a small quantity of mAb. Ascites/glycerol (1:1 ratio) containing mAb SM40 (batch 8AC2 M1) was spun at 60,000 g for 30 min (10°C) in a Beckman Avanti™ 30 centrifuge, and a 750 µl aliquot - free of lipid or cell debris - was diluted 1/4 in ImmunoPure® IgG Binding Buffer. A 2.5-ml Protein A AffinityPak™ Column was equilibrated with 12 ml of the binding buffer, and the diluted ascites was then added to allow isolation of the mAb.

Following a wash with 6 ml of binding buffer, the antibody was eluted with 6 ml ImmunoPure® IgG Elution Buffer, and IgG was quantified by absorbance at 280 nm on a Hitachi U-1100 spectrophotometer (total yield ≈ 1 mg). The complete eluate was transferred into CelluSep® T1 cellulose dialysis tubing [(Membrane Filtration Products) - molecular weight cut-off = 3500 Da], and dialysed overnight at 4°C (with gentle stirring) against 240 ml of digestion buffer (20 mM sodium acetate buffer, pH 4.5). Immunoglobulin was concentrated into a volume of 0.4 ml using a Urifil-10 concentrator unit (Millipore).

Immobilised pepsin (Pierce) in the form of agarose beads (100 µl) was washed twice by adding 1.6 ml digestion buffer, spinning the suspension at 20,800 g for 5 min in a microcentrifuge (RT°C), and discarding the supernatant. The entire IgG concentrate was added to the pepsin following resuspension of the prepared beads in 0.2 ml of digestion buffer, and the pre-warmed sample was incubated at 37°C on a rotary wheel for 4 hr. Immobilised enzyme was separated from the digested immunoglobulin by centrifugation at 20,800 g for 5 min (RT°C); and after one rinse of the beads with 0.6 ml of binding buffer, the wash solution was pooled with the primary digest supernatant in a clean vessel.

An AffinityPak™ column [previously regenerated with 10 ml 0.1 M citric acid ("GPR" grade, BDH), pH 3.0] was washed with 12 ml of binding buffer; and application of the crude digest allowed removal of Fc fragments (bound by protein A) from the desired $F(ab')_2$ fraction (collected as eluate). After a rinse of the column with 2.4 ml of binding buffer, the $F(ab')_2$ concentration of the eluate was determined by spectrophotometry ($A_{280}$), and the fragments were stabilised by the addition of FCS to 2%.

## *2H* - Extraction & Preparation Of Template DNA For The Polymerase Chain Reaction

2H(i) *Ammonium Hydroxide Extraction Of DNA*: Ammonium hydroxide solution, diluted to ~1.5 M in $dH_2O$ UHQ, was added to pellets or small aliquots (<20 µl) of template source material at 200 µl per sample. The sealed, screw-cap microcentrifuge tubes were heated at 90°C in a water bath for 10 min, followed by an additional 70 min after removal of the caps to allow evaporation of ammonia. The resultant ~80-µl extracts were stored frozen at -20°C or below; and if necessary, particulate debris was removed by centrifugation (2000 g, 5 min) prior to use of the template in the polymerase chain reaction (PCR).

2H(ii) *Digestion Of Tissue Specimens With Proteinase K And Phenol-Chloroform Extraction Of DNA*: Frozen (-70°C) endometrial biopsies were transferred from storage tubes to centrifuge tubes, and each source tube was thoroughly rinsed with 1.5 ml of digestion buffer [100 mM sodium chloride, 25 mM EDTA (pH 8), 0.5% (w/v) SDS, and 0.1 mg/ml proteinase K (Boehringer Mannheim) in 10 mM Tris-hydrochloride, pH 8], which was then added to the respective tissue sample. The biopsies were incubated in a water bath at 50°C overnight, and vortex mixing was used to disperse the disrupted tissue. To remove particulate matter, tubes were spun at 3300 g in an IEC Centra MP4 centrifuge for 10 min (RT°C), and supernatants (750 µl) were transferred to clean tubes.

An equal volume of 25:24:1 phenol/chloroform/isoamyl alcohol (Sigma; saturated with 10 mM Tris, pH 8.0, 1 mM EDTA) was added to each supernatant, and the samples were homogenised by vigorous vortex mixing for 1 min. Separation of aqueous and organic phases was achieved by centrifugation at 1700 g (RT°C) for 10 min, and the

upper aqueous layer of each sample (500 µl) was transferred to a clean tube. The DNA was precipitated by addition of 250 µl 7.5 M ammonium acetate and 1 ml absolute ethanol ("AR quality", Hayman) to each extract, followed by vortex mixing; and the DNA was recovered by centrifugation for 2 min at 1700 g (RT°C). Each pellet was washed once with 2 ml 70% ethanol and air-dried before resuspension in 200 µl $dH_2O$ UHQ. To facilitate dissolution, the DNA extracts were warmed in a water bath for 1 hr at 65°C, and then thoroughly vortex-mixed prior to storage at -20°C.

## *2I* - Conventional PCR Assays For The Detection Of Sexually Transmitted Bacteria

2I(i) *Design Of PCR Primers*: The DNA sequences of primers used in diagnostic assays were either obtained from published methods, or were based on relevant sequence information retrieved from the European Molecular Biology Laboratory or GenBank databases (see Table *2·3*). Genome sequences were compared between bacterial strains and species using the Genetics Computer Group "Align" (twin alignments) and "Pile-Up" (multiple alignments) programs, which were accessed via the Biotechnology & Biological Sciences Research Council Seqnet facility. Thermodynamic melting temperatures [$T_m$ (calculated by nearest-neighbour analysis)] and secondary structure data for the oligonucleotides were obtained using the Gene Runner package (Hastings Software). Within the constraints imposed by assay specificity, primers for use within a single reaction were designed to minimise the potential for primer-dimer formation, whilst ensuring that $T_m$ values were similar.

2I(ii) *Synthesis & Deprotection Of Oligonucleotides*: Oligonucleotides were synthesised by β-cyanoethyl phosphoramidite chemistry on the Expedite™ Nucleic Acid Synthesis System (PerSeptive Biosystems), using MemSyn (PerSeptive Biosystems) membrane discs as the solid support. Nucleotide derivatives were obtained from Cruachem, and acetonitrile (Romil) was used as the solvent. For cleavage of primers from the matrix and removal of β-cyanoethyl, isobutyryl, and benzoyl protecting groups, each membrane was placed in a gas-tight polypropylene vial containing 800 µl concentrated ammonium hydroxide (~15 M). The vials were transferred into carefully sealed glass vessels and incubated overnight in a water bath at 55°C. After cooling the containers on

**TABLE 2·3: Target Regions & Oligonucleotide Sequences For Primers Used In Diagnostic PCR Assays.**

| *TARGET ORGANISM & AMPLIFIED REGION* | | *PRIMER DESIGNATION* | *DIRECTION OF AMPLIFICATION* | *OLIGONUCLEOTIDE SEQUENCE (5′→3′)* | *EXPECTED AMPLICON SIZE (bp*)* | *REFERENCE* |
|---|---|---|---|---|---|---|
| *Mycoplasma genitalium* MgPa adhesin gene | | Mg1 | Sense | TGTCTATGACCAGTATGTAC | 374 | Palmer *et al.* (1991) |
| | | Mg2 | Antisense | CTGCTTTGGTCAAGACATCA | | |
| | | MGS-1 | Sense | GAGCCTTTCTAACCGCTGC | 673 | de Barbeyrac *et al.* (1993) |
| | | MGS-2 | Antisense | GTGGGGTTGAAGGATGATTG | | |
| | | MgPa-1 | Sense | AGTTGATGAAACCTTAACCCCTTGG | 281 | Jensen *et al.* (1991) |
| | | MgPa-3 | Antisense | CCGTTGAGGGGTTTTCCATTTTTGC | | |
| *Mycoplasma hominis* 16S ribosomal RNA gene | | RNAH1 | Sense | CAATGGCTAATGCCGGATACGC | 334 | Blanchard *et al.* (1993) |
| | | RNAH2 | Antisense | GGTACCGTCAGTCTGCAAT | | |
| *Neisseria gonorrhoeae cppB* | | HO1 | Clockwise^ | GCTACGCATACCCGCGTTGC | 390 | Ho *et al.* (1992) |
| | | HO3 | Anticlockwise^ | CGAAGACCTTCGAGCAGACA | | |
| *N. gonorrhoeae* | *porB* (both alleles) | PorA+B1:S | Sense (outer primer) | CAAGAAGACCTCGGCAACGG | 580 (*porB1a*) 333 (*porB1b*) | Current study |
| | | PorA+B2 | Antisense (outer primer) | GACAACCACTTGGTCY†TAAGTATTGTCGT | | |
| | *porB1b* | PorB-Fw | Sense (inner primer) | *ACGTCTGCAG#*AAAGATACGGCGAAGGCACT | | |
| | *porB1a* | Specific-PorA:S | Sense (inner primer) | CCGCCAATCCTTCATCGGTT | | |
| *Chlamydia trachomatis* conserved region of 7.5 kbp cryptic plasmid | | KL1 | Clockwise~ | TCCGGAGCGAGTTACGAAGA | 241 | Mahony *et al.* (1990) |
| | | KL2 | Anticlockwise~ | ATTCAATGCCCGGGATTGGT | | |
| *C. trachomatis omp1* | | Ct1 | Sense (outer primer) | CTCTTGAAATCGGTATTAGTATTTGCCGCT | 338 | Hayes *et al.* (1992) |
| | | Ct2 | Antisense (outer primer) | TTAGAAGCGGAATTGTGCATTTACGTGAGC | | |
| | | Nest2 | Sense (inner primer) | CATGAGTGGCAAGCAAGTTTA | | M.A. Pickett, unpublished |
| | | Nest4 | Antisense (inner primer) | GCTCTCTCATCGATCAAGCG | | |

*Base pair. ^According to the scheme of Korch *et al.* (1985). †This position is not conserved between *porB1a* and *porB1b* alleles - "Y" denotes random incorporation of cytidine or thymidine during oligonucleotide synthesis. #This primer was originally designed for cloning purposes; the bases in Italics are not complementary to the target sequence. ~According to the scheme of Hatt *et al.* (1988).

ice, the contents were vortex-mixed and the oligonucleotide solution was aspirated from the membranes.

To precipitate the DNA, 45 µl of 3 M sodium acetate (pH 5.0), followed by 1 ml absolute ethanol (pre-cooled to -20°C), was added to each aliquot (400 µl) of the primer preparation. The tubes were then vortex-mixed, incubated for 30 min at -20°C, and spun in a microcentrifuge at 10,600 g (RT°C) for 5 min. Supernatants were discarded, and the DNA pellets were dried for 3 min in a vacuum desiccator prior to resuspension in $dH_2O$ UHQ. The concentration of the oligonucleotide was derived by $A_{260}$ determination on a Hitachi U-1100 spectrophotometer, and stocks were aliquoted in small volumes for storage at -20°C.

2I(iii) *Reaction Compositions & Thermal Cycling Parameters For Diagnostic PCR Assays*: All of the conventional PCR assays for STD agents contained three components at common concentrations: 0.2 mM each deoxyribonucleoside triphosphate (Promega), 2 mM magnesium chloride (Promega), and 0.5 µM each primer (unless otherwise stated). *Thermus aquaticus* (*Taq*) DNA polymerase in Storage Buffer "A" was purchased from Promega and was used with the supplied $Mg^{2+}$-free Reaction Buffer, whilst BIO-X-ACT™ DNA polymerase (a proprietary complex of enzymes) was obtained from Bioline and was used with the accompanying $Mg^{2+}$-free OptiBuffer™. A Peltier Thermal Cycler (PTC-225, MJ Research) was utilised for amplification of PCR products.

The *cppB* PCR for the detection of gonococci was conducted with 1 U/reaction BIO-X-ACT™ polymerase and 5 µl of template in a total volume of 50 µl. The amplification cycle consisted of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min, and extension at 74°C for 30 sec; this was repeated 40 times.

The *porB* assay for detection and typing of gonococci was a semi-nested PCR, requiring 1 U/reaction BIO-X-ACT™ polymerase in both stages of the process. The initial broad-specificity step was undertaken with the outer primer pair (see Table *2.3*) and 5 µl of template in a final volume of 50 µl/reaction. The reaction parameters (ran for 25 cycles) comprised denaturation at 94°C for 30 sec, annealing at 60°C for 1 min, and extension at 74°C for 45 sec. Amplification products from the first phase were added at 3 µl/reaction to the second set of tubes (total volume = 50 µl), which contained the antisense outer primer and both inner primers (see Table *2.3*). The allele-specific amplification step proceeded for 35 cycles under the same conditions as the first round.

The cryptic plasmid assay for diagnosis of chlamydial infection was performed in a total volume of 20 µl, incorporating *Taq* polymerase at 0.75 U/reaction, each primer at 1 µM, and 2 µl of template. Amplification was conducted over 40 cycles as follows: denaturation at 94°C for 15 sec, annealing at 50° for 15 sec, and extension at 72°C for 20 sec.

The chlamydial *omp1* assay (nested PCR) used 0.75 U/reaction *Taq* polymerase in both stages of the procedure. For the first step, the outer primer pair (see Table *2.3*) and 2 µl/reaction of template were included in a final volume of 20 µl. The initial amplification cycle (repeated 35 times) comprised denaturation at 94°C for 15 sec, annealing at 40°C for 20 sec, and extension at 72°C for 1.5 min. The second set of PCRs contained 1 µl of products from the first stage, plus the inner pair of primers (see Table *2.3*), in a total volume of 20 µl/reaction. For generation of the final diagnostic product, 35 rounds of amplification were conducted as follows: denaturation at 94°C for 15 sec, annealing at 46°C for 20 sec, and extension at 72°C for 30 sec.

All assays for *Mycoplasma genitalium* and *M. hominis* shared common reaction parameters. The 50-µl reactions contained 1.5 U *Taq* polymerase and 10 µl of template. Products were amplified over 40 cycles that comprised denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec.

2I(iv) *Detection Of Amplicons By Agarose Gel Electrophoresis & Ultraviolet Transillumination*: To resolve PCR products, SeaKem® LE agarose (FMC) was dissolved in heated Tris-acetate-EDTA buffer [(TAE) - 40 mM Tris-acetate, pH 8.0, with 2 mM EDTA] to a concentration of 2% (w/v), and gels of ~5 mm thickness were cast. The gels were submerged under TAE buffer in a DNA Sub Cell™ or Wide Mini Sub™ Cell (Bio-Rad) electrophoresis apparatus, and loaded with PCR aliquots and molecular size markers [0.5 µg/lane 1 Kb DNA Ladder (Gibco-BRL) or 0.65 µg/lane 100 bp DNA Ladder (Promega)] in combination with Blue/Orange Loading Dye (Promega). Electrophoresis was conducted under constant voltage conditions at 5-6 V/cm, and migration of DNA was estimated by reference to the dye front. Following electrophoresis, the gels were stained with 1 µg/ml ethidium bromide (ICN Biomedicals) for 30 min on a rocking platform. Fluorescent amplicons were visualised on an ultraviolet transilluminator (Ultra-Violet Products) at an excitation wavelength of 302 nm.

## *2J* - TaqMan® PCR Assays For The Detection Of STD Agents & Determination Of Infectious Load

2J(i) *Quantitative PCR Methods & The TaqMan® Assay Principle*: The accuracy of methods such as densitometry for the quantification of PCR products in agarose gels is limited by the characteristics of the amplification process. Initially, the reaction proceeds through an exponential phase as each cycle generates template for successive rounds of amplicon production. This can be described by the equation: $N = N_0(1+E)^n$, where $N$ is the cumulative amplicon copy number, $N_0$ is the starting quantity of template molecules, $E$ is the replication coefficient (efficiency), and $n$ is the number of cycles (Cross, 1995). Thus, an $E$ value of 1 represents the ideal conditions under which the quantity of PCR products doubles during every cycle. However, progressive consumption or degradation of reaction components, coupled with competition between primers and denatured products during the annealing step, eventually slows the rate of amplification until a plateau ($E$ = 0) is reached (Cross, 1995). At this stage, amplicon quantity is essentially independent of the initial template copy number, restricting the usefulness of "endpoint determinations" for PCR.

These considerations led to the development of real-time monitoring, in which the exponential profile of product accumulation can be recorded as it is generated. The prototype methodology (Higuchi *et al.*, 1993) exploited the increase in fluorescence displayed by ethidium bromide on intercalation into double-stranded DNA, and images of the process were captured during each PCR cycle by a computer-controlled video camera system. Analysis of normalised fluorescence profiles revealed that a ten-fold decrease in initial template copy number resulted in a delay of ~3 cycles before a signal was detected. This confirmed that exponential amplification was being measured ($2^{3.32} \approx 10$), and the relationship between fluorescence intensity and starting template quantity was found to be linear over five orders of magnitude. However, because ethidium bromide also binds to non-specific reaction products (such as primer-dimers), the limit of reliable quantification was >100 copies of starting material (Higuchi *et al.*, 1993).

An alternative method of amplicon detection circumvents this problem by replacing the intercalating dye with an oligonucleotide probe, which is specific for the template DNA sequence. The probes are designed to hybridise to denatured amplicons within the region flanked by the primers, and are thus encountered by *Taq* DNA polymerase during synthesis of the nascent strand. The enzyme partially displaces the 5' terminus of the probe and proceeds to exert 5'→3'-exonuclease activity, cleaving the

oligonucleotide into small fragments that are released into the milieu (Holland *et al.*, 1991). As the very process of amplification generates probe cleavage products, 5' labelling of probes enables product accumulation to be indirectly measured.

The first technique to exploit this property of *Taq* polymerase used radioactive labelling and chromatographic analysis of probe fragments (Holland *et al.*, 1991), but this approach has been superseded by TaqMan® technology (developed by PE Applied Biosystems) based on fluorometric detection. TaqMan® probes are labelled at the 5' end with a "reporter" fluor such as 6-carboxy-fluorescein (FAM), whilst the 3' end is modified with a "quencher" [6-carboxy-tetramethyl rhodamine (TAMRA)]. While the oligonucleotide is intact, the quencher is in close proximity to the reporter, and fluorescence is abated. However, digestion of the probe by *Taq* polymerase releases the labels into solution, negating the effect of the quencher and generating a fluorescent signal from the reporter.

In the ABI PRISM™ 7700 Sequence Detector, emitted light is collected in real time by a fibre-optic array mounted above the thermal cycler, and a spectrograph separates the characteristic wavelengths before detection by a charge-coupled device camera linked to a computer. The inclusion of a "passive reference" fluor (carboxy-X-rhodamine) in the PCR buffer allows normalisation of the specific signal produced by the reporter, and each sample is assigned a cycle threshold ($C_T$) value. This represents the onset of the exponential phase, defined as the first point during the PCR in which the fluorescence intensity of the reporter is significantly greater than the baseline signal.

As initial template quantity is inversely related to $C_T$, the samples within a PCR run can be ranked without further analysis. However, a $C_T$ difference of +1 can only be translated into a halving of original copy number if $E \approx 1$, which can be verified by plotting $C_T$ against $\log_{10}$ starting quantity and calculating the slope. For most assays designed following the rules outlined in Section *2J(ii)*, PCR efficiency is close to 100%. Although relative quantification can be achieved by comparison with a calibrator sample or a standard curve based on a dilution series of crude nucleic acid extract, absolute determination of initial template copy number requires carefully prepared standards. These are often purified PCR products, cloned constructs, or synthetic oligonucleotides, which must be quantified by an independent method such as spectrophotometry.

The potential for absolute quantification is not the only advantage of real-time TaqMan® PCR. Analysis of the amplification profiles can also facilitate rapid

optimisation and troubleshooting of PCR assays; as the effects of primer, cation, or inhibitor concentrations can be ascertained throughout the entire run (Higuchi *et al.*, 1993). Furthermore, throughput is increased because downstream procedures such as agarose gel electrophoresis and Southern blotting are no longer required. In addition, amplicon contamination (which can lead to false positive results) is greatly reduced, since monitoring of amplification does not necessitate the unsealing of tubes. Finally, the availability of several reporter dyes provides the means to design multiplex assays, which use differentiable probes to simultaneously detect distinct targets.

2J(ii) *Design Of TaqMan® PCR Primers & Probes*: The software package Primer Express™ (PE Applied Biosystems) was utilised in the design of primers and probes for TaqMan® PCR assays, following guidelines included in the program. In particular, guanosines were excluded from the 5' termini of probes (this nucleotide exhibits weak quenching properties), and strand specificity was selected on the basis of minimising guanosine content. For all oligonucleotides, polyguanosine runs (>3 adjacent guanine bases) were avoided.

To promote PCR efficiency, amplicon size was limited to 50-200 bp. The $T_m$ of each oligonucleotide was calculated using the nearest-neighbour algorithms of Primer Express™ or Gene Runner, and was confined to 58-60°C for primers and 65-67°C for probes. All primers for TaqMan® PCR assays (see Table *2·4*) were obtained as desalted solutions from Cruachem, whilst dual-labelled probes (see Table *2·5*) were supplied as desalted lyophilised solids from Scandinavian Gene Synthesis.

2J(iii) *Reaction Composition & Thermal Cycling Parameters For TaqMan® PCR Assays*: Reactants shared by all the fluorescence detection assays were supplied in the TaqMan® PCR Core Reagent Kit (PE Applied Biosystems), and were used at standard concentrations throughout. Thus, each PCR (conducted in TaqMan® Buffer "A") contained 3.5 mM magnesium chloride, 0.2 mM deoxyadenosine triphosphate, 0.2 mM deoxycytidine triphosphate, 0.2 mM deoxyguanosine triphosphate, 0.4 mM deoxyuridine triphosphate (dUTP), 0.25 U/reaction AmpErase® uracil *N*-glycosylase (UNG), and 0.63 U/reaction AmpliTaq Gold™ heat-activated DNA polymerase. Final oligonucleotide concentrations were 0.3 μM for the primers and 0.1 μM for probes in a total volume of 25 μl. The multiplex gonococcal *porB* assay incorporated two sets of three oligonucleotides

**TABLE *2.4*: Primers Used In TaqMan® PCR Assays For The Detection Of STD Pathogens.**

| *Target Organism & Amplified Region* | | *Primer Designation* | *Direction Of Amplification* | *Oligonucleotide Sequence (5' -3')* |
|---|---|---|---|---|
| *Neisseria gonorrhoeae* | *porB1a* | PIA-forward | Sense | CGAATCTTACCATGCAGGCTTC |
| | | PIA-reverse | Antisense | AAY[1]CTGGTGY[1]TTCTCAGTCGTG |
| | *porB1b* | PIB-forward | Sense | ATACGCCGGCTTGTTCCA |
| | | PIB-reverse | Antisense | CGTACAGGGCATTATTGTCGTAAC |
| *Chlamydia* genus 16S rRNA | | *Chlam*Genus-1344F | Sense | CGGAATTGCTAGTAATGGCGTG |
| | | *Chlam*Genus-1489R | Antisense | CCTCACCTTGGGCGCCT |
| *C. trachomatis* fragment of 7.5 kbp cryptic plasmid | | *Ctrach*Plas-11F | Clockwise[2] | CAGCTTGTAGTCCTGCTTGAGAGA |
| | | *Ctrach*Plas-119R | Anticlockwise[2] | CAAGAGTACATCGGTCAACGAAGA |
| *C. trachomatis* 60 kDa CrP[3] gene | | *Ctrach*60K-127F | Sense | GACACCAAAGCGAAAGACAACAC |
| | | *Ctrach*60K-232R | Antisense | ACTCATGAACCGGAGCAACCT |
| *C. pneumoniae* 60 kDa CrP[3] gene | | *Cpneum*-870F | Sense | AAAGCCAGCACCTGTTCCTATG |
| | | *Cpneum*-964R | Antisense | CACAAAAAGCACCACGGCT |
| *Mycoplasma genitalium* MgPa adhesin gene | | *genitalium*-forward | Sense | TGAGAAATACCTTGATGGTCAG |
| | | *genitalium*-reverse | Antisense | AAAGATAGGCTCGCCATTT |

[1]These positions are variable within the *porB1a* genotype - "Y" denotes random incorporation of cytidine or thymidine during oligonucleotide synthesis. [2]According to the scheme of Hatt *et al.* (1988). [3]Cysteine-rich protein.

**TABLE *2.5*: Probes Used In TaqMan® PCR Assays For The Detection Of STD Pathogens.**

| *Target Organism & Amplified Region* | | *Probe Designation* | *Strand Specificity*[1] | *Oligonucleotide Sequence (5' -3')* |
|---|---|---|---|---|
| *Neisseria gonorrhoeae* | *porB1a* | PIA-probe:598-680 | Antisense | FAM-CGTGCAATATGCCGGCTY[2]CTATAAAAGACT[3]-TAMRA |
| | *porB1b* | PIB-probe:622-655 | Antisense | TET[4]-AAGATACGGCGAAGGCACTAAAAAAATCGAATACT[3]-TAMRA |
| *Chlamydia* genus 16S rRNA | | *Chlam*Genus-1410P | Sense | FAM-AAACCAACTCCCATGATGTGACGGGCT[3]-TAMRA |
| *C. trachomatis* fragment of 7.5 kbp cryptic plasmid | | *Ctrach*Plas-57T | Anticlockwise[5] | FAM-CCCCACCATTTTTCCGGAGCGAT[3]-TAMRA |
| *C. trachomatis* 60 kDa CrP gene | | *Ctrach*60K-159T | Antisense | FAM-AAGCAAAAAAGCAAGAAAAAACCACAGCAAAGAGT[3]-TAMRA |
| *C. pneumoniae* 60 kDa CrP gene | | *Cpneum*-907P | Antisense | FAM-AGACTTGTCCGTAGAAATAAACAACCAGTTGAACAAT[3]-TAMRA |
| *Mycoplasma genitalium* MgPa adhesin gene | | *genitalium*-probe | Antisense | FAM-ATGGAAAACCCCTCAACGGTGT[3]-TAMRA |

[1]The template strand to which the probe was designed to anneal. [2]This position is not conserved within the *porB1a* genotype - "Y" denotes random incorporation of cytidine or thymidine during oligonucleotide synthesis. [3]The 3' thymidine nucleotide (not complementary to the target sequence) was added as a support for the TAMRA fluor, and this terminus was also phosphorylated to prevent extension by DNA polymerase. [4]TET = tetrachloro-6-carboxy-fluorescein. [5]According to the scheme of Hatt *et al.* (1988).

in each reaction; one set consisted of a pair of primers and a FAM-labelled probe for the identification of *porB1a* strains, whilst the other comprised a primer pair and a TET-labelled probe for the detection of *porB1b* (see Tables *2·4* & *2·5*).

All TaqMan® PCRs were subjected to identical thermal parameters on an ABI PRISM™ 7700 Sequence Detector, including two initial steps that preceded amplification. Firstly, a 2-min hold at 50°C allowed AmpErase® UNG to digest potential amplicon contaminants from previous TaqMan® runs, since any such products would contain dUTP in place of deoxythymidine triphosphate and would serve as substrates for the enzyme.

Secondly, a 10-min hold at 95°C was required for activation of AmpliTaq Gold™ and partial degradation of AmpErase® UNG. Fifty cycles of amplification were then conducted, consisting of denaturation at 95°C for 15 sec and annealing-extension at 60°C for 1 min.

# CHAPTER 3

## *EVALUATING THE DIAGNOSIS OF SEXUALLY TRANSMITTED BACTERIAL INFECTIONS IN DEVELOPING COUNTRIES*

### *3A* - Sexually Transmitted Agents In Asymptomatic Thai Men: An Investigation Into Diagnostic Methodology

3A(i) *Epidemiology Of STD Among Young Thai Men*: Recent high-quality data on the risk factors associated with STD acquisition among young men come from studies conducted with military recruits in the upper-northern provinces of the country. As conscription for military service is undertaken by lottery, and neither same-sex behaviour nor HIV-positive status result in exemption or discharge, this population is considered representative of Thai men aged 20-22 years from the lower socio-economic strata (Beyrer *et al.*, 1995).

Beyrer *et al.* (1995) discovered that same-sex behaviour, reported by 6.5% of their 1993 study group, served as a marker characteristic for a high-risk subset within the conscript population. The majority of the men in this sub-population (97%) also had female partners, and had twice the mean number of lifetime sex partners than their exclusively heterosexual counterparts. Additionally, they were more likely to have had sex with a female commercial sex worker (CSW), with a greater proportion neglecting to use a condom on the most recent occasion. Same-sex behaviour was associated with a greater likelihood of reporting ever having had any STD (odds ratio (OR) = 2.71); and more specifically, a history of gonorrhoea (OR = 2.05) and non-gonococcal urethritis (OR = 4.54).

The impact of the "100% Condom Program", instigated by the Thai Ministry of Public Health in 1991 to combat HIV transmission in commercial sex establishments, was monitored in cohorts of conscripts in the same setting (Nelson *et al.*, 1996; Celentano *et al.*, 1998). In 1991, 57.1% of new recruits reported having sex with a CSW during the previous year, whist by 1995, this had fallen to 23.8% (Nelson *et al.*, 1996). Furthermore, 92.6% of the 1995 cohort claimed to have used a condom during their most recent sexual activity with a CSW, compared to only 61.0% of the 1991 intake. Concomitantly, the proportion of men who disclosed that they had had a urethral discharge at any time decreased from 36.0% (1991) to 15.0% [(1995) - Nelson *et al.*, 1996].

Celentano *et al.* (1998) investigated the incidence of several STD during the two-year conscription period for men enlisted in 1991 or 1993. Notably, the incidence of gonorrhoea declined from 8.8 per 100 person-years (PY) in the 1991 cohort to 0.9 per 100 PY for the 1993 group. In addition, the rate for non-gonococcal urethritis dropped five-fold, from 3.9 per 100 PY (1991-1993) to 0.7 per 100 PY (1993-1995). The principal risk factors associated with STD acquisition, such as brothel visits and inconsistent condom use with CSW, also decreased significantly between the two periods, and the "100% Condom Program" was deemed to be significant in engendering these changes (Celentano *et al.*, 1998).

The behavioural trends observed in military conscripts in northern Thailand appear to be mirrored in other groups of young men, such as vocational students. Mills *et al.* (1997) interviewed a total of 1799 male students from within the Bangkok Metropolitan Administration between 1993 and 1996. The mean age of the study population was 18, and 99% were single. Patronage of CSW was 13% in 1993, falling to 5% in 1996; and the proportion of students reporting condom use during their most recent visit to a CSW was consistently greater than 90%. The students were also unlikely to have casual sex partners (that is, non-regular partners other than CSW) at either the beginning (3% in 1993) or the end (1% in 1996) of the study (Mills *et al.*, 1997).

Evidently, risky behaviour was less common among students than among military recruits, even at the beginning of the study period, and several factors can be identified to explain this divergence. On average, the students were three years younger than the conscripts, and thus were less sexually experienced. Furthermore, as deferments from military service are granted to individuals in higher education, a bias towards a different socio-economic bracket is introduced through the conscription process (Beyrer *et al.*, 1995). Finally, regional variation in sexual practices exists within Thailand, as evidenced by the higher HIV prevalence in the north where the Royal Thai Army studies were conducted (Beyrer *et al.*, 1995). Therefore, although specific data are lacking, young students in southern Thailand are probably less likely to acquire a STD than any other male group of a similar age.

3A(ii) *Study Population, Sample Collection, & STD Screening In Thailand*: This STD prevalence study involved 480 male technical college students from Songkla province (southern Thai peninsula) who did not exhibit signs of current genitourinary infection. Sample collection, processing and diagnosis were undertaken by the research

group of Dr. Verapol Chandeying at Prince of Songkla University, Hat Yai. Urine specimens collected from each student were centrifuged, and the sediment stored at -20°C. These samples were subsequently thawed, suspended in Tris-EDTA buffer, and mixed with the wash and lysis buffers from the AMPLICOR™ *Chlamydia trachomatis/Neisseria gonorrhoeae* PCR kit (Roche Diagnostic Systems) according to the manufacturer's instructions.

The research group screened the specimens for both pathogens simultaneously using the commercial multiplex assay. There were seven (1.5%) positive reactions for gonococci and 34 (7.1%) for *C. trachomatis* (including two dual infections). These values were unexpectedly high for an asymptomatic population (particularly for gonococcal infection), and did not support the apparent trend of decreasing STD prevalence in Thailand discussed above. Furthermore, seventeen of the individuals diagnosed with either or both of the infections reported that they were virgins; a situation with potentially adverse socio-cultural consequences for those involved.

Therefore, Southampton University Molecular Microbiology Group was invited to re-test the presumptively positive specimens with independent PCR methods. In addition, the prevalence of *Mycoplasma genitalium*, a potential pathogen for which diagnostic facilities were not available in Thailand, was determined by assaying all of the urine extracts. The samples were received as 2-ml aliquots that had been processed for the AMPLICOR™ kit, stored at -20°C in Thailand, and then transported on dry ice to the UK.

3A(iii) *Protocols For PCR Analysis Of Urine Extracts*: Diagnostic assays for the three genitourinary pathogens were based on published primer sequences, with some modifications to the reported reaction composition and/or conditions. Several oligonucleotide pairs targeting the MgPa adhesin gene of *M. genitalium* were discovered in the literature (see Table *2·3*), and they were evaluated by comparing sensitivity and efficiency using a dilution series of *M. genitalium* DNA (this template was prepared by ammonium hydroxide extraction of a liquid culture). Three primer sets were investigated: "MGS-1/MGS-2" [developed by de Barbeyrac *et al.* (1993)], "Mg1/Mg2" [reported by Palmer *et al.*, (1991)], and "MgPa-1/MgPa-3" [designed by Jensen *et al.* (1991)]. The assays were subjected to common reaction conditions - which previously had been found to have broad efficacy - and the "Mg1/Mg2" primers consistently demonstrated the greatest sensitivity with no additional stochastic products. Adjustment of the annealing temperature did not enhance the performance of this assay; hence the common conditions

were retained [see Section *2I(iii)*]. Specificity was confirmed by running PCRs against ammonium hydroxide preparations of mollicute broth cultures (*Mycoplasma hominis* and *Ureaplasma urealyticum*). No cross-reactivity was observed.

To limit potential inhibition, the 480 urine extracts received from Thailand were diluted with an equal volume of distilled water (UHQ) before being aliquoted (10 µl/well) into 96-well CyclePlates™ (Robbins Scientific). These were sealed with aluminium foil lids, rapidly accelerated to 750 g in a Beckman GS-6KR centrifuge to recover droplets, and immediately decelerated. Following storage at -70°C, the CyclePlates™ were placed on ice and the lids removed promptly to prevent aerosol transfer between wells; PCR master-mix was then added to each sample. Control reactions, run in duplicate on each plate, consisted of *M. genitalium* DNA extract diluted 1/100 (positive), or distilled water (UHQ) in place of template material (negative). The wells were covered with a CycleSeal® PCR plate sealer (Robbins Scientific) before thermal cycling commenced.

For confirmation of *C. trachomatis* infection in the 34 specimens deemed to be positive by the commercial assay, a fragment of the 7.5 kbp cryptic plasmid was amplified, utilising primers designed by Mahony *et al.* (1990) in an adaptation of the published protocol [see Table *2·3* & Section *2I(iii)*]. Two urine extracts, which had been found to be positive for gonococci alone by the AMPLICOR™ kit, were run as negative controls; whilst duplicate positive controls contained a $1/10^6$ dilution of chlamydial DNA from elementary bodies processed with ammonium hydroxide [see Section *2H(i)*].

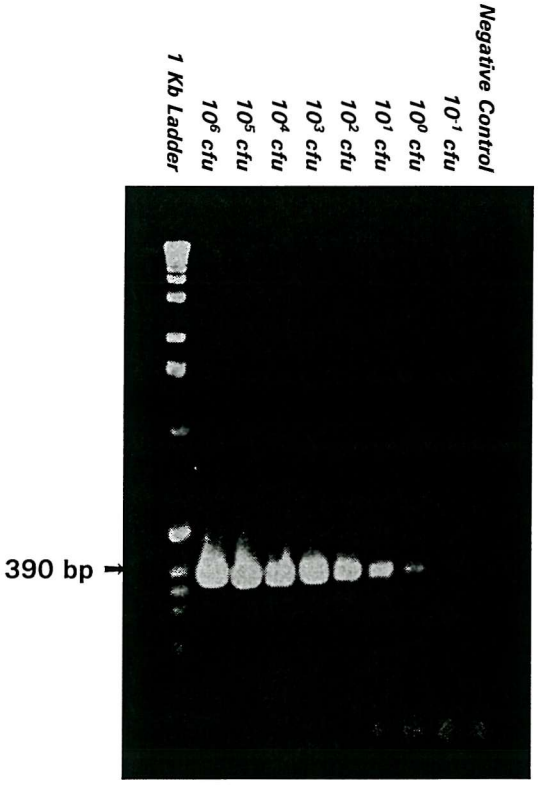
In addition, a nested PCR assay based on the *C. trachomatis ompl* gene was applied to these samples [see Section *2I(iii)*]. The outer primers "Ct1/Ct2", also used in the study of Hayes *et al.* (1992), annealed to highly conserved portions of the gene; whilst the inner set amplified the 3' end of the open-reading frame, maintaining broad specificity within the species (M.A. Pickett, unpublished data). The putatively *Chlamydia*-positive urine extracts were run alongside five samples that did not contain chlamydial DNA according to the AMPLICOR™ kit, and positive controls were included as for the plasmid-based assay.

The seven specimens that required confirmation for gonorrhoea were re-tested using a published protocol (Ho *et al.*, 1992) targeting the *cppB* gene of the 4.2 kbp gonococcal cryptic plasmid [see Table *2·3* & Section *2I(iii)*]. In addition to two standard negative controls, in which distilled water (UHQ) replaced clinical sample, seven samples that had been positive for *C. trachomatis* only (when analysed with the AMPLICOR™ method) were included in this PCR run. The positive control consisted of approximately

1000 copies/reaction of the cryptic plasmid from *N. gonorrhoeae* P9. This was purified from colonies grown on agar, according to the manufacturer's instructions, using the Wizard™ Minipreps DNA purification system (Promega); and quantified by agarose gel electrophoresis through visual comparison with the 1636 bp fragment of the 1 Kb DNA ladder [see Section *2I(iv)*].

To determine the sensitivity of this assay, PCRs were run against ammonium hydroxide extracts of serially diluted gonococcal cells (strain SU96). The expected range of amplicon intensities was seen on the stained agarose gel, from an extremely bright band ($1x10^6$ cfu-equivalents), to a faint but distinct product for ~$1x10^0$ cfu-equivalents (see Figure *3·1*).

**FIGURE *3·1*: Sensitivity Of A PCR Assay Targeting The Gonococcal Cryptic Plasmid – *protocol adapted from Ho* et al. *(1992).***




Amplicons from the *M. genitalium*, *N. gonorrhoeae* and *C. trachomatis* PCRs were loaded on agarose gels and visualised by ethidium bromide post-staining followed by ultraviolet transillumination [see Section *2I(iii)*].

3A(iv) *Development & Evaluation Of A Semi-Nested PCR Assay For Detection & Typing Of Gonococci*: As rare strains of *N. gonorrhoeae* lacking the cryptic plasmid do exist (Roberts *et al.*, 1979), a novel semi-nested PCR assay was developed which targeted the *porB* locus. An outer primer pair was designed using published sequence information for both the *porB1a* (Mee *et al.*, 1993) and *porB1b* alleles (Cooke *et al.*, 1997). As far as possible, primer binding sites were selected to encompass the anomalous *porB* alleles described by Cooke *et al.* (1998), whilst excluding porin genes from the commensal species *N. lactamica* and *N. sicca* (Ward *et al.*, 1992).

Two sense oligonucleotides, targeting either *porB1a* or *porB1b*, were synthesised for use as class-specific inner primers that would be used in conjunction with the antisense outer primer during the semi-nested step. Therefore, typing of gonococcal strains would occur simultaneously with detection. It was predicted that this assay would react with all gonococcal isolates, with the exception of exceedingly rare intermediate serovars which exhibit a number of sequence differences to *porB1a* strains (Cooke *et al.*, 1998).

To investigate the sensitivity and specificity of the PCRs, template DNA was prepared from gonococcal strains SU96 (PorB1a) and SU72 (PorB1b) as follows. The bacteria were cultured as described in Section *2B(ii)*, suspended in PBS with additional mineral salts ("Dulbecco B", Oxoid), and quantified by the standard procedure [Section *2B(iv)*]. Suspensions of each strain were serially diluted and spun for 30 min at 60,000 g (20°C) in a Beckman Avanti™ 30 centrifuge. The supernatants were discarded and the pellets processed by the ammonium hydroxide method [see Section *2H(i)*].

Prior to full evaluation of the semi-nested assay, the two stages of the PCR were tested independently on $1x10^4$ cfu-equivalents of SU96 or SU72, plus duplicate negative control preparations containing no cells. The outer primers detected both the PorB1a and the PorB1b strain, and produced amplification products that differed in size by ~60 bp as expected (the *porB1a* gene has a deletion). However, the PorB1b template gave rise to minor additional products that suggested reaction conditions might not have been optimal. In reactions containing a single specific inner primer plus the antisense outer primer, amplicons were only visible with the target template and were of the anticipated size, differing by ~250 bp. The negative controls did not produce products in all cases.

Products from the first round of PCR were added to reactions containing both of the specific inner primers with additional antisense outer primer [see Section *2I(iii)*]. As both gonococcal strains could be detected at the theoretical limit of ~$1x10^0$ cfu-

equivalents/reaction, assay sensitivity was excellent. In addition, when $5x10^5$ PorB1a and $5x10^5$ PorB1b cells were added to a single PCR, both specific products were clearly visible. The specificity of the assay was maintained up to $1x10^4$ PorB1b organisms, at which point the predicted *porB1a* amplicon became discernible. Stochastic bands and carry-over products from the first stage also became apparent at high template concentrations. However, duplicate no-cell controls were negative.

As large quantities of template were not anticipated to be present in the urine extracts, the *porB* semi-nested assay was applied to the seven samples that were believed to contain gonococcal material, plus seven specimens reported to be positive for *Chlamydia* alone by the AMPLICOR™ PCR. The positive control consisted of amalgamated gonococcal DNA from $5x10^0$ PorB1a and $5x10^0$ PorB1b cells, whereas template was substituted with distilled water (UHQ) in negative reactions.

3A(v) *Analysis Of AMPLICOR™-Positive Specimens For Inhibitors Of DNA Polymerase*: The presumptive *Neisseria gonorrhoeae*-positive samples for confirmatory testing were screened for polymerase inhibitors as follows. Purified cryptic plasmid was introduced into the PCR master-mix so that each reaction contained approximately 1000 copies. The master-mix was then added to the urine extracts, and PCRs proceeded as normal.

In addition, potential inhibition of PCR was also considered for the specimens that were thought to contain chlamydiae. An assay for *Mycoplasma hominis* - based on primers targeting the 16S rRNA gene (see Table *2·3*) - was utilised as an independent means of detecting inhibitors, as template DNA was available from this organism. Prior to the addition of the clinical specimens, *M. hominis* DNA extract was introduced into the PCR master-mix at a final dilution of 1/1000. The intensity of stained products was compared to that of amplicons from control reactions, which contained distilled water (UHQ) in place of urine extract.

3A(vi) *Prevalence Of* Mycoplasma genitalium *& Comparison Of PCR Findings With Those Of Thailand*: Positive reactions for *M. genitalium* were found for 11 (2.3%) of the urine extracts. The positive and negative controls for this assay generated the anticipated outcome in all PCR runs.

Although the cryptic plasmid preparation produced a product of the expected size, all seven samples that were previously found to be positive for gonococci in Thailand

were negative in the plasmid assay. Moreover, none of these specimens were positive for either PorB class following further analysis by semi-nested PCR, despite unambiguous control reactions. Without exception, amplified products were visualised on the gel after spiking of the reaction mix with purified plasmid, indicating that the extracts were not inhibitory.

The nested *ompl* PCR for *C. trachomatis* confirmed twenty (58.8%) of the 34 *Chlamydia*-positive results that were diagnosed by the AMPLICOR™ method. Concordance with the Thai findings was not increased further by the cryptic plasmid assay, which produced a visible product with eight of the *ompl*-reactive specimens. In both of these PCR runs, the control reactions produced the anticipated outcome. In addition, there was no evidence that the remaining 14 discrepant extracts inhibited the *M. hominis* test reactions.

## *3B* - Detection Of *Neisseria gonorrhoeae* And *Chlamydia trachomatis* In A Population Of Sri Lankan STD Clinic Attendees

3B(i) *Demography Of Sri Lanka*: Sri Lanka is a heavily populated island, 270 miles long by 140 miles wide, situated approximately 30 miles from the south-eastern tip of India (Catterall, 1982). The estimated 1996 population was 18.1 million, with a life expectancy at birth of 73.1 years, and a infant mortality rate of 14.8 per 1000 live births (WHO, 1998). Thus, the health statistics of Sri Lanka are exemplary within the region of south and south-east Asia, and are accompanied by high literacy rates, low total fertility rates, an average age at first marriage of 25 for women, and high adoption rates for all methods of contraception (Gunaserera & Wijesinghe, 1996; Caldwell *et al.*, 1998).

In contrast to other south Asian cultures, premarital sexual relations are not regarded as the ultimate social stigma, and Sinhalese society has largely moved away from arranged marriages (Caldwell *et al.*, 1998). Nevertheless, family planning programs confront considerable resistance in providing contraceptives to the unmarried, particularly female adolescents (Caldwell *et al.*, 1998). Furthermore, as predicted by Catterall (1982), the expansion of the Sri Lankan tourist industry has led to new challenges in the control of sexually transmitted diseases. In particular, tourists, business men and military troops from Japan, Europe, the USA, and Australia have fed a burgeoning sex trade in the entire region, which is largely supplied by women migrating from poorer rural areas in search of earnings to send to their families (Lee, 1991).

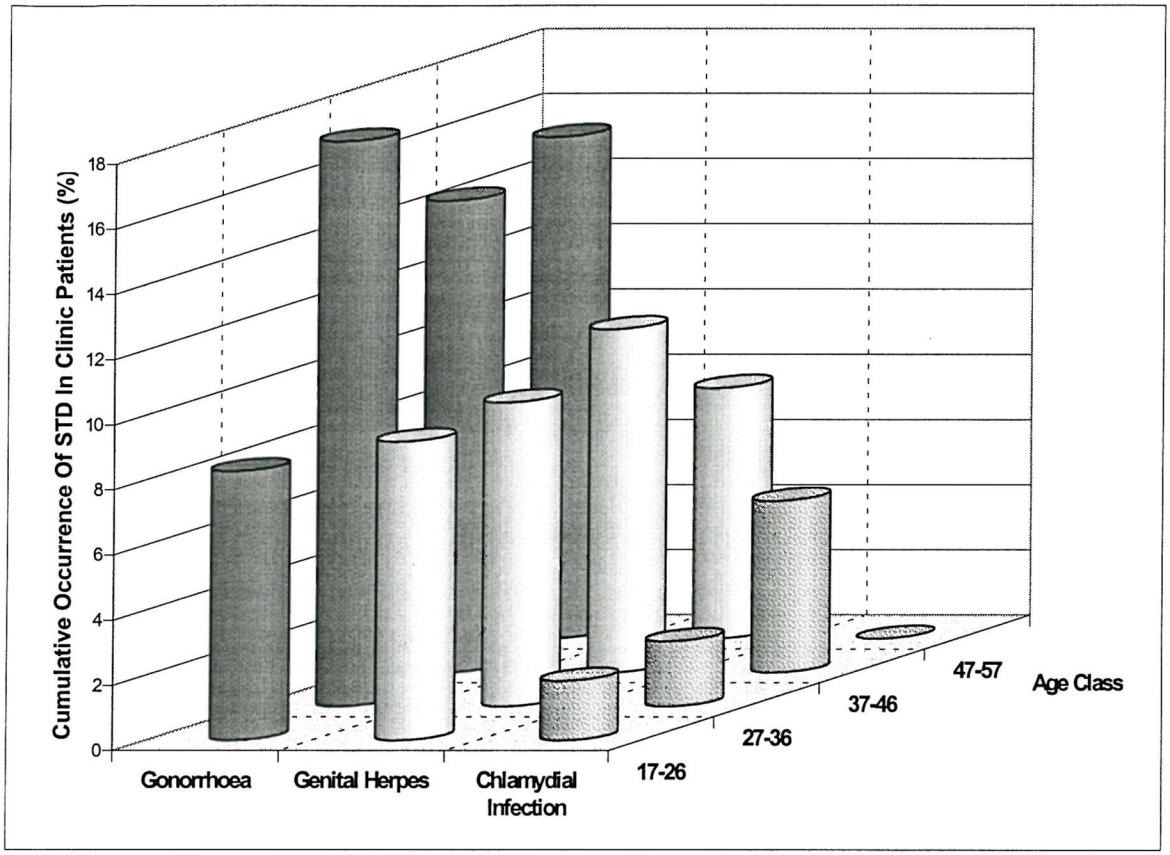
3B(ii) *Definition & Characterisation Of The Study Population*: Against this background, cervical specimens were collected from attendees of the central STD clinic in the National Hospital at Colombo. These became available to the Southampton University Molecular Microbiology Group through a short research project on cervical human papillomavirus infection conducted by Dr. Shamila Amarasekara (under the supervision of Dr. Iyanthimala Abeyewickereme of the National STD/AIDS Control Programme). Between September to December 1997, all women attending the clinic were invited to partake in the study. This population included self-referrals, patients with symptomatic STD who had been referred from other departments, sexual contacts of male urethritis cases, and prostitutes or "vagrants" that were referred by magistrates. The exclusion criteria were pregnancy, previous hysterectomy, and current menstruation. All participants in the study were volunteers who gave informed consent.

During an interview conducted in Sinhalese or Tamil, the 356 women enrolled in the study respond to a structured questionnaire designed by Dr. Amarasekara. This questionnaire was designed to quantify several risk factors implicated in the development of cervical cancer, and included demographic data, gynaecological history and sexual history. In addition, the results of a standardised examination of the external genitalia, vagina and cervix were recorded on the questionnaire form. Evidence indicating vaginal inflammation, ulceration or warts; cervical inflammation, and the presence and nature of any cervical discharge, was noted (see Appendix *1*).

The Colombo clinic maintained records for each patient detailing previous episodes of genitourinary disease. Routine methods employed at the centre included Gram staining of urethral and vaginal smears, Papanicolaou staining of cervical smears for detection of chlamydial inclusions, and the culture of swabs for *Neisseria gonorrhoeae*. Bacterial vaginosis (indicated by clue cells) and candidal vaginitis were diagnosed by smears from the lateral fornix, whilst screening for trichomonal infection was conducted on posterior fornix smears in saline. Finally, syphilis serology was undertaken on blood specimens by means of the Venereal Disease Research Laboratory reaginic test.

The age of the study participants ranged between 17 and 57 years, with a mean of 31.1 years (median = 30.0). Questionnaire responses received from the women were analysed for characteristics that may be relevant to STD acquisition. Data were incomplete for eight women, who were excluded from all subsequent analyses (revised $n$ = 348).

**FIGURE *3·2*: Instances Of Gonorrhoea, Genital Herpes & Chlamydial Infection In The Clinical History Of Sri Lankan STD Patients – *reports ordered by age.***




This was an extremely high-risk population for STD, as the vast majority (81.9%) of women attending the clinic had a history of at least one previous genitourinary infection (see Appendix *1*). There were high levels of both cervical (31.9%) and vaginal (53.7%) abnormalities among these patients, supporting the sexual history data and suggesting that many women might have been infected at the time of examination. Furthermore, during the interview nearly half (45.2%) of the women disclosed that they were commercial sex workers, and it is likely that this underestimates the true proportion. The median number of lifetime sex partners for those who denied prostitution was 1, whilst for affirmed sex workers, this figure was 1424.

Analysis of the patient records (see Appendix *1*) revealed that 48 (13.8%) had had gonorrhoea, 9 (2.6%) chlamydial infection, and 33 (9.5%) genital herpes. These findings, organised by age group, are presented in Figure *3·2*. Although herpes history was similar across all age classes, gonorrhoea was much less frequently recorded amongst the youngest group (see Figure *3·2*). Infection with *Chlamydia* was uncommon in this

population as judged by the indigenous diagnostic system, with the greatest proportion of cases being reported from the 37-46 years-old age class.

In a population that was regularly exposed to a wide spectrum of STDs, containing a subset of individuals which report high levels of partner exchange, the low cumulative occurrence of chlamydial infection was unexpected. Indeed, it highlights the potential shortcomings of a diagnostic service with limited facilities, as *Chlamydia* is particularly difficult to detect without expensive reagents or cell culture systems. To investigate the point prevalence of chlamydial infection and gonorrhoea in the clinic attendees, cervical specimens transported to the UK were analysed by TaqMan® PCR techniques. In particular, the frequency of asymptomatic infections that might have been substantially underestimated by traditional methods was determined.

3B(iii) *Sample Collection Protocol & Processing For PCR*: For subsequent PCR analysis in the UK, epithelial cells were retrieved from the cleaned cervix using a plastic "cytobrush" (Cellpath), which was then transferred into a tube containing 1 ml of 0.2% sodium azide in PBS. Specimens were stored and transported at -20°C.

The tubes received from Sri Lanka, each containing a cervical sample, were thawed and vortex-mixed vigorously to detach cells from the sampling device. To retrieve particles, the suspensions were transferred to microcentrifuge tubes and spun at 60,000 g (20°C) for 30 min in a Beckman Avanti™ 30 centrifuge. The cell pellets were processed for PCR by the ammonium hydroxide technique [see Section *2H(i)*]; and the resultant DNA solutions were diluted, stored and handled as for the Thai urine specimens [see Section *3A(iii)*], except that MicroAmp® Optical 96-well reaction plates (PE Applied Biosystems) were used in place of CyclePlates™. These were sealed with MicroAmp® Optical caps (PE Applied Biosystems) prior to thermal cycling.

3B(iv) *Design & Validation Of A Multiplex TaqMan® PCR Targeting The Gonococcal* porB *Locus*: The availability of clinical specimens from a high-risk population prompted the development of a multiplex PCR for gonococci based on TaqMan® technology [see Section *2J(i)*]. A primer pair and fluorescently labelled probe were designed independently for the *porB1a* and *porB1b* alleles, using published data for the respective sequences [Mee *et al.*, 1993 (*porB1a*); Cooke *et al.*, 1997 (*porB1b*)]. In addition, gene sequences encoding anomalous PorB molecules (Cooke *et al.*, 1998) were also analysed during construction of the assay.

The PCRs were validated using the gonococcal DNA templates that were prepared from strains SU96 (PorB1a) and SU72 (PorB1b), as described in Section *3A(iv)*. In order to confirm the species specificity of the assays, a clinical isolate of *Neisseria meningitidis* was processed in a similar manner.

Primer specificity was evaluated in the absence of fluorescently labelled probe by applying each primer pair to the three DNA templates ($5x10^4$ SU96 cfu-equivalents, $5x10^4$ SU72 cfu-equivalents, and the meningococcal preparation). Probe solution was substituted with water in each reaction, and the PCRs proceeded as detailed in Section *2J(iii)*, except that amplification products were detected by agarose gel electrophoresis followed by ethidium bromide staining and ultraviolet transillumination [see Section *2I(iv)*]. Both primer pairs produced the expected product only in the presence of the intended target DNA. Addition of the respective probe to each assay maintained this specificity; and positive reactions were not observed with either ammonium hydroxide-boiled *Escherichia coli* cells, similarly processed human skin cells with normal flora, or 5 ng/reaction purified human genomic DNA (PE Applied Biosystems).

**TABLE *3·1*: Performance Of A Multiplex TaqMan® Assay For Gonococcal *porB1a/porB1b* Alleles – *cycle threshold values# for PCRs run against a matrix of mixed DNA templates.***

| | | *PorB1A Gonococcal Template* (cfu-equivalents/reaction)* | | | |
|---|---|---|---|---|---|
| ***PorB1B Gonococcal Template~ (cfu-equivalents/reaction)*** | ***Fluorescent Reporter Specificity*** | **$2.5x10^4$** | **$2.5x10^2$** | **$2.5x10^0$** | **Zero** |
| **$2.5x10^4$** | ***porB1A*$^{FAM}$** | 21.31 | 30.09 | Negative | Negative |
| | ***porB1B*$^{TET}$** | 22.16 | 22.61 | 22.42 | 22.51 |
| **$2.5x10^2$** | ***porB1A*$^{FAM}$** | 21.15 | 27.39 | 35.79 | Negative |
| | ***porB1B*$^{TET}$** | Negative | 30.76 | 30.45 | 30.19 |
| **$2.5x10^0$** | ***porB1A*$^{FAM}$** | 21.17 | 27.83 | 34.83 | Negative |
| | ***porB1B*$^{TET}$** | Negative | Negative | 36.99 | 37.15 |
| **Zero** | ***porB1A*$^{FAM}$** | 22.05 | 29.12 | 36.16 | Negative |
| | ***porB1B*$^{TET}$** | Negative | Negative | Negative | Negative |

#The $C_T$ values are the mean of duplicate reactions. *These DNA extracts were prepared from strain SU96 expressing PorB1a. ~These DNA extracts were prepared from strain SU72 expressing PorB1b.

Finally, the two PCRs were combined into a single multiplex assay [see Section *2J(iii)*]. This was validated against gonococcal DNA extracts from PorB1a and PorB1b organisms, which were amalgamated in different ratios to produce a template matrix.

Multiplexing was technically feasible as the *porB1a*-specific probe was labelled with the reporter 6-carboxy-fluorescein (FAM), whilst the *porB1b*-specific probe was modified with tetrachloro-6-carboxy-fluorescein (TET). Nevertheless, in practice it might have been difficult to reliably differentiate these fluors, and the two primer pairs or probes could have competed or interacted during PCR amplification. The results displayed in Table *3·1* demonstrated that gonococci could be simultaneously detected and typed in a single reaction. However, in the unlikely event of a dual infection with both a PorB1a and a PorB1b strain, the minority allele might not be detected, particularly if *porB1a* genomes were dominant (see Table *3·1*).

3B(v) *Design, Validation, & Comparison Of Two TaqMan® PCR Assays For The Diagnosis Of Chlamydial Infection*: For detection of *Chlamydia trachomatis* in the Sri Lankan specimens, two PCRs that amplified sequences from different regions were compared in assays using a panel of chlamydial phenol-chloroform extracts. The first PCR was designed using the published sequence of the *C. trachomatis* 60-kDa cysteine-rich protein (CrP) gene (Watson *et al.*, 1989), and targeted positions of divergence from the homologous gene in other *Chlamydia* species (Watson *et al.*, 1991; primer sequences differed from those reported in this paper). A second assay was based on the nucleotide sequence of the 7.5 kbp *C. trachomatis* cryptic plasmid (Hatt *et al.*, 1988).

The relative sensitivity and specificity of these assays were assessed by cycle threshold ($C_T$) analysis on the ABI PRISM™ 7700 Sequence Detector [see Sections *2J(i) & 2J(iii)*], using reconstituted *C. pneumoniae* or *C. psittaci* extracts, and solutions of *C. trachomatis* or *C. muridarum* DNA diluted 1/100. Although neither assay reacted with *C. pneumoniae* DNA, the cryptic plasmid PCR unequivocally detected *C. psittaci* template, and produced a weak positive reaction with the *C. muridarum* extract (see Table *3·2*). However, greater sensitivity was exhibited by the plasmid-based system, as it consistently reached the net fluorescence threshold three cycles earlier than did the alternative assay (see Table *3·2*).

The principal objective of testing the Sri Lankan cervical specimens was detection of infections that might have been missed at a clinic with limited laboratory facilities. Therefore, the more sensitive PCR targeting the cryptic plasmid was chosen for diagnosis.

3B(vi) *Prevalence Of Gonorrhoea &* Chlamydia trachomatis *Infection As Determined By PCR*: To determine the point prevalence of these infections by molecular

methods, the DNA extracts of the cervical specimens were assayed by PCR. TaqMan® assays targeting the *porB1a/porB1b* alleles of *N. gonorrhoeae* were run with separate positive controls (three each) for PorB1a and PorB1b. These reactions contained DNA from either $1x10^4$ PorB1a-expressing gonococci, or $1x10^4$ PorB1b cells. No-template controls (NTCs), in which template DNA was substituted with distilled water (UHQ), were included six-fold on each MicroAmp® plate. For the *C. trachomatis* assay, NTCs were run in duplicate, and positive reactions consisted of ten dilutions of a cloned construct (pCTL12A) containing the complete cryptic plasmid sequence (Hatt *et al.*, 1988).

**TABLE 3·2: Relative Sensitivity & Specificity Of Two PCR Assays For *Chlamydia trachomatis* – comparison utilising a range of chlamydial DNA preparations.**

| | CYCLE THRESHOLD* | |
|---|---|---|
| CHLAMYDIAL TEMPLATE DNA (species/strain) | 60 KDA CRP ASSAY | CRYPTIC PLASMID ASSAY |
| *C. PSITTACI* EAE-AG | Negative | 29.32 |
| *C. PNEUMONIAE* IOL-207 | Negative | Negative |
| *C. MURIDARUM*# | Negative | 37.82 |
| *C. TRACHOMATIS* SEROVAR E NI-1 | 17.94 | 15.08 |
| *C. TRACHOMATIS* SEROVAR A SA1 | 17.37 | 14.64 |
| *C. TRACHOMATIS* SEROVAR E DK20 | 20.67 | 17.44 |
| *C. TRACHOMATIS* SEROVAR C UW-1 | 17.94 | 14.60 |
| *C. TRACHOMATIS* SEROVAR D IOL-1883 | 20.56 | 18.06 |

*The $C_T$ values are the mean of duplicate reactions. #*Chlamydia muridarum* sp. nov. EVERETT, BUSH & ANDERSEN was formerly known as the mouse pneumonitis biovar of *C. trachomatis* (Everett *et al.*, 1999).

Both the positive and negative control reactions displayed the anticipated outcome on all the gonococcal PCR runs. *Neisseria gonorrhoeae* expressing PorB1b were detected in 19 (5.5%) of the specimens, but PorB1a organisms were entirely absent. In contrast, the prevalence of *C. trachomatis* was 25.6% (89 positive samples, including seven instances of co-infection with gonococci). Positive controls for the cryptic plasmid produced the expected profile; but on two occasions, one NTC produced a weak positive reaction. In both cases, these negative controls were directly adjacent to PCRs containing

large quantities of pCTL12A DNA. As extract volumes were insufficient for re-testing of the specimens included on these plates, Fisher's exact test was used to compare the frequency of *Chlamydia*-positive reactions in the row neighbouring the plasmid-containing controls with that observed across the remainder of the plate. Proximity to the control row had no significant effect on the proportion of samples that were positive. Herpes simplex virus type 2 DNA was detected in only two (0.6%) of the samples (S.A. Lanham, unpublished data).

3B(vii) *Analysis Of Gonococcal & Chlamydial Infection In Relation To Cervical Abnormalities*: To determine whether the presence of pathological signs on cervical examination was associated with a positive PCR diagnosis for *C. trachomatis* or *N. gonorrhoeae*, the two parameters were crosstabulated and analysed by means of the $\chi^2$ test statistic. Abnormalities including inflammation, discharge, erosion, ulceration, and the presence of cysts or polyps (see Appendix *1*) were grouped into a single category, producing a 2x2 contingency table for each infection. Yates' Continuity Correction was applied to the $\chi^2$ statistic, as each test had only one degree of freedom (Fowler & Cohen, 1990).

The $\chi^2$ analyses revealed that cervical pathology was not significantly associated with either organism. In addition, the sensitivity of cervical signs relative to PCR was only 38.2% for the detection of *Chlamydia* [positive predictive value (PPV), 30.6%], and 52.6% for gonococci (PPV, 9.0%). Although the relative increase in likelihood of infection in the presence of detectable abnormalities (odds ratio) was 1.461 for *Chlamydia* and 2.508 for gonococci, these values were not statistically significant.

## *3C* - Conclusions

In this investigation, two very different study populations – a low-risk group of asymptomatic males in Thailand, and a high-risk group of female STD clinic patients in Sri Lanka – were found to present distinct diagnostic challenges to the local health services. In Hat Yai, facilities were available for the detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in urine specimens using the AMPLICOR™ multiplex assay. However, repeat testing in the UK, using two independent assays for each pathogen, did not confirm the results obtained with AMPLICOR™ for 41% of the *Chlamydia*-positive

diagnoses and 100% of the positive reactions for *N. gonorrhoeae*, suggesting that they were false-positives. As nested PCR assays and appropriate controls for DNA polymerase inhibition were conducted in the current work, false-negative diagnoses relative to AMPLICOR™ were extremely unlikely. These results may indicate that the commercial assay is inappropriate for use in a low-risk setting without prior evaluation in the local population.

The limited diagnostic resources available in Sri Lanka presented quite a different problem in the STD clinic at Colombo. Although culture of swabs appeared to provide a reliable means of detecting gonococcal infection, the prevalence of chlamydial cervicitis was vastly underestimated by the use of Papanicolaou-stained smears to visualise inclusions. In addition, the fact that nearly two-thirds of *Chlamydia*-infected patients were asymptomatic might have prevented the scale of the problem from being recognised.

The absence of *N. gonorrhoeae* among the Thai students, and the relatively low prevalence of gonorrhoea in the Sri Lankan STD clinic population (despite a high proportion of affirmed commercial sex workers) suggests that the Asian HIV epidemic has led to a marked decrease in unprotected sexual activity. Gonococcal infections were exclusively of the PorB1b class in the Sri Lankan women, but only half of these patients had cervical abnormalities. The interplay between host and pathogen during cervical inflammation will be investigated further in the following chapter.

# CHAPTER 4

## *PATHOGENESIS OF ACUTE MUCOPURULENT CERVICITIS IN 90 THAI GYNAECOLOGICAL PATIENTS: RELATION TO ASCENDING INFECTION*

4(i) *Mucopurulent Cervicitis - Definition, Aetiology & Clinical Significance*: Characterisation and investigation of cervicitis has been hampered by the lack of reproducible, widely accepted criteria for clinical diagnosis, and by a confusing and inconsistent nomenclature (Brunham *et al.*, 1984; Sweet, 1998). However, some initial distinctions can be made on the basis of cervical structure. Cervical inflammation affecting the columnar epithelium of the endocervix, usually caused by bacterial infections, is known as endocervicitis or *mucopurulent cervicitis* (MPC). Conversely, ulceration or lesion-formation in the stratified squamous epithelium of the ectocervix, associated with viral agents and trichomonads, is termed ectocervicitis or exocervicitis (Holmes, 1990; Sweet, 1998).

The authoritative text of K.K. Holmes *et al.*, *Sexually Transmitted Diseases*, defines MPC as:

> "*...the appearance of the inflamed cervix on physical examination – optimally by colposcopy – with manifestations such as yellow endocervical discharge, oedema, erythema of the zone of ectopy, and easily induced endocervical bleeding...*" (Holmes, 1990)

A widely-accepted cytological criterion, based on the presence of ≥30 polymorphonuclear neutrophils (PMNs) per 400X microscopic field in a Gram-stained preparation of endocervical mucus, is also cited by this text and others as a diagnostic standard for MPC (Holmes, 1990; Sweet, 1998).

As it is not a reportable disease in the USA (Sweet, 1998), estimates of the prevalence of MPC are limited. However, Brunham *et al.* (1984) found that 40% of randomly selected women attending an STD clinic in Seattle had visible mucopus and/or elevated PMN numbers on smears, and a prevalence of 26% was found in a subsequent larger study on a similar population (Holmes, 1990).

The aetiology of MPC is complex, comprising infectious and non-infectious causes (Sweet, 1998). The latter include autoimmune diseases, malignancy, physical trauma (Sweet, 1998), and inflammation related to oral contraceptive use, which is correlated with cervical ectopy (Paavonen *et al.*, 1986; Holmes, 1990). Among the

infectious agents, *Chlamydia trachomatis* is undoubtedly the most important. Brunham *et al.* (1984) reported that 50% of their patient population attending a STD clinic who had either mucopus or elevated PMNs on examination were found to have *C. trachomatis* infections. Additionally, Holmes (1990) discussed a study in which *C. trachomatis* or *Neisseria gonorrhoeae* was isolated from 45% of women in a similar group with mucopurulent cervical discharge. However, the characteristics of the study population are likely to affect the isolation rate for chlamydiae; for instance, 39% of MPC patients attending a university health centre were found to be infected in one study, in contrast to 48-67% of STD clinic attendees (Paavonen *et al.*, 1987; cited in Sweet, 1998).

The role of gonococci in MPC is less clear. Brunham *et al.* (1984) discovered that none of eight patients in their study infected with *N. gonorrhoeae* alone had visible mucopus, and neither were PMN numbers on smears significantly elevated compared to women without a detectable pathogen. Although cervical signs are considered a classic component of the clinical presentation of gonorrhoea (Curran *et al.*, 1975), more recent studies examining a greater spectrum of potential aetiologies have not found a correlation between gonococcal infection and MPC (Paavonen *et al.*, 1986).

Several other infectious causes have been proposed for MPC. Herpes simplex virus (HSV) infection is certainly correlated with ectocervical ulceration and necrosis (Sweet, 1998), but Brunham *et al.* (1984) found that only 20% of HSV-infected patients had visible mucopus, and PMNs were non-existent on smears. Some organisms that are commonly isolated from the female genital tract, such as *Mycoplasma hominis*, *Ureaplasma urealyticum* and *Trichomonas vaginalis*, appear to be associated with MPC, but controlling for co-infection with *C. trachomatis* nullifies this relationship (Sweet, 1998). However, *M. genitalium* has been found to be a minor but significant cause of non-chlamydial cervicitis (Uno *et al.*, 1997). Furthermore, anaerobic bacteria associated with bacterial vaginosis probably contribute to the aetiology of cervicitis, as treatment targeted at the former improves clinical resolution of the latter (Schwebke *et al.*, 1995; cited in Sweet, 1998).

In addition to acting as a reservoir for transmission of pathogens to sexual partners and neonates, cervicitis can give rise to two categories of serious complications. Ascending infections involving the endometrium and salpinges can lead to infertility, ectopic pregnancies and chronic pelvic pain (Sweet, 1998); whilst during pregnancy, the risks are increased for chorioamnionitis, premature membrane rupture, premature delivery, and puerperal infection (Holmes, 1990).

4(ii) *Patient Population & Study Objectives*: Evidently, MPC is a clinical diagnosis that encompasses a variety of causes, and hence may develop in entirely different ways between affected individuals. Therefore, in the knowledge that an investigation into the aetiology and immunobiology of cervicitis might lead to improved management and treatment of MPC patients, an on-going collaboration with the University Hospital of Hat Yai, Thailand, was utilised as a source of gynaecological samples from a relevant patient group. This population consisted of 100 consecutive, sexually active women (age range 18-35) attending the Obstetrics & Gynaecology Department of the hospital, who presented with symptoms of acute MPC.

Three objectives of the study were identified. Firstly, procurement of ultra-frozen endocervical swabs would allow testing of the hypothesis that a final common pathway of proinflammatory cytokine production exists in MPC, irrespective of aetiology. Secondly, as several genitourinary sites in each patient were to be sampled, the presence of pathogens in locations other than the endocervix could be studied in infectious MPC. Thirdly, biopsy material was to be available for the detection of endometrial involvement in women with identified infections of the lower genital tract, thereby providing a marker for the potential development of pelvic inflammatory disease or chorioamnionitis.

4(iii) *Sample Collection Protocol (Thailand)*: Sample material for laboratory analysis was to be obtained from the ectocervix, endocervix, urethra, and endometrium of patients. Exclusion criteria for study candidates were administration of antibiotics in the previous 7 days or subsequent to onset of symptoms, and any factor that prevented the collection of an endometrial biopsy.

A standard cervical examination was conducted on each patient, and any abnormal discharge was sampled by ectocervical swab. Following cleaning of the ectocervix, endocervical columnar epithelial cells were retrieved by rotating a swab in the endocervical canal for approximately 10 sec. A third specimen was recovered by inserting a swab approximately 2 cm into the urethra, and rotating to detach cells as for the endocervix. Finally, a Pipelle® suction curette (EuroSurgical Ltd.) was used to obtain an endometrial biopsy, following the method recommended by the manufacturer. All four specimens were immediately placed in preservation solution [10% (v/v) glycerol, 1% (w/v) BSA, and 0.1% (w/v) sodium azide in PBS], and held at <4°C pending transferral to -70°C storage within the same day. The samples were transported to the UK in liquid nitrogen, and maintained at -70°C until use.

All women enrolled in this study had microscopic mucopus detected in cervical smears. The vast majority of subjects (96.7%) had ≥30 PMN per 400X microscopic field, whilst in the remaining women, ≥10 PMN per field were observed (see Appendix *2*).

4(iv) *Processing Of Endocervical Specimens For Cytokine Assays*: The gynaecological samples received from Thailand were sorted by anatomical site; any patient that lacked a specimen from any one site was excluded from laboratory analysis, which produced a final sample size of 90. Swabs in sealed tubes were defrosted at room temperature, and 0.8 ml of assay buffer [see Section *2E(ii)*] containing 0.1% (v/v) polyoxyethylene ether W-1 was added to each tube prior to vigorous shaking of the samples on a vibrating platform for 10 min. The suspended material recovered from each sample was then transferred into microcentrifuge tubes and spun at 60,000 g (20°C) in a Beckman Avanti™ 30 centrifuge for 15 min.

Supernatants separated from the endocervical cell pellets (900 µl) were organised by the 12x8 format in an assay block, which was sealed and stored at -70°C prior to analysis. Subsequently, six cytokines (IL-1β, IL-6, IL-8, MIP-1α, RANTES and TNF-α) were quantified in the cervical secretions by fluorometric sandwich immunoassay [see Section *2F(ii)*].

4(v) *Processing Of Swab Samples For PCR Assays*: Following thawing of the ectocervical and urethral swabs, 0.8 ml of PBS was added to each tube, and agitation of the specimens proceeded as for the endocervical samples. Suspended cells and debris were aspirated from the swabs and spun at 60,000g (20°C) for 5 min in a Beckman Avanti™ 30 centrifuge. The resultant supernatants were discarded.

Cell pellets recovered from the endocervical, ectocervical and urethral specimens were prepared for PCR by the ammonium hydroxide technique [see Section *2H(i)*], and DNA extracts (70 µl/well) were stored in sealed 96-well "U"-bottomed cell culture plates at -70°C. Potential cross-contamination between wells was controlled by unsealing the cell culture plates whilst the extracts were deep-frozen. Template DNA (5 µl/well) was added to PCR master-mix in MicroAmp® Optical 96-well reaction plates, which were sealed with MicroAmp® Optical caps and agitated on a vibrating platform prior to thermal cycling.

4(vi) *Comparison Between Alternative Methods Of DNA Extraction Applied To Endometrial Biopsy Tissue*: The ammonium hydroxide PCR template preparation method [see Section *2H(i)*] has been found to be consistently reliable with a range of biological secretions, eukaryotic cells and bacteria. However, extraction of DNA from tissue samples presents particular problems due to high concentrations of PCR inhibitors; difficulties in penetrating cells within the tissue block; the need to remove extraneous matter such as lipids, mucus and proteins; and the presence of nucleases which are released from cells during processing.

In a series of preliminary investigations, elementary bodies (EBs) of *Chlamydia trachomatis* were artificially added to small blocks of endometrial tissue prior to overnight incubation with proteinase K. Although the samples were effectively digested, the resultant unfractionated supernatants contained potent DNA polymerase inhibitors, which were not inactivated by boiling in ammonium hydroxide. Proteinase K itself did not inhibit PCR when subjected to an identical processing procedure.

An alternative approach was attempted in which particulate material resulting from tissue digestion was spun down and extracted with ammonium hydroxide after discarding the supernatant. Again, spiking of the samples with chlamydial EBs was used to determine loss of DNA during processing and the effects of inhibition. Although this technique greatly reduced the inhibitory properties of the extracts, template detection was significantly compromised by the loss of template DNA discarded with the supernatants.

Therefore, it was decided that DNA from the Thai endometrial biopsies would be purified by the phenol-chloroform method [see Section *2H(ii)*] after digestion of the tissue. For optimisation of the entire procedure, eight biopsies (taken from patients known to be negative for pathogens of interest at all sites within the reproductive tract) were used in a validation experiment. The specimens were organised into pairs based on similarity in gross characteristics, including mass (~0.7g each) and blood content.

The conventional proteinase K digestion method uses a buffer containing the detergent SDS, which solubilises cell membrane lipids. Although this undoubtedly facilitates extraction of DNA, emulsified lipids might contribute to PCR inhibition. Hence, the original protocol was applied to one specimen from each pair [see Section *2H(ii)*]; whilst in an alternative system, the second biopsy was digested overnight at 37°C on a rotary shaker (180 revs/min), using SDS-free buffer.

Neither protocol completely digested the biopsy material overnight. The remaining tissue in tubes containing SDS buffer disintegrated upon vortex mixing and,

unlike tissue incubated in SDS-free buffer, did not prevent the formation of a clear aqueous phase during the phenol-chloroform extraction.

4(vii) *Determining The Detection Limits For Identification Of Infected Biopsy Specimens*: To ascertain the efficiency of the DNA template extraction procedure on endometrial tissue, suspensions of three mucosal pathogens were used as exogenous controls. These comprised a crude lysate of *Neisseria gonorrhoeae* SU96 (equivalent to $6x10^4$ cells), purified *C. trachomatis* L1 EBs (approximately $7x10^4$ cells), and purified *C. pneumoniae* VR1310 EBs [~$2x10^5$ inclusion-forming units (ifu)]. The latter organism was included as a candidate internal positive control that would not interfere with diagnosis of sexually transmitted infections in the Thai endometrial biopsy specimens. Control preparations were introduced into three of the four samples allocated to each of the DNA extraction protocols. Since ~40% of each digested biopsy extract was processed by the phenol-chloroform technique, and DNA was precipitated from the clear upper two-thirds of the aqueous phase, it was assumed that only ~25% of total DNA could be recovered.

Cycle threshold ($C_T$) analysis by TaqMan® PCR allowed relative quantification of the recovered templates. To ensure that the standards were calibrated for the estimated loss of template incurred during processing, they each contained ~25% of the relevant bacterial suspension added to the test biopsy specimens. Thus, if the $C_T$ values of these reference templates and the processed samples were identical, no undefined losses or inhibition had occurred; whereas a $C_T$ difference equal to 1 would indicate an apparent 50% loss in template copy number. Only the method involving digestion in SDS buffer was fully evaluated, as the alternative technique (in which SDS was omitted) produced heavily contaminated phenol-chloroform extracts.

The results summarised in Table *4·1* indicated that variable extraction efficiency and significant reductions in exogenous DNA were generated by this procedure, even when unavoidable losses of the original material were taken into account. However, contrary to expectation, ostensible losses in control template correlated with large DNA pellets following phenol-chloroform extraction, suggesting that a vast excess of non-target DNA reduced PCR efficiency. To determine whether PCR inhibition could mimic a decline in template copy number, human DNA extracted from the unadulterated biopsy specimen was added in a 1:1 ratio to duplicate reactions containing *C. pneumoniae* DNA. An inhibitory effect equivalent to a 24.2% reduction in chlamydial DNA was observed.

Combining the defined wastage of each specimen with the additional decrease in efficiency, it was possible to calculate the detection parameters for a pathogen that had reached the endometrium. Assuming a conservative reproducible PCR sensitivity of 10 molecules of template DNA, this protocol would detect infection in biopsies containing $3.3x10^3$ copies in the worst scenario, and ~100 copies in the best case. Therefore, it was judged that this method was acceptable for use with the Thai endometrial specimens, although a requirement for monitoring the efficiency of the DNA extraction was identified.

**TABLE *4·1*: Efficiency Of DNA Extraction From Endometrial Biopsies – *assessment of proteinase K digestion followed by phenol-chloroform extraction.***

| | CYCLE THRESHOLD* | | APPARENT REDUCTION IN TEMPLATE COPY NUMBER (%)# |
|---|---|---|---|
| ORGANISM | REFERENCE TEMPLATE | SPIKED SPECIMEN | |
| NEISSERIA GONORRHOEAE+ | 30.4 | 36.8 | 98.8 |
| CHLAMYDIA TRACHOMATIS~ | 19.6 | 21.0 | 62.1 |
| C. PNEUMONIAE^ | 23.3 | 28.3 | 96.9 |

*The $C_T$ value is the mean of duplicate reactions. #Assuming a PCR efficiency of ~1. +The *porB* locus assay was used to detect this organism [see Section *2J(iii)*]. ~Detected using the cryptic plasmid PCR [see Section *2J(iii)*]. ^Detected using the 60 kDa CrP gene PCR [see Section *2J(iii)*].

4(viii) *The Construction Of A Specific PCR For The Detection Of* Chlamydia pneumoniae *Exogenous Control DNA*: An exogenous internal positive control consists of template DNA that is artificially introduced ("spiked") into samples in defined amounts. This DNA must not be naturally present in the specimens, and should be added in a form similar to that of the diagnostic target.

Therefore, *Chlamydia pneumoniae* elementary bodies were chosen for this purpose, as *C. trachomatis* was predicted to be the commonest pathogen present in these samples, and the former species is unlikely to be found in the female reproductive system. Accordingly, a TaqMan® assay specific for *C. pneumoniae* was designed, utilising the published sequence (Watson *et al.*, 1990) of the 60-kDa cysteine-rich protein (CrP) gene [see Section *2J(iii)*]. This was evaluated in PCRs using ammonium hydroxide-boiled templates prepared from purified EBs of *C. pneumoniae* (2 strains), *C. psittaci* (1 strain)

or *C. trachomatis* (3 strains). Only the *C. pneumoniae* strains produced positive reactions with this assay.

4(ix) *Design & Validation Of A TaqMan® PCR Assay For* Mycoplasma genitalium: To determine the significance of *Mycoplasma genitalium* as a cervical pathogen in the study population, a TaqMan® assay was developed based on the MgPa adhesin gene [see Section *2J(iii)*]. This sequence, in conjunction with multiple copies of MgPa operon fragments, constitutes 4.7% of the *M. genitalium* genome (Fraser *et al.*, 1995). The gene product is a critical virulence determinant for this organism (Razin *et al.*, 1998), and is analogous to the P1 adhesin of *M. pneumoniae*, with which it shares over 40% identity at the DNA sequence level. Therefore, the primers and probe were selected to encompass regions of divergence between the two species.

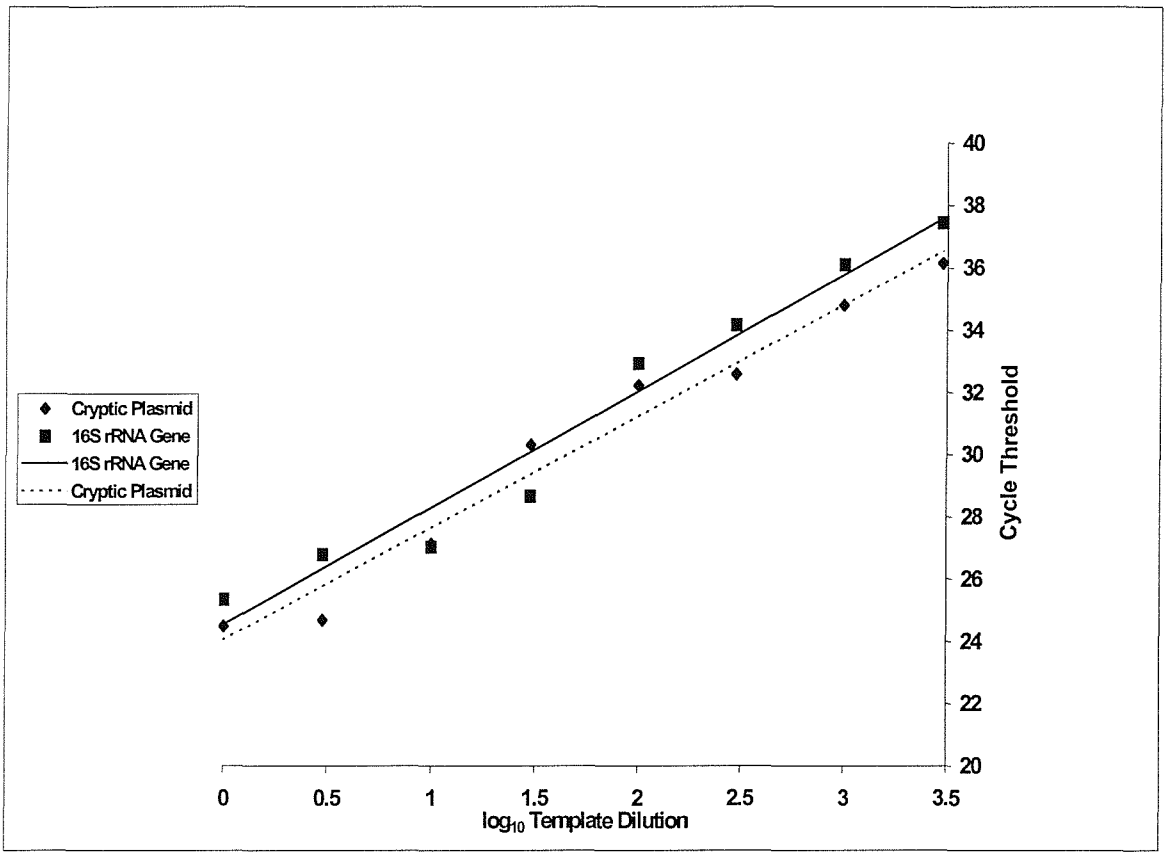
A preliminary assessment of the *M. genitalium* TaqMan® assay was conducted on ammonium hydroxide-boiled extracts of three mollicutes (*M. genitalium, M. hominis* and *Ureaplasma urealyticum*) that had been cultured in liquid medium, plus a purified preparation of human genomic DNA. No cross-reactions with organisms other than the intended target were observed.

4(x) *Comparative Performance Of Three PCR Assays For The Quantification Of Chlamydial Templates*: In addition to the TaqMan® assays for *Chlamydia trachomatis* designed previously, which targeted either the cryptic plasmid or the 60 kDa CrP gene [see Section *3B(v)*], a third PCR - based on the 16S rRNA gene sequence (Pettersson *et al.*, 1997) – was developed [see Section *2J(iii)*]. The relative sensitivity of these assays was compared in parallel on ammonium hydroxide-boiled *C. trachomatis* EBs; which were diluted in either distilled water (UHQ), or ammonium hydroxide extract of uninfected buffalo green monkey kidney (BGMK) cells.

Cycle threshold analysis of these TaqMan® assays [see Section *2J(i)*] established that the BGMK cell extract had little effect on the efficiency or sensitivity of the plasmid PCR; the $C_T$ values for the parallel dilutions were usually within one cycle, even at the lower limit of detection. The plasmid PCR detected template 0.76 cycles earlier, on average, than did the 16S rRNA gene assay (see Figure *4·1*), corresponding to a sensitivity ratio of 59%. Therefore, for a given $C_T$ value, the cryptic plasmid PCR would detect template from approximately half the quantity of chlamydiae required by the 16S rRNA gene PCR. Additionally, at the extreme low end of the dilution series, reactions

based on these targets detected three-fold fewer templates than did the 60-kDa CrP gene PCR. Taking the dilution series as a whole and averaging cycle threshold differences, this assay reacted with template 3.58 cycles later than did the cryptic plasmid PCR; a sensitivity ratio of 8%. Thus, the latter assay had an approximately ten-fold greater likelihood of detection per organism, and was selected for the quantification of chlamydiae where this was appropriate.

**FIGURE *4·1*: Comparative Sensitivity Of Two PCR Assays For The Detection Of *Chlamydia trachomatis* – *dilution series prepared in distilled water.***

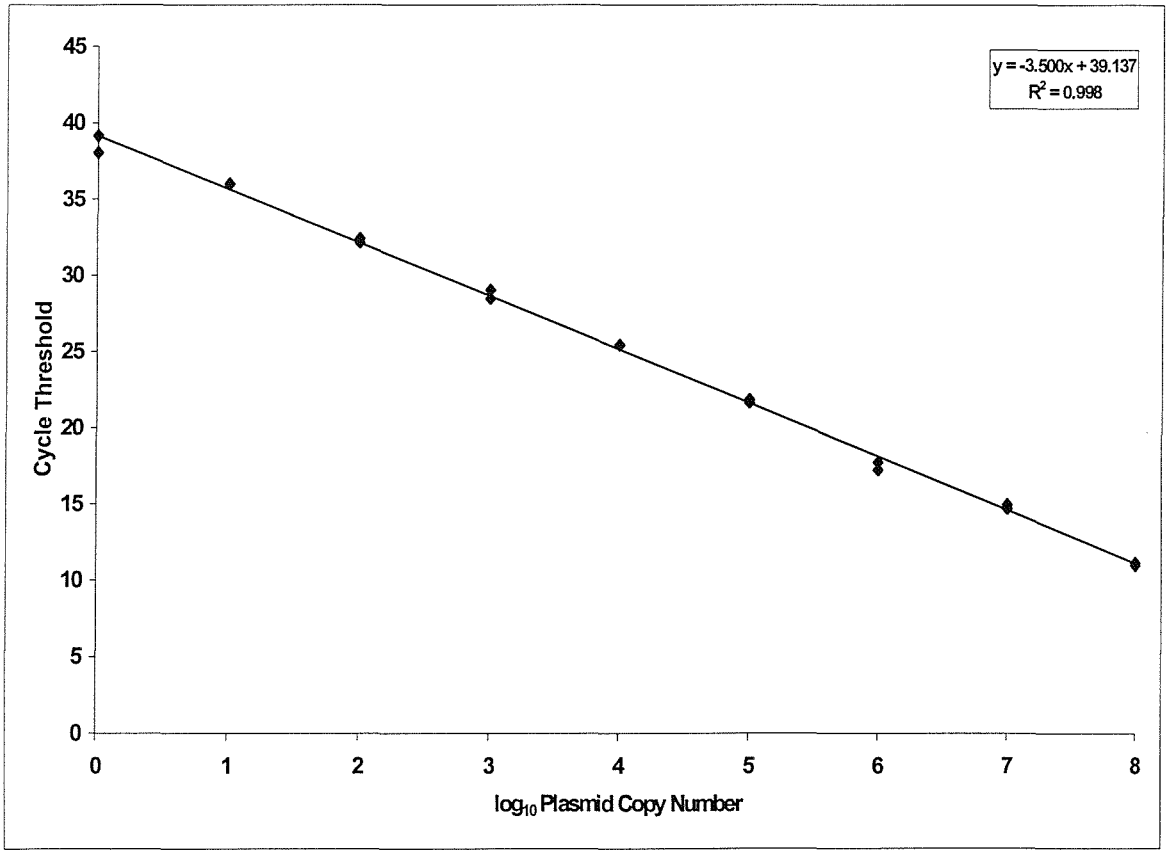



4(xi) *Quantification Of The Chlamydial Cryptic Plasmid For Application As A Standard In TaqMan® PCR*: The availability of an instrument that monitors DNA amplification during PCR in real-time [see Section *2J(i)*] enabled the development of an absolute quantitative method for assessment of chlamydial infection. The construct pCTL12A, containing the entire sequence of plasmid pLGV440 from *Chlamydia trachomatis* L1/440/LN (Hatt *et al.*, 1988), was used as a standard in TaqMan® PCR assays. Recombinant plasmid copy number was calculated from the concentration of the

stock preparation - determined by spectrophotometric absorption at 260 nm ($A_{260}$) - and the estimated molecular weight (6.81 mDa). Nucleic acid purity, expressed as the $A_{260}$:$A_{280}$ ratio, was 1.69.

The pCTL12A standard was evaluated by the TaqMan® PCR assay described in Sections *2J(iii)* & *3B(v)*, using ammonium hydroxide preparations of BGMK cells, or cells infected with *C. trachomatis* L1 for 16 hr or 41 hr. Both these samples and the standards were assayed in duplicate, and the plasmid dilutions encompassed $1x10^{8}$-$1x10^{-1}$ copies per reaction.

**FIGURE *4·2*: Standard Curve For A Quantitative *Chlamydia trachomatis* TaqMan® Assay Targeting The Cryptic Plasmid – *performance using recombinant plasmid as template.***




Analysis of the standard curve generated on the ABI PRISM™ 7700 Sequence Detector demonstrated that the correlation coefficient was 0.998, with a PCR efficiency of ~95% (see Figure *4·2*). The cycle threshold at which one plasmid copy would be detected was estimated by the *y*-intercept as 39.14 (see Figure *4·2*), and both dilutions containing plasmids close to extinction produced such a result, although this must be

interpreted in terms of the Poisson distribution that describes the outcome of sampling rare objects. Whilst the uninfected BGMK cells were negative for the cryptic plasmid as expected, the cells infected for 16 hr produced an interpolated copy number of ~$2.4x10^6$ plasmids/µl, and the 41 hr post-infection template contained >$2x10^7$ copies/µl (~$7.2x10^7$ by extrapolation). Therefore, the quantitative cryptic plasmid assay generated plausible data when applied to impure sources of template DNA.

4(xii) *Prevalence Of Genitourinary Pathogens*: Specimens from the urethra, ectocervix and endocervix of 90 women with a clinical diagnosis of mucopurulent cervicitis were run in TaqMan® PCR assays, in which appropriate positive and negative controls were included. In all cases, negative controls consisted of duplicate no-template reactions (NTCs) in which distilled water (UHQ) replaced template DNA. For the *N. gonorrhoeae porB* locus PCR [see Sections *2J(iii) & 3B(iv)*], positive controls consisted of one reaction containing DNA from ~50 PorB1a organisms (strain SU96), one reaction containing ~5 PorB1a organisms, and two parallel reactions containing equivalent amounts of PorB1b cells (strain SU72). The cryptic plasmid assay for *C. trachomatis* was run with duplicate controls containing ~30 and ~3 EBs from an infected BGMK cell preparation. Finally, two ten-fold dilutions of a whole-cell *M. genitalium* extract (not quantified) were used as controls for amplification of the MgPa adhesin gene from this organism. All of these DNA preparations were processed by the ammonium hydroxide technique [see Section *2H(i)*]. The control reactions generated the expected outcome in all cases, with the exception of two runs in which one or both of the replicates containing ~3 EBs was negative.

No gonococci of either PorB class were detected in any of the patients. However, six women (6.7%) were infected with *Mycoplasma genitalium*, which was discovered in the endocervix of four patients and the ectocervix of five. Although none of the urethral swabs contained this organism, two ascending infections were identified in endometrial biopsies. In contrast, *Chlamydia trachomatis* was found in a much larger proportion (26.7%) of the study population. The distribution of this organism within the genitourinary systems of the 24 infected women is summarised in Table *4·2*. In three individuals (3.3% of the population), cervical co-infections with chlamydiae and *M. genitalium* were evident. Therefore, in summary, one or more pathogens were identified in 27 (30%) of cervicitis patients.

4(xiii) *Detection Of* Chlamydia trachomatis *In Endometrial Biopsy Specimens*: Endometrial biopsy specimens from patients that had a positive diagnosis for *C. trachomatis* at one or more sites (24 samples) were spiked with ~$2x10^5$ *C. pneumoniae* ifu/sample, and processed by the validated method [see Section *2H(ii)*]. One exceptionally mucoid and bloody specimen was not extracted, as it failed to separate into distinct phases during the phenol-chloroform process. The remaining 23 extracts were assayed for *C. trachomatis* and *C. pneumoniae* by TaqMan® PCR. Both the positive controls for *C. trachomatis* and the NTCs were the same as those used in the assays on the specimens from the three other sites. For *C. pneumoniae*, an ammonium hydroxide extract of ~$2x10^5$ ifu was diluted five-fold (four times) to provide a standard curve.

With the exception of one replicate of a *C. trachomatis* control containing ~3 EBs that was negative, the control reactions produced the anticipated outcome. All the biopsies were positive for the exogenous control, but cycle thresholds varied greatly between the extracts. Relative to the reference reaction containing the same total amount of *C. pneumoniae* DNA that was spiked into the biopsies prior to processing, the median apparent recovery was 1.3%.

The *C. trachomatis* assay revealed that eight (34.8%) of the biopsies were positive for this pathogen. While two of these patients had chlamydiae detected in the endocervix only, the remainder was infected in all of the additional sites (see Table *4·2*).

4(xiv) *Quantification Of* Chlamydia trachomatis *In Endocervical Specimens*: In order to determine whether the number of chlamydiae in the endocervix correlated with cytokine production and/or infection elsewhere within the genitourinary system, positive specimens from this site ($n$ = 18) were assayed using the quantitative TaqMan® PCR. All endocervical extracts, standards and no-template controls were run in duplicate.

The results of the plasmid quantification are displayed in Table *4·2*; control reactions produced the expected result in all cases, and the correlation coefficient of the standard curve was 0.997. The number of chlamydiae present in each endocervical sample was calculated by multiplying the quantity of plasmids detected in a 5 µl aliquot by 16 (total extract volume = 80 µl), and dividing this value by 4 (chlamydiae contain ~4 plasmids per cell on average; M.A. Pickett, unpublished data). Endocervical specimens containing less than 10 chlamydiae (nine samples) were not designated precise copy numbers - these included seven samples in which one or both replicates were negative in the quantitative assay, although they had been positive in the diagnostic PCR. One of

these specimens was inhibitory for PCR, as assessed by its effect on the ROX signal measured by the ABI PRISM™ 7700 Sequence Detector.

It was clear from the quantitative data that women with detectable infection of the endocervix could be divided into two groups: those with specimens containing fewer than 20 chlamydiae, and those with greater than 10,000 organisms (see Table *4·2*). Consequently, the $\chi^2$ statistic was utilised to test the hypothesis that chlamydial numbers in the endocervix were associated with detection of organisms elsewhere in the

**TABLE *4.2*: *Chlamydia*-Positive MPC Patients Ordered By Site Of Infection & Endocervical *Chlamydia* Load.**

| *DETECTION OF* CHLAMYDIA TRACHOMATIS *BY ANATOMICAL SITE* | | | |
|---|---|---|---|
| ***ENDOCERVIX (chlamydial load recovered)*** | ***ECTOCERVIX*** | ***URETHRA*** | ***ENDOMETRIUM~*** |
| $1.4x10^6$ | + | + | + |
| $4.7x10^4$ | + | + | - |
| $3.3x10^4$ | + | + | + |
| $1.8x10^4$ | + | + | + |
| $1.5x10^4$ | + | + | + |
| $1.4x10^4$ | + | + | + |
| $1.5x10^1$ | - | - | + |
| $1.4x10^1$ | + | - | - |
| $1.2x10^1$ | + | - | - |
| $<1x10^1$ | + | + | + |
| $<1x10^1$ | - | - | - |
| $<1x10^1$ | - | - | - |
| $<1x10^1$ | - | - | - |
| $<1x10^1$ | - | - | - |
| $<1x10^1$ | - | - | - |
| $<1x10^1$ | - | - | - |
| $<1x10^1$ | - | - | - |
| Not Quantifiable* | - | - | + |
| Not Detected | - | + | - |
| Not Detected | + | - | - |
| Not Detected | - | + | - |
| Not Detected | + | - | Inadequate Sample# |
| Not Detected | - | + | - |
| Not Detected | - | + | - |

~Only those endometrial biopsies from patients that were previously found to be positive for at least one pathogen in at least one anatomical site were processed for PCR. *This sample was positive in the qualitative assay, but inhibitory factors interfered with quantification. #It was not possible to extract DNA from this mucoid and bloody specimen, as it failed to separate into aqueous and organic phases during the phenol-chloroform procedure.

genitourinary system. Presence or absence of infection in the urethra, ectocervix or endometrium was separately crosstabulated against endocervical chlamydial load -

categorised as "not detected", "low shedders", or "high shedders" – in 2x3 contingency tables. Because of the small sample size, significance levels were calculated using the exact rather than the asymptotic distribution.

Infection of the ectocervix was significantly associated with endocervical *Chlamydia* load ($P < 0.05$); in particular, a positive ectocervical diagnosis was associated with high shedders. There was also a very highly significant positive association between the presence of chlamydiae in the urethra, and the number of organisms in the endocervix of affected patients ($P < 0.001$). Moreover, a highly significant association ($P < 0.01$) between endometritis and endocervical *Chlamydia* load was revealed: women classified in the "high shedder" group were more likely to have organisms detected in the upper reproductive tract. Despite these findings, however, a similar $\chi^2$ analysis demonstrated that neither endocervical *Chlamydia* infection *per se*, nor chlamydial load, was significantly associated with clinical severity as assessed by the presence of macroscopic mucopus on cervical examination.

4(xv) *Quantification Of Cytokines In Endocervical Secretions*: To investigate the mediation of the inflammatory response in the endocervix, cell-free supernatants prepared from the swab specimens were assayed for six cytokines (IL-1β, IL-6, IL-8, MIP-1α, RANTES, and TNF-α) known to trigger inflammation and the associated influx of phagocytic cells. Quantities calculated for supernatants that were visibly contaminated with blood (eight specimens) were excluded from the analyses to prevent spurious skewing of the sample distributions. In addition, production of TNF-α was at the lower limit of detection for almost all specimens, and was not investigated further.

The patient population displayed a very wide range of production for all cytokines analysed. This was most evident in the observed scatter of IL-1β levels (range = 2.64 log units), whereas the lowest variability was exhibited by MIP-1α (range = 0.92 log units). Since disease severity was assessed in Thailand by recording the observed cervical discharge in one of three categories [clear, cloudy, or mucopurulent (see Appendix *2*)], the hypothesis that copious mucopus production would be linked to increased quantities of proinflammatory cytokines was investigated using independent *t*-tests. For this comparison, the patient population was divided into two groups (see Figure *4.3*): individuals with macroscopic mucopus, and those with clear or cloudy secretions that indicated microscopic inflammation only.

**FIGURE *4.3*: Influence Of Clinical Severity On The Mean Endocervical Proinflammatory Cytokine Response In Thai Women With Mucopurulent Cervicitis.**

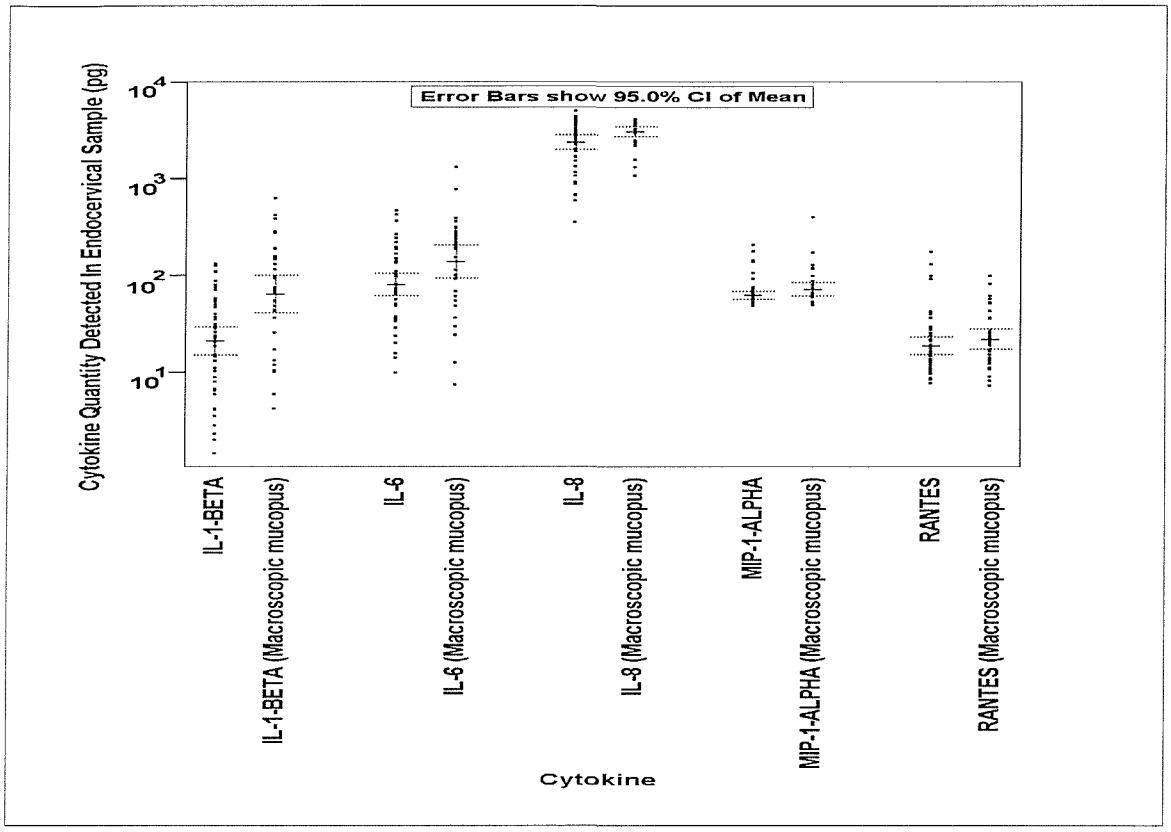
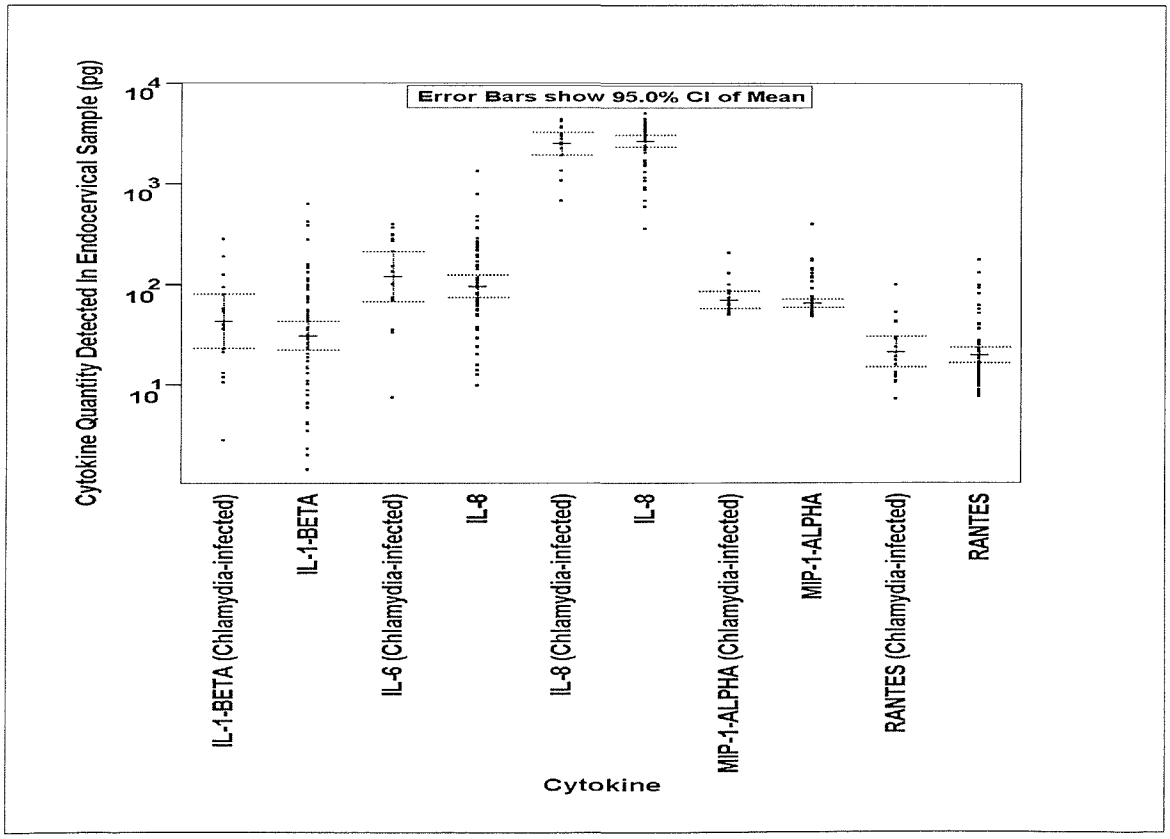



**FIGURE *4.4*: Influence Of Chlamydial Infection On The Mean Endocervical Proinflammatory Cytokine Response In Thai Women With Mucopurulent Cervicitis.**



Several cytokines were present in significantly greater amounts in specimens from women with a macroscopic mucopurulent discharge, as compared to patients with only elevated PMN numbers on smears. Although the production of MIP-1α and RANTES was not significantly affected by clinical severity, the difference in mean IL-1β levels between the two groups was very highly significant ($P$ <0.001), and IL-6 and IL-8 were also significantly elevated in supernatants from women with overt disease ($P$ <0.05 in both cases). Therefore, IL-1β, IL-6, and IL-8 are probably involved in the pathogenesis of MPC, and clinical presentation may be mediated through a cytokine cascade that is ultimately independent of aetiology.

To test this hypothesis, *t*-tests were also conducted between the mean amounts of each cytokine measured in *Chlamydia*-infected and uninfected endocervical specimens (see Figure *4·4*). In addition, mean cytokine responses were compared between the three categories of chlamydial load [see Section *4(xiv)*] by one-way analysis of variance.

The population means for the infected samples versus *Chlamydia*-negative samples were not significantly different from each other in all cases (see Figure *4·4*). Furthermore, there was no statistical evidence that numbers of chlamydiae in the endocervix affected cytokine responses. Thus, patients with MPC of unknown aetiology (70% of this study population) exhibited a proinflammatory cytokine profile that was indistinguishable from that of women who had a demonstrable infectious cause.

4(xvi) *Conclusions*: In this investigation into the pathogenesis of mucopurulent cervicitis in Thailand, the causative roles of bacterial pathogens and levels of proinflammatory cytokine production were analysed by PCR and immunoassay, respectively. Contrary to expectations, only about one-third of the women in this study were infected with one or more of the organisms that are frequently associated with this disease. Although over one-quarter of the study participants were found to have chlamydial infection, there was no significant connection between this pathogen and disease severity. *Mycoplasma genitalium* was detected in a much smaller proportion of women and is unlikely to be a major factor in the development of MPC, whilst *Neisseria gonorrhoeae* was entirely absent from this population.

According to the quantitative data obtained regarding chlamydial load in the endocervix, infected women could be classified as "high shedders" of chlamydiae, "low shedders", or without detectable endocervical organisms. Statistical analysis revealed that the presence of chlamydiae in other regions of the genitourinary system was associated

with "high shedder" status at the endocervix. In particular, potentially serious ascending infections, which were identified in approximately one-third of patients with positive specimens at other sites, were more frequently detected in women from this category.

Cytokine profiles in this study population were difficult to interpret without post-treatment specimens or samples from an endemic control group. Nevertheless, it was clear that significantly elevated production of IL-1β, IL-6, and IL-8 occurred in individuals with an overt mucopurulent discharge, suggesting a role for these proinflammatory cytokines in the pathogenesis of MPC. In contrast, chlamydial infection did not generate a unique inflammatory response in the endocervix, indicating that many of the stimuli responsible for cytokine induction - and the mechanism by which they cause disease - have yet to be identified.

# CHAPTER 5

## *INTERACTIONS BETWEEN* NEISSERIA GONORRHOEAE *& DIFFERENTIATED HUMAN MACROPHAGES: RELEASE OF PROINFLAMMATORY CYTOKINES*

### *5A* - Macrophages As Mediators Of Acute Inflammation In Gonorrhoea: A Hypothesis

The current model of gonococcal pathogenesis during acute infection is founded on the interaction of two types of cell in the human host: epithelial cells and neutrophils [see Sections *1D(i)* & *1D(iii)*]. Gonococci attach to and are subsequently internalised by urethral epithelium cells (Apicella *et al.*, 1996), generating the release of proinflammatory cytokines (Naumann *et al.*, 1997). Consequently, neutrophils are recruited to the site of infection, where gonococci are phagocytosed but not necessarily eliminated (Shafer & Rest, 1989; Meyer, 1998). The resultant purulent discharge is the hallmark of gonococcal urethritis or cervicitis (Harkness, 1948; Handsfield & Sparling, 1995).

Since tissue-resident macrophages are found in the mucosae of both the urinary tract (El-Demiry *et al.*, 1986) and the cervix (Tay *et al.*, 1987), gonococci and gonococcal antigens probably come into contact with these cells early in infection. Furthermore, since the proportion of CD14-positive cells is greater in the fallopian tubes than in the cervix (Givan *et al.*, 1997), macrophage products may be particularly important in the pathogenesis of pelvic inflammatory disease. However, very few recent studies have been conducted on the interaction of gonococci with mononuclear phagocytes. Although Knepper *et al.* (1997) investigated the role of gonococcal surface phenotype in the mediation of phagocytosis, their experiments used peripheral blood monocytes, not differentiated macrophages. Similarly, the brief report of Harper *et al.* (1996) described the cytokine response of fresh adherent monocytes stimulated with gonococcal components.

Therefore, in this study, monocytes were matured *in vitro* until they resembled differentiated macrophages. The interaction of these cells with gonococci was investigated in a series of experiments that examined adherence and internalisation, the cytokine response to viable organisms, and the production of cytokines following stimulation with purified gonococcal components.

## *5B* - The Generation Of Differentiated Macrophages *In Vitro*

5B(i) *Development, Classification & Identification Of Macrophages*: The mononuclear phagocyte system comprises monoblasts and promonocytes in the bone marrow; monocytes in the peripheral blood; and a number of different macrophage types found in body cavities, tissues, and sites of inflammation (van Furth, 1992). Monoblasts, which are derived from granulocyte-macrophage colony forming cells (GM-CFC), divide once to generate two promonocytes; these cells undergo a further single division to produce a total of four monocytes per monoblast (van Furth, 1992). Monocytes in the bone marrow are released into the circulation within 24 hours of production, and form two pools - a circulating population, located within the lumen of the vasculature; and a marginating group, which loosely adheres to the endothelium (van Furth, 1992).

Under normal conditions, peripheral blood monocytes in humans have a half-time in the circulation of ~71 hours (Whitelaw, 1972). However, this transit time is decreased when monocytes are recruited to acute inflammatory foci, where they are transformed into exudate macrophages (Takahashi *et al.*, 1996). Antigens that are refractory to digestion following phagocytosis give rise to the state of chronic inflammation observed in granulomas; in which macrophages develop into epithelioid cells, coalesce in the vicinity of the foreign body, and become multinucleated giant cells (van Furth, 1992; McNally *et al.*, 1996). These cells have a severely impaired phagocytic function (Kaplan & Gaudernack, 1982).

A growing body of controversial evidence suggests that tissue-resident macrophages have a distinct ontology that largely bypasses the monocytic stage (Takahashi *et al.*, 1996). Foetal macrophages develop directly from GM-CFC in yolk sacs, migrate to embryonic tissues, and proliferate to form tissue-specific sub-populations. This process occurs before hepatic haematopoiesis and the release of monocytes into the circulation, although eventually monocyte-derived macrophages do appear in certain tissues. In the adult, GM-CFC directly enter the peripheral blood, lodge in tissues, and differentiate *in situ* into resident macrophages. These cells are long-lived and are replenished by self-renewal (Takahashi *et al.*, 1996). Surface phenotype analysis supports the proposal that tissue-resident macrophages are distinctly different from macrophages found in exudates (Andreesen *et al.*, 1988).

The production of mature macrophages from monocyte precursors *in vitro* requires both human serum (Andreesen *et al.*, 1990; Krause *et al.*, 1998) and the autocrine secretion of macrophage colony-stimulating factor (Brugger *et al.*, 1991). Macrophage

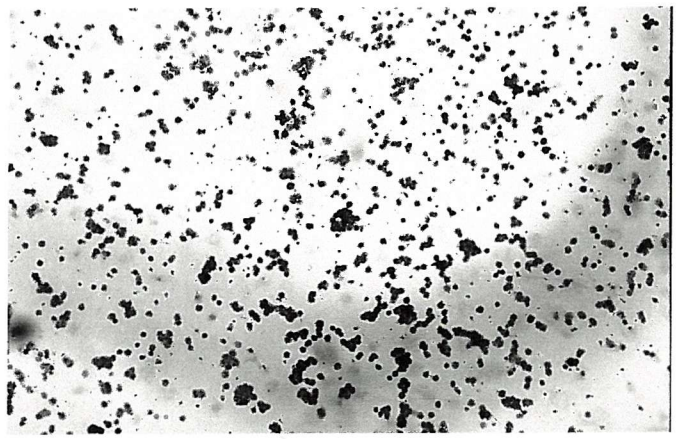
differentiation is associated with changes in surface receptors (Andreesen *et al.*, 1990), an increase in lysozyme secretion and tumour cytotoxicity (Andreesen *et al.*, 1983), and a shift in the cytokine response (Scheibenbogen & Andreesen, 1991). Most of the important components of this process occur by the seventh day *in vitro* (Scheibenbogen & Andreesen, 1991; Audran *et al.*, 1996), although the culture substrate has a profound effect on macrophage morphology and function. While cells grown on glass develop into exudate-type macrophages and ultimately multinucleated giant cells; monocytes cultured on collagen gels exhibit the fusiform morphology of tissue macrophages, and remain efficient at phagocytosis of particles (Kaplan & Gaudernack, 1982).

A rapid method for the identification of cells within the mononuclear phagocyte system is the cytological demonstration of α-naphthyl acetate esterase (ANAE), in which a diazonium salt coupler is used to highlight sites of enzyme activity. This technique produces granular deposits in monocytes and histiocytes, but also gives a strong reaction with megakaryocytes, and much weaker reactions with granulocytes and lymphocytes (Yam *et al.*, 1971). However, whilst the monocyte enzyme is inhibited by the addition of sodium fluoride to the reaction, the lymphocyte equivalent is unaffected (Li *et al.*, 1973). Macrophages isolated from various tissues display only partial esterase inhibition with fluoride (Ennist & Jones, 1983).

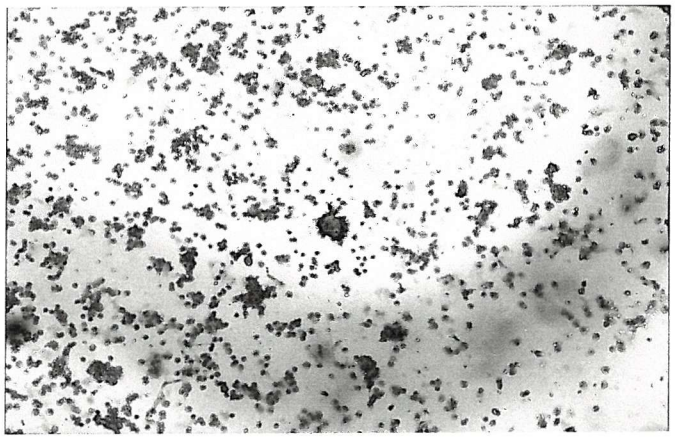
Before studies on the interaction of macrophages with gonococci could be initiated, it was necessary to investigate the morphology and phenotype of monocyte-derived macrophages that had been produced *in vitro*. Plastic and glass culture substrates were coated with a thin layer of Matrigel® basement membrane matrix to encourage differentiation along a tissue-resident macrophage pathway. In addition, ANAE activity was monitored before and after eight days' incubation with human serum, which was interspersed with vigorous washing to remove loosely attached cells.

5B(ii) *Detection Of α-Naphthyl Acetate Esterase Activity In Adherent Peripheral Blood Mononuclear Cells Cultured For Different Periods* In Vitro: Following density gradient centrifugation, the average (mean) composition of the leukocyte suspension was 75.4% lymphocytes, 7.2% neutrophils, and 14.8% monocytes. The platelet-to-monocyte ratio in buffy coats was 1000:1, which was reduced by 2-3 orders of magnitude by centrifugation of the peripheral blood mononuclear cells (PBMC) through FCS.

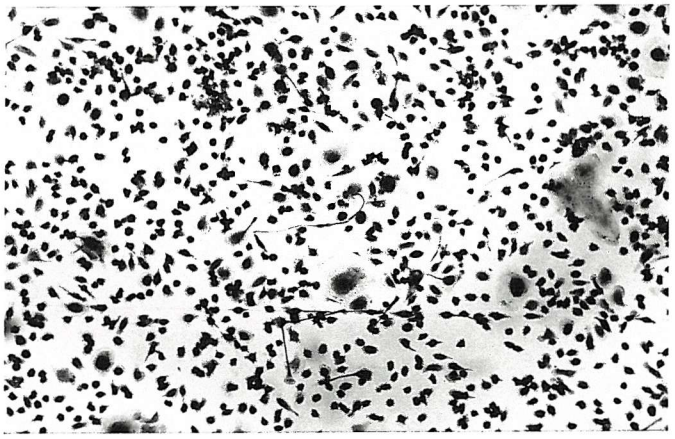
**FIGURE *5·1*: α-Naphthyl Acetate Esterase Activity In Adherent Peripheral Blood Mononuclear Cells Cultured *In Vitro – the effect of fluoride inhibition.***



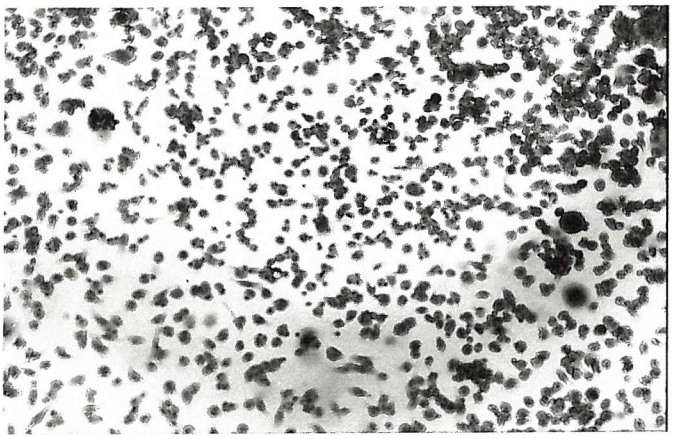
***A*:** Adherent peripheral blood mononuclear cells incubated for 18 hr. The cells were agitated and washed prior to staining for ANAE activity, and counterstained with Mayer's haematoxylin. Original magnification = X100.



***B*:** Cells from the same preparation as in "*A*", with sodium fluoride inhibition. Original magnification = X100.



***C*:** Staining pattern of ANAE after 8 days' incubation in medium containing human serum. Original magnification = X100.



***D*:** Cells from the same preparation as in "*C*", with sodium fluoride inhibition. Original magnification = X100.

When the ANAE cytological procedure [see Section *2E(i)*] was applied to PBMC that remained attached to the substrate after 18 hrs' incubation, gentle shaking, and washing; most of the cells displayed intense black granulation, indicating enzyme activity (see Figure *5·1A*). Furthermore, the addition of sodium fluoride to the reaction resulted in a marked reduction of the black deposits, demonstrating enzyme inhibition in many cells (see Figure *5·1B*). Retention of staining for ANAE was largely restricted to clumps of cells, where lymphocytes remained adherent despite the washing procedure (see Figure *5·1B*).

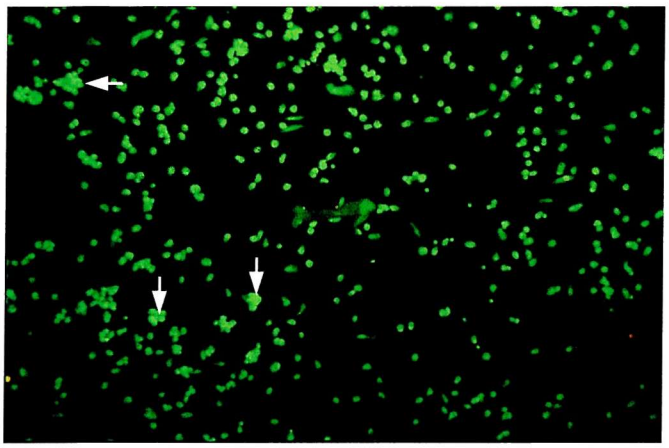
The composition of the culture was remarkably different after incubation in the presence of human serum for eight days, which incorporated two further rounds of shaking and washing. By this stage, contaminating platelets were rare due to detachment or phagocytic elimination. Although cell morphology was heterogeneous - comprising rounded cells, giant cells with large cytoplasmic haloes, and elongated fibroblast-like cells – there was an overall increase in size, accompanied by extremely high levels of ANAE activity which overwhelmed the counterstain (see Figure *5·1C*). Sodium fluoride did not completely inhibit the reaction at this stage, but intense staining was retained in only the largest cells (see Figure *5·1D*).

5B(iii) *Estimating The Quantity & Viability Of Macrophages Cultured* In Vitro: For the design of experiments involving gonococcal interactions with phagocytes, an estimate of the number of macrophages contained in each culture unit was required. This was achieved by extraction of DNA from cell monolayers, followed by quantification of nuclei - using the PicoGreen™ reagent – against a standard curve consisting of serially-diluted PBMC [see Section *2C(iii)*]. The mean number (± standard deviation) of cell nuclei in a 24-well culture plate was 23826±9970 per well.

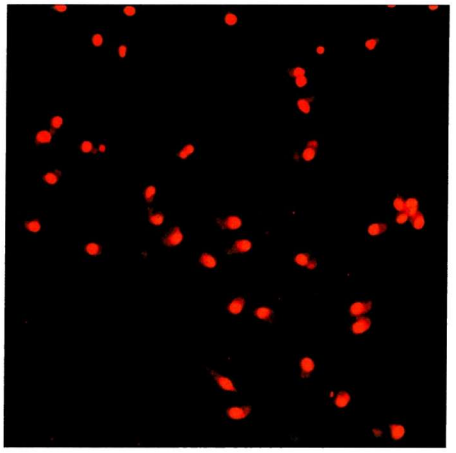
Cell viability was determined by staining unfixed cells with the Molecular Probes LIVE/DEAD® Viability/Cytotoxicity Kit [see Section *2E(i)*]. This kit comprises of two fluorescent nucleic acid stains: a green dye (SYTO® 10) that permeates the plasma membranes of all cells, irrespective of viability; and a red dye (DEAD Red™/ethidium monoazide) which only labels cells with damaged membranes (Haugland, 1996). The dye concentrations are optimised to allow differential staining of live (green) versus dead (red) cells (Haugland, 1996).

After one week of development *in vitro*, the macrophage culture consisted of >99% viable cells (see Figure *5·2A*). The principle of the Molecular Probes assay was

**FIGURE 5·2: Determination Of Macrophage Viability Using The Molecular Probes LIVE/DEAD® Kit – *validation of the principle with saponin permeabilisation.***

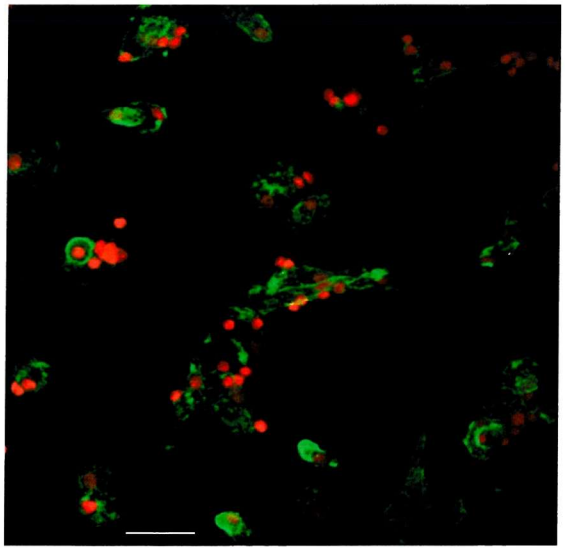


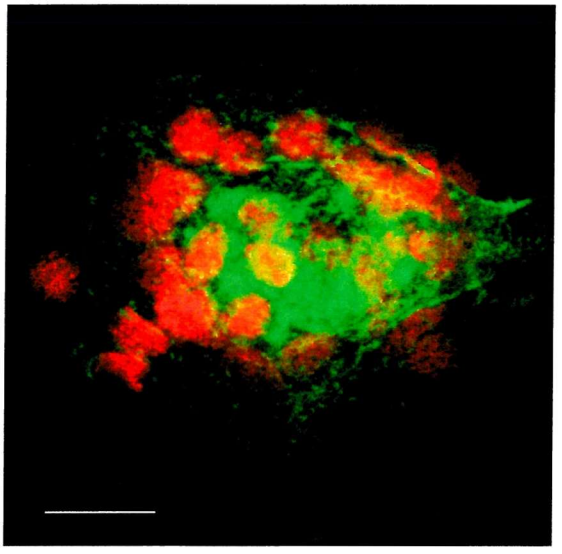
◀ ***A*:** Macrophages stained with SYTO® 10 (green) and DEAD Red™ nucleic acid stains after 7 d *in vitro*. The arrows indicate multinucleated cells. Original magnification = X100.



▶ ***B*:** Cells from the same preparation as in "*A*", permeabilised with saponin prior to staining with the LIVE/DEAD® kit. Original magnification = X200.

**FIGURE 5·3: Morphology Of Differentiated Macrophages Visualised By Confocal Microscopic Analysis Of Phagocytic Glycoprotein-1 Expression.**

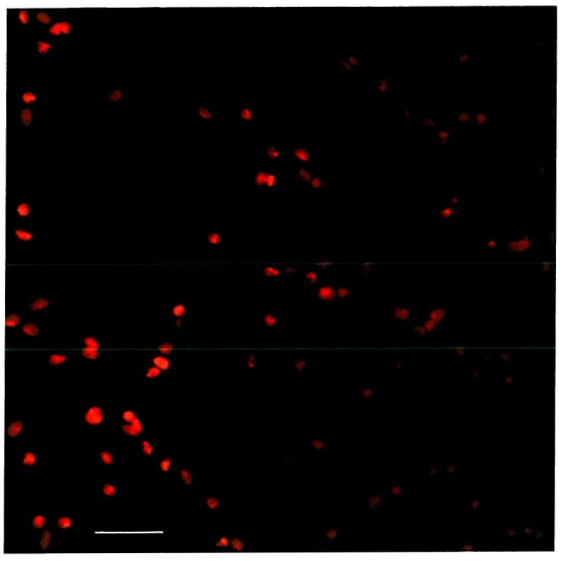




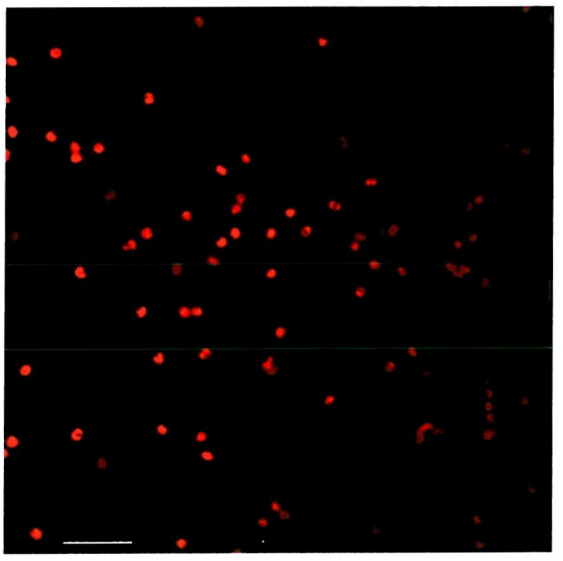
***A*:** A low power field illustrating the expression of phagocytic glycoprotein-1 (FITC conjugated secondary antibody) on macrophages cultured for 11 d. Nuclei are counterstained with propidium iodide. The scale bar represents 50 μm.

***B*:** A single giant cell stained for Pgp-1 expression. A large number of nuclei are revealed by the propidium iodide counterstain. The scale bar represents 20 μm.

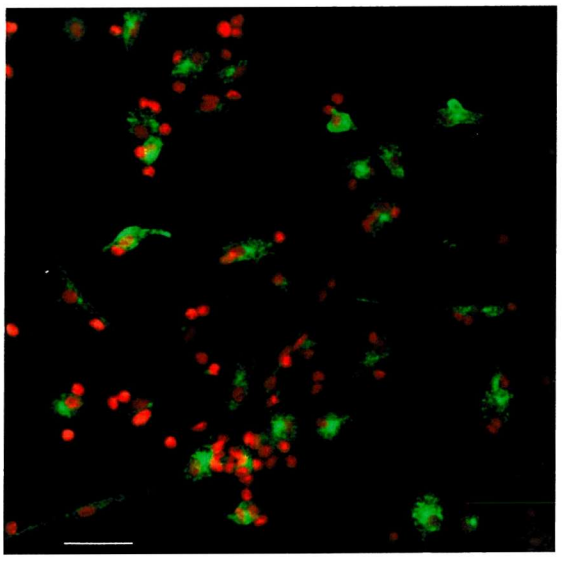
**FIGURE 5-4: Monocyte-To-Macrophage Differentiation Revealed By Confocal Microscopic Analysis Of Mannose Receptor Expression.**



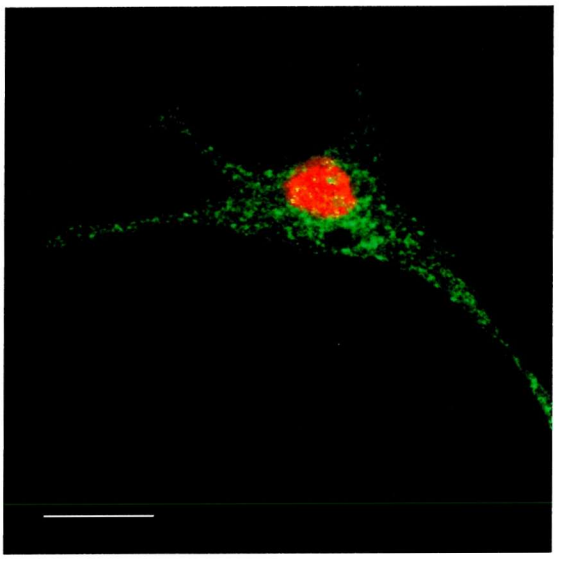
***A*:** Low power field displaying 11-d old macrophages incubated with an isotype-matched control primary antibody (anti-HPV type 16 E7 protein) and FITC-labelled secondary antibody. Nuclei are counterstained with propidium iodide. The scale bar represents 50 μm.



***B*:** Adherent peripheral blood mononuclear cells incubated for 20 hr prior to agitation, washing, and staining for mannose receptor expression. Nuclei are counterstained with propidium iodide. The scale bar represents 50 μm.



***C*:** Low power field displaying macrophages cultured for 11 d and labelled with an anti-mannose receptor antibody. Nuclei are counterstained with propidium iodide. The scale bar represents 50 μm.



***D*:** High magnification image of a single stellate macrophage cell demonstrating mannose receptor expression (FITC-conjugated secondary antibody). The nucleus is counterstained with propidium iodide. The scale bar represents 20 μm.

verified by incubation (37°C) of macrophages with a permeabilising agent (0.1% saponin in PBS with 2% FCS) for 15 min prior to staining. Although many cells detached from the substrate during this procedure, the remaining macrophages were stained exclusively by the DEAD Red™ dye as predicted (see Figure *5·2B*).

5B(iv) *Macrophage Morphology & Differentiation Visualised By Confocal Microscopic Analysis Of Cell Surface Receptor Expression*: Immunological labelling of phagocytic glycoprotein-1 (Pgp-1, CD44), a ubiquitous cell surface receptor which undergoes increased expression during monocyte-to-macrophage differentiation (Levesque & Haynes, 1996), was utilised in a microscopic study of cell morphology [see Sections *2E(ii)* & *2E(iv)*]. Varying degrees of expression between cells (see Figure *5·3A*) were revealed after staining with a FITC-conjugated secondary antibody. However, heterogeneous macrophage morphology was clearly indicated by this marker; which reacted with rounded cells, the processes of elongated cells, and several cells containing two or more nuclei (see Figure *5·3A*). At higher magnification, giant cells (~100 μm in diameter) that displayed intense staining for Pgp-1 were occasionally seen; and large numbers of nuclei were sometimes detected within the perimeter of a single cell by the propidium iodide counterstain (see Figure *5·3B*).

In contrast to Pgp-1, expression of the mannose receptor is restricted to differentiated macrophages and immature dendritic cells, being completely absent from circulating monocytes (Noorman *et al.*, 1997). Accordingly, adherent PBMC cultured for 20 hr prior to incubation with an anti-mannose receptor monoclonal antibody did not react with the fluorescent conjugate as expected (see Figure *5·4B*). However, after 11 d *in vitro*, most cells exhibited high levels of receptor expression, which extended along the processes of fusiform cells (see Figure *5·4C*). At higher magnification, the punctate distribution of this glycoprotein was revealed (see Figure *5·4D*). Macrophages from the same donor were not fluorescently labelled when tested with an isotype-matched control primary antibody (anti-HPV type 16 early protein 7), demonstrating the specificity of the method (see Figure *5·4A*).

## *5C* - Gonococcal Interactions With Macrophages: Attachment & Internalisation

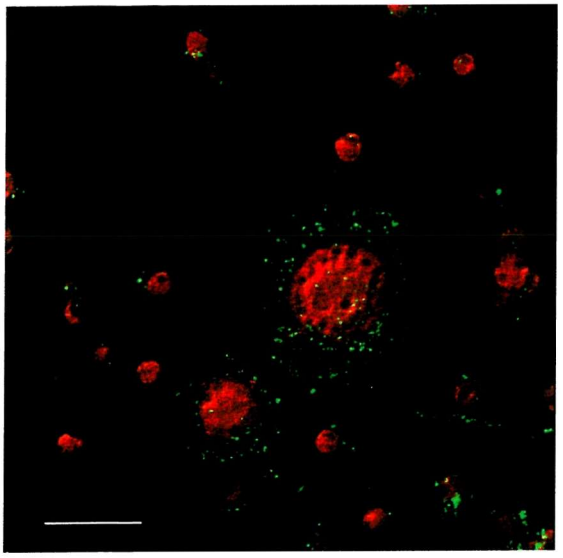
5C(i) *Confocal Microscopic Analysis Of Binding & Uptake Of Gonococci By Macrophages – Experimental Design*: During the characterisation of *in vitro*-grown macrophages described in the previous section, it became apparent that at least two cell types coexisted in cultures maintained for over one week. To ascertain whether macrophages and multinucleated giant cells responded similarly to gonococci, confocal microscopy was utilised to investigate phagocytosis in a culture challenged after 11 days' maturation.

The hypothesis that the macrophage mannose receptor might be involved in non-opsonic phagocytosis (Fraser *et al.*, 1998) of gonococci was investigated by confocal microscopy. This experiment required macrophages to be briefly challenged with a bacterial suspension, washed, and then incubated for different periods prior to fixation and fluorescent labelling. A large inoculum dose was necessary to ensure that the macrophages had a sufficient chance of gonococcal contact, particularly as the period of co-incubation was short, and subsequent washing steps might have removed some loosely attached bacteria.

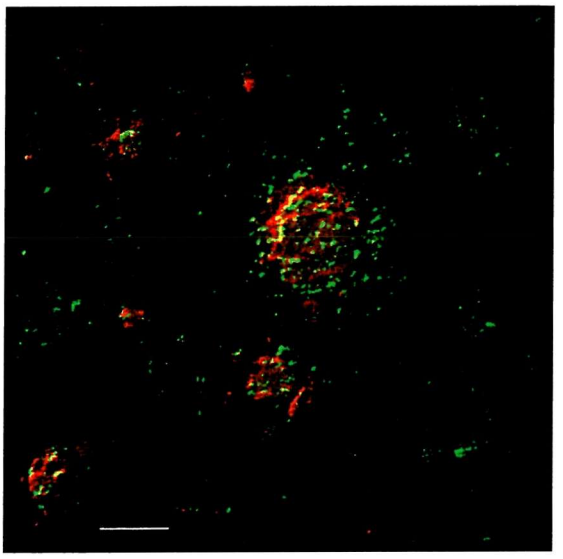
Fluorescent gonococci of the $Opa^-/Pil^-$ phenotype were selected and quantified as described in Sections *2B(iii) & 2B(iv)*, and a suspension in experimental medium was prepared at $1x10^9$ cfu/ml. Macrophage cultures grown on Matrigel®-coated cover slips were agitated and washed [see Section *2C(vi)*] with experimental medium - containing 5 μg/ml ampicillin - before the addition of 0.5 ml gonococcal inoculum/well. The culture plates were then spun at 500 g for 5 min in a Beckman GS-6KR centrifuge, following which the inoculum was gently replaced with fresh medium. One plate was incubated (37°C, 5% $CO_2$) for 10 min only, whilst a second culture was left for 6 hr and rinsed once with fresh medium prior to fixation and staining [see Section *2E(iii)*]. A permeabilisation step was included during fluorescent labelling to facilitate visualisation of the mannose receptor in intracellular stores (Wileman *et al.*, 1984).

5C(ii) *Differential Responses To Gonococcal Challenge Exhibited By Macrophages & Multinucleated Giant Cells*: Following centrifugation and 10 mins' further incubation, gonococci were visible in a "halo" around the periphery of the larger (multinucleated) cells (see Figure *5·5A*). Fewer bacteria adhered to the smaller macrophages, although a higher affinity for these cells compared to the

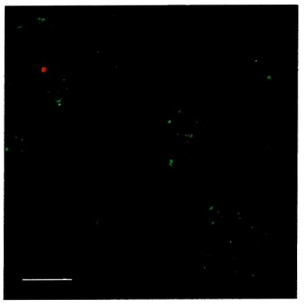
**FIGURE 5·5: Gonococcal Adherence To The Macrophage Surface After Short (10 Minutes) & Prolonged (6 Hours) Periods Of Incubation.**



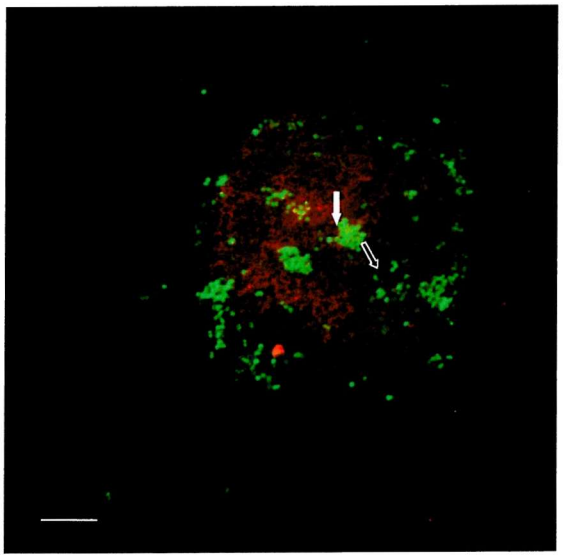
▲ ***A*:** Low power field illustrating mannose receptor expression (TRITC-conjugated secondary antibody) and the distribution of $Opa^-/Pil^-$ $GFP^+$ gonococci on both macrophages and multinucleated cells. The preparation was fixed after 10 mins' incubation. The scale bar represents 50 μm.



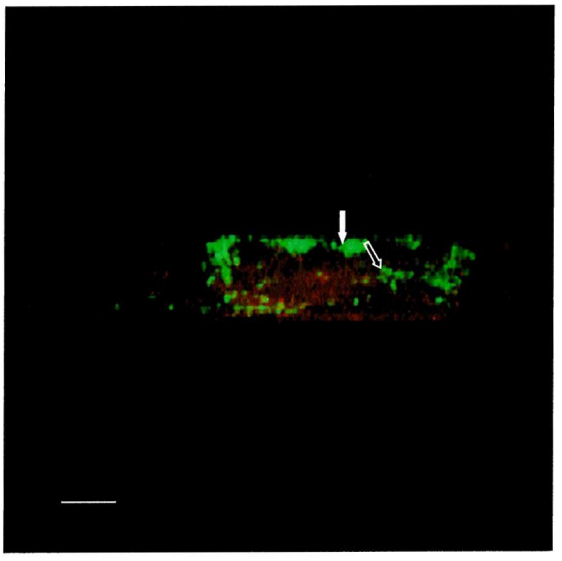
▲ ***B*:** Adherent $GFP^+$ gonococci on mannose receptor-expressing cells (TRITC conjugate) following 6 hrs' incubation. The scale bar represents 50 μm.



⇗ ***C*:** A control preparation labelled with an isotype-matched primary antibody (anti-HPV type 16 E7 protein) in place of anti-mannose receptor antibody. The scale bar represents 50 μm.



▲ ***D*:** A single giant cell stained for mannose receptor expression after 6 hrs' incubation with adherent $GFP^+$ gonococci (apical view). The simulated fluorescence-processing algorithm revealed a non-uniform cell surface, with "peaks" (filled arrow) and "troughs" (arrow outline). The scale bar represents 10 μm.



▲ ***E*:** The same cell as shown in "*D*", rotated through 90° (lateral view). The hemispherical shape of the cell is apparent; and gonococci can be seen attached to the apical surface, on the sides of the cell, and around the periphery. The points labelled in the apical view are also indicated on this image. The scale bar represents 10 μm.

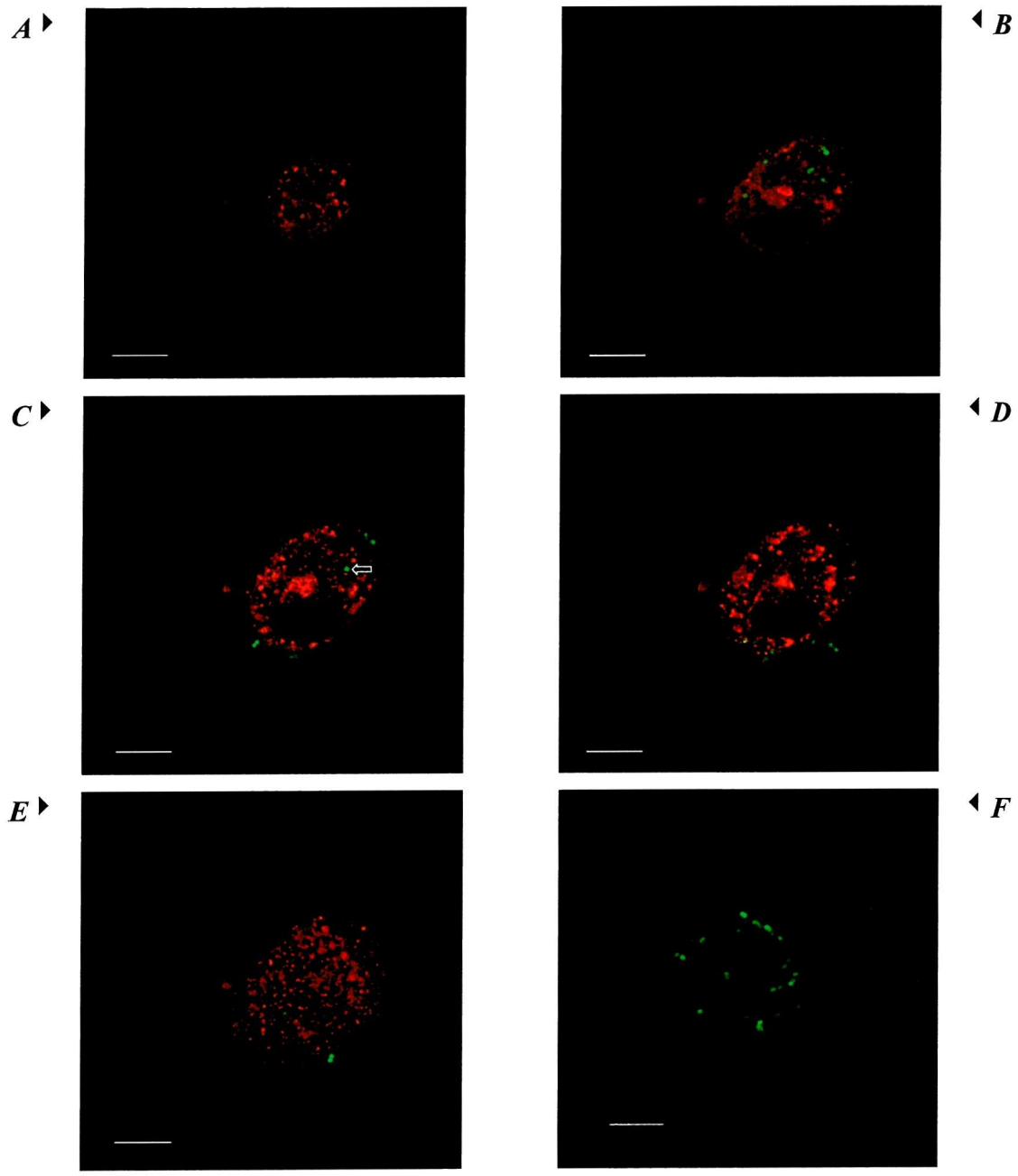
Matrigel®substrate was evident. In contrast to uninfected macrophages (see Figures *5·3 & 5·4*), the challenged cells were rounded and contracted, lacking elongated processes (see Figure *5·5A*). By 6 hr post-inoculation, the larger cells were frequently covered with bacterial aggregates, and more gonococci were visible on the background than was observed previously (see Figure *5·5B*). A control preparation of infected cells, incubated with an anti-HPV type 16 E7 protein monoclonal antibody (mAb) instead of the anti-mannose receptor mAb, confirmed that the TRITC labelling was specific for the intended target (see Figure *5·5C*).

In order to locate cell-associated gonococci precisely, single cells were scanned at high magnification. One method for examining the relationship between two fluors is co-localisation analysis, which detects and semi-quantifies superimposition. Therefore, as the emission profile of TRITC overlaps with that of green fluorescent protein (GFP), preparations reserved for high-power scanning were labelled with a secondary antibody conjugated to cyanine-5 (Cy5). This fluor is more photostable than TRITC, and has a far-red emission maximum (670 nm).

Co-localisation of Cy5 (labelling the anti-mannose receptor mAb) and GFP was not observed with either the cells fixed after 10 min or at 6 hr. Therefore, alternative methods were utilised to determine the position of gonococci. Large numbers of gonococci were frequently associated with giant cells, even after 6 hrs' incubation, but the bacteria appeared to be surface-exposed in images that accentuated depth perception [simulated fluorescence processing (SFP); see Figure *5·5D* & Section *2E(iv)*]. Although gonococci were present at more than one level in relation to the cell, as revealed by computer-simulated rotation of the image through 90° (see Figure *5·5E*), this was more consistent with adherence to a ruffled surface than with internalisation. In addition, optical sectioning through the giant cell supported the interpretation that several gonococci were located in a trough-like depression (data not shown).

In contrast to the observations of multinucleated cells, evidence for gonococcal phagocytosis was obtained with macrophages after only 10 min of interaction. A single representative cell was scanned in 0.96-μm intervals, and the resultant optical sections were examined for internalised bacteria. Whereas the apical portion of the cell did not contain gonococci within the perimetric boundary (see Figure *5·6A*), two sections taken two-thirds of the way into the macrophage revealed several phagocytosed organisms (see Figures *5·6B & C*). The disappearance of these bacteria from subsequent sections provided further support for their intracellular location (see Figures *5·6D & E*).

**FIGURE 5·6: Optical Sectioning Through A Macrophage Fixed After 10 Minutes' Incubation With Fluorescent Gonococci – *evidence for rapid phagocytosis.***



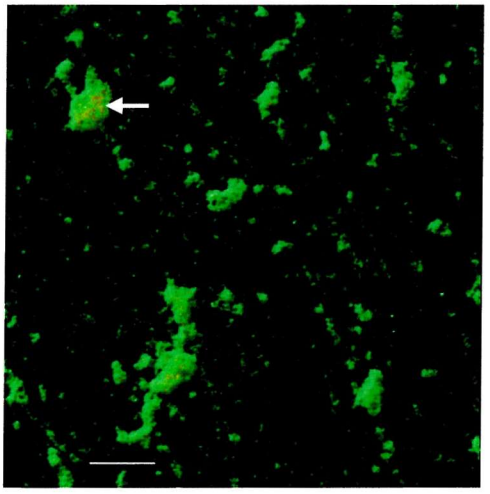

***A*:** The apex of a single macrophage, stained with a Cy5-conjugated secondary antibody to reveal mannose receptor expression. This image is a composite of seven sections, representing a slice 6.7 μm thick. ***B*:** The first bacteria that appear to lie within the perimeter of the macrophage are seen at a depth of 14.4 μm. The large unstained region of the cell indicates the position of the nucleus. This image represents a single 0.96 μm-thick section. ***C*:** In contrast to the previous image, this single section (depth 16.3 μm) displays only a single internalised gonococcus (arrow), demonstrating the precision of the technique for localising bacteria. ***D*:** At a depth of 18.2 μm, gonococci are restricted to the periphery of the macrophage (single-section image). ***E*:** The base of the cell (22.1 μm from the apex; single-section image). ***F*:** Control image displaying a different infected macrophage fixed at the same time-point. The anti-mannose receptor mAb was substituted with anti-HPV type 16 E7 protein to demonstrate the specificity of fluorescent (Cy5) staining. This image is a 3-D construction of the entire cell.

*- All scale bars represent 10 μm. The cell height ("A"-"E") was 24 μm, indicating a parabolic shape. -*

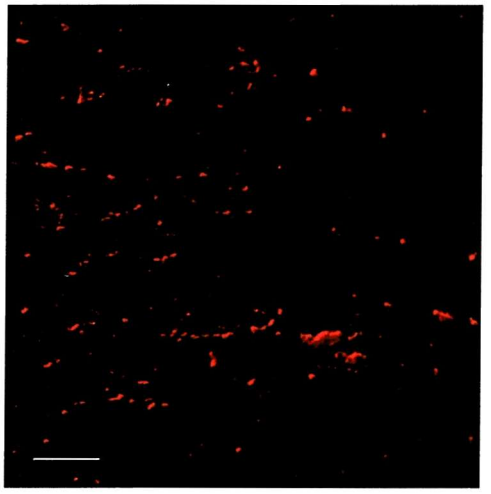
5C(iii) *Viability Of Macrophages, Multinucleated Giant Cells, & Gonococci Following 6 Hours' Co-incubation*: To determine the outcome of gonococcus-macrophage interactions after 6 hrs' incubation, a macrophage culture was challenged with Opa-b$^{+}$/Pil$^{-}$ fluorescent gonococci, as described for the phagocytosis study. Unfixed cells were then stained with the DEAD Red™ dye from the Molecular Probes LIVE/DEAD® Viability/Cytotoxicity Kit [see Section *2E(iii)*], enabling visualisation of both dead gonococci and dead macrophages. Thus, if the two types of monocyte-derived cells differed in their ability to destroy gonococci, this would be identified.

The applicability of the LIVE/DEAD® kit to bacteria expressing GFP was confirmed in a validation study. Gonococci (~5x10$^{8}$ cfu/well) were spun onto Matrigel®-coated cover slips without macrophages, and incubated in parallel with the other plate. The adherent organisms were then either stained immediately in the same manner as the

**FIGURE *5·7*: Viability Of Gonococci Determined By The Molecular Probes LIVE/DEAD® Kit – *validation experiment.***



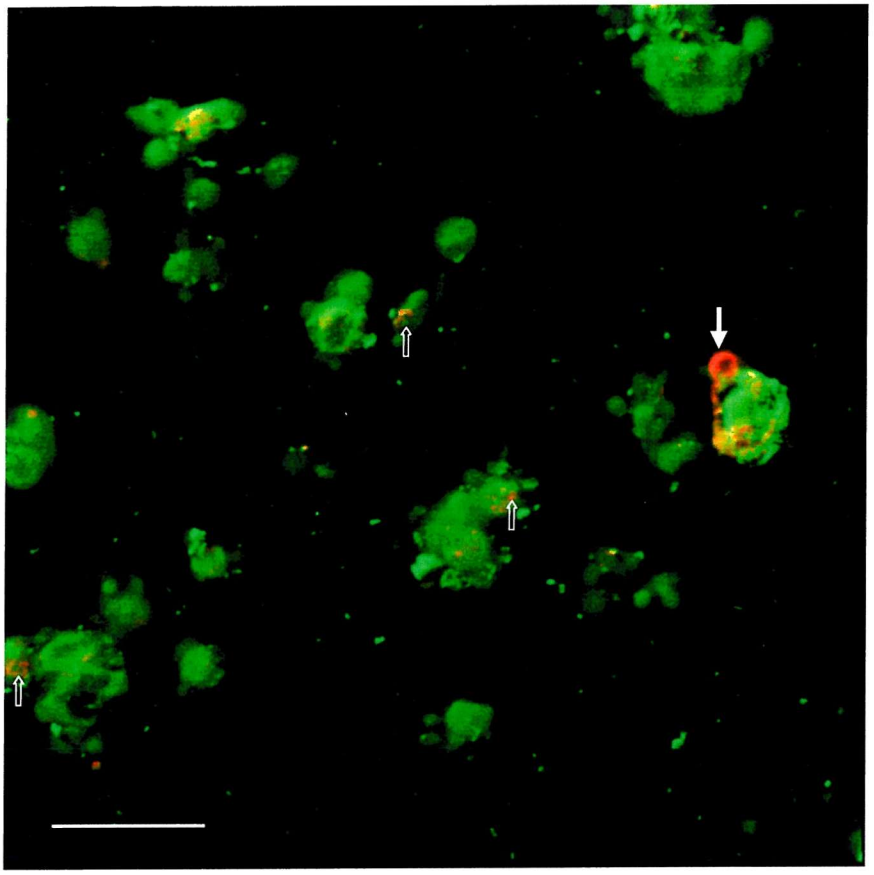
***A*:** Fluorescent gonococci (Opa-b$^{+}$/Pil$^{-}$) attached to the Matrigel® substrate following 6 hrs' incubation in the absence of macrophages. The DEAD Red™ dye has stained several organisms contained in the largest aggregates (arrow). The scale bar represents 50 μm.



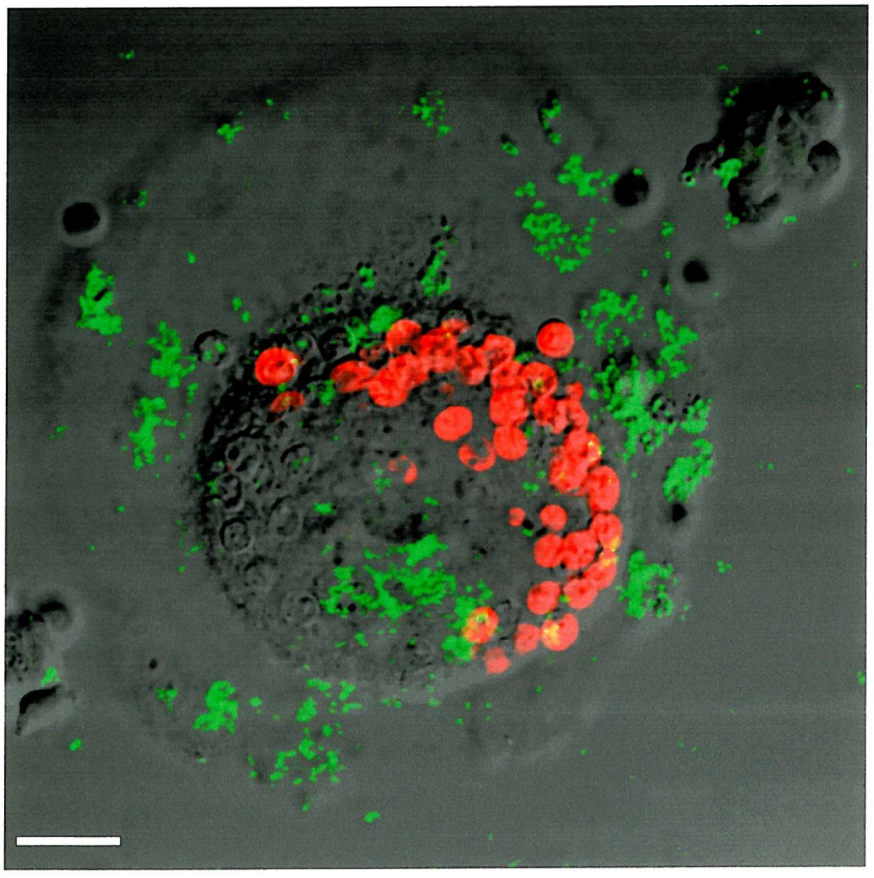
***B*:** A similar preparation of gonococci to that shown in "*A*", exposed to isopropanol for 15 min prior to incubation with the LIVE/DEAD® kit components. The bacteria display uniform red staining. The scale bar represents 50 μm.

phagocytes [see Section *2E(i)*], or incubated for 15 min (RT°C) with 200 μl/well pure isopropanol before addition of the kit components. The outcome was unequivocal, as displayed in Figure *5·7*, supporting the proposed experimental design.

**FIGURE 5·8: Application Of The DEAD Red™ Nucleic Acid Stain To A Culture Of Macrophages & Giant Cells Infected With Gonococci.**



***A*:** Macrophages incubated with Opa-b$^{+}$/Pil$^{-}$ GFP$^{+}$ gonococci for 6 hr prior to staining with the DEAD Red™ dye. The natural autofluorescence of these cells was enhanced using the SFP algorithm, producing a less vivid green than that exhibited by GFP. The filled arrow indicates the stained nucleus of a dead cell, the remnants of which are obscured by aggregated gonococci. Small clusters of red gonococci are associated with many of the viable macrophages (arrow outlines). The scale bar represents 50 μm.



***B*:** A single multinucleated giant cell from the same culture as the macrophages in "*A*". The extent of the cytoplasm was visualised by differential interference contrast microscopy, and this greyscale image was combined with the green (GFP) and red (DEAD Red™) channels. Both stained and unstained nuclei are present in the central region, but red gonococci are absent. The scale bar represents 20 μm.

Low power fields were scanned for cell nuclei stained with DEAD Red™. Very few dead macrophages were observed in the infected cultures, indicating that despite their rounded appearance, most phagocytes maintained membrane integrity during interactions with gonococci. The occurrence of cell death appeared to be associated with large numbers of adherent bacteria that formed a "blanket" over the macrophage surface (see Figure *5·8A*). In contrast, viable macrophages were frequently associated with red-stained organisms (see Figure *5·8A*), suggesting a process of gonococcal destruction.

Conversely, in gonococcus-challenged giant cells, nuclei stained with DEAD Red™ were observed on numerous occasions, despite the high rate of viability preceding infection (see Figure *5·2A*). The affected cells were often surrounded by a "halo" of aggregated gonococci that adhered to the extensive peripheral cytoplasm (see Figure *5·8B*), providing an explanation for the distribution of gonococci seen in the low power image at 10 min (see Figure *5·5A*). Some of the giant cells were found to contain both stained and unstained nuclei, implying ongoing cell death within the syncytium; but red-fluorescent gonococci were not apparent (see Figure *5·8B*).

## *5D* - Investigating The Effects Of Gonococcal Surface Phenotype On The Macrophage Cytokine Response: Stimulation With Viable Bacteria.

5D(i) *Cytokine Profiles Over Time - Experimental Design*: In the preceding experiments, several key components of gonococcal-macrophage interaction were elucidated. The microscopic studies demonstrated that macrophages bound gonococci and internalised them rapidly, leading to the death of cell-associated organisms. To determine whether macrophages responded to viable gonococci by releasing proteins involved in the mediation and regulation of inflammation, cell culture medium was sampled at five time-points following challenge and assayed for several cytokines. Although several variant organisms were used to stimulate macrophage cultures, the principal purpose of this experiment was to identify an optimum incubation period for a single time-point study, which would compare the effects of gonococcal surface phenotype on cytokine induction in more detail.

The fluorescent gonococcal phase-variants described in Section *2B(i)* were cultured, maintained, suspended and quantified as explained in Sections *2B(ii)*, *2B(iii)* & *2B(iv)*. Macrophages grown in 96-well plates were agitated, washed with experimental medium [see Section *2C(vi)*], and covered with 100 µl of fresh medium (containing 5

μg/ml ampicillin throughout) prior to the addition of bacteria. A separate plate of cells was prepared for each of the time-points studied. The gonococcal inocula (100 μl) in experimental medium were added to four replicate wells, to give challenge concentrations of both $1x10^7$ and $1x10^6$ cfu/ml for all five variants. Diluent medium was added to eight control wells on each plate, which were then incubated at 37°C with 5% $CO_2$.

At 0 hr, 1 hr, 3 hr, 9 hr and 27 hr post-infection, one plate was removed from the incubator and 150 μl of medium was transferred "well-for-well" into a 96-well, "V"-bottomed plate. This was sealed with adhesive foil and spun at 1500 g for 10 min in a Beckman GS-6KR centrifuge. Supernatants (100 μl/well) were transferred to 96-well assay blocks; these were sealed with adhesive foil and stored frozen at -70°C. To permit ready screening of the experiment, the supernatant from one well of each set of replicates was stored in a single assay block.

5D(ii) *Preliminary Results - Processing And Analysis Of Induced Cytokine Data*: Time-resolved fluorescence immunoassays [see Section *2F(ii)*] were conducted for nine cytokines on the aliquots of macrophage medium reserved for screening. Evaluation of the raw data [relative fluorescence units (RFU)] revealed that four cytokines (IL-1β, IL-10, IL-12 and GM-CSF) were not detected at any time-point and with all gonococcal phenotypes; these were not pursued further. Five cytokines (IL-6, IL-8, TNF-α, RANTES and MIP-1α) displayed time-dependent increases in RFU; these were converted into concentrations (pg/ml) and the mean "time-zero" value was subtracted from all the results (see Figure *5·9*).

Cytokine release from stimulated cells is regulated by a complex series of signal transduction pathways, synergistic interactions between inflammatory mediators, and autocrine feedback loops (Dinarello, 1997; Trinchieri, 1997). Therefore, minute changes in the cellular microenvironment may be expected to result in considerable variability between replicates in a single treatment group. Accordingly, statistical analyses were performed on cytokine concentrations that had undergone a $\log_{10}(x+1)$ transformation; which is appropriate when the variance of a sample is greater than the mean, and the data includes values of zero (Fowler & Cohen, 1990). This enabled the use of analysis of variance (ANOVA) - a statistically powerful parametric test suitable for the simultaneous comparison of a large number of means - whilst avoiding conflict with assumptions regarding data distribution (Fowler & Cohen, 1990). The significance of the difference between particular mean-pairs was then determined by post-hoc tests.

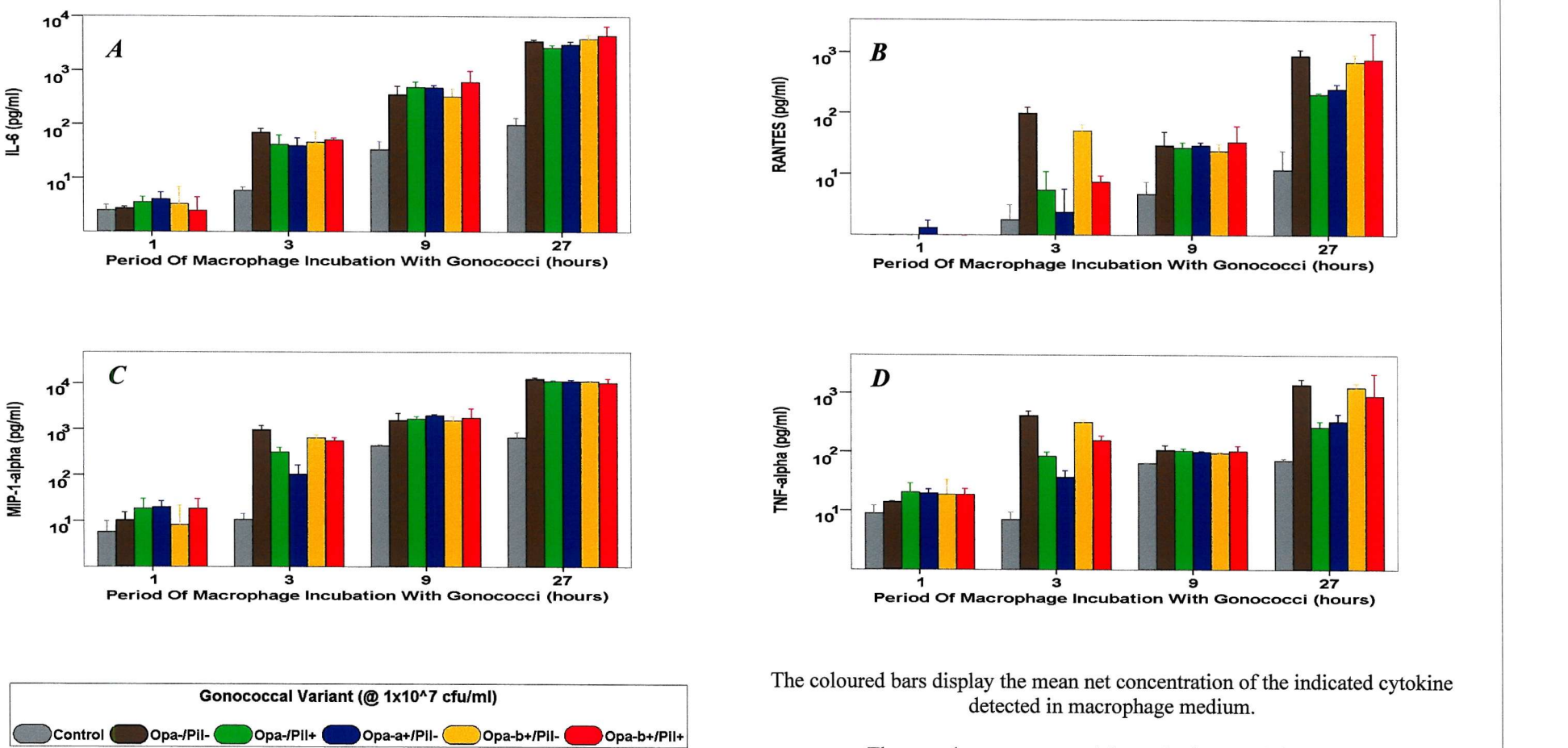
When cells are exposed to bacteria for prolonged incubation periods, growth of organisms in the culture medium and the ensuing toxic by-products are likely to interfere with cytokine production. Therefore, macrophage responses at later time-points were anticipated to be more variable than at the beginning of the experiment. This effect was taken into account by performing Levene's test for homogeneity of variances prior to the ANOVA, with the outcome determining the choice of post-hoc test: Tukey's honestly significant difference test (where variances were equal), or the more stringent Tamhane's T2 test (where variances were significantly different).

Finally, to determine whether bacterial concentration significantly affected macrophage responses, the data was split into separate groups for each gonococcal variant at each time-point, and the mean levels of each cytokine were compared between the $1x10^7$ and $1x10^6$ cfu/ml inocula by independent *t*-tests. Because of the large number of comparisons, statistical significance was only accepted at the $P < 0.01$ level. In the vast majority of cases, no significant effect of gonococcal concentration was found. However, exceptions were seen with the $Opa^-/Pil^-$ variant at 3 hr post-stimulation for RANTES, MIP-1α and TNF-α; and at 27 hours for IL-8. At these points, $1x10^7$ cfu/ml produced a significantly greater cytokine response than did $1x10^6$ cfu/ml ($P < 0.01$ for all comparisons). Therefore, for simplicity, subsequent analyses were restricted to the higher concentration only.

5D(iii) *The Macrophage Cytokine Response To Gonococcal Inocula Of Different Phenotypes – Profiles Over A 27-Hour Time Course*: The results were split into four groups based on time-point for each cytokine, and the mean cytokine concentrations for each stimulus ($n = 3$) were compared to each other (see below) and the control ($n = 6$) by one-way ANOVA. Critical probabilities for the analysis with respect to the controls are displayed in **Table *A3·1*, Appendix *3***. At 1 hr post-stimulation, none of the cytokines were significantly elevated above control levels. However, one or more of the gonococcal variants induced statistically significant production of IL-6, TNF-α, MIP-1α, and RANTES at all the subsequent time-points relative to unchallenged cells. In contrast, IL-8 levels were only significantly increased in stimulated cells at the 3-hr time-point, and the mean concentration in control supernatants by 27 hr exceeded 4 ng/ml.

The response profiles over time for IL-6, TNF-α, MIP-1α, and RANTES are displayed in Figure *5·9*. A two-way ANOVA, with time-point and gonococcal phenotype as fixed factors, enabled changes in the cytokine profiles over the course of the

**FIGURE 5·9: Production Of Proinflammatory Cytokines By Macrophages In Response To Viable Gonococcal Cells Of Different Phenotypes – *profiles over time.***




The coloured bars display the mean net concentration of the indicated cytokine detected in macrophage medium.

The error bars represent +1.0 standard error of the mean.

experiment to be investigated independently of the different stimuli applied to the cells. Significant increases in the concentrations of IL-6, IL-8, MIP-1α, RANTES, and TNF-α were identified during both the 1-3 hr and 9-27 hr intervals ($P$ <0.001 for all comparisons). However, only IL-6, IL-8, and MIP-1α exhibited significant net production within the 3-9-hr period ($P$ <0.001 in all cases).

Significant differences in the stimulatory capacities of the five gonococcal phenotypes were restricted to the early phase (3 hr) of the experiment - at later time-points, the responses became more homogeneous (see Figure *5·9*). Expression of Opa-a was associated with a reduction in the intensity of the macrophage cytokine response when compared with variants expressing Opa-b or no Opa proteins. Thus, stimulation by Opa-a$^{+}$/Pil$^{-}$ gonococci was less effective than with either the Opa-b$^{+}$/Pil$^{-}$ or Opa$^{-}$/Pil$^{-}$ variant for: RANTES ($P$ <0.05, $P$ <0.01; respectively), MIP-1α (both $P$ <0.01), and TNF-α ($P$ <0.01, $P$ <0.001; respectively). In addition, organisms expressing Opa-a were not as potent as the Opa-negative phenotype in generating IL-8 ($P$ <0.05).

Piliation was also found to have a negative effect on cytokine output from stimulated cells. The Opa$^{-}$/Pil$^{+}$variant induced significantly lower concentrations of both RANTES ($P$ <0.05) and TNF-α ($P$ <0.01) relative to Opa$^{-}$/Pil$^{-}$ organisms.

The observed rate of cytokine release and the preliminary comparisons between gonococcal phenotypes indicated that a more detailed analysis should take place at an early time-point. However, although interim plateaux were evident between 3 hr and 9 hr for RANTES and TNF-α (see Figures *5·9B & D*), substantial increases in concentration were demonstrated during the same interval by IL-6, IL-8, and to some extent MIP-1α. Therefore, 6 hr was judged to be the optimum incubation period, as it would allow several cytokines to reach appreciable levels in the cell medium, and might enable detection of additional cytokines with delayed expression. Additionally, the confounding influences of autocrine feedback mechanisms and bacterial replication in the medium would be minimised.

5D(iv) *The Effects Of Gonococcal Surface Phenotype On The Binding Capacity, Killing Efficiency, & Cytokine Response Of Macrophages – Experimental Design*: In this study conducted at 6 hr post-challenge, sampling of the culture medium for cytokines was undertaken in parallel with quantification of bacteria, which were classified as "cell-associated" or "intracellular". Hence, the hypothesis that macrophage responses were correlated with the avidity of particular gonococcal phenotypes for these cells was

investigated. The methodology comprised saponin-mediated lysis of challenged macrophage cultures before and after addition of the antibiotic gentamicin, followed by inoculation of agar plates with the lysate. Discrimination between cell-associated and intracellular organisms was possible because uptake of gentamicin by phagocytes is extremely limited (Vaudaux & Waldvogel, 1979); whilst the detergent saponin disrupts eukaryotic cell membranes without significantly affecting bacterial viability (Read *et al.*, 1996).

Five gonococcal phase-variants ($Opa^-/Pil^-$, $Opa\text{-}a^+/Pil^-$, $Opa\text{-}b^+/Pil^-$, $Opa^-/Pil^+$, and $Opa\text{-}b^+/Pil^+$) were selected, cultured, suspended and quantified as detailed in Sections *2B(ii)*, *2B(iii)* & *2B(iv)*; following which each variant was diluted to a presumptive concentration of $2x10^7$ cfu/ml in experimental medium. In order to correct for viability differences between the five preparations, each gonococcal suspension was diluted, inoculated onto GC-agar in triplicate, and incubated overnight for subsequent counting. During the day preceding the infection experiment, the ampicillin-containing medium in 24-well macrophage cultures was replaced with antibiotic-free experimental medium, allowing overnight equilibration. Additionally, macrophages were shaken and washed as normal [see Section *2C(vi)*] immediately before the addition of the gonococcal variants or control medium at 0.5 ml/well (six replicates per phenotype/uninfected control). In parallel, gonococci were also added to a Matrigel®-coated control plate, from which macrophages were absent (four replicates per phenotype).

All of the incubations in this experiment were conducted at 37°C in 5% $CO_2$. Washes were undertaken with pre-warmed 2% FCS in PBS (1 ml/well), whilst the gonococcal diluent was 2% FCS in Hanks' balanced salt solution. Following 6 hrs' incubation, aliquots of cell culture medium were transferred from the 24-well plates to a 96-well assay block - entailing a one-in-two dilution in assay buffer [see Section *2E(ii)*] containing 0.1% (v/v) polyoxyethylene ether W-1 – and stored at -70°C. Subsequently, each replicate group was divided in half to allow differential processing. One subset was washed four times and incubated with 0.5 ml/well 1% sterile-filtered saponin in wash solution for 15 min. The surface of these wells was then scraped, and the suspensions were diluted 1/100 and 1/1000 before inoculation onto GC-agar (50 µl/plate).

The remaining wells were washed twice prior to incubation with 0.5 ml/well gentamicin solution (200 µg/ml in experimental medium) for 1.5 hr. This subset was then subjected to the washing and saponin-treatment procedure, and suspensions were spread onto agar without dilution (macrophage and control plates), or were diluted 1/10

(occupied wells only). Agar plates were incubated for 36 hr, prior to enumeration of gonococcal colonies using a ProtoCOL automated colony counter (Synbiosis).

5D(v) *Quantification Of Binding & Uptake Of Gonococci By Macrophages – Differences Between Variants*: The counts for the optimal dilution from each well were converted to cfu/well, adjusting for differences in viability by comparison with the Opa$^-$ /Pil$^-$ inoculum. Geometric means, which are less affected by extreme values than arithmetic means, were calculated for viable counts before and after the application of gentamicin. Although a proportion of gonococcal binding was clearly attributable to the Matrigel® coating, the mean recovery from occupied wells was always much greater than from empty wells (see Table *5·1*). Furthermore, incubation of control wells with gentamicin completely eliminated substrate-adherent gonococci of all five phenotypes (see Table *5·1*).

**TABLE *5·1*: Relative Affinity Of Various Gonococcal Variants For Macrophages Or Matrigel® Basement Membrane Matrix – *effect of gentamicin treatment.***

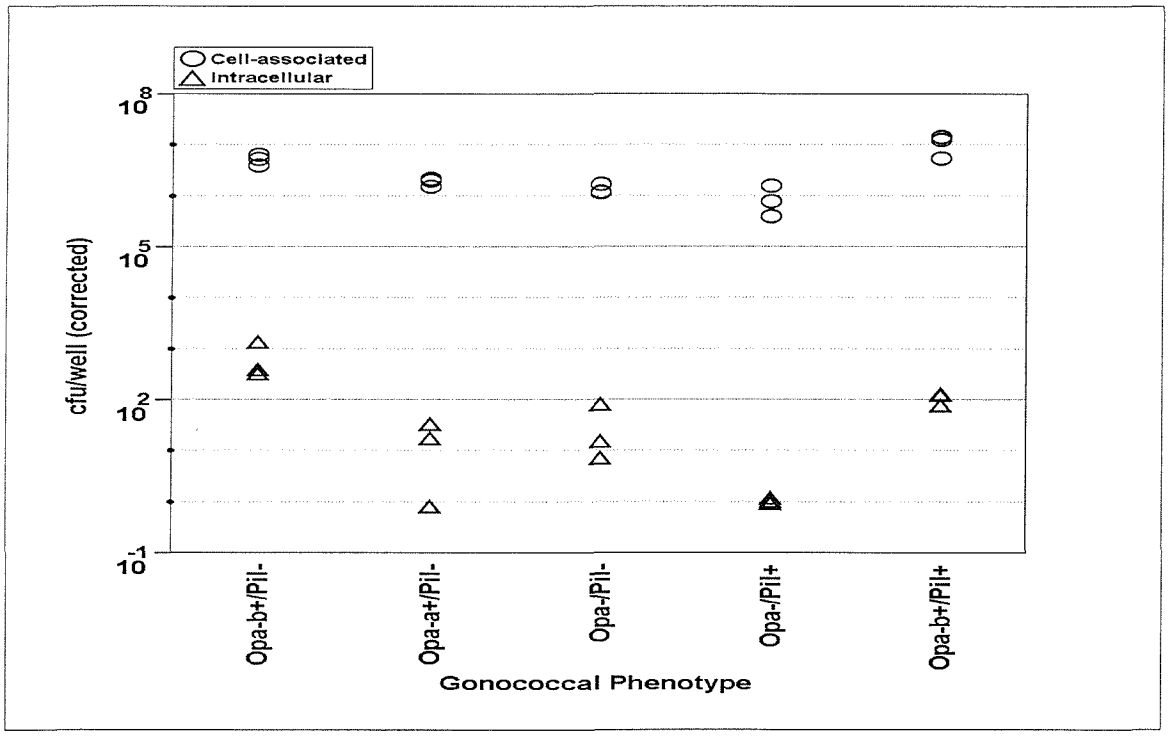
| | GEOMETRIC MEAN CFU/WELL (x/÷ standard error) | | | |
|---|---|---|---|---|
| | GENTAMICIN ABSENT | | GENTAMICIN PRESENT | |
| GONOCOCCAL VARIANT | MACROPHAGES PRESENT* | MACROPHAGES ABSENT# | MACROPHAGES PRESENT* | MACROPHAGES ABSENT# |
| OPA$^-$/PIL$^-$ | $2.0\times10^6$ x/÷ 1.13 | $2.1\times10^4$ x/÷ 10.75 | $2.8\times10^1$ x/÷ 1.93 | 0 |
| OPA-A$^+$/PIL$^-$ | $2.8\times10^6$ x/÷ 1.11 | $5.0\times10^5$ x/÷ 1.56 | $7.5\times10^0$ x/÷ 3.12 | 0 |
| OPA-B$^+$/PIL$^-$ | $5.6\times10^6$ x/÷ 1.16 | $3.8\times10^5$ x/÷ 1.56 | $5.0\times10^2$ x/÷ 1.69 | 0 |
| OPA$^-$/PIL$^+$ | $1.2\times10^6$ x/÷ 1.45 | $5.2\times10^4$ x/÷ 2.32 | 0 | 0 |
| OPA-B$^+$/PIL$^+$ | $9.2\times10^6$ x/÷ 1.31 | $1.2\times10^6$ x/÷ 5.92 | $1.3\times10^2$ x/÷ 1.29 | 0 |

*$N = 3$. #$N = 2$.

In order to compare the cell-specific component of total attachment between gonococcal variants, adjusted counts were calculated for each occupied well by subtraction of the relevant geometric mean count for empty wells (see Figure *5·10*). This produced a conservative estimate of cell-associated attachment, as the area of exposed substrate matrix in occupied wells was considerably less than in wells lacking macrophages. The role of Opa phenotype in determining gonococcal avidity for macrophages was investigated in a comparison between the non-piliated variants by

independent *t*-tests. This revealed that expression of Opa-b was associated with a significantly greater mean level of adherence than were the $Opa^-$ and $Opa\text{-}a^+$ phenotypes ($P < 0.05$ in both cases; see Figure *5·10*). In contrast, there was no significant difference between the mean numbers of cell-associated $Opa\text{-}a^+$ and Opa-negative organisms. Piliated organisms were not included in the analysis of cell-associated counts because their degree of adherence to Matrigel® was variable (see Table *5·1*) and, unlike $Pil^-$ variants, could not be distinguished in *t*-tests from attachment in the presence of cells. However, visual inspection of the data (see Figure *5·10*) suggested that pili had little effect on gonococcal avidity for macrophages.

**FIGURE *5·10*: The Effects Of Gonococcal Phenotype On Association With Macrophages & Intraphagocytic Survival.**




With the exception of $Opa^-/Pil^+$ gonococci, all of the variants survived in gentamicin-treated macrophages (see Figure *5·10*). However, the mean number of survivors never exceeded ~0.01% of the mean cell-associated level, equivalent to an average of only one viable intracellular bacterium ($Opa\text{-}b^+/Pil^-$ variant) per 50 macrophages. Microscopic examination of the colonies produced by the intracellular gonococci provided no evidence indicating that a phenotypic shift from the dominant inoculum type had occurred.

In order to identify possible differences in the mean number of survivors between variants, one-way ANOVA was conducted on viable counts following $\log_{10}(x+1)$ transformation. The significance of the difference between particular mean-pairs was then ascertained using Tamhane's T2 test. A greater mean number of Opa-b$^+$/Pil$^+$ organisms survived gentamicin treatment compared to the Opa$^-$/Pil$^+$ variant ($P$ <0.05). There was also a ~20-fold greater geometric mean number of intracellular Opa-b$^+$/Pil$^-$ colonies than Opa$^-$/Pil$^-$ colonies - which corresponded to a ~65-fold excess compared to the Opa-a$^+$/Pil$^-$ variant - but these differences were not significant (see Table *5·1*). Conversely, pilus expression was associated with a decrease in intracellular counts relative to equivalent non-piliated phenotypes, although this trend was not significant (see Figure *5·10*).

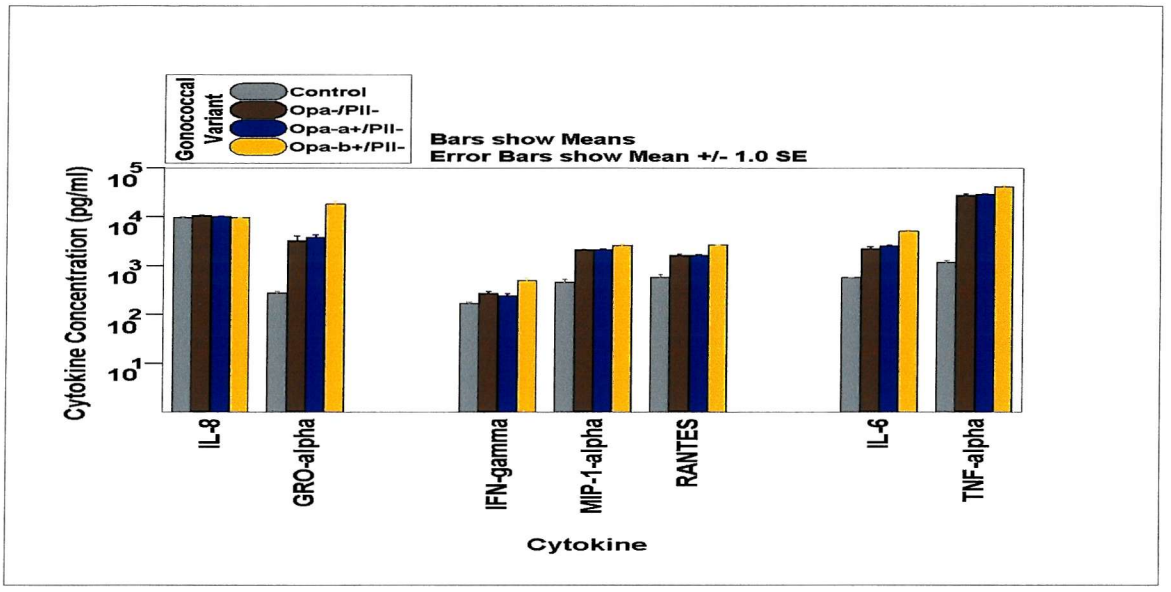
5D(vi) *The Effects Of Gonococcal Surface Phenotype On The Macrophage Cytokine Response – Comparisons At 6 Hours*: To ascertain whether macrophage responses were linked to the avidity of gonococci for these cells, the levels of six cytokines involved in inflammation (IL-6, IL-8, TNF-α, IFN-γ, RANTES, and MIP-1α) were determined by fluorescence immunoassay [see Section *2F(ii)*]. In addition, colorimetric enzyme immunoassay kits were used for the detection of ENA-78 and GRO-α [see Section *2F(iii)*].

Examination of the raw data revealed that ENA-78 was not present in any of the samples collected during this experiment. The concentrations of the remaining seven cytokines were calculated, and one-way ANOVA (with gonococcal phenotype as the factor) was conducted on $\log_{10}(x)$-transformed values. The significance of the difference between particular mean-pairs was determined by post-hoc tests as described previously [see Section *5D(ii)*]. To simplify analysis and discussion of the findings, the cytokines were allocated to three groups based on functional overlap: "inflammatory mediators" (IL-6 & TNF-α), neutrophil-chemoattractive "CXC chemokines" (IL-8 & GRO-α), and a third group that has important effects on "monocyte/macrophage" function (MIP-1α, RANTES, & IFN-γ). All critical probabilities referred to in this section are summarised in **Table *A3·2*, Appendix *3***.

The inflammatory mediators exhibited statistically highly significant production with all the non-piliated variants when compared to the uninfected controls ($P$ <0.001). Comparisons between these variants revealed that expression of Opa-b led to greater mean responses for IL-6 and TNF-α (see Figure *5·11*), relative to both Opa$^-$ ($P$ <0.001, $P$ <0.01; respectively) and Opa-a$^+$ ($P$ <0.001, $P$ <0.01; respectively). However, there were

no significant differences between the levels of these cytokines induced by the Opa-a$^+$ variant and Opa$^-$ gonococci.

**FIGURE *5·11*: The Effect Of Gonococcal Opacity Protein Phenotype On Proinflammatory Cytokine Release From Macrophages – *comparisons at 6 hours.***




Within the "monocyte/macrophage" group, the mean concentrations of MIP-1α and RANTES were significantly greater than control levels for all non-piliated gonococcal types ($P$ <0.001 for all comparisons, $P$ <0.01 for all comparisons; respectively). However, IFN-γ was induced in significant mean amounts by the Opa-negative and Opa-b$^+$ variants ($P$ <0.05, $P$ <0.001; respectively), but not by Opa-a$^+$ organisms (see Figure *5·11*). Analysis of the responses to non-piliated gonococci demonstrated that Opa-b was a more potent stimulator of RANTES and IFN-γ (see Figure *5·11*) than either Opa$^-$ ($P$ <0.01 for both cytokines), or Opa-a$^+$ ($P$ <0.01, $P$ <0.001; respectively). However, no such differences were observed for MIP-1α. In accordance with the inflammatory mediator group, Opa-a$^+$ had no significant effects on the responses for "monocyte/macrophage" cytokines when compared with Opa$^-$.

The CXC chemokines IL-8 and GRO-α displayed divergent responses in stimulated versus control cells (see Figure *5·11*). While IL-8 levels were uniformly high in all treatment groups, including the control, GRO-α exhibited specific induction in response to all non-piliated gonococcal variants ($P$ <0.001 in all cases). In addition, Opa-b significantly elevated GRO-α production (see Figure *5·11*) relative to Opa$^-$

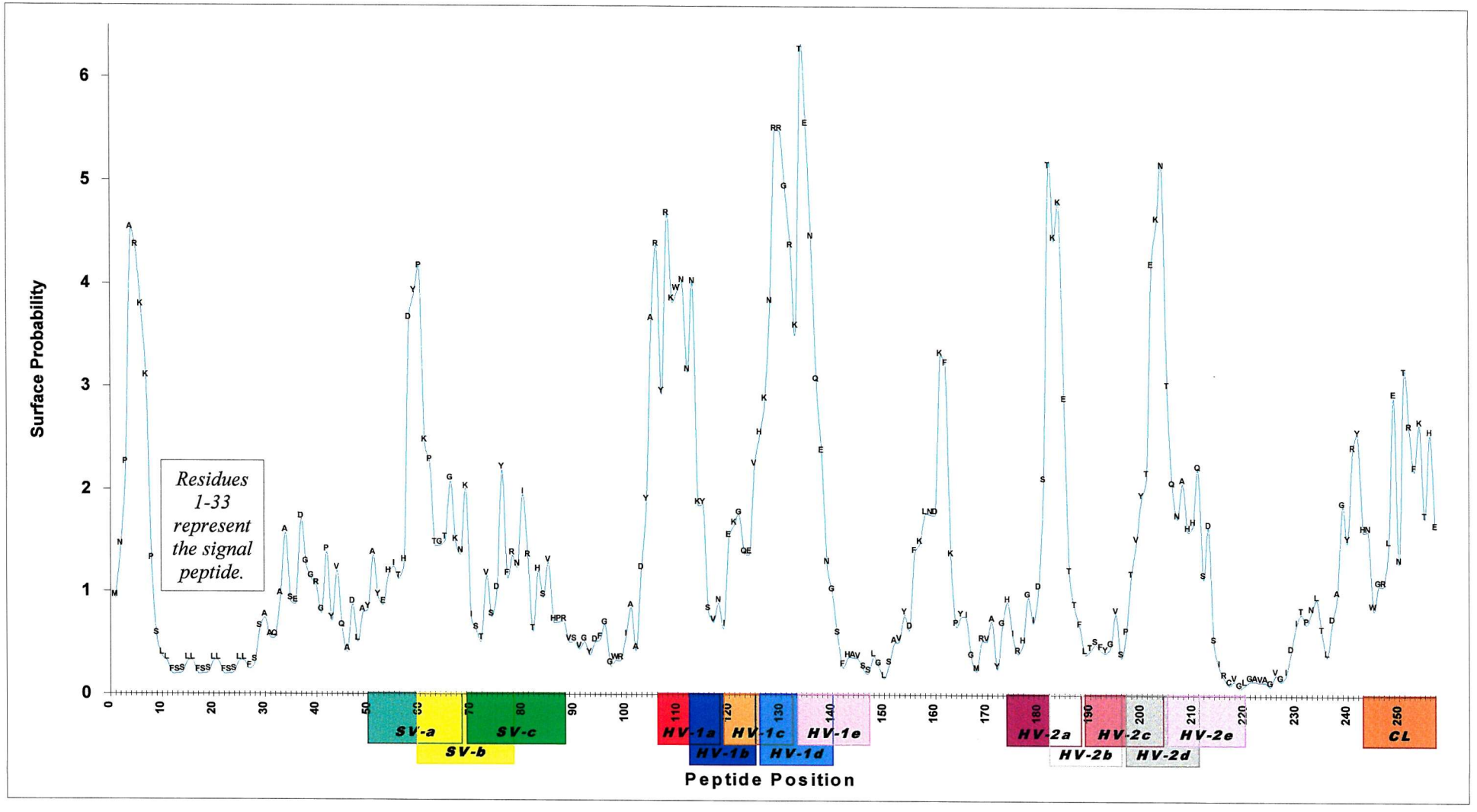
gonococci ($P$ <0.001) and the Opa-a$^+$ variant ($P$ <0.001). There was no significant difference in GRO-α release from cells stimulated with Opa$^-$ gonococci or Opa-a$^+$ organisms.

Piliated gonococcal variants induced significant concentrations of all three cytokine groups compared to unchallenged macrophages (see Table *3.2*, Appendix *3*). The increased stimulatory capacity of Opa-b$^+$ gonococci was supported in an analysis that included these phenotypes. Relative to Opa$^-$/Pil$^+$ gonococci, the Opa-b$^+$/Pil$^+$ variant stimulated increased production of TNF-α ($P$ <0.01), all of the "monocyte/macrophage" group ($P$ <0.05 for RANTES; $P$ <0.01 for MIP-1α & IFN-γ), and GRO-α ($P$ <0.001). The effect of pili on the cytokine response was investigated by comparison of the phenotype pairs Opa$^-$/Pil$^-$ versus Opa$^-$/Pil$^+$, and Opa-b$^+$/Pil$^-$ versus Opa-b$^+$/Pil$^+$. No significant differences between the mean responses were detected.

5D(vii) *Synthetic Peptides For the Study Of Interactions Between Gonococcal Opacity Protein B & Macrophages*: In the preceding experiments, divergent macrophage responses to gonococci were observed following binding and uptake of Opa-a$^+$ or Opa-b$^+$ organisms. While expression of Opa-a led to equivalent or reduced cytokine levels compared to Opa$^-$ gonococci, the Opa-b$^+$ phenotype stimulated equivalent or increased production. These opposing tendencies resulted in consistently elevated responses to Opa-b$^+$ organisms relative to the Opa-a$^+$ phenotype. Moreover, this difference in stimulatory capacity was supported by the viable count data, which indicated that Opa-b expression conferred an increase in avidity for the macrophage surface.

A preliminary investigation was conducted to identify the particular determinant(s) that were responsible for the enhanced binding conferred by Opa-b. Since regions of the protein that protrude from the gonococcal outer membrane presumably determine its specificity for macrophages, a series of peptides representing these domains was required. The two-dimensional structure of Opa proteins predicted by hydrophobicity analysis, strand prediction, and comparisons with porin crystal structure (Bhat *et al.*, 1991; Malorny *et al.*, 1998) comprises four surface-exposed loops, which are termed semivariable (SV), hypervariable (HV) 1 and 2, and conserved (CL). Consequently, the position of these domains in P9-Opa-b was ascertained by alignment of the amino-acid sequence with those of Opa proteins from other strains (Bhat *et al.*, 1991; Kupsch *et al.*, 1993; Malorny *et al.*, 1998). To confirm the validity of the model when applied to P9-Opa-b, surface probability was calculated by the method of Emini *et al.* (1985) for the

**FIGURE *5·12*: Surface Probability Profile Of Gonococcal Opacity Protein B – *correlation between regions represented by synthetic peptides & surface exposure.***



complete protein sequence (see Figure *5·12*). Fourteen overlapping peptides were then synthesised [see Section *2G(i)*] to encompass the regions exhibiting a high likelihood of surface exposure, which corresponded to the locations of the four loops (see Figure *5·12*).

Peptides representing the HV-1 region were selected for use in this experiment for two reasons. Firstly, this loop contains several residues that vary between P9-Opa-a and P9-Opa-b (Y. Deng, unpublished data); and secondly, HV-1 has been identified as the critical domain that enables adherence of gonococcal strain MS11mk to Chang epithelial cells (Grant *et al.*, 1999).

5D(viii) *The Affinity Of Gonococcal Opacity Protein B Hypervariable Region 1 Peptides For The Macrophage Surface*: Macrophage cultures grown on cover slips were washed and agitated according to the normal procedure (see Section *2C(vi)*], and the biotinylated peptides were added at 10 μg/ml (0.5 ml/well) in standard incubation medium without serum or other supplements. The experiment was performed in duplicate, and peptides were omitted from negative control wells. Sodium azide (0.05% w/v) was present throughout the 1.5 hr incubation period (37°C, 5% $CO_2$) to inhibit metabolic activity, which might otherwise have resulted in peptide internalisation or degradation. The cover slips were then rinsed gently with fresh medium, transferred to a separate plate, and fixed with methanol. For fluorescent labelling, a modification of the standard procedure [see Section *2E(ii)*] was followed. To block non-specific binding, PBS containing 1% BSA/5% sucrose was used instead of goat serum, as the latter contains biotin. For the detection of bound peptides, a streptavidin-FITC conjugate (Sigma) was applied at 5 μg/ml; this was co-incubated with anti-Pgp-1 mAb [see Section *5B(iv)*] to allow subsequent counterstaining using a TRITC-labelled secondary antibody.

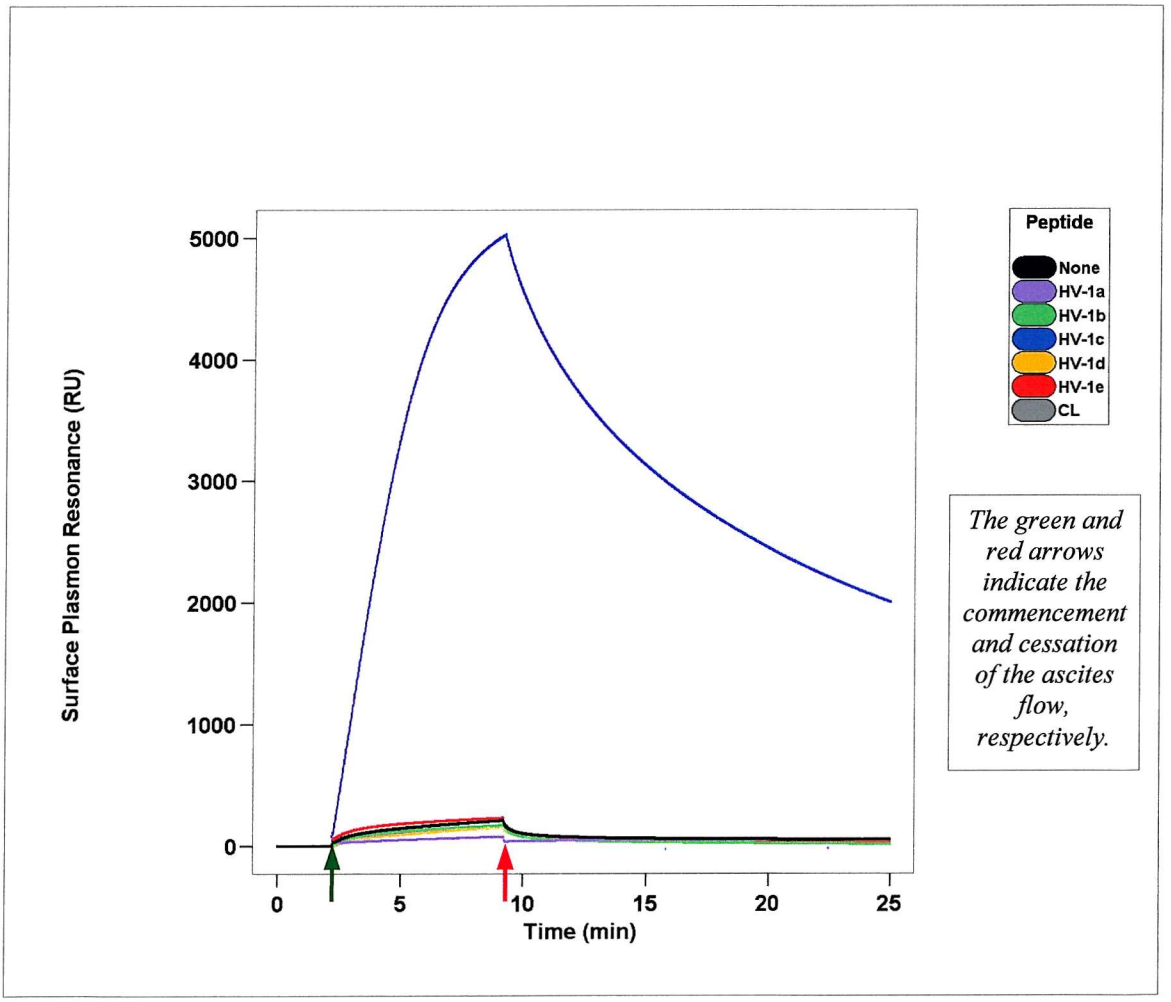
Fluorescent staining of the cells with the FITC conjugate was not visualised above control levels for any of the peptides. However, it was not known whether the biotin tags were fully accessible to the fluorescent label; therefore, non-reactivity of the peptides with the macrophage surface could not be confirmed. Omission of the anti-Pgp-1 mAb did not influence the outcome of this experiment.

5D(ix) *Characterisation Of An Anti-Opacity Protein B Monoclonal Antibody*: Since binding of Opa-b peptides to macrophages could not be detected, studies on this protein were continued in their stead utilising an anti-Opa-b mAb, designated SM40 [see Section *2G(iii)*]. Blocking of gonococcal attachment to macrophages by this antibody

could confirm that the observed cytokine response was directly related to the degree of bacterial adherence.

To assess the suitability of SM40 for the intended application, its precise specificity was ascertained by real-time biomolecular interaction analysis [(BIA) - see Sections *2G(ii)* & *2G(iii)*] using the biotinylated Opa-b peptides. Preliminary investigations by immunoassay (M.A. Pickett, unpublished data) suggested that SM40 bound to an epitope within HV-1. Therefore, all five peptides from this domain were included in the BIA, and the conserved loop peptide was used as a control. Additional controls included a flow cell without peptide, and a parallel run in which SM40 was substituted with 10Eii (an anti-chlamydial mAb).

**FIGURE *5·13*: Specificity Of The Anti-Gonococcal Opacity Protein B Monoclonal Antibody SM40 As Determined By Biomolecular Interaction Analysis.**



The specificity of SM40 was determined unequivocally in this experiment, as it displayed significant reactivity with only a single peptide, HV-1c (see Figure *5·13*). The surface plasmon resonance signal for this reaction was 1-2 orders of magnitude greater than for the other peptides, which exhibited weak signals indistinguishable from that of the no-peptide control (see Figure *5·13*). Furthermore, no reactivity of 10Eii with HV-1c or the other peptides was observed (data not shown). Inspection of the amino-acid sequences of Opa-a and Opa-b revealed that the HV-1c position is not conserved between the two proteins (M.A. Pickett, unpublished data), indicating that SM40 binds to a potentially important region of the molecule.

5D(x) *Binding Of Opa-b$^+$ Gonococci & Release Of Cytokines By Macrophages In The Presence Of Monoclonal Antibody SM40*: The BIA result supported the proposed application of SM40 in an inhibition experiment. The study described in Section *5D(iv)*, in which gonococcal avidity and the macrophage cytokine response were simultaneously investigated, was repeated with a single variant (Opa-b$^+$/Pil$^-$) in the presence of antibody. Since opsonised gonococci might be bound by macrophages via one or more of the receptors for the Fc portion of IgG (Aderem & Underhill, 1999), SM40 F(ab')$_2$ fragments were prepared [see Section *2G(iv)*] and used in parallel with untreated ascites. The experimental design incorporated infected cells, uninfected control cells, and Matrigel®-coated control wells (containing gonococci but not macrophages); which were assigned to four treatment groups [no antibody, 1 μg/ml F(ab')$_2$, 7.5 μg/ml F(ab')$_2$, and 1/500 dilution of ascites].

The F(ab')$_2$ fragments were dialysed overnight (4°C) against antibiotic-free experimental medium and filter-sterilised before use. Ascites fluid, stored at -70°C in 50% glycerol, was spun in a Beckman Avanti™ 30 centrifuge at 60,000 g (10°C) for 30 min, and an aliquot free from lipid or cellular debris was diluted in experimental medium. Gonococci were suspended in each of the three antibody preparations (and in control medium) to a concentration of $2x10^7$ cfu/ml, and were added immediately to the culture plates to limit the formation of cross-linked bacterial agglutinates. Gonococcal adherence was then determined as previously, using six (macrophages present) and three (macrophages absent) replicates per treatment group. Sampling of cell culture medium for cytokines was also undertaken [see Section *5D(iv)*]; and subsequently, assays for IL-6, TNF-α, RANTES, and MIP-1α were conducted. Finally, following saponin treatment, the gonococcal suspensions were diluted 1/100 and 1/2000 before inoculation of agar plates.

The purpose of this experiment was to determine whether a mAb directed against Opa-b could reduce attachment of gonococci to macrophages, and thereby decrease the magnitude of the cytokine response. However, the detection of antibody effects that were specific to the gonococcus-macrophage interface could be confounded by non-specific factors. For instance, antibodies might affect bacterial adherence to the Matrigel® substrate, whether or not macrophages were present; whilst the cytokine response could be influenced by the antibody preparation, even in the absence of other stimuli. Therefore, a statistical method was required that could discriminate between the specific effects and the confounding effects, and two-way ANOVA was chosen for this purpose. The statistic analysed the influence of antibody in the presence or absence of macrophages (viable count data), and in the presence or absence of gonococci (cytokine data). Separate tests were conducted on the results pertaining to ascites (whole IgG) or to $F(ab')_2$ fragments; but since the lower concentration of digested antibody had little apparent effect, it was excluded from the analyses. To ensure that the data fulfilled the statistical assumptions of ANOVA, viable counts were subjected to a $\log_{10}$ transformation, while cytokine concentrations were converted to $x^{-0.6}$ values.

**TABLE *5·2*: The Effects Of An Anti-Opacity Protein B Antibody On Binding Of Gonococci & Cytokine Release By Macrophages – *findings for intact and digested immunoglobulins.***

| | Gonococcal Adherence (geometric mean cfu/well +/- SE*) | | Cytokine Concentration (geometric mean pg/ml +/- SE*) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | IL-6 | | TNF-α | | RANTES | | MIP-1α | |
| Treatment Group | Presence/Absence Of Cells | | Presence/Absence Of Gonococci | | | | | | | |
| | + | - | + | - | + | - | + | - | + | - |
| No Antibody | $6.2x10^6$ +/- 1.11 | $3.4x10^6$ +/- 1.22 | 946x/÷ 1.07 | 12x/÷ 1.12 | 8272x/÷ 1.11 | 558x/÷ 1.01 | 1101x/÷ 1.06 | 315x/÷ 1.01 | 13137x/÷ 1.11 | 516x/÷ 1.03 |
| Ascites | $8.6x10^6$ +/- 1.14 | $2.9x10^6$ +/- 137 | 826x/÷ 1.24 | 13x/÷ 1.17 | 7495x/÷ 1.21 | 557x/÷ 1.04 | 982x/÷ 1.19 | 333x/÷ 1.07 | 10899x/÷ 1.27 | 532x/÷ 1.02 |
| F(ab')2 | $6.8x10^5$ +/- 1.40 | $6.9x10^6$ +/- 1.13 | 589x/÷ 122 | 9x/÷ 1.18 | 7193x/÷ 1.20 | 554x/÷ 1.02 | 783x/÷ 1.18 | 307x/÷ 1.02 | 9221x/÷ 1.24 | 659x/÷ 1.05 |

*SE = standard error of the mean.

In order to visualise the influence of whole SM40 IgG on gonococcal adherence to macrophages, the geometric mean counts for control wells were subtracted from the respective counts from occupied wells (see Table *5·2*). A greater mean number of gonococci were associated with macrophages in the presence of anti-Opa-b mAb ($5.7x10^6$ cfu/well) than in its absence ($2.8x10^6$ cfu/well), which was consistent with the anticipated effect of opsonisation. However, when all of the relevant data was incorporated into the

analysis, the increase in gonococcal binding attributable to macrophages was not significantly different in the presence or absence of whole antibody ($P$ = 0.168). In addition, undigested IgG had no significant effects on the net induction of IL-6, TNF-α, RANTES, and MIP-1α by gonococci. Indeed, any binding of bacteria to phagocytes via Fcγ receptors did not enhance cytokine production, since the mean levels of all assayed cytokines in infected cultures were reduced by the addition of anti-Opa-b mAb (see Table *5·2*).

The $F(ab')_2$ fragments of SM40 appeared to have a greater inhibitory effect on cytokine production than did intact IgG (see Table *5·2*). Taking all replicate measurements into account, the net release of MIP-1α induced by gonococcal stimulation was significantly reduced in the presence of antibody fragments ($P$ <0.001; ~1.5-fold difference between corrected means). This trend was mirrored by IL-6 (1.6-fold difference), TNF-α (1.2-fold difference), and RANTES (1.7-fold difference) – see Table *5·2* - but in these cases statistical significance was not attained. Therefore, there was some evidence that anti-Opa-b $F(ab')_2$ fragments inhibited the stimulatory potency of Opa-$b^+$ gonococci.

At least three hypotheses could be proposed to explain the observed inhibitory effect, which were not mutually incompatible. Firstly, antibody fragments might have decreased the affinity of Opa-$b^+$ gonococci for macrophages by specific blockade of Opa-b-mediated binding, thus negating the enhanced capacity of this protein to stimulate cytokine release. Secondly, $F(ab')_2$ fragments bound to the gonococcal surface could have had a general inhibitory effect on bacterium-macrophage interactions, reducing the probability of contact between phagocytic receptors and bacterial ligands such as LOS or PorB. Finally, the addition of antibody might have confounded the experiment through effects on gonococcal aggregation (Blake *et al.*, 1995), replication, or interactions with the Matrigel® substrate; and any one of these factors could have indirectly modified macrophage responses.

The first step in discriminating between these possibilities was the viable count analysis for the effect of $F(ab')_2$ fragments. The addition of digested antibody did not significantly affect the difference in gonococcal binding between control wells and those containing macrophages ($P$ = 0.283). However, in contrast to the results for whole IgG, a significant increase in bacterial adherence that could be attributed to macrophages was not apparent. This was because digested antibody enhanced binding of gonococci to the Matrigel® coating of unoccupied wells by a factor of two ($P$ <0.05, independent $t$-test).

Thus, to estimate macrophage-specific bacterial attachment accurately, it would be necessary to determine the surface area occupied by these cells in the culture vessel, which was not possible due to heterogeneous macrophage morphology [see Section *5B(iv)*] and the indefinable effect of cellular protrusions on the area available for interactions with gonococci. Therefore, to determine whether the effects of Opa-b could be reproduced in an entirely different context, isolated outer membranes containing this protein were included in the subsequent series of experiments.

## *5E* - Stimulation Of Macrophages With Purified Gonococcal Surface Components

5E(i) *Cytokine Profiles Over Time – Experimental Design*: The experiments described in the previous section, in which macrophages were stimulated with viable bacteria, demonstrated that these cells responded to gonococcal challenge by releasing several proinflammatory cytokines. However, it was not clear whether cytokine induction was dependent on gonococcal metabolic activity and/or the phagocytic uptake of whole organisms. Viable count data indicated a special role for Opa-b in gonococcus-macrophage interactions, as it conferred increased avidity for phagocytes and induced elevated cytokine levels; but a number of confounding factors restricted the information that could be obtained from these studies. Challenge of macrophages was not undertaken for prolonged periods as this led to variable responses, probably because of bacterial replication and subsequent cell toxicity. In addition, the incubation time of the experiments had to be limited in order to prevent a shift in the dominant gonococcal phenotype, which could otherwise have occurred through phase and antigenic variation. Finally, the effect of gonococcal surface components [such as lipooligosaccharide (LOS) and outer membrane blebs] released into the cellular microenvironment could not be taken into account, even though these materials might stimulate a significant cytokine response from macrophages independently of intact diplococci.

Therefore, to investigate the role of specific gonococcal structures in cytokine induction, the purified surface components described in Section *2D* were incubated with macrophages. Outer membranes and LOS were directly diluted into standard incubation medium with reduced supplements (2% FCS & 25 μg/ml ampicillin only), but pili were spun at 60,000 g for 15 min (20°C) in a Beckman Avanti™ 30 centrifuge prior to resuspension. Stimulatory materials were introduced into washed macrophage culture

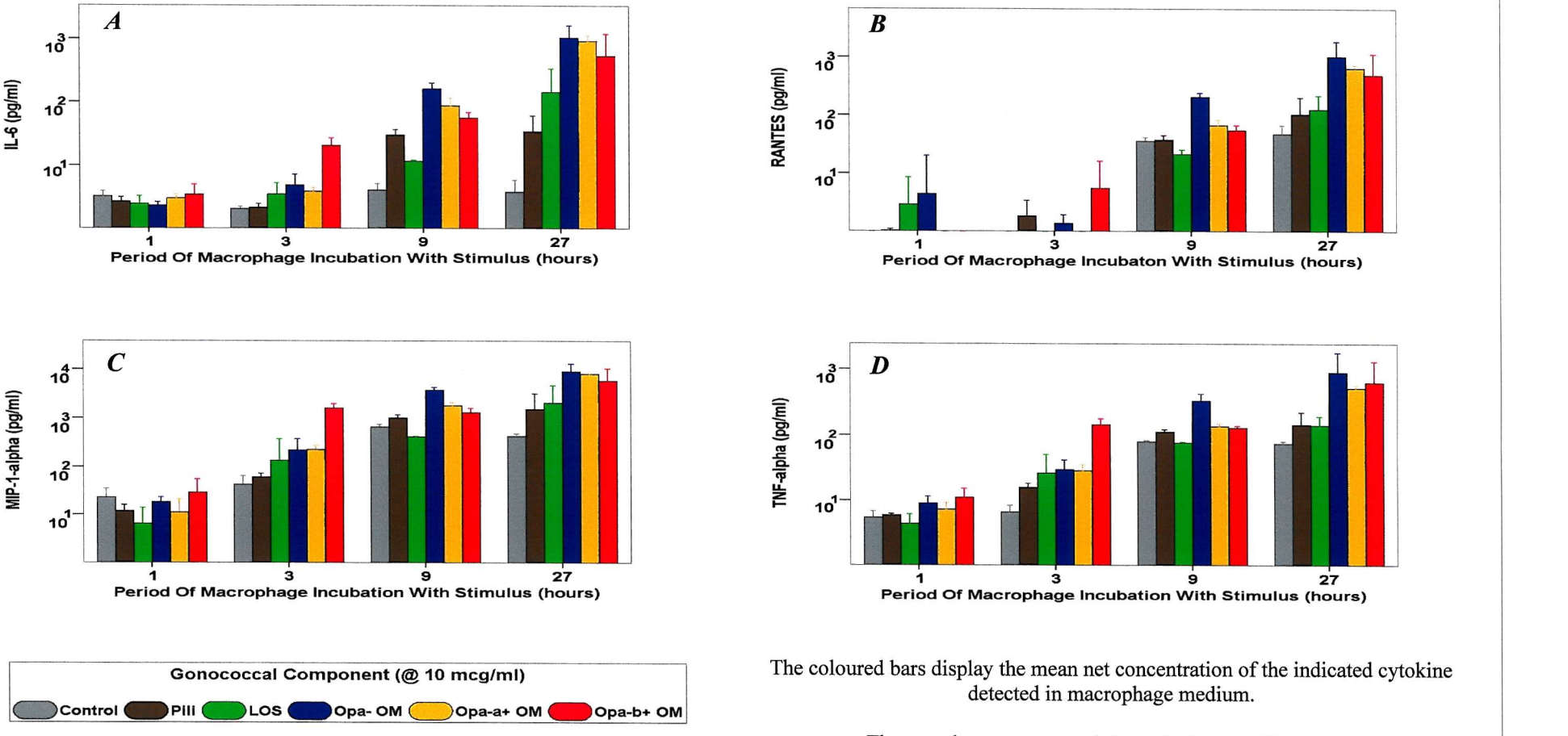
wells in an identical manner to the viable gonococci [see Section *5D(i)*], producing final concentrations of 10 μg/ml and 1 μg/ml (LOS and outer membranes), or 100 μg/ml and 10 μg/ml (pili). Cell culture medium was collected over a five-point time scale as previously described [see Section *5D(i)*], but the centrifugation step was omitted.

5E(ii) *Preliminary Results - Processing And Analysis Of Induced Cytokine Data*: The reserved aliquots of macrophage medium were screened for ten cytokines by time-resolved fluorescence immunoassay [see Section *2F(ii)*]. Examination of the raw RFU data indicated that GM-CSF, IL-1β, IL-10, IL-12 and TGF-$\beta_1$ were not induced by any of the stimuli at any point during the experiment. Production of RANTES, IL-6, IL-8, TNF-α and MIP-1α increased over time, so data for these cytokines were processed as for the viable gonococci [see Section *5D(ii)*]. Comparison of the mean cytokine responses between the two concentrations of each stimulus at each time-point revealed no significant differences, except in two instances. The Opa-$a^+$ outer membranes (OM) at 10 μg/ml induced significantly greater IL-6 and RANTES production for the 27-hr time-point than did the lower concentration ($P$ <0.01 in both cases). The data for the 10-μg/ml concentration (including pili) were selected for analysis to allow for valid comparisons between all components.

5E(iii) *The Macrophage Cytokine Response To Purified Gonococcal Components – Profiles Over A 27-Hour Time Course*: All critical probabilities for comparisons between stimulated and control macrophages are presented in **Table *A3·3*, Appendix *3***. None of the induced cytokines were significantly above control levels by 1 hr post-stimulation. By 3 hr, however, production of IL-6, MIP-1α, and TNF-α was significantly elevated by one or more of the gonococcal components relative to unchallenged cells. In contrast, significant induction of RANTES did not occur until the 9 hr time-point, whilst IL-8 production in stimulated cultures was indistinguishable from the high levels (3.8 ng/ml by 27 hr) observed in controls throughout the experiment.

Analysis of the IL-8, MIP-1α, and TNF-α data by time (see Figure *5·14C & D*) revealed that mean production was significantly different between all adjacent time-points. The greatest increases in cytokine output occurred between the 1 and 3 hr time-points ($P$ <0.001 for these three cytokines). Subsequently, net release in the 3-9 hr ($P$ <0.001 for IL-8 and TNF-α; $P$ <0.01 for MIP-1α) and 9-27 hr ($P$ <0.001 for IL-8; $P$

**FIGURE *5:14*: Production Of Proinflammatory Cytokines By Macrophages In Response To Purified Gonococcal Components – *profiles over time.***




The coloured bars display the mean net concentration of the indicated cytokine detected in macrophage medium.

The error bars represent +1.0 standard error of the mean.

<0.05 for MIP-1α and TNF-α) intervals became progressively smaller. However, the release of both IL-6 and RANTES was delayed until 9 hr post-stimulation, as there were no overall significant differences between the 1 hr and 3 hr profiles (see Figure *5·14A & B*). Net production of these cytokines was greatest in the 3-9 hr period ($P$ <0.001 for both RANTES and IL-6), then slowed down between the 9 and 27 hr time-points ($P$ <0.01 for both cytokines).

When the cytokine responses generated by each gonococcal fraction were compared with each other, significant differences were generally restricted to the 3 and 9 hr time-points. At 3 hr, OM containing Opa-b exhibited significantly increased potency in relation to Opa-a$^+$ OM for MIP-1α ($P$ <0.05), IL-6 ($P$ <0.01), and TNF-α ($P$ <0.05). In addition, Opa-b$^+$ OM induced higher mean concentrations of IL-6 and TNF-α compared to Opa-negative OM at this time-point ($P$ <0.05 for both cytokines).

5E(iv) *Relative Stimulatory Capacities Of Gonococcal Components In Macrophage Cytokine Production – Design & Analysis*: Clearly, induction of a cytokine response in macrophages was not dependent on gonococcal viability. However, compared to the parallel study conducted with live organisms [see Section *5D(iii)*], cytokines in macrophage medium accumulated at a slower rate when gonococcal components were used for stimulation. This consideration, together with the elimination of bacterial replication, permitted an overnight incubation period in an expanded experiment (six replicates per treatment group) comparing the stimulatory potencies of the purified fractions.

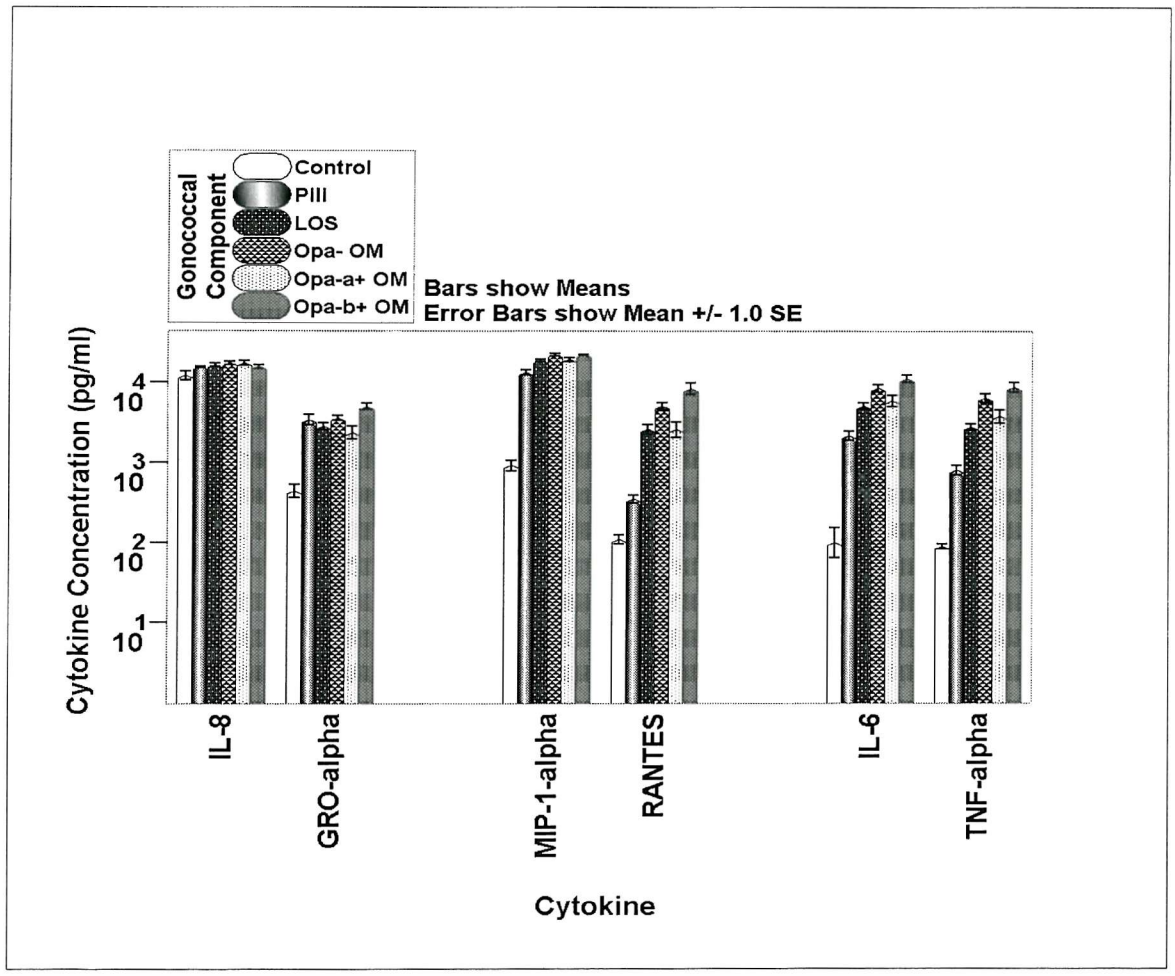
Macrophages grown in 24-well cell culture plates were stimulated with gonococcal components as described previously [see Section *5E(i)*], except that only a single concentration (10 μg/ml) of each stimulus was applied. After 18 hrs' incubation at 37°C (5% $CO_2$), 150-μl aliquots of cell medium were transferred from each well into sterile micro-tubes, and stored undiluted at -70°C. Five cytokines (IL-6, IL-8, MIP-1α, RANTES and TNF-α) were then measured by time-resolved fluorescence immunoassay [see Section *2F(ii)*]. Additionally, production of GRO-α and ENA-78 was assessed using colorimetric ELISA kits [see Section *2F(iii)*].

Optical density readings for the ENA-78 ELISA indicated uniformly low concentrations in all macrophage supernatants (including controls), and these values were not processed further. The remaining raw data were converted to cytokine concentrations and subjected to log($x$) transformation. The means of each replicate group were then

compared by post-hoc tests following one-way ANOVA, as described previously [see Section *5D(ii)*]; and all critical probabilities quoted in the following section are summarised in **Table *A3·4*, Appendix *3***.

5E(v) *Differences In The Stimulatory Capacities Of Gonococcal Surface Components Following 18 Hours' Incubation With Macrophages*: The use of a larger number of macrophages per sample, and six replicates in each experimental group, allowed for greater differentiation between stimuli than was possible in the time-course experiment. The isolated outer membranes used for stimulation of macrophages were quantified by protein content (see Section *2D*), but since 50% of the Gram-negative outer membrane is composed of LPS (Hitchcock, 1984), vesicles suspended at 10 μg protein/ml should be directly comparable to the purified LOS solution (used at 10 μg/ml). Therefore, the different preparations would be expected to induce similar cytokine responses if LOS, rather than outer membrane proteins, was the critical stimulus.

**FIGURE *5·15*: Relative Potencies Of Purified Gonococcal Components As Stimulators Of Macrophage Cytokine Production – *comparisons at 18 hours.***



Compared to unchallenged cells, LOS and Opa$^-$ OM generated significantly elevated production of IL-6 ($P$ <0.01 for both), TNF-α ($P$ <0.001 for both), MIP-1α ($P$ <0.001 for both), RANTES ($P$ <0.001 for both), and GRO-α ($P$ <0.001 for both) – see Figure *5·15*. Interestingly, a two-fold greater mean responses to Opa$^-$ OM relative to LOS were observed for TNF-α ($P$ <0.01) and RANTES ($P$ <0.05) – see Figure *5·15*. A similar difference (1.7-fold) between these stimuli was also apparent for IL-6, but this was not statistically significant ($P$ = 0.052; see Figure *5·15*). Hence, outer membrane proteins - particularly PorB - might amplify cytokine induction compared to LOS alone. Additionally, stimulatory potency could be affected by a difference in the physical structure of LOS in membrane vesicles versus purified LOS dissolved in the culture medium.

To determine whether differences in Opa protein type affected the cytokine response when examined in isolation from viable organisms, the stimulatory potencies of vesicles containing Opa-a or Opa-b were compared. Cytokine release following challenge with the two preparations was significantly in excess of control levels for IL-6 ($P$ <0.01 for both), RANTES ($P$ <0.001 for both), MIP-1α ($P$ <0.001 for both), TNF-α ($P$ <0.001 for both), and GRO-α ($P$ <0.001 for both) – see Figure *5·15*. The Opa-b$^+$ OM were associated with increased (two-to-three-fold) cytokine production relative to Opa-a$^+$ OM for TNF-α ($P$ <0.01), RANTES ($P$ <0.001), and GRO-α ($P$ <0.01) – see Figure *5·15*. Interleukin-6 also conformed to this trend (1.9-fold difference between means), but statistical significance was not attained ($P$ = 0.124; see Figure *5·15*). The Opa-a$^+$ OM stimulated equivalent or diminished responses compared to Opa$^-$ OM (two-fold difference between means for RANTES, $P$ <0.05), whereas Opa-b$^+$ OM tended to induce equivalent or elevated cytokine production. These findings were consistent with the observed effects of Opa-a and Opa-b phenotypes on macrophage responses when viable gonococci were used to challenge the cells [see Section *5D(vi)*].

In relation to unchallenged macrophages, purified pili were found to stimulate significant production of IL-6 ($P$ <0.01), RANTES ($P$ <0.001), MIP-1α ($P$ <0.001), TNF-α ($P$ <0.001), and GRO-α ($P$ <0.001) - see Figure *5·15*. However, the levels of IL-6, RANTES, MIP-1α, and TNF-α induced by pili were considerably lower than for all the other components (see Figure *5·15* & Table *A3·4*, Appendix *3*). For instance, when compared to Opa$^-$ OM, pili induced a 1.7-fold lower mean concentration of MIP-1α, 3.9-fold less IL-6, 7.9-fold less TNF-α, and 14.4-fold less RANTES. This weak stimulatory

effect was in accordance with the negligible or slightly suppressive influence of piliation on the response of macrophages to viable bacteria [see Sections *5D4(i)* & *5D(vi)*].

5E(vi) *The Effects Of Autologous Human Serum On The Rate Of Cytokine Production Following Stimulation With Gonococcal Components – Experimental Design*: The preceding studies demonstrated that macrophage responses could be induced by isolated gonococcal surface components, producing a similar cytokine profile to that observed when live organisms were used as stimuli. In these experiments, macrophages were challenged in culture medium containing a low concentration of FCS, but human serum was absent. However, since plasma exudation constitutes one aspect of inflammation (Persson *et al.*, 1998), the cytokine response to gonococcal components may be enhanced in the early stages of gonorrhoea by interactions with natural antibodies (Trautmann *et al.*, 1998), LPS-binding protein (Schumann *et al.*, 1990), or other serum factors.

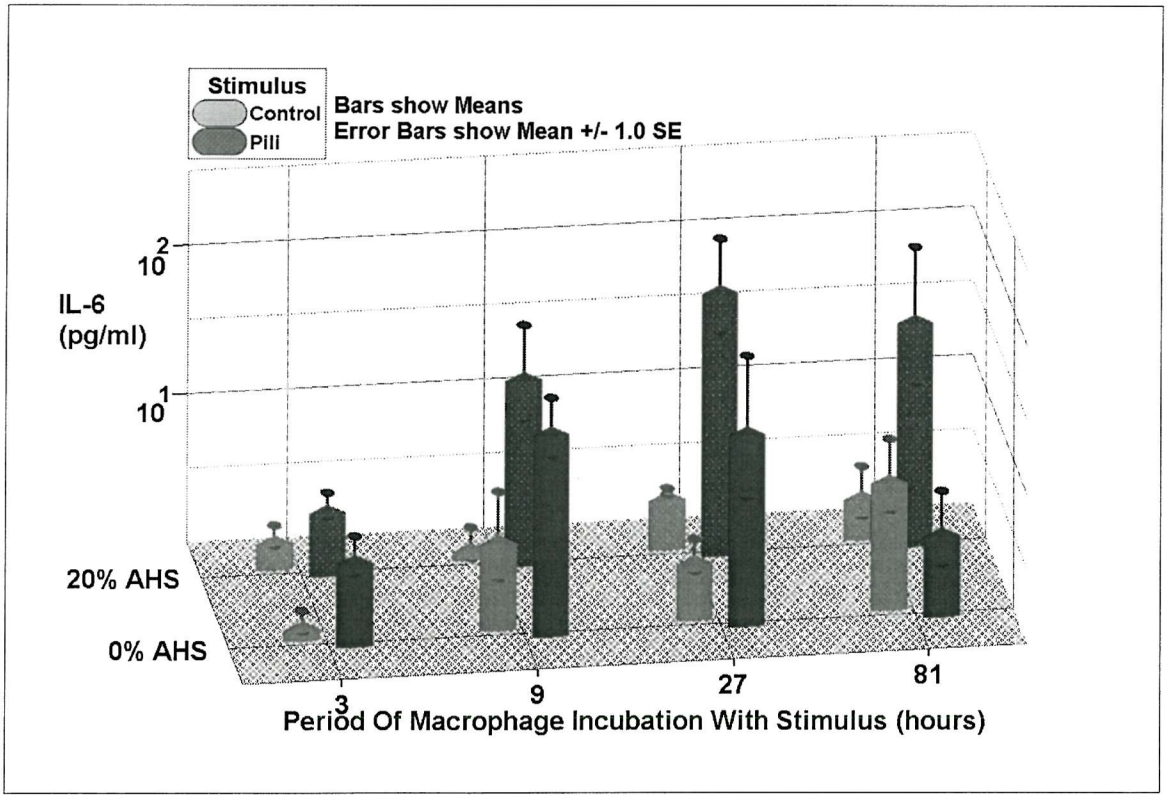
Macrophages were challenged with gonococcal surface components in the presence of autologous human serum (AHS), and key proinflammatory cytokines were measured over an 81-hour period. This extended time course was designed to enable detection of prolonged effects on cytokine production. Pili, LOS, and Opa-negative outer membrane vesicles were selected for use as stimuli, as these components are common to many Gram-negative bacteria and are recognised by antibodies present in normal human serum (Hamadeh *et al.*, 1995; Apicella *et al.*, 1986; Henriksen *et al.*, 1998).

The methodology employed in Section *5E(i)* was applied to this experiment, with minor modifications. Gonococcal components were pre-incubated (1 hr, RT°C) as five-fold concentrates in two parallel groups; the diluents were standard incubation medium without supplements, and heat-inactivated AHS (undiluted). The stimuli were then added to the supplemented (2% FCS, 25 μg/ml ampicillin) medium of washed macrophage cultures, producing final concentrations of 100 μg/ml (pili) or 10 μg/ml (LOS & vesicles) in two treatment groups (20% AHS and 0% AHS). These groups included appropriate control replicates that lacked gonococcal stimuli. Macrophage medium was collected from separate culture plates at 0 hr (controls only), 3 hr, 9 hr, 27 hr, and 81 hr; and all samples were assayed for IL-6, TNF-α, and MIP-1α. In addition, aliquots from >1 d post-stimulation were screened for IL-10 and IL-12, but these cytokines were not detected.

5E(vii) *The Macrophage Cytokine Response To Pili In The Presence Of Autologous Human Serum – Profiles Over Time*: The results obtained from this experiment are summarised in **Table *A3·5*, Appendix *3***. To determine whether a more in-depth study on the effects of human serum was warranted, a statistical analysis of the data collected for pili was undertaken. The three factors involved in this experiment – time, presence/absence of stimulus, and presence/absence of AHS – were included in a three-way ANOVA. This statistic was capable of differentiating a specific effect of AHS on the response to pili from any confounding effect identified in the AHS controls.

In general, the mean concentrations of IL-6, TNF-α, and MIP-1α increased up to 27 hr, but by 81 hr, declines were apparent in all three cases - presumably as a result of proteolysis. Net production in the 9-27 hr interval was statistically significant for TNF-α and MIP-1α ($P$ <0.001 for both). As noted in Section *5E(v)*, pili demonstrated moderate but statistically significant stimulation of IL-6 ($P$ <0.001), TNF-α ($P$ <0.01), and MIP-1α ($P$ <0.001) release; irrespective of serum components. When separated from the other factors, AHS had no significant effects on the mean levels of IL-6 and MIP-1α, although it was associated with a significant decrease in TNF-α production ($P$ <0.01).

**FIGURE *5·16*: The Effect Of Autologous Human Serum On The Macrophage IL-6 Response To Gonococcal Pili – *profile over time.***



Taking the respective controls as baseline, AHS significantly increased the stimulatory potency of pili for induction of IL-6 ($P$ <0.05; see Figure *5·16*) and MIP-1α ($P$ <0.01) over the entire incubation period. However, this effect was more pronounced during the latter half of the experiment for both IL-6 (see Figure *5·16*) and MIP-1α. The influences of AHS on macrophage responses to LOS and Opa$^-$ OM were equivocal (see Table *A3·5*, Appendix *3*), and further elucidation of the role played by serum factors would require a detailed study incorporating an optimum period for macrophage challenge. Therefore, an 18-hr sampling point was selected for the following experiments, as the effects of AHS became evident after 9 hr, whereas protein degradation took >1 d to reverse the preceding accumulation of cytokines in the culture medium.

5E(viii) *Stimulation Of Macrophages With Gonococcal Components In The Presence Of Normal Sera From Several Individuals – Experimental Design*: In the preceding time-course study, human serum was shown to have positive impacts on cytokine responses following macrophage incubation with purified pili. To determine whether this was a general characteristic of normal human serum, and not just a chance event specific to a single blood donor, six sera were obtained for use in a more detailed experiment. In addition, the increase in scale would enable the possible effects of serum factors on the stimulatory potencies of LOS and gonococcal outer membranes to be ascertained.

Serum was obtained from the donor of macrophages used in this experiment (autologous), a different blood donor whose cells had previously been used for macrophage preparation (allogeneic), and four sexually active individuals [(SAI) - specimens provided by P.J. Watt]. The heat-inactivated sera were diluted to 20% in standard incubation medium without other supplements, and each gonococcal component was pre-incubated (1.5 hr, RT°C) at 200 μg/ml (pili) or 20 μg/ml (LOS and Opa$^-$ OM) in both diluent alone and in each serum preparation. Macrophages cultured in 96-well format were shaken and washed as normal, and the stimuli were added to an equal volume of medium in triplicate wells, producing a final concentration of 10% human serum (where applicable) in other supplements as previously described [see Section *5E(vi)*]. To enable correction for potential stimulatory or suppressive effects in the absence of gonococcal components, each serum was also added at the same concentration, in triplicate, to control cells. Samples of macrophage medium (150 μl)

were collected at 18 hr post-stimulation, and following storage at -70°C, immunoassays were conducted for TNF-α, IL-6, and MIP-1α.

In order to simplify the statistical analysis, the data set for each cytokine [transformed into $\log_{10}(x)$ values] was processed in three stages. Firstly, one-way ANOVA and Tukey's HSD test were used to determine whether the mean uncorrected responses for gonococcal stimulus-plus-serum were significantly greater than for the stimulus alone. Where such differences were identified, the tests were repeated following subtraction of the geometric mean control concentrations (that is, for serum only) from the respective replicate values for stimulus-plus-serum. This procedure was validated by ensuring that each mean control value was significantly smaller than the corresponding mean response for stimulus-plus-serum; but it should be noted that this correction resulted in a conservative estimate of the interactive effect, as the serum-only response included constitutive (baseline) cytokine expression. Finally, where a single serum significantly increased the stimulatory capacities of more than one gonococcal fraction, the magnitude of the effects was compared by two-way ANOVA.

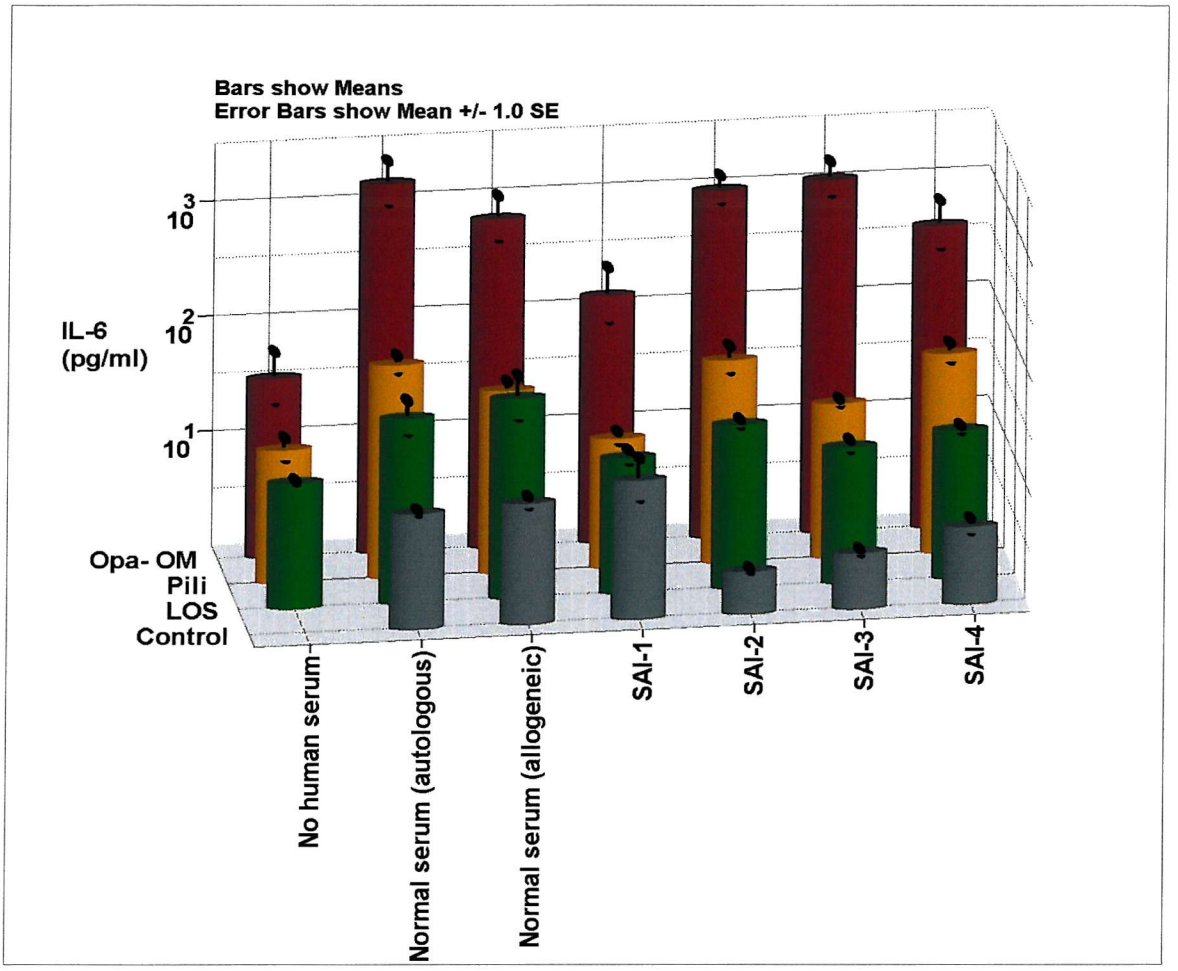
5E(ix) *The Effects Of Normal Sera From Different Donors On The Macrophage Cytokine Response To Gonococcal Components*: In Section *5E(vii)*, serum from the macrophage donor was found to enhance macrophage responses to isolated pili, even though the probability of exposure to gonococci would be extremely low for such an individual. To test the hypothesis that ubiquitous serum factors (such as natural antibodies) mediated the observed effect, macrophages were incubated with sera from six healthy adults in this experiment. The results and calculations referred to in this section are summarised in **Table *A3·6*, Appendix *3***.

Following pre-analysis and subtraction of the control responses for sera alone, the macrophage IL-6 response to pili was significantly increased by autologous serum ($P$ <0.01), and sera from SAI-2 ($P$ <0.01) and -4 ($P$ <0.05) – see Figure *5·17*. These sera also enhanced the stimulatory capacity of Opa$^-$ OM for IL-6 release ($P$ <0.001 for AHS, $P$ <0.01 for SAI-2, $P$ <0.05 for SAI-4); as did allogeneic normal serum ($P$ <0.01) and serum from SAI-3 ($P$ <0.01) – see Figure *5·17*. In contrast, there was no evidence that the sera elevated IL-6 levels during stimulation of macrophages with LOS (see Figure *5·17*).

When pili were compared with Opa$^-$ OM, the increases in IL-6 induction attributable to the interaction between gonococcal stimulus and certain sera were not equal. Thus, statistically greater effects of AHS ($P$ <0.05), allogeneic normal serum ($P$

<0.05), and SAI-2 serum ($P$ <0.05) were observed on vesicles relative to pili (see Figure *5·17*), even though pili were used at a ten-fold greater concentration than the membranes. This implies that the serum factor(s) responsible for the elevated response to

**FIGURE *5·17*: The Effects Of Sera From Six Different Donors On The Macrophage IL-6 Response To Gonococcal Components.**




outer membranes are present in larger amounts, or exhibit greater avidity, than the factor(s) which act upon pili. Furthermore, the absence of a significant impact of serum on the macrophage response to soluble LOS suggests that PorB or membrane-dependent LOS structures are the target of opsonins.

Human serum displayed similar effects on macrophage production of MIP-1α, and to a lesser extent, TNF-α, during challenge of cells with gonococcal components. Addition of AHS significantly increased the release of TNF-α mediated by Opa$^-$ OM ($P$ <0.05), but other comparisons for this cytokine and MIP-1α were not significant. To

visualise the overall influence of the six sera on the magnitude of cytokine production, the geometric mean concentrations induced by each serum alone and each gonococcal component alone were subtracted from the corresponding geometric mean response to gonococcal stimulus-plus-serum (see Table *A3·6*, Appendix *3*). Grand arithmetic means were then calculated for each stimulus (see Table *5·3*); these were conservative estimates of changes in cytokine levels attributable to the interaction of serum with the specified component.

**TABLE *5·3*: The Effects Of Normal Human Serum On The Macrophage Cytokine Response Following Challenge With Gonococcal Components.**

| | *NET CHANGE IN INDUCED CYTOKINE LEVELS (mean pg/ml ± SE*)* | | |
|---|---|---|---|
| *GONOCOCCAL COMPONENT* | *IL-6* | *TNF-α* | *MIP-1α* |
| LOS | +8.6±6.5 | -61.1±20.5 | +193.0±135.1 |
| PILI | +21.0±10.3 | -62.4±21.8 | +1359.5±488.3 |
| OPA⁻ OM | +824.3±223.7 | +665.5±218.2 | +6971.8±822.6 |

*SE = standard error of the mean.

For all three cytokines, serum factors had a considerably greater impact on the response to vesicles compared to the other stimuli (see Table *5·3*). Several sera exhibited intrinsic stimulation of TNF-α release which exceeded that of pili or LOS alone, but this was nullified when the sera and components were combined, resulting in negative values (see Table *5·3*). However, the positive interaction of serum and Opa⁻ OM on TNF-α production occurred despite this effect.

5E(x) *The Influence Of Immune Sera On The Macrophage Cytokine Response To Gonococcal Components*: Cytokine production elicited by gonococcal outer membrane vesicles and pili was clearly elevated in the presence of normal human serum. One explanation for these effects was that antibodies modified the interaction between macrophages and gonococcal components, leading to changes in the cytokine response. As sera from naturally infected gonorrhoea patients would be expected to contain a higher titre of anti-gonococcal antibodies than those of uninfected individuals (Zak *et al.*, 1984), specimens from four British cases with different infection histories were acquired (courtesy of J.E. Heckels) for use in a macrophage challenge experiment. They comprised a male presenting with typical acute symptoms (five days' discharge and dysuria), a

female with cervicitis and urethritis who had been infected for at least three months, an asymptomatic male infected for at least three months, and a male with disseminated gonococcal infection (DGI) manifesting as arthritis. For three of the patients, the PorB phenotype of the recovered gonococcal isolate was known (see Table *5·4*). This experiment used the same methodology and statistical analyses employed in the normal serum study [see Section *5E(viii)*], except that the DGI serum was used at 5% final concentration because stocks were limited.

Correcting for cytokine levels observed in the serum-only controls, stimulation of macrophages by Opa$^-$ OM was enhanced by all of the sera for all three cytokines (see Table *5·4*). However, only the IL-6 and TNF-α responses in the presence of the DGI serum reached statistical significance ($P$ <0.01, $P$ <0.05; respectively), despite the lower concentration used for this specimen. Generally, the immune sera did not increase cytokine production following challenge with the other gonococcal stimuli, although the specimen from the chronically infected female elevated MIP-1α release elicited by pili (see Table *5·4*). The considerable intrinsic stimulatory capacity of the sera in the absence of gonococcal components (see Table *5·4*) might have complicated their combined interaction, as noted in the normal serum study [see Section *5E(ix)*].

**TABLE *5·4*: The Effects Of Various Immune Sera On The Macrophage Cytokine Response To Gonococcal Surface Components.**

| | *SOURCE OF SERUM* | | | | |
|---|---|---|---|---|---|
| | NO SERUM | ♂ DGI (PorB unknown) | ♀ CHRONIC (PorB1a) | ♂ ACUTE (PorB1b) | ♂ CHRONIC (PorB1a) |
| *STIMULUS* | *IL-6 (geometric mean pg/ml x/÷ SE*)* | | | | |
| CONTROL | - | 10x/÷1.03 | 11x/÷1.02 | 9x/÷1.31 | 5x/÷1.08 |
| LOS | 13x/÷1.01 | 14x/÷1.09 | 15x/÷1.08 | 17x/÷1.17 | 17x/÷1.05 |
| PILI | 15x/÷1.22 | 14x/÷1.05 | 22x/÷1.07 | 16x/÷1.14 | 18x/÷1.21 |
| OPA$^-$ OM | 37x/÷1.63 | 533x/÷1.29 | 116x/÷1.56 | 192x/÷1.39 | 109x/÷1.14 |
| | *TNF-α (geometric mean pg/ml x/÷ SE*)* | | | | |
| CONTROL | - | 47x/÷1.01 | 51x/÷1.01 | 121x/÷1.04 | 101x/÷1.03 |
| LOS | 53x/÷1.02 | 68x/÷1.12 | 64x/÷1.11 | 69x/÷1.18 | 61x/÷1.05 |
| PILI | 76x/÷1.26 | 61x/÷1.04 | 84x/÷1.04 | 57x/÷1.07 | 67x/1.15 |
| OPA$^-$ OM | 270x/÷1.47 | 1054x/÷1.11 | 359x/÷1.34 | 541x/÷1.17 | 425x/÷1.06 |
| | *MIP-1α (geometric mean pg/ml x/÷ SE*)* | | | | |
| CONTROL | - | 207x/÷1.01 | 231x/÷1.02 | 627x/÷1.13 | 360x/÷1.28 |
| LOS | 234x/÷1.06 | 382x/÷1.20 | 324x/÷1.12 | 400x/÷1.45 | 367x/÷1.08 |
| PILI | 376x/÷1.47 | 452x/÷1.21 | 1142x/÷1.18 | 368x/÷1.28 | 582x/÷1.53 |
| OPA$^-$ OM | 1125x/÷1.84 | 9185x/÷1.10 | 3812x/÷1.30 | 6330x/÷1.23 | 5068x/÷1.11 |

*SE = standard error of the mean.

Clearly, the effects of sera from naturally infected individuals on macrophage responses to gonococcal components did not exceed those observed with normal human serum. Therefore, either antibodies induced by infection were not responsible for increased cytokine production; or other factors in immune sera, such as anti-Rmp blocking antibodies, abated opsonisation. Since the latter effect should not interfere with the binding of immunoglobulins to pili, macrophages were incubated in 10% anti-pilus antiserum during stimulation of the cells with homologous antigen. The human antiserum was obtained by immunisation of a volunteer with purified α-pili (strain P9), following adsorption of the preparation onto Alhydrogel® (Superflos) adjuvant. Specific antibody titres were determined by radioimmunoassay, demonstrating an increase from 1/100 (pre-immunisation) to ~1/4000 (seven weeks post-immunisation). The experimental protocol was identical to that used with the normal sera [see Section *5E(viii)*], and the data set for each cytokine (IL-6, TNF-α, and MIP-1α) was analysed by two-way ANOVA. This would ascertain whether the post-immune serum caused a significant increase in the stimulatory potency of pili relative to the pre-immune serum, whilst taking serum-only control values into account.

The high titre of anti-pilus antibodies in the post-immune serum was associated with an increase in MIP-1α induction by pili, but this was not significant (see Table *5·5*). Production of IL-6 and TNF-α following challenge with pili was unaffected by the immunological status of the serum (see Table *5·5*). Therefore, the factor(s) in serum that enhanced macrophage responses to purified pili (see Sections *5E(vii)* & *5E(ix)*] were unlikely to have been specific IgG antibodies to pilus components.

**TABLE *5·5*: The Influence Of Anti-Pilus Antiserum On The Macrophage Cytokine Response To Purified Gonococcal Pili.**

| | *CYTOKINE CONCENTRATION (geometric mean pg/ml x/÷ SE*)* | | | | | |
|---|---|---|---|---|---|---|
| | *CONTROL* | | *+ PILI* | | *NET INCREASE* | |
| *CYTOKINE* | *PRE-IMMUNE* | *POST-IMMUNE* | *PRE-IMMUNE* | *POST-IMMUNE* | *PRE-IMMUNE* | *POST-IMMUNE* |
| IL-6 | 11x/÷1.03 | 10x/÷1.02 | 15x/÷1.16 | 15x/÷1.05 | 4 | 4 |
| TNF-α | 50x/÷1.01 | 48x/÷1.01 | 65x/÷1.08 | 67x/÷1.06 | 15 | 19 |
| MIP-1α | 221x/÷1.02 | 222x/÷1.05 | 512x/÷1.27 | 726x/÷1.34 | 291 | 504 |

*SE = standard error of the mean.

## *5F* - Conclusions

The first objective of this investigation was the production of differentiated macrophages *in vitro*. Phenotypic analysis of adherent mononuclear cells cultured for 1-2 weeks *in vitro* confirmed that full maturation had occurred, although a minor population of multinucleated giant cells was also present. Confocal microscopic analysis of macrophages challenged with gonococci demonstrated that the bacteria were rapidly phagocytosed, and neither pilus nor Opa protein expression was necessary for efficient internalisation. This implies that differentiated macrophages can bind gonococci via receptors recognising LOS and/or PorB. In addition, the vast majority of the phagocytes remained viable for the duration of the experiment.

Viable gonococci rapidly induced the elevated production of several cytokines (IL-6, TNF-α, RANTES, MIP-1α, IFN-γ, and GRO-α) that are recognised as mediators of inflammation. Analysis of cytokine secretion in conjunction with viable counts of cell-associated bacteria supported the hypothesis that gonococcal interactions with macrophages were not dependent on pili or Opa. Indeed, if pilus expression had any effect on macrophage responses, it was suppressive. However, Opa-$b^+$ gonococci displayed enhanced avidity for macrophages, both in terms of cell-association and stimulation of cytokine release; whilst Opa-$a^+$ organisms resembled the $Opa^-$ phenotype or even generated a weaker response.

Interestingly, the induction of cytokine secretion was independent of gonococcal metabolic activity, as purified surface components generated a qualitatively similar profile in stimulated macrophage cultures to that observed with viable bacteria. Isolated outer membrane vesicles lacking Opa proteins were more potent inducers of cytokine production than was soluble LOS, suggesting that a particular LOS conformation and/or essential outer membrane proteins (particularly PorB) were required for maximal stimulation. The use of $Opa^+$ vesicles supported the data obtained with whole organisms, since the Opa-$b^+$ membranes were more effective stimuli than were those containing Opa-a. Similarly, purified pili generated a relatively weak response from macrophages, supporting the observations of the previous experiments.

When macrophage medium was supplemented with normal human serum during stimulation experiments, cytokine production in the presence of pili or vesicles was considerably amplified. However, no such enhancement was apparent with soluble LOS. Surprisingly, pre-incubation of gonococcal components with immune sera did not lead to

further increases in stimulatory capacity, implying that either antibodies were not responsible for opsonisation, or blocking antibodies negated their effects.

# CHAPTER 6

## *DISCUSSION OF THE PRESENTED FINDINGS*

### *6A* - The Aetiology Of Sexually Transmitted Bacterial Infections: Clinical Aspects & Pathogenesis

6A(i) *Objectives Of The Investigation & Study Requirements*: The original intention of this project was to investigate the diagnosis and pathogenesis of gonorrhoea using specimens collected from a developing country. Of particular interest were the factors determining symptomatic versus asymptomatic outcomes, and localised versus ascending infection, especially in connection with cytokine production and infectious load. Ultimately, this might have led to the identification of specific triggers for serious sequelae, which could have informed future development of a diagnostic/prognostic test.

This research proposal had three minimum requirements. Firstly, a collaborative connection with an institution located in the developing world would be needed in order to obtain specimens; and for analysis of ascending infection, ethical approval for sampling of the female upper genital tract would be essential. Secondly, a high prevalence of gonorrhoea in the study population would be necessary, as there was a limited period in which to collect specimens, and identification of risk factors would require an adequate sample size. Finally, any analysis of inflammation-related proteins - or RNA from either pathogen or host - would be dependent on reliable storage and transport of the specimens at -70°C or below.

A long-standing partnership between Southampton University Molecular Microbiology Group and the Department of Obstetrics-Gynaecology at Prince of Songkla University, Hat Yai, Thailand, ensured that the first condition was fulfilled before the project commenced. However, with regard to the other two requirements, problems were encountered that limited the scope of the clinical research programme. The progress that was made despite this is discussed in the following subsections.

6A(ii) *False Positive Diagnoses Of STD In Asymptomatic Male Students From Southern Thailand*: The collaborative link with Prince of Songkla University resulted in the receipt of 480 urine specimens from asymptomatic male technical college students. These samples had already been processed and assayed using the AMPLICOR™ *Chlamydia trachomatis/Neisseria gonorrhoeae* PCR kit, following which they had been

stored at –20°C. The test results reported from Thailand, indicating a prevalence of 1.5% for asymptomatic gonococcal infection and 7.1% for chlamydial infection, suggested that this population would provide an opportunity to study asymptomatic carriage of STD agents. Confirmation of unapparent gonococcal infection in a naïve population would have important consequences for the understanding of gonorrhoeal epidemiology, and would lend support to the study of Grosskurth *et al.* (1996), who discovered such infections among men in Tanzania.

Re-testing in the UK using two independent PCR methods could not confirm the presence of gonococci in any of the putatively positive samples. This finding was reminiscent of that obtained by Bassiri *et al.* (1997), who tested over 3000 urine specimens from asymptomatic women for *C. trachomatis* and *N. gonorrhoeae* using the AMPLICOR™ multiplex assay. Although nine of their samples were positive for gonococci according to AMPLICOR™, which targets the putative methyltransferase gene, all of the specimens were negative in a second assay based on a 16S rRNA gene sequence. Furthermore, Farrell (1999) found that the AMPLICOR™ *N. gonorrhoeae* assay exhibited low specificity (86%) when applied to urine and genital swab specimens collected from a high-risk Australian aborigine population. The probable explanation was cross-reactivity with the commensal species, *N. subflava* (Farrell, 1999). In contrast, assays targeting the *cppB* gene of the cryptic plasmid (used in the current study) display excellent specificity for gonococci (Ho *et al.*, 1992; Farrell, 1999). Although gonococcal strains lacking this plasmid have been reported (Roberts *et al.*, 1979), the Thai samples did not react in a second PCR that was based on a locus (*porB*) essential for gonococcal viability; and inhibition of DNA polymerase was also ruled out.

Fourteen of the 34 specimens identified as *Chlamydia*-positive in Thailand were negative in two independent confirmatory assays. Compared to the gonococcal test, the AMPLICOR™ assay for *C. trachomatis* [which detects the cryptic plasmid (Peterson *et al.*, 1997)] has demonstrated greater reliability in a number of studies on urine specimens (Stary *et al.*, 1996; Quinn *et al.*, 1996; Bassiri *et al.*, 1997). However, in the report of Pasternack *et al.* (1999), false-positive diagnoses were identified by discrepant analysis in 1% of urine samples. Although detailed analyses of reproducibility are lacking for urine specimens, Mulcahy *et al.* (1998) reported that only 47% of "weak positive" endocervical samples produced consistent results on repeat testing. In addition, Peterson *et al.* (1997) subjected 13 endocervical specimens exhibiting discrepant or equivocal outcomes in the AMPLICOR™ assay to five rounds of confirmatory testing using two different primer

pairs; ten of these samples failed to generate consistent results. In this study as in the current work, an assay targeting *ompl* was used to exclude the possibility of missed infections caused by plasmid-free chlamydiae (Peterson *et al.*, 1990). It is unlikely that inhibition was responsible for the unconfirmed Thai results, but it is possible that very low template copy numbers precluded a consistent outcome.

Screening of the students' urine specimens for *Mycoplasma genitalium* identified 11 infections, a prevalence of 2.3%. However, this finding needs to be interpreted with caution. This organism is isolated more frequently from men with non-gonococcal urethritis (NGU) than from demographically equivalent control groups, but a prevalence of 6% has been reported for the latter in the UK (Horner *et al.*, 1993). Therefore, *M. genitalium* may represent a potential risk for the future development of NGU, and would presumably be transmitted to sexual partners who may themselves develop symptoms.

It is very likely that the scarcity of sexually transmitted agents in these students was due to condom promotion campaigns instigated by the Thai authorities in the wake of the HIV epidemic. In 1987, over 100,000 cases of gonorrhoea in men were reported nationally from government clinics (Rojanapithayakorn & Hanenberg, 1996), and *N. gonorrhoeae* accounted for three times the number of urethritis cases seen in Bangkok than did *C. trachomatis* (Kuvanont *et al.*, 1989). The "100% Condom Program", which focused on the enforcement of condom use in commercial sex establishments (CSE), was initiated in selected provinces in 1989, and was adopted at the national level in 1991 (Rojanapithayakorn & Hanenberg, 1996). Together with a mass advertising campaign targeted at the patrons of CSE, the program had a huge impact on the incidence and aetiology of STD. By 1994, the number of male gonorrhoea cases reported from across the country had dropped to ~10,000; whilst from 1993, NGU was more frequently seen than gonorrhoea because of a slower decline in incidence (Rojanapithayakorn & Hanenberg, 1996). The momentum of the campaign appears to have been successfully maintained, as Mills *et al.* (1997) documented a low level of risk behaviours for STD acquisition among male vocational students in Bangkok between 1993 and 1996.

6A(iii) *Gonorrhoea & Chlamydial Infection In A High-Risk Population Of Sri Lankan STD Clinic Attendees*: Cervical specimens from a population that was likely to have a high prevalence of gonorrhoea were obtained through a short research project conducted in Sri Lanka by Dr. S. Amarasekara. The study group comprised STD clinic attendees in Colombo, with a high proportion (45%) of affirmed commercial sex workers.

Despite these factors, gonococci were detected by PCR in only 19 (5.5%) of the samples, and all infections were caused by *porB1b* strains. Although data on the epidemiology of gonorrhoea in Sri Lanka are limited, the prevalence amongst women attending the Colombo clinic in the mid-1980s was 10% (Abeyewickereme, 1989). During the subsequent decade, penicillinase-producing *N. gonorrhoeae* disappeared completely and 4-fluroquinolone resistance became apparent (Abeyewickereme *et al.*, 1996). However, the total number of gonococcal isolates recovered from the Colombo clinic patients declined from 1104 in 1986 to 318 in 1995 (Abeyewickereme *et al.*, 1996). Although cumulative data are difficult to interpret, the fact that only half the number of 17-26-year-olds had ever had gonorrhoea compared to the 27-36-years old age class might reflect a recent decrease in incidence.

In contrast, the prevalence of chlamydial infection (25.6%) was much higher than the indigenous diagnostic system had detected, and two probable explanations can be proposed for this. Firstly, Papanicolaou-stained smears used for the visualisation of chlamydial inclusions demonstrate low concordance with the results of cell culture - one reason being that only ~60% of smears may contain an adequate sample of cells on which to base a diagnosis (Dorman *et al.*, 1983). Secondly, in a developing country facility with limited resources, priority for diagnosis is inevitably given to symptomatic patients. However, the sensitivity of cervical abnormalities for the detection of chlamydial infection was only 38%, suggesting that a clinical "pre-screen" would lead to underestimation of the scale of the problem. This tenuous link between infection with sexually transmitted agents and cervical inflammation is recognised as a challenge to the development of syndromic protocols for the management of cervicitis (van Dam *et al.*, 1998).

Although these specimens could have potentially yielded information regarding the molecular basis for clinical outcome in gonorrhoea - particularly as nearly 50% of infections were subclinical - logistical problems prevented further investigation. To demonstrate meaningful differences among only 19 samples, specimen quality would have been critical. However, only -20°C storage facilities were available in Sri Lanka. Furthermore, trained personnel, resources, and ethical approval for sampling of the upper genital tract were not obtainable.

6A(iv) *The Pathogenesis Of Mucopurulent Cervicitis & Its Relation To Other Infections Of The Female Genitourinary System*: The aforementioned collaboration with

Prince of Songkla University in Thailand enabled a study of the aetiology and pathogenesis of mucopurulent cervicitis (MPC) in a population presenting with acute symptoms of this condition. For several reasons, this project had the potential to reveal important associations between gonococcal characteristics and the clinical manifestations of gonorrhoea in women. Firstly, the occurrence of gonorrhoea in this population was expected to be high. Based on data collected between 1993 and 1994, several years after implementation of the "100% Condom Program", the prevalence of gonococcal infection of the cervix among antenatal clinic attendees in Bangkok was estimated to be 1.4% (Chaisilwattana *et al.*, 1997). This figure reflects the prevalence in asymptomatic women, and it was reasonable to assume that patients with symptomatic cervicitis would be more likely to be infected (Curran *et al.*, 1975). The second factor supporting the study rationale was the availability of facilities and ethical approval for sampling of the endometrium, which would enable an analysis of the risks associated with ascending infection in this population. Finally, inflammatory mediators could be assayed in cervical secretions because all specimens were frozen at -70°C on the day of collection, and were transported to the UK in liquid nitrogen.

There was a complete absence of gonococcal infection among these women. Clearly, the gonorrhoea prevalence estimate for Bangkok does not apply to the city of Hat Yai, located several hundred miles to the south. Since significant regional variation in HIV prevalence has been documented in Thailand (Beyrer *et al.*, 1995), it is probable that the occurrence of other STD is similarly dependent on epidemiological differences between provinces. Indeed, a recent study of women with abnormal vaginal discharge attending Prince of Songkla University Hospital revealed that only 0.4% had gonorrhoea (Chandeying *et al.*, 1998). It appears that the decline in gonorrhoea associated with the "100% Condom Program" has been so rapid in Thailand (Rojanapithayakorn & Hanenberg, 1996) that prevalence estimates are outdated by the time they reach publication.

In contrast, 20% of the cervicitis patients in the present study had *Chlamydia trachomatis* infections of the endocervix, and inclusion of positive samples from other genitourinary sites increased detection to 27%. The slower decline of NGU relative to gonorrhoea in Thai men (Rojanapithayakorn & Hanenberg, 1996) appears to be mirrored by cervicitis in women. Thus, the prevalence of cervical *Chlamydia* infection among antenatal clinic attendees has been reported to be seven-fold greater than that of gonococcal infection in Bangkok (Chaisilwattana *et al.*, 1997); whilst in Hat Yai, ten-fold

more cases of chlamydial infection than gonorrhoea were seen in women with abnormal vaginal discharge (Chandeying *et al.*, 1998).

Studies on the role of *C. trachomatis* in MPC are predominantly restricted to the USA. The proportion of cases associated with this organism has been reported to be 50% for randomly selected STD clinic attendees (Brunham *et al.*, 1984), and 42% for STD patients referred for suspected cervicitis (Paavonen *et al.*, 1986). However, chlamydiae are less frequently isolated from students with MPC attending university health clinics; whether they are referred for suspected cervicitis (32%; Paavonen *et al.*, 1986), or are diagnosed during pelvic examinations for related or unrelated complaints (36%; Moscicki *et al.*, 1987). In developing countries, data on MPC are very limited. Herrmann *et al.* (1996) reported that chlamydial infection of the cervix was detected in 15% of routine clinic attendees and sex workers in Nicaragua with macroscopic mucopus. However, in Hat Yai, only 9% of women in the vaginal discharge study who had visible or microscopic cervical mucopus were diagnosed with chlamydial or gonococcal infection (Chandeying *et al.*, 1998).

The reasons for this wide range in the proportion of MPC cases associated with *C. trachomatis* are not known. Differences in diagnostic sensitivity are one possibility, although one study reporting many *Chlamydia*-negative cases (Herrmann *et al.*, 1996) used PCR assays, which would be expected to be at least as sensitive as cell culture. Use of the oral contraceptive pill (OCP) is independently associated with MPC (Paavonen *et al.*, 1986), and has been identified as a risk factor for the acquisition of *C. trachomatis* (Herrmann *et al.*, 1996) – probably via its positive effect on cervical ectopy (Hobson *et al.*, 1980). Therefore, differences in the prevalence of OCP use may influence the proportion of MPC cases attributed to chlamydial infection. In the North American studies, 28-46% of the study population used this method (Brunham *et al.*, 1984; Paavonen *et al.*, 1986); whereas in Nicaragua, OCP was used by only 12% of women (Herrmann *et al.*, 1996). This is similar to the figure of 17% determined from the patient records for the Thai MPC population. Race is another factor that may affect the positive predictive value of MPC for chlamydial infection. In the study of students conducted by Moscicki *et al.* (1987), 53% of black patients with MPC were infected with *Chlamydia*, compared to only 13% of non-black subjects. It is equally likely that the aetiology of MPC in Thai women differs from that recorded for other races.

The association of *C. trachomatis* with MPC in different settings may also depend on the relative importance of other STD agents in a given population. For instance,

Paavonen *et al.* (1986) discovered a significant role for *Ureaplasma urealyticum* in the aetiology of MPC after correcting for the occurrence of chlamydial infection. Similarly, Uno *et al.* (1997) established that 9% of Japanese women with *Chlamydia*-negative cervicitis were infected with *Mycoplasma genitalium*, compared to none of the asymptomatic pregnant controls. In the current work, this organism was discovered in 7% of all women, and 5% of patients without demonstrable chlamydial infection. However, as this study was restricted to symptomatic women, the prevalence in a demographically similar control group was not determined. Interestingly, Uno *et al.* (1997) reported that 4% of their subjects with *Chlamydia*-negative adnexitis had positive endocervical specimens for *M. genitalium*. Among Thai MPC patients with *M. genitalium* detected in the lower genital tract, two (33%) also had endometrial infections, indicating a potential role for this organism in the aetiology of pelvic inflammatory disease.

Of the 24 Thai women with chlamydial infection, four (17%) had positive urethral specimens without detectable chlamydial DNA in the endocervix. Paavonen (1979) determined that 25% of *Chlamydia*-infected Finnish women attending Obstetrics & Gynaecology outpatient clinics had positive urethral cultures but negative cervical cultures. The proportion of urethra-only infections is probably affected by the sensitivity of detection for cervical involvement; but nevertheless, paired assays on specimens from both sites will reduce false-negative diagnoses. Despite the existence of infections confined to the urethra, the current study revealed that a positive urethral diagnosis was associated with a high infectious load in the endocervix.

In the present study, 35% of women with chlamydiae detected in the lower genital tract also had endometrial infections. Paavonen *et al.* (1985) used transcervical endometrial aspiration to isolate *C. trachomatis* from the uteri of North American STD clinic attendees with suspected cervicitis. Positive cultures were obtained from 23% of women who also had endocervical *Chlamydia* infection, although the presence of organisms in the aspirate did not correlate with histological evidence of plasma cell endometritis. In a study of women with pelvic pain, Chernesky *et al.* (1998) applied a similar methodology to that used in the current work (proteinase K digestion of biopsies, phenol-chloroform extraction, and plasmid-based PCR) to diagnose chlamydial infection of the endometrium, although the specimens in this case were formalin-fixed and paraffin-embedded. Chlamydial DNA was detected in 29% of biopsies from women with positive endocervical cultures for *C. trachomatis*.

Thus, the proportion of ascending infections in the Thai MPC populations was higher than reported by other investigators, but this might be due to superior diagnostic sensitivity rather than to a genuine epidemiological difference. Chernesky *et al.* (1998) used a PCR directed to human β-globin DNA to control for false-negative assays, yet several women who were negative for chlamydiae in the PCR had evidence of infection by other methods. Since biopsy extracts will contain a vast excess of human DNA relative to target sequences, the use of an exogenous positive control at $2x10^5$ templates per specimen - as in the present study - is more likely to detect false-negative reactions. However, the very large ratio of human DNA to template affected assay sensitivity, probably because phosphate groups bind the magnesium ions required by DNA polymerase for optimal activity (Harris & Jones, 1997).

Quantification of chlamydiae in endocervical specimens revealed that women with a high infectious load were more likely to have a positive diagnosis for the endometrium. This may be because a high-grade infection reflects failure of the immune system to control and localise chlamydial replication. However, two caveats must be considered in the interpretation of this apparent association. Firstly, although the suction curettes used for endometrial sampling were sheathed to minimise contamination from the lower genital tract, the extreme sensitivity of the PCR technique prevents exclusion of this possibility for heavily infected cervices. Secondly, a positive diagnosis for an endometrial sample would require 100-3300 template copies, whilst for endocervical specimens, ten plasmids or less could be detected. Therefore, if chlamydial numbers in endometrial biopsies were proportional to endocervical infectious load, a spurious association between high-grade cervical infections and the presence of organisms in the upper genital tract would result.

Hobson *et al.* (1980) used inclusion counts in cell culture to quantify endocervical *Chlamydia* load in STD patients attending a UK (Liverpool) clinic. While this group found infectious load to be normally distributed, all counts determined in the current study could be classified as less than 20 or greater than 10,000. However, cell culture might not have detected very low levels of chlamydiae, as this technique requires both viable organisms and the successful formation of inclusions. Hobson *et al.* (1980) did not find a correlation between chlamydial numbers and clinical signs of salpingitis - although few of the women had such symptoms - but a positive association with the presence of mucopus was reported. This contrasts with the present study, in which neither chlamydial

infection *per se*, nor chlamydial infectious load, were associated with clinical severity of MPC.

Several factors may explain this discrepancy. In the Liverpool study, the number of chlamydiae recovered was increased through a synergism between OCP and cervical ectopy, which might also have affected the cervical response to infection. While 53% of Liverpool STD patients used OCP, only 17% of the Thai women did so. Furthermore, Hobson *et al.* (1980) reported that young age was positively associated with inclusion counts, perhaps through hormonal effects or immunological naivety. Although a significant proportion (28%) of the Liverpool patients were under 20 years of age, only 2% of the Thai subjects fell into this category. Finally, the Liverpool study included chlamydial infections that were not associated with cervical mucopus, whereas the current work involved the severity of cervicitis in a population of symptomatic women.

The levels of proinflammatory cytokines detected in endocervical specimens were not affected by the presence of *C. trachomatis* or by the infectious load of this organism. However, overt disease (manifested as macroscopic mucopus) was associated with elevated production of IL-1β, IL-6, and IL-8. Published investigations of cervical cytokine secretion in STD are very limited. Hedges *et al.* (1998) quantified several cytokines in the cervical mucus of women attending a North American STD clinic. In the majority of women, levels of IL-1β, IL-6, and IL-8 were below the limits of detection; and no differences were found between individuals without gonococcal infections, patients with gonococcal cervicitis, and cases of co-infections involving *N. gonorrhoeae* plus *C. trachomatis* or trichomonads. No attempt to correlate cytokine secretion with clinical findings was reported. Although the absence of a pathogen-specific cytokine response accords with the current work, direct comparisons are invalid because women in the American study might have been predominantly asymptomatic. Furthermore, the methodology of Hedges *et al.* (1998) included sequential ELISA (serial testing of individual samples for different cytokines) and colorimetric detection, which is likely to be less sensitive than parallel assays utilising time-resolved fluorometry.

The involvement of IL-1β, IL-6, and IL-8 in MPC is to be expected from their established roles in the inflammatory response. Interleukin-1β induces the cellular enzymes responsible for secretion of other inflammatory mediators, such as prostaglandin-$E_2$, leukotrienes, and nitric oxide (Dinarello, 1997). In addition, this cytokine stimulates the release of IL-6 and IL-8 (Dinarello, 1997), and causes infiltrating neutrophils to undergo various phenotypic changes [("priming") - Dallegri & Ottonello,

1997]. While IL-6 may act in concert with IL-1β to increase proteinase expression (Ito *et al.*, 1992; cited in Richards, 1998), the primary function of IL-8 is chemoattraction of neutrophils, which results in the formation of a purulent discharge (Mikami *et al.*, 1998). However, it is impossible to differentiate cause from effect in specimens that represent "snapshots" of natural disease, and recruited leukocytes might have produced these cytokines following an unidentified primary phase.

Microbiological triggers of the inflammatory response were not identified for 70% of the MPC patient population. Despite its undisputed dominant role in infectious MPC, *C. trachomatis* is rarely isolated from even half the cases in a given population (Moscicki *et al.*, 1987), and therefore "cryptic" aetiologies must account for much of the disease. This was highlighted in the vaginal discharge study conducted in Hat Yai by Chandeying *et al.* (1998). Only 56% of women treated for MPC with ofloxacin and doxycycline exhibited clinical cure (partners were also treated), and the frequencies of both spontaneous resolution and new instances of MPC at re-assessment were high. In women with both MPC and bacterial vaginosis, administration of antibiotics directed against the latter improves clinical resolution of the former, implying a role for anaerobes in cervical inflammation (Schwebke *et al.*, 1995; cited in Sweet, 1998). It is also likely that hormonal influences are important in the pathogenesis of MPC. Paavonen *et al.* (1986) reported that MPC was associated with the use of OCP in currently uninfected women who had serum antibody to *C. trachomatis*, suggesting a state of chronic inflammation. This may be related to the effects of hormones on chemokine expression, since progesterone appears to regulate the secretion of IL-8 in the female genital tract throughout the menstrual cycle (García-Velasco & Arici, 1999).

## ***6B*** **- The Interaction Of *Neisseria gonorrhoeae* With Human Macrophages: Generation Of Proinflammatory Cytokines & Implications For Pathogenesis**

6B(i) *The Proposed Role Of Macrophages In Gonorrhoea*: In the Thai MPC study, IL-1β, IL-6, and IL-8 were implicated in the pathogenesis of cervical inflammation. *Neisseria gonorrhoeae* was absent from this study population, but the independence of the observed response to aetiology suggests that gonococcal cervicitis would have been associated with a similar spectrum of cytokines. The detection of cytokines in endocervical specimens raises the question of their source. Mononuclear phagocytes represent a small but significant component of the normal human cervix,

accounting for ~1% of all cells in this tissue (Givan *et al.*, 1997), or ~20 macrophages per $mm^2$ of epithelium and ~50 per $mm^2$ of stroma (Tay *et al.*, 1987). While *in vitro* experiments support a role for cervical epithelium cells in the generation of proinflammatory cytokines (Naumann *et al.*, 1997), the contribution of tissue-resident macrophages to the inflammatory response has not been determined.

In men, symptoms of gonorrhoea are usually apparent within 2-5 days of natural infection (Handsfield & Sparling, 1995) - long before a specific immune response could be mounted in a naïve individual. Human volunteer experiments have revealed that secretion of IL-6, IL-8, and TNF-α in urine occurs 2-14 hours after gonococcal challenge, whilst IL-1β production is associated with disease onset (Ramsey *et al.*, 1995). Tissue-resident macrophages, which have been detected in normal human urothelium (El-Demiry *et al.*, 1986), are a likely source of local cytokine generation during the pre-symptomatic phase of gonorrhoea. In addition, urethral symptoms persist for an average of eight weeks if left untreated (Holmes, 1974). Thus, the inflammatory response in gonococcal urethritis, although acute, can be sustained over a prolonged period. This may involve macrophages located in the subepithelial lamina propria of the male urinary system, which are more numerous than their counterparts in the urothelium (El-Demiry *et al.*, 1986).

A significant proportion (10-40%) of women with untreated gonococcal infection subsequently develops pelvic inflammatory disease [(PID) - Sciarra, 1997]. The pathology of PID involves swelling of the fallopian tubes, congestion of the tubal mucosa, fibrin deposition, adhesion between pelvic organs, and abscess formation (Weström & Mårdh, 1990). *Ex vivo* models of gonococcal salpingitis have determined that the initial mucosal damage occurs through sloughing of ciliated epithelial cells (Ward *et al.*, 1974), which is mediated by the release of TNF-α (McGee *et al.*, 1992). Mononuclear phagocytes constitute more than 2% of the resident cell population in fallopian tube tissue (Givan *et al.*, 1997); hence, gonococci are likely to encounter this cell type during ascending infection.

In summary, it is probable that tissue-resident macrophages interact with gonococci during localised infections in both sexes, and in ascending infections involving the female upper genital tract. However, the vast majority of studies on gonococcal pathogenesis have focused on only two types of cell: epithelial cells (van Putten & Duensing, 1997) and neutrophils (Shafer & Rest, 1989). Therefore, the association of macrophages with gonococci was observed *in vitro*, and an investigation of the cytokine

response was conducted to elucidate the possible role of these cells in the pathogenesis of gonococcal infections.

6B(ii) *Phagocytosis Of Gonococci – Effects On Macrophages & Bacteria*: To date, most studies on the interaction of gonococci with human mononuclear phagocytes have been restricted to undifferentiated adherent monocytes (Harper *et al.*, 1996; Knepper *et al.*, 1997; Lorenzen *et al.*, 1999). Although Mosleh *et al.* (1998) used macrophages that had been matured in the presence of human serum, only morphological criteria were used to characterise their cells. In the current work, *in vitro* culture progressed for at least eight days to allow development of a mature surface phenotype (Andreesen *et al.*, 1990). Fluorescence microscopy was utilised to demonstrate the expression of relevant receptors on the macrophage plasma membrane.

The macrophage mannose receptor is not expressed on peripheral blood monocytes, but can be detected after 3 days' culture in the presence of human serum, and is expressed by macrophages located in various tissues (Noorman *et al.*, 1997). This glycoprotein is a critical determinant of macrophage function, responsible for non-opsonic interactions with numerous bacteria and other pathogens (Fraser *et al.*, 1998). The binding of certain ligands to the mannose receptor, such as myeloperoxidase, induces enhanced bactericidal activity via increased production of reactive oxygen intermediates (Lefkowitz *et al.*, 1997). Furthermore, phagocytosis mediated by this receptor results in the secretion of proinflammatory cytokines (Aderem & Underhill, 1999). Thus, the detection of the mannose receptor on cultured cells in the current study confirmed that functionally mature macrophages had been successfully produced.

*In vitro* culture of monocytes with human serum is also associated with increased expression of phagocytic glycoprotein-1 [(Pgp-1, CD44) - Levesque & Haynes, 1996]. This receptor binds several components of the extracellular matrix - such as hyaluronan (Levesque & Haynes, 1996) - and is involved in the fusion of macrophages that culminates in multinucleated giant cell formation (Sterling *et al.*, 1998). Spontaneous coalescence of macrophages into multinucleated cells with distinctive nuclear rings (Sorimachi *et al.*, 1998) occurs during contact with glass, and these cells dominate the culture after 2-3 weeks *in vitro* (Kaplan & Gaudernack, 1982). Since giant cells are unlikely to be involved in the pathogenesis of gonorrhoea, their formation was minimised by the culture of macrophages on Matrigel®-coated surfaces for a maximum of 11 days (significant numbers of these cells were not observed before day 10). Matrigel® basement

membrane matrix may inhibit macrophage fusion by blockade of Pgp-1 (Sterling *et al.*, 1998).

Following centrifugation to facilitate cell-bacterium contact, gonococci were phagocytosed by macrophages within 10 minutes. Similarly, Read *et al.* (1996) observed that the majority of capsulate and non-capsulate meningococci incubated with differentiated human macrophages were internalised by 10 minutes. However, in their experiments, bacteria were pre-opsonised with complement component 3. This research group also used gentamicin to determine the number of opsonised meningococci that survived phagocytosis, and they reported a mean recovery of only one non-capsulate organism per ~120 macrophages after three hours' warm incubation. Although the methodology of this study differed in some respects to the current work, their estimate is similar to that determined for gonococci: a maximum mean recovery of two bacteria per 100 macrophages after a total of 7½ hours.

Evidently, uptake and killing of neisseriae by macrophages is an efficient process, and it seems unlikely that destruction of gonococci is dependent on neutrophil-mediated "surface phagocytosis" of organisms bound to the macrophage surface, as has been reported in the liver (Gregory & Wing, 1998). Indeed, neutrophils may be less effective at eliminating gonococci than macrophages. Hence, Casey *et al.* (1986) determined that 3% of cell-associated gonococci survived within neutrophils for over 2½ hours, compared to a maximum of ~0.01% in macrophages after 7½ hours in the present study. This residual survival of internalised gonococci may be linked to a delay in macrophage phagolysosome formation, which is mediated by the incorporation of PorB protein into the phagosomal membrane (Mosleh *et al.*, 1998). However, Bessen & Gotschlich (1986) found that ~0.01% of gonococci associated with HeLa cells resisted gentamicin treatment in the presence of a phagocytosis inhibitor (cytochalasin *b*), implying that organisms located in membranous depressions (such as those observed on giant cells in the current study) can be protected from antibiotic activity.

Another limitation of the gentamicin exclusion assay is that it cannot discriminate between effective killing of bacteria by phagocytes, and efficient destruction of cells by bacteria, since damaged cells will be permeable to the antibiotic. To circumvent this problem, the viability of gonococcus-challenged phagocytes was determined microscopically using the Molecular Probes LIVE/DEAD viability/cytotoxicity kit. Macrophage death in the presence of gonococci was uncommon, although membrane disruption appeared to be associated with large numbers of bacteria on the phagocyte

surface. Müller *et al.* (1999) demonstrated that viable gonococci and purified PorB proteins induce apoptosis following prolonged incubation (15 hours) with epithelial and monocytic cell lines. However, the results presented in the current study represent necrotic cell death (which leads to membrane permeability) rather than programmed cell death (apoptosis), and Müller *et al.* (1999) did not detect any increase in the former during the time-frame of their experiments. It is possible that apoptosis was responsible for the dramatic change in macrophage morphology noted after gonococcal challenge, although gonococcal strain P9 (expressing PorB1b) is likely to be less effective at inducing this process than PorB1a strains (Müller *et al.*, 1999).

In contrast, a large proportion of giant cells that bound gonococci became necrotic. Multinucleation shares many features in common with apoptosis (Sorimachi *et al.*, 1998), but this cannot explain the rapid loss of membrane integrity exhibited by infected giant cells. These cells appeared to be overwhelmed with bacteria that were not effectively ingested (Kaplan & Gaudernack, 1982), which might have led to cytotoxicity via LOS (Cooper *et al.*, 1986) or other gonococcal components.

6B(iii) *The Roles Of The Major Gonococcal Surface Components In Interactions With Human Macrophages*: Cytokines have critical roles in both the initiation and termination of the inflammatory response, and thereby determine the course of disease progression (Dinarello, 1997). The present study was designed to detect a wide range of cytokines, as mononuclear phagocytes produce numerous pro- and anti-inflammatory mediators in response to a variety of stimuli. Thus, monocytes and macrophages generate three of the most important proinflammatory cytokines (IL-1β, TNF-α, and IL-6) following exposure to LPS (Scheibenbogen & Andreesen, 1991). Interleukin-12, which is also involved in the development of inflammation, is released by macrophages stimulated with CD40 ligand (Trinchieri, 1997); whilst alveolar macrophages are a source of IFN-γ during mycobacterial infection (Fenton *et al.*, 1997). Furthermore, cytokines such as GM-CSF can be produced by monocytes in response to stimulation by Fc fragments of IgG (Ishiguro *et al.*, 1991).

The generation of CXC chemokines (IL-8, ENA-78, and GRO subfamily) has been documented for alveolar macrophages challenged with LPS (Goodman *et al.*, 1998), and the CC chemokine MIP-1α is also induced by this stimulus (Nibbs *et al.*, 1998). Another CC chemokine, RANTES, is released from monocytes as part of the proinflammatory cascade (Nelson *et al.*, 1998). However, under certain conditions,

macrophages secrete anti-inflammatory mediators. For instance, infection with respiratory syncytial virus leads to IL-10 production in alveolar macrophages (Panusak *et al.*, 1995; cited in Trinchieri, 1997), whilst proinflammatory stimuli such as IL-12 elicit the release of TGF-β (Sutterwala & Mosser, 1999). Therefore, the implications of gonococcus-macrophage interactions for the pathogenesis of gonorrhoea were investigated by including assays for all of these cytokines in the current work.

Viable gonococci exclusively induced proinflammatory cytokines (comprising IL-6, TNF-α, MIP-1α, RANTES, GRO-α, and IFN-γ), and the levels of several of these proteins exceeded the baseline by one-to-two orders of magnitude following three hours of infection. Remarkably, purified gonococcal components generated a similar cytokine profile, although macrophage responses were generally less rapid with these stimuli. Thus, secretory products of live organisms - such as IgA1 protease - might have contributed to the rate or scale of cytokine release (Lorenzen *et al.*, 1999), but they did not appear to elicit a distinct spectrum of inflammatory mediators. Furthermore, *de novo* transcription or metabolic activity in the gonococcus was not required for induction of the observed response, which contrasts with the manipulation of cytokine regulation by virulent *Listeria monocytogenes* located in the macrophage cytosol (Kuhn & Goebel, 1998).

Macrophages efficiently bound and internalised gonococci lacking both pili and Opa proteins, and this was followed by a considerable cytokine response. Conversely, T.F. Meyer's group at Tübingen (Knepper *et al.*, 1997) reported that the non-piliated, transparent phenotype of strain MS11 (PorB1b) exhibited a very low level of association with adherent monocytes, which led to a weak respiratory burst compared to $Opa^{+}$ variants. There are three possible explanations for this discrepancy. Clearly, differences in LOS serotype (Preston *et al.*, 1996) between MS11 and strain P9 might have resulted in dissimilar interactions with mononuclear phagocytes.

Secondly, the Tübingen group conducted phagocytosis assays in the absence of foetal calf serum (FCS), whereas in the present study, cell culture medium contained 2% FCS. Serum contains a glycoprotein termed LPS binding protein [(LBP) - Schumann *et al.*, 1990]; this forms a complex with LPS that is subsequently recognised by the CD14-antigen located on the surface of mononuclear phagocytes (Wright *et al.*, 1990; Couturier *et al.*, 1991). Soluble CD14 molecules present in serum also bind to LPS and interact as a unit with an unidentified monocytic receptor (Blondin *et al.*, 1997). In contrast, serum factors are not required for recognition of LPS by a binding site on complement receptor

3 (Wright *et al.*, 1989), or for hydrophobic interactions with the monocyte plasma membrane mediated by lipid A, although these mechanisms appear to be of low avidity (Couturier *et al.*, 1991). Indeed, the concentration of meningococcal LOS required to induce half-maximal secretion of TNF-α is more than 1000 times greater for CD14-deficient murine macrophages than for $CD14^+$ cells (Gangloff *et al.*, 1999). The concentration of FCS used in the present study might have been sufficient for serum-dependent interactions with the LOS of intact gonococci, since Couturier *et al.* (1991) detected CD14-mediated binding in assays containing 2.5 μg/ml meningococcal LOS and only 5% FCS.

The third important methodological difference between the Tübingen study and the current work was that the former used adherent peripheral blood monocytes without a prior period of maturation *in vitro*. Macrophages express several surface glycoproteins involved in phagocytosis that display broad specificity, such as the mannose receptor and class-A scavenger receptors (Aderem & Underhill, 1999). Scavenger receptors bind a wide spectrum of polyanionic ligands (Aderem & Underhill, 1999), whilst the carbohydrate recognition profile of the mannose receptor includes *N*-acetylglucosamine (Shepherd *et al.*, 1982). This oligosaccharide is a major component of LOS molecules from serum-sensitive gonococcal strains (Rice, 1989), although co-localisation of the mannose receptor with non-piliated, transparent organisms was not observed by confocal microscopy in the present study. Nevertheless, differentiated macrophages have a greater potential for LOS-mediated interactions with gonococci than do fresh monocytes.

There was no evidence that factors in normal or immune human serum enhanced macrophage responses to purified LOS, suggesting that sufficient LBP might have been present in 2% FCS for interactions with dissolved LOS. Antibodies present in normal human serum (NHS) recognise antigenic determinants that are common to gonococcal LOS and enterobacterial LPS, but the dominant isotype is IgM (Apicella *et al.*, 1986). As the ability to bind IgM-coated antigens in the absence of complement is highly restricted within the mononuclear phagocyte system (Roubin & Zolla-Pazner, 1979), such antibodies are unlikely to act as opsonins in heat-inactivated sera. Furthermore, the bactericidal activity of anti-LOS IgG may be blocked by serum IgA (Apicella *et al.*, 1986); and natural antibodies appear to inhibit the secretion of proinflammatory cytokines from monocytes, possibly via interference with LPS binding (Abe *et al.*, 1994).

Interestingly, when equivalent amounts of LOS were administered to macrophages as either a pure solution or as part of an outer membrane preparation, the

vesicles induced greater production of several cytokines. Although Jones *et al.* (1980) noted that whole gonococci elicited a stronger LOS-specific antibody response in rabbits than did purified LOS, few studies on the effect of LOS conformation on stimulatory potency have been conducted. However, Kitchens & Munford (1998) reported that aggregated *Escherichia coli* LPS was internalised much more rapidly by THP-1 cells than was monomeric LPS. Despite this, the physical state of the LPS did not affect the IL-8 response of these cells when challenged for one or two hours in the presence of LBP. Additionally, the present study revealed that factors in NHS enhance cytokine generation induced by outer membrane vesicles, which contrasts with the results obtained with pure LOS. This may involve (alone or in combination) natural antibodies that recognise conformation-dependent epitopes, cross-linking of Fcγ receptors by opsonised vesicles (Lee & Rikihisa, 1997), or antibodies specific for gonococcal outer membrane proteins (OMPs).

It is likely that OMPs contributed to the superior stimulatory potency of the vesicles relative to dissolved LOS. Harper *et al.* (1996) reported that the IL-8 and TNF-α responses of fresh human monocytes to gonococcal whole cell lysates (strain MS11) were at least two-fold greater than those observed with purified LOS, despite a lower concentration of LOS in the former preparation. By far the most abundant gonococcal OMP is PorB (Johnson & Gotschlich, 1974), which has been found to mediate Opa-independent binding and invasion of Chang epithelial cells (van Putten *et al.*, 1998). This ability appeared to be limited to strains expressing PorB1a, and occurred only when phosphate availability was limited during the infection experiments (van Putten *et al.*, 1998). Although the data presented in the current work are compatible with expression of a macrophage receptor recognising PorB1b of strain P9, cytokine production may not require specific binding of this protein, since Mosleh *et al.* (1998) demonstrated that isolated PorB becomes incorporated into the plasma membrane of differentiated human macrophages. This event is accompanied by rapid intracellular calcium fluxes in monocytic cells that ultimately result in apoptosis (Müller *et al.*, 1999), and it seems probable that cytokines would be released in parallel.

The opsonin most likely to be responsible for the increased stimulatory capacity of gonococcal vesicles in the presence of NHS is anti-PorB antibodies. Zak *et al.* (1984) discovered low levels of anti-PorB IgG in normal donors; and Henriksen *et al.* (1998) identified an epitope on an *E. coli* porin that is shared by neisserial porins, indicating a potential origin for such antibodies. Remarkably, the enhancement of the vesicle-

mediated cytokine response by sera from gonorrhoea patients was not greater than that observed with NHS. Possibly, high levels of anti-Rmp blocking antibodies in immune sera might have obscured binding sites for anti-PorB IgG (Rice *et al.*, 1986), preventing opsonisation.

The expression of Opa proteins on both viable gonococci and purified outer membranes clearly affected cytokine production by macrophages. Relative to transparent organisms, the P9-Opa-b phenotype was associated with a two-fold increase in gonococcal avidity for macrophages, and this was mirrored by a proportional increment in cytokine release. This protein has also been shown to confer a similar enhancement in gonococcal avidity for Chang epithelial cells (Virji & Everson, 1981), although Lambden *et al.* (1979) reported that the Opa-$b^+$ variant associated poorly with granulocytes. As the *opa* genes of *N. gonorrhoeae* undergo sequence variation via several diversifying mechanisms (Bhat *et al.*, 1991), direct comparisons of Opa protein characteristics between different strains are not valid. However, P9-Opa-b may represent a similar subtype of protein to MS11-$Opa_{50}$, since the latter also triggers uptake by monocytes and Chang epithelial cells while exhibiting weak affinity for granulocytes (Kupsch *et al.*, 1993; Knepper *et al.*, 1997). It is possible that these attributes relate to the distribution of charged amino-acid residues in the variable regions (Kupsch *et al.*, 1993). Knepper *et al.* (1997) has also presented evidence suggesting that MS11-$Opa_{50}$ utilises different receptors on epithelial cells and monocytes, as heparin blocks gonococcal interactions with the former (mediated by heparan sulphate-proteoglycans), but not with the latter.

In contrast, variants expressing P9-Opa-a display efficient associations with granulocytes (Lambden *et al.*, 1979) and primary human endometrial cells (Christodoulides *et al.*, 2000). The receptor for Opa on both these cell types is likely to be biliary glycoprotein, since this is the principal CD66-antigen responsible for granulocytic responses to $Opa^+$ gonococci (Gray-Owen *et al.*, 1997b), and it binds to P9-Opa-a with greater avidity than to Opa-b (M. Virji, unpublished data). The role of CD66-antigens in the interaction of mononuclear phagocytes with gonococci has yet to be established, although they are known to be expressed on alveolar macrophages (Bordessoule *et al.*, 1993).

In the current study, Opa protein expression modified the degree of gonococcal association with macrophages and consequent cytokine production, but the magnitude of the effects was relatively small. Furthermore, the addition of $F(ab')_2$ fragments of anti-Opa-b monoclonal antibody SM40, which have been shown to inhibit the respiratory

burst in granulocytes exposed to Opa-b$^+$ gonococci (Virji & Heckels, 1986), resulted in only a modest reduction of macrophage responses. This contrasts with the report of Naumann *et al.* (1997), who found that significant cytokine induction in epithelial cell lines was totally dependent on Opa-mediated interactions when the gonococcal inoculum was non-piliated. Therefore, rather than binding to distinct macrophage surface molecules, Opa proteins might have increased (Opa-b) or decreased (Opa-a) the avidity of adherence to phagocytic receptors recognising LOS or PorB. This is supported by the fact that macrophage challenge with Opa-a$^+$ membrane vesicles, or early interactions with viable Opa-a$^+$ organisms, resulted in diminished cytokine secretion compared to Opa$^-$ stimuli. The findings of van Putten *et al.* (1997) provide a potential mechanism for this phenomenon, as Opa$^+$ gonococci may accrete negatively charged polysaccharides from agar or FCS, thus inhibiting their capacity to associate with human cells until unmasked proteins are synthesised. Furthermore, different Opa proteins expressed by a single gonococcal strain may differ markedly in their ability to bind polyanions, depending on the extent of positively charged regions in surface-exposed loops (Swanson, 1994).

Piliation of whole organisms did not enhance gonococcal associations with macrophages, nor did it result in elevated cytokine secretion from stimulated cells - indeed, there was some evidence for a negative effect on these processes. A consensus view on the role of pili in gonococcal interactions with phagocytes has yet to be established. Ofek *et al.* (1974) reported that piliation inhibited the phagocytosis of whole organisms by human monocytes and granulocytes; whilst Virji & Heckels (1986) - using strain P9 - found that granulocytic bactericidal activity and respiratory burst were entirely dependent on Opa expression, with pilus phenotype neither enhancing nor reducing these responses. In contrast, Knepper *et al.* (1997) determined that several piliated variants of strain MS11 (all Opa$^-$), expressing distinct pilin proteins, associated in greater numbers with monocytes and granulocytes than did a non-piliated control, although oxidative defences were not elicited by these organisms. However, it is possible that the different outcomes described in this study and the current work are the result of inter-strain variation in pilus composition or differences in receptor expression between monocytes and macrophages.

In the present study, highly purified pili were found to induce modest but significant cytokine production in macrophages. Similarly, van de Verg *et al.* (1996) stimulated IL-6 and IL-8 secretion in ME180 cervical carcinoma cells using isolated gonococcal pili, but the observed response was lower than that generated after challenge

with outer membrane complexes containing Opa and pili. In epithelial cells, pili are essential for the Opa-independent induction of several proinflammatory cytokines by viable gonococci (Naumann *et al.*, 1997; Christodoulides *et al.*, 2000). The key pilus receptor on epithelial cells is membrane cofactor protein (Källström *et al.*, 1997); therefore, it is possible that differences in expression of this glycoprotein between epithelial cells and professional phagocytes account for their distinct interactions with pili.

Factors present in NHS increased macrophage cytokine responses to purified pili. Hamadeh *et al.* (1995) discovered that natural anti-α1,3-galactosyl antibodies (anti-Gal) of the IgG, IgM, and IgA isotypes bind to meningococcal pili, and it is probable that the *N*-acetylglucosamine-α1,3-galactose domains on gonococcal pili are also recognised by these antibodies (Parge *et al.*, 1995). Therefore, IgG antibodies in NHS might have opsonised pili and increased the efficiency of attachment to the macrophage surface. However, neither immune serum from gonorrhoea patients nor anti-pilus antiserum obtained from an immunised volunteer elicited further enhancement of cytokine release. This was unexpected, since immunisation of rabbits with whole bacteria generates highly opsonic anti-pilus antibodies that facilitate phagocytosis of gonococci by murine peritoneal macrophages (Jones *et al.*, 1980). Hamadeh *et al.* (1995) reported that IgA anti-Gal in NHS can prevent complement-mediated killing of piliated meningococci, and it is possible that natural or artificial immunisation increased the levels of these antibodies, ablating opsonisation. Alternatively, the opsonin in NHS might have been the mannose-binding protein - the ligands of which include *N*-acetylglucosamine (Fraser *et al.*, 1998) – and thus the immune status of the serum would not affect cellular responses.

6B(iv) *The Cytokine Profile Generated By Gonococcal Stimulation Of Human Cells*: Macrophages stimulated with viable *Neisseria gonorrhoeae* or surface components from this organism secreted only a proportion of the cytokines from the repertoire assayed in the current work. Thus, while levels of IL-6, TNF-α, MIP-1α, RANTES, GRO-α, and IFN-γ were elevated in response to challenge, constitutive IL-8 production was only affected in the initial phase of infection; and IL-1β, IL-10, IL-12, GM-CSF, TGF-$\beta_1$, and ENA-78 were not detected. Although there are no published studies on the induction of cytokines in differentiated macrophages by gonococci or gonococcal fractions, other human cell types have been stimulated *in vitro*, enabling some comparisons with the current findings.

Harper *et al.* (1996) determined that fresh adherent monocytes secreted IL-8, TNF-α, and IL-1β following challenge with LOS or whole cell lysates, but GM-CSF was not produced. Granulocytes were also found to release IL-8 when exposed to the same stimuli. In a more detailed study, Lorenzen *et al.* (1999) discovered that peripheral blood mononuclear cells (PBMC) generated TNF-α, IL-1β, IL-6, IL-8, and GM-CSF in response to IgA1 protease, but substantial cytokine production in monocytes was dependent on direct contact with T-lymphocytes. However, the gonococcal enzyme did not induce significant amounts of IL-10 or IFN-γ in PBMC cultures.

Several research groups have used epithelial cells derived from relevant tissues to characterise the cytokine profile generated by gonococcal infection. Accordingly, van de Verg *et al.* (1996) stimulated ME180 cervical carcinoma cells with viable organisms or purified gonococcal components, which led to the release of IL-6 and IL-8 in most cases (purified LOS was ineffective). Naumann *et al.* (1997) utilised three different epithelial-like cervical cell lines in a comprehensive investigation of cytokine gene transcription. Piliated or $Opa^+$ gonococci, but not endotoxin, triggered increased synthesis of messenger RNA (mRNA) transcripts encoding TGF-β, GM-CSF, TNF-α, IL-1β, IL-6, IL-8, and IL-12; whilst immunoassays for GM-CSF, IL-8, and TNF-α detected protein secretion. However, transcription of IL-10 and IFN-γ genes was not induced, and the constitutive production of mRNA encoding RANTES was unaffected by gonococcal challenge. Primary endometrial cells have been found to secrete TNF-α following stimulation with several phenotypic variants of strain P9 (including $Pil^-/Opa^-$); but IL-1β, GM-CSF, RANTES, and MIP-1α were not found (Christodoulides *et al.*, 2000). Interestingly, a piliated gonococcal inoculum was required for both the increased generation of IL-8 and the suppression of constitutive IL-6 release by these cells (Christodoulides *et al.*, 2000).

The dominant inflammatory mediators produced by mononuclear phagocytes are TNF-α, IL-6, and IL-1β - all of which are secreted following exposure to endotoxin (Scheibenbogen & Andreesen, 1991). Hence, the generation of TNF-α and IL-6 observed in the present study was expected, although the results of Christodoulides *et al.* (2000) suggest that under certain conditions, gonococci may have differential effects on the regulation of these cytokines. Additionally, macrophage responses were not entirely dependent on LOS, since highly pure pili were capable of inducing significant amounts of TNF-α and IL-6.

The complete absence of IL-1β recorded in the current work probably relates to the differentiation state of the macrophages. Whereas freshly isolated monocytes produce considerable amounts of this cytokine after stimulation with endotoxin, the capacity for secretion is greatly reduced in cells matured *in vitro* (Scheibenbogen & Andreesen, 1991). Another key inflammatory product of mononuclear phagocytes is IL-12, a cytokine involved in IFN-γ induction and the regulation of specific immune responses (Sutterwala & Mosser, 1999). Synthesis of IL-12 can be suppressed at the level of transcription by ligation of macrophage surface proteins such as Fcγ receptors, complement receptor 3, and membrane cofactor protein (Sutterwala & Mosser, 1999). Thus, it is possible that binding of gonococci inhibits rather than promotes IL-12 generation.

Interleukin-8 is the prototype member of the CXC family of chemokines, which exert specific effects on neutrophils (Mukaida *et al.*, 1998). Despite the observation that viable gonococci caused a transient initial increase over constitutive levels in infected cultures, IL-8 secretion by macrophages reached a maximum without exogenous stimulation. Accordingly, Kasahara *et al.* (1991) demonstrated that the adherence of monocytes to culture substrates in the absence of other stimuli induced transcription of the IL-8 gene, which was followed by peak protein levels after 24 hours. Thus, the enhanced generation of IL-8 in monocytes or PBMC incubated with gonococcal components [recorded by Harper *et al.* (1996) and Lorenzen *et al.* (1999), respectively] indicates that in the current study, monocyte-to-macrophage maturation in Matrigel®-coated culture vessels elevated constitutive production of this chemokine. Indeed, a vast range of physical, microbiological, and biochemical stimuli are capable of triggering IL-8 expression (Lindley, 1998), complicating *in vitro* investigations of its role in pathogenesis.

Conversely, GRO-α levels displayed up to sixty-fold increases over baseline secretion following challenge with viable organisms, and gonococcal components also stimulated production. This chemokine has not been previously investigated in connection with gonococcal pathogenesis, but Goodman *et al.* (1998) reported rapid release of GRO proteins in alveolar macrophages exposed to endotoxin. Although the functionally related chemokine ENA-78 is also generated in response to endotoxin challenge, substantial protein concentrations in monocyte cultures are not apparent until at least 20 hours post-stimulation, since *de novo* synthesis of a protein intermediate is required (Schnyder-Candrian & Walz, 1997). Consequently, detection of this chemokine in the cell medium of gonococcus-infected macrophage cultures might have required

time-course experiments conducted for prolonged periods; but such studies were impracticable due to uncontrolled bacterial replication in the culture milieu.

In contrast to their apparent insignificance in the cytokine profile of epithelial cells challenged with gonococci (Naumann *et al.*, 1997; Christodoulides *et al.*, 2000), RANTES and MIP-1α (members of the CC chemokine family) appear to be important components of the macrophage response to *N. gonorrhoeae*. Although these chemokines are classically associated with chemoattraction of lymphocytes and monocytes (Nelson *et al.*, 1998; Nibbs *et al.*, 1998), they also demonstrate important autocrine effects on mononuclear phagocytes. Thus, RANTES regulates the expression of integrins (Vaddi & Newton, 1994; cited in Nelson *et al.*, 1998) – which are involved in the binding of both endotoxin and complement component C3bi (Wright *et al.*, 1989) - whilst MIP-1α induces the release of other cytokines (Fahey *et al.*, 1992; cited in Nibbs *et al.*, 1998).

Two of the cytokines assayed in the current work – IFN-γ and GM-CSF – are triggers of the macrophage activation process, during which anti-microbial defences (such as the generation of reactive oxygen intermediates) become enhanced (de Maeyer & de Maeyer-Guignard, 1998; Jones, 1993). The absence of GM-CSF noted in the present study accords with the only other report of cytokine production in purified mononuclear phagocytes stimulated with gonococcal components (Harper *et al.*, 1996). As the main cellular sources of IFN-γ are T-lymphocytes and natural killer cells (de Maeyer & de Maeyer-Guignard, 1998), the detection of this cytokine in the cell medium of gonococcus-stimulated macrophages was unexpected. However, IFN-γ production can be triggered in human alveolar macrophages by mycobacteria, IL-12, and IFN-γ itself; but not by endotoxin (Fenton *et al.*, 1997). Therefore, although significant IFN-γ expression has not been found in other cell types following challenge with gonococci or their products (Naumann *et al.*, 1997; Lorenzen *et al.*, 1999), induction of this cytokine in macrophages may represent a specific attribute of *N. gonorrhoeae*.

The acute proinflammatory cytokine response is frequently brought under control by the suppressive qualities of IL-10 (Trinchieri, 1997), but the current study and other reports (Naumann *et al.*, 1997; Lorenzen *et al.*, 1999) suggest that this cytokine is not generated following gonococcal infection. Another cytokine with the potential to exert anti-inflammatory effects is TGF-β (Koj, 1998), and this was also absent from the macrophage cytokine profile. Although TGF-β mRNA transcripts have been detected in epithelial cells challenged with gonococci (Naumann *et al.*, 1997), further studies are

required to ascertain whether this multifunctional protein is a significant component of the host response to *N. gonorrhoeae*.

6B(v) *The Role Of Macrophages In The Pathogenesis Of Gonococcal Infections*: In order for their relevance to be ascertained, the described *in vitro* studies require comparison with *in vivo* and *ex vivo* investigations on human subjects and organs. Experimental gonococcal urethritis in men is associated with the rapid secretion of IL-8 and IL-6 in urine, which is soon followed by TNF-α production and the ultimate release of IL-1β at disease onset (Ramsey *et al.*, 1995). However, GM-CSF is not detected at any time (Ramsey *et al.*, 1995). In contrast, natural cervical infection in STD clinic patients does not appear to be correlated with a qualitatively or quantitatively unique cytokine response (Hedges *et al.*, 1998), but a high occurrence of previous exposure to STD may partially explain this finding.

Cannon *et al.* (1996) reported that a gonococcal deletion mutant, which was unable to express pilin, successfully colonised the urethras of male experimental subjects but failed to generate symptomatic disease. The implication of this study is that the acute inflammatory response is dependent on pilus-mediated interactions - presumably dominated by epithelial cells. However, the evident role of TNF-α in the initial phase of cytokine production suggests the involvement of macrophages at this stage. It has been determined that ~$1.3x10^5$ endometrial epithelium cells generate 15-30 pg of TNF-α by 24 hours post-inoculation when challenged with $1.5x10^7$ P9 gonococci (Christodoulides *et al.*, 2000; J.S. Everson, unpublished data), whereas ~$2.4x10^4$ macrophages produce 14-21 ng of this cytokine after 6 hours' stimulation with $1x10^7$ P9 organisms. Therefore, on a "cell-for-cell" basis, the secretory potential of macrophages for rapid and sustained release of TNF-α is probably several thousand times greater than that of epithelial cells. As resident macrophages are more abundant in the subepithelial layers of both the urinary tract and the cervix than they are in the epithelium (El-Demiry *et al.*, 1986; Tay *et al.*, 1987), significant TNF-α production may require epithelial exfoliation (Mosleh *et al.*, 1997). This is consistent with the slightly delayed appearance of TNF-α in the urine (Ramsey *et al.*, 1995).

The relative importance of the different CXC chemokines in neutrophil chemotaxis is difficult to establish, since the concentrations of these proteins have not been compared in natural or experimental gonorrhoea. Goodman *et al.* (1998) determined that alveolar macrophages generated IL-8, GRO proteins, and ENA-78 in response to

endotoxin; but the neutrophil chemotactic activity of the culture medium could be completely abrogated by anti-IL-8 antibodies. Nevertheless, macrophages resident in the genitourinary system may behave very differently to alveolar macrophages. In addition, the properties of Opa-b-like opacity proteins could be important *in vivo*, where the release of physiologically active concentrations of GRO-α and other cytokines might be dependent on the avidity of the gonococcus-macrophage encounter.

Once attracted to the site of infection, neutrophils are likely to be "primed" by TNF-α and other factors, resulting in the exocytosis of granule contents and the expression of novel receptors (Dallegri & Ottonello, 1997). Furthermore, these cells may represent the source of IL-1β (Tiku *et al.*, 1986; cited in Dallegri & Ottonello, 1997) that is detected at the onset of discharge in gonococcal urethritis (Ramsey *et al.*, 1995). Neutrophil defensive products released during phagocytosis of large numbers of gonococci, such as elastase, probably contribute to gonorrhoeal symptoms by promoting tissue damage and plasma exudation (Dallegri & Ottonello, 1997). Therefore, macrophage clearance of apoptotic neutrophils is important in the control of acute inflammation (Savill, 1992; cited in Dallegri & Ottonello, 1997).

Experimental evidence for the critical role of TNF-α in the pathogenesis of pelvic inflammatory disease (PID) has been obtained using an *ex vivo* fallopian tube organ culture model (McGee *et al.*, 1992). The addition of gonococci to this system results in the sloughing of ciliated epithelial cells, which is mediated by the LOS-induced production of TNF-α (Cooper *et al.*, 1986; McGee *et al.*, 1992). Two lines of evidence implicate macrophages in this process. Firstly, homogenisation of fallopian tube tissue releases large amounts of TNF-α from cells located below the epithelial layer (McGee *et al.*, 1992). Secondly, *in vitro* experiments indicate that epithelial cells do not release TNF-α in response to endotoxin stimulation (van de Verg *et al.*, 1996; Naumann *et al.*, 1997). Contact between macrophages and outer membrane blebs released from growing bacteria may be particularly important in this context, since serum-sensitive gonococcal strains produce vesicles that are enriched for LOS in relation to the source cells (Pettit & Judd, 1992).

Other cytokines generated by macrophages may contribute to the pathogenesis of PID. One characteristic of salpingitis is granulocytic infiltration, followed by an influx of mononuclear cells and plasma cells (Weström & Mårdh, 1990; Lan *et al.*, 1995). Chemokines such as GRO-α (Dallegri & Ottonello, 1997), RANTES (Nelson *et al.*, 1998), and MIP-1α (Nibbs *et al.*, 1998) are likely to be involved in this process.

Furthermore, the effects of IL-6 on extracellular matrix regulation and the growth of fibroblasts (Richards, 1998) may indicate a role in pathological scar formation, which can result in occlusion of the fallopian tubes (Weström & Mårdh, 1990).

Very few investigators have attempted to measure cytokine levels in women with PID. However, support for the importance of TNF-α in the pathogenesis of ascending infection was provided by the study of Toth *et al.* (1992), in which fallopian tube fluid from patients with or without salpingitis was retrieved during laparoscopy and subjected to specific immunoassay. While TNF-α was completely absent in the control group, the levels in women with salpingitis were associated with the severity of pathology; although the only positive cultures from damaged salpinges yielded chlamydiae rather than gonococci. In addition, Mori *et al.* (1991) determined that IL-1β and TNF-α concentrations in peritoneal fluid were elevated in individuals with acute PID (aetiology not stated) compared to "normal pelvis" controls. No correlation was found between the concentrations of these two cytokines, or between the level of either of these cytokines and the concentration of peritoneal macrophages. Furthermore, production of TNF-α and IL-1β *in vitro* by macrophages recovered from the PID patients did not parallel the levels detected in their peritoneal fluid. Mori *et al.* (1991) concluded that their findings might be explained by the dynamics of macrophage activation, cytokine secretion by other leukocytes in the peritoneum, and/or an inability to replicate *in vivo* stimulatory conditions *in vitro*. In contrast, Koyama *et al.* (1993) reported that peritoneal IL-1β was undetectable in several women with "post-PID" of unspecified aetiology, and IL-6 levels were also low in this group.

Regulation of the macrophage cytokine response is probably important in determining the severity of the sequelae associated with ascending infection. The asymmetric nature of the host response to gonococcal stimuli, in which potent proinflammatory cytokines are released without an associated regulatory protein such as IL-10, has been noted by other investigators (Lorenzen *et al.*, 1999). In the present study, stimulation of macrophages with gonococcal components triggered cytokine secretion that reached a maximum within 1-3 days. This *in vitro* finding suggests that proinflammatory cytokine generation can be regulated in the absence of classic "anti-inflammatory" factors – an interpretation that is supported by the study of Schindler *et al.* (1990), who discovered that suppression of IL-1β and TNF-α release in PBMC could be mediated by IL-6. However, new populations of macrophages may be continually

exposed to gonococcal challenge through epithelial shedding *in vivo*, and thus cytokine production is likely to be perpetuated until the pathogen is eliminated.

## *6C* - Topics For Future Investigation

6C(i) *Host-Pathogen Interactions Determining The Clinical Outcome Of Pelvic Inflammatory Disease & Cervicitis*: At the beginning of the 1990s, Rice & Schachter (1991) summarised the key research questions that they believed were priorities in the study of pelvic inflammatory disease. These included the influence of reproductive hormones in the pathogenesis of PID (particularly the potential association between oral contraceptives and subclinical disease), the role of infiltrating inflammatory cells in the mediation of oxidative tissue damage, and the mechanism by which gonococcal LOS exerts toxic effects on the fallopian tube mucosa. Chemokines are now known to be involved in various aspects of female reproductive physiology (García-Velasco & Arici, 1999), and neutrophil products are recognised as key determinants of inflammation-related pathology (Dallegri & Ottonello, 1997), but the direct relevance of such findings to PID has yet to be determined. In contrast, the discovery that LOS-induced TNF-α was cytotoxic for ciliated epithelial cells (McGee *et al.*, 1992) laid the foundation for a molecular model of fallopian tube damage. However, the cellular source of TNF-α has not been identified, and it is not known whether LOS structure affects toxicity or how it accumulates within epithelial cells (Cooper *et al.*, 1986; Rice & Schachter, 1991).

Very few studies using material recovered from PID cases have been conducted in the last decade. To date, only a single report has been published in which cytokine levels were directly assayed in fallopian tube fluid obtained during laparoscopy (Toth *et al.*, 1992). Although this study was important in demonstrating an association between *in vivo* TNF-α production and the severity of tubal damage, only seven subjects with PID were investigated, gonococci were absent from reproductive tract cultures, and no other cytokines were measured. It would be interesting to obtain fallopian tube swabs from patients with gonococcal PID to identify which cytokines correlated with disease severity and/or infectious load. In this context, the hypothesis that ectopic pregnancy can result from chronic inflammatory damage long after the causative organism has been eliminated is particularly relevant (Lan *et al.*, 1995).

In the current work, determination of infectious load by quantitative PCR proved an effective method for probing the relationship between cervicitis, urethritis, and endometritis. Unfortunately, the absence of gonorrhoea in the Thai study population, coupled with inclusion criteria based on evidence of symptomatic disease, precluded an investigation of how host and pathogen factors interact to produce the clinical characteristics of cervicitis. Future research could focus on potential differences in cervical cytokine responses, bacterial load, and the attributes of the infecting strain in symptomatic versus asymptomatic women. For instance, is cryptic gonococcal infection caused by a suppressed inflammatory response, fewer bacteria in the cervix, organisms expressing particular LOS or PorB serotypes, or some combination of these factors?

In a population with a high prevalence of gonorrhoea, cytospin preparations of urine specimens, urethral exudates, or endocervical scrapings could provide interesting data regarding the sources of cytokine secretion. Simultaneous immunological labelling of cell-associated bacteria and cytokines, perhaps in conjunction with cell differentiation markers, would allow localisation of cytokine production using fluorescent conjugates (Agace *et al.*, 1993). In particular, the three cytokines found to be significantly associated with the clinical severity of cervicitis (IL-1β, IL-6, and IL-8) require further investigation in such a system. The critical questions are: which cells generate these cytokines, are the secreting cells always in contact with gonococci or their products, and do these features differ between subjects with and without symptoms?

6C(ii) *Host Cell Signalling In Binding & Internalisation Of* Neisseria gonorrhoeae *– Do Gonococcal Surface Structures Manipulate The Response?* One question that was not resolved in the present study was whether Opa-b of strain P9 binds to macrophages via a specific mechanism, or rather modifies the avidity of attachment mediated by other gonococcal components – perhaps by an effect on surface charge. The precise nature of the receptor-ligand interaction determines which signalling pathway is activated in the host cell, and this affects downstream events such as phagocytosis, cytokine release and oxidative defence mechanisms. Thus, in Chang conjunctival epithelium cells, binding of heparan sulphate-proteoglycans (HSPG) by a specific Opa protein leads to the activation of acidic sphingomyelinase, an enzyme thought to control gonococcal uptake via production of ceramide (Grassmé *et al.*, 1997; cited in Meyer, 1999). However, gonococci expressing this Opa can also invade HeLa cervical carcinoma cells by adsorption of vitronectin from serum - which facilitates interactions with both HSPG and

integrins - resulting in stimulation of protein kinase C and engulfment of adherent organisms without the involvement of ceramide (Meyer, 1999).

The Opa-dependent internalisation of gonococci by neutrophils proceeds by a third distinct pathway. Binding of CD66-antigens on the phagocyte surface triggers phosphorylation of Src-related protein tyrosine kinases, which subsequently activate Rac1 - a guanosine triphosphatase involved in the reorganisation of actin filaments that proceeds phagocytosis (Hauck *et al.*, 1998). Although the signalling cascade stimulated by ligation of Fcγ receptors shares initial similarities with the CD66-specific pathway, the latter does not involve Syk kinase, a critical intermediary in the uptake of IgG-opsonised particles (Hauck *et al.*, 1998; Aderem & Underhill, 1999). Gonococci may have evolved to utilise host cell receptors (and thus signalling pathways) that suppress intercellular communication or intracellular defences. Hence, uptake of transparent, piliated gonococci by professional phagocytes does not appear to be accompanied by a respiratory burst (Knepper *et al.*, 1997), implying a role for complement receptors in this process (Aderem & Underhill, 1999). Furthermore, the oxidative burst elicited by $Opa^+$ gonococci is considerably less intense than that generated by certain commensal neisseriae, even though the pathogenic species is internalised more readily (Hauck *et al.*, 1997).

In an extension of the current study, similar investigations could be applied to macrophages in order to determine the signalling pathways that result in cytokine production. In epithelial cells, gonococcal attachment to the plasma membrane is sufficient to induce the rapid activation of nuclear factor-κB, which mediates the transcription of several cytokine genes via binding to enhancer elements within the promoter regions (Naumann *et al.*, 1997). The activation of transcription factors can be monitored by incubation of nuclear protein extracts from stimulated cells with labelled oligonucleotides representing specific binding sites; DNA-protein complexes are then detected by electrophoretic mobility shift assays (Naumann *et al.*, 1997). Similarly, signal transduction preceding transcriptional events may be investigated by immunocomplex kinase assays, in which autophosphorylation is used to determine the activation status of immunoprecipitated tyrosine kinases from challenged cells (Hauck *et al.*, 1998).

A particularly interesting finding in the present study was that macrophages could bind and internalise gonococci lacking both pili and Opa proteins with a high level of efficiency. Does this mean that these phagocytes can circumvent the adaptive repertoire of the gonococcus? Certainly, macrophages respond to gonococcal challenge with a potent cytokine response, and seem to kill phagocytosed organisms effectively. This

would appear to exclude primary roles for scavenger receptors [involved in the clearance of apoptotic cells (Aderem & Underhill, 1999)] or complement receptors in gonococcus-macrophage interactions. However, PorB may be able to subvert the macrophage armoury by interfering with the formation of the phagolysosome (Mosleh *et al.*, 1998) and inducing calcium influxes that herald the onset of apoptosis (Müller *et al.*, 1999).

# APPENDICES

# APPENDIX 1

## PATIENT RECORDS FOR SRI LANKAN STD CLINIC STUDY GROUP

| ID | Sample ID | GC PCR | CT PCR | HPV | AGE | COITARCHE | SEX PARTNERS | PARITY | SMOKING | CSW | STD'S | CONTRACEPTION | CX | VAGINA | PAP REPORT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | A01 | 0 | 1 | | 38 | 20 | 272 | 2 | no | yes | NSC | condom | NAD | NAD | NDCS |
| 2 | A02 | 0 | 1 | | 25 | 21 | 1 | 2 | no | no | S3 | no | NAD | NAD | NDCS |
| 3 | A03 | 0 | 1 | 6 | 45 | 14 | 86 | 2 | no | yes | NSV | LRT | inflamed | inflamed, ulcers | NDCS |
| 4 | A04 | 0 | 0 | | 44 | 17 | 350 | 4 | no | yes | chlamydia, TV, GC, GV, NSV | condom | NAD | NAD | NDCS |
| 5 | A05 | 0 | 0 | | 30 | 20 | 1316 | 1 | no | yes | TV | condom | inflamed, eroded, discharge | inflamed | NDCS |
| 6 | A06 | 0 | 0 | | 38 | 14 | 48 | 7 | no | yes | NSV | condom | NAD | NAD | inflammatory nuclear changes |
| 7 | A07 | 0 | 0 | 16, 33 | 23 | 16 | 4248 | 0 | yes | yes | GC | condom | NAD | NAD | NDCS |
| 8 | A08 | 0 | 0 | | 28 | 22 | 1 | 1 | no | no | GC | IUCD | inflamed | discharge | NDCS |
| 9 | A09 | 1 | 0 | 59, 66 | 26 | 18 | 1593 | 1 | no | yes | GC | condom | NAD | discharge | NDCS |
| 10 | A10 | 0 | 0 | | 31 | 16 | 4956 | 6 | no | yes | S4E | condom | inflamed, eroded | inflamed | PHX of CIN1, inflammatory nuclear changes |
| 11 | A11 | 0 | 1 | 16 | 26 | 22 | 16 | 2 | no | yes | NSV | depo | NAD | NAD | NDCS |
| 12 | A12 | 0 | 0 | | 30 | 23 | 2 | 1 | no | no | NSV | condom | NAD | discharge | NDCS |
| 13 | A13 | 0 | 1 | 35, 33 | 29 | 19 | 20 | 3 | yes | yes | GC, NSV | LRT | NAD | NAD | NDCS |
| 14 | A14 | 0 | 0 | 6 | 24 | 24 | 1 | 0 | no | no | nil | nil | NAD | discharge | NDCS |
| 15 | A15 | 0 | 0 | | 24 | 23 | 1 | 0 | no | no | GW | condom | NAD | NAD | NDCS |
| 16 | A16 | 0 | 0 | 18 | 24 | 23 | 1 | 1 | no | no | BV | condom | inflamed | inflamed, discharge, warts | NDCS |
| 17 | A17 | 0 | 0 | | 38 | 16 | 1316 | 3 | no | yes | TV, S4L, NSC | LRT | NAD | NAD | NDCS |
| 18 | A18 | 0 | 0 | | 43 | 23 | 2 | 3 | no | no | nil | LRT | NAD | NAD | NDCS |
| 19 | A19 | 0 | 0 | | 28 | 15 | 2 | 2 | no | no | S4L | depo | inflamed | NAD | NDCS |
| 20 | A20 | 0 | 1 | 6, 45 | 19 | 18 | 20 | 0 | no | yes | TV | condom | inflamed, eroded | inflamed, discharge | NDCS |
| 21 | A21 | 0 | 1 | | 35 | 14 | 12,744 | 1 | no | yes | TV, GC, S4L | OCP | NAD | discharge | inflammatory nuclear changes |
| 22 | A22 | 1 | 1 | 16 | 26 | 14 | 2124 | 2 | no | yes | TV, S4L, GC | LRT | inflamed, eroded, | inflamed, discharge | NDCS |
| 23 | A23 | 0 | 0 | 16 | 40 | 23 | 1062 | 4 | no | yes | TV, HSV | IUCD | NAD | NAD | NDCS |
| 24 | A24 | 0 | 0 | 66 | 35 | 22 | 2 | 0 | no | no | NSV | condom | NAD | discharge | |
| 25 | A25 | 0 | 0 | 56 | 45 | 24 | 1770 | 7 | no | yes | NSV | LRT | inflamed | NAD | inflammatory nuclear changes |
| 26 | A26 | 0 | 0 | | 22 | 20 | 3 | 0 | no | no | nil | condom | NAD | NAD | NDCS |
| 27 | A27 | 0 | 0 | | 34 | 26 | 1 | 1 | no | no | nil | condom | NAD | NAD | NDCs |
| 28 | A28 | 0 | 0 | | 26 | 23 | 1 | 1 | no | no | HSV | depo | NAD | inflamed, discharge | NDCS |
| 29 | A29 | 0 | 0 | | 21 | 16 | 1316 | 2 | no | yes | NSC | IUCD | inflamed | inflamed, discharge | NDCS |
| 30 | A30 | 0 | 0 | | 43 | 17 | 4 | 5 | no | no | S4L | LRT | NAD | NAD | CIN1 |
| 31 | A31 | 1 | 1 | 18 | 20 | 17 | 120 | 0 | no | yes | nil | condom | NAD | NAD | NDCS |
| 32 | A32 | 0 | 1 | | 46 | 22 | 180 | 1 | no | yes | S4L | LRT | NAD | NAD | NDCS |
| 33 | A33 | 0 | 0 | 31 | 30 | 16 | 432 | 4 | no | yes | GC, S4L, BV | condom | NAD | NAD | CIN 1-2 |
| 34 | A34 | 0 | 0 | | 26 | 19 | 7476 | 1 | no | yes | BV | condom | NAD | NAD | NDCS |
| 35 | A35 | 0 | 1 | 67 | 34 | 12 | 1204 | 2 | yes | yes | NSV | LRT | inflamed | inflamed, discharge | |
| 36 | A36 | 0 | 0 | | 28 | 18 | 2848 | 0 | no | yes | nil | condom | NAD | NAD | NDCS |
| 37 | A37 | 0 | 0 | 18 | 37 | 21 | 1 | 2 | yes | no | nil | LRT | inflamed | NAD | CIN 1-2 |
| 38 | A38 | 0 | 0 | | 23 | 15 | 2136 | 1 | no | yes | S3 | condom | inflamed | discharge | NDCS |
| 39 | A39 | 0 | 0 | | 25 | 25 | 1 | 0 | no | no | NSV | nil | inflamed, discharge | NAD | NDCS |
| 40 | A40 | 0 | 0 | | 46 | 23 | 6408 | 5 | no | yes | chlamydia, GC | LRT | NAD | NAD | NDCS |
| 41 | A41 | 0 | 0 | 59 | 18 | 18 | 21 | 0 | no | yes | nil | condom | NAD | NAD | NDCS |
| 42 | A42 | 0 | 0 | | 34 | 16 | 3 | 1 | no | no | GV | condom | NAD | discharge | NDCS |
| 43 | A43 | 0 | 1 | | 40 | 21 | 3 | 2 | no | no | GC, TV, S4L | LRT | NAD | NAD | NDCS |
| 44 | A44 | 0 | 0 | | 40 | 16 | 10,680 | 0 | yes | yes | TV | | NAD | inflamed, discharge | NDCS |
| 45 | A45 | 0 | 1 | | 35 | 21 | 1 | 2 | no | no | S4L | nil | NAD | NAD | NDCS |
| 46 | A46 | 0 | 0 | | 35 | 18 | 3120 | 3 | yes | yes | S4L | depo | inflamed | inflamed, discharge | NDCS |
| 47 | A47 | 0 | 0 | 18 | 25 | 13 | 156 | 0 | no | yes | S4E | condom | NAD | discharge | NDCS |
| 48 | A48 | 0 | 0 | | 26 | 26 | 1 | 0 | no | no | HSV | condom | inflamed, ulcerated | discharge | NDCS |
| 49 | A49 | 0 | 0 | 18 | 35 | 15 | 1 | 6 | no | no | HSV, GV | nil | inflamed | inflamed, ulcers, discharge | NDCS |
| 50 | A50 | 0 | 0 | | 30 | 19 | 1 | 3 | no | no | TV, S4L | condom | NAD | NAD | NDCS |
| 51 | A51 | 0 | 1 | | 42 | 16 | 3660 | 2 | no | yes | GC, S4L, BV, NSC | OCP | inflamed | inflamed, discharge | NDCS |
| 52 | A52 | 0 | 1 | | 27 | 18 | 1424 | 3 | yes | yes | TV, GC, S4L | OCP | NAD | NAD | |
| 53 | A53 | 0 | 1 | 45, 35, 43 | 46 | 22 | 2136 | 3 | no | yes | GC, TV, BV, Chlamydia | LRT | NAD | NAD | NDCS |
| 54 | A54 | 0 | 0 | | 27 | 12 | 12,810 | 0 | yes | yes | TV | condom | NAD | discharge | NDCS |
| 55 | A55 | 0 | 0 | 58 | 27 | 17 | 180 | 2 | no | yes | GC | depo | NAD | discharge | NDCS |
| 56 | A56 | 0 | 0 | 6, 43, 45 | 20 | 20 | 1 | 0 | no | no | GW | nil | NAD | warts | NDCS |
| 57 | A57 | 1 | 1 | han831 | 22 | 18 | 180 | 1 | no | yes | nil | LRT | inflamed | discharge | NDCS |
| 58 | A58 | 0 | 1 | | 19 | 16 | 2 | 0 | no | no | chlamydia | condom | NAD | NAD | NDCS |
| 59 | A59 | 0 | 1 | | 29 | 18 | 3660 | 2 | no | yes | TV, GV, S4L | LRT | NAD | discharge | NDCS |
| 60 | A60 | 0 | 1 | | 45 | 19 | 1 | 4 | no | no | nil | LRT | inflamed, eroded, discharge | discharge | CIN 2-3 |
| 61 | A61 | 0 | 1 | | 24 | 15 | 300 | 1 | no | yes | NAD | OCP | NAD | NAD | NDCS |
| 62 | A62 | 0 | 1 | | 38 | 32 | 1 | 1 | no | no | GV, S4L | nil | inflamed | inflamed, discharge | |
| 63 | A63 | 0 | 1 | 16 | 35 | 20 | 1 | 2 | no | no | S4L | nil | inflamed | inflamed, discharge | inflammatory nuclear changes |
| 64 | A64 | 0 | 0 | | 38 | 16 | 3 | 3 | no | no | S4L | LRT | inflamed | inflamed, ulcers, discharge | NDCS |
| 65 | A65 | 0 | 0 | | 39 | 17 | 3194 | 7 | no | yes | NSV | IUCD | NAD | NAD | NDCS |
| 66 | A66 | 0 | 0 | | 19 | 17 | 1 | 1 | no | no | nil | condom | NAD | NAD | NDCS |
| 67 | A67 | 0 | 0 | | 44 | 27 | 1 | 2 | no | no | nil | vasectomy | NAD | discharge | NDCS |
| 68 | A68 | 1 | 0 | | 32 | 16 | 7120 | 2 | yes | yes | TV, S4L, GC | LRT | inflamed | discharge | |
| 69 | A69 | 0 | 1 | | 49 | 24 | 1 | 5 | no | no | S2 | LRT | NAD | NAD | NDCS |
| 70 | A70 | 0 | 0 | | 22 | 16 | 2 | 1 | no | no | TV, HSV | depo | NAD | NAD | CIN 1-2 |
| 71 | A71 | 0 | 1 | mixed | 34 | 16 | 712 | 2 | no | yes | TV, S4L, GC, NSC | condom | NAD | discharge | NDCS |
| 72 | A72 | 0 | 1 | 33 | 35 | 17 | 5340 | 2 | yes | yes | S4L, TV, NSC | condom | NAD | NAD | NDCS |
| 73 | A73 | 0 | 1 | 53, 45 | 43 | 21 | 1 | 5 | no | no | S4L | LRT | NAD | NAD | |
| 74 | A74 | 0 | 0 | | 28 | 15 | 1424 | 3 | no | yes | GC, chlamydia, NSV, NSC | LRT | NAD | NAD | NDCS |
| 75 | A75 | 0 | 0 | | 30 | 18 | 10 | 0 | no | yes | nil | condom | NAD | NAD | NDCS |
| 76 | A76 | 0 | 0 | 18, 11 | 32 | 18 | 80 | 3 | no | yes | NSV | condom | NAD | NAD | CIN 1-2 |
| 77 | A77 | 0 | 0 | cp6304 | 48 | 18 | 3660 | 6 | no | yes | TV | LRT | Inflamed, discharge | NAD | inflammatory nuclear changes |
| 78 | A78 | 0 | 1 | 70 | 32 | 24 | 3660 | 3 | no | yes | TV | condom | NAD | inflamed, discharge | NDCS |
| 79 | A79 | 0 | 0 | | 29 | 21 | 1424 | 2 | yes | yes | S4L | condom | NAD | NAD | NDCS |
| 80 | A80 | 0 | 0 | 56, 31 | 19 | 18 | 1 | 1 | no | no | GW | nil | NAD | inflamed, ulcers, warts | NDCS |
| 81 | B01 | 0 | 1 | | 28 | 24 | 1 | 0 | no | no | nil | nil | NAD | NAD | NDCS |
| 82 | B02 | 0 | 1 | | 26 | 21 | 500 | 1 | no | yes | TV | nil | inflamed | inflamed, discharge | |
| 83 | B03 | 0 | 1 | | 22 | 19 | 14 | 2 | no | yes | nil | condom | NAD | NAD | NDCS |
| 84 | B04 | 0 | 0 | | 33 | 21 | 1 | 3 | no | no | HSV | nil | inflamed, eroded, discharge | inflamed, ulcers | NDCS |
| 85 | B05 | 0 | 0 | 51 | 32 | 17 | 1 | 1 | no | no | S4L | nil | inflamed | NAD | NDCS |
| 86 | B06 | 0 | 1 | | 34 | 17 | 4984 | 3 | yes | yes | S4E | LRT | inflamed | NAD | NDCS |
| 87 | B07 | 0 | 0 | | 34 | 22 | 1602 | 0 | no | yes | TV, S4L, NSV | LRT | inflamed | discharge | NDCS |
| 88 | B08 | 1 | 1 | new sit | 37 | 15 | 712 | 4 | no | yes | S4L | condom | inflamed | inflamed, discharge | NDCS |
| 89 | B09 | 0 | 0 | | 28 | 21 | 890 | 1 | yes | yes | S4L | condom | NAD | discharge | NDCS |
| 90 | B10 | 0 | 0 | 16, 42 | 23 | 23 | 1 | 0 | no | no | GW | nil | NAD | warts | NDCS |
| 91 | B11 | 0 | 1 | 56 | 19 | 17 | 240 | 0 | no | yes | NSV | condom | NAD | NAD | NDCS |
| 92 | B12 | 0 | 0 | 16 | 26 | 19 | 2136 | 2 | yes | yes | NSC | condom | NAD | NAD | NDCS |
| 93 | B13 | 0 | 0 | mixed | 47 | 23 | 7272 | 5 | no | yes | GC, S4L, NSC, BV | OCP | NAD | NAD | NDCS |
| 94 | B14 | 1 | 1 | | 24 | 18 | 1 | 1 | no | no | nil | condom | inflamed | inflamed, discharge | NDCS |
| 95 | B15 | 0 | 0 | | 38 | 28 | 1 | 1 | no | no | HSV | condom | NAD | discharge | NDCS |
| 96 | B16 | 0 | 0 | | 44 | 22 | 1 | 6 | no | no | S4L,TV | LRT | inflamed | discharge | NDCS |
| 97 | B17 | 0 | 0 | | 31 | 18 | 2 | 2 | no | no | S4L, TV, chlamydia, GC, GV | condom | NAD | discharge | |
| 98 | B18 | 0 | 0 | | | | | | | | | | | | |
| 99 | B19 | 0 | 0 | | 44 | 20 | 1 | 7 | no | no | HSV, TV, NSV | nil | inflamed | inflamed, discharge | NDCS |
| 100 | B20 | 0 | 0 | | 29 | 29 | 1 | 0 | no | no | HSV | nil | NAD | NAD | NDCS |
| 101 | B21 | 0 | 1 | mixed | 20 | 19 | 16 | 1 | no | yes | BV | condom | inflamed, eroded | inflamed, discharge | NDCS |
| 102 | B22 | 0 | 0 | 16 | 24 | 19 | 1 | 1 | no | no | NSC | nil | inflamed, eroded | NAD | NDCS |
| 103 | B23 | 0 | 0 | | 40 | 14 | 1 | 3 | no | no | S4L | LRT | NAD | NAD | NDCS |
| 104 | B24 | 0 | 0 | 58 | 25 | 14 | 712 | 4 | no | yes | NSV, BV | LRT | inflamed, eroded | NAD | inflammatory nuclear changes |
| 105 | B25 | 0 | 1 | | 41 | 28 | 1 | 4 | no | no | HSV | condom | NAD | NAD | |
| 106 | B26 | 0 | 0 | | 33 | 28 | 416 | 2 | no | no | nil | depo | NAD | NAD | |
| 107 | B27 | 0 | 0 | | 24 | 20 | 1 | 1 | no | no | nil | IUCD | NAD | discharge | |
| 108 | B28 | 0 | 0 | | 25 | 22 | 1 | 1 | no | no | GW | IUCD | inflamed, eroded, discharge | inflamed, discharge, wart | NDCS |
| 109 | B29 | 0 | 0 | | | | | | | | | | | | |
| 110 | B30 | 0 | 0 | 35, 66 | 24 | 14 | 180 | 2 | no | yes | S4E | condom | NAD | NAD | |
| 111 | B31 | 0 | 0 | 66 | 24 | 24 | 1 | 0 | no | no | HSV, BV | OCP | NAD | NAD | |
| 112 | B32 | 0 | 0 | 16 | 37 | 15 | 1 | 3 | no | no | HSV | LRT | inflamed, eroded, discharge | inflamed, ulcers | CA CX stage 2b |
| 113 | B33 | 0 | 0 | 45 | 38 | 18 | 712 | 2 | no | yes | TV, S4L, NSC | condom | NAD | discharge | NDCS |
| 114 | B34 | 0 | 0 | | 33 | 25 | 6405 | 0 | no | yes | TV, GC, S4L, AV | condom | NAD | NAD | |
| 115 | B35 | 0 | 0 | | 22 | 17 | 1158 | 2 | no | no | GW, S4E, NSC | OCP | NAD | discharge | |
| 116 | B36 | 1 | 0 | | 30 | 20 | 1 | 0 | no | no | BV | nil | NAD | NAD | |
| 117 | B37 | 0 | 0 | 33 | 30 | 19 | 1 | 4 | no | no | S4L | depo | inflamed | NAD | |
| 118 | B38 | 1 | 0 | 16, 66 | 18 | 18 | 1 | 0 | no | no | nil | nil | NAD | NAD | |

# APPENDIX 1

## *PATIENT RECORDS FOR SRI LANKAN STD CLINIC STUDY GROUP (continued)*

| ID | Sample ID | GC PCR | CT PCR | HPV | AGE | COITARCHE | SEX PARTNERS | PARITY | SMOKING | CSW | STD'S | CONTRACEPTION | CX | VAGINA | PAP REPORT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 119 | B39 | 0 | 0 | | 39 | 25 | 1 | 2 | no | no | nil | depo | inflamed, eroded | NAD | |
| 120 | B40 | 0 | 1 | | | | | | | | | | | | |
| 121 | B41 | 0 | 0 | | 34 | 28 | 1 | 2 | no | no | S4L | OCP | NAD | discharge | NDCS |
| 122 | B42 | 0 | 0 | | 32 | 31 | 1 | 0 | no | no | nil | nil | inflamed | inflamed, discharge | inflammatory nuclear changes |
| 123 | B43 | 0 | 0 | | 28 | 10 | 946 | 2 | yes | yes | S4E | condom | inflamed | inflamed, discharge | NDCS |
| 124 | B44 | 0 | 0 | | 27 | 13 | 3560 | 1 | yes | yes | S4E | LRT | NAD | NAD | |
| 125 | B45 | 0 | 0 | | 40 | 17 | 10,680 | 2 | no | yes | TV, S4L, NSC | condom | inflamed | discharge | |
| 126 | B46 | 0 | 0 | 33 | 26 | 24 | 1 | 0 | no | no | nil | nil | eroded | NAD | |
| 127 | B47 | 0 | 0 | | 38 | 19 | 2 | 4 | no | no | TV | LRT | inflamed | discharge, warts | NDCS |
| 128 | B48 | 0 | 0 | | 36 | 34 | 1 | 1 | no | no | nil | nil | NAD | NAD | |
| 129 | B49 | 0 | 0 | | 30 | 17 | 2848 | 1 | no | yes | S3, GC, NSGI, NSC | LRT | NAD | NAD | |
| 130 | B50 | 1 | 0 | 39 | 37 | 16 | 3194 | 6 | no | yes | NSC, TV, GC, BV | condom | NAD | NAD | |
| 131 | B51 | 0 | 0 | 59 | 27 | 26 | 1 | 0 | no | no | nil | nil | NAD | ulcers | |
| 132 | B52 | 0 | 0 | | 25 | 19 | 1 | no | no | 2 | NSV | depo | inflamed | inflamed | NDCS |
| 133 | B53 | 0 | 0 | | 37 | 13 | 1880 | 3 | no | yes | BV, NSV | LRT | NAD | discharge | NDCS |
| 134 | B54 | 1 | 0 | | 25 | 14 | 60 | 4 | yes | yes | NSC | condom | inflamed, eroded | discharge | NDCS |
| 135 | B55 | 0 | 1 | | 34 | 15 | 2 | 3 | no | no | TV | LRT | NAD | NAD | |
| 136 | B56 | 0 | 1 | | 25 | 22 | 2 | 0 | no | no | HSV | nil | NAD | NAD | |
| 137 | B57 | 0 | 0 | | 22 | 21 | 1 | 1 | no | no | NSV | nil | inflamed, eroded | inflamed, discharge | |
| 138 | B58 | 0 | 0 | | 39 | 18 | 1 | 3 | no | no | S4L | LRT | NAD | NAD | |
| 139 | B59 | 0 | 0 | | 44 | 19 | 1 | 2 | no | no | S4L | LRT | NAD | NAD | |
| 140 | B60 | 0 | 0 | 16 | | | | | | | | | | | |
| 141 | B61 | 0 | 0 | | 29 | 20 | 10,680 | 0 | no | yes | S4L, TV | depo | NAD | discharge | NDCS |
| 142 | B62 | 0 | 0 | | 38 | 18 | 2 | 1 | no | no | S4L | condom | NAD | discharge | NDCS |
| 143 | B63 | 0 | 0 | 56 | 35 | 19 | 6408 | 3 | no | yes | GC, TV, NSC, BV | LRT | NAD | discharge | NDCS |
| 144 | B64 | 0 | 0 | 45, 43, 42 | 18 | 18 | 24 | 0 | no | yes | TV | condom | NAD | discharge | |
| 145 | B65 | 0 | 1 | 16 | 31 | 22 | 1 | 3 | no | no | S4L | condom | NAD | NAD | |
| 146 | B66 | 0 | 0 | 42 | 33 | 25 | 1 | 1 | no | no | nil | condom | NAD | discharge | |
| 147 | B67 | 1 | 0 | | 47 | 22 | 10,560 | 2 | no | yes | NSC, S4L | OCP | NAD | discharge | |
| 148 | B68 | 0 | 0 | | 32 | 27 | 2 | 2 | no | no | nil | condom | inflamed | inflamed, discharge | |
| 149 | B69 | 0 | 1 | 18 | 26 | 18 | 5 | 2 | no | no | NSV | depo | NAD | NAD | |
| 150 | B70 | 0 | 1 | | 27 | 27 | 1 | 0 | no | no | nil | nil | NAD | discharge | |
| 151 | B71 | 0 | 0 | | 33 | 20 | 3972 | 2 | no | yes | S4L | LRT | NAD | discharge | NDCS |
| 152 | B72 | 0 | 0 | | 33 | 33 | 1 | 0 | no | no | HSV | nil | NAD | ulcers | |
| 153 | B73 | 0 | 0 | | 40 | 20 | 1 | 3 | no | no | S4L | condom | NAD | NAD | NDCS |
| 154 | B74 | 0 | 0 | | 28 | 19 | 520 | 0 | no | yes | NSGI, S4L, TV, BV | condom | NAD | NAD | |
| 155 | B75 | 0 | 0 | 42, 58 | 54 | 15 | 1 | 5 | no | no | GC | abdominal hysterectomy | NAD | NAD | |
| 156 | B76 | 0 | 0 | | 29 | 15 | 3972 | 4 | no | yes | TV, NSC | condom | NAD | NAD | |
| 157 | B77 | 0 | 0 | | 33 | 19 | 2 | 2 | no | no | NSV | depo | NAD | discharge | |
| 158 | B78 | 0 | 0 | | 23 | 20 | 1424 | 2 | no | yes | nil | depo | NAD | NAD | |
| 159 | B79 | 0 | 0 | | 22 | 20 | 120 | 1 | no | yes | TV | LRT | NAD | NAD | |
| 160 | B80 | 0 | 0 | 67 | 18 | 16 | 712 | 0 | no | yes | NSV | condom | NAD | NAD | |
| 161 | C01 | 0 | 0 | | 37 | 15 | 7120 | 1 | no | yes | TV, S4L, BV, NSC | LRT | NAD | NAD | NDCS |
| 162 | C02 | 0 | 1 | | 46 | 17 | 1424 | 5 | no | yes | NSV, AV | LRT | NAD | NAD | NDCS |
| 163 | C03 | 0 | 1 | | 27 | 17 | 1 | 1 | no | no | NV | condom | eroded | discharge | NDCS |
| 164 | C04 | 0 | 0 | | 27 | 15 | 1 | 2 | no | no | nil | condom | NAD | NAD | |
| 165 | C05 | 0 | 0 | 43, 45 | 28 | 18 | 200 | 1 | yes | yes | nil | LRT | NAD | discharge | NDCS |
| 166 | C06 | 0 | 1 | | 18 | 17 | 1 | 0 | no | no | nil | OCP | NAD | discharge | |
| 167 | C07 | 0 | 0 | 16 | 29 | 22 | 260 | 0 | yes | yes | TV, GC, S4L | condom | inflamed | NAD | |
| 168 | C08 | 0 | 1 | | 28 | 22 | 1 | 2 | no | no | NSC | OCP | NAD | NAD | |
| 169 | C09 | 0 | 0 | | 28 | 16 | 1 | 3 | no | no | GC | LRT | NAD | discharge | |
| 170 | C10 | 0 | 0 | | 23 | 15 | 3972 | 2 | no | yes | S4L | condom | NAD | NAD | |
| 171 | C11 | 0 | 0 | | 30 | 13 | 2 | 3 | no | no | S4L | LRT | inflamed | discharge | NDCS |
| 172 | C12 | 0 | 0 | | 35 | 19 | 2 | 4 | no | yes | NSV | condom | inflamed | discharge | NDCS |
| 173 | C13 | 0 | 0 | | 26 | 13 | 2136 | 1 | no | yes | HSV | condom | NAD | NAD | NDCS |
| 174 | C14 | 0 | 0 | 45 | 43 | 17 | 3320 | 6 | yes | yes | S4L | LRT | NAD | discharge | |
| 175 | C15 | 0 | 1 | 43, 45, 33 | 19 | 16 | 178 | 2 | no | yes | NSV, TV | condom | eroded | discharge | |
| 176 | C16 | 0 | 0 | | 24 | 24 | 1 | 0 | no | no | nil | nil | NAD | discharge | |
| 177 | C17 | 0 | 0 | 59, 33 | 37 | 21 | 2136 | 1 | yes | yes | nil | condom | NAD | discharge | |
| 178 | C18 | 0 | 0 | | 32 | 13 | 780 | 3 | no | yes | NSV | LRT | NAD | NAD | |
| 179 | C19 | 0 | 0 | 66 | 23 | 17 | 2748 | 1 | no | yes | chlamydia, NSC | IUCD | NAD | discharge | |
| 180 | C20 | 0 | 0 | | 47 | 19 | 3560 | 2 | no | yes | S4E, TV, NSGI | LRT | NAD | NAD | |
| 181 | C21 | 0 | 0 | | 36 | 24 | 1 | 3 | no | no | S4L | LRT | NAD | NAD | NDCS |
| 182 | C22 | 0 | 0 | | 22 | 19 | 24 | 2 | no | yes | nil | depo | NAD | NAD | |
| 183 | C23 | 0 | 0 | | 33 | 15 | 2136 | 3 | no | yes | NSV, NSC | LRT | NAD | NAD | NDCS |
| 184 | C24 | 0 | 0 | | 27 | 22 | 1 | 1 | no | no | HSV | OCP | NAD | discharge | NDCS |
| 185 | C25 | 0 | 0 | 67 | 40 | 13 | 1780 | 5 | no | yes | S4L | LRT | NAD | NAD | |
| 186 | C26 | 0 | 0 | 35 | 21 | 19 | 1 | 1 | no | no | TV | nil | NAD | NAD | NDCS |
| 187 | C27 | 0 | 0 | 16 | 35 | 16 | 48 | 3 | yes | yes | NSV | condom | NAD | discharge | |
| 188 | C28 | 0 | 0 | hard31 | 30 | 19 | 3 | 1 | no | no | TV | nil | inflamed | discharge | |
| 189 | C29 | 0 | 0 | | 30 | 23 | 1 | 3 | no | no | S4E | depo | NAD | NAD | |
| 190 | C30 | 0 | 0 | 16 | 24 | 19 | 712 | 0 | no | yes | NSV, GC, TV, GW | condom | NAD | NAD | |
| 191 | C31 | 0 | 0 | | 45 | 25 | 1 | 3 | no | no | S4L | LRT | NAD | NAD | NDCS |
| 192 | C32 | 0 | 1 | 6 | 18 | 16 | 1 | 0 | no | no | NSV | condom | NAD | NAD | NDCS |
| 193 | C33 | 0 | 0 | 39, 51 | 34 | 26 | 2 | 0 | no | no | NSV, AV | condom | NAD | NAD | NDCS |
| 194 | C34 | 0 | 0 | 42 | 39 | 17 | 3194 | 7 | no | yes | NSV | IUCD | NAD | NAD | NDCS |
| 195 | C35 | 0 | 0 | | 50 | 20 | 2 | 5 | no | no | nil | IUCD | NAD | NAD | NDCS |
| 196 | C36 | 0 | 0 | | 29 | 15 | 2 | 5 | no | no | S4L | LRT | NAD | NAD | |
| 197 | C37 | 0 | 0 | | 37 | 17 | 1 | 3 | no | no | chlamydia | IUCD | inflamed, eroded | inflamed, discharge | |
| 198 | C38 | 0 | 1 | 16 | 20 | 19 | 1 | 0 | no | no | NSV | nil | NAD | NAD | NDCS |
| 199 | C39 | 0 | 0 | | 20 | 18 | 1 | 1 | no | no | S4L, NSV | depo | NAD | NAD | |
| 200 | C40 | 0 | 0 | | 27 | 18 | 2 | 3 | no | no | NSV | condom | NAD | NAD | |
| 201 | C41 | 0 | 0 | 32 | 33 | 14 | 1424 | 4 | no | yes | NSC | depo | NAD | NAD | NDCS |
| 202 | C42 | 0 | 0 | | 37 | 23 | 3 | 1 | no | no | NSV | condom | NAD | NAD | NDCS |
| 203 | C43 | 0 | 0 | | 27 | 16 | 832 | 2 | no | yes | NSV | OCP | NAD | discharge | inflammatory nuclear changes |
| 204 | C44 | 0 | 0 | | 35 | 30 | 1 | 1 | no | no | HSV | condom | NAD | inflamed, ulcers, discharge | NDCS |
| 205 | C45 | 0 | 0 | 31, 35, 16 | 30 | 27 | 5 | 1 | no | no | HIV, GW | OCP | NAD | warts | NDCS |
| 206 | C46 | 0 | 0 | | 44 | 29 | 1 | 7 | no | no | HSV, TV, NSV | condom | inflamed | inflamed, discharge | NDCS |
| 207 | C47 | 1 | 1 | | 23 | 18 | 2 | 1 | no | no | TV, GW | condom | eroded | NAD | NDCS |
| 208 | C48 | 0 | 0 | | 30 | 15 | 3560 | 1 | no | yes | nil | LRT | NAD | NAD | NDCS |
| 209 | C49 | 0 | 0 | 51, 45, 43, 33 | 20 | 16 | 1 | 3 | no | no | TV | condom | NAD | NAD | NDCS |
| 210 | C50 | 1 | 0 | 6, 67 | 25 | 22 | 1 | 1 | no | no | GW | OCP | eroded | warts | NDCS, koilocytic atypia |
| 211 | C51 | 0 | 0 | | 34 | 22 | 1 | 6 | no | no | S4L, TV | OCP | inflamed | discharge | NDCS |
| 212 | C52 | 0 | 0 | | 37 | 16 | 1 | 2 | no | no | S4L | condom | NAD | NAD | NDCS |
| 213 | C53 | 0 | 0 | 53 | 27 | 18 | 156 | 2 | no | yes | S4L, TV, NSC | condom | NAD | discharge | NDCS |
| 214 | C54 | 0 | 1 | 70 | 28 | 23 | 712 | 2 | no | yes | S4L, GC | condom | NAD | NAD | NDCS |
| 215 | C55 | 0 | 0 | 52 | 20 | 10 | 1092 | 0 | yes | yes | nil | depo | NAD | discharge | NDCS |
| 216 | C56 | 0 | 1 | | 20 | 19 | 936 | 0 | no | yes | NSV | condom | inflamed | discharge | NDCS |
| 217 | C57 | 0 | 1 | | 33 | 23 | 1 | 3 | no | no | S4L | IUCD | inflamed, eroded | inflamed, dischareg | NDCS |
| 218 | C58 | 0 | 0 | | 48 | 27 | 180 | 1 | no | yes | NSV | condom | NAD | NAD | NDCS |
| 219 | C59 | 0 | 1 | 16 | 29 | 14 | 16 | 5 | no | no | nil | LRT | NAD | discharge | NDCS |
| 220 | C60 | 0 | 1 | | 37 | 30 | 1 | 2 | nno | no | GC | nil | inflamed | discharge | NDCS |
| 221 | C61 | 0 | 1 | | 41 | 19 | 2 | 4 | no | no | GC | LRT | NAD | discharge | NDCS |
| 222 | C62 | 0 | 0 | | 48 | 19 | 1 | 5 | no | no | S4L | LRT | NAD | discharge | NDCS |
| 223 | C63 | 0 | 0 | | 27 | 16 | 15 | 0 | no | no | NSV | nil | NAD | discharge | NDCS |
| 224 | C64 | 0 | 0 | 16 | 21 | 16 | 1 | 1 | no | no | S4E | depo | NAD | NAD | NDCS |
| 225 | C65 | 0 | 0 | 35, 33 | 38 | 18 | 2 | 0 | no | no | TV, S4E | condom | NAD | NAD | NDCS |
| 226 | C66 | 0 | 0 | | 23 | 22 | 1 | 0 | no | no | nil | nil | NAD | discharge | NDCS |
| 227 | C67 | 0 | 1 | | 27 | 22 | 2 | 1 | no | no | nil | nil | NAD | NAD | NDCS |
| 228 | C68 | 0 | 0 | | 38 | 38 | 1 | 0 | no | no | nil | nil | NAD | discharge | NDCS |
| 229 | C69 | 0 | 1 | | 35 | 23 | 8900 | 5 | no | yes | TV, GC, BV | condom | NAD | discharge | NDCS |
| 230 | C70 | 0 | 0 | 58 | 27 | 19 | 1 | 2 | no | no | S4L | OCP | NAD | discharge | NDCS |
| 231 | C71 | 0 | 1 | new s1t | 40 | 17 | 12,460 | 3 | no | yes | GC, S4L, GV, TV, NSC | LRT | NAD | NAD | NDCS |
| 232 | C72 | 0 | 1 | | 27 | 16 | 1 | 3 | no | no | S4L | LRT | NAD | discharge | NDCS |
| 233 | C73 | 0 | 0 | mixed | 28 | 22 | 624 | 1 | no | yes | GC | condom | NAD | NAD | |
| 234 | C74 | 0 | 0 | | 23 | 20 | 1 | 2 | no | no | HSV | depo | NAD | NAD | NDCS |
| 235 | C75 | 0 | 1 | | 22 | 13 | 43 | 2 | no | yes | no | condom | NAD | discharge | NDCS |
| 236 | C76 | 0 | 1 | | 29 | 18 | 24 | 3 | no | yes | BV, NSV | OCP | NAD | NAD | NDCS |
| 237 | C77 | 0 | 1 | | 33 | 17 | 8900 | 1 | yes | yes | NSV | condom | inflamed | inflamed, discharge | NDCS |

# APPENDIX 1

## *PATIENT RECORDS FOR SRI LANKAN STD CLINIC STUDY GROUP (continued)*

| ID | Sample ID | GC PCR | CT PCR | HPV | AGE | COITARCHE | SEX PARTNERS | PARITY | SMOKING | CSW | STD'S | CONTRACEPTION | CX | VAGINA | PAP REPORT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 238 | C78 | 0 | 0 | | 32 | 22 | 2 | 5 | no | no | HSV, TV | LRT | inflamed | inflamed, ulcers, discharge | NDCS |
| 239 | C79 | 0 | 0 | | 40 | 17 | 7120 | 2 | no | yes | NSV, S4L | LRT | NAD | discharge, ulcers | NDCS |
| 240 | C80 | 0 | 0 | | 28 | 16 | 4272 | 4 | yes | yes | NSV | LRT | inflamed | NAD | NDCS |
| 241 | D01 | 0 | 1 | | 41 | 26 | 2 | 4 | no | no | NSV | LRT | NAD | NAD | NDCS |
| 242 | D02 | 0 | 0 | | 39 | 12 | 5340 | 4 | no | yes | nil | condom | NAD | discharge | NDCS |
| 243 | D03 | 0 | 0 | | 25 | 14 | 1 | 2 | no | no | BV | depo | NAD | NAD | NDCS |
| 244 | D04 | 0 | 0 | 16 | 22 | 18 | 160 | 1 | no | yes | NSV | condom | NAD | discharge | NDCS |
| 245 | D05 | 0 | 1 | | 32 | 23 | 468 | 8 | no | yes | NSV, GC, S4L | OCP | NAD | NAD | NDCS |
| 246 | D06 | 0 | 0 | 66 | 22 | 19 | 1 | 1 | no | no | S4L | nil | NAD | discharge | CIN 1, koilocytic atypia |
| 247 | D07 | 0 | 1 | | 29 | 16 | 1 | 2 | no | no | S4L, TV | LRT | inflamed | inflamed, discharge | NDCS |
| 248 | D08 | 0 | 0 | | 26 | 23 | 1 | 1 | no | no | HSV | depo | inflamed | ulcers | NDCS |
| 249 | D09 | 0 | 0 | | 33 | 30 | 1 | 1 | no | no | HSV | OCP | inflamed | inflamed, discharge | NDCS |
| 250 | D10 | 0 | 1 | han831 | 21 | 12 | 1424 | 1 | no | yes | BV, TV | OCP | inflamed | inflamed, discharge | NDCS |
| 251 | D11 | 0 | 0 | 42 | 43 | 18 | 1 | 2 | no | no | S3 | IUCD | NAD | dicharge | NDCS |
| 252 | D12 | 0 | 0 | | 28 | 19 | 2848 | 1 | no | yes | GC | LRT | NAD | NAD | NDCS |
| 253 | D13 | 0 | 0 | cp6108 | 24 | 13 | 7120 | 1 | no | yes | NSC | condom | inflamed | discharge | NDCS |
| 254 | D14 | 0 | 0 | 42 | 34 | 20 | 1 | 2 | no | no | nil | nil | inflamed | discharge | NDCS |
| 255 | D15 | 0 | 0 | | 27 | 25 | 3 | 3 | no | no | S4L | depo | NAD | NAD | |
| 256 | D16 | 0 | 0 | | 39 | 31 | 156 | 1 | no | yes | TV | condom | inflamed | discharge | NDCS |
| 257 | D17 | 0 | 1 | 56, 6 | 25 | 22 | 1 | 1 | no | no | GW | OCP | eroded | warts | CIN1, koilocytic atypia |
| 258 | D18 | 0 | 0 | | 29 | 14 | 1 | 4 | no | no | S4L | depo | NAD | NAD | NDCS |
| 259 | D19 | 0 | 1 | | 29 | 20 | 8900 | 2 | no | yes | NSV, TV | LRT | inflamed, eroded | discharge | NDCS |
| 260 | D20 | 0 | 1 | cp6304 | 22 | 12 | 144 | 2 | no | yes | TV, GW, GC | OCP | inflamed, eroded | inflamed, discharge | NDCS |
| 261 | D21 | 0 | 0 | 56 | 34 | 19 | 1 | 1 | no | no | S4L | LRT | NAD | NAD | NDCS |
| 262 | D22 | 0 | 0 | | 20 | 10 | 300 | 0 | yes | yes | BV, HSV, TV | nil | NAD | discharge | NDCS |
| 263 | D23 | 0 | 0 | | 20 | 15 | 1424 | 1 | no | yes | nil | depo | NAD | discharge | NDCS |
| 264 | D24 | 0 | 0 | 45 | 18 | 15 | 48 | 1 | no | yes | HSV, GC | condom | inflamed | discharge | NDCS |
| 265 | D25 | 0 | 0 | | 30 | 20 | 1 | 2 | no | no | NSV | nil | inflamed | discharge | inflammatory nuclear changes |
| 266 | D26 | 0 | 1 | 18 | | | | | | | | | | | |
| 267 | D27 | 0 | 0 | | 21 | 16 | 1 | 2 | no | no | nil | condom | NAD | discharge | NDCS |
| 268 | D28 | 0 | 0 | | 30 | 17 | 1 | 3 | no | no | TV, S4L | LRT | NAD | discharge | inflammatory nuclear changes |
| 269 | D29 | 0 | 0 | | 32 | 20 | 156 | 1 | no | yes | TV | IUCD | inflamed | discharge | NDCS |
| 270 | D30 | 0 | 0 | | 27 | 14 | 1 | 1 | no | no | TV, S4L | nil | inflamed | inflamed, discharge | NDCS |
| 271 | D31 | 0 | 0 | | 40 | 20 | 624 | 2 | no | yes | S4L | condom | NAD | NAD | NDCS |
| 272 | D32 | 0 | 1 | 42 | 27 | 19 | 1 | 1 | no | no | nil | OCP | NAD | discharge | NDCS |
| 273 | D33 | 0 | 0 | | 45 | 19 | 2 | 1 | no | no | S4L, GC | nil | NAD | discharge | NDCS |
| 274 | D34 | 0 | 0 | | 30 | 26 | 2 | 0 | no | no | nil | condom | NAD | discharge | NDCS |
| 275 | D35 | 0 | 0 | | 36 | 17 | 8274 | 2 | no | yes | S4L, GC, HSV, TV | LRT | NAD | NAD | NDCS |
| 276 | D36 | 0 | 0 | | 19 | 17 | 1 | 1 | no | no | GC | depo | NAD | discharge | NDCS |
| 277 | D37 | 0 | 0 | mixed | 40 | 21 | 1 | 3 | no | no | TV | vasectomy | inflamed, eroded | discharge | NDCS |
| 278 | D38 | 0 | 0 | not type | 33 | 18 | 4680 | 2 | yes | yes | TV, NSC, GV, GC | OCP | NAD | NAD | NDCS |
| 279 | D39 | 0 | 0 | | 35 | 19 | 624 | 1 | no | yes | S4L, TV, NSC | LRT | inflamed, eroded | inflamed, discharge | NDCS |
| 280 | D40 | 0 | 0 | 35 | 28 | 14 | 2808 | 3 | yes | yes | S4E | condom | inflamed | inflamed, discharge | NDCS |
| 281 | D41 | 0 | 0 | 16 | 23 | 17 | 20 | 0 | no | yes | NSV | nil | NAD | discharge | NDCS |
| 282 | D42 | 0 | 0 | | 24 | 17 | 2 | 0 | no | no | nil | nil | NAD | NAD | inflammatory nuclear changes |
| 283 | D43 | 1 | 0 | 56, 6, 11 | 30 | 18 | 40 | 4 | no | yes | GC | LRT | NAD | NAD | NDCS |
| 284 | D44 | 0 | 0 | | 33 | 24 | 1 | 0 | no | no | S4E | nil | NAD | discharge | inflammatory nuclear changes |
| 285 | D45 | 0 | 1 | 45 | 20 | 15 | 712 | 2 | no | yes | NSV | OCP | NAD | discharge | |
| 286 | D46 | 0 | 0 | | 38 | 13 | 3 | 3 | no | no | S4L, NSC | nil | NAD | discharge | NDCS |
| 287 | D47 | 0 | 0 | 56 | 38 | 26 | 1 | 4 | no | no | TV | nil | NAD | inflamed, discharge | NDCS |
| 288 | D48 | 0 | 0 | 42 | 22 | 20 | 420 | 1 | yes | yes | NSV, TV | condom | inflamed | discharge | NDCS |
| 289 | D49 | 0 | 0 | | 36 | 22 | 1 | 2 | no | no | HSV | OCP | inflamed, eroded, ulcers | inflamed, ulcers, discharge | NDCS |
| 290 | D50 | 0 | 0 | | 27 | 23 | 1 | 1 | no | no | nil | IUCD | inflamed, discharge | discharge | NDCS |
| 291 | D51 | 0 | 0 | | 20 | 15 | 1 | 0 | no | no | TV | nil | NAD | discharge | NDCS |
| 292 | D52 | 0 | 0 | | 25 | 18 | 2136 | 1 | no | yes | S4L, BV | condom | inflamed | inflamed, discharge | NDCS |
| 293 | D53 | 0 | 0 | new s11 | 50 | 33 | 1 | 2 | no | no | nil | nil | NAD | NAD | NDCS |
| 294 | D54 | 0 | 0 | | 26 | 20 | 2136 | 0 | no | yes | nil | condom | NAD | NAD | NDCS |
| 295 | D55 | 0 | 0 | 70 | 34 | 21 | 2 | 2 | no | no | S4L | condom | NAD | NAD | NDCS |
| 296 | D56 | 1 | 0 | | 26 | 19 | 3660 | 1 | no | yes | S4L | condom | inflamed | inflamed, discharge | NDCS |
| 297 | D57 | 0 | 0 | | 28 | 19 | 1 | 4 | no | no | HSV | OCP | inflamed, cystic | inflamed, discharge | NDCS |
| 298 | D58 | 0 | 0 | | 33 | 16 | 5696 | 2 | yes | yes | nil | condom | NAD | discharge | NDCS |
| 299 | D59 | 0 | 0 | | 45 | 12 | 7120 | 1 | no | yes | GC, TV, GV, S4L, NSC, BV | LRT | NAD | discharge | NDCS |
| 300 | D60 | 0 | 0 | | 43 | 19 | 1 | 3 | no | no | TV | nil | NAD | discharge | inflammatory nuclear changes |
| 301 | D61 | 0 | 1 | | 40 | 19 | 1 | 5 | no | no | S4L | OCP | inflamed, eroded | discharge | |
| 302 | D62 | 0 | 1 | cp6304 | 57 | 19 | 2 | 9 | no | no | NSV | LRT | NAD | NAD | |
| 303 | D63 | 0 | 1 | | | | | | | | | | | | |
| 304 | D64 | 0 | 0 | | 47 | 17 | 1 | 5 | no | no | nil | LRT | NAD | NAD | |
| 305 | D65 | 0 | 0 | 16 | 33 | 18 | 2 | 5 | no | no | BV, NSV | LRT | inflamed | NAD | |
| 306 | D66 | 0 | 0 | | 32 | 29 | 1 | 1 | no | no | TV | OCP | inflamed, ulcers | ulcers | |
| 307 | D67 | 0 | 0 | | 34 | 33 | 1 | 0 | no | no | HSV, GW | nil | NAD | NAD | |
| 308 | D68 | 0 | 1 | 16 | 36 | 17 | 4 | 2 | no | no | TV | LRT | inflamed, discharge | discharge | |
| 309 | D69 | 0 | 0 | | 34 | 28 | 90 | 4 | no | yes | NSC | condom | NAD | NAD | |
| 310 | D70 | 0 | 0 | | 29 | 25 | 1 | 1 | no | no | NSC | IUCD | NAD | discharge | |
| 311 | D71 | 0 | 0 | | | | | | | | | | | | |
| 312 | D72 | 1 | 0 | | 32 | 16 | 1248 | 1 | no | yes | TV, S2, GV, GC | LRT | NAD | NAD | |
| 313 | D73 | 0 | 1 | 67 | 34 | 19 | 2 | 2 | no | no | S4L | LRT | NAD | NAD | |
| 314 | D74 | 0 | 1 | | 39 | 19 | 1 | 3 | no | no | NSC | nil | inflamed, discharge | NAD | |
| 315 | D75 | 0 | 1 | | 35 | 25 | 2 | 1 | no | no | chlamydia | nil | inflamed | discharge | |
| 316 | D76 | 0 | 0 | | 20 | 17 | 1 | 2 | no | no | NSV | nil | polyp | discharge | |
| 317 | D77 | 0 | 0 | | 42 | 16 | 1 | 4 | no | no | HSV | LRT | inflamed, discharge | inflamed, discharge | |
| 318 | D78 | 0 | 0 | 62 | 29 | 26 | 1 | 0 | no | no | NSC | nil | NAD | discharge | |
| 319 | D79 | 0 | 1 | | 23 | 15 | 1424 | 2 | no | yes | S4L | condom | NAD | discharge | |
| 320 | D80 | 1 | 1 | | 30 | 20 | 712 | 1 | yes | yes | NSV, BV | condom | inflamed | discharge | |
| 321 | E01 | 0 | 0 | | 32 | 28 | 1 | 0 | no | no | NSV | OCP | inflamed | discharge | |
| 322 | E02 | 0 | 1 | | 40 | 17 | 1 | 2 | no | no | HSV, S4L | LRT | inflamed, eroded, discharge | NAD | |
| 323 | E03 | 0 | 1 | | 25 | 15 | 1602 | 1 | yes | yes | NSV, GW, GC | condom | NAD | discharge | |
| 324 | E04 | 0 | 1 | | 33 | 18 | 1 | 4 | no | no | GC | depo | NAD | NAD | |
| 325 | E05 | 0 | 0 | | 32 | 16 | 4272 | 3 | yes | yes | NSV, BV | depo | NAD | discharge | |
| 326 | E06 | 0 | 1 | | 38 | 19 | 4 | 4 | no | no | NSV | OCP | atrophic, prolapsed | NAD | |
| 327 | E07 | 1 | 0 | 30 | 18 | 17 | 1 | 0 | no | no | GC | nil | NAD | NAD | |
| 328 | E08 | 0 | 0 | | 26 | 25 | 1 | 1 | no | no | nil | depo | NAD | discharge | |
| 329 | E09 | 0 | 0 | | 30 | 19 | 2 | 4 | no | no | NSV | nil | inflamed, eroded, discharge | NAD | |
| 330 | E10 | 0 | 0 | 45 | 22 | 19 | 120 | 0 | no | yes | S3 | condom | NAD | discharge | |
| 331 | E11 | 0 | 0 | | 39 | 20 | 1 | 4 | no | no | S4L | nil | inflamed | discharge | |
| 332 | E12 | 0 | 0 | | 38 | 30 | 2 | 1 | no | no | nil | nil | NAD | NAD | |
| 333 | E13 | 0 | 0 | | 21 | 15 | 30 | 3 | no | yes | TV, S4E | nil | inflamed, discharge | discharge | |
| 334 | E14 | 0 | 1 | | 36 | 23 | 7120 | 2 | no | yes | TV, BV, GC | condom | inflamed, eroded | discharge | |
| 335 | E15 | 0 | 0 | | 32 | 28 | 1 | 2 | no | no | HSV | nil | inflamed | inflamed, ulcers | |
| 336 | E16 | 0 | 1 | 56, 51 | 23 | 17 | 7 | 2 | no | no | BV | LRT | NAD | discharge | |
| 337 | E17 | 0 | 0 | | 33 | 14 | 520 | 1 | no | yes | NSV | nil | inflamed, discharge | NAD | |
| 338 | E18 | 0 | 0 | 70 | 30 | 15 | 1 | 2 | no | no | S4L | LRT | NAD | discharge | |
| 339 | E19 | 0 | 0 | | 31 | 23 | 1 | 2 | no | no | TV | OCP | NAD | NAD | |
| 340 | E20 | 0 | 0 | | 36 | 14 | 1424 | 0 | no | yes | S4L, NSV | condom | NAD | discharge | |
| 341 | E21 | 0 | 0 | | 36 | 34 | 1 | 0 | no | no | nil | condom | discharge | inflamed, discharge | |
| 342 | E22 | 0 | 0 | | 42 | 19 | 2 | 3 | no | no | S4L | LRT | inflamed, cystic | discharge | |
| 343 | E23 | 0 | 0 | | 24 | 14 | 416 | 2 | no | yes | S4L | IUCD | NAD | NAD | |
| 344 | E24 | 0 | 0 | | 42 | 14 | 4272 | 2 | no | yes | nil | condom | NAD | NAD | |
| 345 | E25 | 0 | 0 | 16 | | | | | | | | | | | |
| 346 | E26 | 0 | 0 | 16 | 17 | 16 | 2 | 0 | no | no | nil | nil | inflamed | discharge | |
| 347 | E27 | 0 | 0 | han831 | 21 | 18 | 1780 | 3 | no | yes | nil | condom | NAD | NAD | |
| 348 | E28 | 0 | 1 | | 25 | 20 | 720 | 1 | no | yes | BV | condom | NAD | NAD | |
| 349 | E29 | 0 | 0 | | 22 | 18 | 156 | 0 | no | yes | BV | condom | NAD | NAD | NDCS |
| 350 | E30 | 0 | 1 | 16 | 18 | 17 | 1 | 0 | no | no | nil | condom | NAD | NAD | |
| 351 | E31 | 0 | 0 | | 28 | 16 | 2 | 2 | no | no | nil | depo | NAD | NAD | |
| 352 | E32 | 0 | 0 | han831 | 27 | 25 | 1 | 1 | no | no | HSV | depo | NAD | ulcers | |
| 353 | E33 | 0 | 0 | han831 | 46 | 24 | 1 | 2 | no | no | HSV | IUCD | NAD | ulcers | |
| 354 | E34 | 0 | 0 | | 22 | 17 | 1 | 4 | no | no | S4L | depo | NAD | NAD | |
| 355 | E35 | 0 | 1 | | 26 | 20 | 14,952 | 2 | no | yes | NSC, S4L | depo | NAD | discharge | |
| 356 | E36 | 0 | 0 | 35 | 32 | 24 | 1 | 2 | no | no | S4L, NSC | depo | NAD | discharge | |

# APPENDIX 2

## *PATIENT RECORDS FOR THAI MUCOPURULENT CERVICITIS STUDY GROUP*

| Study_No | Hospital_No | Date_test | Age | Occupat | Race | Religious | Parity | Spont_abort | Criminal_abort | Dead_fetus | Contreception | Smoking | Previous_STD | Patient | Previous_STD_Husband |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 755699 | 12/09/96 | 33 | house wife | Thai | Bhudist | 1 | 0 | - | 0 | none | no | no | no | no |
| 2 | 687940 | 12/09/96 | 31 | | Thai | Bhudist | 0 | 0 | - | 0 | none | no | no | no | |
| 3 | 4537 | 12/16/96 | 33 | farmer | Thai | Bhudist | 1 | 0 | - | 0 | pills | no | no | no | |
| 4 | 756882 | 12/16/1996 | 34 | house wife | Thai | Bhudist | 1 | 0 | - | 0 | none | no | no | no | |
| 5 | 757149 | 12/17/1996 | 27 | house wife | Thai | Bhudist | 0 | 0 | - | 0 | DMPA | no | no | no | |
| 6 | | 12/17/1996 | 30 | farmer | Thai | Bhudist | 2 | 0 | - | 0 | none | no | no | no | |
| 7 | 757017 | 12/17/1996 | 28 | house wife | Thai | Bhudist | 1 | 1 | no | 0 | Tubal steril | no | no | no | |
| 8 | 499683 | 12/20/1996 | 42 | gov officer | Thai | Bhudist | 2 | 0 | - | 0 | coitus intrup | no | herpes | no | herpes |
| 9 | 757840 | 12/23/1996 | 43 | gov officer | Thai | Bhudist | 4 | 0 | - | 0 | condom | no | no | no | gonorrhea, NGU |
| 10 | 572110 | 12/23/1996 | 34 | | Thai | Bhudist | 3 | 0 | no | 0 | Tubal steril | no | no | no | |
| 11 | 709093 | 12/24/1996 | 19 | | Thai | Bhudist | 0 | - | - | 1 | pills | no | no | no | |
| 12 | 167770 | 12/24/1996 | 41 | | Thai | Bhudist | 4 | 0 | - | 0 | Tubal steril | no | cervicitis | no | |
| 13 | 614663 | 12/24/1996 | 26 | | Thai | Bhudist | 1 | 0 | no | 0 | pills | no | no | no | |
| 14 | 803421 | 12/24/1996 | 31 | gov officer | Thai | Bhudist | 2 | 0 | - | 0 | none | no | no | no | |
| 15 | 364171 | 01/06/96 | 26 | gov officer | Thai | Bhudist | 0 | 0 | - | 0 | none | no | no | no | |
| 16 | 468605 | 01/07/97 | 45 | farmer | Muslim | Islam | 6 | 0 | - | 1 | pills | yes | no | no | |
| 17 | 383851 | 01/07/97 | 47 | | Thai | Bhudist | 2 | 0 | - | 0 | Tubal steril | no | no | no | |
| 18 | 760159 | 01/13/1997 | 19 | | Thai | Bhudist | 1 | 0 | - | 0 | none | no | no | no | |
| 19 | 759922 | 01/20/1997 | 46 | | Thai | Bhudist | 3 | 1 | no | 0 | Tubal steril | no | no | no | |
| 20 | 32045 | 01/20/1997 | 29 | | Thai | Bhudist | 2 | 1 | no | 0 | none | no | no | no | |
| 21 | 764533 | 01/27/1997 | 45 | | Thai | Bhudist | 2 | 0 | - | 0 | Tubal steril | no | no | no | |
| 22 | 523186 | 01/27/1997 | 37 | | Thai | Bhudist | 2 | 0 | - | 0 | implant | no | no | no | |
| 23 | 763270 | 01/27/1997 | 28 | | Thai | Bhudist | 2 | 0 | - | 0 | none | no | no | no | |
| 24 | 360526 | 01/27/1997 | 38 | | Thai | Bhudist | 2 | 0 | - | 0 | condom | no | no | no | |
| 25 | 274974 | 01/27/1997 | 30 | | Thai | Bhudist | 2 | 1 | no | 0 | none | no | no | no | |
| 26 | 468605 | 01/27/1997 | 30 | business | Thai | Bhudist | 2 | 0 | - | 0 | Tubal steril | no | no | no | |
| 27 | 289661 | 02/12/1997 | 25 | farmer | Thai | Bhudist | 1 | 2 | - | 0 | DMPA | no | no | no | |
| 28 | 706037 | 02/24/1997 | 35 | worker | Thai | Bhudist | 2 | 0 | - | 0 | pills | no | no | no | |
| 29 | 769244 | 02/24/1997 | 28 | farmer | Thai | Bhudist | 4 | 1 | yes | 0 | Tubal steril | no | no | no | |
| 30 | 595246 | 02/25/1997 | 42 | farmer | Thai | Bhudist | 3 | 0 | - | 0 | condom | no | no | no | |
| 31 | 769468 | 02/25/1997 | 32 | business | Thai | Bhudist | 4 | 1 | yes | 0 | none | no | no | no | |
| 32 | 108855 | 03/03/97 | 45 | | Thai | Bhudist | 0 | 0 | - | 0 | Tubal steril | no | no | no | |
| 33 | 243222 | 03/10/97 | 45 | | Thai | Bhudist | 7 | 0 | - | 0 | Tubal steril | no | no | no | |
| 34 | 361372 | 03/10/97 | 33 | house wife | Thai | Bhudist | 2 | 2 | no | 0 | Tubal steril | no | no | no | |
| 35 | 391219 | 03/11/97 | 30 | house wife | Thai | Bhudist | 2 | 2 | yes | 0 | none | no | no | no | |
| 36 | 772548 | 03/31/1997 | 32 | house wife | Thai | Bhudist | 1 | 0 | - | 0 | none | no | no | no | |
| 37 | 780607 | 04/28/1997 | 32 | house wife | Thai | Bhudist | 2 | 0 | no | 0 | Tubal steril | no | no | no | |
| 38 | 782997 | 05/12/97 | 39 | | Thai | Bhudist | 2 | 0 | no | 0 | none | no | no | no | |
| 39 | 111605 | 05/12/97 | 43 | gov officer | Thai | Bhudist | 3 | 2 | - | 0 | Tubal steril | yes | gonorrhea | no | gonorrhea |
| 40 | 224198 | 05/12/97 | 35 | gov officer | Thai | Bhudist | 3 | 2 | - | 0 | none | no | bartholinitis | no | |
| 41 | 4464 | 05/12/97 | 37 | gov officer | Thai | Bhudist | 2 | 2 | - | 0 | Tubal steril | no | no | no | NGU |
| 42 | 760543 | 05/19/1997 | 42 | worker | Thai | Bhudist | 6 | 0 | - | 0 | none | no | no | no | herpes |
| 43 | 784723 | 06/02/97 | 40 | gov officer | Thai | Bhudist | 2 | 0 | - | 1 | pills | no | herpes | no | |
| 44 | 244514 | 06/03/97 | 37 | gov officer | Muslim | Islam | 2 | 2 | yes | 1 | none | no | TV | no | |
| 45 | 415165 | 06/10/97 | 45 | farmer | Thai | Bhudist | 3 | 2 | - | 0 | none | no | no | no | |
| 46 | 562760 | 06/10/97 | 27 | house wife | Thai | Bhudist | 3 | 0 | - | 1 | none | no | no | no | NGU |
| 47 | 788290 | 06/10/97 | 44 | worker | Thai | Bhudist | 3 | 2 | - | 0 | Tubal steril | no | no | no | |
| 48 | 788243 | 06/10/97 | 41 | business | Thai | Bhudist | 3 | 2 | - | 0 | Tubal steril | no | no | no | no |
| 49 | 697869 | 06/16/1997 | 41 | gov officer | Thai | Bhudist | 2 | 2 | - | 0 | coitus intrup | no | gonorrhea | no | gonorrhea |
| 50 | 195785 | 06/23/1997 | 41 | gov officer | Thai | Bhudist | 2 | 2 | - | 0 | Tubal steril | no | no | no | no |
| 51 | 461742 | 07/07/97 | 25 | worker | Thai | Bhudist | 0 | 3 | - | 1 | pills | no | no | no | no |
| 52 | | | 32 | gov officer | Thai | Bhudist | 3 | 2 | - | 0 | Tubal steril | no | no | no | no |
| 53 | 559343 | 07/28/1997 | 32 | gov officer | Thai | Bhudist | 3 | 2 | - | 0 | none | no | no | no | no |
| 54 | 248184 | 07/28/1997 | 32 | house wife | Thai | Bhudist | 3 | 2 | - | 0 | Tubal steril | no | gonorrhea | no | gonorrhea |
| 55 | 789518 | 07/28/1997 | 27 | business | Thai | Bhudist | 1 | 3 | - | 0 | none | no | no | no | no |
| 56 | 496845 | 07/28/1997 | 34 | | Muslim | Islam | 1 | 3 | yes | 0 | pills | no | no | no | gonorrhea |
| 57 | 698420 | 08/11/97 | 42 | farmer | Thai | Bhudist | 4 | 2 | - | 0 | coitus intrup | no | no | no | |
| 58 | | 08/25/1997 | 27 | worker | Thai | Bhudist | 2 | 1 | - | 0 | none | no | no | no | no |
| 59 | 803107 | 08/08/97 | 27 | worker | Thai | Bhudist | 0 | 0 | - | 0 | none | no | no | no | no |
| 60 | 511851 | 10/13/1997 | 31 | farmer | Thai | Bhudist | 2 | 2 | - | 0 | pills | no | no | no | no |
| 61 | 388713 | 10/13/1997 | 45 | farmer | Thai | Bhudist | 3 | 1 | - | 0 | Tubal steril | no | no | no | no |
| 62 | 388774 | 10/20/1997 | 36 | gov officer | Thai | Bhudist | 1 | 3 | no | 0 | none | no | herpes | no | herpes |
| 63 | 578196 | 10/20/1997 | 33 | | Thai | Bhudist | 3 | 3 | - | 0 | safe period | no | gonorrhea | no | gonorrhea |
| 64 | 486261 | 12/01/97 | 39 | house wife | Chinese | Christ | 3 | 3 | - | 0 | Tubal steril | no | no | no | no |
| 65 | 474103 | 12/08/97 | 42 | business | Thai | Bhudist | 3 | 3 | - | 0 | none | no | no | no | no |
| 66 | 799348 | 12/08/97 | 37 | farmer | Thai | Bhudist | 3 | 3 | - | 0 | vasectomy | no | MPC | no | NGU |
| 67 | 819265 | 12/15/1997 | 36 | farmer | Thai | Bhudist | 3 | 3 | - | 0 | Tubal steril | yes | no | no | no |
| 68 | 377767 | 12/15/1997 | 32 | house wife | Thai | Bhudist | 2 | 2 | - | 0 | pills | yes | no | no | no |
| 69 | 377767 | 12/15/1997 | 32 | gov officer | Thai | Bhudist | 3 | 1 | no | 0 | none | yes | no | no | no |
| 70 | 821493 | 12/29/1997 | 27 | farmer | Thai | Bhudist | 1 | 3 | - | 0 | coitus intrup | no | no | no | no |
| 71 | 823098 | 01/12/98 | 43 | business | Thai | Bhudist | 3 | 3 | - | 0 | Tubal steril | no | no | no | no |
| 72 | 824322 | 01/19/1998 | 45 | | Thai | Bhudist | 3 | 0 | - | 2 | pills | no | TV | no | no |
| 73 | 462825 | 01/19/1998 | 33 | farmer | Thai | Bhudist | 2 | 0 | - | 0 | none | no | no | no | no |
| 74 | 649272 | 01/26/1998 | 49 | business | Thai | Bhudist | 1 | 0 | - | 0 | Tubal steril | no | no | no | no |
| 75 | 40899 | 01/26/1998 | 37 | gov officer | Thai | Bhudist | 3 | 0 | - | 0 | none | no | no | no | no |
| 76 | 314408 | 02/23/1998 | 38 | gov officer | Thai | Bhudist | 2 | 2 | - | 0 | condom | no | no | no | no |
| 77 | 469903 | 03/30/1998 | 35 | farmer | Thai | Bhudist | 2 | 2 | - | 0 | none | no | no | no | no |
| 78 | 484869 | 04/27/1998 | 42 | worker | Thai | Bhudist | 2 | 2 | - | 0 | none | no | no | no | no |
| 79 | 484869 | 04/27/1998 | 45 | | Thai | Bhudist | 1 | 3 | - | 0 | none | no | no | no | no |
| 80 | 838607 | 04/27/1998 | 31 | | Thai | Bhudist | 3 | 1 | - | 0 | condom | no | no | no | no |
| 81 | 665709 | 05/04/98 | 39 | gov officer | Thai | Bhudist | 3 | 0 | - | 0 | Tubal steril | no | no | no | no |
| 82 | 510830 | 05/04/98 | 34 | gov officer | Thai | Bhudist | 3 | 1 | yes | 0 | none | no | no | no | no |
| 83 | 777309 | 05/26/1998 | 38 | gov officer | Thai | Bhudist | 2 | 1 | yes | 0 | condom | no | no | no | no |
| 84 | 719173 | 06/08/98 | 26 | | Thai | Bhudist | 2 | 1 | yes | 0 | pills | no | no | no | no |
| 85 | 362381 | 06/27/1998 | 45 | farmer | Thai | Bhudist | 2 | 2 | - | 0 | condom | no | no | no | no |
| 86 | 29433 | 07/13/1998 | 42 | worker | Thai | Bhudist | 2 | 2 | - | 0 | DMPA | no | no | no | no |
| 87 | 158951 | 07/20/1998 | 34 | gov officer | Thai | Bhudist | 1 | 2 | no | 0 | pills | no | no | no | n |

| Study_No | Drug allergy | History of Infertiliy | symptom_hypersecretion | symptom_foul_discharge | symptom_yellow_discharge | Symp_Pelvic_pain | Symp_Pelvic_discomfort | Other_symptom | Character_vag_discharge | Character_endocerv_discharge | TV_wet_mount |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | no | no | yes | no | yes | no | yes | no | floccular | cloudy | no |
| 2 | no | yes | yes | yes | yes | no | no | itching | floccular | mucopus | no |
| 3 | yes, cotrimoxazole | no | yes | yes | no | no | yes | no | floccular | cloudy | no |
| 4 | no | no | yes | no | no | no | no | itching | floccular | cloudy | no |
| 5 | no | no | yes | yes | yes | no | yes | itching | homogeneous | mucopus | no |
| 6 | no | no | yes | no | yes | no | yes | no | floccular | mucopus | no |
| 7 | no | no | yes | no | yes | no | yes | no | floccular | mucopus | no |
| 8 | no | no | yes | no | no | no | no | itching | floccular | cloudy | no |
| 9 | no | no | yes | no | no | no | yes | itching | floccular | mucopus | no |
| 10 | no | no | yes | no | no | no | no | no | floccular | cloudy | no |
| 11 | no | no | yes | yes | yes | no | yes | itching | homogeneous | cloudy | no |
| 12 | no | no | yes | no | no | no | yes | no | floccular | clear | no |
| 13 | no | no | yes | no | no | no | yes | no | floccular | cloudy | no |
| 14 | no | no | yes | no | no | no | no | no | floccular | cloudy | no |
| 15 | no | yes | yes | no | no | no | no | no | floccular | mucopus | no |
| 16 | no | no | yes | no | no | no | no | no | floccular | cloudy | no |
| 17 | no | yes | yes | no | no | no | no | no | floccular | cloudy | no |
| 18 | no | no | yes | no | no | no | no | itching | homogeneous | mucopus | no |
| 19 | no | no | yes | no | yes | no | no | itching | floccular | mucopus | no |
| 20 | no | yes | yes | no | yes | no | yes | no | homogeneous | mucopus | no |
| 21 | no | no | yes | yes | yes | no | yes | itching | homogeneous | mucopus | no |
| 22 | no | yes | yes | no | yes | no | yes | no | homogeneous | mucopus | no |
| 23 | no | no | yes | yes | yes | no | yes | itching | homogeneous | mucopus | yes |
| 24 | no | yes | yes | no | no | no | yes | itching | floccular | cloudy | no |
| 25 | no | no | yes | no | yes | no | no | no | floccular | cloudy | no |
| 26 | no | yes | yes | no | yes | no | yes | itching | floccular | cloudy | no |
| 27 | no | yes | yes | no | yes | no | no | itching | floccular | cloudy | no |
| 28 | no | no | yes | no | no | no | no | no | homogeneous | cloudy | no |
| 29 | no | yes | yes | yes | yes | no | yes | itching | floccular | mucopus | no |
| 30 | no | no | no | no | no | no | yes | no | floccular | mucopus | no |
| 31 | no | yes | yes | yes | yes | no | no | no | homogeneous | cloudy | no |
| 32 | no | no | yes | no | no | no | no | itching | floccular | cloudy | no |
| 33 | no | yes | yes | no | no | no | no | no | floccular | cloudy | no |
| 34 | no | yes | yes | no | no | yes | no | no | floccular | mucopus | no |
| 35 | no | no | yes | no | no | yes | no | no | floccular | mucopus | no |
| 36 | no | no | yes | no | no | no | no | itching | homogeneous | cloudy | yes |
| 37 | no | no | yes | no | no | no | no | no | floccular | cloudy | no |
| 38 | no | no | yes | no | no | no | yes | itching | floccular | mucopus | yes |
| 39 | no | no | yes | no | no | no | no | itching | floccular | cloudy | no |
| 40 | no | no | no | no | yes | no | yes | no | floccular | cloudy | no |
| 41 | no | yes | yes | yes | yes | no | yes | itching | floccular | clear | no |
| 42 | yes, metronid | no | yes | yes | no | yes | no | no | floccular | cloudy | no |
| 43 | no | no | no | no | yes | no | no | no | homogeneous | mucopus | no |
| 44 | yes, metronid | yes | yes | yes | yes | no | yes | no | floccular | cloudy | no |
| 45 | no | no | yes | no | no | no | no | no | floccular | mucopus | no |
| 46 | no | no | yes | yes | yes | no | yes | ulcer | floccular | cloudy | no |
| 47 | no | no | yes | no | no | no | yes | no | floccular | mucopus | no |
| 48 | no | no | yes | yes | no | no | no | no | floccular | mucopus | no |
| 49 | yes, penicillin | no | yes | no | no | no | yes | no | floccular | cloudy | no |
| 50 | yes, sulfa | no | no | no | yes | no | yes | no | floccular | mucopus | no |
| 51 | no | no | no | no | yes | no | no | no | floccular | cloudy | no |
| 52 | yes, sulfa | no | yes | no | no | no | yes | no | floccular | cloudy | no |
| 53 | yes, sulfa | yes | no | no | yes | no | no | no | floccular | cloudy | no |
| 54 | no | no | yes | no | no | no | no | no | homogeneous | mucopus | no |
| 55 | no | no | yes | no | no | yes | no | no | floccular | cloudy | no |
| 56 | no | no | yes | no | yes | no | no | no | floccular | cloudy | no |
| 57 | no | no | yes | no | yes | no | no | no | homogeneous | cloudy | no |
| 58 | no | no | yes | yes | yes | no | no | no | homogeneous | cloudy | no |
| 59 | no | no | yes | no | no | no | no | no | floccular | clear | no |
| 60 | no | no | yes | no | yes | no | yes | no | floccular | mucopus | no |
| 61 | no | no | yes | no | yes | no | yes | no | floccular | mucopus | no |
| 62 | no | no | yes | no | no | no | no | no | floccular | cloudy | no |
| 63 | no | no | yes | no | yes | no | yes | itching | floccular | cloudy | no |
| 64 | no | no | yes | no | yes | yes | no | no | floccular | mucopus | no |
| 65 | yes, tetra, penicil | yes | no | yes | yes | no | no | no | homogeneous | cloudy | no |
| 66 | no | yes | yes | no | no | no | no | no | floccular | cloudy | no |
| 67 | no | no | yes | no | no | no | no | no | floccular | cloudy | no |
| 68 | no | no | no | no | yes | no | yes | no | floccular | cloudy | no |
| 69 | yes, penicillin | no | yes | yes | yes | no | no | no | homogeneous | cloudy | no |
| 70 | no | no | yes | no | yes | no | no | no | floccular | cloudy | no |
| 71 | no | no | yes | no | no | no | yes | no | floccular | cloudy | no |
| 72 | no | no | yes | no | yes | no | yes | no | floccular | mucopus | no |
| 73 | no | no | yes | no | yes | no | no | no | floccular | mucopus | no |
| 74 | no | no | yes | no | no | no | no | no | floccular | cloudy | no |
| 75 | no | no | yes | no | no | no | yes | no | floccular | cloudy | no |
| 76 | no | no | yes | no | no | no | no | no | homogeneous | mucopus | no |
| 77 | no | no | no | no | yes | no | no | no | homogeneous | mucopus | no |
| 78 | no | no | yes | no | no | no | no | itching | homogeneous | mucopus | no |
| 79 | no | no | yes | no | yes | no | no | no | floccular | mucopus | no |
| 80 | no | yes | yes | no | yes | no | yes | no | floccular | clear | no |
| 81 | no | no | no | no | yes | no | no | no | homogeneous | cloudy | no |
| 82 | no | no | no | no | yes | no | yes | itching | floccular | cloudy | no |
| 83 | no | no | yes | no | yes | no | no | itching | homogeneous | cloudy | yes |
| 84 | no | no | yes | no | yes | no | no | no | homogeneous | clear | no |
| 85 | no | no | yes | no | no | no | yes | no | floccular | cloudy | no |
| 86 | no | no | yes | no | no | no | no | no | floccular | cloudy | no |
| 87 | no | no | no | no | yes | no | no | no | floccular | clear | no |
| 88 | no | no | yes | yes | yes | no | yes | no | floccular | mucopus | no |
| 89 | no | no | yes | no | yes | no | no | no | floccular | mucopus | no |
| 90 | yes, metronid | yes | no | yes | no | no | no | no | floccular | mucopus | no |
| 91 | no | no | yes | no | no | no | no | no | floccular | mucopus | no |
| 92 | no | no | yes | yes | no | yes | no | no | floccular | mucopus | no |

| Study_No | Fungus_wet_mount | pH_vag_discharge | foul_smell-or_amine_test-Positive | Clue_cell>20% | lactobacilli_scanty_absent | microscopic_mucopus >10 | microscopic_mucopus >30 | PMN:Epithelial > 1: 1 | other_clinical_diagnosis | *C. trachomatis* PCR | *M. genitalium* PCR |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | no | 3.8 | no | no | yes | yes | yes | no | no | neg | neg |
| 2 | no | 3.8 | no | no | yes | yes | yes | yes | no | neg | neg |
| 3 | no | more than 4.5 | yes | yes | yes | yes | yes | no | BV | neg | pos |
| 4 | yes | 3.8 | no | no | yes | yes | yes | yes | Vaginal candidiasis | neg | neg |
| 5 | no | more than 4.5 | yes | yes | yes | yes | yes | yes | BV | pos | neg |
| 6 | no | 4.2 | no | no | yes | yes | yes | yes | no | neg | neg |
| 7 | no | 4.2 | no | no | no | yes | yes | yes | no | neg | neg |
| 8 | no | 3.8 | no | no | no | yes | yes | no | no | neg | neg |
| 9 | no | 4.2 | no | no | yes | yes | yes | yes | no | neg | neg |
| 10 | no | 4.2 | no | no | yes | yes | yes | no | no | neg | neg |
| 11 | no | more than 4.5 | no | yes | yes | yes | yes | no | BV | pos | pos |
| 12 | no | 3.8 | no | no | no | yes | no | no | no | neg | neg |
| 13 | yes | 3.8 | no | no | yes | yes | yes | no | Vaginal candidiasis | pos | neg |
| 14 | no | 3.8 | no | no | no | yes | yes | yes | no | pos | neg |
| 15 | no | 4.2 | no | no | yes | yes | yes | yes | no | neg | neg |
| 16 | no | 3.8 | no | no | no | yes | yes | no | no | neg | neg |
| 17 | no | 3.8 | no | no | no | yes | yes | no | no | neg | neg |
| 18 | no | more than 4.5 | no | yes | yes | yes | yes | yes | BV | pos | pos |
| 19 | no | 3.8 | no | no | yes | yes | yes | yes | no | neg | neg |
| 20 | no | 4.2 | no | no | yes | yes | yes | yes | no | neg | neg |
| 21 | no | 4.2 | no | no | yes | yes | yes | yes | no | neg | neg |
| 22 | no | more than 4.5 | no | no | no | yes | yes | yes | no | neg | neg |
| 23 | no | more than 4.5 | yes | yes | yes | yes | yes | yes | TV and BV | neg | neg |
| 24 | no | 3.8 | no | no | yes | yes | yes | no | no | neg | neg |
| 25 | no | 3.8 | no | no | yes | yes | yes | yes | no | neg | neg |
| 26 | no | more than 4.5 | no | no | yes | yes | yes | yes | no | neg | neg |
| 27 | yes | 3.8 | no | no | no | yes | yes | no | Vaginal candidiasis | neg | neg |
| 28 | no | 4.2 | no | no | yes | yes | yes | yes | no | neg | neg |
| 29 | no | 3.8 | no | no | yes | yes | yes | no | no | neg | neg |
| 30 | no | 3.8 | no | no | yes | yes | yes | yes | no | neg | neg |
| 31 | no | more than 4.5 | yes | yes | yes | yes | yes | yes | BV | pos | neg |
| 32 | no | 3.8 | no | no | yes | yes | yes | no | no | pos | neg |
| 33 | no | more than 4.5 | no | no | yes | yes | yes | yes | no | pos | neg |
| 34 | no | 3.8 | no | no | no | yes | yes | yes | no | pos | neg |
| 35 | no | more than 4.5 | no | no | yes | yes | yes | yes | no | pos | neg |
| 36 | no | more than 4.5 | yes | yes | yes | yes | yes | yes | TV and BV | pos | neg |
| 37 | no | 3.8 | no | no | no | yes | yes | yes | no | pos | neg |
| 38 | no | 3.8 | no | no | yes | yes | yes | yes | TV | neg | neg |
| 39 | no | 4.2 | no | no | no | yes | yes | no | no | neg | neg |
| 40 | no | 3.8 | no | no | no | yes | yes | yes | no | pos | pos |
| 41 | no | 4.2 | no | no | yes | yes | yes | no | no | neg | neg |
| 42 | no | more than 4.5 | yes | yes | yes | yes | yes | no | BV | pos | neg |
| 43 | no | 3.8 | no | no | yes | yes | yes | yes | no | neg | neg |
| 44 | no | 3.8 | no | no | yes | yes | yes | no | no | neg | neg |
| 45 | no | 3.8 | no | no | yes | yes | yes | no | no | neg | neg |
| 46 | no | more than 4.5 | no | no | yes | yes | yes | yes | no | neg | neg |
| 47 | no | 3.8 | no | no | no | yes | yes | no | no | neg | neg |
| 48 | no | more than 4.5 | no | no | yes | yes | yes | yes | no | pos | neg |
| 49 | no | 3.8 | no | no | yes | yes | yes | yes | no | neg | neg |
| 50 | no | 3.8 | no | no | yes | yes | yes | yes | no | neg | neg |
| 51 | no | 3.8 | no | no | yes | yes | yes | yes | no | neg | neg |
| 52 | no | 3.8 | no | no | yes | yes | yes | yes | no | pos | neg |
| 53 | no | 4.2 | no | no | yes | yes | yes | yes | no | neg | neg |
| 54 | no | more than 4.5 | yes | yes | yes | yes | yes | yes | BV | neg | neg |
| 55 | no | 3.8 | no | no | yes | yes | yes | yes | no | neg | neg |
| 56 | no | more than 4.5 | no | no | yes | yes | yes | yes | no | neg | neg |
| 57 | no | more than 4.5 | yes | yes | yes | yes | yes | yes | BV | pos | neg |
| 58 | yes | more than 4.5 | yes | yes | yes | yes | yes | yes | BV and Vaginal candid | neg | neg |
| 59 | no | less than 3.8 | no | no | yes | yes | yes | yes | no | neg | neg |
| 60 | no | more than 4.5 | no | no | yes | yes | yes | yes | no | neg | neg |
| 61 | no | 4.2 | no | no | no | yes | no | no | no | neg | pos |
| 62 | no | more than 4.5 | yes | yes | yes | yes | yes | yes | BV | neg | neg |
| 63 | no | more than 4.5 | no | no | yes | yes | yes | no | no | pos | neg |
| 64 | no | 3.8 | no | no | yes | yes | yes | no | no | pos | neg |
| 65 | no | more than 4.5 | yes | yes | yes | yes | yes | yes | BV | pos | neg |
| 66 | no | less than 3.8 | no | no | no | yes | yes | no | no | neg | pos |
| 67 | no | 3.8 | no | no | yes | yes | yes | yes | no | neg | neg |
| 68 | no | 3.8 | no | no | yes | yes | no | yes | no | neg | neg |
| 69 | no | more than 4.5 | yes | no | yes | yes | yes | yes | BV | neg | neg |
| 70 | no | less than 3.8 | no | no | no | yes | no | no | no | neg | neg |
| 71 | yes | 4.2 | no | no | yes | yes | yes | yes | Vaginal candidiasis | neg | neg |
| 72 | no | 3.8 | no | no | yes | yes | yes | yes | no | pos | neg |
| 73 | no | more than 4.5 | no | yes | yes | yes | yes | no | BV | neg | neg |
| 74 | no | 3.8 | no | no | yes | yes | yes | no | no | neg | neg |
| 75 | no | more than 4.5 | no | no | yes | yes | yes | yes | no | neg | neg |
| 76 | no | 3.8 | no | no | yes | yes | yes | yes | no | neg | neg |
| 77 | no | more than 4.5 | yes | no | yes | yes | yes | yes | BV | neg | neg |
| 78 | no | more than 4.5 | yes | yes | yes | yes | yes | yes | BV | neg | neg |
| 79 | no | more than 4.5 | no | no | no | yes | yes | yes | no | pos | neg |
| 80 | no | 3.8 | no | no | yes | yes | yes | yes | no | pos | neg |
| 81 | no | more than 4.5 | no | no | yes | yes | no | yes | no | neg | neg |
| 82 | yes | more than 4.5 | no | no | yes | yes | yes | no | Vaginal candidiasis | neg | neg |
| 83 | no | more than 4.5 | yes | yes | yes | yes | yes | yes | BV and TV | neg | neg |
| 84 | no | more than 4.5 | yes | yes | yes | yes | yes | yes | BV | neg | neg |
| 85 | no | more than 4.5 | no | no | yes | yes | yes | yes | no | neg | neg |
| 86 | yes | 4.2 | no | no | yes | yes | yes | yes | Vaginal candidiasis | neg | neg |
| 87 | no | more than 4.5 | no | no | yes | yes | yes | no | no | neg | neg |
| 88 | no | more than 4.5 | no | no | yes | yes | yes | yes | no | pos | neg |
| 89 | no | more than 4.5 | no | no | yes | yes | yes | yes | no | neg | neg |
| 90 | no | more than 4.5 | no | no | yes | yes | yes | yes | no | incomplete | incomplete |
| 91 | no | more than 4.5 | no | no | yes | yes | yes | yes | no | incomplete | incomplete |
| 92 | no | more than 4.5 | yes | yes | yes | yes | yes | yes | BV | neg | neg |

# APPENDIX 3

## *SUMMARIES & ANALYSES OF MACROPHAGE CYTOKINE PRODUCTION*

**TABLE *A3·1*: Macrophage Cytokine Production In Response To Viable Gonococci – *critical probabilities for stimulated versus control cells*[#]**

| | *IL-8* | | | | *IL-6* | | | |
|---|---|---|---|---|---|---|---|---|
| | *INCUBATION PERIOD (hr)* | | | | *INCUBATION PERIOD (hr)* | | | |
| *GONOCOCCAL VARIANT* | 1 | 3 | 9 | 27 | 1 | 3 | 9 | 27 |
| OPA$^-$/PIL$^-$ | NS | $P$ <0.001 | NS | NS | NS | $P$ <0.001 | $P$ <0.01 | $P$ <0.01 |
| OPA$^-$/PIL$^+$ | NS | $P$ <0.05 | NS | NS | NS | $P$ <0.001 | $P$ <0.01 | $P$ <0.01 |
| OPA-A$^+$/PIL$^-$ | NS | NS | NS | NS | NS | $P$ <0.01 | $P$ <0.01 | $P$ <0.001 |
| OPA-B$^+$/PIL$^-$ | NS | $P$ <0.01 | NS | NS | NS | $P$ <0.001 | $P$ <0.01 | $P$ <0.001 |
| OPA-B$^+$/PIL$^+$ | NS | $P$ <0.05 | NS | NS | NS | $P$ <0.001 | $P$ <0.001 | $P$ <0.05 |

| | *RANTES* | | | | *MIP-1α* | | | |
|---|---|---|---|---|---|---|---|---|
| | *INCUBATION PERIOD (hr)* | | | | *INCUBATION PERIOD (hr)* | | | |
| *GONOCOCCAL VARIANT* | 1 | 3 | 9 | 27 | 1 | 3 | 9 | 27 |
| OPA$^-$/PIL$^-$ | NS | $P$ <0.01 | NS | $P$ <0.05 | NS | $P$ <0.001 | NS | $P$ <0.001 |
| OPA$^-$/PIL$^+$ | NS | NS | NS | NS | NS | $P$ <0.001 | NS | $P$ <0.01 |
| OPA-A$^+$/PIL$^-$ | NS | NS | NS | NS | NS | $P$ <0.001 | $P$ <0.001 | $P$ <0.01 |
| OPA-B$^+$/PIL$^-$ | NS | $P$ <0.01 | NS | $P$ <0.05 | NS | $P$ <0.001 | NS | $P$ <0.01 |
| OPA-B$^+$/PIL$^+$ | NS | NS | $P$ <0.05 | NS | NS | $P$ <0.001 | NS | $P$ <0.01 |

| | *TNF-α* | | | |
|---|---|---|---|---|
| | *INCUBATION PERIOD (hr)* | | | |
| *GONOCOCCAL VARIANT* | 1 | 3 | 9 | 27 |
| OPA$^-$/PIL$^-$ | NS | $P$ <0.001 | NS | $P$ <0.05 |
| OPA$^-$/PIL$^+$ | NS | $P$ <0.001 | NS | NS |
| OPA-A$^+$/PIL$^-$ | NS | $P$ <0.01 | $P$ <0.05 | NS |
| OPA-B$^+$/PIL$^-$ | NS | $P$ <0.001 | NS | $P$ <0.05 |
| OPA-B$^+$/PIL$^+$ | NS | $P$ <0.001 | NS | NS |

[#]NS = not significant. Statistics were calculated by one-way ANOVA followed by Tukey's HSD or Tamhane's T2 post-hoc tests.

**TABLE *A3·2*: Comparison Between Various Gonococcal Phenotypes As Stimulators Of Macrophage Cytokine Production After 6 Hours' Incubation – *critical probabilities*[#]**

*IL-8*

| GONOCOCCAL PHENOTYPE | GEOMETRIC MEAN CYTOKINE CONCENTRATION (pg/ml) | GONOCOCCAL PHENOTYPE: NONE (control) | OPA⁻/PIL⁻ | OPA⁻/PIL⁺ | OPA-A⁺/PIL⁻ | OPA-B⁺/PIL⁻ | OPA-B⁺/PIL⁺ |
|---|---|---|---|---|---|---|---|
| NONE (control) | 9698 | N/A | | | | | |
| OPA⁻/PIL⁻ | 10745 | NS | N/A | | | | |
| OPA⁻/PIL⁺ | 10276 | NS | NS | N/A | | | |
| OPA-A⁺/PIL⁻ | 10184 | NS | *P* <0.001 | NS | N/A | | |
| OPA-B⁺/PIL⁻ | 9609 | NS | *P* <0.01 | *P* <0.05 | NS | N/A | |
| OPA-B⁺/PIL⁺ | 9523 | NS | *P* <0.01 | *P* <0.05 | NS | NS | N/A |

*IL-6*

| GONOCOCCAL PHENOTYPE | GEOMETRIC MEAN CYTOKINE CONCENTRATION (pg/ml) | GONOCOCCAL PHENOTYPE: NONE (control) | OPA⁻/PIL⁻ | OPA⁻/PIL⁺ | OPA-A⁺/PIL⁻ | OPA-B⁺/PIL⁻ | OPA-B⁺/PIL⁺ |
|---|---|---|---|---|---|---|---|
| NONE (control) | 572 | N/A | | | | | |
| OPA⁻/PIL⁻ | 2248 | *P* <0.001 | N/A | | | | |
| OPA⁻/PIL⁺ | 2737 | *P* <0.01 | NS | N/A | | | |
| OPA-A⁺/PIL⁻ | 2536 | *P* <0.001 | NS | NS | N/A | | |
| OPA-B⁺/PIL⁻ | 5076 | *P* <0.001 | *P* <0.001 | *P* <0.05 | *P* <0.001 | N/A | |
| OPA-B⁺/PIL⁺ | 4748 | *P* <0.001 | *P* <0.001 | NS | *P* <0.001 | NS | N/A |

*RANTES*

| GONOCOCCAL PHENOTYPE | GEOMETRIC MEAN CYTOKINE CONCENTRATION (pg/ml) | GONOCOCCAL PHENOTYPE: NONE (control) | OPA⁻/PIL⁻ | OPA⁻/PIL⁺ | OPA-A⁺/PIL⁻ | OPA-B⁺/PIL⁻ | OPA-B⁺/PIL⁺ |
|---|---|---|---|---|---|---|---|
| NONE (control) | 585 | N/A | | | | | |
| OPA⁻/PIL⁻ | 1631 | *P* <0.01 | N/A | | | | |
| OPA⁻/PIL⁺ | 1745 | *P* <0.01 | NS | N/A | | | |
| OPA-A⁺/PIL⁻ | 1603 | *P* <0.01 | NS | NS | N/A | | |
| OPA-B⁺/PIL⁻ | 2686 | *P* <0.01 | *P* <0.01 | NS | *P* <0.01 | N/A | |
| OPA-B⁺/PIL⁺ | 2741 | *P* <0.01 | *P* <0.01 | *P* <0.05 | *P* <0.001 | NS | N/A |

*MIP-1-alpha*

| GONOCOCCAL PHENOTYPE | GEOMETRIC MEAN CYTOKINE CONCENTRATION (pg/ml) | GONOCOCCAL PHENOTYPE: NONE (control) | OPA⁻/PIL⁻ | OPA⁻/PIL⁺ | OPA-A⁺/PIL⁻ | OPA-B⁺/PIL⁻ | OPA-B⁺/PIL⁺ |
|---|---|---|---|---|---|---|---|
| NONE (control) | 467 | N/A | | | | | |
| OPA⁻/PIL⁻ | 2188 | *P* <0.001 | N/A | | | | |
| OPA⁻/PIL⁺ | 2109 | *P* <0.001 | NS | N/A | | | |
| OPA-A⁺/PIL⁻ | 2122 | *P* <0.001 | NS | NS | N/A | | |
| OPA-B⁺/PIL⁻ | 2604 | *P* <0.001 | *NS* | NS | NS | N/A | |
| OPA-B⁺/PIL⁺ | 2734 | *P* <0.001 | *P* <0.01 | *P* <0.01 | *P* <0.01 | NS | N/A |

*TNF-alpha*

| GONOCOCCAL PHENOTYPE | GEOMETRIC MEAN CYTOKINE CONCENTRATION (pg/ml) | GONOCOCCAL PHENOTYPE: NONE (control) | OPA⁻/PIL⁻ | OPA⁻/PIL⁺ | OPA-A⁺/PIL⁻ | OPA-B⁺/PIL⁻ | OPA-B⁺/PIL⁺ |
|---|---|---|---|---|---|---|---|
| NONE (control) | 1161 | N/A | | | | | |
| OPA⁻/PIL⁻ | 27910 | *P* <0.001 | N/A | | | | |
| OPA⁻/PIL⁺ | 27837 | *P* <0.001 | NS | N/A | | | |
| OPA-A⁺/PIL⁻ | 28769 | *P* <0.001 | NS | NS | N/A | | |
| OPA-B⁺/PIL⁻ | 41048 | *P* <0.001 | *P* <0.01 | *P* <0.01 | *P* <0.01 | N/A | |
| OPA-B⁺/PIL⁺ | 41543 | *P* <0.001 | *P* <0.01 | *P* <0.01 | *P* <0.001 | NS | N/A |

*GRO-alpha*

| GONOCOCCAL PHENOTYPE | GEOMETRIC MEAN CYTOKINE CONCENTRATION (pg/ml) | GONOCOCCAL PHENOTYPE: NONE (control) | OPA⁻/PIL⁻ | OPA⁻/PIL⁺ | OPA-A⁺/PIL⁻ | OPA-B⁺/PIL⁻ | OPA-B⁺/PIL⁺ |
|---|---|---|---|---|---|---|---|
| NONE (control) | 281 | N/A | | | | | |
| OPA⁻/PIL⁻ | 3302 | *P* <0.001 | N/A | | | | |
| OPA⁻/PIL⁺ | 4404 | *P* <0.001 | NS | N/A | | | |
| OPA-A⁺/PIL⁻ | 3788 | *P* <0.001 | NS | NS | N/A | | |
| OPA-B⁺/PIL⁻ | 18312 | *P* <0.001 | *P* <0.001 | *P* <0.001 | *P* <0.001 | N/A | |
| OPA-B⁺/PIL⁺ | 17532 | *P* <0.001 | *P* <0.001 | *P* <0.001 | *P* <0.001 | NS | N/A |

*IFN-gamma*

| GONOCOCCAL PHENOTYPE | GEOMETRIC MEAN CYTOKINE CONCENTRATION (pg/ml) | GONOCOCCAL PHENOTYPE: NONE (control) | OPA⁻/PIL⁻ | OPA⁻/PIL⁺ | OPA-A⁺/PIL⁻ | OPA-B⁺/PIL⁻ | OPA-B⁺/PIL⁺ |
|---|---|---|---|---|---|---|---|
| NONE (control) | 168 | N/A | | | | | |
| OPA⁻/PIL⁻ | 276 | *P* <0.05 | N/A | | | | |
| OPA⁻/PIL⁺ | 262 | *P* <0.05 | NS | N/A | | | |
| OPA-A⁺/PIL⁻ | 242 | NS | NS | NS | N/A | | |
| OPA-B⁺/PIL⁻ | 499 | *P* <0.001 | *P* <0.01 | *P* <0.01 | *P* <0.001 | N/A | |
| OPA-B⁺/PIL⁺ | 473 | *P* <0.001 | *P* <0.01 | *P* <0.01 | *P* <0.001 | NS | N/A |

[#]N/A = not applicable, NS = not significant. Statistics were calculated by one-way ANOVA followed by Tukey's HSD or Tamhane's T2 post-hoc tests.

**TABLE *A3·3*: Macrophage Cytokine Production In Response To Gonococcal Components – *critical probabilities for stimulated versus control cells*[#]**

| | *IL-8* | | | | *IL-6* | | | |
|---|---|---|---|---|---|---|---|---|
| | *INCUBATION PERIOD (hr)* | | | | *INCUBATION PERIOD (hr)* | | | |
| *GONOCOCCAL COMPONENT* | 1 | 3 | 9 | 27 | 1 | 3 | 9 | 27 |
| PILI | NS | NS | NS | NS | NS | NS | $P < 0.001$ | NS |
| LOS | NS | NS | NS | NS | NS | NS | $P < 0.05$ | $P < 0.01$ |
| OPA⁻ OM | NS | NS | NS | NS | NS | NS | $P < 0.001$ | $P < 0.001$ |
| OPA-A⁺ OM | NS | NS | NS | NS | NS | NS | $P < 0.001$ | $P < 0.001$ |
| OPA-B⁺ OM | NS | NS | NS | NS | NS | $P < 0.001$ | $P < 0.001$ | $P < 0.001$ |
| | *RANTES* | | | | *MIP-1α* | | | |
| | *INCUBATION PERIOD (hr)* | | | | *INCUBATION PERIOD (hr)* | | | |
| *GONOCOCCAL COMPONENT* | 1 | 3 | 9 | 27 | 1 | 3 | 9 | 27 |
| PILI | NS | NS | NS | NS | NS | NS | NS | NS |
| LOS | NS | NS | NS | NS | NS | NS | NS | NS |
| OPA⁻ OM | NS | NS | $P < 0.001$ | $P < 0.01$ | NS | NS | $P < 0.001$ | NS |
| OPA-A⁺ OM | NS | NS | NS | $P < 0.05$ | NS | NS | $P < 0.01$ | $P < 0.001$ |
| OPA-B⁺ OM | NS | NS | NS | $P < 0.05$ | NS | $P < 0.01$ | $P < 0.05$ | NS |
| | *TNF-α* | | | | | | | |
| | *INCUBATION PERIOD (hr)* | | | | | | | |
| *GONOCOCCAL COMPONENT* | 1 | 3 | 9 | 27 | | | | |
| PILI | NS | NS | NS | NS | | | | |
| LOS | NS | $P < 0.05$ | NS | NS | | | | |
| OPA⁻ OM | NS | $P < 0.05$ | NS | NS | | | | |
| OPA-A⁺ OM | NS | $P < 0.05$ | NS | $P < 0.001$ | | | | |
| OPA-B⁺ OM | NS | $P < 0.001$ | $P < 0.05$ | NS | | | | |

[#]NS = not significant. Statistics were calculated by one-way ANOVA followed by Tukey's HSD or Tamhane's T2 post-hoc tests.

**TABLE *A3·4*: Comparison Between Various Gonococcal Surface Components As Stimulators Of Macrophage Cytokine Production After 18 Hours' Incubation – *critical probabilities*[#]**

*IL-8*

| *GONOCOCCAL COMPONENT* | *GEOMETRIC MEAN CYTOKINE CONCENTRATION (pg/ml)* | *GONOCOCCAL COMPONENT* | | | | | |
|---|---|---|---|---|---|---|---|
| | | NONE (control) | PILI | LOS | OPA⁻ OM | OPA-A⁺ OM | OPA-B⁺ OM |
| NONE (control) | 11937 | N/A | | | | | |
| PILI | 15381 | NS | N/A | | | | |
| LOS | 16311 | NS | NS | N/A | | | |
| OPA⁻ OM | 17229 | NS | NS | NS | N/A | | |
| OPA-A⁺ OM | 17227 | NS | NS | NS | NS | N/A | |
| OPA-B⁺ OM | 15446 | NS | NS | NS | NS | NS | N/A |

*IL-6*

| *GONOCOCCAL COMPONENT* | *GEOMETRIC MEAN CYTOKINE CONCENTRATION (pg/ml)* | *GONOCOCCAL COMPONENT* | | | | | |
|---|---|---|---|---|---|---|---|
| | | NONE (control) | PILI | LOS | OPA⁻ OM | OPA-A⁺ OM | OPA-B⁺ OM |
| NONE (control) | 100 | N/A | | | | | |
| PILI | 2175 | *P* <0.01 | N/A | | | | |
| LOS | 5064 | *P* <0.01 | *P* <0.01 | N/A | | | |
| OPA⁻ OM | 8437 | *P* <0.01 | *P* <0.001 | NS | N/A | | |
| OPA-A⁺ OM | 5870 | *P* <0.01 | *P* <0.05 | NS | NS | N/A | |
| OPA-B⁺ OM | 11065 | *P* <0.01 | *P* <0.001 | *P* <0.01 | NS | NS | N/A |

*RANTES*

| *GONOCOCCAL COMPONENT* | *GEOMETRIC MEAN CYTOKINE CONCENTRATION (pg/ml)* | *GONOCOCCAL COMPONENT* | | | | | |
|---|---|---|---|---|---|---|---|
| | | NONE (control) | PILI | LOS | OPA⁻ OM | OPA-A⁺ OM | OPA-B⁺ OM |
| NONE (control) | 110 | N/A | | | | | |
| PILI | 350 | *P* <0.001 | N/A | | | | |
| LOS | 2597 | *P* <0.001 | *P* <0.001 | N/A | | | |
| OPA⁻ OM | 5042 | *P* <0.001 | *P* <0.001 | *P* <0.05 | N/A | | |
| OPA-A⁺ OM | 2580 | *P* <0.001 | *P* <0.001 | NS | *P* <0.05 | N/A | |
| OPA-B⁺ OM | 8282 | *P* <0.001 | *P* <0.001 | *P* <0.001 | NS | *P* <0.001 | N/A |

*MIP-1-alpha*

| *GONOCOCCAL COMPONENT* | *GEOMETRIC MEAN CYTOKINE CONCENTRATION (pg/ml)* | *GONOCOCCAL COMPONENT* | | | | | |
|---|---|---|---|---|---|---|---|
| | | NONE (control) | PILI | LOS | OPA⁻ OM | OPA-A⁺ OM | OPA-B⁺ OM |
| NONE (control) | 923 | N/A | | | | | |
| PILI | 13192 | *P* <0.001 | N/A | | | | |
| LOS | 18601 | *P* <0.001 | *P* <0.05 | N/A | | | |
| OPA⁻ OM | 21936 | *P* <0.001 | *P* <0.01 | NS | N/A | | |
| OPA-A⁺ OM | 19188 | *P* <0.001 | *P* <0.05 | NS | NS | N/A | |
| OPA-B⁺ OM | 21898 | *P* <0.001 | *P* <0.05 | NS | NS | NS | N/A |

*TNF-alpha*

| *GONOCOCCAL COMPONENT* | *GEOMETRIC MEAN CYTOKINE CONCENTRATION (pg/ml)* | *GONOCOCCAL COMPONENT* | | | | | |
|---|---|---|---|---|---|---|---|
| | | NONE (control) | PILI | LOS | OPA⁻ OM | OPA-A⁺ OM | OPA-B⁺ OM |
| NONE (control) | 90 | N/A | | | | | |
| PILI | 810 | *P* <0.001 | N/A | | | | |
| LOS | 2781 | *P* <0.001 | *P* <0.001 | N/A | | | |
| OPA⁻ OM | 6416 | *P* <0.001 | *P* <0.001 | *P* <0.01 | N/A | | |
| OPA-A⁺ OM | 3778 | *P* <0.001 | *P* <0.001 | NS | NS | N/A | |
| OPA-B⁺ OM | 8702 | *P* <0.001 | *P* <0.001 | *P* <0.001 | NS | *P* <0.01 | N/A |

*GRO-alpha*

| *GONOCOCCAL COMPONENT* | *GEOMETRIC MEAN CYTOKINE CONCENTRATION (pg/ml)* | *GONOCOCCAL COMPONENT* | | | | | |
|---|---|---|---|---|---|---|---|
| | | NONE (control) | PILI | LOS | OPA⁻ OM | OPA-A⁺ OM | OPA-B⁺ OM |
| NONE (control) | 440 | N/A | | | | | |
| PILI | 3393 | *P* <0.001 | N/A | | | | |
| LOS | 2834 | *P* <0.001 | NS | N/A | | | |
| OPA⁻ OM | 3560 | *P* <0.001 | NS | NS | N/A | | |
| OPA-A⁺ OM | 2344 | *P* <0.001 | NS | NS | NS | N/A | |
| OPA-B⁺ OM | 4965 | *P* <0.001 | NS | NS | NS | *P* <0.01 | N/A |

[#]N/A = not applicable, NS = not significant. Statistics were calculated by one-way ANOVA followed by Tukey's HSD or Tamhane's T2 post-hoc tests.

**TABLE *A3·5*: The Effects Of Autologous Human Serum On Macrophage Responses To Gonococcal Surface Components – *cytokine production over an 81-hour time course.***

| | *STIMULUS (μg/ml)* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | CONTROL | CONTROL + HS* | PILI (100) | PILI (100) + HS* | LOS (10) | LOS (10) + HS* | OPA⁻ OM (10) | OPA⁻ OM (10) + HS* |
| *TIME (hr)* | *IL-6 (geometric mean pg/ml x/÷ SE˜)* | | | | | | | |
| 3 | 0.35x/÷ 1.17 | 0.70x/÷ 1.17 | 3.13x/÷ 1.33 | 2.01x/÷ 1.20 | 0.10x/÷ 1.05 | 12.30x/÷ 1.63 | 5.57x/÷ 1.75 | 7.76x/÷ 1.15 |
| 9 | 3.38x/÷ 1.95 | 0.30x/÷ 1.27 | 24.90x/÷ 1.56 | 19.22x/÷ 2.06 | 29.88x/÷ 1.70 | 9.87x/÷ 1.57 | 41.78x/÷ 1.05 | 128.02x/÷ 1.52 |
| 27 | 1.74x/÷ 1.28 | 1.45x/÷ 1.05 | 21.22x/÷ 2.98 | 65.65x/÷ 2.05 | 66.85x/÷ 1.73 | 23.30x/÷ 1.31 | 396.42x/÷ 1.43 | 130.10x/÷ 3.43 |
| 81 | 7.19x/÷ 1.74 | 1.13x/÷ 1.47 | 2.99x/÷ 1.75 | 35.13x/÷ 2.87 | 17.54x/÷ 1.35 | 5.58x/÷ 1.32 | 229.04x/÷ 1.39 | 78.10x/÷ 2.70 |
| | *TNF-α (geometric mean pg/ml x/÷ SE˜)* | | | | | | | |
| 3 | 55.99x/÷ 1.81 | 3.52x/÷ 1.52 | 194.48x/÷ 1.43 | 30.02x/÷ 1.07 | 40.65x/÷ 1.39 | 64.82x/÷ 1.74 | 97.14x/÷ 1.40 | 108.10x/÷ 1.24 |
| 9 | 29.30x/÷ 1.20 | 5.04x/÷ 1.19 | 155.67x/÷ 2.23 | 43.73x/÷ 1.90 | 113.13x/÷ 1.14 | 21.11x/÷ 1.39 | 181.95x/÷ 1.06 | 367.39x/÷ 1.60 |
| 27 | 130.96x/÷ 1.59 | 176.17x/÷ 3.58 | 355.02x/÷ 1.95 | 347.05x/÷ 1.55 | 491.04x/÷ 1.16 | 193.11x/÷ 1.26 | 1481.91x/÷ 1.77 | 734.59x/÷ 2.29 |
| 81 | 88.49x/÷ 1.35 | 64.18x/÷ 1.07 | 79.14x/÷ 1.18 | 112.64x/÷ 1.55 | 106.91x/÷ 1.23 | 60.85x/÷ 1.09 | 461.90x/÷ 1.18 | 201.20x/÷ 1.73 |
| | *MIP-1α (geometric mean pg/ml x/÷ SE˜)* | | | | | | | |
| 3 | 232.11x/÷ 1.74 | 37.31x/÷ 1.07 | 384.99x/÷ 1.59 | 120.12x/÷ 1.11 | 124.79x/÷ 1.44 | 507.83x/÷ 1.52 | 495.37x/÷ 2.09 | 590.25x/÷ 1.38 |
| 9 | 154.91x/÷ 1.19 | 13.68x/÷ 1.39 | 779.17x/÷ 1.86 | 972.65x/÷ 2.16 | 703.93x/÷ 1.35 | 528.52x/÷ 1.76 | 1209.49x/÷ 1.12 | 4713.36x/÷ 1.27 |
| 27 | 600.48x/÷ 1.29 | 464.60x/÷ 1.08 | 1628.85x/÷ 1.85 | 5767.12x/÷ 1.29 | 2276.47x/÷ 1.23 | 2799.05x/÷ 1.31 | 11066.32x/÷ 1.10 | 6555.58x/÷ 1.75 |
| 81 | 412.94x/÷ 1.04 | 525.57x/÷ 1.14 | 530.82x/÷ 1.15 | 3260.84x/÷ 1.86 | 817.61x/÷ 1.19 | 940.43x/÷ 1.30 | 6192.62x/÷ 1.25 | 5939.80x/÷ 2.02 |

*Human serum at 20%. ˜Standard error of the mean.

## TABLE *A3·6*: The Effects Of Sera From Six Different Donors On Macrophage Responses To Gonococcal Components – *calculation of net change in induced cytokine levels.*

| IL-6 STIMULUS | SERUM CODE | N | Geometric Mean | Serum only controls | Stimulus only | Corrected means | Grand mean Standard Error |
|---|---|---|---|---|---|---|---|
| Control | Normal serum (autologous) | 3 | 10.2148 | | | | |
| | Normal serum (allogeneic) | 3 | 11.365 | | | | |
| | SAI-1 | 3 | 16.2409 | | | | |
| | SAI-2 | 3 | 2.3676 | | | | |
| | SAI-3 | 3 | 3.0976 | | | | |
| | SAI-4 | 3 | 4.7445 | | | | |
| | Total | 18 | 6.3507 | | | | |
| LOS | No human serum | 3 | 12.5459 | | | | |
| | Normal serum (autologous) | 3 | 41.4116 | 10.2146 | 12.5459 | 18.661 | 8.6402 |
| | Normal serum (allogeneic) | 3 | 55.9421 | 11.365 | 12.5459 | 32.0312 | 8.482937938 |
| | SAI-1 | 3 | 15.3206 | 16.2409 | 12.5459 | -13.4662 | |
| | SAI-2 | 3 | 27.0487 | 2.3676 | 12.5459 | 12.1362 | |
| | SAI-3 | 3 | 15.6209 | 3.0976 | 12.5459 | -0.0226 | |
| | SAI-4 | 3 | 19.803 | 4.7445 | 12.5459 | 2.5126 | |
| | Total | 21 | 23.2979 | | | | |
| PIII | No human serum | 3 | 14.5489 | | | | |
| | Normal serum (autologous) | 3 | 72.3654 | 10.2146 | 14.5489 | 47.6219 | 21.00363333 |
| | Normal serum (allogeneic) | 3 | 39.0754 | 11.365 | 14.5489 | 13.1615 | 10.2619541 |
| | SAI-1 | 3 | 13.4947 | 16.2409 | 14.5489 | -17.2951 | |
| | SAI-2 | 3 | 58.7685 | 2.3676 | 14.5489 | 41.852 | |
| | SAI-3 | 3 | 22.3845 | 3.0976 | 14.5489 | 4.738 | |
| | SAI-4 | 3 | 55.2363 | 4.7445 | 14.5489 | 35.9429 | |
| | Total | 21 | 32.7434 | | | | |
| Opa- OM | No human serum | 3 | 36.7581 | | | | |
| | Normal serum (autologous) | 3 | 1647.9477 | 10.2146 | 36.7581 | 1600.975 | 624.3184033 |
| | Normal serum (allogeneic) | 3 | 733.5239 | 11.365 | 36.7581 | 685.4008 | 223.7315198 |
| | SAI-1 | 3 | 141.9894 | 16.2409 | 36.7581 | 88.9904 | |
| | SAI-2 | 3 | 1065.515 | 2.3676 | 36.7581 | 1026.3093 | |
| | SAI-3 | 3 | 1194.152 | 3.0976 | 36.7581 | 1154.2963 | |
| | SAI-4 | 3 | 431.3617 | 4.7445 | 36.7581 | 389.8591 | |
| | Total | 21 | 445.1329 | | | | |

| MIP-1-alpha STIMULUS | SERUM CODE | N | Geometric Mean | Serum only controls | Stimulus only | Corrected means | Grand mean Standard Error |
|---|---|---|---|---|---|---|---|
| Control | Normal serum (autologous) | 3 | 214.7406 | | | | |
| | Normal serum (allogeneic) | 3 | 218.0154 | | | | |
| | SAI-1 | 3 | 365.0937 | | | | |
| | SAI-2 | 3 | 114.302 | | | | |
| | SAI-3 | 3 | 124.8747 | | | | |
| | SAI-4 | 3 | 118.7672 | | | | |
| | Total | 18 | 175.2555 | | | | |
| LOS | No human serum | 3 | 233.8418 | | | | |
| | Normal serum (autologous) | 3 | 1021.3057 | 214.7406 | 233.8418 | 572.7233 | 192.0833 |
| | Normal serum (allogeneic) | 3 | 1036.4114 | 218.0154 | 233.8418 | 584.5542 | 135.1207569 |
| | SAI-1 | 3 | 375.4524 | 365.0937 | 233.8418 | -223.4831 | |
| | SAI-2 | 3 | 474.6508 | 114.302 | 233.8418 | 126.507 | |
| | SAI-3 | 3 | 283.5059 | 124.8747 | 233.8418 | -75.2106 | |
| | SAI-4 | 3 | 525.418 | 118.7672 | 233.8418 | 172.809 | |
| | Total | 21 | 487.7862 | | | | |
| PIII | No human serum | 3 | 375.5714 | | | | |
| | Normal serum (autologous) | 3 | 3171.4712 | 214.7406 | 375.5714 | 2681.1592 | 1359.529217 |
| | Normal serum (allogeneic) | 3 | 1670.17 | 218.0154 | 375.5714 | 1076.5632 | 488.2569767 |
| | SAI-1 | 3 | 631.0827 | 365.0937 | 375.5714 | -109.5824 | |
| | SAI-2 | 3 | 2042.1943 | 114.302 | 375.5714 | 1552.3209 | |
| | SAI-3 | 3 | 739.0353 | 124.8747 | 375.5714 | 238.5892 | |
| | SAI-4 | 3 | 3312.4436 | 118.7672 | 375.5714 | 2818.1052 | |
| | Total | 21 | 1300.046 | | | | |
| Opa- OM | No human serum | 3 | 1125.4332 | | | | |
| | Normal serum (autologous) | 3 | 9932.2318 | 214.7406 | 1125.4332 | 8592.058 | 6871.801517 |
| | Normal serum (allogeneic) | 3 | 7880.2664 | 218.0154 | 1125.4332 | 6536.8178 | 822.6481153 |
| | SAI-1 | 3 | 4958.4204 | 365.0937 | 1125.4332 | 3367.8936 | |
| | SAI-2 | 3 | 9702.5073 | 114.302 | 1125.4332 | 8462.7721 | |
| | SAI-3 | 3 | 9648.8342 | 124.8747 | 1125.4332 | 8396.5263 | |
| | SAI-4 | 3 | 7719.9419 | 118.7672 | 1125.4332 | 6474.7414 | |
| | Total | 21 | 6085.8108 | | | | |

| TNF-alpha STIMULUS | SERUM CODE | N | Geometric Mean | Serum only controls | Stimulus only | Corrected means | Grand mean Standard Error |
|---|---|---|---|---|---|---|---|
| Control | Normal serum (autologous) | 3 | 50.0258 | | | | |
| | Normal serum (allogeneic) | 3 | 49.34 | | | | |
| | SAI-1 | 3 | 110.0323 | | | | |
| | SAI-2 | 3 | 102.4545 | | | | |
| | SAI-3 | 3 | 99.5643 | | | | |
| | SAI-4 | 3 | 99.3604 | | | | |
| | Total | 18 | 80.6542 | | | | |
| LOS | No human serum | 3 | 53.1424 | | | | |
| | Normal serum (autologous) | 3 | 100.0699 | 50.0258 | 53.1424 | -3.0783 | -61.06666333 |
| | Normal serum (allogeneic) | 3 | 112.2422 | 49.34 | 53.1424 | 9.7598 | 20.63775507 |
| | SAI-1 | 3 | 62.9739 | 110.0323 | 53.1424 | -100.2008 | |
| | SAI-2 | 3 | 66.3799 | 102.4545 | 53.1424 | -89.217 | |
| | SAI-3 | 3 | 55.4048 | 99.5643 | 53.1424 | -97.3019 | |
| | SAI-4 | 3 | 68.1769 | 99.3604 | 53.1424 | -86.3259 | |
| | Total | 21 | 71.0615 | | | | |
| PIII | No human serum | 3 | 75.7966 | | | | |
| | Normal serum (autologous) | 3 | 144.6231 | 50.0258 | 75.7966 | 18.8007 | -62.39651667 |
| | Normal serum (allogeneic) | 3 | 96.703 | 49.34 | 75.7966 | -28.4336 | 21.804811 |
| | SAI-1 | 3 | 62.8819 | 110.0323 | 75.7966 | -122.947 | |
| | SAI-2 | 3 | 94.2336 | 102.4545 | 75.7966 | -84.0175 | |
| | SAI-3 | 3 | 66.1462 | 99.5643 | 75.7966 | -109.2147 | |
| | SAI-4 | 3 | 126.59 | 99.3604 | 75.7966 | -48.567 | |
| | Total | 21 | 91.23 | | | | |
| Opa- OM | No human serum | 3 | 270.344 | | | | |
| | Normal serum (autologous) | 3 | 1817.0627 | 50.0258 | 270.344 | 1496.6929 | 665.5326333 |
| | Normal serum (allogeneic) | 3 | 951.3435 | 49.34 | 270.344 | 631.6595 | 218.2019013 |
| | SAI-1 | 3 | 351.5397 | 110.0323 | 270.344 | -28.8366 | |
| | SAI-2 | 3 | 1078.4255 | 102.4545 | 270.344 | 705.627 | |
| | SAI-3 | 3 | 1312.0762 | 99.5643 | 270.344 | 942.1679 | |
| | SAI-4 | 3 | 615.5895 | 99.3604 | 270.344 | 245.8851 | |
| | Total | 21 | 757.4901 | | | | |

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