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ANTIBODY MEDIATED MUCOSAL DEFENCES IN THE FEMALE GENITAL TRACT

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This thesis is dedicated to the memory of Jack

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ABSTRACT

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Infections that breach the mucosal defences of the female genital tract pose a major threat to the reproductive health of young women, are associated with significant mortality, and are a major drain on limited public health resources. Progress into the primary prevention of these infections through vaccine development is now gaining momentum and there is an evolving literature reporting vaccination strategies for antibody driven protection of the human female genital tract.

The objective of this study was to investigate antibody mediated protection of the mucosal surfaces of both the upper and lower human female genital tracts. To this end model vaccines were given to volunteer women parenterally, orally, and onto the endocervical mucosa. Mucosal washings were then analysed for vaccine specific antibody responses.

Four weeks prior to total abdominal hysterectomy for dysfunctional uterine bleeding 12 women were vaccinated by intramuscular injection, and 12 women by endocervical vaccine administration with 1 ml. of formaldehyde inactivated Hepatitis A virus adsorbed onto aluminium hydroxide. 17 of the women also received a live attenuated *Salmonella typhi* (Ty 21a) vaccine orally.

Total IgG1, IgG2, IgG3, IgG4 and IgA1 were assayed using commercial radial immunodiffusion kits. Immunoassays for IgA2, secretory IgA and vaccine-specific IgG and IgA antibodies were developed using time resolved fluorometry.

Significant Hepatitis A specific IgA and IgG responses were detected in serum four weeks after vaccination both locally and parenterally. High levels of specific IgG and IgA were found throughout the genital tract but particularly in the vagina, cervix and endometrium. The highest concentrations of specific IgG and IgA were detected in the vagina and cervix following cervical vaccination and these responses were significantly greater than those generated by parenteral vaccination. Similar levels of specific IgG and IgA were found throughout the upper and lower genital tracts, suggesting that IgG is at least as important as IgA in providing humoral immunity at the mucosal surface. Immunocytochemical studies confirmed that immune cell populations capable of both antigen uptake and processing, and local antibody production, were present in the lower genital tract following cervical vaccination.

Oral vaccination is clearly a highly acceptable route for vaccine administration. However, low levels of specific IgG and IgA to *Salmonella typhi* LPS 09 antigen were detected throughout the genital tract in those women who had seroconverted following oral immunisation.

In contrast to the dominance of secretory IgA in secretions in other mucosal sites we have identified IgG as the dominant isotype throughout the female genital tract. Higher levels of total IgG than IgA were detected in both the upper and lower genital tracts. Approximately 50% of antibody detected in the vagina was IgG1 or IgG2, and in the cervix and fallopian tube this figure approached 70%. Secretory IgA was consistently detected in all mucosal washings but in low concentration.

These studies imply that maximum antibody driven responses to sexually transmitted diseases would ideally involve local mucosal vaccination but there are practical difficulties and doubts over the acceptability of cervical vaccination in the early teenage years, before the onset of sexual activity. Importantly however, the specific antibody distribution following parenteral vaccination suggests that significant concentrations of serum antibodies are translocated to the mucosal surfaces of the genital tract and that parenteral vaccination could be as effective as cervical vaccination at least in the protection of the upper genital tract.

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LIST OF ABBREVIATIONS

AIDS	Acquired immuno-deficiency syndrome
APC	Antigen presenting cell
BAM	Biotinylated anti-mouse
BAR	Biotinylated anti-rabbit
BSA	Bovine serum albumin
CD	Cluster differential
CIN	Cervical intra-epithelial neoplasia
CTB	Cholera toxin B subunit
DAB	Diaminobenzidine
EB	Elementary body
HPV	Human papilloma virus
HSP	Heat shock protein
HIV	Human immunodeficiency virus
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin
IEL	Intra-epithelial lymphocyte
LC	Langerhans cell
M cells	Microfold cells
MAdCAM	Mucosal addressin cell adhesion molecule
pIg	Polymeric immunoglobulin
PID	Pelvic inflammatory disease
RID	Radial immunodiffusion
RB	Reticulate body
RO	Reverse osmosis
SC	Secretory component
SIg	Secretory immunoglobulin
STD	Sexually transmitted disease
SIV	Simian immunodeficiency virus
TBS	Tris buffered saline
VAP	Vascular adhesion protein
VCAM	Vascular cell adhesion molecule

1.INTRODUCTION

1.1 INTRODUCTION

The mucosal surfaces of the human present a vast surface area to potential pathogens and indeed are frequently the portal of entry for such infectious microorganisms. It has been established over the last two decades that the mucosal immune system is a separate component of the host's immune apparatus and represents a separate entity from the systemic immune system.

Mucosal immunity in the female reproductive tract has evolved to maintain an environment suitable for effective reproduction. This immune system mounts an effective response against potentially pathogenic micro-organisms but appropriately adapts to the antigenic stimuli of fertilisation, implantation, pregnancy and parturition. The mucosal immunology of the female reproductive tract is thus unique and differs in several important areas from that in sites such as the buccal mucosa, respiratory and gastrointestinal tracts.

Progress into the primary prevention of pelvic inflammatory disease, human papilloma virus (HPV) and human immunodeficiency virus (HIV) infection through vaccine development is now gaining momentum. However such vaccines will need to be effective at the mucosal surfaces of the female genital tract necessitating a better understanding of mucosal immunology at this site.

These infections not only pose a major threat to the reproductive health of young women but are associated with a significant mortality and are a major drain on limited public health resources.

This introduction firstly presents a discussion of the aetiology, clinical consequences and current treatments of those infections of the human female reproductive tract that are associated with more serious morbidity and mortality. The focus is on pelvic inflammatory disease because that was the main remit of the Medical Research

Council programme grant within which my research fellowship was taken up. There then follows a detailed discussion of the mucosal immunology of the female reproductive tract culminating in an examination of the optimal vaccination routes to achieve an effective immune response at that site.

1.2 PELVIC INFLAMMATORY DISEASE

Pelvic inflammatory disease (PID) is an acute clinical syndrome associated with ascending spread of micro-organisms from the vagina or cervix to the endometrium, fallopian tubes, and/or contiguous structures (Westrom & Wolner-Hanssen 1993).

1.2.1 EPIDEMIOLOGY

The public health significance of PID is indisputable, with up to one in nine American women of reproductive age reporting that they have received treatment for pelvic infection (Aral et al 1991). An estimated one million women seek treatment for PID in the United States at an annual cost of $4.2 billion (Washington & Katz 1991). The situation in the non-industrialised world is undoubtedly worse, although a lack of accurate epidemiological data makes it hard to quantify. Certainly in sub-Saharan Africa bilateral fallopian tube occlusion secondary to salpingitis is the predominant cause of infertility.

The incidence of PID is strongly correlated with the prevalence of sexually transmitted diseases (STDs), and has increased in most countries, reflecting the worldwide epidemic of STDs.

This is predominantly a disease of young sexually active women, with at least 75% of cases occurring in women under the age of 25. Of the estimated 10-15% of women of reproductive age in the United States who have had at least one episode of PID, the highest annual incidence is among sexually active women in their teenage years. Of those hospitalised with acute PID in 1988, it was notable that 43% were younger than 25 years of age and 34% underwent major surgery as a direct result of the infection (National Institutes of Health 1991).

Although there has been a downward trend in the cumulative incidence of self reported PID in the United States in recent years an increase in the proportion of asymptomatic PID might account for it (Aral et al 1991).

Chlamydia trachomatis and *Neisseria gonorrhoeae* are probably the most prevalent sexually transmitted bacteria in the western world, and one or both of these micro-organisms have been implicated in at least 60% of cases of PID in young women. In the United Kingdom, uncomplicated gonococcal infections in women have halved in frequency during the last decade, while at the same time the rate of chlamydial infections has continued to rise (Catchpole 1992), such that *C. trachomatis* is probably now the major aetiological pathogen in PID.

The incidence of PID in the developing world is more difficult to assess, available data often being based on point prevalence studies of the different STDs. Gonococcal infection rates of up to 15% have been reported in pregnant women in many African countries, and as high as 20% in Zaire and Kenya (Miotti et al 1992). Surveys of Ugandan women have found the prevalence of PID to range from 6% to up to 19% (Arya et al 1980).

The evidence for a role for ulcerating STDs, especially chancroid, as cofactors in HIV transmission, is strong (Piot & Laga 1989). Studies involving non-ulcerative STDs are less conclusive, but if found to be associated with an increased risk of HIV transmission the overall risk in the developing world would be large, since they are highly prevalent.

1.2.2 MICROBIOLOGY AND PATHOGENESIS

C. trachomatis and *N. gonorrhoeae* are described as primary pathogens in PID, as it is believed that they damage the protective mechanisms within the endocervix and the tubal mucosa by causing an acute inflammatory response. This subsequently allows endogenous bacteria from the vagina and cervix to ascend into the upper genital tract, where they act as secondary invaders. Identification of microorganisms from fallopian tube samples by culture appears to be directly related to the severity of disease. STDs are usually only isolated in women who present early and have relatively mild disease, whereas the isolation of endogenous microorganisms occurs more frequently in women with advanced disease.

The presence of a mixed polymicrobial infection leads to tissue destruction, which favours the formation of tubo-ovarian abscesses where strictly anaerobic microorganisms predominate and STDs are rarely isolated.

Neisseria gonorrhoeae

Gonococci selectively adhere to and invade non-ciliated secretory cells in the mucosa of the fallopian tube, where they are protected from the host's immune defence mechanisms. After crossing the epithelium, gonococci are released from the basal surface by exocytosis and then invade the sub-epithelial space. Whereas only the secretory cells are infected, it is the ciliated cells of the tubal mucosa that are damaged in gonococcal PID. Either they slough off, or ciliary stasis occurs, probably as a result of gonococcal toxin production. Gonococcal antigen also activates the complement cascade, producing an acute polymorphonuclear response that in turn switches on the 'common inflammatory pathways'. Cell death and tissue destruction ensue, providing the ideal situation for invasion by endogenous anaerobic microorganisms.

Chlamydia trachomatis

Chlamydiae are small bacteria that are obligate intracellular parasites.

C. trachomatis adheres to the columnar epithelial cells of the endocervix and the epithelial surfaces of the endometrium and fallopian tubes. Endocytosis results in the metabolically inert but infectious elementary body (EB) being taken into the cytoplasm of the cell. Within the endosomal vacuole, which is apparently protected from immune surveillance, the EB changes into the larger vegetative reticulate body (RB) over a period of 24 hours. The RB multiplies by binary fission, producing intracellular inclusions that contain thousands of RBs. The RBs subsequently condense again within the inclusion into EBs and, in 48-72 hours, cell lysis occurs, releasing the infectious forms into the extracellular fluid, where they can then attach to new cells and repeat the cycle.

It is unlikely that the upper genital tract damage seen in chlamydial PID is caused by the local effects of chlamydial replication alone. More probably the host's humoral and cell-mediated immune response to *C. trachomatis* determines the extent of tubal damage. Chlamydiae appear to be capable of influencing the immune response in ways that are beneficial to their survival, such that persistent - i.e. viable but non-replicating - forms remain in the cells of the upper genital tract, serving as sub-clinical pockets of antigenic stimulation. Primary chlamydial infection in monkeys is self-limiting, but repeat inoculation produces extensive tubal scarring (Patton et al 1989). This may well be a delayed hypersensitivity-type response, either to the presence of persistent chlamydial antigen in the genital tract or perhaps provoked by ubiquitous heat shock proteins (HSP). Similar mechanisms may be responsible for much of the morphological damage seen in women following chlamydial PID, as there does appear to be a strong correlation between the finding of antibodies to HSP and PID, tubal factor infertility and ectopic pregnancy (Paavonen & Lehtinen 1994).

The relative contribution of humoral versus cell-mediated immunity for host resistance to chlamydiae is controversial, and a fuller understanding of the factors governing protective immunity in the genital tract would aid vaccine development. Johansson et al (1997) showed that B-cell-deficient mice develop complete immune protection against genital tract infection with *Chlamydia trachomatis*, confirming that cell-mediated immunity, in particular that owing to $CD4^+$ T helper 1 (Th1)-type cells, is critical for host resistance against *C trachomatis* in mice. Kelly and Rank (1997) have also demonstrated a short-lived protective immunity in the murine genital tract following intravaginal infection with *C trachomatis* mediated primarily by Th1 $CD4^+$ cells, and have identified homing receptors that mediate $CD4^+$ T cell recruitment to the genital tract

Mycoplasmas

The role that mycoplasmas may play in the aetiology of PID is uncertain, as they are part of the normal vaginal flora in the genital tract of more than 50% of healthy sexually active women. Mycoplasmas are rarely isolated from fallopian tube specimens of women with PID and, although acute antibody responses do occur in a minority of women, this may simply reflect exposure of the immune system to the microorganism as a result of epithelial damage in the genital tract.

1.2.3 SEQUELAE

Most women recover completely following an episode of PID, although extrapolation from infertility data (Hull 1985) and the reported levels of symptomatic sequelae following hospitalisation for PID, indicate that the long term outcome for some women has considerable implications for the provision of gynaecological services (Buchan et al 1993).

The likelihood of developing tubal factor infertility following PID is determined by the severity and number of episodes of disease. Follow-up of the cohort in Lund, Sweden indicates that 8% of women attempting to conceive after one episode of PID have tubal factor infertility, whereas after two episodes 19.5% are affected and after three or more episodes the figure rises to 40%. Only 0.6% of women experience tubal factor infertility after a single episode of mild disease, but this figure rises to 21.4% after an episode of severe disease.

Damage to the fallopian tubes after PID is a well documented risk factor for ectopic pregnancy, and in this same series the ectopic pregnancy rate was 9.1% compared to a rate of 1.4% in the control group (Westrom et al 1992).

Although attention has focused on tubal infertlity and ectopic pregnancy following PID, other serious sequelae are common. Chronic abdominal pain occurs frequently and contributes to the high rates of hospitalisation and the 10-fold increase in hysterectomy rate seen in women following PID (Buchan et al 1993).

1.2.4 TREATMENT

Because PID is frequently asymptomatic, and in many women presenting with symptoms of acute PID in whom substantial tubal damage has already occurred, therapeutic interventions have been largely disappointing.

Many antibacterial regimens have been assessed for the treatment of PID, but invariably focus on short-term objectives rather than their efficacy in preventing long-term sequelae. The choice of regimen tends to be governed by local prescribing guidelines, patterns of antibacterial resistance, predicted patient compliance and accurate microbiological diagnosis. Whichever regimen is chosen, evaluation and treatment of symptomatic PID should be prompt. Delay of care for three or more days in gonorrhoea or chlamydia associated PID leads to a threefold increased risk of ectopic pregnancy or tubal infertility (Hillis et al 1993).

Should patients resume sexual intercourse with an untreated, infected partner, the chances of reinfection are high. More than 50% of the sexual partners of women with PID will have evidence of a genital tract infection.

Although radical surgery is rarely necessary in the management of acute PID, aggressive laparoscopic surgery should be considered. Laparoscopic surgical techniques can be utilised to drain purulent fluid, lyse bowel adhesions and excise acute and necrotic tubo-ovarian adhesions. There is some evidence that early laparoscopic treatment of tubo-ovarian abscess leads to more rapid short-term recovery and preservation of normal anatomy (Reich & McGlynn 1987)

As an adjunct to the antibacterial and surgical management of acute PID, the role of non-steroidal anti-inflammatory agents in the prevention of long-term sequelae has been considered, but as yet remains unclear. The development of new, highly specific anti-inflammatory agents may offer better prospects.

1.2.5 PREVENTION

Because PID and its sequelae cause widespread suffering and drain limited health care resources, investments in prevention are likely to pay dividends. Successful strategies have been employed, particularly in Sweden, to control genital chlamydia and gonorrhoea and significantly reduce the occurrence of symptomatic PID.

Primary prevention of PID is dominated by preventing both exposure to and acquisition of chlamydial and gonococcal infection. Intense, culturally sensitive educational campaigns should aim to raise professional and public awareness of healthy sexual behaviour. These should employ both the mass media and a community based approach. Young people can be targeted in schools from as early as 12 years of age, and the message continually reinforced later. Health care providers should be encouraged to update their knowledge about the prevention, recognition and proper treatment of PID and associated STDs. Clinicians must routinely enquire about high risk sexual behaviour, and encourage screening tests for those at risk.

Finally and most importantly the search for bacterial STD vaccines is being pursued with renewed vigour and offers hope for future primary prevention of PID. The development and delivery of vaccines that induce specific immune responses in the genital tract secretions would also have far-reaching implications for the prevention of HIV transmission and other sexually transmitted diseases. This long term goal requires an improved understanding of mucosal immunology in the female genital tract.

1.3 HUMAN PAPILLOMA VIRUS

Infectious agents known to affect the female genital tract have been implicated in the aetiology of cervical neoplasia and recent research has focused on several subtypes of human papilloma virus (HPV) suggesting that these are now the main risk factor for invasive cervical cancer.

The HPV types which infect the lower reproductive tract can be divided into two groups. One is found in condylomata acuminata and low grade cervical intra-epithelial neoplasia (CIN). The other, including types 16,18 and 33 is associated with high grade CIN and invasive tumours. HPV 16 seems to predominate in squamous cell tumours and HPV 18 in adenosquamous tumours. The presence of HPV 16/18 DNA in CIN does appear to increase the risk of progression to more advanced CIN lesions, although neither the presence of nor the type of HPV DNA seem to have any prognostic significance in established cervical carcinoma (Kristensen et al 1996).

The question is whether present evidence is sufficient for making firm recommendations on HPV screening and although several retrospective studies and smaller prospective studies suggest that HPV testing may well improve cervical cancer screening, only large prospective screening studies on the association between HPV infection and the development of CIN and cervical cancer will enable firm conclusions to be drawn (van Ballegooijen et al 1997).

Unfortunately, should a firm case be developed for HPV screening in cervical cancer, there is no evidence that anti viral therapy completely prevents viral replication, indeed there is evidence to suggest that HPV 16 infection may often be very transient (Fairley et al 1994).

Once again, it seems that the key will be the prevention of infection with HPV through vaccine development and current research is focused on the E6 and E7 oncoproteins expressed in HPV tumours.

1.4 HUMAN IMMUNODEFICIENCY VIRUS

Although I will not enter into a detailed discussion of infection with HIV and acquired immunodeficiency syndrome (AIDS), it is clear that this disease is providing the driving force behind much of the work into reproductive tract mucosal immunology in the search for an effective vaccine.

In the developed world HIV transmission is predominantly through intravenous drug abuse and homosexual contact, whilst in the developing world transmission is mainly through heterosexual contact. Indeed, it has been estimated by the World Health Organisation that 80% of HIV transmission worldwide is heterosexual and this is becoming increasingly important in the developed world as the epidemic progresses.

Although combination anti-viral therapy seems to be very promising, there is an urgent need for an effective HIV vaccine. Since most homosexual and heterosexual transmission of the virus involves passage across mucosal surfaces, such a vaccine will need to be effective at mucosal membranes and its evolution will require a detailed understanding of reproductive tract mucosal immunology.

2 MUCOSAL IMMUNOLOGY IN THE FEMALE REPRODUCTIVE TRACT

2.1 INTRODUCTION

Within the humoral immune system there is a characteristic distribution of antibodies in two main compartments. Antibodies found in blood and lymph belong predominantly to the immunoglobulin (Ig) G class, whereas those found in external mucosal secretions are predominantly of the IgA class.

Human IgA exists in both monomeric and polymeric forms, although the dimeric form predominates in most mucosal secretions. A distinguishing feature of polymeric IgA (and pIgM) is the presence of J chain, a polypeptide synthesised by antibody producing cells, which is incorporated into pIgA prior to its secretion from plasma cells. The presence of secretory component (SC) distinguishes secretory IgA (SIgA) from serum IgA and plays a crucial role in the transport of pIgA to mucosal secretions. SC also protects SIgA from proteolysis. More than 90% of serum IgA is in the monomeric form whereas SIgA predominates in mucosal secretions (Mestecky & McGhee 1987).

There are two IgA subclasses, IgA1 and IgA2, which differ in several structural respects but particularly in their susceptibility to microbial proteases. There is proportionately more IgA2 in mucosal secretions than serum where its greater protease resistance would presumably be advantageous (Crago et al 1984).

IgM in mucosal secretions is also associated with SC although its concentration is substantially lower than that of secretory IgA. The concentration of IgG in mucosal secretions is usually somewhat greater than that of IgM and is not thought to be associated with a specific transport mechanism.

The majority of secretory IgA is derived from local mucosal synthesis and not, to a significant degree, from the circulation (Mestecky 1987). Most of the monomeric

serum IgA is produced in the bone marrow and although small amounts of polymeric IgA are present in serum these probably originate in mucosal sites.

A high proportion of Ig-producing cells in the mucosal lymphoid tissue, which represents a huge surface area, are committed to the IgA isotype and this is reflected in the fact that more IgA is produced in humans than the combined synthesis of all other isotypes (Conley & Delacroix 1987).

Antibodies in mucosal secretions, predominantly IgA, combine with microorganisms to reduce their motility, growth and adhesive properties thus reducing their chances of breaching the mucosal epithelium. During their transport to the mucosal epithelium polymeric IgA antibodies can also combine with microorganisms that have entered the mucosal epithelial cells, giving IgA a role in host defence that has traditionally been reserved for cell-mediated immunity (Mazanec et al 1992). In addition to the polymeric immunoglobulin receptor on mucosal epithelial cells, IgA antibodies can bind to receptors on a variety of leucocytes and have been shown in some experimental systems to be capable of activating the alternative complement pathway, although IgA has traditionally been viewed as non-inflammatory or even anti-inflammatory (Lamm 1997).

2.2 EVIDENCE FOR MUCOSAL IMMUNITY IN THE HUMAN FEMALE REPRODUCTIVE TRACT

The first barrier in mucosal defences is provided by antibodies in secretions. In most mucosal surfaces actively transported secretory IgA is the dominant isotype and in mucosa associated lymphoid tissues the synthesis of IgA antibodies is independant from the systemic immune system. However, in some mucosal sites, including the lungs and genital tract, IgG antibodies have been reported as the major isotype and seem to be predominantly serum derived (Hocini et al 1995).

Both tissues and appropriate washings from the human female genital tract have demonstrated that all effector components of the humoral mucosal immune system are present.

SC has been detected with a high frequency on the surface of and in the columnar epithelial cells of the fallopian tube, along with IgA and J chain containing plasma cells in the lamina propria, suggesting that the fallopian tube is a potential site for mucosal immunity (Kutteh et al 1988, 1990, 1994). The endometrium contains SC and IgA in glandular epithelia and IgA in the glandular lumina although IgA and IgG plasma cells are rare (Kelly & Fox 1979, Rebello et al 1975). The endocervix, which is lined by columnar epithelium contains a significant number of IgA plasma cells (as well as IgG and IgM plasma cells) and SC on the luminal and glandular epithelium. An approximately equal distribution of IgA1 and IgA2 plasma cells have been demonstrated both in the endocervix as well as the other effector sites in the female reproductive tract. This suggests that the endocervix may well be a critical site for mucosal immunity in this region.

The ectocervix and vagina are lined by stratified squamous epithelium and display a few IgA plasma cells in the stromal lamina propria. A high proportion of these contain IgA2 with J chain. SC has not been confidently identified in the vagina or ectocervix (Kutteh et al 1988).

Johansson et al (1999) have noted that leucocytes are not randomly scattered in the human cervix and vagina but are organised in a distinct pattern. T cells were found clustered in a distinct band beneath the epithelium and also dispersed in the epithelium and lamina propria, whereas CD38+ plasma cells were present only in the lamina propria. Major histocompatibility complex class II+ cells (MHCII+) were numerous in the lamina propria and epithelium and morphologically resembled dendritic cells with a probable role in antigen presentation. Lymphoid aggregates containing CD19+ and CD20+ B cells as well as CD3+, CD4+ and CD8+ cells were also found in the cervix.

Both IgA and IgG have been identified in fluid from the cervix and vagina and in cervical mucus there are higher levels of IgG than IgA which contrasts with other external secretions such as tears, saliva, milk and intestinal fluids in which secretory IgA is the dominant isotype (Mestecky & Fultz 1999). Human cervical mucus contains albumin / IgG ratios that approximate to those in serum, suggesting that the IgG is serum-derived although no active transport mechanism for IgG has been described in the genital tract (Tjokronegoro & Sirisinha 1975). Of the IgA measured in cervical mucus, approximately 80% seems to be locally produced SIgA (Kutteh & Mestecky 1994).

Analysis of vaginal washings in the human has shown a large proportion of IgG and a low amount of IgA including monomers, secretory IgA and fragments. IgM is at a very low level and free secretory component molecules are abundant. Vaginal IgG may cross the epithelium by passive diffusion or an active Fc receptor associated mechanism.

In women who have undergone hysterectomy, IgA levels in vaginal fluid are <10% of normal, suggesting an endometrial/cervical origin for IgA (Jalanti & Isliker 1977). However, IgG levels were not significantly altered by hysterectomy suggesting significant local derivation from the vagina. In some of these studies it was difficult however to exclude serum contamination of the local fluids and thus the origin of the Ig isotypes measured.

The composition of genital secretions is dependant on hormonal and inflammatory factors. Evidence for the former will be presented later. It is clear that local inflammation markedly influences the levels of immunoglobulins found in genital tract secretions and patients with abnormal cervical cytology show increases in IgG and more markedly IgA in cervical mucus (Coughlan & Skinner 1972). This is an important point in that vaccine induced inflammation in the cervix is also likely to lead to greater diffusion of serum derived antibody across the cervical epithelium.

2.3 MUCOSAL IMMUNOLOGY IN THE ANIMAL MODEL

In a physiological sense it is important to remember that considerable inter-species variation exists, particularly when studying the immunology of the genital tract. Caution should therefore be exercised before making extrapolations from animal data. Some brief observations of differences between the most commonly used animal model and humans will be made in prelude to consideration of the animal model in mucosal vaccination strategies later.

The most frequently used animal model is the mouse

Many IgA plasma cells are found concentrated in the uterine horns and body. In the fimbria and infundibulum there are some IgA plasma cells in the stroma but these features are not observed in the rest of the oviduct. The mouse cervix and vagina are lined by stratified squamous epithelium where there are few IgA plasma cells (Parr & Parr 1985). Most of the IgA production seems to originate from the uterus since levels are dramatically reduced in hysterectomised mice whereas IgG levels are relatively unaffected suggesting a vaginal origin (Parr & Parr 1990). These findings contrast with the human female genital tract in which the endocervix seems to be the most important immune effector site.

Less is known about secretory immunity in the genital tracts of other species. IgG and IgA plasma cells have been identified in the genital tracts of monkeys as well as IgG and IgA in genital tract secretions (Miller et al 1992).

2.4 INDUCTION OF IMMUNITY IN THE FEMALE REPRODUCTIVE TRACT

In the gastro-intestinal tract the Peyer's patches of the small intestine are major sites of induction of both cell-mediated and humoral immune responses. The epithelium overlying the Peyer's patches contains specialised M (microfold) cells which transport orally administered antigen to the lymphoid tissue of the Peyer's patches which contain antigen presenting cells (APCs), B and T lymphocytes. After proliferation and partial differentiation, B and T cells enter the regional mesenteric lymph nodes and after further differentiation eventually enter the circulation. Most T and B cells activated in the intestine migrate to the lamina propria of the intestine but a significant proportion end up in mucosal tissues outside the intestine (Holmgren et al 1992).

Induction of immunity in the female genital tract has not been described as clearly as in the gastro-intestinal tract and M cells or specialised APCs have not been described in the vagina or cervix. The vaginal mucosa consists of non-keratinised stratified squamous epithelium with an underlying vascular submucosa, and the ectocervix has a similar architecture. Langerhans cells and macrophages are present in the vaginal mucosa and cervix and it seems likely that they act as APCs. Macrophages are general antigen presenting cells for primed lymphocytes but cannot stimulate naive T cells. Dendritic cells which process antigen migrate to draining lymph nodes and as interdigitating dendritic cells powerfully initiate primary T cell responses.

Antigen reaching the submucosa of the cervix or vagina would be taken up by APCs followed by antigen processing by these cells and migration to draining lymph nodes. In the lymph nodes the APCs stimulate B and T lymphocytes which enter the circulation via the lymphatics and thoracic duct. These T and B cell populations including memory cells migrate to the genital tract and may on exposure to antigen participate in a secondary immune response.

Langerhans cells (LCs) and intraepithelial T lymphocytes (IELs) are present in the vagina and cervix where they are most concentrated in the transformation zone, and close contact has been observed between LCs, IELs, capillary endothelium and macrophages suggesting a role in antigen presentation. The uterus and fallopian tube also contain IELs, mainly of the cytotoxic/suppressor subtype (Edwards & Morris 1985; Morris et al 1983; Morris et al 1986). Macrophages have been demonstrated throughout the endometrium and they do not seem to undergo menstrual cycle related alterations in number. The function of endometrial macrophages is unknown but their location within T lymphocyte aggregates and close to endometrial glands suggests a role in antigen presentation. The majority of endometrial T lymphocytes are of the cytotoxic/suppressor (CD8+) subset and it may be that they play a role in endometrial immunosuppression during pregnancy. In addition, a population of suppressor cells (CD2+, CD3-) has been identified which appears to be hormone dependant and whose numbers expand during the secretory phase of the menstrual cycle (Bulmer et al 1988).

Classical NK cells are only present in negligible numbers in normal human endometrium although there is a population of phenotypically unusual endometrial granulated lymphocytes (CD56+ CD16-) which are present in large numbers, increasing significantly in the late secretory phase, which are capable of lymphoproliferative and cytotoxic activity and are likely to be concerned with endometrial defence (Jones et al 1998).

Mucosal lymphoid nodules containing B lymphocytes are far less frequently found in the female genital tract than in intestinal or respiratory mucosa. Those lymphoid nodules that are described in the endometrium are mainly aggregates of T lymphocytes. Germinal centers and mantle zones containing B lymphocytes may only exist as the remnant of exposure to invasive microorganisms. There are also few lymphoid nodules in the fallopian tube and cervix, suggesting that IgA plasma cells in the genital tract originate from another source (Bulmer et al 1988).

2.5 HORMONAL REGULATION OF GENITAL TRACT IMMUNITY

The female genital tract is unique amongst mucosal sites in that it must be able to tolerate the antigenic challenge of spermatozoa, fetus and trophoblast whilst mounting an effective immune response against potentially pathogenic microorganisms. Spermatozoa and seminal plasma contain over 30 antigens that can elicit strong antibody responses, and although immunity to spermatozoa is not an all or nothing phenomenon, enough motile sperm to lead to fertilisation usually reach the upper genital tract. In some women sperm agglutination and sperm-immobilising antibodies in cervical mucus can lead to infertility, although this is the exception rather than the rule. There are clearly fundamental mechanisms in place which can moderate such an immune reaction and these relate to both the seminal fluid and the female genital tract. Much evidence has been presented for the existence of immunosuppressive factors within the seminal fluid and indeed as an inherent component of sperm function. The effect of the menstrual cycle on female genital tract immunity has not been extensively investigated in the human model. In the mouse the vaginal stratified epithelium has been demonstrated to be permeable to various proteins except at estrus which would minimise sensitisation to sperm and seminal fluid proteins at the time of mating (Parr & Parr 1990). In the rat, epithelial and stromal cells at proestrus present significantly more antigen than epithelial cells in the uterus at other stages of the reproductive cycle. In contrast, antigen presentation by vaginal cells is lowest at proestrus and inhibited by estradiol (Wira et al 1995). Although cells capable of antigen presentation have been described in the human vagina and cervix such a resistance to antigen uptake at the time of ovulation has not been described. However, hormonal fluctuations during the menstrual cycle do affect the stratified epithelium of the vagina and ectocervix altering epithelial thickness, endocytic activity of epithelial cells and the distribution of dendritic cells. The fact that antigen uptake and processing across stratified epithelia requires close collaboration between epithelial

and dendritic cells suggests that the effectiveness of vaginal/ectocervical vaccines may depend on the stage of the menstrual cycle (Neutra et al 1996).

Hormone dependant changes in humoral immunity have been described in both animal and human models. Estradiol and progesterone regulate uterine and vaginal levels of IgA, IgG and SC in the rat. Estradiol leads to an elevation of immunoglobulins and SC in the uterus of the rat whilst leading to a decrease in concentration in vaginal secretions. Progesterone tends to inhibit some of these estradiol-dependant changes (Wira et al 1983).

In the human endometrium the concentrations of SC and IgA fluctuate with the menstrual cycle, being highest in the secretory phase. The number of IgA containing plasma cells in the cervix is far more abundant than in the endometrium and increases in the late secretory phase but decreases during pregnancy (Murdoch et al 1982). Cervical IgA and IgG levels have been shown to be depressed in women using a combined oral contraceptive (Schumacher 1980).

From these observations it seems likely that both afferent and efferent arms of a mucosal immune response are under the control of sex steroids in the human female genital tract although the complete picture is not as yet clear.

Other factors have also been shown to influence genital tract immunity. Dexamethasone raises total IgA levels in serum and lowers IgA in reproductive tract secretions. It may be that this represents a stress response to boost protection in systemic sites once the mucosal barrier has been breached (Wira et al 1990). Little is known about the role of cytokines in the regulation of genital tract immunity in the human, although selected cytokines clearly play an important role in the mouse and rat. There is also an evolving literature on the neuropeptide regulation of mucosal immunity, although mainly focused on the gastrointestinal tract (for review: Stead et al 1987).

2.6 COMMON MUCOSAL IMMUNITY: ORIGIN OF IMMUNOGLOBULIN PRODUCING CELLS IN THE FEMALE GENITAL TRACT

The existence of a localised mucosal immune response in the female genital tract is apparent in that antibodies are generated against locally invasive microorganisms (Schumacher 1980). However, antigen presentation in remote sites can also stimulate an antibody response in the genital tract.

External secretions of glands that are remote from the site of antigen stimulation have been shown to contain natural SIgA antibodies to such antigens. For example, colostrum and tears contain high levels of antibody to *Streptococcus mutans* which is an oral bacterium and this is in the absence of a significant titre of corresponding antibodies in the serum (Arnold et al 1976).

The Peyer's patches in the small intestine are a potent source of IgA precursor B cells and, after antigen uptake and processing, isotype and antigen committed cells exit the Peyer's patches. After maturation in mesenteric lymph nodes these cells enter the circulation through the thoracic duct and then home to mucosal surfaces and glands, both in the small intestine and at other remote mucosal sites, where they mature into IgA producing plasma cells (for review see Mestecky et al 1994).

Other IgA inductive sites may also be important in this context and include the oropharyngeal lymphoid tissue, bronchus associated lymphoid tissue, rectal lymphoid tissue and lymphoid cells from the peritoneal cavity.

The origin of precursor cells that populate the human female reproductive tract has not been conclusively investigated, however there may be some leads from circumstantial evidence and experiments in the animal model. The similarity of the proportion of plasma cells producing IgA1 and IgA2 between the lamina propria of the large intestine and the genital mucosal tissues suggests that the rectal lymphoid tissue may be an important source of IgA precursors destined for the genital tract (Crago et al 1984). It should also be noted that the regional lymph nodes (obturator and iliac

nodes) draining the large bowel also drain tissues from the reproductive tract. In the mouse model radiolabelled mesenteric lymph node cells have been shown to repopulate recipient cervix and vagina within 24 hours with 60% IgA and 25% IgG positive cells, suggesting an important contribution from the small bowel (McDermott & Bienenstock 1979). The contribution of precursors from the peritoneal cavity has only been demonstrated for the intestine although intraperitoneal immunisation of animals is effective in inducing antibodies in genital secretions (Scicchitano et al 1988)

Further discussion of induction of immunity in the female genital tract through local, systemic and remote mucosal vaccination follows, although in the human model these investigations are not comprehensive.

2.7 INDUCTIVE SITES FOR MUCOSAL IMMUNITY IN THE GENITAL TRACT

Immunisation against the invasive microorganisms that penetrate the mucosal defences of the female genital tract would have a major impact on both reproductive morbidity and mortality. To date however, immunisation strategies have not demonstrated an effective, sustained antibody response in the mucosal surfaces of the genital tract. Researchers have investigated several inductive sites for genital tract immunity including immunisation locally, at other mucosal sites and parenterally. Much of this work has been in the animal model although there is an expanding literature in the response to experimental vaccination in the human female genital tract.

2.7.1 LOCAL MUCOSAL IMMUNISATION

The presence of specific antibodies in the cervical and vaginal secretions from women with sexually transmitted diseases suggests that a mucosal immune response can be induced in the genital tract and this observation has led to experimental immunisation of the uterus and vagina. Ogra was the first to investigate the local antibody response in the human female genital tract to inactivated poliovaccine administered into the vagina, uterus, nasopharynx, and by intramuscular injection. Intravaginal and intrauterine immunisation consistently resulted in the appearance of secretory antibody to poliovirus in the genital tract. The vaginal response was predominantly of IgA, whilst the uterine response was mainly of IgG, in the absence of a significant IgG titre in the serum, suggesting local secretory antibody production. In reponse to oral and intramuscular immunisation the genital tract response was limited to IgG and this coincided with high serum titres at least three weeks later (Ogra & Ogra 1973).

Kozlowski et al (1997 and 1999) demonstrated that oral, rectal and vaginal immunisation with a recombinant cholera toxin B-containing vaccine increased specific IgG in serum and IgA in saliva, although only the four women immunised via the vaginal route increased specific IgA antibodies in lower genital tract secretions and could be shown to induce a local IgG response. Using the same vaccine applied to the posterior vaginal fornix three times at two weekly intervals, Wassén et al showed that six out of seven women vaccinated vaginally responded with specific genital tract antibodies. Specific IgG and IgA were found in cervical mucus in higher titres than following oral immunisation with the same vaccine.

Animal studies have generally shown that large doses of antigen and repeated administration are necessary to achieve a significant specific secretory antibody reponse in the genital tract. Indeed, a comparative study of the secretory antibody response in mouse vaginal fluid after pelvic, parenteral and vaginal immunisation demonstrated a poor response to the vaginal route (Thapar et al 1990). Haneberg et al (1994) confirmed this finding in that mice vaginally immunised with cholera toxin produced neither local nor distant IgA responses. In contrast Johansson et al (1998) found that the strongest antibody responses in mice were induced after vaginal and intra-nasal immunisations with the same cholera toxin, and that these routes also gave rise to specific IgA and IgG antibody secreting cells in the genital mucosa. If female mice are immunised with homologous sperm both orally and with a vaginal booster, vaginal IgA antisperm antibodies have been demonstrated in high titres along with reduced fertility (Allardyce & Rademaker 1987).

The possible mechanism of antigen uptake in the human female genital tract and influence of sex hormones have already been discussed and are no doubt important in determining the optimum timing of vaccination. This has been demonstrated in the mouse where immune responses may be optimal when vaccination occurs at diestrus or in early pregnancy (Parr & Parr 1990). Likewise, infection of the genital tract with invasive microorganisms may be dependant on the menstrual cycle phase. Another problem encountered when attempting to present vaccine to the uterus, cervix or

vagina is that most preparations readily leak out, reducing contact time with mucosal tissues.

Several strategies are being developed to improve vaccine delivery to mucosal tissues. These include the use of bioadhesive microspheres, attenuated bacterial and viral vectors and absorption enhancing agents (Staats et al 1994). We have attempted to improve vaccine delivery to the human cervix using a contraceptive cervical cap and this will be discussed in more detail within the methodology section.

2.7.2 ORAL IMMUNISATION

The advantages of using the oral route to deliver vaccines are obvious, particularly because of ease of administration. However, more effective immune responses are generally produced in the intestine by replicating organisms rather than non-replicating ones, perhaps because the latter are inactivated by stomach enzymes, acidity, or are unable to penetrate the mucous lining of the gastro-intestinal tract. These problems are being overcome by exploiting different methods of antigen delivery. Antigen carriers have been developed which use organisms that naturally colonise the gastro-intestinal tract but have been genetically modified to avoid virulence. The organism's own defences can thus avoid destruction by enzymes or stomach acids. Antigens can also be encapsulated in biodegradable microspheres and delivered to Peyer's patches avoiding stomach defences. Chemical adjuvants can also be used which stimulate a more potent immune response to their accompanying antigens.

The role of oral immunisation in eliciting antibody responses in the genital tract has been addressed in the human. In Ogra's study, oral immunisation with live attenuated poliovaccine led to a weak IgG response in the human reproductive tract which was probably serum derived (Ogra & Ogra 1973). Wassén's group found that oral cholera vaccination resulted in weak IgG and IgA genital tract responses in less than 50% of vaccinees (Wassén et al 1996). Kozlowski et al confirmed these findings (1997),

demonstrating specific IgG and IgA in the cervix after oral cholera toxin B subunit vaccination. The cervical IgA response was short-lived and this route failed to increase specific antibodies of either isotype in the vagina.

Oral immunisation in mice and rats has generally led to disappointing results, although LaScolea demonstrated a significant secretory antibody response in the mouse genital tract in response to oral immunisation with *Chlamydia trachomatis*. Subsequent chlamydial intravaginal challenges led to a dramatic booster effect on cervical IgA and protection from infection (LaScolea et al 1991). Mice, orally immunised with cholera toxin demonstrated genital tract antibody responses that were 10 times weaker than following nasal immunisation (Johansson et al 1998) In macaques orally immunised with simian immune deficiency virus (SIV) incorporated into microspheres a protective immune response against vaginal challenge with SIV has been demonstrated following systemic priming (Marx et al 1993).

The effectiveness of intra-peritoneal vaccination in inducing a secretory antibody response in mouse vaginal washings has already been discussed. The effect of intra-rectal immunisation on inducing a genital tract response seems to be promising in inducing SIV-specific secretory antibodies in non-human primates (Lehner et al 1993). The effect of rectal and nasal vaccination on genital tract responses in two small groups of women has now been addressed and is discussed below.

2.7.3 PARENTERAL IMMUNISATION

Since the systemic and mucosal immune systems seem to be functionally compartmentalised, parenteral vaccine administration does not consistently stimulate significant mucosal IgA responses. Local mucosal administration of vaccines tends to be more effective as has been demonstrated in the gastro-intestinal tract with the success of the live attenuated polio vaccine. However there is increasing evidence that IgG is the major isotype in the female genital tract and this may favour systemic vaccination in the induction of cervico-vaginal protection. It may well be the case that

in the female genital tract both mucosal and systemic immunisation is necessary for the induction of an effective antigen specific immune response.

In Ogra's study a weak serum derived IgG response was generated in human genital tract secretions after intramuscular immunisation with inactivated polio vaccine (Ogra & Ogra 1973). Bouvet et al also demonstrated specific antibodies in human vaginal secretions following parenteral vaccination with the tetanus toxoid vaccine. These were predominantly of the IgG isotype and showed a dramatic increase after booster injection (Bouvet et al 1994).

It seems, at least in the animal model, that parenteral immunisation in the vicinity of a mucosal organ can elicit significant IgA secretion at that mucosal site. In female mice vaccinated at two parenteral sites within the pelvis there were higher and longer sustained IgA responses than following non-pelvic parenteral vaccination. These responses may have been generated in the iliac and para-aortic nodes draining the reproductive tract. All vaccination routes produced vaginal IgG responses (Thapar et al 1990).

The issue of whether IgA is more important than IgG in protecting the female genital tract against bacterial and viral infections is unclear and may be dependant on characteristics of the individual microorganisms as much as immunological factors.

2.8 CONCLUSION

The investigation of stimulation of immune responses in the human female genital tract is clearly gaining momentum and it seems likely that the optimal route of vaccination will involve parenteral and local mucosal presentation of antigen. The question however is also a pragmatic one. When is the best time to vaccinate against pelvic inflammatory disease, HPV and HIV? If the answer to this is at puberty then is cervical, vaginal or rectal immunisation likely to be acceptable to a large percentage of the teenage female population, particularly if repeated administration is required. If

reasonably effective responses to parenteral vaccination can be demonstrated would this not be far more acceptable?

The five studies quoted above which address the issue of stimulation of antibody mediated immunity in the genital tract in the human model suffer from similar drawbacks. Numbers are very limited and do not allow for meaningful numerical comparisons of vaccination by different routes. None of these studies exceed ten cases in total. The focus from each of these studies has been on the lower genital tract and antibody responses to vaccination in the fallopian tube have been overlooked despite the importance of this site in the primary prevention of PID. Techniques used to sample mucosal surface antibody are variable and we have tried to standardise this by using a similar technique in the upper and lower genital tracts to gently wash out mucosal secretions.

We attempt to address these issues by looking at genital tract immune responses to parenteral, cervical and oral vaccinations in a much larger cohort of women than has previously been studied. We also try to investigate in greater depth the distribution of humoral immunity in the female genital tract and its response to menstrual cycle variation.

3. METHODS

3.1 INTRODUCTION AND HYPOTHESES

Despite the recent advances in our understanding of the mucosal immunology of the female reproductive tract, there are several areas of research still to be explored before rational immunisation strategies can be developed. Whilst much has been learnt from the animal model, it is preferable, within the constraints of ethical considerations, patient choice and safety, to focus on the human. The experimental work within this study solely concentrated on the human reproductive tract. The two main hypotheses are summarised below.

1. An investigation of the distribution of IgA and IgG (including subclasses) and secretory IgA in the human upper and lower genital tracts, and to assess the influence of menstrual cycle phase on this distribution.

2. An investigation of the optimal routes of stimulation of immune responses in the female genital tract. This arm of the study examined antigen specific antibody responses in vaginal, cervical, endometrial and fallopian tube washings, and serum, to vaccines given orally, parenterally and onto the endocervix.

3.2 LABORATORY ANALYSIS

3.3 ANTIBODY ISOTYPE AND SUBCLASS ANALYSIS

For each participant in the trial antibody isotype and subclass concentration were estimated for each site (i.e. vagina, cervix, endometrium, fallopian tube and serum) using radial immunodiffusion (RID). Where there was conflict between menstrual dates, serum progesterone/estradiol levels and endometrial histology, the latter was used as the arbiter of menstrual phase.

3.3.1 RADIAL IMMUNODIFFUSION

The radial immunodiffusion studies were carried out using a commercial kit (BIND A RID or NANORID. The Binding Site Limited, Birmingham).

Radial immunodiffusion is routinely used for measuring the concentrations of soluble antigens in biological fluids. It is derived from the work of Mancini et al and Fahey and McKelvey (Mancini et al 1965, Fahey & McKelvey 1965).

The method involves antigen diffusing radially from a cylindrical well through an agarose gel containing an appropriate mono-specific antibody. Antigen-antibody complexes are formed, which form a precipitin ring. The ring size increases until equilibrium is reached between the formation and breakdown of these complexes, this point being termed completion. A linear relationship exists between the square of the ring diameter and the antigen concentration. Using samples of known concentration and measuring their ring diameters, a calibration curve is constructed. The concentrations of antigen in unknown samples may then be determined by measuring their ring diameters and reading off the calibration curve. Reference tables are however provided based on the ideal linear calibration curve which convert ring diameters directly to protein concentrations.

Where low level antibody concentrations were suspected (IgG3 and IgG4), the ultra-low level detection RID plates (Nanorid) were used. Otherwise, low level detection RID plates (Bind A Rid) were used.

The plates have 14 cylindrical wells, and so that number of samples (including calibrator) can be run on each plate. Because it was not necessary to construct a calibration curve the neat/high calibrator was run on each plate to ensure that they were performing correctly.

For the serum samples recommended dilutions were specified and normal serum ranges given (i.e. IgG1 4.22-12.93 g/l, IgG2 1.17-7.47 g/l, IgG3 0.41-1.29 g/l and IgG4 0.01-2.91 g/l.). The serum dilutions for IgG1, IgG2, and IgA1 used were 1:1000, for IgG3 and IgG4 1:100, and for IgA2 1:50. For each of the genital tract washings estimates were made of the likely required dilutions and then adjusted once a feel for the early results was attained. The initial dilutions used were as follows.

IgG1	IgG2	IgG3	IgG4	IgA1	IgA2
V 1:3-5	V 1:3	V neat	V neat	V 1:3-5	V neat
C 1:3-5	C 1:3	C neat	C neat	C 1:3-5	C neat
U 1:5	U 1:3	U neat	U neat	U 1:3	U neat
F neat	F neat	F neat	F neat	F neat	F neat

Table 1. V=vagina, C=cervix, U=uterus/endometrium, F=fallopian tube

If antigen (immunoglobulin) in particular samples was undetectable, one of the following measures was taken, in the order specified.

i) A less dilute or more concentrated sample was used.

ii) A double fill of the well was made (i.e. using double the sample volume)

iii) A plate with a lower measuring range was used, if available.

If the samples were too concentrated with precipitin rings at completion beyond the recommended scale further dilutions were made as appropriate.

The plates were opened for 10-15 mins to allow for evaporation of any condensation in the wells or on the gel surface. The test samples, control and neat/high calibrator samples were then applied to the wells in 10 or 20 microlitre aliquots using a micropipette. The plate lids were then reapplied and stored flat at room temperature in a moist box to avoid drying of the gel. The minimum diffusion time for completion was usually 96 hours but varied according to the plate used.

Ring diameter measurement at completion was initially attempted using a jeweller's eyepiece and callibrator with bright sidelighting and a dark background. This was found to be difficult and inaccurate and so was eventually performed using a RID plate reader and expressed to the nearest 0.1mm (Fig. 1). The antibody concentrations were then read off the RID reference table and adjusted for dilutions made and any double fills of wells. Values obtained for the control samples were deemed to be acceptable if within 10% of the concentration stated on the vial label.

IgA2 was consistently undetectable or detected in very low concentrations in patient specimens, controls and callibrators using the commercial radial immunodiffusion kits. Thus IgA2 and secretory IgA were separately assayed using time-resolved fluorimetry (see section 3.4.3).

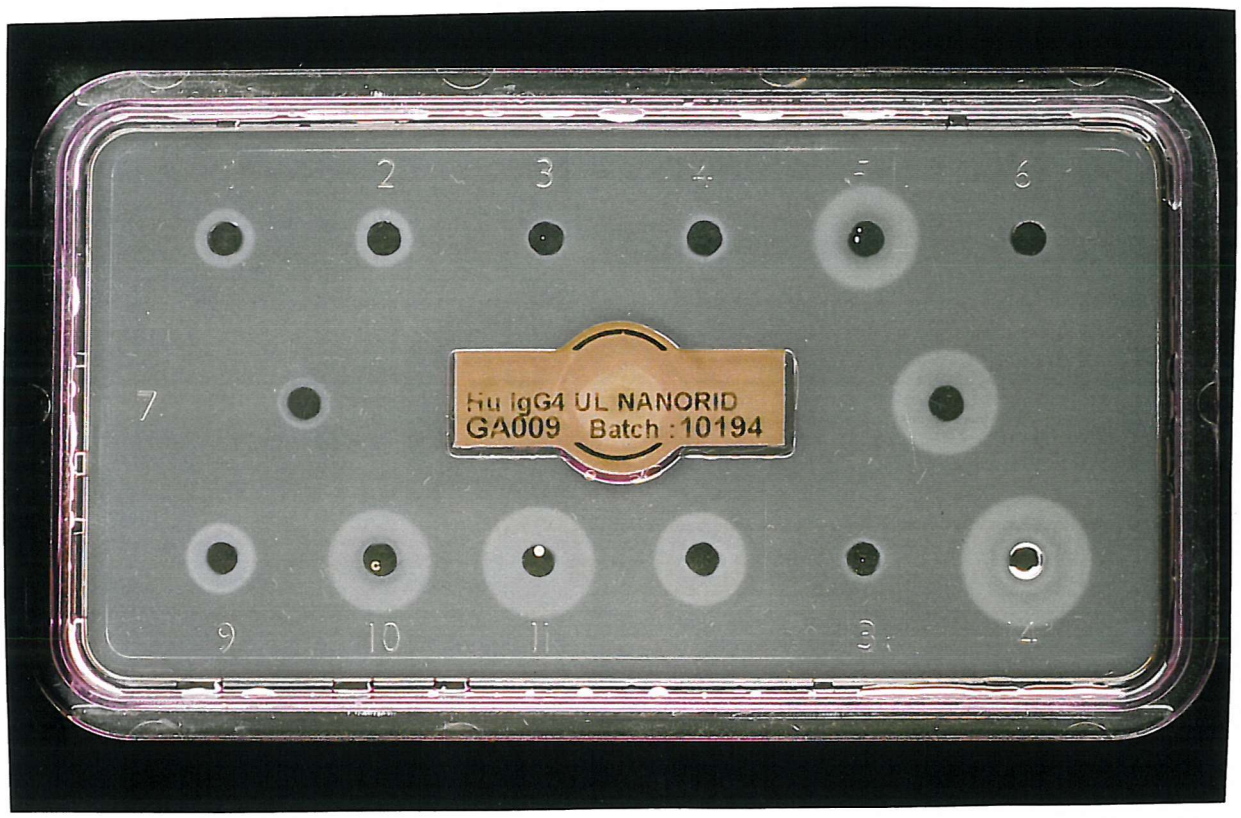


Figure 1. A radial immunodiffusion plate for detecting human IgG4. The plate is at "completion" and the antibody levels are calculated by measuring the precipitin ring diameters and reading concentrations off a RID reference table.

3.4 IMMUNOASSAYS

3.4.1 TIME-RESOLVED FLUOROMETRY, INTRODUCTION

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.

3.4.2 ASSAY PROTOCOL

All incubations took place at 37°C, and reagents (100 µl/well) were added to plates at the concentrations recorded for the individual assays. Washes were conducted between each step with 250 µl/well of 25 mM Tris-phosphate, pH 8.0 containing 100 mM sodium chloride and 0.05% Tween 20.

To coat the polystyrene surface of 96-well FluoroNunc™ Maxisorp plates, antigens or capture antibodies were applied in carbonate buffer, pH 9.6 (35 mM sodium hydrogen carbonate, 15 mM sodium carbonate and 0.05% sodium azide). The plates were then sealed, incubated overnight at room temperature, and subsequently aspirated prior to the addition of 250 µl/well blocking solution (PBS with 1% bovine serum albumin (RIA grade; Sigma) and 5% sucrose. Following two hours incubation, the wells were washed three times immediately before transfer of the samples and standards to the plates.

Patient samples stored at -70°C were thawed and diluted with assay buffer [25 mM Tris-HCl, pH 7.8 with 150 mM sodium chloride, 0.1% polyoxyethylene ether W-1 (Sigma), 0.023% bovine gamma-globulins (Cohn fraction II; NBS Biologicals), 10 μM diethylenetriaminepentaacetic acid (Sigma), 0.0245% sodium azide and 0.25% BSA (Sigma)], which was used as the diluent throughout the remainder of the protocol. All samples were assayed in a dilution series as follows.

Vagina	1:4,	1:20,	1:100
Cervix	1:10,	1:50,	1:250
Endometrium	1:10,	1:50,	1:250
Fallopian tube	1:4,	1:20,	1:100
Serum	1:50,	1:250,	1:1250

'Blanks' consisted of assay buffer only. The samples and standards (100 μl/well) were incubated for 2 hours 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 hours. Four washes were conducted before the Europium labelled streptavidin (100 ng/ml) was applied to the plate and incubated for 1 hour. Following a series of five washes, Delfia^{R} Enhancement Solution was dispensed into the wells, after which the plate was agitated for 10 minutes on a Titertek^{R} shaker (Flow Laboratories). Fluorescence was measured on a Wallac 1234 Delfia^{R} fluorometer.

3.4.3 SPECIFIC ASSAYS

3.4.3.a Secretory IgA

Trap: Anti-human secretory component (Dako A/S Denmark) diluted to 1:500.

Standards: Human colostral IgA (Sigma-Aldrich Co. Ltd. Poole). Dilutions: 10, 5, 2.5, 1.25, 0.6, 0.3, 0.15, 0.075 µg/ml.

Detector Antibody: Affinity purified goat anti-human IgA, biotin labelled (KPL, Maryland USA).

3.4.3.b IgA2

Trap: Mouse monoclonal anti-human IgA2 (Nordic Tilbury, Netherlands) diluted to 5 µg/ml.

Standards: Purified human IgA2 (Chemicon International, Inc., Temecula, CA). Dilutions: 5, 2.5, 1.25, 0.6, 0.3, 0.15, 0.075 µg/ml.

Detector Antibody: Biotinylated goat anti-human IgA (KPL Maryland, USA).

3.4.3.c Salmonella typhi

Antibodies to Salmonella typhi lipo-polysaccharide were assayed essentially by the method described above.

Salmonella typhi endotoxin (Sigma) was dissolved at 10 mg/ml in grade Dimethylsulphoxide (BDH Poole) and then diluted to 1 mg/ml in carbonate buffer. This was mixed with an equal volume of methylated bovine albumen 1 mg/ml in carbonate buffer and incubated for 20 minutes at RT. For coating the plates the lipo-polysaccharide antigen was diluted to a concentration of 5 µg/ml in carbonate buffer.

The assay was standardised using rabbit antisera specific for the Salmonella typhi lipo-polysaccharide 09 antigen (PHLS Collingdale) with 04 and 08 antisera as negative controls.

3.4.3.d Hepatitis A

Formalin inactivated Hepatitis A virus antigen (Sorin Biomedica Diagnostics, Sallugia, Italy) was diluted to 1:10 in 0.1 M carbonate buffer at a pH of 8.5.

FluoroNunc Maxisorb plates were then coated with 0.1 nl. of antigen overnight at room temperature.

These were blocked for two hours at 37°C with 5% sucrose/ 1% BSA. Three washes were then carried out.

The samples were then added (three dilutions of each as detailed above) and incubated at 37°C for 2 hours. Four washes were then carried out.

Affinity purified goat anti-human IgG (and IgA) kpl alpha antibodies diluted 1:1000 were then applied and incubated for 2 hours at 37°C

Application of Europeum streptavidin, enhancement and reading were then carried out as described above.

Positive and negative controls were found from patient assays with proven Hepatitis A status (Abbot Axsym).

3.5 IMMUNOCYTOCHEMISTRY

In order to illustrate the antibody studies and gain further information about immune cell populations in the genital tract, biopsies were taken from endocervix, endometrium and fallopian tube in one of the cervically vaccinated cases, and in one non-immunised subject with cervical intra-epithelial neoplasia, an intra-uterine contraceptive device and chlamydial lower genital tract infection at the time of hysterectomy, for immunostaining using the panel of markers below.

Half of the biopsies were preserved in a paraffin block and half were frozen in liquid nitrogen. The panel of markers used is listed below.

CD3	Pan T cell marker
CD8	Suppressor/cytotoxic subset of T cells
CD45RA	Naive T cells and many B cells
CD45RO	Primed or memory T cells; these constitute approximately 70% CD4+ and 35% CD8+ in peripheral blood lymphocytes
CD20	Pan B cell marker (excludes plasma cells)
CD21	Reacts with the CR2 receptor staining follicular dendritic cells and mature B cell lymphoid aggregates
CD68	Macrophages
S100	Langerhans cells

The primary antibodies used to detect these markers are listed in table 2, along with antibody source and concentrations, method of antigen retrieval from paraffin section and whether monoclonal or polyclonal antibodies were used.

surface marker	primary antibody	concentration	antigen retrieval	monoclonal/ polyclonal
CD3	aCD3*	1/200	trypsin	polyclonal
CD8	aCD8#	1/400	microwave	monoclonal
CD45RA	4KB5*	1/100	none	monoclonal
CD45RO	UCHL1*	1/200	none	monoclonal
CD20	L26*	1/1000	none	monoclonal
CD21	1F8*	1/20	trypsin	monoclonal
CD68	KP1*	1/40	trypsin	monoclonal
S100	aS100*	1/1000	trypsin	polyclonal

Table 2. Immunostaining for fixed, paraffin embedded sections. Antibody characteristics.

* DAKO, Glostrup, Denmark

Oxford university, non-commercial. Available from DAKO, Glostrup, Denmark

3.5.1 IMMUNOSTAINING TECHNIQUES

A Streptavidin-Biotin Peroxidase Complex (StABCPx) immunostaining technique for fixed, paraffin embedded sections was used, using monoclonal or polyclonal antibodies. The principle is that antigen binds to either a monoclonal or polyclonal antibody which is then detected using a three stage Streptavidin-Biotin peroxidase complex technique. The enzyme label peroxidase is demonstrated using the chromogen diaminobenzidine (DAB).

The procedure used is described below. For each case (antibody profile) a section was included in which the primary antibody was replaced with TRIS buffered saline pH 7.6 (TBS). Control sections were used in which there was likely to be positive and negative staining. Positive control was usually provided by tonsillar tissue.

1. Sections de-paraffinised in xylene (2 x 5 mins.) and taken through 100% to 70% alcohol (1 minute each).

2. Endogenous peroxidase inhibited by treatment with freshly prepared inhibitor (0.5% H_2O_2 in methanol) for 10 minutes.

3. Wash in RO water.

4.

a) Antigen retrieval by microwave (see below).

b) Antigen retrieval using trypsin (see below).

c) If antigen retrieval unnecessary, 1% bovine serum albumin (BSA) in TBS applied for 30 minutes.

5. Primary antibody applied correctly diluted in TBS (see table 2) and incubated at 4°C for 18-24 hours (overnight).

6. Sections warmed to room temperature before washing in TBS (3 x 5 minutes).

7. Application of either biotinylated sheep anti-mouse Ig (BAM) for monoclonal antibodies or biotinylated swine anti-rabbit Ig (BAR) for polyclonal antibodies, diluted in TBS, for 30 minutes at room temperature.

8. Peroxidase labelled complexes prepared at a dilution of 1:200 in TBS. Left to complex on bench for 20 minutes.

9. TBS wash 3 x 5 minutes.

10. Prepared Streptavidin/Biotin peroxidase complexes applied for 30 minutes at room temperature.

11. TBS wash 3 x 5 minutes.

12. DAB substrate applied for 10 minutes. The principle being that the peroxidase label reacts with the hydrogen peroxidase and diaminobenzidine (DAB) to form an insoluble brown precipitate at the site of the antigen / antibody reaction.

13. TBS rinse followed by a wash in running tap water for two minutes.

14. Rinse in 70% alcohol.

15. Counterstain with Harris' haematoxylin for 1-2 minutes. Rinse in tap water for one minute. Differentiate in 1% acid alcohol for 5 seconds. Blue in running tap water for at least 5 minutes.

16. Dehydrate, clear and mount in DPX.

This technique is adapted from that described by Mepham & Britten (1990).

In those cases listed in table 2 above which required antigen retrieval using microwave or trypsin, the methods used were as follows.

3.5.2 ANTIGEN RETRIEVAL - MICROWAVE PRETREATMENT

In fixed paraffin-embedded tissue sections demonstration of some antigens is enhanced after pretreatment in a microwave oven using a buffer (citrate, EDTA or urea). Suitable sections were de-paraffinised and endogenous peroxidase blocked as described above. The following steps were then taken.

1. Plastic staining racks were filled with 24 slides and placed in a polythene box. To maintain a constant load three polythene boxes were always used, together with 72 slides. Blank slides, without sections, were used to make up this number.

2. Each box was filled with 330ml. of 0.01M citrate buffer. This was prepared using 2.1g of citric acid crystals, 1000ml. of RO water, and adjusting the pH to 6.0 with 1M sodium hydroxide (approximately 25ml.). The perforated lids were firmly closed.

3. A panasonic microwave oven NN-6450 (800 watts) was used and the three boxes were placed on markers, always on the same site on the oven plate. The oven was set for 25 minutes on medium power.

4. Once finished the boxes were carefully removed. The lid was removed and placed under cold running water for 2-3 minutes.

5. The sections were placed in staining trays and washed in TBS (2 x 5 minutes) before continuing with the immunostaining technique described above.

3.5.3 ANTIGEN RETRIEVAL - TRYPSIN PRETREATMENT

In fixed paraffin-embedded tissue sections the demonstration of some antigens is enhanced after pretreatment with the proteolytic enzyme trypsin. Sections were de-paraffinised and endogenous peroxidase blocked as before. The following steps were then taken.

1. Waterbath set at 37°C. Lidded glass trough filled with RO water and an empty lidded glass trough placed within the waterbath for the trypsin once prepared.

2. 0.5g of trypsin and 0.5g of calcium chloride mixed with 500 mls. of RO water in a beaker.

3. Beaker placed on hotplate with magnetic stirring bar and temperature gently raised to 37°C.

4. Slides placed in staining racks, rinsed in tap water and transferred to the RO water in the waterbath. Sections left for at least five minutes to reach 37°C.

5. As the temperature of the trypsin approaches 30°C, switch to pH meter and adjust the pH to 7.8 by adding 0.1M sodium hydroxide drop by drop.

6. Trypsin solution at pH 7.8 and 37°C quickly transferred to the pre-warmed empty trough in the waterbath. Pre-warmed sections transferred straight into the trypsin and left for appropriate time.

7. Sections placed in cold running water to arrest proteolysis.

8. Sections moved to staining trays, washed in TBS (2 x 5 minutes) before continuing with the immunostaining technique described above.

This technique was adapted from those described by Curran & Gregory (1977), and Mepham et al (1979).

4 STUDY DESIGN

4.1 VACCINE DELIVERY

Following local ethics committee approval, 24 women were recruited from the waiting list at the Princess Anne Hospital, Southampton, to undergo vaccination one month prior to abdominal hysterectomy. The aim was to achieve an approximately equal distribution of women in the proliferative and secretory phases of the menstrual cycle at the time of hysterectomy. The vaccination study groups were subdivided as follows.

1. 12 women were vaccinated parenterally with one dose (1ml.) of Havrix Monodose (SmithKline Beecham). This is a suspension of formaldehyde-inactivated hepatitis A virus adsorbed onto aluminium hydroxide. A 1 ml. prefilled syringe contains 1440 ELISA units. The vaccinations were given by the intramuscular route into the deltoid region.

2. 12 women were vaccinated endocervically with one dose (1ml.) of Havrix Monodose (SmithKline Beecham). This dose was given onto the endocervical mucosa using the prefilled syringe with a quill.

3. All of the women in the above two groups were asked to take Vivotif (Evans). This is a live attenuated *Salmonella typhi* (Ty 21a) vaccine. The vaccine is contained within an enteric-coated capsule and three capsules are taken on alternate days. Each capsule contains 2 x 10^{9} viable *Salmonella typhi* Ty 21a Berna cells. 17 women agreed to take this oral vaccine.

All vaccinations were given four weeks prior to hysterectomy, regardless of type or route of administration. The intramuscular and endocervical Havrix Monodose vaccinations were all given by the author. The Vivotif oral typhoid vaccinations were

self administered at home according to the manufacturer's instructions. The vaccinations were generally well tolerated and there were no significant side effects. There were occasional minor cutaneous reactions to the intramuscular hepatitis A vaccine but no treatments were required. One woman suffered with significant nausea after two doses of Vivotif and omitted the third dose.

Although the Havrix Monodose vaccine is fairly viscous, following endocervical administration there was some inevitable leakage into the vagina and some of the doses may have penetrated into the endometrial cavity.

In an attempt to improve vaccine delivery to the endocervical mucosa a short pilot study was carried out in which contraceptive cervical caps containing methylene blue dyed gel were applied to the cervix 24 hours prior to hysterectomy. In the five women agreeable to this intervention there was negligible contact with the endocervix as evidenced by a lack of blue dye and so this technique was abandoned in favour of that described above.

4.2 PATIENT RECRUITMENT

Patients on the waiting list for abdominal hysterectomy at the Princess Anne Hospital, Southampton, were selected as potential participants if they fulfilled the following inclusion and exclusion criteria.

a) Listed for total abdominal hysterectomy for menorrhagia (preferably dysfunctional uterine bleeding), pelvic pain or dysmenorrhoea.

b) No pre-operative evidence of significant pelvic pathology.

c) Normal cervical cytology history.

d) No history of previous tubal sterilisation.

e) Less than 44 years of age.

f) Not taking a combined oral contraceptive pill or progestogens.

g) Not previously vaccinated against hepatitis A or typhoid.

Patients identified from the medical records as potential participants were telephoned and then, if agreeable, were personally counselled by the author. Full medical histories were taken and information sheets were provided. Those patients who elected to participate in the trial gave written consent and were then seen again in the ensuing few days for administration of the vaccine. General practitioners were informed in writing that their patients had agreed to participate. There was no formal

randomisation as to the route of delivery of the hepatitis A vaccine and this tended to be guided by patient choice, with the endocervical administration being less popular. The only patients considered for entry into the trial were those who would benefit from immunity to Hepatitis A and/or Typhoid. These were identified at interview as:

1) Those who may visit areas of medium or high endemicity for hepatitis A in the next two years (i.e. anywhere outside northern and western Europe, N. America, Australia and New Zealand).

2) Institutional staff (i.e. nurseries, schools and institutions for the mentally handicapped).

3) Food packers or handlers, drain and sewerage workers.

It should be noted that if the trial dose of hepatitis A vaccine was administered onto the endocervix, then an immunising parenteral dose was offered at the time of surgery. More persistent hepatitis A immunity can be achieved for up to 10 years with a booster at 6-12 months, and this was offered to all participants in the trial.
One woman agreed to a booster cervical immunisation with Havrix Monodose which was given 24 hours prior to surgery.
The formaldehyde inactivated hepatitis A vaccine (Havrix Monodose) was chosen because of low immunity in the local population (approx. 12%) and for potential advantages in the event of foreign travel to those areas specified above. It also generates an antibody driven immune response which suited the methodology of this study, concentrating on humoral immunity. The oral typhoid vaccine (Vivotif) is one of two licensed vaccines for oral use in the United Kingdom. An important advantage of oral typhoid is that genetic engineering of Salmonella is well established and a wide range of other antigens have been inserted into salmonella to generate novel vaccines against a wide range of infectious diseases. Salmonella may provide an oral

vector for chlamydial and gonococcal epitopes. Oral polio was the alternative vaccine considered but rejected on two grounds. Firstly the universal immunisation with this vaccine in the U.K. would limit the percentage of responders and secondly the constraints of the viral capsid structure prevent the engineering of complete antigens from other pathogens into the vaccine strains.

4.3 SPECIMEN COLLECTION

Patients were seen on the day before surgery in order to document any vaccine-related side effects.

It is usual practise to perform salpingectomy in conjunction with oophorectomy and so if ovarian removal was planned at the same time as hysterectomy there was no problem in retrieving undamaged fallopian tubes. If ovarian removal was not planned, patients were separately consented for bilateral salpingectomy which requires only a slight modification of operative technique.

At the time of surgery 20 ml. of blood was taken and centrifuged for serum estradiol and progesterone levels. The rest of the sample was stored for analysis as described below. Washings were taken from the vagina, endocervix, endometrial cavity and fallopian tube as detailed below. I attended theatre for each case and collected the blood and vaginal washings. The uterus and fallopian tubes were then taken immediately to a laboratory to collect washings from the sites specified above.

The diluent/preservative used was Dulbecco A phosphate buffered saline (PBS) without calcium/magnesium and sodium bicarbonate, containing:

10% glycerol

1% RIA grade bovine serum albumin

0.1% sodium azide

0.1% tween 20

Washings were obtained from each site as follows:

1) Vagina. Once the patient was anaesthetised but prior to surgery, the vaginal fornices were thoroughly swabbed using two large cotton wool swabs. The tips of the swabs were cut off into 3 ml.of PBS and shaken for five minutes.

2) Cervix. The cervix was excised from the uterus and any blood carefully cleared from the cut surface. 5 ml. of PBS was circulated through the endocervical canal at 42 ml./hour for 40 minutes using a peristaltic P-3 pump (Fig.2).

3) Fallopian tube. 5 ml. of PBS was circulated at 42 ml./hour for 40 minutes through the excised but otherwise undamaged fallopian tube, using a peristaltic P-3 pump (Fig.3).

4) Endometrium. The cornua were occluded using Spencer Wells forceps. A foley catheter was then passed into the endometrial cavity and the balloon inflated with 2-3 ml. of water. The catheter was then drawn downwards to exclude leakages and 5 ml. of PBS was circulated in the endometrial cavity for 10 minutes using a syringe via the foley catheter (Fig.4).

5) Serum. 10 ml. of serum was sent for estimation of estradiol and progesterone levels in the hospital service endocrine laboratory. A 2% serum dilution in PBS (20 microl. of serum in 1 ml. of PBS) was stored in a refridgerator at 2-6 ^{o} C. 4 ml. of serum was stored neat at - 70 ^{o} C.

For each woman menstrual cycle phase was estimated using menstrual dates, serum progesterone and estradiol levels and retrospective histopathological analysis. The classification used was proliferative, secretory or menstrual phase.

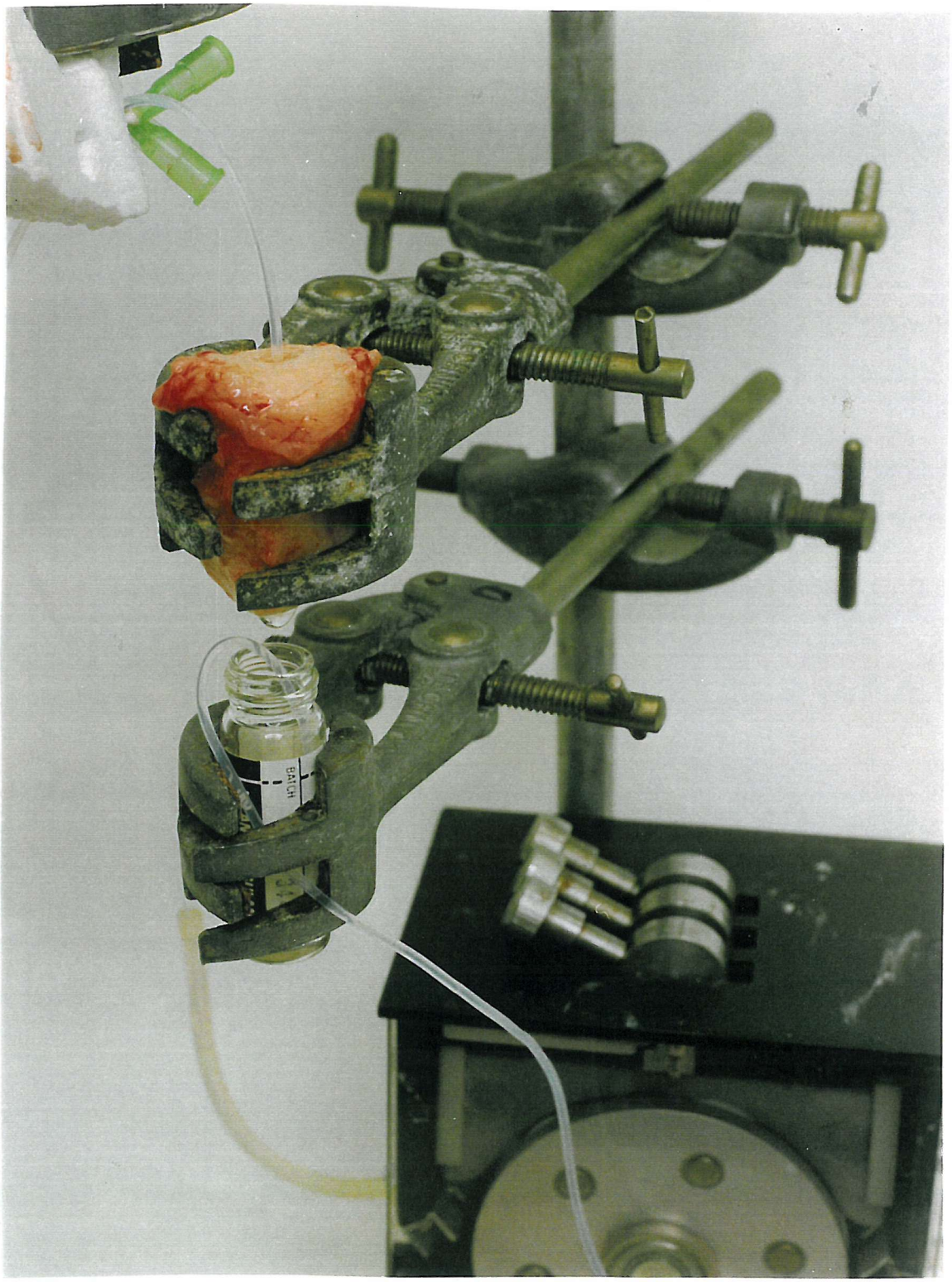


Fig. 2. Cervical washings. PBS is circulated through the endocervical canal using a peristaltic pump and capillary tubing in a continuous circuit for 40 minutes.

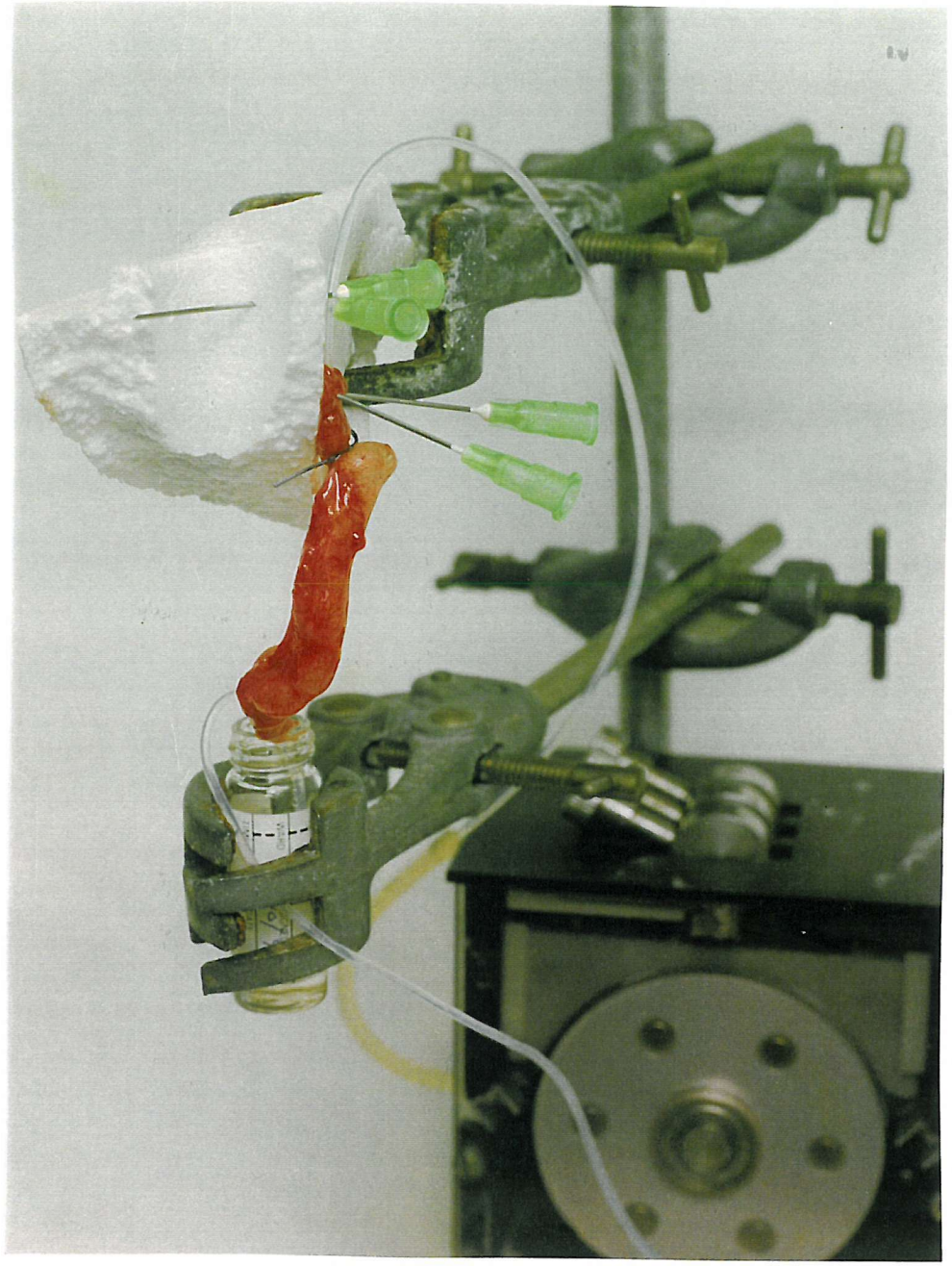


Fig. 3. Fallopian tube washings. The capillary tube is inserted into the cornual end of the tube and secured using vicryl suture material. PBS is circulated through the tube using a peristaltic pump in a continuous circuit for 40 minutes.

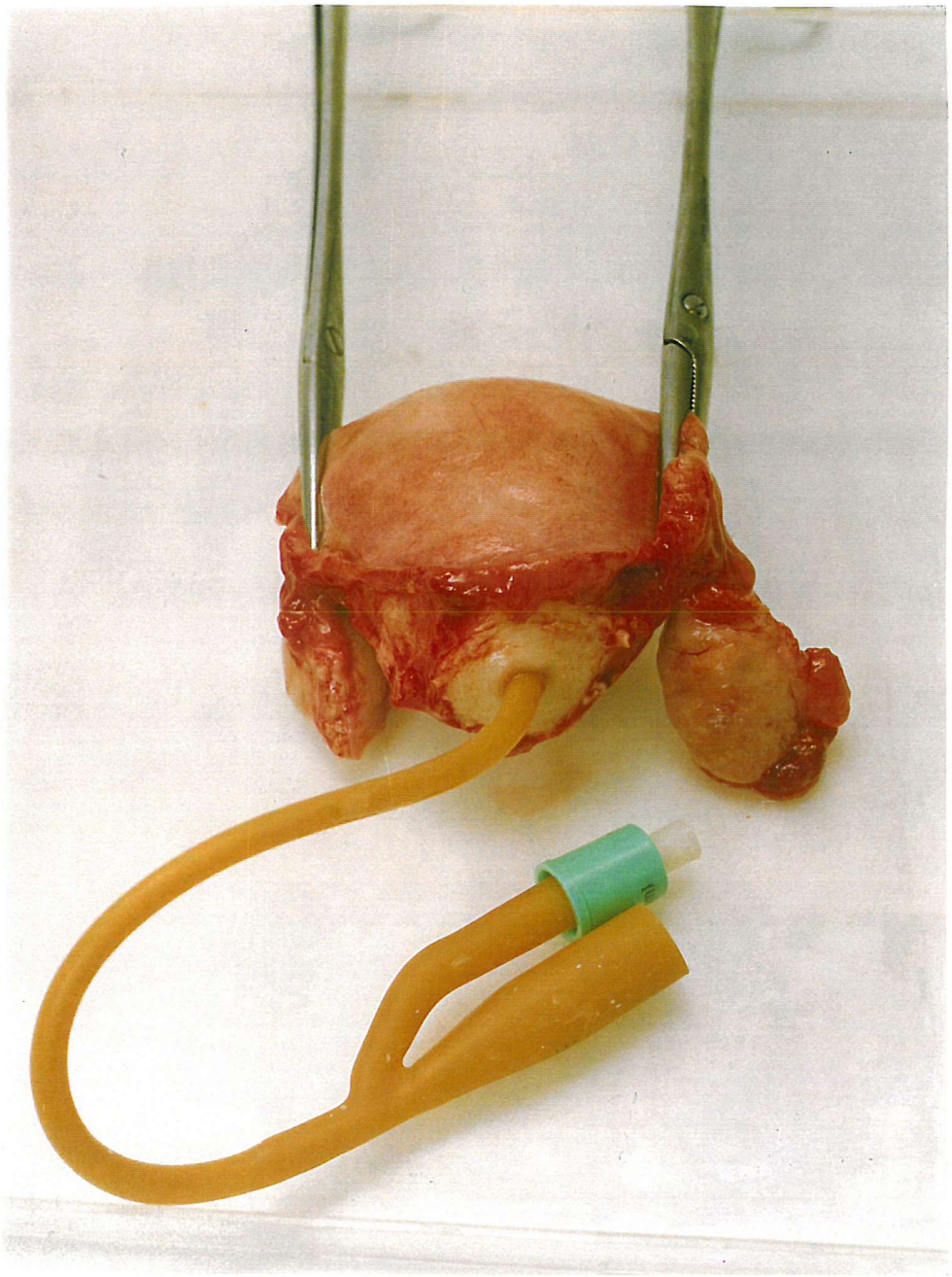


Fig. 4. Endometrial washings. The fallopian tubes are occluded at the cornua with Spencer Wells forceps. The foley catheter balloon is inflated with 2-3 ml. of PBS and drawn downwards. 5 ml. of PBS is drawn in and out of the uterus using a modified bladder syringe.

4.4 SAMPLE PROCESSING

The washings from the vagina, cervix, endometrium and fallopian tube were centrifuged to remove any cell debris and 1 ml. of each sample was stored in a refridgerator at 2-6 ° C and two 2 ml. samples were stored at - 70 ° C.

In four selected cases, biopsies were taken from the endocervix, endometrium and fallopian tube for immunocytochemical analysis as detailed later. One of these cases was randomly chosen from the cervically vaccinated subjects, there were two cases from healthy non-immunised women which acted as controls, and one of the cases had CIN3, an intra-uterine contraceptive device, and confirmed chlamydial infection of the lower genital tract at the time of hysterectomy.

Once all the washings and biopsies had been taken, the relevant histopathology request forms were completed and the surgical specimens were transported in formalinised saline to the pathology department for routine histopathological analysis. Prior agreement had been attained from the histopathologists that this sequence of events would not affect the quality of pathological analysis.

Inevitably some difficulties were encountered in gaining entirely undamaged surgical specimens for each site in each woman and these were mainly due to surgical difficulties and unexpected pelvic pathology. These anomalies are listed below:

1. Subtotal hysterectomy performed and so no cervical washings were obtained.
2. Vaginal hysterectomy performed and the fallopian tubes were too badly damaged to be useful.
3. The uterus was inadvertently opened by the surgeon after removal.
4. The cervix was extensively scarred from previous obstetric trauma and washings could not be obtained using the peristaltic pump.
5. No vaginal swabs were obtained.

In those cases above where washings could not be reliably collected using the peristaltic pump mechanism swabs were taken and processed as described for the vaginal washings.

5.RESULTS

5.1 HEPATITIS A SPECIFIC ANTIBODIES

The broad aim of this study was to evaluate antibody driven immune responses in the female genital tract to model vaccines introduced via different mucosal and parenteral sites. An evaluation of specific antibody responses in the lower genital tract (vagina and cervix) is a practical option in women not undergoing hysterectomy by collecting cervical and vaginal secretions *in vivo*. This can provide valuable information on potentially clinically useful immune responses against organisms such as the potentially oncogenic human papilloma viruses which breach mucosal defences and cause pathology predominantly in the lower genital tract. However in order to prevent pelvic inflammatory disease which implies an infection ascending into the upper genital tract an immune response against the primary pathogens (particularly *Chlamydia* and *N. gonorrhoeae*) in the endometrium and fallopian tubes would be beneficial. In order to analyse upper genital tract responses women undergoing elective abdominal hysterectomy were immunised four weeks prior to surgery.

In each case the Havrix monodose vaccine had been administered via either route exactly four weeks prior to hysterectomy in which the cervix, uterus and fallopian tubes were carefully excised undamaged. These results therefore demonstrate a "snapshot" of the humoral immune response throughout the genital tract after four weeks rather than a longitudinal observation of evolving or declining immunity.

The antibody responses to Hepatitis A vaccination are shown on scattergram plots in figures 5 and 6. Total specific IgA and IgG is plotted on a logarithmic scale for each patient against genital tract site and serum. Results for each anatomical site are depicted in red or blue dependant on whether the Hepatitis A vaccine (Havrix monodose) was administered cervically or parenterally. Statistical results and analysis are shown beneath each figure.

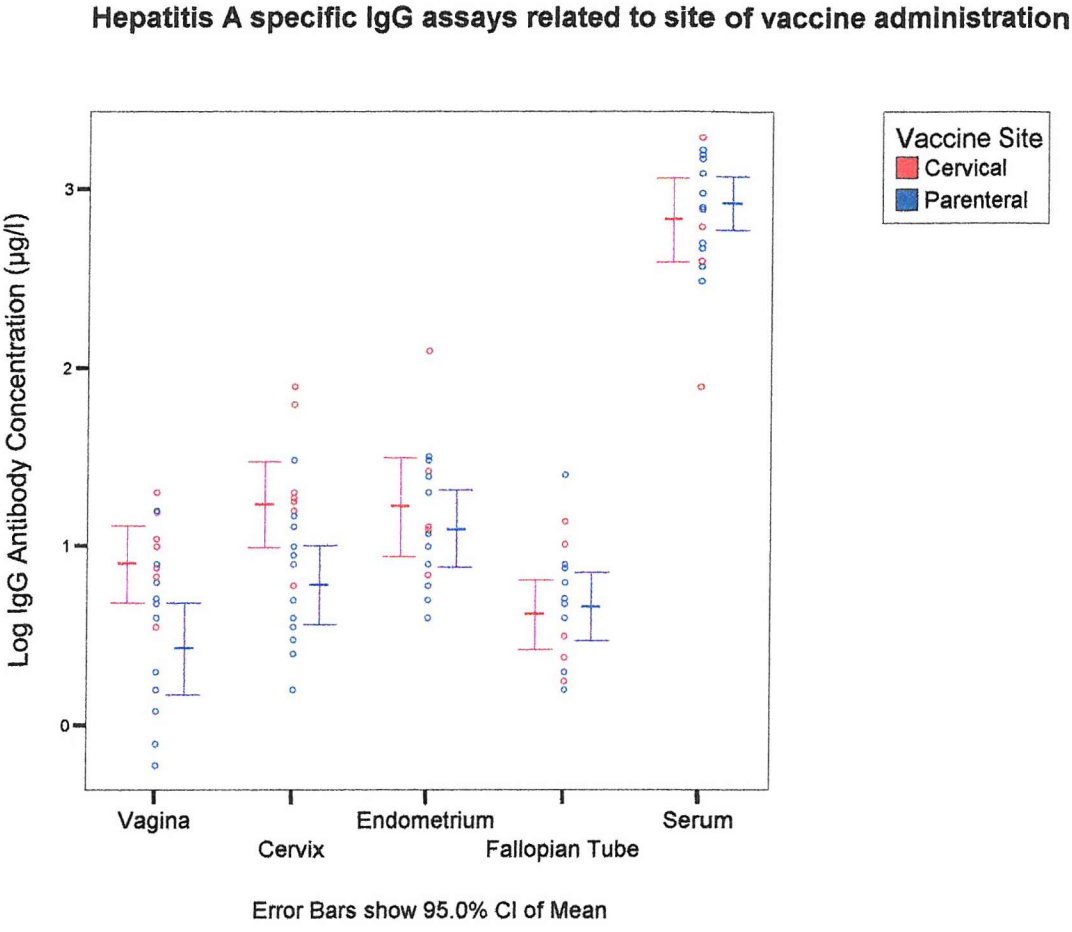


Figure 5. An independent samples student t test was used on the means. Significantly more vaccine-specific IgG antibody was detected in the vagina (p=0.006) and cervix (p=0.005) following cervical vaccine administration.

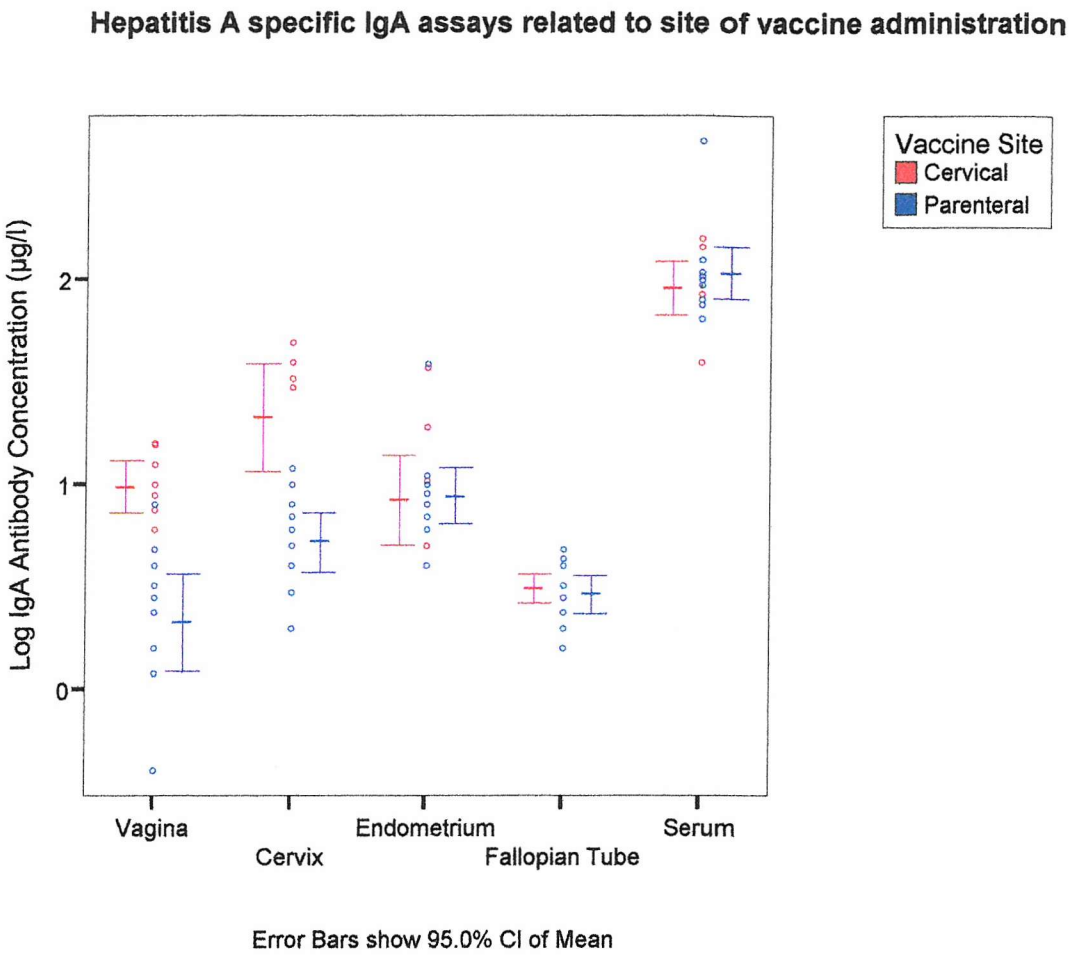


Figure 6. An independant samples student t test was used on the means. Significantly more vaccine-specific IgA antibody was detected in the vagina (p=0.00007) and cervix (p=0.0001) following cervical vaccine administration.

5.1.1 SPECIFIC HUMORAL RESPONSE

In this arm of the study, Hepatitis A (Havrix monodose) vaccine has been used to demonstrate both a serum and mucosal immune response throughout the female genital tract following local cervical or parenteral vaccination. There were significant IgA and IgG responses in serum four weeks after vaccination which developed following both cervical and intra-muscular vaccination. No significant differences in mean antibody concentration were observed when comparing vaccination route. The implication is that antigen presenting cells in the cervix can initiate a serum response which is as effective as that observed following intra-muscular vaccination. It would seem likely that macrophages and dendritic cells transfer viral antigens into the reticulo-endothelial system.

High levels of specific IgG and IgA were found, particularly in the vagina, cervix and endometrium. A lesser but significant response was noted in the fallopian tube. The highest levels of specific IgA and IgG were found in the vagina and cervix following cervical vaccination. These responses were significantly greater than those generated by parenteral vaccination (IgG vagina $p=0.006$; IgG cervix $p=0.005$. IgA vagina $p=0.00007$; IgA cervix $p=0.0001$). Consistently high levels of specific antibody (IgA and IgG) were found in the endometrium regardless of vaccination route and this probably reflects its larger surface area and the slightly different method employed to wash out antibodies. No significant differences were seen for either isotype in the upper genital tract when comparing vaccination route.

In general similar levels of specific IgG and IgA were observed throughout the genital tract and it would seem that IgG (whether serum-derived or locally produced) is at least as important as IgA in providing humoral immunity at the mucosal surface. We cannot be certain of the origin of these antibodies which may reflect passive transfer of serum-derived antibodies or local antibody synthesis. Because of deficiencies in the

commercially available reagents we were unable to assay Hepatitis A specific secretory IgA which would confirm local synthesis. However IgG and IgA-containing cells and secretory component have all been identified in the female genital tract (Kutteh et al 1994, Bjercke & Brandtzaeg 1993).

The Havrix monodose vaccine was administered to the endocervix using a quill and although the vaccine is fairly viscous, thus prolonging exposure time to the endocervix, it is inevitable that some vaccine contacted endometrial tissue and some leaked into the vagina.

The dominant site of antigen uptake and processing is however the cervix and both our own and other immunocytochemical studies lend weight to this theory. Assuming that the cervix is responsible for antigen uptake and processing this would probably be influenced by existing cervical inflammation secondary to infection (possibly chlamydial, gonococcal or bacterial vaginosis), cervical intra-epithelial neoplasia, cervical ectropion, menstrual cycle phase and use of hormonal contraceptives. These factors could not be controlled for in our study but will clearly need to be addressed in the future to optimise clinically useful vaccine administration in the genital tract. Our data do not demonstrate significant menstrual cycle variation in IgG or IgA subclasses which would tend to exclude that factor from the equation although these results are at variance with other animal and human studies which would suggest that immunity in the genital tract is influenced by sex steroids. This will be discussed in greater depth below.

Two interactive factors must be considered in explaining the responses that we have demonstrated, persisting four weeks after immunisation. The first is that the Hepatitis A vaccine caused local inflammation of the cervix. This would allow the passive leakage of specific antibody into the cervico-vaginal secretions. The second is that cervical vaccination stimulated significant local mucosal immune responses. The answer probably lies in a combination of both and our data confirms that of previous studies demonstrating that greatest levels of antigen-specific IgA and IgG are generated in mucosal regions

closest to the site of antigen exposure (Haneberg et al 1994, Ogra & Karzon 1969, Kozlowski et al 1997), although remote mucosal vaccination produces a lesser serum and local antibody response. Our observations confirm data both in humans (Ogra & Ogra 1973, Bouvet et al 1994 and Kozlowski et al 1997) and non-human primates (Marx et al 1994 and Lehner et al 1993) demonstrating humoral responses in the genital tract to vaccines delivered parenterally, to the vagina or in remote mucosal sites. Importantly, Wassén et al (1996) noted that cervico-vaginal vaccine administration is more effective than other routes in generating a specific local IgG and IgA response. However, direct quantitative comparisons between genital tract antibody responses to cervical and parenteral vaccinations in the human model have not previously been reported.

We have demonstrated not only a significant IgA but also IgG response in the lower genital tract, particularly following cervical vaccination. Whilst this may be passively serum derived there is evidence to suggest local synthesis. A significant percentage of the B cells in human cervical mucosa are IgG positive and the distribution of IgG subclasses in cervico-vaginal secretions differs from that in serum (Hocini et al 1995, Kutteh et al 1993). Local vaginal vaccination has also demonstrated a cervico-vaginal IgG response in the absence of a specific serum response (Ogra & Ogra 1973).

Regardless of the exact origin of the specific IgG and IgA antibodies that we have demonstrated, there was a markedly greater response in the lower genital tract following cervical vaccination and this finding would tend to support the fact that for maximum responses to sexually transmitted disease vaccines the need is to involve local mucosal vaccination.

5.2 IMMUNOCYTOCHEMISTRY

Cellular immunity in the female genital tract has been incompletely described in the human model.

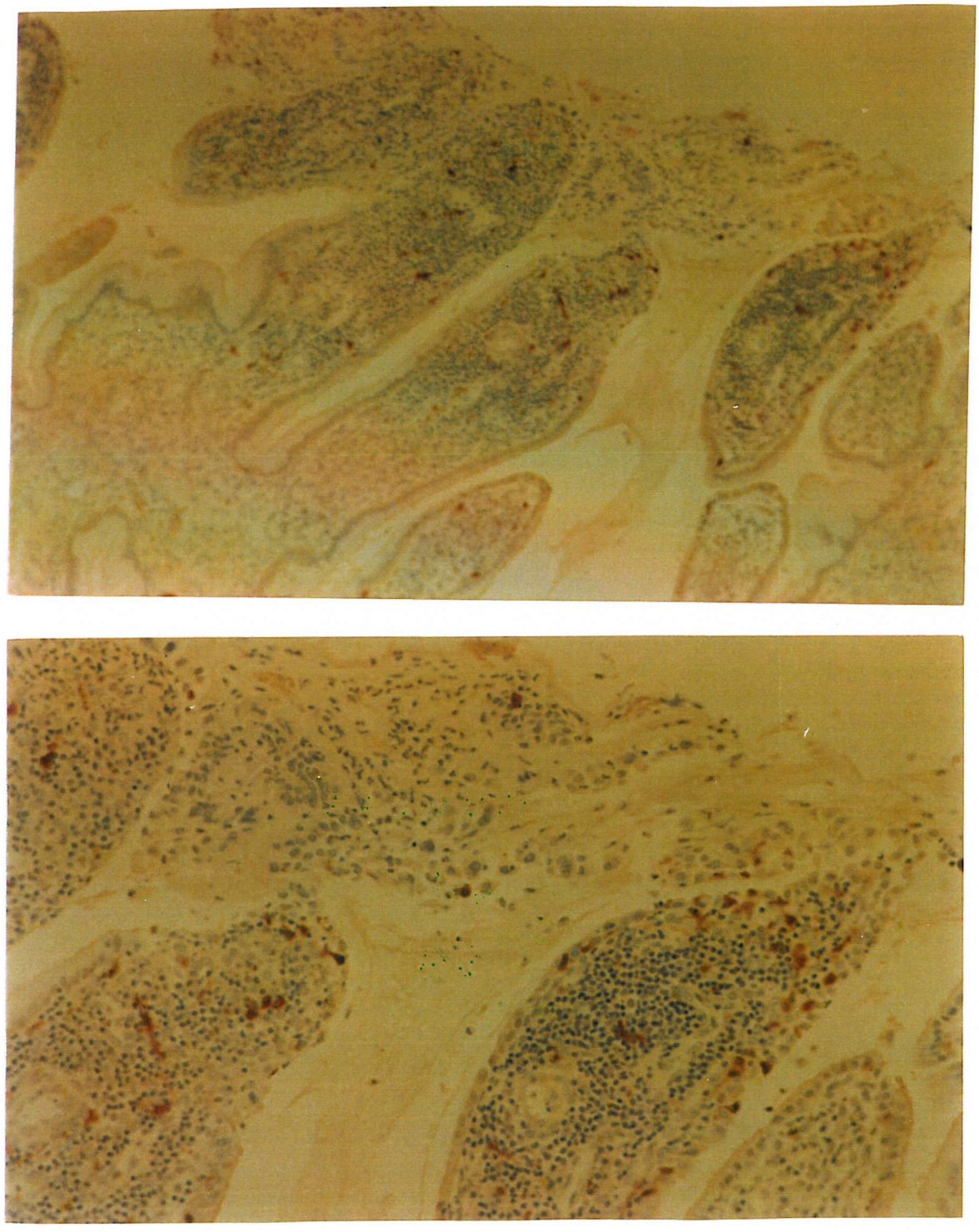
In order to demonstrate that the afferent and efferent limbs necessary for a humoral immune response in the female genital tract are present and to lend weight to a local origin for the specific antibody responses described above, an immunocytochemical examination of the uterus and cervix was performed on one of the cervically vaccinated subjects. In addition biopsies from a patient with cervical intra-epithelial neoplasia, an intra-uterine contraceptive device and confirmed chlamydial infection of the lower genital tract at the time of hysterectomy were analysed as representative of a focal natural immunological reaction. Control sections from healthy non-immunised cervix and endometrium were taken and did not demonstrate the changes shown below.

Markers for macrophages (CD68+) and Langerhans cells (S100) were used to explore potential antigen presentation. CD3+ and CD8+ T lymphocytes were investigated as well as naive T cells (CD45RA+) and memory or activated T cells (CD45RO). A pan B lymphocyte marker (CD20+) was used and organised B cells were demonstrated using CD21+ which stains dendritic networks and B cell lymphoid aggregates.

The results are depicted in figures 7 to 18.

Figures 7 and 8 demonstrate sections of glandular endocervical epithelium following immunostaining for S100 which is expressed by Langerhans cells. The Langerhans cells are stained brown and show characteristic dendritic morphology. In the right side of the higher power view Langerhans cells are seen in close apposition to intra-epithelial and sub-epithelial lymphocytes and glandular epithelium suggesting a role in antigen presentation in the endocervix.

Antigen presenting cells in endocervical epithelium four weeks after cervical Hepatitis A vaccination



Figures 7 and 8. Sections of endocervical glandular epithelium. Magnification x 100 and x250

Figure 9 shows a section of cervical squamous cell epithelium following immunostaining for S100 which is expressed by Langerhans cells. The surface epithelium is at the top of the section and the basement membrane can be seen in the lower left quadrant. Langerhans cells, stained black and showing characteristic dendritic morphology can be seen just above the basement membrane in close apposition to lymphocytes which are clustered around the basement membrane.

Figure 10 shows a section of glandular endocervical epithelium following immunostaining for CD68 which is expressed by macrophages. The macrophages are stained black and arrowed. Their position, close to glandular epithelium and in the sub-epithelium could be consistent with antigen transport from the epithelial surface, antigen processing and presentation.

Antigen processing and presenting cells in the endocervix four weeks after cervical Hepatitis A vaccination

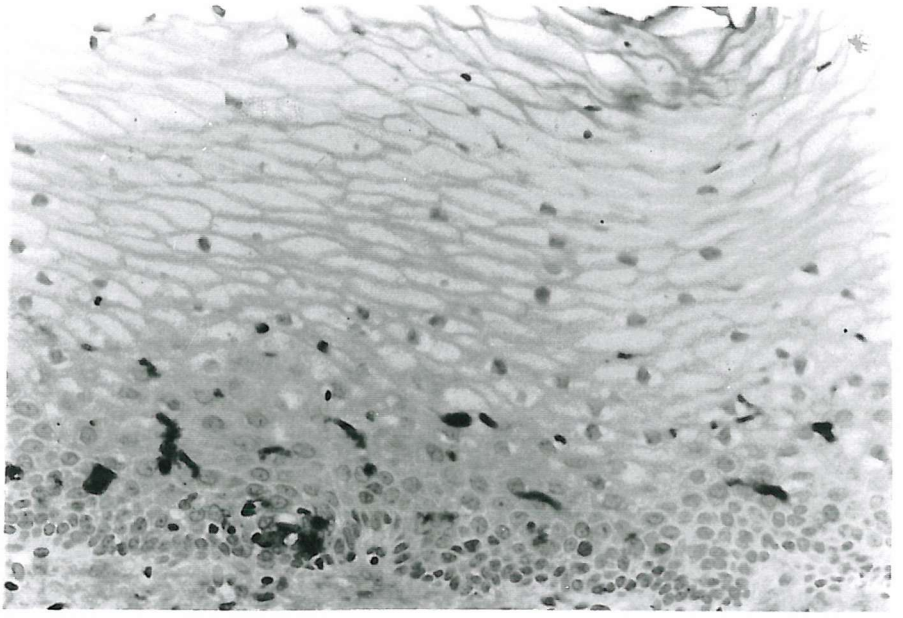


Figure 9. Section of squamous cell cervical epithelium adjacent to the transformation zone. Magnification x 400

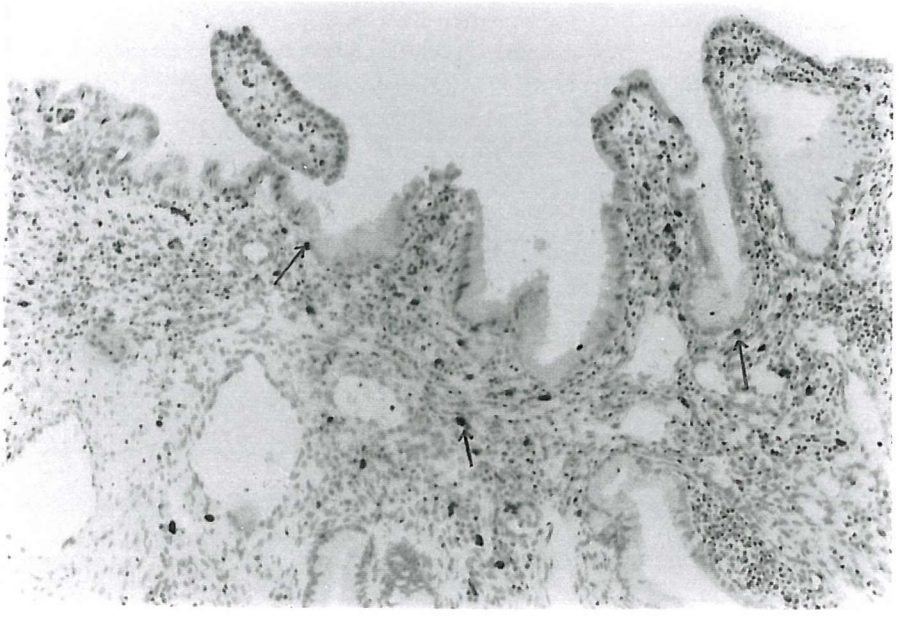


Figure 10. Section of endocervical glandular epithelium adjacent to the transformation zone. Magnification x 400

Figure 11 shows a section of proliferative phase endometrium following immunostaining for CD20 which is a pan B cell marker but excludes plasma cells. Endometrial glands are shown on the left and right sides of the section. There is a diffuse aggregate of B cells between the endometrial glands.

Figure 12 shows a section of endocervix following immunostaining for CD20. A diffuse aggregate of B cells stained black is seen below the epithelium. A capillary is seen in cross section on the left side of the section (arrowed).

Antibody producing cells in endometrium and endocervix four weeks after cervical Hepatitis A vaccination

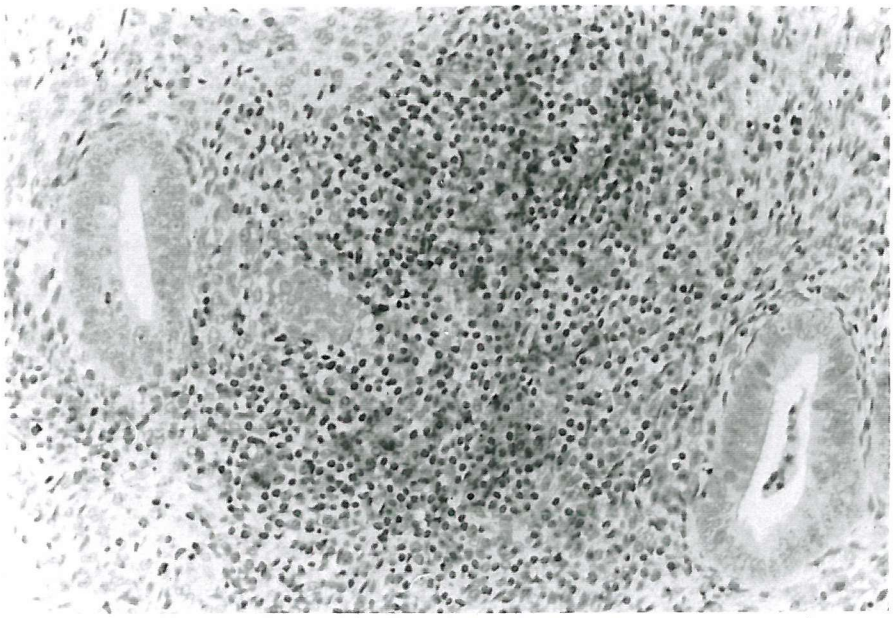


Figure 11. Section of proliferative phase endometrium. Magnification x 100

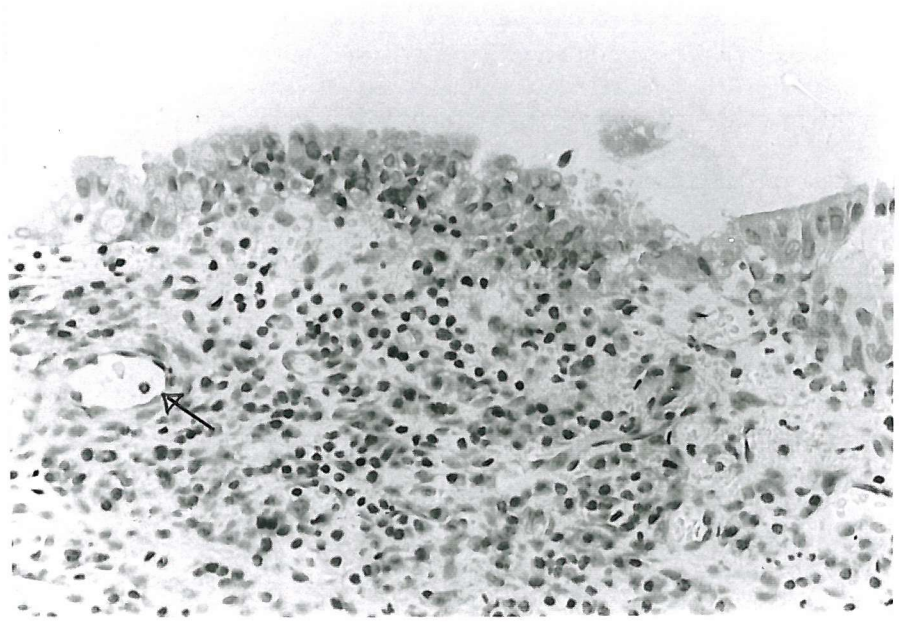


Figure 12. Section of endocervical epithelium adjacent to the transformation zone. Magnification x 200

Figure 13 shows a section of ectocervical squamous epithelium following immunostaining for CD45RO which is expressed by primed or memory T cells. The positively stained (black) T lymphocytes are clustered around the basement membrane which is arrowed. The ectocervical epithelium is seen at the top of the section.

Figure 14 shows a section of endocervical glandular epithelium following immunostaining for CD8, a marker for suppressor/cytotoxic sub-set of T cells. Positively stained cells (arrowed) are diffusely scattered beneath the epithelium.

T lymphocytes in sections of ecto- and endocervical epithelium four weeks after cervical Hepatitis A vaccination

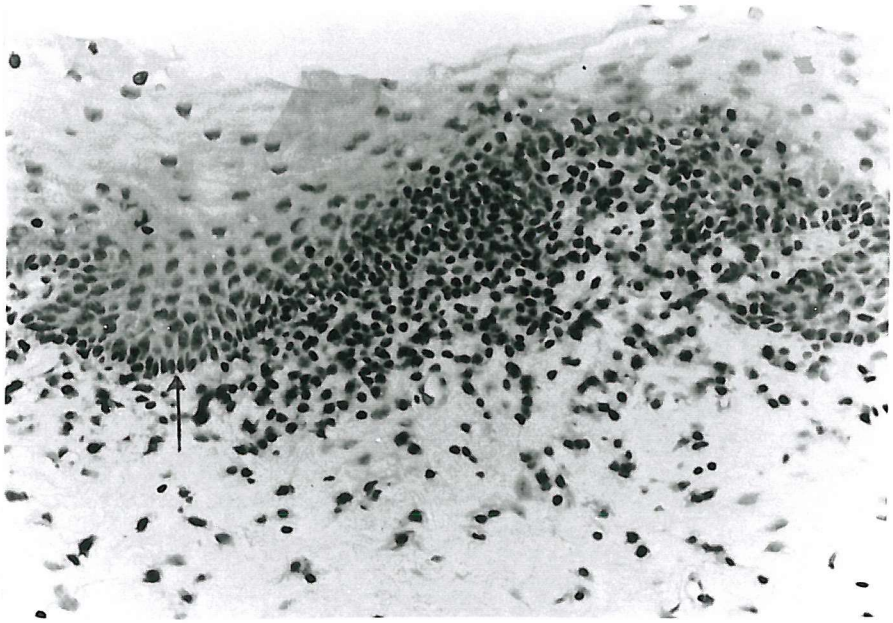


Figure 13. Section of squamous cell cervical epithelium from the ectocervix. Magnification x 200

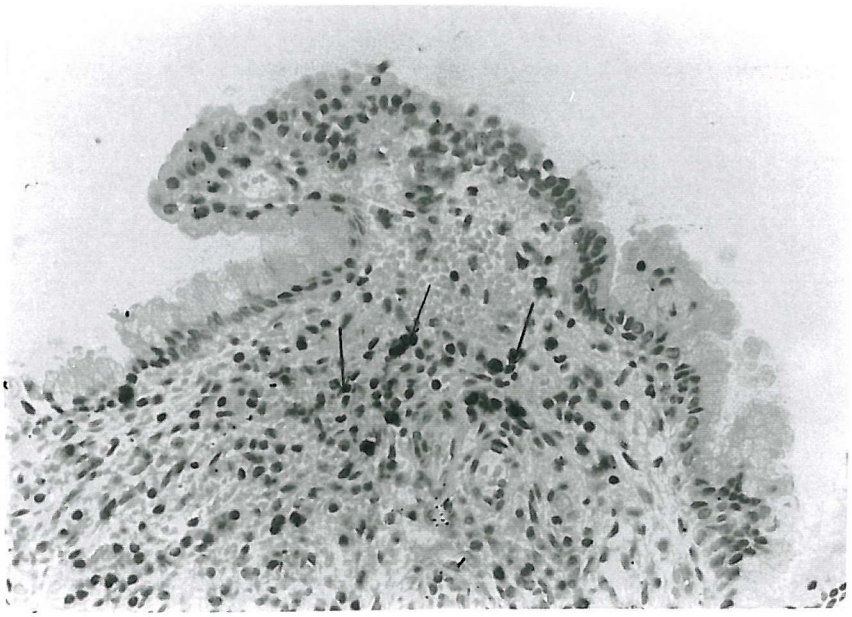


Figure 14. Section of endocervical glandular epithelium. Magnification x 100

Figure 15 shows a section of late secretory phase endometrium following immunostaining for CD3 which is a pan T cell marker. There are three T cell aggregates, stained brown, and several convoluted endometrial glands are visible.

Figure 16 shows a section of transitional zone cervix following immunostaining for CD3. The squamous epithelium is on the right superior border of the section. There is a diffuse aggregate of T cells, stained brown, in the sub-epithelium.

Aggregates of T lymphocytes in endometrial and endocervical sections associated with natural chlamydial infection and an intra-uterine contraceptive device

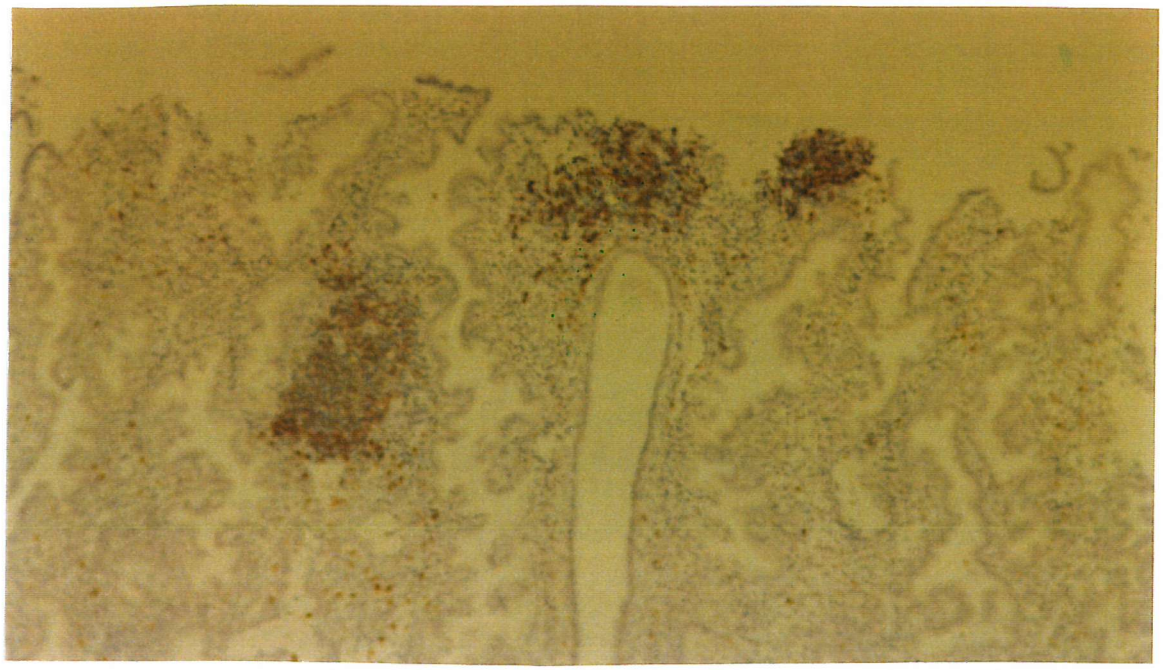


Figure 15. Section of late secretory phase endometrium. Magnification x 100

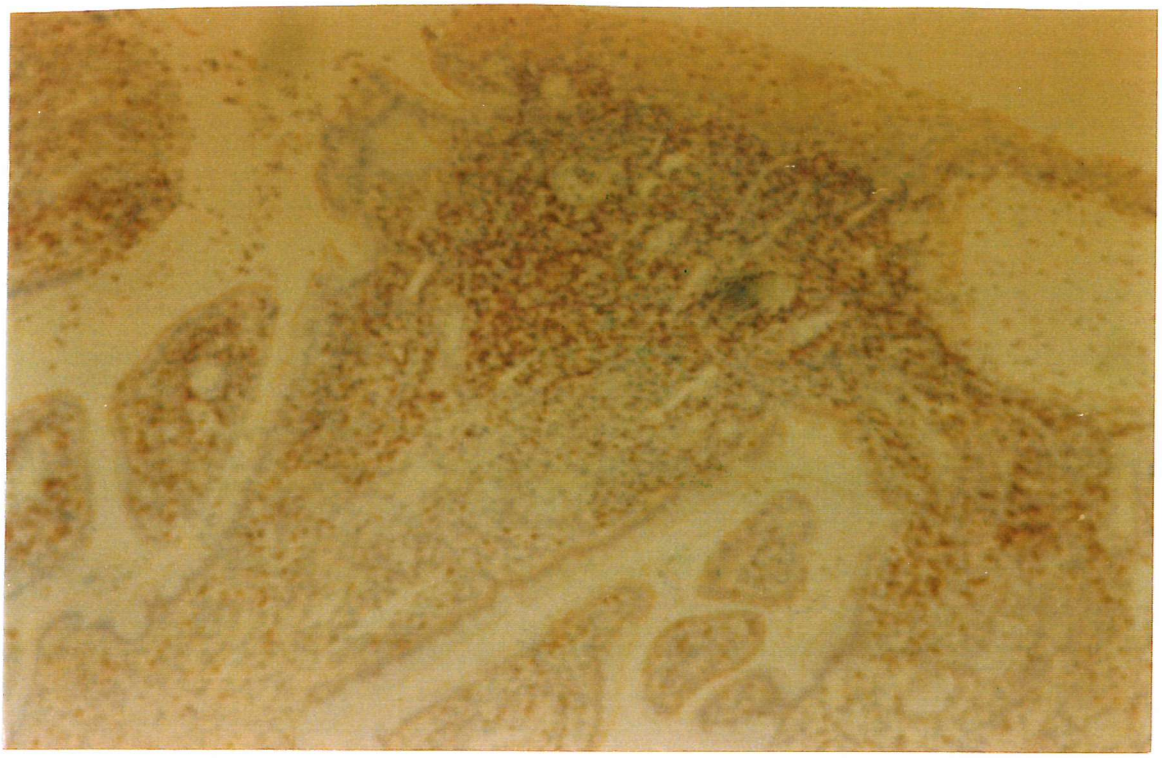
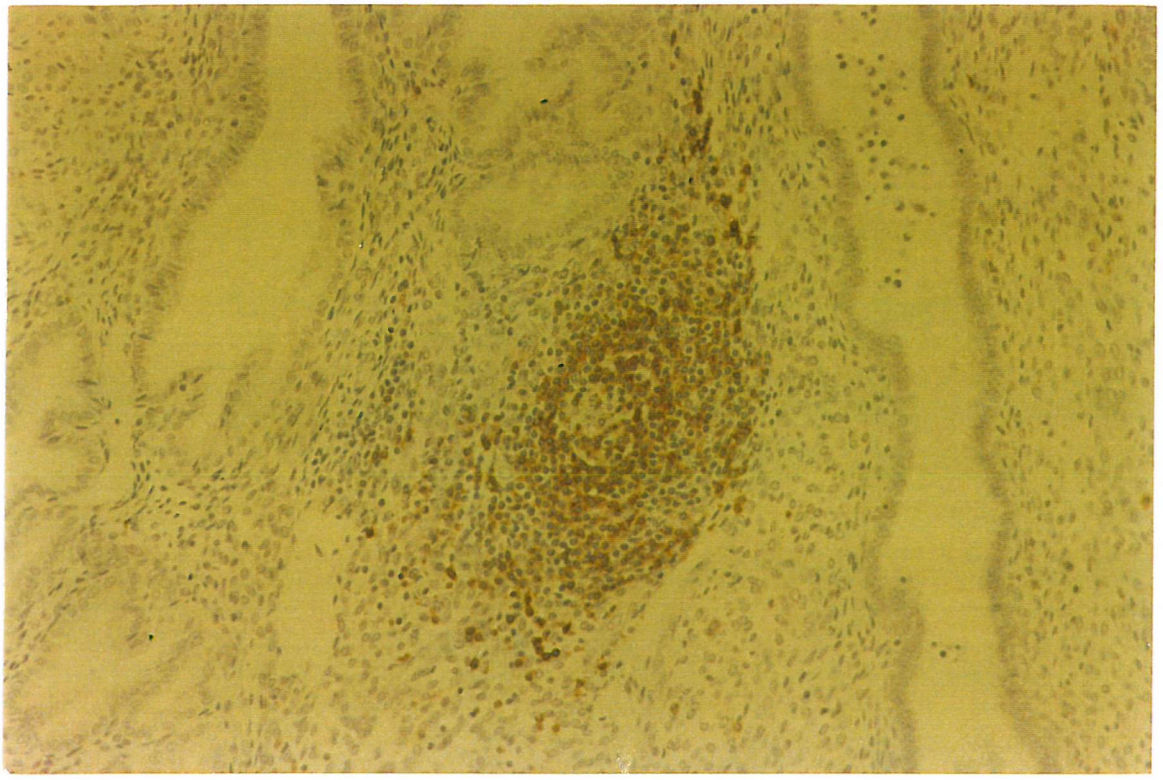
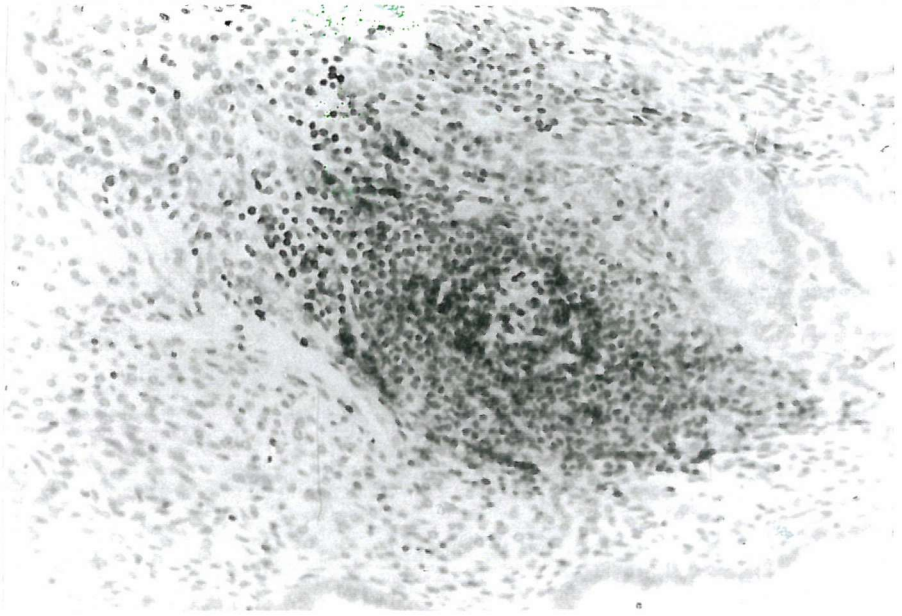


Figure 16. Section of transitional zone of endocervix. Magnification x 100

Figures 17 and 18 show sections of late secretory phase endometrium following immunostaining for CD21 which reacts with the CR2 receptor staining follicular dendritic cells and mature B cell lymphoid aggregates. B cell follicles are seen in each section stained brown (fig.17) and black (fig.18).

Mature B cell lymphoid aggregates/follicular dendritic networks in endometrial sections associated with natural chlamydia infection and an intra-uterine contraceptive device





Figures 17 and 18. Sections of late secretory phase endometrium. Magnification x 100

Induction of immunity in the female genital tract has not been described as clearly as in the gastro-intestinal tract and M cells or specialised antigen presenting cells (APCs) have not been described. Dendritic antigen presenting cells (Langerhans cells) and macrophages are present in the cervical epithelium and lamina propria as demonstrated in Figures 7-10 and are most concentrated in the transformation zone. Close contact is observed between Langerhans cells, intra-epithelial lymphocytes, capillary and glandular epithelium, and macrophages suggesting a role in antigen presentation which has been confirmed in an in vitro setting (Fahey et al 1999).

Diffuse aggregates of B lymphocytes are noted in the endometrium following cervical vaccination in close apposition to endometrial glands and also within and below the cervical epithelium, suggesting local antibody production (figures 11 and12). Primed or memory T lymphocytes (CD45RO) are shown in figure 13 clustered in a distinct band beneath the epithelium and dispersed in the epithelium and lamina propria. A similar distribution of CD3+, CD8+ and CD4+ lymphocytes have been described by Johanssen et al (1999) who also noted CD38+ plasma cells in the lamina propria of the cervix and vagina, and lymphoid aggregates containing CD19+ and CD20+ B cells in the cervix.

CD8+ T lymphocytes are demonstrated within the cervix in figure 14 where they may play a role in immunosuppression facilitating sperm entry to the upper genital tract. The majority of endometrial T lymphocytes are CD8+ and may play a role in endometrial immunosuppression during early pregnancy.

Figures 15 to 18 demonstrate a focal immune response to chlamydial infection, CIN, and an intra-uterine contraceptive device. Well defined CD3+ lymphoid aggregates are noted both in the endometrium (Figure 15) and cervix (Figure 16). B cells organised into mature lymphoid aggregates/dendritic networks (CD21+) are shown in the endometrium (Figures 17 and 18). No such lymphoid nodules were demonstrated in those subjects who

had undergone cervical vaccination and have previously only been demonstrated in reponse to natural infection in the genital tract.

These results suggest that the afferent and efferent arms of a local humoral reponse to locally presented antigen is present in the lower genital tract.

5.3 SALMONELLA TYPHI SPECIFIC IgG ANTIBODY RESPONSE

To investigate the humoral immune response in the genital tract following oral immunisation, genital tract washings and serum were analysed for antibodies to *Salmonella typhi* 09 antigen which is the dominant lipopolysaccharide antigen of *Salmonella typhi*. Antibody responses to this antigen have been widely used to monitor the success of oral typhoid vaccination. Nevertheless the 09 antigen is not an ideal antigen since it is shared with *Salmonella enteritidis* and *Salmonella dublin*. Antibody responses to *Salmonella typhi* are sometimes measured using the Vi capsular polysaccharide but again this is not specific for typhoid since *S.dublin* carries the Vi antigen.

Genital tract washings were taken at the time of hysterectomy, four weeks after vaccination with a live attenuated *Salmonella typhi* Ty 21a oral vaccine (Vivotif, Evans). 17 women were vaccinated in this arm of the study. The 7 women who did not receive this vaccine acted as controls.

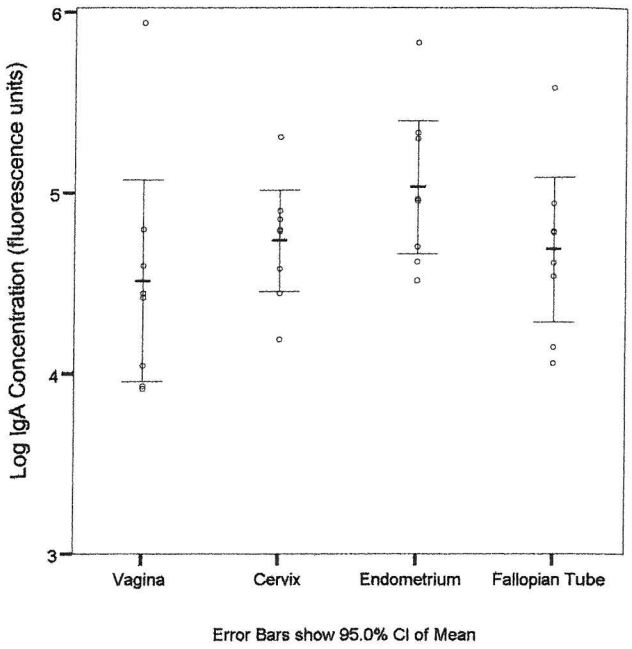
Specific IgG and IgA was assayed using time resolved fluorometry as previously described. Secretory IgA was also assayed but the only commercial source of monospecific antibody to human secretory component is available from DAKO (Glostrup, Denmark). For use in immunoassay this rabbit antibody is available coupled to horseradish peroxide, readily detectable in Elisa systems. Unfortunately we found this antibody to be non-specifically sticky giving high backgrounds and thus obscuring weak specific sIgA reponses to the vaccine.

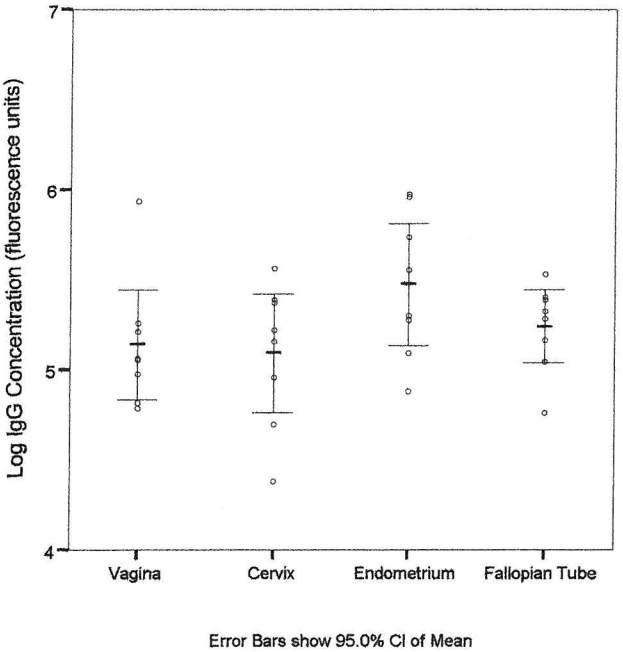
A population background level of antibodies to the salmonella 08 antigen was established for the controls (fluorescence units). The percentage of women immunised orally who had generated a significant serum IgG response was then evaluated in comparison to the population background, expressed as the mean serum count of the controls plus two standard deviations. Eight of the 17 women vaccinated (47%) had seroconverted.

For those women who had seroconverted, specific IgG and IgA levels, expressed in fluorescence units, were compared for each genital tract site. The results are shown in figure 19.

Low levels of specific IgG and IgA to the 09 antigen were detected and no significant differences were demonstrated in responses between different genital tract sites.

Figure 19. IgG and IgA antibodies specific to salmonella typhi LPS 09 antigen in the female genital tract





Oral vaccination is clearly a highly acceptable route for vaccine administration. However, whilst specific antibody is often detected in the female genital tract following oral vaccination, IgG and IgA levels are usually low. Following oral vaccination with the cholera toxin B-subunit, only three of seven women responded with detectable IgA and IgG anti-CTB antibodies in the genital tract as opposed to a generally greater response in six out of seven women vaccinated vaginally (Wassén et al 1996). Using the same oral vaccine, Kozlowski et al (1999) demonstrated a weak and transient rise in specific IgG in cervical secretions. Again using a cholera toxin B subunit oral vaccine, Rudin et al (1998) demonstrated 5-fold CTB-specific IgA and 20 fold specific IgG responses in vaginal secretions associated with a strong serum response. However, nasal vaccination was superior to the oral route in eliciting genital tract responses.

An important implication of both the Hepatitis A and Salmonella vaccination studies is that both IgG and IgA are present at the mucosal cell surface since they can be removed by non-traumatic lavage. If it can be demonstrated that significant concentrations of serum antibodies are translocated to the surfaces of the upper and lower genital tracts parenteral vaccination could be equally effective to cervical or rectal vaccination in the prevention of infection in the female genital tract.

5.4 GENITAL TRACT AND SERUM DISTRIBUTION OF IgG AND IgA SUBCLASSES

The first barrier encountered by potential mucosal pathogens is provided by antibody in secretions. Secretory IgA is actively transported through epithelial cells and in the gastro-intestinal tract is the dominant isotype. Because of rapidly evolving interest in mucosal immune protection against sexually transmitted organisms in the genital tract, knowledge regarding local secretory and sytemic humoral immunity is increasing. It may be that serum derived or locally produced IgG present at mucosal surfaces in the genital tract is at least as important as secretory IgA. In an attempt to further define humoral immunity in the genital tract we have analysed mucosal washings from the upper and lower genital tracts for the distribution of IgG and IgA antibody subclasses.

30 women undergoing abdominal hysterectomy for benign causes, usually dysfunctional uterine bleeding, were evaluated. Menstrual cycle phase was determined for each woman to assess whether humoral immunity in the genital tract is under menstrual cycle control.

Techniques were developed as previously described to obtain washings from the endocervix, endometrium and fallopian tube, and swabs were taken from the vaginal fornices and shaken in the PBS medium. Care was taken to use a gentle washing technique from intact mucosal surfaces and to avoid serum contamination. IgG1-4 and IgA1 were assayed using commercial radial immunodiffusion kits. IgA2 and secretory IgA were assayed using time-resolved fluorometry.

The results are presented in scattergram format comparing antibody subclass concentration for each genital tract site. Menstrual phase comparisons for the means are shown with error bars showing 95% confidence intervals. Figures 20 to 23 show the IgG subclasses, figures 24 to 26 IgA subclasses and secretory IgA, and figure 27 serum IgG and IgA subclasses.

Figure 20. Genital tract distribution of IgG1

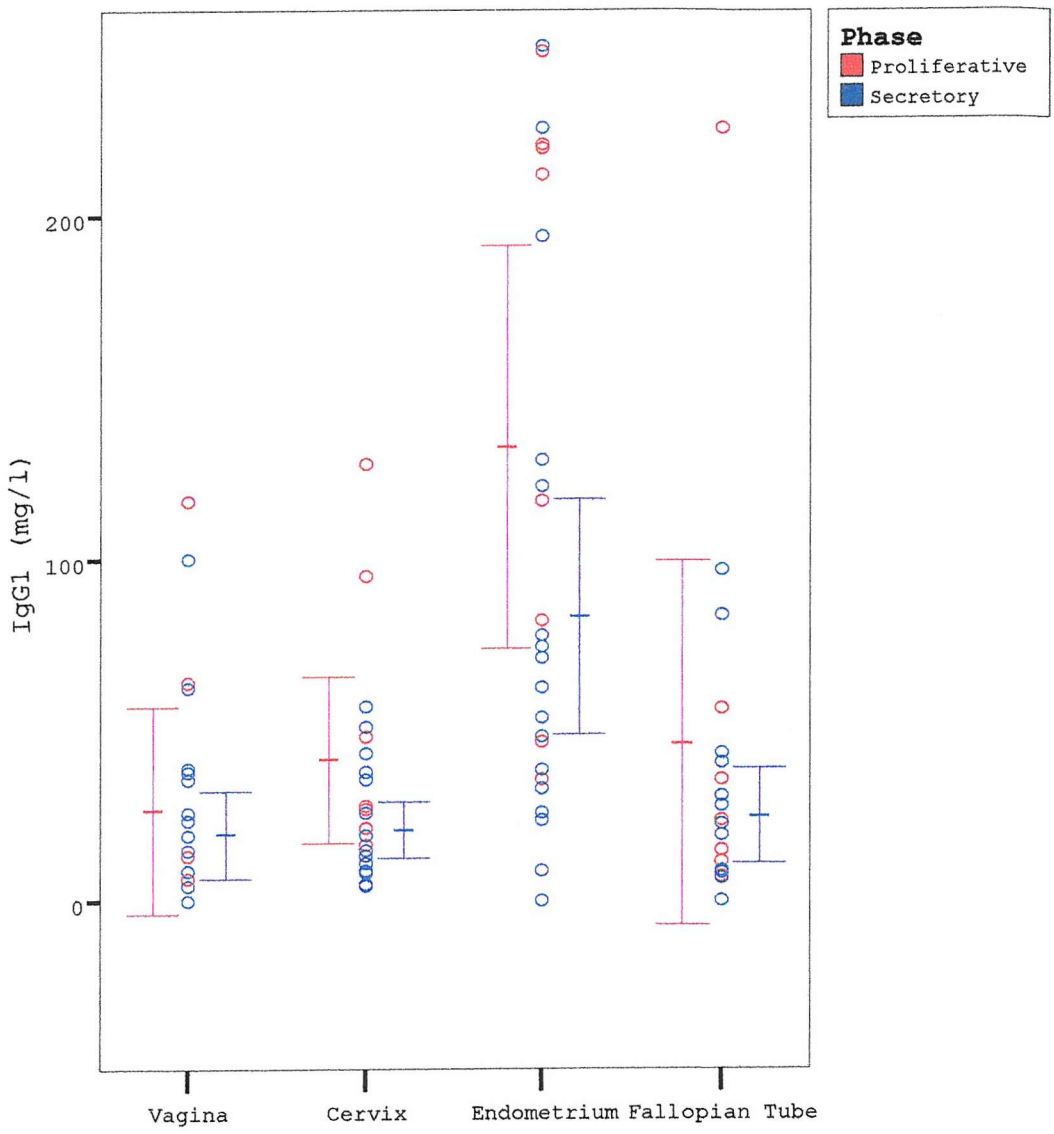


Figure 21. Genital tract distribution of IgG2

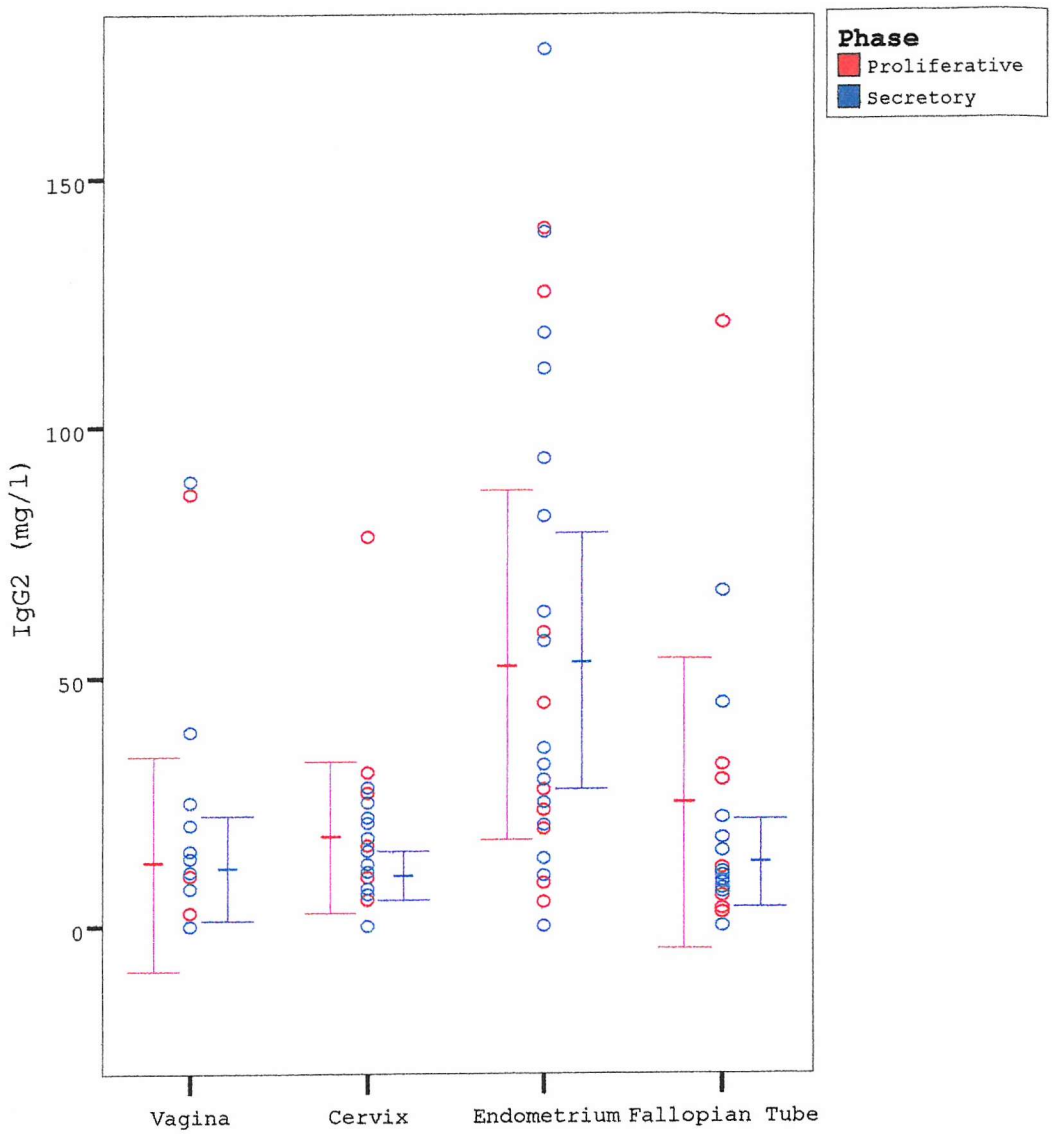


Figure 22. Genital tract distribution of IgG3

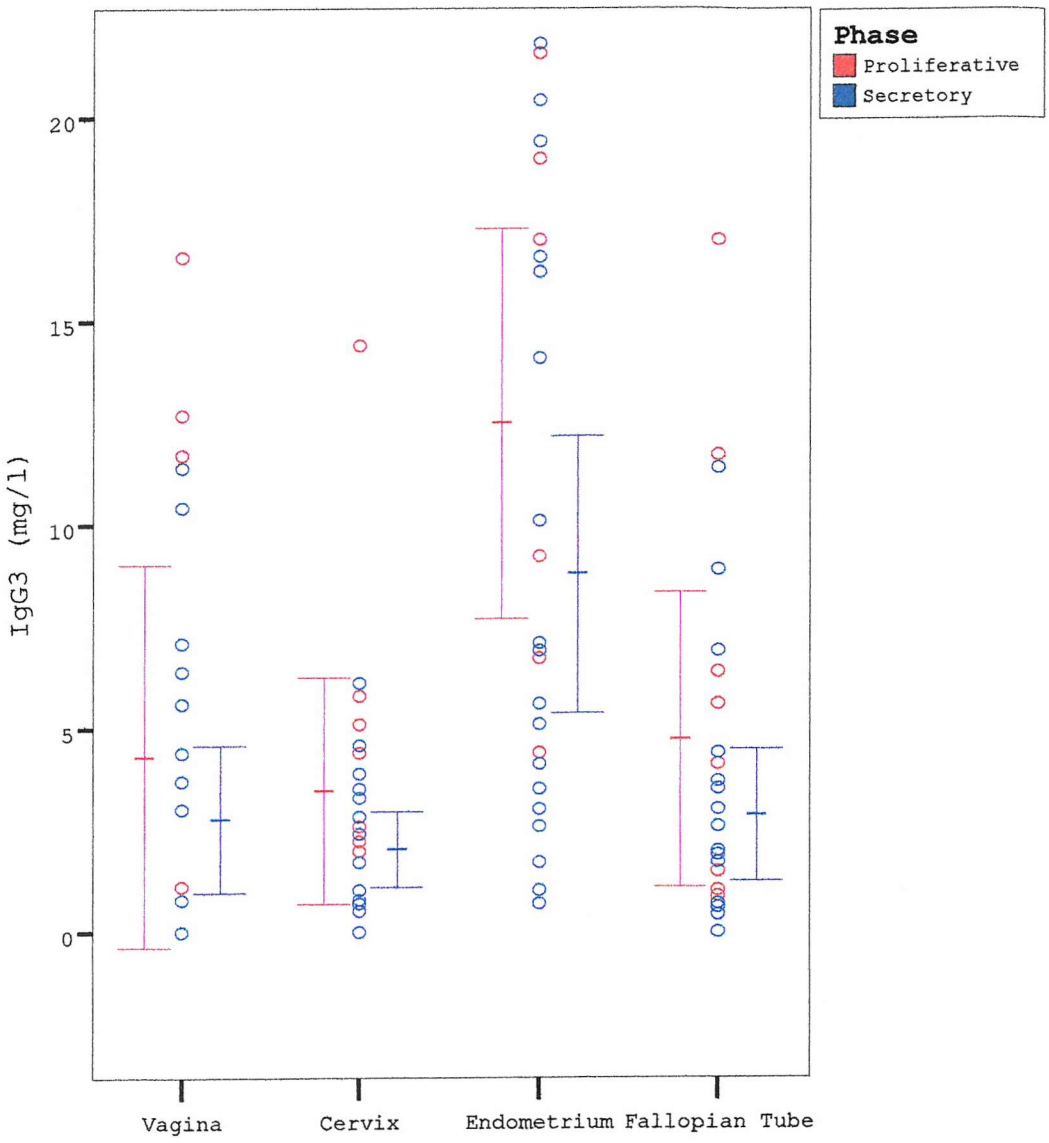


Figure 23. Genital tract distribution of IgG4

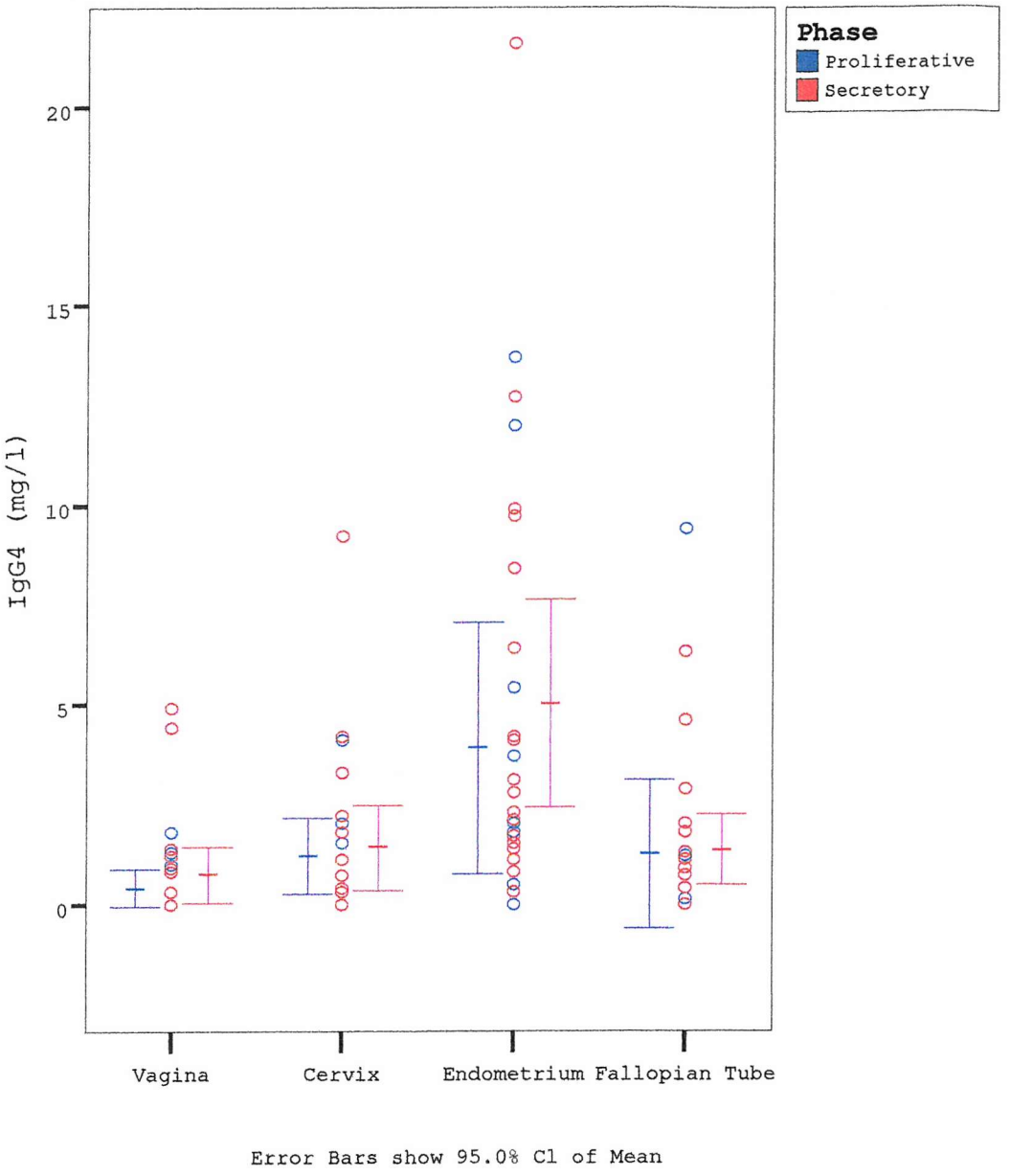


Figure 24. Genital tract distribution of IgA1

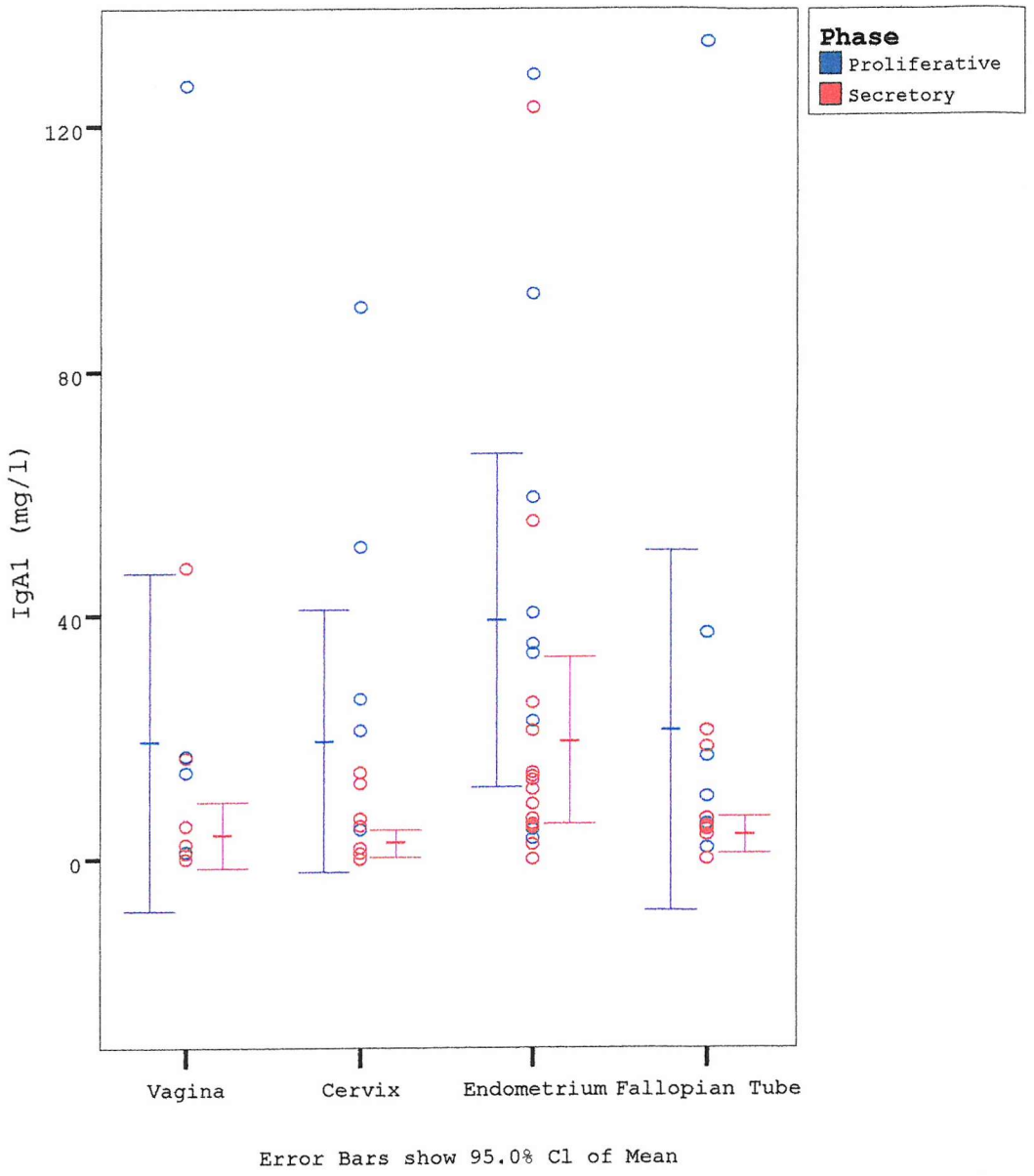


Figure 25. Genital tract distribution of IgA2

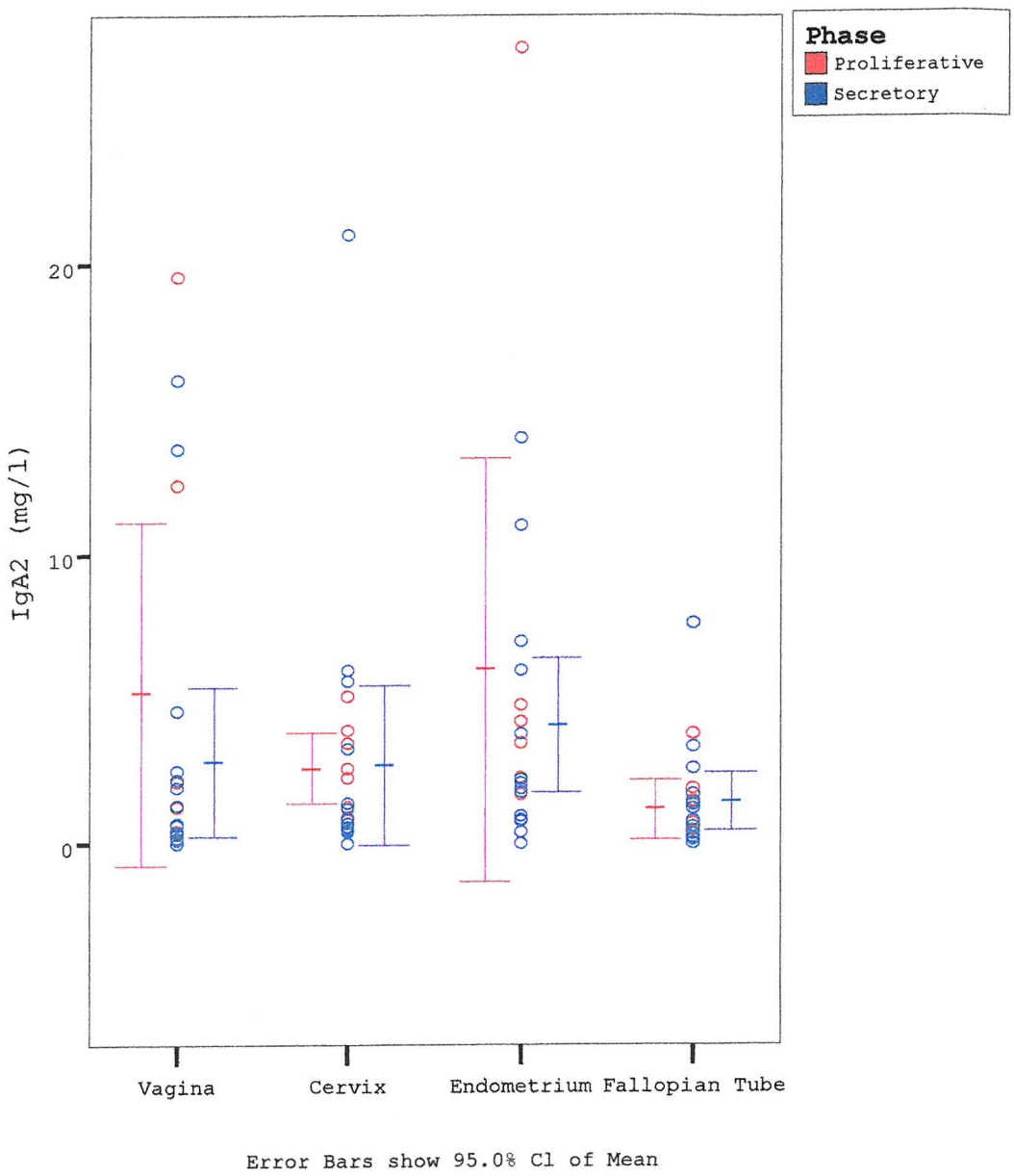


Figure 26. Genital tract distribution of secretory IgA

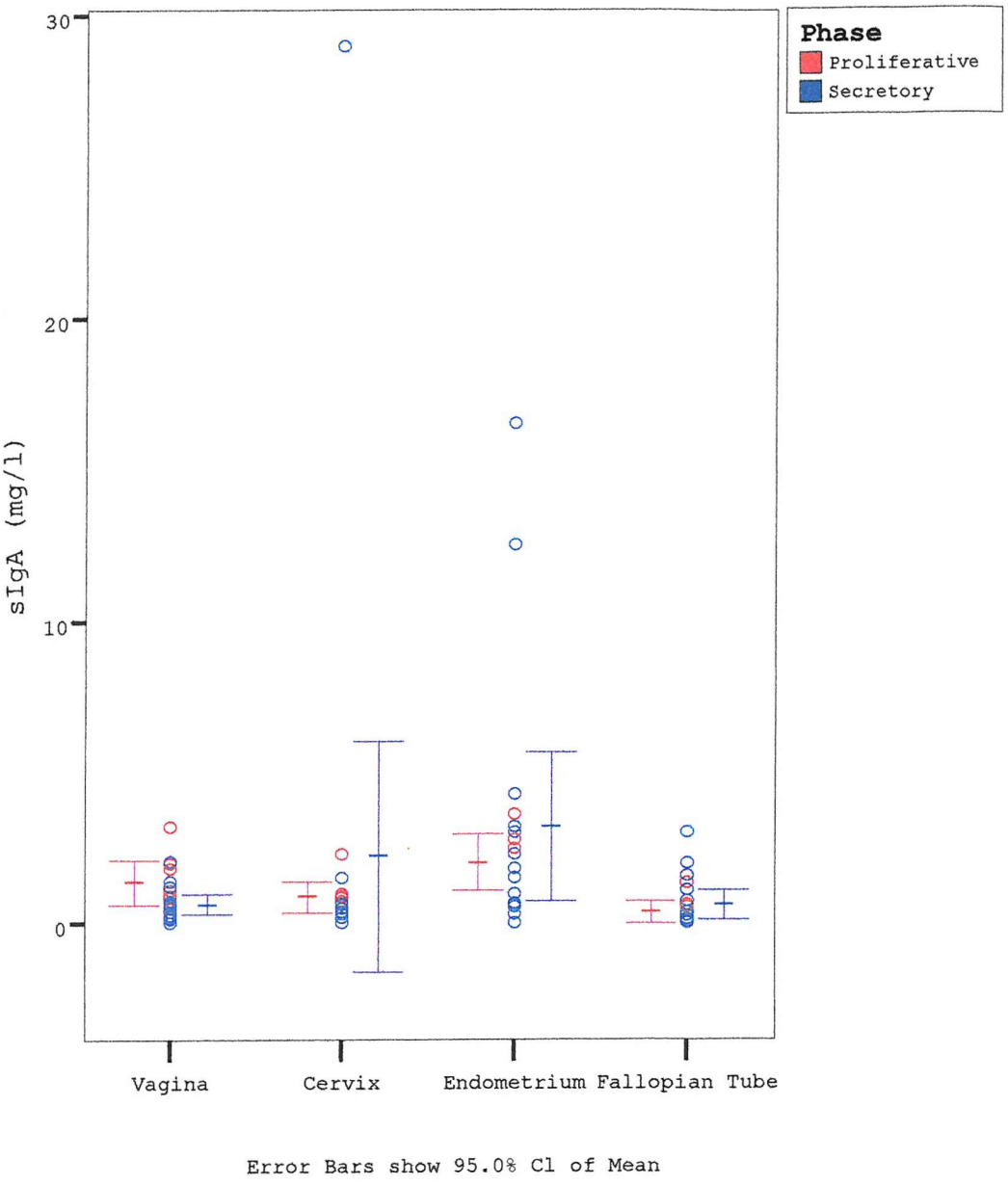
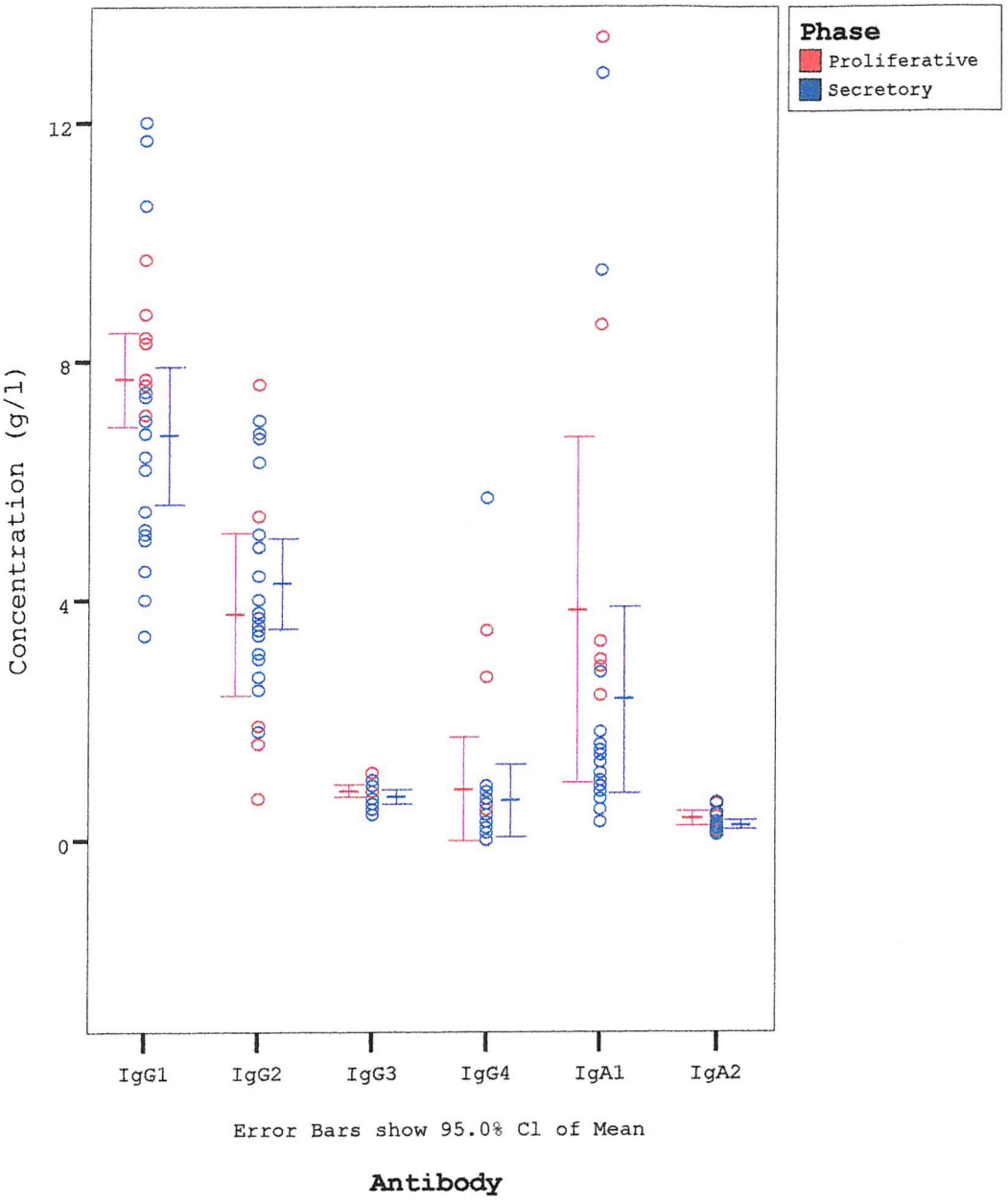


Figure 27. Serum levels of IgG and IgA subclasses



IgG which is grouped into four isotypic subclasses is the most well-studied isotype at both structural and functional levels. The different IgG subclasses share a long half-life which facilitates the maintenance of high serum IgG levels. The most well-known function of IgG is complement activation via the classical cascade. Complement is fixed most effectively by IgG1 and IgG3 and less so by IgG2. The second mode of IgG action entails targeting of bound antigen for destruction through phagocytosis (macrophages, granulocytes) or antibody-dependent cellular cytotoxicity (lymphocytes, natural killer cells). These functions are mediated by Fcγ -specific receptors on the effector cells. The IgG subclasses differ in their abilities to bind to Fcγ receptors. FcγRI (on monocytes, macrophages and neutrophils) binds IgG1 and IgG3 most avidly and IgG2 to a lesser degree. FcγRII (on macrophages, monocytes, neutrophils and B cells) and FcγRIII (on monocytes, macrophages, NK cells, neutrophils and some T cells) selectively bind with low affinity IgG1 and IgG3. IgG isotypes also bind to placental Fc receptors which facilitate transport of maternal IgG into the fetal circulation.

Different antigenic stimuli induce the production of different IgG subclasses. Viruses induce mostly IgG1 and IgG3 responses and IgG2 antibodies are associated with carbohydrate antigens. Repeated immunisation tends to induce humans to produce IgG4 antibodies. Normal serum levels of the IgG subclasses in the adult are listed below.

IgG1	4.2-12.9 g/l.
IgG2	1.2-7.5 g/l.
IgG3	0.4-1.3 g/l.
IgG4	0.01-2.9 g/l.

IgA which has a short half life represents 10-15% of serum immunoglobulin and is the dominant isotype in some mucosal secretions. IgA fixes complement via the alternative pathway, through a specific FcγR on macrophages it can serve as an opsonin for

phagocytosis, and it can induce eosinophil degranulation. IgA antibodies are highly resistant to digestion by proteolytic enzymes and their oligomeric nature enhances their ability to interact with high avidity with viruses and bacteria present in secretions. It is notable that the incidence of isolated IgA deficiency in humans is approximately 0.1% but most IgA-deficient individuals have no obvious deficiency in immune function. Normal adult serum levels of IgA are listed below.

IgA1	0.5-3.0 g/l.
IgA2	0.0-0.2 g/l.

Our results demonstrate a systematic survey of immunoglobulin isotype and subclass throughout the human female genital tract. Probably the most striking finding is that there were higher levels of IgG than IgA throughout the upper and lower genital tract. In the vagina, approximately 50% of the antibody detected was IgG1 or IgG2 and in the cervix this figure approached 70%. The highest antibody concentrations were detected in the endometrium for all the isotypes and subclasses studied. This is probably reflective of the much larger surface area within the endometrial cavity from which washings were taken. Our results demonstrate total antibody washed out rather than the concentration at the mucosal surface. In the fallopian tube, the majority of antibody detected was of the IgG isotype; again approximately 70% of antibody detected was IgG1 or IgG2.

Secretory IgA was detected throughout the upper and lower genital tracts but in low concentration. We detected significantly more IgA1 than IgA2 throughout the genital tract and serum (ratio of approximately 4:1, IgA1:IgA2). This equates with findings in the respiratory and upper gastro-intestinal tracts, whereas in the rectum and large intestine approximately equal proportions of IgA1 and IgA2 are detected.

The serum distribution of IgG and IgA subclasses were as expected with total IgG levels of approximately 10g/l. and total IgA levels of approximately 2g/l. In four cases very high levels of IgA1 were detected which is difficult to explain.

For each antibody isotype and subclass, mean antibody concentrations were compared for secretory and proliferative menstrual cycle phase and no significant differences were identified.

In contrast to the dominance of sIgA in secretions in other mucosal sites we have identified IgG as the dominant isotype throughout the female genital tract, an observation which has been confirmed by other authors (Hocini et al 1995). The necessary structural and cellular components for active trans-epithelial transport of polymeric IgA have been identified. The mechanisms involved in the movement of IgG into mucosal secretions have not been described but it seems that the presence of IgG in mucosal secretions, whether serum or locally derived, is at least as important as sIgA in providing humoral mucosal immunity. Hocini et al have investigated IgG subclasses in the lower genital tract and found that the percentage of IgG1 was highly increased and significantly higher than found in serum. This would suggest an active transport mechanism rather than passive transudation.

Immunoglobulin secreting cells (IgG and IgA) have been identified in greatest concentration in the endocervix and this would seem to be the most important site for antibody production (Crowley-Nowick et al 1995). It is likely that a significant proportion of the antibody we detected in the vagina and endometrium originated from the endocervix.

Our results did not show any significant menstrual phase variation in antibody levels in the upper or lower genital tract. Animal data, particularly in the mouse and rat has shown that genital tract immunity is under hormonal control and human studies have shown that cervical IgA and IgG levels are depressed in women on the combined contraceptive pill, suggesting sex steroid control of humoral immunity (Schumacher et al 1980). However, a

systematic analysis of the entire human genital tract has not previously been reported. Previous animal and human data has been reviewed in section 2.5.

Overall our work has shown that humoral immunity in the genital tract involves serum derived and locally produced antibody of which the most important isotype is IgG. Secretory IgA is present at low levels and would seem to less important. The effect of sex steroid hormones, particularly on the induction of immune responses in the human female genital tract clearly needs to be explored to optimise the timing of local mucosal vaccination.

6. DISCUSSION

6.1 MUCOSAL DEFENCES

The genital tract is colonised by a wide range of organisms forming a normal microbial flora and is constantly exposed to potential pathogens. Despite this the incidence of infection is relatively low. This is due to the presence of effective defence mechanisms that protect the mucosal surfaces of the genital tract. Specific immunity aside the mucosal surfaces are not an inert barrier but are protected by antimicrobial agents in the genital tract secretions.

The lower genital tract is lined by a single layer of columnar cells in the endocervix and by stratified squamous epithelium in the vagina and ectocervix. Interestingly the vagina and ectocervix are heavily colonised with bacteria whilst the uterus only two or three centimetres away is usually sterile. The mucosal immune system has evolved to maintain an equilibrium with such a normal microbial flora and to protect the upper genital tract from infection.

There are a number of mucosal defence mechanisms which do not require specific responses to micro-organisms by the immune system but play an important role in protection at mucosal surfaces. These include the maintenance of an environment that is nutritionally hostile to the invading micro-organism (eg by depriving bacteria of iron), the production of a fluid or mucus flow that washes the organism away and production of peptides and proteins with anti-bacterial activity such as lysozyme and defensins.

Mucosal surfaces are named for their ability to secrete a covering of mucus, highly viscous fluid containing dissolved mucopolysaccharides produced by goblet cells. The viscosity of the mucus itself is an important barrier coupled to the flow of secretions away from the epithelial surface. This flow is generated by the constant synthesis of fresh mucus, peristalsis and in ciliated epithelia such as the fallopian tube the motion of ciliated cells. Mucosal secretions contain substantial amounts of lactoferrin, an iron-binding protein which reduces the concentration of ferric ions to levels unable to

support bacterial growth. The secretions also contain lysozyme, an enzyme capable of digesting the peptidoglycan layer of bacteria. The mucosal secretions of the intestine contain cysteine rich peptides which are similar to defensins, bactericidal peptides found in granules of leukocytes. Defensin 5, a tissue specific antimicrobial peptide, has recently been identified in human cervix and endometrium although its exact role in local protection has not yet been established (Svinarich et al 1997). Bronchial secretions contain similar peptides. The spectrum of action and the role of these peptides in host defences has not been fully elucidated.

The mucosal barrier also depends on defences requiring specific recognition by the immune system. The mucus secretions contain high levels of immunoglobulin of a number of classes. Defence of the airways against bacterial infection is essential for survival and is the subject of intensive research. Parallels can be drawn between antibody mediated protection of the airways and female genital tract. In the upper respiratory tract the predominant isotype is IgA. In contrast in the lower respiratory tract the dominant isotype is IgG and we have demonstrated a similar dominance of IgG in the female genital tract. Mucosal secretions also contain small quantities of secretory IgM which increase in those with selective IgA deficiency.

Secretory IgA is particularly stable in mucosal secretions largely due to the secretory component. The polymeric IgA and secretory component complex is stabilised by covalent linkages which contributes to its resistance to proteolytic enzymes, a property of particular importance in the hostile environment of the gastro-intestinal tract.

IgA can directly inactivate toxins, enzymes or microbial adherence factors by simple binding thereby preventing entry into mucosal tissues. Aggregated IgA binds to polymorphs and can activate the alternative complement pathway although the level of complement components in secretions is low. Where the mucosal barrier is breached or inflammation occurs, both mucosal SIgA and submucosal pIgA could have an important anti-inflammatory regulatory activity, providing not only immune defense but also damage-limiting capability.

The ideology relating to humoral defences at mucosal surfaces is dominated by secretory IgA, yet inherited IgA deficiency is encountered with relative frequency (1:700) and is usually asymptomatic. In the airways, patients with selective IgA deficiency can suffer from recurrent but relatively minor upper respiratory tract viral infections, whereas the importance of IgG is suggested by the occurence of recurrent bacterial pneumonia in those with hypogammaglobulinaemia. Antibodies to bacterial capsules are predominantly IgG2 and similar infections present in patients with subtle deficiencies of IgG2 alone or in combination with IgA deficiency (Bjorkander et al 1985).

Much of the successful work on mucosal vaccination has focused on the respiratory tract and this has been extensively reviewed by Taylor et al (1993). In the proximal airways the bronchial wall contains more plasma cells staining for IgA than IgG whereas in the lower airways the converse is true. Correspondingly, secretions in the trachea and main bronchi contain relatively more IgA than IgG and in the bronchioles and alveoli IgG dominates. The mechanisms of transport of IgG from the submucosal plasma cells to the epithelial surface is not known but it is thought that at least 50% of IgG is locally produced. The presence of IgA as the major isotype in the proximal airways suggests an important role but much of the information about IgA function at this site is indirect. The role of IgG in the lower respiratory tract is clearer where IgG3 and IgG4 are most abundant which may reflect their importance in opsonization for macrophage phagocytosis. IgG1 and IgG2 are present in lung lavage in concentrations similar to their serum concentrations whereas IgG3 and IgG4 are increased in lavage related to serum suggesting local synthesis (Merrill et al 1985). The most important role for IgG in the lung may be as secondary defence after initial penetration of antigen and entry to the tissues with resulting inflammation which increases protein transudation, including antibodies, and increases local numbers of IgG plasma cells. Bacterial proliferation in the lung can be prevented by simultaneous administration of IgG systemically (Toews et al 1985). The success of the pneumococcal and influenza

vaccines shows that parenteral vaccination can be effective in protecting against pathogens invading mucosal surfaces.

Less research has been focused on antibody mediated immune responses in the genital tract but significant analogies can be drawn between our findings in the genital tract and established literature on the respiratory tract. Mucosal immunologists have tended to view the antibody defence of all mucosal surfaces as dominated by secretory IgA. Within the gastro-intestinal tract secretory IgA is essential because of its resistance to proteolytic enzymes which are in abundance. However, on the respiratory and genito-urinary mucosal surfaces resistance to proteolytic destruction is not an essential requirement of antibody function. IgG has been shown to dominate humoral immunity in the lower airways and parenteral vaccination can lead to useful IgG-driven immunity to potential lower respiratory tract pathogens. We have demonstrated a similar picture in the female genital tract where the demonstration of high levels of IgG on the mucosal surfaces seems to drive antibody mediated protection of the upper and lower genital tracts from potential pathogens.

The Hepatitis A vaccination studies showed that high serum levels of specific IgG and IgA can be induced by either cervical or parenteral vaccination, and the immunocytochemical studies demonstrated the presence of cells known to be capable of antigen uptake and processing within the cervix. High levels of specific IgG and IgA were detected throughout the genital tract following vaccination by either route but superior responses were generated in the lower genital tract following cervical vaccination. The implication is that maximum antibody driven responses to sexually transmitted diseases would ideally involve local mucosal vaccination but there are practical difficulties and doubts over acceptability of cervical vaccination in the early teenage years. The specific antibody distribution following parenteral vaccination suggests that significant concentrations of serum antibodies are translocated to the surfaces of the upper and lower genital tracts and that parenteral vaccination could be as effective as cervical vaccination at least in the protection of the upper genital tract.

The success of parenteral influenza and pneumococcal vaccines in the respiratory tract could be mirrored in protection of the upper genital tract.

Our analysis of the distribution of immunoglobulin isotype and subclass throughout the human female genital tract used a technique comparable to broncho-alveolar lavage and confirms the importance of IgG in antibody driven protection of the mucosal surfaces and extends the parallels with the respiratory tract. In general higher concentrations of IgG were found in the upper and lower genital tracts. Between 50% and 70% of antibody washed from the mucosal surfaces of the upper and lower genital tracts was IgG1 or IgG2 and concentrations of secretory IgG were generally low. The ratio of IgA1 to IgA2 in serum and genital tract washings was approximately 4:1 which equates with similar findings in the respiratory tract.

The best studied example of transepithelial immunoglobulin transport is of polymeric IgA by the polymeric immunoglobulin receptor across many types of epithelial cells. The transport of IgG into genital tract secretions has not been described and whilst a significant proportion is passively derived from serum there is likely to be a specific transport mechanism. The mechanism of IgG transport by the human placental trophoblast has been described following studies of IgG uptake by purified trophoblast maintained in culture. These studies suggest the presence of a novel trophoblast Fcγ receptor and demonstrated the ability to bind and endocytose human IgG with clear specificity for the IgG subclasses from human serum (Esterman et al 1995).

The fact that application of antigens to mucosal surfaces distant from the genital tract results in detectable specific antibodies in the genital tract as demonstrated in our *Salmonella typhi* vaccination studies is compatible with common mucosal homing mechanisms. In order to recruit leucocytes to the tissue, the endothelium of the vessels express adhesion molecules or addressins that serve as ligands for homing receptors on the cells. The only characterised mucosal addressin is the mucosal addressin cell adhesion molecule-1 (MAdCAM-1), which is expressed on the endothelium in the gut associated lymphoid tissue and recruits leucocytes to the intestinal mucosa. Johansson

et al (1999) have described the distribution of adhesion molecules in human cervix and vagina and found that MAdCAM-1 was not expressed on the vascular endothelium in the cervical or vaginal mucosa. However intercellular adhesion molecule-1 (ICAM-1), vascular adhesion protein-1 (VAP-1) and P-selectin were expressed in all of their tissue samples and vascular cell adhesion molecule-1 (VCAM-1) and E-selectin were variably expressed. The conclusion is that lymphocyte homing mechanisms to the human female genital tract are present but different to that seen in the intestine. This implies that there may be no advantages of vaccination via the gastro-intestinal tract over parenteral vaccination in the induction of protective antibodies in the female genital tract.

6.2 ALTERNATIVE VACCINATION STRATEGIES

We have investigated humoral immune responses in the human female genital tract to vaccines presented orally, parenterally and to the cervix. The only mucosal vaccines licensed for use today are given by the oral route and although no vaccines are given by the nasal route experimental studies with nasal vaccines have recently been performed in humans. Experiments initially done in rodents, rhesus monkeys and chimpanzees demonstrated that viral or bacterial antigens presented to the nasal cavity induced significant immune responses in local secretions but also in the genital tract. Rudin et al (1998) recently extended this work to the human model. Eight subjects were vaccinated nasally once and seven twice with cholera toxin B subunit (CTB). Strong serum responses to CTB were observed and there were marked specific IgG and IgA responses in nasal secretions. However, in contrast to recent findings in animals (Johansson et al 1998), specific IgG and IgA responses in vaginal secretions were only comparable to those elicited in orally vaccinated controls although the antibody responses were of longer duration. Because nasal immunisation has induced substantial antibody responses in the vagina in animals, further studies in humans are

indicated, particularly comparing nasal vaccination against parenteral and cervico-vaginal routes.

Strategies for inducing antibodies in both the rectum and genital tract clearly need to be developed for effective immunisation against HIV since most HIV infections are acquired sexually and involve contact of virus with rectal or genital tract mucosal epithelium. Rectal immunisation has recently been performed experimentally to investigate its ability to generate specific antibody both in the rectum and genital tract secretions. Recent results in non-human primates on the effectiveness of various immunisation routes for inducing simian immunodeficiency virus-specific antibodies in secretions of the female genital tract have demonstrated the superiority of intra-rectal immunisation (Lehner et al 1992). In humans rectal immunisation has been shown to be superior to other routes for inducing high levels of specific IgG and IgA in rectal secretions but was ineffective in inducing antibodies in genital tract secretions. Likewise, vaginal immunisation fails to produce significant responses in the rectum (Kozlowski et al 1999). Other groups have demonstrated vaginal IgG and IgA antibodies in women rectally immunised with viral or bacterial vaccines (Nardelli-Haefliger et al 1996, Hordnes et al 1996) It may be that generation of optimal immune responses against STDs and HIV in the rectum and lower genital tract requires vaccination in both these sites.

Our Hepatitis A studies demonstrated a significant serum and genital tract response to both cervically and parenterally administered vaccine, although the genital tract response was more marked with local immunisation. We have also demonstrated that IgG is the dominant isotype in the female genital tract. An important implication of these observations is that if significant concentrations of serum antibodies can be translocated to the surfaces of the upper and lower genital tracts parenteral vaccination could be as effective as cervical or rectal vaccination in the prevention of infection in the genital tract. Since vaccines against STDs, HIV and potentially oncogenic human papilloma virus would need to be given at puberty or in the early teenage years to predate sexual activity, parenteral administration would have clear benefits over

cervical and rectal administration which may be seen as socially unacceptable until sexual maturity has been reached and many genital tract infections have already taken place.

It would clearly be desirable to conduct longitudinal studies looking at the longer term evolution of antibody driven immunity in the upper and lower female genital tracts to model vaccines administered at different sites. It is currently however impractical to gain serial samples from the mucosal surfaces of the upper genital tract and so longitudinal data will inevitably focus on the lower genital tract.

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8. APPENDIX

CONTENTS

Table A: Hepatitis A specific IgA and IgG levels in genital tract washings and serum four weeks after vaccination

Patient	IgG	Site *	A/b Conc.	Vaccine #	Patient	IgA	Site *	A/b Conc.	Vaccine #
1	G	1	2.0	2	1	A	1	3.2	2
1	G	2	13.0	2	1	A	2	10.0	2
1	G	3	32.0	2	1	A	3	11.0	2
1	G	4	8.0	2	1	A	4	4.8	2
1	G	5	775.0	2	1	A	5	100.0	2
2	G	1	8.0	2	2	A	1	1.6	2
2	G	2	4.0	2	2	A	2	6.0	2
2	G	3	8.0	2	2	A	3	8.0	2
2	G	4	2.0	2	2	A	4	2.0	2
2	G	5	475.0	2	2	A	5	105.0	2
3	G	1	16.0	2	3	A	1	2.4	2
3	G	2	15.0	2	3	A	2	12.0	2
3	G	3	32.0	2	3	A	3	9.0	2
3	G	4	25.2	2	3	A	4	4.4	2
3	G	5	1250.0	2	3	A	5	125.0	2
4	G	1	11.2	1	4	A	1	12.4	1
4	G	2	6.0	1	4	A	2	12.0	1
4	G	3	12.5	1	4	A	3	8.0	1
4	G	4	5.2	1	4	A	4	2.8	1
4	G	5	625.0	1	4	A	5	75.0	1
5	G	1	5.2	2	5	A	1	4.0	2
5	G	2	3.6	2	5	A	2	6.0	2
5	G	3	25.0	2	5	A	3	10.0	2
5	G	4	1.6	2	5	A	4	2.4	2
5	G	5	500.0	2	5	A	5	110.0	2
6	G	1	4.0	2	6	A	1	4.8	2
6	G	2	3.0	2	6	A	2	7.0	2
6	G	3	31.0	2	6	A	3	9.0	2
6	G	4	5.2	2	6	A	4	4.0	2
6	G	5	312.5	2	6	A	5	475.0	2
7	G	1	3.6	1	7	A	1	7.6	1
7	G	2	4.0	1	7	A	2	3.0	1
7	G	3	10.0	1	7	A	3	8.0	1
7	G	4	3.2	1	7	A	4	2.8	1
7	G	5	775.0	1	7	A	5	40.0	1
8	G	1	1.2	1	8	A	1	6.0	1
8	G	2	31.0	1	8	A	2	40.0	1
8	G	3	8.0	1	8	A	3	7.0	1
8	G	4	4.0	1	8	A	4	2.4	1
8	G	5	625.0	1	8	A	5	80.0	1
9	G	1	20.0	1	9	A	1	15.6	1
9	G	2	10.0	1	9	A	2	30.0	1
9	G	3	27.0	1	9	A	3	19.0	1
9	G	4	10.4	1	9	A	4	3.2	1
9	G	5	1975.0	1	9	A	5	65.0	1
10	G	1	4.8	2	10	A	1	2.8	2
10	G	2	4.0	2	10	A	2	3.0	2
10	G	3	20.0	2	10	A	3	8.0	2
10	G	4	6.4	2	10	A	4	2.4	2

10	G	5	1500.0	2	10	A	5	100.0	2
11	G	1	.6	2	11	A	1	.4	2
11	G	2	10.0	2	11	A	2	6.0	2
11	G	3	5.0	2	11	A	3	4.0	2
11	G	4	4.0	2	11	A	4	1.6	2
11	G	5	1575.0	2	11	A	5	65.0	2
12	G	1	6.4	2	12	A	1	8.0	2
12	G	2	8.0	2	12	A	2	5.0	2
12	G	3	4.0	2	12	A	3	6.0	2
12	G	4	4.8	2	12	A	4	2.4	2
12	G	5	1500.0	2	12	A	5	125.0	2
13	G	1	1.2	2	13	A	1	.4	2
13	G	2	31.0	2	13	A	2	8.0	2
13	G	3	25.0	2	13	A	3	10.0	2
13	G	4	7.6	2	13	A	4	4.0	2
13	G	5	1675.0	2	13	A	5	100.0	2
14	G	1	10.0	1	14	A	1	4.8	1
14	G	2	79.0	1	14	A	2	30.0	1
14	G	3	125.0	1	14	A	3	37.0	1
14	G	4	8.0	1	14	A	4	3.2	1
14	G	5	1975.0	1	14	A	5	160.0	1
15	G	1	.8	2	15	A	1	1.2	2
15	G	2	2.5	2	15	A	2	4.0	2
15	G	3	6.0	2	15	A	3	6.0	2
15	G	4	4.0	2	15	A	4	2.0	2
15	G	5	975.0	2	15	A	5	80.0	2
16	G	1	1.2	2	16	A	1	3.2	2
16	G	2	1.6	2	16	A	2	2.0	2
16	G	3	4.0	2	16	A	3	7.0	2
16	G	4	4.0	2	16	A	4	3.2	2
16	G	5	775.0	2	16	A	5	95.0	2
17	G	1	10.0	1	17	A	1	15.6	1
17	G	2	18.0	1	17	A	2	30.0	1
17	G	3	7.0	1	17	A	3	4.0	1
17	G	4	2.4	1	17	A	4	4.0	1
17	G	5	775.0	1	17	A	5	80.0	1
18	G	1	15.6	1	18	A	1	16.0	1
18	G	2	20.0	1	18	A	2	50.0	1
18	G	3	10.0	1	18	A	3	6.0	1
18	G	4	6.4	1	18	A	4	4.0	1
18	G	5	400.0	1	18	A	5	85.0	1
19	G	1	7.6	1	19	A	1	8.0	1
19	G	2	19.0	1	19	A	2	10.0	1
19	G	3	13.0	1	19	A	3	5.0	1
19	G	4	1.8	1	19	A	4	4.0	1
19	G	5	800.0	1	19	A	5	125.0	1
20	G	1	6.8	1	20	A	1	5.2	1
20	G	2	19.0	1	20	A	2	9.0	1
20	G	3	10.0	1	20	A	3	10.0	1
20	G	4	1.6	1	20	A	4	2.4	1
20	G	5	625.0	1	20	A	5	80.0	1
21	G	1	7.6	1	21	A	1	10.0	1
21	G	2	16.0	1	21	A	2	30.0	1

21	G	3	12.0	1	21	A	3	4.0	1
21	G	4	3.2	1	21	A	4	2.0	1
21	G	5	400.0	1	21	A	5	145.0	1
22	G	1	1.6	2	22	A	1	1.2	2
22	G	2	9.0	2	22	A	2	2.0	2
22	G	3	10.0	2	22	A	3	39.0	2
22	G	4	2.0	2	22	A	4	4.0	2
22	G	5	800.0	2	22	A	5	80.0	2
23	G	1	2.0	2	23	A	1	4.0	2
23	G	2	5.0	2	23	A	2	6.0	2
23	G	3	12.0	2	23	A	3	8.0	2
23	G	4	4.0	2	23	A	4	2.8	2
23	G	5	375.0	2	23	A	5	75.0	2
24	G	1	20.0	1	24	A	1	8.8	1
24	G	2	63.0	1	24	A	2	33.0	1
24	G	3	125.0	1	24	A	3	10.5	1
24	G	4	14.0	1	24	A	4	3.2	1
24	G	5	1575.0	1	24	A	5	125.0	1

= route of vaccine administration. 1 = cervical. 2 = parenteral.

* = site of mucosal washings.
1 = vagina
2 = cervix
3 = endometrium
4 = fallopian tube
5 = serum

Antibody concentrations are expressed in microgrammes/litre

Flow diagram showing overview of vaccinations given and routes, menstrual phase and samples collected

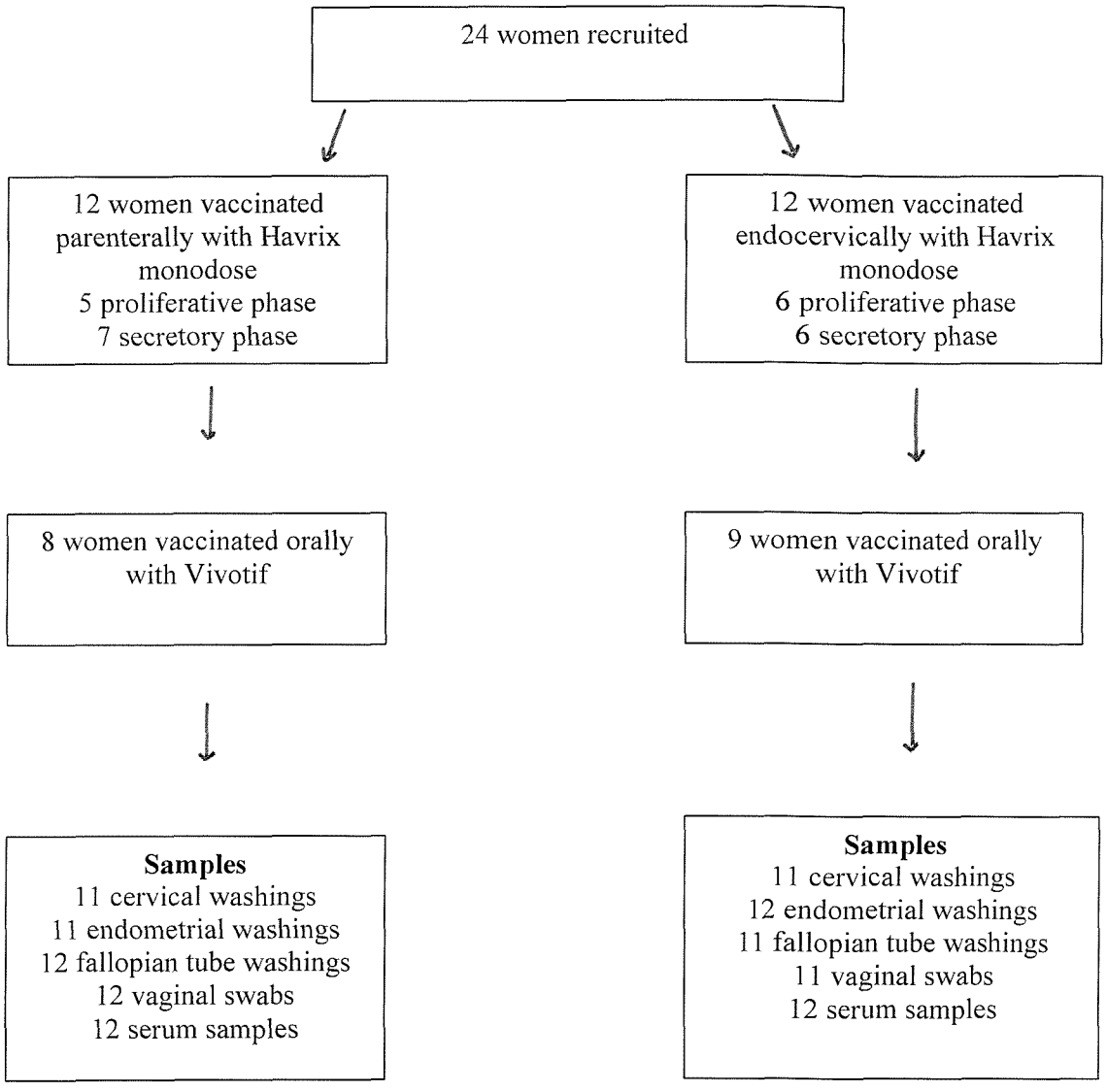

Table B: Immunoglobulin isotype and subclass concentrations throughout the genital tract and serum related to phase of menstrual cycle

Patient	Mens Phase*	sample #	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2	sIgA
1	2	0	6.6	11.0	1.1	1.8	16.5	.68	3.20
1	2	1	95.0	26.6	5.8	1.5	26.0	2.60	2.30
1	2	2	194.0	140.0	17.0	12.0	4.9	4.80	2.80
1	2	3	11.8	3.5	.0	.0	1.9	.44	.16
1	2	4	8.3	7.6	.8	.6	13.4	.27	.00
2	3	0	38.9	13.5	6.4	4.4	16.5	16.00	.20
2	3	1	57.0	17.7	6.1	1.1	6.5	3.30	.48
2	3	2	38.4	9.9	4.1	1.7	5.7	1.80	1.00
2	3	3	22.6	8.9	3.5	.0	3.9	.76	.24
2	3	4	6.8	3.5	1.0	.3	9.5	.19	.00
3	2	0	6.6	2.6	1.1	1.0	16.5	4.60	1.12
3	2	1	26.9	9.7	2.6	2.0	90.4	1.40	.39
3	2	2	82.0	27.4	6.7	2.1	92.5	2.20	.55
3	2	3	15.2	5.9	1.5	.0	16.8	.60	.16
3	2	4	7.6	2.7	1.1	2.7	.	.37	.00
4	2	0	64.0	2.6	12.7	.0	126.6	12.40	1.80
4	2	1	16.5	12.2	2.2	.4	51.2	2.30	.40
4	2	2	46.6	19.5	5.1	1.4	40.3	2.30	.55
4	2	3	.	.	11.7	1.8	134.0	3.80	1.32
4	2	4	7.1	4.9	.7	3.5	8.6	.37	.00
5	3	0	100.0	89.1	11.4	1.4	47.6	13.60	1.40
5	3	1	9.2	20.5	.8	1.1	12.3	.90	.40
5	3	2	71.1	57.1	6.9	1.4	21.1	7.00	3.00
5	3	3	83.4	67.2	6.9	1.3	18.3	7.60	2.00
5	3	4	6.4	5.1	.6	.1	2.8	.29	.00
6	2	0	117.0	86.7	11.7	1.3	14.1	2.16	.92
6	2	1	48.4	30.7	4.4	.7	.	3.50	.85
6	2	2	212.0	127.0	19.0	.0	35.2	3.50	1.50
6	2	3	35.6	32.3	4.1	.0	.	.72	.56
6	2	4	8.8	5.4	.9	.1	1.4	.22	.00
7	2	0	.	.	.0	.0	16.8	19.60	2.00
7	2	1	26.0	17.6	1.0	3.3	.0	3.90	.75
7	2	2	248.0	5.0	19.4	2.0	128.5	27.50	2.50
7	2	3	.	.	.0	1.2	.0	.32	.00
7	2	4	8.4	.7	.5	.	2.9	.60	.00
8	2	0	13.2	.0	.0	.0	.0	.	.
8	2	1	26.0	.0	.0	.0	.0	.	.
8	2	2	220.0	119.0	16.6	5.4	33.5	.	.
8	2	3	226.0	121.0	17.0	9.4	36.9	.	.
8	2	4	9.7	4.9	.6	.3	1.8	.	.
9	3	0	26.0	14.9	3.7	.0	.9	2.00	1.40
9	3	1	11.2	12.3	3.9	1.1	.0	5.60	.70
9	3	2	33.0	32.3	2.6	3.1	2.5	14.00	12.50
9	3	3	9.0	.0	.7	.7	.0	3.32	1.60
9	3	4	6.8	7.0	.6	.4	.3	.45	.00
10	2	0	23.8	9.9	16.6	.0	1.1	.	.
10	2	1	28.3	16.2	5.1	1.1	.0	.	.
10	2	2	117.0	59.0	16.6	3.7	3.2	.	.
10	2	3	7.3	11.6	.9	.1	.0	.	.

10	2	4	7.0	3.6	1.0	.3	.3	.	.
11	3	0	38.0	20.3	5.6	4.9	2.3	1.32	.68
11	3	1	35.6	21.8	3.9	4.2	1.7	.70	.50
11	3	2	194.0	119.0	19.4	9.7	12.8	.00	.00
11	3	3	.	.	.	.	.	.00	.00
11	3	4	12.0	6.3	1.0	.7	12.8	.18	.00
12	3	0	35.6	24.6	7.1	.3	.9	4.60	2.08
12	3	1	38.0	24.6	3.3	.3	.9	6.00	.70
12	3	2	250.0	176.0	20.4	8.4	13.5	11.00	16.50
12	3	3	28.3	14.9	1.7	.4	.0	2.60	3.00
12	3	4	10.6	6.7	1.0	.3	.5	.40	.00
13	3	0	15.1	.0	.8	.9	.0	.68	.26
13	3	1	26.0	6.4	1.7	2.2	5.5	21.00	29.00
13	3	2	9.0	.0	.7	.8	.0	.40	.55
13	3	3	40.6	11.0	2.6	2.9	5.5	1.72	.36
13	3	4	11.7	4.4	.7	.6	2.8	.63	.00
14	3	0	.0	.0	3.0	.0	.0	.12	.60
14	3	1	43.1	14.9	4.6	1.8	5.5	.45	1.50
14	3	2	74.2	29.1	6.9	2.3	9.0	.95	4.30
14	3	3	.0	.0	.4	.0	.0	.12	.12
14	3	4	6.4	2.7	.7	.2	1.3	.08	.00
15	3	0	.0	.0	.0	.0	.0	.20	.72
15	3	1	.	.	.0	.0	.	.00	.00
15	3	2	48.4	13.6	7.1	1.4	5.5	2.20	3.20
15	3	3	30.8	7.5	4.4	.9	4.8	1.72	1.08
15	3	4	7.5	3.0	1.0	.2	1.0	.12	.00
16	2	0	.	.	.	.	.	1.28	.88
16	2	1	19.3	.0	.7	.0	4.8	.85	.70
16	2	2	74.2	23.2	9.2	.5	22.6	4.20	2.80
16	2	3	56.6	14.9	6.4	.0	10.3	1.92	.16
16	2	4	7.5	3.0	.8	.1	3.3	.30	.00
17	3	0	23.8	7.5	4.4	1.2	.0	.	.
17	3	1	13.2	7.5	3.5	1.1	.0	.	.
17	3	2	.0	.0	1.0	.3	.0	.	.
17	3	3	.0	.0	.0	.0	.0	.	.
17	3	4	6.8	3.8	1.0	.3	1.3	.	.
18	3	0	.0	.0	.0	.0	.0	.40	.24
18	3	1	9.0	.0	1.0	.7	.0	.40	.20
18	3	2	23.8	.0	3.0	2.1	5.0	.75	.70
18	3	3	96.6	8.6	11.4	6.3	21.1	1.32	.76
18	3	4	3.4	6.8	.5	5.7	.8	.12	.00
19	3	0	.0	.0	.0	.0	.0	.00	.00
19	3	1	8.8	.0	.0	.4	.0	1.20	.40
19	3	2	62.2	35.5	3.5	4.2	11.5	2.10	2.30
19	3	3	8.4	.0	.0	.7	.0	.32	.00
19	3	4	6.2	3.7	.4	.4	1.6	.19	.00
20	3	0	8.8	.0	.0	.0	.0	2.20	.56
20	3	1	8.4	.0	.5	.3	.0	.60	.20
20	3	2	26.0	.0	1.7	1.1	6.6	.85	.00
20	3	3	.0	.0	.0	.0	.0	.24	.36
20	3	4	5.0	1.8	.4	.2	1.1	.20	.00
21	2	0	.0	.0	.0	.0	.0	.48	.48
21	2	1	19.3	5.3	2.0	.0	.0	1.30	1.00

21	2	2	35.6	8.6	4.4	1.8	9.0	1.70	3.60
21	2	3	9.2	2.6	1.0	.0	.0	.24	.28
21	2	4	5.2	1.9	.8	.2	2.4	.13	.00
22	3	0	.0	.0	.0	.0	.0	.12	.16
22	3	1	51.0	27.6	3.5	9.2	14.1	.50	.30
22	3	2	226.0	139.0	16.6	21.6	55.3	1.90	.65
22	3	3	6.6	6.6	.6	1.8	.0	.00	.08
22	3	4	5.1	3.1	.5	.9	1.3	.08	.00
23	3	0	.0	.0	.0	.0	.0	.68	.40
23	3	1	19.3	17.6	2.8	3.3	.0	.60	.00
23	3	2	71.1	82.2	10.1	12.7	25.6	1.80	.35
23	3	3	19.3	21.8	3.7	4.6	6.6	.56	.04
23	3	4	4.0	4.9	.6	.8	1.4	.16	.00
24	3	0	4.8	.0	.0	.0	.0	.72	.28
24	3	1	4.8	.0	.0	.0	.0	1.40	.60
24	3	2	71.1	20.3	5.6	6.4	14.1	6.00	1.80
24	3	3	9.2	7.5	2.0	1.8	.0	.76	.12
24	3	4	5.2	3.6	.6	.6	1.5	.40	.00
25	2	0	.0	.0	.0	.0	.0	.40	.72
25	2	1	128.0	77.8	14.4	4.1	21.1	5.10	.90
25	2	2	9.2	.0	1.7	.3	.0	1.90	1.80
25	2	3	30.8	29.1	5.6	1.3	5.0	1.48	.64
25	2	4	7.7	5.1	.9	.2	1.3	.45	.00
26	3	0	62.2	38.9	10.4	.8	5.5	2.52	1.24
26	3	1	11.2	.0	2.4	.0	.0	.60	.50
26	3	2	121.0	93.8	14.1	1.5	25.6	3.80	1.50
26	3	3	9.2	10.1	1.9	.0	.0	1.40	.10
26	3	4	7.0	5.1	.9	.0	1.8	.23	.00
27	3	0	.0	.0	.0	.0	.0	.36	.27
27	3	1	9.2	.0	.0	.0	.0	.40	.30
27	3	2	128.6	111.8	21.8	9.9	123.0	11.00	3.00
27	3	3	40.6	17.6	3.0	1.1	5.1	1.20	.10
27	3	4	7.4	3.4	.6	.0	.9	.17	.00
28	3	0	.0	.0	.0	.0	.0	.	.
28	3	1	15.1	11.0	.0	.0	.0	.	.
28	3	2	77.8	63.0	16.2	4.1	25.6	.	.
28	3	3	43.1	44.5	8.9	2.0	5.2	.	.
28	3	4	4.5	4.0	1.0	.2	1.0	.	.
29	2	0	9.2	.0	.0	.0	.0	.	.
29	2	1	21.6	.0	.0	.0	.0	.	.
29	2	2	221.0	44.5	21.6	13.7	59.3	.	.
29	2	3	23.8	.0	3.7	.0	5.7	.	.
29	2	4	7.4	1.6	.9	.5	3.0	.	.
30	3	0	19.3	11.0	.0	.0	.0	.	.
30	3	1	5.0	.0	.7	.0	.0	.	.
30	3	2	53.8	24.6	5.1	2.8	5.5	.	.
30	3	3	.0	.0	.0	.0	.0	.	.
30	3	4	5.5	2.5	.5	.3	.7	.	.

* = menstrual cycle phase. 2 = proliferative. 3 = secretory

= sample site. 0 = vagina. 1 = cervix. 2 = endometrium. 3 = fallopian tube. 4 = serum.

Antibody concentrations are expressed in mg/l (g/l for serum)

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INVESTIGATIONS INTO THE RESPONSE OF MUCOSAL SURFACES TO VACCINATION

PATIENT CONSENT FORM

I,....., agree to having typhoid and hepatitis A vaccinations one month before my hysterectomy as part of the investigations to develop vaccines against pelvic infection.
The details of the project have been fully explained to me by Dr. A. Moors and I have read the relevant information sheet.

Name of patient: .....

(signed) ..... Date: .....

Name of investigator: .....

(signed) ..... Date: .....

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Dear Dr.

One of your patients..... has kindly agreed to take part in a research study that I am running at the Princess Anne hospital. I write to summarise the basis of this study.

The departments of academic gynaecology and molecular microbiology have a joint five year programme grant from the MRC to study the immunobiology of the female reproductive tract. The long term aim of this study will be to develop vaccines against the common causative agents of pelvic inflammatory disease, namely Chlamydia trachomatis and Neisseria gonorrhoeae. However, the results will have implications for vaccine development against any pathogen invading via the genital tract.

More specifically, I am attempting to define the immunology of the genital tract more clearly. The main aim of this work is to assess the best route of administration for a potential vaccine and to see how the reproductive tract fits into the concept of a "common mucosal immune system".

We intend to model the problem using two vaccines in common use, given via different routes one month prior to hysterectomy. The vaccines used will be the Typhoid vaccine, "Vivotif", given orally, and the monodose Hepatitis A vaccine, given either parenterally or as a gel into the endocervix.

It is intended to administer these vaccines only to those women who would benefit from them. In the case of the Hepatitis A vaccine this would include those women intending foreign travel outside North-West Europe or North America. If the initial dose of vaccine is non-immunising (i.e. the intra-cervical route is used) then a later immunising dose will be offered.

Following hysterectomy the cervix, vaginal fornices, fallopian tubes, endometrium, tears and saliva will be analysed to assess their immune responses to these vaccines. The specimens will still be available for full histological analysis in the usual way.

We intend to study the responses of forty women in this way. The participants need to be

under the age of forty-five years, not sterilised, and with no evidence of pelvic pathology. The bulk of these will be hysterectomies for dysfunctional uterine bleeding or dysmenorrhoea.

Participants are fully counselled in a personal interview by myself and give written consent. There is, of course, full ethics committee approval for the study.

I hope that you do not object to your patients being approached to help in this research and am sure that you will appreciate the importance of this work.

If there are any further queries that you or your patient would like to take up with me, please do not hesitate to ring or write to me at the Princess Anne Hospital.

Yours sincerely,

Mr. Adam Moors MRCOG

Information Sheet for Pelvic Infection Study

Your assistance in this study, which is aiming to find vaccines to prevent pelvic infection or pelvic inflammatory disease, would be much appreciated. This information sheet outlines the basis of the project.

Pelvic infection or pelvic inflammatory disease (PID) is an infectious condition caused by several organisms. However, the two most common causative organisms have been identified. PID is a common disorder, affecting 1 in 10 women in the United States, and probably a similar number here.

If a woman has suffered from PID there is a substantial risk of damage to the fallopian tubes leading either to infertility (difficulty conceiving or childlessness) or to ectopic pregnancy (pregnancy in the fallopian tube). In the longer term, women who have suffered from PID are more likely to experience pain in the abdomen or pelvis, longer and more painful periods, and pain during sexual intercourse. These women are more likely to require a hysterectomy to control their symptoms.

The consequences of this disease can clearly be devastating and that is why the Medical Research Council have given us a large grant to try and develop vaccines against the organisms which cause it.

The work on this project is split between two departments at Southampton general hospital. These are the molecular microbiology unit and the gynaecology unit. The scientists in the molecular microbiology unit are working on developing the new vaccines. We in the gynaecology unit are trying to establish the best way of giving these vaccines once they are developed. In order to do this we are giving well established and safe vaccines, which are in everyday use, to volunteer women prior to their hysterectomy.

The vaccines used will be against Typhoid and Hepatitis A. The Typhoid vaccination is in tablet form (3 tablets taken over 6 days). The hepatitis A vaccine is given either as one injection into the upper arm or as a gel into the neck of the womb (cervix). The latter is very similar to having a smear performed. Both of these vaccines would be administered approximately one month prior to your operation. Any side effects are mild and uncommon; Dr. Moors will discuss these with you. There are many reasons why being vaccinated against these conditions may be useful to you, the most obvious of these being foreign travel. These indications will also be discussed with you.

After your hysterectomy, the womb (uterus and cervix) and fallopian tubes will be washed to see if they have produced antibodies against the vaccines that you took. We may also need a sample of saliva after the operation, during which we will need to take a small sample of blood to estimate your response to the vaccinations. The anaesthetist will take this at the time that the drip is inserted and so no unnecessary injections will be performed.
Your uterus and tubes will then go to the pathologist to be studied - this is normal

practise for all operations performed.

If you so wished we would offer you a booster of the Hepatitis A vaccine giving long term immunity. This would be given several months later.

I do hope that you decide to participate in this study. Obviously we do not offer any financial incentives to take part although we can pay for your travel expenses.

If there are any areas of the project that you feel muddled about or do not understand please do not hesitate to ask.

Dr. Adam Moors MBBS MRCOG