UNIVERSITY OF SOUTHAMPTON

Early life exposure to a dietary allergen

Characteristics, and consequences for allergic sensitisation and disease

Gillian Helen Sarah Vance

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Department of Child Health
Allergy and Inflammation Sciences Division
Faculty of Medicine, Health and Biological Sciences

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES
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Doctor of Philosophy

EARLY LIFE EXPOSURE TO A DIETARY ALLERGEN
CHARACTERISTICS, AND CONSEQUENCES FOR ALLERGIC SENSITISATION AND DISEASE

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Allergic diseases have become an important public health issue. While the genetic basis for allergy is well established, genetic factors must interact with environmental influences to determine disease development. This complex interaction most likely occurs in early life since distinctive alterations of immune responsiveness, which may predict an allergic phenotype, are apparent at birth. Demonstration of raised IgE and allergen-specific T-cell reactivity at birth has inferred that exposure of the naïve immune system to allergens may occur antenatally. Animal models have shown that the dose, timing, route and form of antigen exposure regulate whether tolerance or immune priming occurs. Also long-term T_H1-like (tolerant) and T_H2-like (allergic) memory against individual antigens occurs during the first exposures, and once consolidated, is not readily reversible. Consequently, the nature of early life exposure to an allergen may contribute to the complex process that determines allergic sensitisation and disease manifestation. Therefore, the hypothesis of this thesis was that *'the characteristics of early life exposure to dietary egg allergen determine infant atopic phenotype'*.

Hen egg ovalbumin (OVA) was the dietary allergen investigated. Egg allergy is common in infancy & has implications for later inhalant sensitisation and respiratory allergic disease. An OVA detection *ELISA* was developed. By this method OVA was found in maternal blood throughout pregnancy, cord blood, amniotic fluid and breast milk, thus confirming direct exposure to dietary allergen in early life. The dose, timing & routes of allergen passage were described & OVA form in breast milk, maternal & cord blood evaluated by gel filtration sample separation and *ELISA*. OVA was found only in free form in breast milk, but in blood was present both as free antigen and in complex with IgG, the form depending on specific IgG concentration. Thus maternal IgG may determine mode of fetal allergen presentation. Maternal IgG could also inhibit antigen detection, an effect dependent on concentration and functional affinity. This raises the possibility that maternal IgG may block presentation of allergenic epitopes *in vivo*, with implications for immune regulation.

Women, with a personal or partner history of atopy, were randomised to dietary egg exclusion or a normal healthy diet from 17-20 weeks of pregnancy till the end of breast-feeding. OVA was present in as many blood & breast milk samples of egg avoiding as control women. Atopic women had higher levels of serum OVA than non-atopic women, while atopic & egg-avoiding women more often had OVA in breast milk, and in higher quantities, than non-atopic, egg avoiding women. These data suggest that dietary exclusion, particularly by atopic women, does NOT eliminate allergen exposure in early life. Antenatal OVA exposure, in the context of an egg-avoiding & atopic mother, was associated with a greater risk of an atopic phenotype at 6 months of age. Also, exposure via breast milk from an atopic mother suggested a greater risk of later atopy. Maternal serum OVA IgG concentration was shown to mark compliance to an egg exclusion diet & differences in cord concentrations were related to subsequent atopy. These data imply modulatory influences of maternal IgG & atopic environment over developing immune responses and raise the possibility that dietary exclusion as a primary allergy prevention strategy may have *adverse* consequences. Postnatally, differences in OVA IgG and IgG subclasses were identified for persistently egg sensitised children & elevated OVA IgG1 was associated with later asthma. This suggests that serum OVA IgG1 measurement might be used as an adjunct to skin testing and serum IgE measurement to predict allergic respiratory disease.

This work has provided insight into mechanisms that may modulate early life programming of atopy and has proposed factors for consideration in primary allergy prevention strategies. Furthermore, the potential for a serological measurement in infancy to predict long-lasting respiratory disease offers the prospect of early implementation of secondary allergy prevention measures.

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ABBREVIATIONS

AD	Atopic dermatitis
AF	Amniotic fluid
APC	Antigen presenting cell
AU	Arbitrary unit
BCR	B-cell receptor
BLG	β-lactoglobulin
BLG G	β-lactoglobulin specific IgG
BM	Breast milk
BSA	Bovine serum albumin
BSAP	B-cell specific activator protein
C	Constant (region)
Chi^2	Chi-square
CBMC	Cord blood mononuclear cell
CLA	Cutaneous-Lymphocyte-associated Antigen
Conc	Concentration
cSMAC	Central supramolecular activation clusters
CSR	Class switch recombination
CTACK	Cutaneous T-cell Attracting ChemoKine
CV	Chorionic villi
D	Diversity (region)
DC	Dendritic cell
DELFIA	Dissociation-enhanced lanthanide fluorescence immunoassay
dsb	Double strand break
DTH	Delayed type hypersensitivity
ECL	Enhanced chemiluminescence
ELISA	Enzyme linked immunosorbent assay
ETS	Environmental tobacco smoke
FcεRI	(high affinity) IgE receptor
HDM	House-dust mite
HSA	Human serum albumin
ICOS	Inducible costimulator
IDO	Indoleamine 2,3 dioxgenase
IFN-	Interferon-
Ig	Immunoglobulin
IL-	Interleukin-
ISAAC	International Study of Asthma and Allergies in Childhood
ITAM	Immunoreceptor tyrosine based activation motif
ITIM	Immunoreceptor tyrosine based inhibitory motif
IU	International Unit
J	Joining (region)
kDa	Kilodalton
L	Ligand
LIF	Leukaemia inhibitory factor
LPR	Late phase reaction
LPS	Lipopolysaccharide
LYS	Lysozyme
MHC	Major Histocompatibility Complex
MUC	Ovomucoid

MUC G	Ovomucoid specific IgG
MW	Molecular Weight
NF-κB	Nuclear factor-kappa B
NK	Natural killer (cell)
OD	Optical density
OVA	Ovalbumin
OVA G	Ovalbumin specific IgG
OVA G1	Ovalbumin specific IgG1
OVA G4	Ovalbumin specific IgG1
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PG	Prostaglandin
pIgR	Polymeric immunoglobulin receptor
PRRs	Pattern recognition receptors
pSMAC	Peripheral supramolecular activation clusters
PTK	Protein tyrosine kinase
RAST	Radioallergosorbent test
ROC	Receiver-operator characteristic (curve)
S	Switch
sCD14	soluble CD14
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
sIgA	Secretory IgA
SDS	Sodium dodecyl sulphate
SPT	Skin prick test
SQ	Standard quality (solution)
T_C	T-cytotoxic cell
TCR	T-cell receptor
TGFβ	Transforming growth factor β
T_H	T-helper cell
TLR	Toll-like receptor
TNF-	Tumour necrosis factor-
Treg	T regulatory cell
V	Variable (region)

Chapter One

General Introduction and hypothesis

Chapter 1

General Introduction and Hypothesis

1.1 The Normal Immune Response

The body is exposed to a host of pathogens with the potential to cause disease, and possibly death. Protection from these agents is provided by a complex array of defensive measures, collectively called the immune system [(1)].

In humans, the immune system comprises two strategic arms - the *innate* and *acquired* systems - which essentially differ in the means, and hence the specificity, by which they recognise pathogens. Phylogenetically older, the innate immune system discriminates potentially noxious substances through recognition of conserved molecular patterns shared by large groups of microorganisms [(2)(3)]. This limited specificity contrasts with the precision that characterises antigen recognition by the acquired immune system: diversity of cell surface receptors and circulating proteins facilitate specific immune responses to an almost infinite number of molecular structures [(4)(5)].

Innate immunity offers first-line defence that rapidly operates to limit microbial infection. Adaptive immunity subsequently provides the specific armamentarium that can effectively eliminate the invading pathogen. However, optimal host defence is dependent on co-operation between the two arms: activation of the innate immune response is a prerequisite for triggering acquired immunity, while products secreted by cells of the acquired immune response (for example, immunoglobulins and cytokines) augment the activity of the innate system [(4)(6)].

1.1.1 Innate Immunity

Innate defence mechanisms include anatomical barriers, such as intact skin, and physiological deterrents, such as high temperature or acidic pH, as well as inducible responses mediated by cellular and humoral components. Cells of the innate system include phagocytes (neutrophils, macrophages, eosinophils), natural killer (NK) and

mast cells [7]. Pathogen recognition is mediated by a set of germline-encoded pattern recognition receptors (PRRs). These include mannose receptors, which recognise the carbohydrate signature of microbes [8]; CD14, which recognises lipopolysaccharide (LPS), a common constituent of Gram-negative bacterial outer membranes [9] and the Toll-like receptor (TLR) family which recognise a variety of microbial components, including LPS (recognised by TLR4) [10], lipoprotein (recognised by TLR2) [11] and bacterial DNA (recognised by TLR9) [12]. The major soluble protein effector of innate immunity is the complement system, an enzyme cascade that has the ability to recognise, opsonize or lyse particulate material, including bacteria and yeasts.

1.1.2 Adaptive Immunity

The adaptive immune system is a diverse, yet specific, defence response that has evolved in vertebrates to cope with the genetic variability of microorganisms [13]. The system may be further divided into humoral and cell-mediated responses. Humoral responses are mediated by antibodies (immunoglobulins) - circulating antigen-specific proteins - that can neutralise soluble antigen or facilitate its elimination. Cell-mediated responses can directly eradicate intracellular pathogens via effector cells – in particular, T-lymphocytes.

1.1.2.1 Cellular component of the adaptive immune response

Lymphocytes are principal immune cells of the adaptive response. They display diversity and specificity but are also characterised by the immunological attributes of memory and self/non-self discrimination. Memory refers to the retention of clones of antigen-specific cells following elimination of an infection that facilitate a more rapid and heightened response on a second exposure to the same organism [14]. The presence of intercellular recognition molecules, such as the proteins coded for by the major histocompatibility complex (MHC) on chromosome 6, permits essential lymphocyte discrimination of self versus non-self, or altered self, cells.

Two main lymphocyte types participate in the adaptive immune response – B-lymphocytes and T-lymphocytes.

B-cells interact with antigen via membrane bound immunoglobulin. When activated they divide and differentiate into memory cells or plasma cells. Plasma cells do not express surface immunoglobulin but secrete antibody into the surrounding medium.

Antigen recognition by T-cells occurs via a specific membrane receptor (TCR) associated with a set of polypeptides – the CD3 complex. T-cells may be further divided into two main subtypes: T-helper (T_H) and T-cytotoxic (T_C) cells. T_H cells express the membrane glycoprotein CD4. They recognise antigen in association with MHC class II proteins, present on antigen presenting cells (APC), via the CD3-TCR complex. Their role is in the regulation of the immune response. T_C cells express the membrane molecule CD8. They recognise antigen in association with MHC class I proteins, present on virtually every nucleated cell, and therefore can destroy altered self-cells.

B- and T-cells recognise different epitopes on the same antigenic molecule. As B-cells recognise soluble antigen their epitopes tend to be accessible sites on the exposed surface of the molecule. They are often located in flexible regions where site mobility maximises complementarity of antigen – receptor interaction. In contrast, T-cells recognise antigen peptides in association with MHC. Since this interaction requires prior antigen processing, so T-cell epitopes are often internal linear sequences [15].

1.1.2.1.1 T-cell receptor

The normal development of B- and T-lymphocytes is dependent on the assembly of a functional receptor complex.

The TCR is a heterodimer consisting of an alpha (α) and beta (β), or less commonly a gamma (γ) and delta (δ), chain. Each chain has a **V**ariable (**V**) and a **C**onstant (**C**) domain. The **V** domain contains 3 hypervariable regions that contact antigenic peptide in the MHC binding groove. Each **C** domain includes a short connecting sequence, a transmembrane region that anchors it in the plasma membrane and which

contains positively charged residues enabling the receptor to interact with the CD3 complex, and a cytoplasmic sequence.

As will be described in more detail for B-cell immunoglobulin assembly, germline DNA encoding individual TCR chains is made up of multigene families comprising a number of gene segments containing many different coding sequences. Receptor diversity is generated through a process of gene segment recombination. During this carefully regulated mechanism, various gene segment combinations are assembled to create unique sequences that code for specific receptor chains.

1.1.2.1.2 Cytokines

Interaction between immune cells is mediated by a group of low molecular weight (MW) proteins or glycoproteins termed cytokines. These molecules elicit their biological effects at low concentration by binding to specific receptors on target cells. Their main action is to regulate the immune response by stimulating or inhibiting cell activation, proliferation or differentiation, and the secretion of antibodies or other cytokines.

The T_H cell is central to the regulation of the adaptive immune response. Two main subsets of T_H cell – referred to as T_H1 and T_H2 - are recognised based on the profile of cytokines released. Originally described for murine cells [16], there is strong evidence for comparable cytokine patterns and functions in humans [17][18]. T_H1 cells typically secrete interferon (IFN)-γ, Interleukin (IL)-2 and tumour necrosis factor (TNF)-β, while T_H2 cells secrete IL-4, IL-5 and IL-13. The T_H1 profile promotes cell-mediated responses and thus provides protection from viral infections and intracellular pathogens. Conversely, the T_H2 arm provides optimal help for humoral immune responses, including IgE and non-complement activating IgG subclass isotype switching, and mucosal immunity through induction of mast cell and eosinophil growth and differentiation and facilitation of IgA synthesis [19].

Mention should be made of other CD4+ T-cell subsets outside the broad classification of T_H1 and T_H2 cells. T_H0 cells produce both T_H1 and T_H2 cytokines, including IL-2,

IL-3, IL-4, IL-5, IL-10 and IFN-γ. CD4+ cells may also suppress the immune response and these cells are collectively known as regulatory T-cells (Treg cells) [20]. These inhibitory T-cell populations include a naturally occurring CD4+CD25+ subset [21], T_H3 cells, induced following oral administration of antigen and which predominantly secrete transforming growth factor (TGF)-β – a potent immunosuppressive cytokine [22], and Tr1 cells, induced *in vitro* by repetitive stimulation of naïve T-cells in the presence of IL-10 and which are able to inhibit T_H1 and T_H2 responses *in vivo* [23]. These regulatory populations may play an essential role in limiting immune pathology.

1.1.2.2 Humoral component of the adaptive immune response

1.1.2.2.1 Immunoglobulin structure

Immunoglobulins (Ig) (antibodies) may either be membrane bound on B-cells, where they have a role in antigen recognition, or may be secreted by plasma cells, and have an effector function in host defence.

They are made up of four peptide chains - two light chains, either kappa (κ) or lamda (λ), and two heavy chains. The chains consist of domains, namely, homologous units of 110 amino acid residues. Light chains have one variable domain ($\mathbf{V}_L$) and one constant domain ($\mathbf{C}_L$), while heavy chains have one variable ($\mathbf{V}_H$) and three or four constant ($\mathbf{C}_H$) domains depending on the antibody class. Five different heavy chain constant regions produce five antibody classes - IgM (μ), IgG (γ), IgA (α), IgD (δ), and IgE (ε). The specific antigen-binding site is found in several hypervariable regions of the **V** domains where amino acid sequence variability is maximal *(figure 1.1)*.

Figure 1.1 Basic immunoglobulin structure.

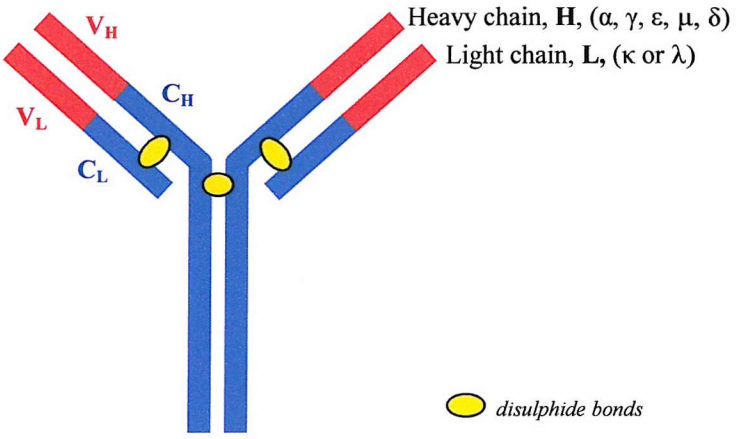


Figure 1.1 The basic structure of an immunoglobulin comprises 4 polypeptide chains – 2 identical Light (L) chains and 2 identical Heavy (H) chains – joined together by disulphide bonds. The Variable (V) domains of the H and L chains have several hypervariable regions that constitute the antigen-binding site. Beyond the V domain is the Constant (C) region. There are 5 sequence patterns of the C_H region -α, γ, ε, μ, δ –corresponding to the 5 Ig classes, IgA, IgG, IgE, IgM & IgD, respectively.

1.1.2.2.2 Immunoglobulin functions

The heavy chain constant region delineates the variety of collaborative interactions with other proteins, cells and tissues that characterise antibody class function.

IgG, MW 150 kilodaltons (kDa), is the most abundant antibody, constituting approximately 80% of total serum immunoglobulin. Four human IgG subclasses exist – IgG1, IgG2, IgG3 and IgG4 – numbered according to their decreasing average serum concentrations. They are distinguished by differences in the γ chain amino acid sequence, which also dictates the biological activity of the molecule. IgG contributes to pathogen elimination by activating the complement cascade and by promotion of phagocytosis via Fcγ receptors on phagocytic cells. Maternal IgG, in particular IgG1, can cross the placenta [(24)]. This antibody class may therefore have the additional, and unique, role of protecting the newborn infant from pathogenic onslaught in the first few months of life.

IgM comprises approximately 10% of the total immunoglobulin pool. In its pentameric form (MW 900 kDa), this antibody activates complement most effectively, and it efficiently binds and neutralises viral infectivity. Pentameric IgM may also be found in external secretions, such as breast milk, saliva, tears and mucus, though secretory IgA (sIgA) is the predominant immunoglobulin class at mucosal surfaces [25]. Secretory IgA occurs primarily as a dimeric complex containing two additional peptides, - the **J** (**J**oining) chain and secretory component (SC). As will be discussed *(1.2.3.2)*, sIgA has an important role in providing the first line of specific immunological defence, particularly for the breast-fed newborn [26][27]. In addition, IgA is the second most prevalent antibody in human serum, constituting 15-20% of total immunoglobulins. Serum IgA is mainly in monomeric form and exists as two subclasses, IgA1 and IgA2, which differ by the presence or absence of a 13-amino acid hinge region. This O-glycosylated region is exclusive to the IgA1 subclass and may increase susceptibility to bacterial proteolysis, but reduce hepatic clearance [28]. The IgA receptor, FcαRI (CD89), is expressed on neutrophils, monocytes, macrophages and eosinophils [29]. A variety of functions have been ascribed to it, including stimulation of phagocytosis and release of inflammatory cytokines [29]. It has been suggested that FcαRI-expressing liver Kupffer cells may provide a secondary line of defence by facilitating phagocytosis of IgA-coated bacteria that have penetrated the gut barrier [30].

IgE (MW 190 kDa) is normally present in extremely low average concentration in serum. Most commonly it is bound to tissue mast cells or blood basophils. Cross-linking of this receptor-bound IgE by antigen results in cell release of pharmacologically active mediators that may have a role in protection from parasitic infections [31]. However, in the western world, this activity more commonly orchestrates the signs and symptoms of allergic disease *(1.3)*. IgD is a membrane molecule expressed by B-cells. It may have a role in antigen induced B-cell activation, but no biological effector function has been identified [32].

1.1.2.2.3 Immunoglobulin diversity

The κ and λ light chains and the heavy chain are coded for by multigene families, located on different chromosomes [33]. In humans the κ chain is found on

chromosome 22, the λ chain on chromosome 2 and the heavy chain on chromosome 14. Each of these multigene families are made up gene segments – **L**, **V**, **J** and **C** gene segments in the light chain families; **L**, **V**, **D**, **J** and **C** in the heavy chain family – and individual segments contain a large number of different coding sequences. The **L** segment codes for a short **L**eader sequence able to guide the heavy or light chain through the endoplasmic reticulum, but which is cleaved before assembly of the immunoglobulin molecule. The **V** (**V**ariable) and **J** (**J**oining) segments encode the variable region, and the **C** segment encodes the **C**onstant region. The **D** (**D**iversity) segment is found only in the heavy chain gene family and codes for a third hypervariable region lying between $\mathbf{V}_H$ and $\mathbf{J}_H$.

The body is able to produce as many as 10^{11} antibodies of different specificity [34]. Diversity of immunoglobulin structure, and hence specificity, is generated by a number of mechanisms. Combinatorial diversity is facilitated by the process of **V(D)J** recombination, during which various **V**, **D** and **J** genes are assembled to create unique sequences. This process is initiated by the introduction of a DNA double strand break (dsb) at specific sequences that flank each gene segment, an event mediated by the action of two recombination activating genes, RAG1 and RAG2 [35]. The dsb is then processed and the modified gene segments joined together. This latter process requires the action of other molecules essential to DNA dsb repair in all cell types – XRCC4, DNA ligase IV, Ku 70, Ku 80, and the catalytic subunit of DNA dependent protein kinase [36]. In addition to **V(D)J** recombination, diversity is also promoted by the opportunity for different light and heavy chains to assemble and other processes such as junctional flexibility, somatic hypermutation and nucleotide addition.

1.1.2.2.4 Immunoglobulin class switching

The generation of immunoglobulin diversity is an essential feature of humoral responses. However, an additional key event for effective humoral immunity is immunoglobulin class switch recombination (CSR), since this ability enables the immune system to express different Ig isotypes, with concomitant different effector functions, whilst still maintaining antigen specificity.

CSR is a DNA recombination process in which the μ switch (S) region of IgM is joined to a downstream S region of another antibody class. The intervening DNA initially loops out and is then excised as 'switch circle DNA', allowing the expression of the downstream antibody isotype. The process is dependent on transcriptional activity and the cooperation of other molecules to transduce signals from the environment to the intracellular compartments and activate the switch machinery [37]. Germline gene transcription is cytokine dependent and the type of the cytokine dictates the region of the Ig locus at which transcription is activated, and hence directs isotype specificity. Thus IL-4 and IL-13 initiate transcription through the ε locus [38][39][40], a function that is inhibited by IFN-γ [41]. Initiation of transcription must be accompanied by activation of switch recombination in order to produce mature transcripts. This requires engagement of the surface molecule CD40 on B-cells with CD40 ligand (L) (CD154) expressed on activated T-cells [42][43]. However, the process is a complex one and an increasing number of other proteins are being reported to participate in CSR. For example, B-cell specific activator protein (BSAP), a transcription factor, may interact with nuclear factor kappa B (NF-κB) to regulate CD40-dependent ε germline transcription [44][45].

1.1.3 Immune activation

1.1.3.1 T-cell priming

Activation of naïve T-cells - termed T-cell priming - is regulated by three distinct signals. The process is initiated by binding of the TCR-CD3 complex to antigenic peptide presented by MHC class II on an APC *('signal 1')*. This TCR-mediated signal is amplified by an interaction with costimulatory and adhesion molecules *('signal 2')*, while soluble or membrane-bound molecules participating in activation polarise cytokine production toward T_H1 or T_H2 patterns *('signal 3')*. 'Professional' antigen presenting cells are dendritic cells (DCs), macrophages and B-cells. Only DCs constitutively express MHC class II and costimulatory molecules and therefore they have a particular role in the activation of naïve T-cells.

1.1.3.1.1 The Immunological Synapse

The point of interaction between a T-cell and an APC is the immunological synapse [46]. This specialised cell-cell junction comprises a central cluster of TCR-MHC complexes (central supramolecular activation clusters – cSMAC) surrounded by adhesion and costimulatory molecules (peripheral SMAC – pSMAC) [47].

1.1.3.1.2 'Signal 1' – TCR-CD3 antigen binding

Antigen is internalised and processed by the APC. The resultant peptide-class II MHC complex is then transported to the cell surface where it is recognised by the TCR. The TCR is associated with a group of polypeptides - collectively known as the CD3 complex - which functions as the intracellular signalling unit. Signalling is initiated when crosslinking of the TCR is effected by engagement with peptide-MHC complexes. This induces the translocation of the coreceptor CD4 to the TCR and the recruitment of protein tyrosine kinases (PTKs) Fyn and Lck. These PTKs are required for the phosphorylation of the immunoreceptor tyrosine based activation motif (ITAM) sequences on the cytoplasmic domain of the CD3 complex, an action that initiates signal transduction from the receptor complex. The subsequent signalling cascade leads to activation of the PTK, ZAP-70, which in turn activates many pathways that culminate in the release of transcription factors necessary for the transcription of genes required for IL-2 and IL-2 receptor production. Binding of IL-2 to its receptor stimulates T-cell proliferation and differentiation, thereby producing a clone of memory or effector T-cells.

1.1.3.1.3 'Signal 2' – Costimulatory molecules

The molecules CD80 and CD86 (B7-1 and B7-2) are members of the immunoglobulin superfamily and are expressed constitutively on dendritic cells and may be induced, or up-regulated, respectively, on macrophages and B-cells. Their ligands are CD28 and CTLA-4 (CD152), both of which are expressed by T-cells. Signalling via CD28 provides a positive costimulatory signal while signalling via CTLA-4 downregulates T-cell activation. Engagement of the TCR with antigen results in induction of CTLA-

4 expression and has an important role in lymphocyte homeostasis [48]. *'Signal 2'* is essential for T-cell clonal expansion and in its absence the T-cell becomes anergic.

While CD28-mediated costimulation appears to be the primary costimulatory signal for naïve T-cells, several other CD28 family members have been identified and shown to regulate T-cell expansion and effector function *(table 1.1)*. Inducible costimulator (ICOS) ligation results in up-regulation of CD40L on T-cells [49] and an increased secretion of IL-4, IL-5, IL-10, IFN-γ and TNF-α [50], indicating a function of ICOS in enhancing T-cell dependent B-cell help and Ig class switching [51].

Programmed death-1 (PD-1), like CTLA-4, mediates an inhibitory signal for T-cells. PD-1 knock-out mice develop autoimmune disease [52], but a delay in the disease onset and the fact that the presence of PD-1 does not protect CTLA-4 deficient animals from severe lymphoproliferation would suggest that PD-1 has a secondary role in lymphocyte homeostasis.

Table 1.1 The CD28 family of costimulatory molecules.

Receptor	Ligand(s)	Biological function
CD28	CD80 (B7-1) & CD86 (B7-2)	Activation
CTLA-4	CD80 (B7-1) & CD86 (B7-2)	Inhibition
ICOS	B7h/B7RP-1/LICOS/B7H2	Activation
PD-1	B7H1/PD-L1	Inhibition

1.1.3.1.4 'Signal 3' – Determinants of T-cell polarisation

The cytokines present during T_H activation strongly influence T-cell differentiation. Thus, IL-12, secreted by macrophages and dendritic cells, promotes T_H1 differentiation and cell-mediated immune responses [53] via the IL-12 receptor, itself

up-regulated by the prototypic T_H1 cytokine IFN-γ [54]. Conversely, T_H2 differentiation, and thereby humoral immunity, is promoted by IL-4 [55][56]. Naïve T_H cells exposed to IL-4 will differentiate into T_H2 cells, and indeed, above a threshold level of IL-4, T_H2 differentiation is favoured over T_H1, even if IL-12 is present [54]. However, a number of other factors also influence T_H polarisation, including the antigen dose [57], the type of APC [57], the nature of the pathogen [58][59], and the type of affected tissue [60]. Dendritic cell APCs may be induced to produce high or low levels of IL-12: exposure of immature DCs to IFN-γ or viral RNA during their initial activation primes for mature DCs capable of high IL-12 production, and consequently a T_H1 driving capacity (Type 1 DC) [61]. Conversely, prostaglandin E2 (PGE_2), as may be produced by helminth infection, primes for low IL-12 production (Type 2 DC) and hence will promote T_H2 cell development [62]. Therefore, based on the nature of their experience in peripheral tissues, DCs may direct appropriate T-cell development for protective immunity. However, if the microenvironment is able to determine functional polarisation of the APC, then it is also possible that aberrant environmental signals may promote T_H skewing, with implications for immune dysregulation.

1.1.3.2 B-cell activation

A signal sequence similar to that described above is also apparent in B-cell activation.

B-cells bind antigen via the B-cell receptor (BCR), comprised of a ligand binding immunoglobulin and a heterodimer – Ig-α/Ig-β – containing the signal transducing ITAM sequence. Binding initiates receptor-mediated endocytosis of the antigen and induces signalling through the BCR *('signal 1')* that leads to the up-regulation of class II MHC molecules and the costimulatory ligands CD80 and CD86 (B7 family). Internalised antigen is processed and presented on the cell membrane in association with class II MHC.

B-cell activation is dependent on interaction with T_H cells. When a T_H cell encounters an appropriate B-cell a T-B conjugate is formed. CD40L expressed on the activated T-cell interacts with CD40 on the B-cell to ensure B-cell activation *('signal 2')*. Cytokines produced by the T_H cell also act on the B-cell to induce proliferation and

differentiation into memory B and plasma cells, immunoglobulin class switching and affinity maturation *('signal 3')*.

1.2 Modulation of Immune Responses through Pregnancy and Early Life

1.2.1 Maternal immune responses through pregnancy

Human pregnancy creates a unique immunological challenge. Despite intimate contact between fetal allogeneic tissue and maternal uterine tissue, the fetus manages to escape maternal immune rejection. Several mechanisms may account for this paradox [(63)]:

The implanting blastocyst has an outer covering of two trophoblast cell layers *(figure 1.2)*, - an inner cytotrophoblast and an outer syncytiotrophoblast. Chorionic villi (CV) (which ultimately form the placenta) develop from these cell layers as outgrowths of cytotrophoblast covered with syncytiotrophoblast. Invasive cytotrophoblast extending beyond the vicinity of the CV is referred to as extravillous trophoblast. The trophoblast is of embryonic origin and therefore bears paternally-derived antigens, which should be recognised as foreign by the immunocompetent mother. Maternal cells only encounter two subpopulations of the trophoblast, - the syncytiotrophoblast of the CV and the extravillous trophoblast. Maternal immune cell activation by trophoblast antigens is limited by the fact that the syncytiotrophoblast does not express HLA class I or II molecules and the expression of non-classical HLA-G on extravillous cytotrophoblast does not appear to stimulate T_C activity [(64)], and actively inhibits NK cells [(65)].

Utero-placental products, such as progesterone [(66)], prostaglandin E2 [(67)] and cytokines such as IL-4, IL-5 and IL-10 [(68)], inhibit maternal T_H1 responses that are detrimental for pregnancy [(69)(70)]. IL-4 and IL-4 receptor expression have been identified in both fetal and maternal endothelial cells [(71)]. IL-4 has a variety of actions, including stimulation of cell growth and differentiation, inhibition of IL-1 production by human monocytes and hence untimely uterine activity [(72)] and inhibition of NK cell recruitment and activation [(73)].

Other soluble factors that may regulate fetal tolerance include leukaemia inhibitory factor (LIF) and indoleamine 2,3 dioxygenase (IDO). LIF is synthesised by the uterine endometrium and is essential for implantation [74]. The action of LIF through pregnancy may induce trophoblast growth and differentiation [75]. IDO, an enzyme that catabolises tryptophan, is secreted by the syncytiotrophoblast and is essential for maintenance of pregnancy in the mouse [76]. This function may relate to an inhibition of maternal immune cell function by tryptophan depletion.

Placental macrophages are able to exert anti-inflammatory effects: they are deficient in the production of oxygen-free radicals and produce more anti-inflammatory IL-10 and IL-1 receptor antagonist. Also, in pregnancy, activation of the complement cascade may be inhibited [77] and expression of CD95 ligand by syncytiotrophoblast may trigger apoptosis of activated CD95+ T-cells with which they come into contact, thereby eliminating potentially damaging maternal T-cells [78][79].

Despite a state of functional immune suppression, the pregnant woman is not notably susceptible to infection. This occurs because of a compensatory activation of the innate immune system. There are an increased number of monocytes and granulocytes, which have an activated phenotype [80] and show increased phagocytosis and respiratory burst activity [81][82]. A unique immunological dysregulation thus provides defence against infection without impairing fetal survival.

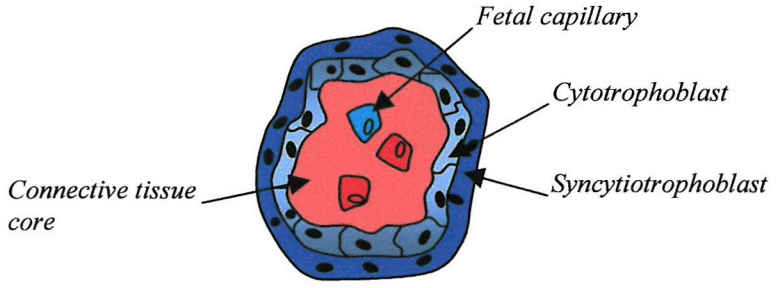


Figure 1.2 A chorionic villus early in gestation.

A continuous layer of syncytiotrophoblast is in contact with the maternal circulation. Beneath this is the cytotrophoblast cell layer. The core is composed of connective tissue and fetal capillaries.

1.2.2 *In utero* immune development

At birth, the newborn baby must deal with the challenge of transfer from an antenatal sterile environment to a postnatal world of microbes. A successful transition is thus dependent on the development of an intact, and functional - albeit immature - immune system.

1.2.2.1 Cell-mediated immune responses

Haematopoiesis begins as early as 3-4 weeks of gestation in the fetal yolk sac and extraembryonic mesenchymal tissue. By 5-6 weeks of gestation the fetal liver has become the main site of haematopoiesis, though this function declines in the third trimester and ceases soon after birth [83]. The thymus and spleen are seeded from the liver and pluripotent stem cells are detectable in the bone marrow at 11-12 weeks of gestation.

The pluripotent stem cell develops into myeloid or lymphoid stem cells. Myeloid stem cells form granulocytes, including eosinophils, basophils, neutrophils and mast cells, and mononuclear cells, including monocytes, macrophages and myeloid dendritic cells. The lymphoid stem cell further develops into T-cells, B-cells, NK cells and lymphoid dendritic cells.

MHC class II + cells have a particular role in adaptive immunity by initiating the immune response through antigen processing and presentation. Macrophages and dendritic cells can be detected in the fetal liver at 7-8 weeks gestation. Langerhans cells (skin-resident DCs) migrate into the epidermis during the first trimester and have a phenotype resembling the adult by the second trimester [84], whilst in the lamina propria of the fetal gut, class II + cells are present by 11 weeks gestation [85][86] and CD83+ dendritic cells detectable in lymphoid aggregates by 14 weeks gestation [86]. Functionally, cord blood monocytes have been shown to phagocytose at a level comparable to adults, but to display reduced chemotaxis and cytokine production [87][88]. Furthermore, monocytes, but not dendritic cells [89], were capable of antigen presentation, as determined by mixed lymphocyte reaction [90].

CD7+ T-cell precursors from the fetal liver seed the thymus by 8-9 weeks gestation. T-cell numbers increase in the fetal spleen and liver between 12 and 23 weeks gestation and T-cells migrate into the circulation by 15-16 weeks gestation [91]. T-cells may be detected in the lamina propria and epithelium of the fetal gut by 12-14 weeks gestation [92] and these cells are phenotypically distinct from circulating T-cells and precede the development of Peyer's patches (intestinal lymphoid follicles), implying that they are not derived from these sources [93]. Furthermore, the presence of costimulatory molecule pairs (CD86-CD28/CD152 and CD40-CD40L) in the gut from 16 weeks gestation suggests that the molecular 'machinery' required for *in utero* initiation of antigen-specific reactivity is in place from the second trimester [86]. Indeed, activation of T-cells in the fetal gut is suggested by the surface expression of activation markers HLA-DR, CD25, CD69 and low CD62L, and the memory marker CD45 RO [93]. Mitogen-induced proliferation of cord blood mononuclear cells has been demonstrated from 17 weeks of gestation [94] and antigen-specific reactivity from 23 weeks gestation [95]. However, umbilical cord T-cells produce lower concentrations of cytokines compared to adult T-cells [96], particularly in relation to T_H1 cytokines [97], a deficit that might have repercussions for the biological activity of other cytokine-dependent immune cells.

1.2.2.2 Humoral responses

B-cell progenitors may be identified in the fetal liver by 8 weeks of gestation. Subsequently B-cells are found in the circulation by 12 weeks, the spleen between 13 and 23 weeks and the bone marrow between 16 and 20 weeks of gestation.

Circulating B-cells express the surface molecules CD19, CD20, CD21, CD22, HLA-DR, IgM and IgD [98]. The majority of these cells are also CD5+, in comparison to adult blood, where only a few B-cells display this marker [98]. This difference may have a functional significance, as CD5+ cell activation is T-cell independent and produces polyreactive antibodies, which may offer a useful first line of defence for the susceptible newborn.

Endogenous production of immunoglobulin by the neonate is impaired: umbilical cord blood IgM, IgA and IgE are extremely low and IgG is primarily of maternal

origin. However, the fetus has the capacity for Ig synthesis and class switching, as evidenced by the spontaneous, and antigen-stimulated, production of parasite-specific IgE and IgG by cord blood mononuclear cells from babies born to helminth-infected mothers [99]. Furthermore, detection of elevated cord blood IgE concentration is a risk factor for later allergic sensitisation [100] and atopic disease [101][102]. The impairment of humoral immunity is thus likely to relate to a deficiency in T-cell-derived cofactors required for the process.

1.2.3 Postnatal immune development

1.2.3.1 Passive humoral immunity – IgG transfer

Since the neonate is deficient in IgG production, the transplacental transfer of maternal specific IgG is essential for the survival of the newborn baby. This mechanism provides the infant with a temporary 'library' of maternal antigenic exposures that have not been experienced during fetal life.

Maternal IgG crosses the placenta by an active transport mechanism [24][99]. The placental barrier across which IgG must pass consists of two cell layers - the syncytiotrophoblast and fetal capillary endothelium - and an intervening stroma [103] *(figure 1.3)*. The syncytiotrophoblast covers the chorionic villi. IgG has been visualised by immunoelectronmicroscopy to cross this layer by transcytosis in vesicles [104], following what is thought to be either receptor-mediated, or fluid phase endocytosis. A number of IgG receptors (FcγR) have been identified in the syncytiotrophoblast, including placental alkaline phosphatase [105], annexin II [106], FcγRIII [107] and the neonatal Fc receptor, FcRn [108]. A mechanism for IgG transfer across the syncytiotrophoblast has been hypothesised [109]: after receptor-mediated, or fluid phase endocytosis, the vesicles containing IgG fuse with endosomes, causing exposure to low pH. IgG binds preferentially to FcRn at low pH and so is targeted for transcytosis. At the basal surface IgG is exocytosed and released from FcRn due to the more neutral pH within the CV stroma. Once across the syncytiotrophoblast, IgG appears to transit the villus interstitium via bulk flow [110]. Transport across the villus endothelium is less well understood, but is likely to occur in intracellular structures

called caveolae [111]. Villus endothelial cells express receptors specific for IgG, identified predominantly to be the FcγRIIb2 isoform [112]. In mice this receptor is capable of mediating transepithelial IgG transport [113], suggesting that it may well have a similar function in humans.

All IgG subclasses cross the placenta in an order of preference IgG1>G3>G4>G2 [114]. Transfer is inefficient in the first trimester, but the rate increases from 20 weeks of gestation throughout the remainder of pregnancy, such that by term fetal IgG concentrations exceed those in the maternal circulation [114].

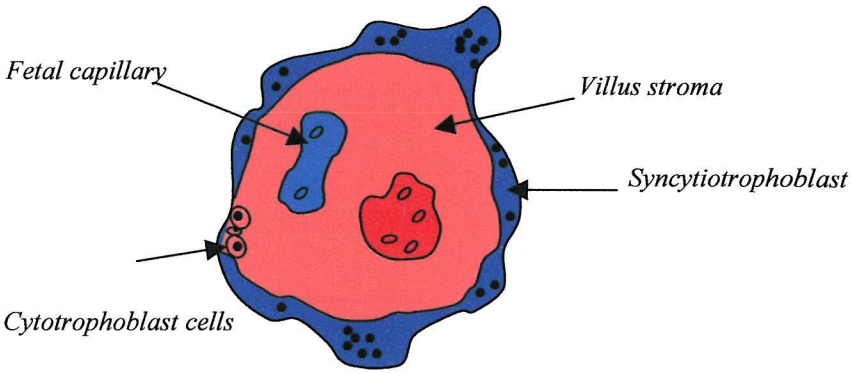


Figure 1.3 A chorionic villus at term.

By term the cytotrophoblast cell layer has almost completely disappeared. The chorionic villus is now made up of a complete layer of synctiotrophoblast and the underlying stroma containing the fetal capillaries.

1.2.3.2 Breast milk

Human milk is considered to be the ideal food for newborns. Not only does it have an appropriate nutritional composition [115], but it also contains bioactive factors that promote immune protection and modulate immune function of the young infant [116].

Milk is a complex fluid containing carbohydrate (in particular lactose), fat, protein and minerals. Minor components include enzymes, vitamins, oligosaccharides, trace elements and growth factors. Cellular components, mostly sloughed epithelial cells, macrophages, neutrophils and lymphocytes, account for a small fraction of the total milk volume. Many of these components promote anti-microbial defence. For

example, lactoferrin, a major milk protein, chelates iron and so is bacteriostatic for siderophilic bacteria and fungi [117]. Lactoferrin also activates NK cells, modulates complement activation and enhances neonatal intestinal growth and probiotic bacterial colonisation, thereby reducing intestinal infection [118].

Specific antibody targeted protection against pathogens in the infant's environment is provided by breast milk immunoglobulins, of which secretory IgA is the predominant class. The plasma cells that produce sIgA home to the interstitium underlying the mammary secretory cells after migrating from lymphoid tissue in the intestine (Peyer's patches) and the bronchial tree. Thus the specific IgA secreted into breast milk appropriately provides the infant with protection against microbes previously encountered at mucosal surfaces. Dimeric secretory IgA crosses the mammary epithelium after binding to the polymeric immunoglobulin receptor (pIgR) expressed at the basolateral surface of the cell. The receptor-IgA complex is endocytosed into a coated pit and the vesicle transferred to the luminal surface, where it fuses with the membrane. The receptor is enzymatically cleaved from the membrane, forming the secretory component, and is secreted with the IgA [26][119].

The polymeric structure of sIgA enables it to cross-link large antigens and so prevent pathogen attachment to mucosal cells, and hence invasion. Furthermore, the antigen-IgA complex may be easily entrapped by mucus and thus eliminated by gut peristaltic activity.

A variety of immunomodulatory agents in breast milk influence the development of the immature neonatal immune system. These include breast milk cytokines: IL-8 is chemotactic for intestinal intraepithelial leucocytes, IL-10 and IFN-γ modulate intestinal epithelial barrier integrity [120], IL-12 enhances the production of inflammatory cytokines [121] and TGF-β promotes the local production of IgA [121].

Breast milk contains high concentrations of soluble CD14 (sCD14) [122], which facilitates the interaction of CD14 (LPS receptor) negative cells, such as epithelial cells, with the bacterial product LPS [123]. In the neonatal gut this may have implications for regulation of the immune response to the first encounter with the germ-laden environment.

Recent research interest has focused on the effects of dietary lipids, especially polyunsaturated fatty acids, and dietary anti-oxidants upon the immune system [124][125]. Polyunsaturated n-6 series fatty acids in breast milk, derived from dietary linoleic acid, may have anti-inflammatory properties [126]. The arachidonic acid metabolite - PGE_2 - suppresses antigen-specific T-cell proliferation in gut-associated lymphoid tissue [127] and promotes an anti-inflammatory milieu through the suppression of T_H1 cell development [62] and stimulation of IL-10 [128] and TGF-β secretion with maintenance of IgA production.

1.2.3.3 Microbial stimulation

The adaptive immune response, deficient at birth, does not fully mature until approximately 5 years of age [129]. During this period, the T_H1/T_H2 balance in all children favours the T_H2 cytokine phenotype, most likely reflecting the differential suppression of T_H1 responses in fetal life. Postnatal maturation of T_H1 competence is dependent on stimuli from the microbial environment, both pathogenic [130] and commensal [131] organisms. In the absence of appropriate microbial contact, the overall balance within the adaptive immune system remains skewed towards a T_H2 profile and immature B-cells are switched to IgE production [132].

1.3 The Abnormal Immune Response – Allergic Disease

The complex structure and function of the immune system means that there is also much opportunity for malfunction. A range of clinical diseases is consequent on this occurrence. For example, immunodeficiency diseases are a group of disorders that arise from the defective functioning, or lack, of any combination of the immune components. Primary immunodeficiencies are due to genetic or developmental defects, whilst secondary immunodeficiencies are caused by a variety of agents, including infection with HIV-1. The clinical sequelae range from mild effects, such as recurrent respiratory infections associated with IgA deficiency, to severe and potentially fatal disease, such as severe combined immunodeficiency (SCID).

The discrimination between self and non-self antigens is a characteristic quality of the adaptive immune system. A loss of this ability results in autoimmune disease, such as Crohns disease and rheumatoid arthritis, whilst retention of this capacity, in the context of therapeutic tissue transplant, may induce graft rejection or graft versus host disease.

An exaggerated immune response to an innocuous antigen is referred to as a hypersensitivity reaction. Hypersensitivity reactions are classified into four types according to the underlying mechanism. Types I, II and III are adverse reactions mediated by the humoral branch of the immune system and are referred to as immediate hypersensitivities, whereas Type IV describes cell-mediated adverse reactions and is referred to as delayed-type hypersensitivity (DTH). Type I hypersensitivity reactions are mediated by overproduction of IgE in response to common ingested or inhaled antigens. These reactions, which predominate in genetically susceptible – or atopic – individuals, are manifest as a group of disorders that are referred to as allergic disease.

1.3.1 Clinical spectrum of allergic disease

The spectrum of allergic disease includes asthma, eczema, allergic rhinoconjunctivitis and food hypersensitivity. Disease severity varies from person to person, ranging from mild and intermittent, to continuous and intractable symptoms. They constitute a major source of suffering, disability and loss of productivity throughout the world [133][134].

Allergic disease most commonly presents in childhood. Food allergies and eczema are usually the first atopic manifestations appearing in the first weeks and months of life. The foods most frequently responsible for allergic symptoms in childhood are cow's milk, hen's egg, wheat, soy and peanuts. Symptoms may appear in the gastrointestinal tract (diarrhoea, vomiting), the skin (urticaria, atopic dermatitis) and less commonly in the respiratory tract (wheezing, rhinorrhoea). While food induced disease often resolves [135][136], food allergy is predictive of later inhalant sensitivity and asthma [137][138].

Atopic dermatitis (AD) is characterised by a symmetrical, itchy, papulovesicular rash that has an age-related, distinctive distribution. Like food allergy, while AD itself is often 'outgrown' [139], it is also a risk factor for the development of persistent allergic disease, such as asthma, particularly if it presents early in life or is severe [140][141][142]. This progression of symptoms from food allergy and atopic eczema to respiratory allergy is often referred to as the 'Allergic March' [143].

1.3.2 Mechanism of atopic allergic disease

Atopic allergic disease is characterised by the production of allergen-specific IgE. The IgE sensitises tissue mast cells and blood basophils by binding to high-affinity IgE receptors (FcεRI) on the cell surface [144]. Further allergen exposure, with antigen binding and cross-linking of the membrane-bound IgE, triggers a cascade that results in cell degranulation and *de novo* synthesis of lipid mediators and cytokines [145]. The clinical effect is an immediate weal and flare reaction, sneezing and runny nose or wheeze, depending on whether the allergen is encountered at the skin, nose or airways, respectively. This immediate response may be followed by a late-phase reaction (LPR), which is slow to peak (6-9 hours) and slow to resolve. In the skin, LPRs are characterised by an oedematous, erythematous and indurated swelling, in the nose, by sustained blockage and in the lung, by further wheezing.

Sensitisation is the first step in the allergic process. Dendritic cells in the lung or gut and Langerhans cells in the skin take up allergen at the epithelial surface and migrate to regional lymph nodes. Here they present peptide-MHC complexes to naïve T-cells and induce T-cell activation and T_H2 differentiation. Following antigen presentation to T-cells, allergen-specific B-cells may be activated to produce specific IgE, which is bound by mast cells and basophils expressing FcεRI on their surface. Antigen uptake and presentation are enhanced, 100-1000-fold, by the presence of IgE receptors on the surface of APCs, - a process known as antigen focusing [146]. In addition, IgE binding to mast cells and basophils up-regulates FcεRI surface expression, an effect that facilitates a lower threshold for cell activation, and secretion of increased amounts of mediators [147]. Thus IgE production enhances both facilitated antigen presentation and the biological activity of the effector cells.

The acute effector reaction – responsible for urticaria, acute rhinoconjunctivitis or asthma and potentially fatal anaphylaxis – is triggered by allergen-induced aggregation of mast cell surface FcεRI complexes. Receptor cross-linking results in a cascade of signal transduction events that lead to the release of preformed granule-associated histamine and tryptase and membrane-derived lipid mediators – prostaglandins, leukotrienes and platelet activating factor. The products induce vasodilation, increased vascular permeability, smooth muscle contraction and mucous hypersecretion. Itch is provoked by stimulation of cutaneous sensory nerves by histamine.

The chronic effector reaction, which includes the late phase reaction, is responsible for chronic eczema, asthma and nasal symptoms. This reaction is characterised by cellular infiltration, in particular, eosinophils and T-cells. The mediators of the immediate reaction promote cell recruitment, and these recruited cells in turn release mediators that contribute to the perpetuation and progression of the inflammatory reaction *(figure 1.4)*.

T_H2 cytokines play a critical part in allergic pathology. In addition to promoting IgE production (IL-4, IL-13), their effects include mast cell development (IL-3, IL-9, stem cell factor), airway hyperresponsiveness (IL-9, IL-13) and mucous hypersecretion (IL-4, IL-9, IL-13). They also facilitate eosinophil activity by promoting eosinophil maturation (IL-5, IL-9), recruitment - through upregulation of the eosinophil selective adhesion molecule VCAM-1 (IL-4, IL-13) [148] - and by prolonging cell survival through delayed apoptosis (IL-3, IL-5, GM-CSF).

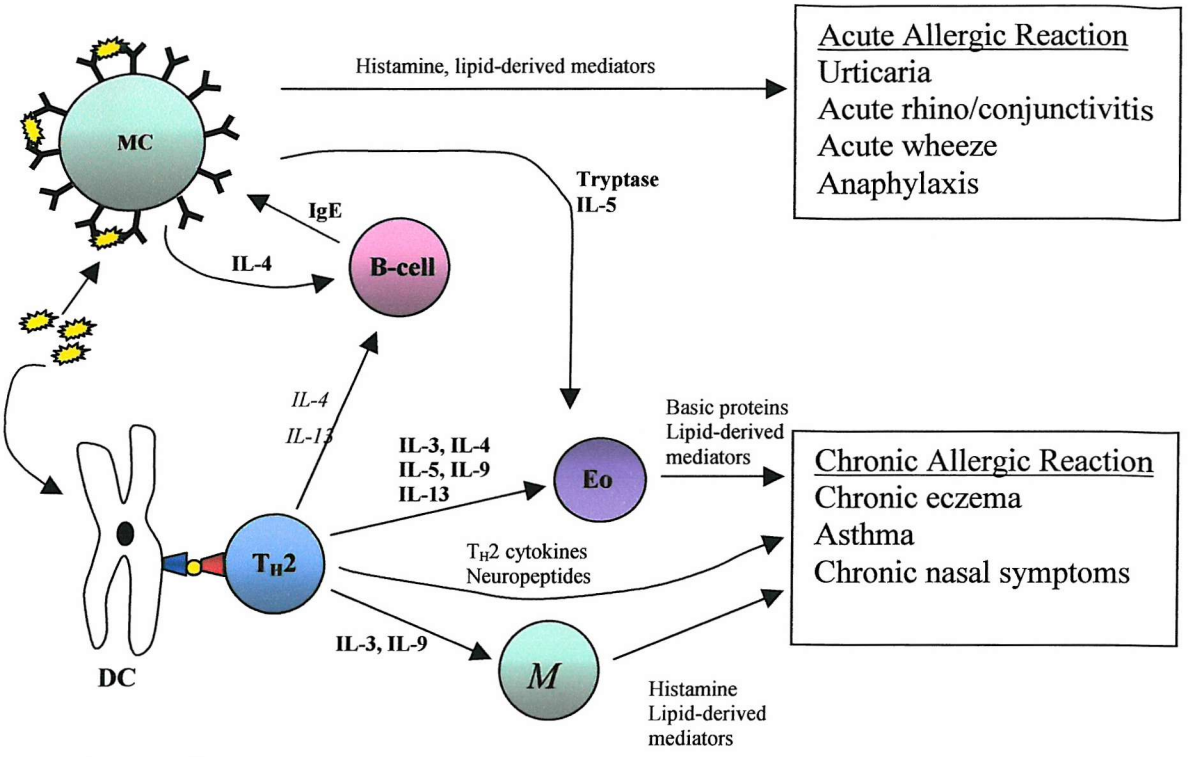


Figure 1.4 Mechanism of atopic allergic disease.

An acute allergic reaction is precipitated by the interaction of an allergen (yellow shape) with membrane-bound IgE on a mast cell (MC) or basophil. The interaction results in MC degranulation with the release of mediators, chemotactic factors and cytokines into the local tissue. The late phase reaction (LPR) (including chronic allergic reactions) can be provoked by mast cell or T-cell activation. T_H2 cells may be activated by MHC class II-restricted allergen presentation by dendritic cells (DC). The LPR is characterised by a cellular infiltrate, particularly eosinophils (Eo) & T-cells. A variety of cytokines, mediators & neuropeptides induce & sustain chronic inflammation. Adapted from [448]

1.3.2.1 Atopic dermatitis

Atopic dermatitis is a chronic inflammatory skin condition with a complex pathogenesis that reflects an interaction between genetic, environmental and psychological factors. Like other atopic allergic diseases, the prevalence of AD has been rising steadily in recent decades [149][150].

AD illustrates the clinico-pathological consequence of cell-mediated inflammation. The skin lesions are characteristically infiltrated by T-lymphocytes [151] - both CD4+ and CD8+ cell types [152]. These cells have a memory phenotype (CD45 RO+) and express the selective skin homing receptor Cutaneous-Lymphocyte-associated Antigen (CLA) [153].

Cell infiltration may be controlled by skin-associated chemokines and cytokines: CCL27 (Cutaneous T-cell Attracting ChemoKine - CTACK) is constitutively produced by keratinocytes and preferentially attracts CLA+ T-cells *in vitro* [154]. Eotaxin – chemotactic for eosinophils and T_H2 cells – is highly expressed in AD skin [155], and acute AD lesions also express high levels of IL-16, which is chemotactic for CD4+ cells [156].

The allergen-specific T-cells produce predominantly T_H2 cytokines (IL-4, IL-5, IL-13) [149][154], although IFN-γ predominates over IL-4 in chronic skin lesions [157][158]. IL-13 is the major cytokine responsible for the increased IgE associated with atopic dermatitis [159], while IL-5 secretion exerts multiple effects on eosinophil activity *(1.3.4)*, including prevention of eosinophil apoptosis [152].

A characteristic histological feature of AD is epidermal spongiosus, caused by loss of keratinocyte cohesion and influx of fluid from the dermis. An increased susceptibility of keratinocytes to apoptosis underlies this appearance [160]. T-cell derived IFN-γ up-regulates Fas receptors on keratinocytes, rendering them susceptible to apoptosis by Fas ligand expressed, or released, by infiltrating T-cells. By contrast, apoptosis of these T-cells is prevented by cytokines and extracellular matrix components of eczematous skin [154]. Dysregulation of apoptosis may thus be a key mechanism in the pathogenesis of atopic dermatitis.

1.3.3 Epidemiology

The prevalence of allergic disease has increased dramatically in recent decades, particularly in English speaking Western countries: a recent survey in western Scotland reported a doubling in the prevalence of atopic asthma in adults over 20 years [161], while in 1998 the International Study of Asthma and Allergies in Childhood (ISAAC) found that 35% of 13-14 year olds in the UK had had asthma symptoms in the previous year, the highest 12 month prevalence of all the countries surveyed [162].

The aetiology of allergy is multifactorial and complex. Genetic inheritance is integral to the pathogenesis, a feature that has been recognised since the early part of the 20th century [163][164]. Atopy, the genetic tendency to generate IgE [165], is the strongest single risk factor for the development of allergic disease: atopic individuals have a 10-20 fold increased risk of developing asthma, as compared to non-atopic individuals [166], and infants born to atopic parents have more than twice the risk of later allergic disease, as compared to those with no family atopy risk [164].

The genetics of allergic disease is complicated by the fact that these disorders are associated with mutations in multiple genes. Using modern molecular techniques, several loci for candidate atopy genes have been determined on various chromosomes *(table 1.2)*.

Table 1.2 Gene regions associated with atopy.

Region	*Candidate gene*	*Phenotype*
2q21-q23	Unknown	specific IgE
5q31.1	IL-4 cluster	total IgE
5q32-q33	Glucocorticoid receptor	total IgE
	β2-Adrenergic receptor	bronchial hyperreactivity, asthma
6p21.3	MHC class II	specific IgE
6p21.3	TNF-α	total IgE
8p23-p21	Unknown	specific IgE
11q13	High affinity IgE receptor	atopy, asthma, total & specific IgE
12q15-q24.1	IGF1, SCF	atopy, total IgE
14q11.2	T-cell receptor	specific IgE
	Mast cell chymase	atopic dermatitis
14q32	IgG heavy chain	atopy
16p11.2-12.1	IL-4 receptor α	total IgE

Genetic factors have only a limited effect on atopic phenotype. For example, there are low concordance rates for asthma observed in twin studies [167] and following the division of Germany, despite a similar genetic predisposition for atopy, there was a marked divergence in the national prevalence rates for allergic disease [168]. These observations suggest that genetic factors must interact with environmental factors to induce clinical disease expression and severity.

The importance of environmental influences is also suggested by the fact that the observed increase in allergic disease prevalence has occurred over only a few decades, a time period that precludes genetic factors in the aetiology. Accordingly, many environmental factors have been implicated in this phenomenon, including changes in diet, air pollution and home living conditions [reviewed in (169)]. Whatever the underlying reasons, the result is a huge number of adults and children with often life-long, and frequently incapacitating, medical conditions, which also have substantial cost implications for health care provision [170]. Allergic disease is undoubtedly a major public health issue and an increased understanding of the mechanisms underlying this process should be considered a priority for research.

1.3.4 Egg allergy

Epidemiological studies in recent years have shown repeatedly that, of all the allergenic foods, egg has a unique place in the natural history of allergic disease. In families with a genetic risk of atopy, egg sensitisation [171][172] or symptomatic egg allergy [173] in infancy are strong risk factors for inhalant sensitisation and asthma in later childhood and adulthood [174].

Hen's egg comprises 8-11% shell, 56-61% white and 27-32% yolk. The white is essentially an aqueous protein solution (10% protein, 88% water) and is the most important source of allergens, though the lipid containing yolk is also allergenic [175].

Egg white contains a number of allergens: ovotransferrin, ovomucoid and ovalbumin [176]. Ovalbumin, a 44 kDa water-soluble glycoprotein, is present in the greatest quantities. Its allergenicity has been confirmed by histamine release assay [177], radioimmunelectrophoresis and radioallergosorbent test (RAST) [178] using the pure

protein. Ovalbumin shows no cross-reactivity with albumins from other species, including human serum albumin *(SWISS-PROT protein sequence data bank* [179]*)*. In fact, there appears to be a lack of cross-reactivity between eggs from different birds, as patients allergic to duck or goose eggs may be tolerant of hen's egg [180].

1.3.5 IgE, parasitic infection and atopy

The classic stimulus for T_H2 mediated immunity is parasitic infection. T_H2 cytokines (IL-4, IL-5, IL-9 and IL-13) and eotaxins promote eosinophilopoiesis and tissue eosinophilia, and an elevated IgE concentration with sensitisation of mast cells [181][182][183]. Parasite antigen-induced degranulation of sensitised mast cells leads to the release of inflammatory mediators and cytokines that contribute to helminth killing [184] and induction of the immune response.

It is likely that in historical times humans were chronically exposed to helminth infection. Since human atopy is promoted by genetic variants within the T_H2 system *(1.3.2)*, it is possible that the relatively high prevalence of such disadvantageous variants in the modern day reflects an original evolutionary benefit, which conferred protection against helminths, and hence a survival advantage. Parasitic infestation of children has been associated paradoxically with a reduced susceptibility to atopy [185], while helminth eradication has unmasked skin sensitivity in previously unresponsive individuals [186]. Such an inverse association has not been reported in all studies [187], and factors such as helminth type, load and socio-economic status may influence the relationship [188].

There are a number of putative mechanisms by which helminth infection may suppress atopic disease expression: helminth infection is associated with excess polyclonal IgE production and saturation of high affinity IgE receptors (FcεRI) on mast cells [189], while the exhaustive production of polyclonal IgE may result in an inability to produce allergen-specific IgE. Furthermore, peripheral blood mononuclear cells (PBMCs) of parasitised children produce high levels of IL-10 when stimulated by parasite antigen [185], a cytokine that is deficient in atopic individuals with active disease [186]. Thus, helminth parasites may trigger powerful

anti-inflammatory mechanisms capable of limiting the magnitude of *in vivo* responses to allergens.

1.4 Programming of the Allergic Phenotype in Early Life

Respiratory allergic disease may be predicted by allergies – in particular, food hypersensitivity and atopic dermatitis - which often manifest within the first weeks of life [190]. Since IgE sensitisation precedes the clinical manifestation, so this relationship suggests that allergic fate may be decided in early life, either *in utero*, or in the postnatal period.

1.4.1 Pregnancy and allergy

Pregnancy has been implicated as a critical time period during which infant atopic phenotype is established. Infants are more likely to be atopic if born to an atopic mother rather than to an atopic father and this difference is not due to a reporting bias [191][192]. Raised serum IgE concentration in adults has been associated with disproportionate fetal growth, as reflected in an increased head circumference at birth [193], and a large head at birth has been associated with raised cord blood IgE and a three-fold increased risk of later asthma [194][195].

These observations suggest that the mother is not only a source of genetic information, but also directs the environment in which the fetus develops and this may have life-long sequelae for allergic disease. As discussed previously *(1.2.1)*, the utero-fetal cytokine environment is skewed to a T_H2 profile in order to ensure the success of pregnancy. Further perturbation of this pro-allergic environment has been described in atopic mothers who were found to produce significantly less IFN-γ in cow's milk β-lactoglobulin (BLG) stimulated cell cultures compared to non-atopic women [196]. Concomitantly, BLG-stimulated umbilical cord mononuclear cells of the corresponding infants produced significantly higher concentrations of IL-13, suggesting that the maternally-determined characteristics of the antenatal environment may modulate the phenotype of the infant's immune response to postnatal contact with allergen.

1.4.1.1 In utero priming

The possibility of fetal immunomodulatory events occurring through pregnancy was given credence by studies reporting polyclonal and allergen-specific proliferative responses by umbilical cord mononuclear cells at birth [197][198][199][200]. These responses are indicative of T-cell antigen-specific priming, which may occur as early as 22-23 weeks of gestation [95][201]. Cord blood mononuclear cell (CBMC) antigen-specific proliferation is a frequent finding and suggests that prenatal priming may be a universal phenomenon. However, babies with a family history of atopy have a higher proliferation index than babies with no genetic risk [202][203] and produce significantly fewer IL-12 secreting cells [204] and lower IFN-γ in culture supernatant [205][206]. The relevance of these characteristic responses for the prediction of the atopic phenotype is a controversial issue, with some [198][207][208], but not all [203][209] authors reporting an association between magnitude of cytokine and proliferation responses at birth and later allergic disease.

1.4.2 Breast-feeding and allergy

Breast-feeding provides a further opportunity for close mother-infant immunological interaction. While the protective effect of human milk for a wide variety of infections, including otitis media, respiratory tract infections, diarrhoea, neonatal sepsis and necrotising enterocolitis, has been reported [210][211][212], the benefit of breast-feeding for infant allergic outcome remains a controversial issue.

As previously discussed *(1.2.3.2)*, breast milk is a rich source of immunological information. Not only does it offer passive protection against infections, but also it actively stimulates the development of the infant immune system. Variation in the composition of breast milk between atopic and non-atopic mothers has been described, and these differences may have implications for infant atopic phenotype: allergic women were found to have significantly higher concentrations of IL-4 in their colostrum than non-allergic women [213]; they also had significantly higher levels of the chemoattractant factors IL-8 and RANTES [214], which are both up-regulated in the blood and airways in respiratory allergic disease [215][216]. These factors are integral to the regulation, and expression, of IgE-mediated hypersensitivity reactions:

IL-4 is potent inducer of a T_H2 response and IgE production, while RANTES is chemotactic for eosinophils, basophils, T-cells with a memory phenotype [217][218] and may enhance IL-4-dependent IgE synthesis [219]. Indeed, breast milk supernatants from atopic, but not from non-atopic women, stimulate IgE production by cord blood lymphocytes [220].

Oral tolerance, defined as immune unresponsiveness to non-pathogenic antigens previously encountered at mucosal surfaces, is a complex phenomenon, but is dependent on gut bacterial colonisation, as indicated by the failure to develop oral tolerance in germ-free pups [131]. Indeed, disturbance of the neonatal gut microflora, with a reduced ratio of bifidobacteria to clostridia, has been associated with the later development of atopy and atopic disease [221]. The probiotic properties of breast milk oligosaccharides, which promote the growth of lactobacillus and bifidobacteria, may thus have a beneficial effect. On the other hand, breast milk containing significantly lower quantities of sCD14 has been associated with infant eczema, and this may relate to a dysregulation of the interaction between gut epithelial cells and bacterial species, mediated by sCD14, in these milks [123]. An alternative explanation for the association between breast milk sCD14 and eczema may be that the varying levels of sCD14 influence phospholipid transport and hence the infants' dietary fat composition [222]. Previous work has associated lower breast milk polyunsaturated fatty acid levels with both maternal atopy and infant allergic sensitisation [126][223].

Clearly, breast milk constitutes an important maternally-derived environmental factor that modulates infant immunity: thus variations within this 'environment' may direct the programming of the infant atopic phenotype.

1.4.3 Infections and allergy

Studies of cytokine profiles at birth have shown that infants respond universally to common environmental allergens in a T_H2 fashion [197]. The impairment in T_H1 function that underlies this profile is restored in non-atopic children, and the adult responder phenotype, characterised by T_H1 cytokine production, is achieved by 5 years of age. In atopic children allergen-specific responses remain T_H2 polarised [224][225]. This switch from a T_H2 biased to a balanced T_H2/T_H1 response reflects the

need for the newborn infant, now no longer sequestered in the sterile pregnancy environment, to develop protective functions against infections. Evidence from work with germ-free animals [131] would suggest that the trigger for this switch is provided by microbial contact - both pathogenic and commensal organisms.

1.4.3.1 Acquired infection.

It is well recognised that children with established allergic disease often experience an exacerbation of their symptoms in the presence of an acquired infection. For example, rhinovirus infection is a common precipitant of an acute asthmatic episode, whilst superimposed staphylococcal infection may promote a deterioration of atopic dermatitis. However, there is also evidence to suggest that early childhood infections may protect against the development of atopic allergic disease – the 'Hygiene hypothesis' [226]. For example, at the time of reunification of Germany, the prevalence of atopy and asthma was significantly less in Leipzig, East Germany, than in Munich, West Germany, despite the fact that Leipzig was heavily polluted. In Leipzig, however, the incidence of respiratory infection was high and indeed, the youngest children of large families, experiencing earlier and more frequent respiratory infections were particularly protected from allergic sensitisation and asthma [227]. The protective influence of this 'sibling effect'- an increased tendency to acquire respiratory infections through household contact - is supported by the observation that children attending day-care nurseries at an early age were less likely to develop asthma [228][229].

Other epidemiological evidence suggesting a counter-atopy effect of infection is provided by surveys reporting an inverse relationship between measles infection in childhood and subsequent aero-allergen sensitisation [230], and seropositivity to hepatitis A virus and atopic status [231]. Similarly, an inverse association between DTH reactivity to tuberculin, suggestive of past exposure to *Mycobacterium tuberculosis*, and later atopy has been described [232], and indeed, more recently, the prevalence of asthma has shown a significant inverse correlation with reported rates of tuberculosis [233].

How infections might confer protection against the development of later atopic disease is not known, but a postulated mechanism is the T_H1 immunity induced by bacterial products, such as LPS and Gram-negative bacteria endotoxin. Children raised on a livestock farm, where bacterial endotoxin abounds, had a reduced risk of atopic allergic disease [234][235] and bacterial endotoxin was found in significantly lower concentrations in the house-dust of homes of sensitised infants compared to non-sensitised infants [236]. LPS stimulates marked IL-12 production, which induces a T_H1 response [236], after binding to its receptor CD14 and induction of the TLR4 signalling cascade. In further support of this mechanism is the association of a polymorphism in the CD14 gene with an increased intensity of atopy [237][238].

1.4.3.2 Intestinal flora

A major microbial assault on the neonatal immune system derives from postnatal intestinal colonisation with commensal organisms.

Since deviation from the default T_H2 immune response in animal studies was dependent on intestinal bacterial colonisation [131], it is not surprising that differences in intestinal microflora have been linked with atopic disease. In Estonia, where prevalence of atopic disease is low, gut colonisation was dominated by lactobacilli, whereas in Sweden, where allergy is common, clostridia colonisation predominated [239]. Children from both countries who developed atopic disease were less often colonised by lactobacilli and had higher counts of aerobic organisms [240]. Furthermore, supplementation of the diet with probiotic strains of bifidobacteria or lactobacilli alleviated symptoms of atopic dermatitis [241][242] and food allergy [243]. A critical step in the postnatal modulation of the atopic responder phenotype may therefore be the acquisition of an appropriate gut commensal flora.

1.4.4 Allergen exposure

Allergen exposure is a prerequisite for allergic sensitisation. Month of birth studies have shown that an infant born just prior to, or during, a time of high aeroallergen load was more likely to develop an atopic phenotype than one born at a time of low allergen exposure: thus asthmatic children born in autumn when house-dust mites

(HDM) were highest had a greater risk of house-dust mite sensitisation [244] and children born just before the birch pollen season in Scandinavia had a greater risk of birch pollen sensitivity than those born just after the season [245]. In large cohort studies allergen exposure was reported to influence sensitisation in a dose-dependent manner: infants born into an environment with high cat and dust mite allergen concentrations were more likely to become sensitised early [246] and to begin wheezing at a younger age [247].

IgE sensitisation is a strong risk factor for disease manifestation [248][249][250]. However, an association between allergen exposure and clinical disease has not been identified: for example, while early environmental exposure to inhalant allergens was found to be directly associated with sensitisation to the same allergens, this exposure had no influence on the prevalence of asthma [247][248]. Indeed, early life exposure to pets in Norway was associated with a reduced risk of atopy-related diseases in early childhood [251].

Allergen exposure may occur by maternal routes. It has long been recognised that breast-feeding may evoke allergic manifestations in the infant [252], and, as previously discussed *(1.4.2)*, adverse allergic consequences may relate to the presence of many immunoregulatory factors found in human milk. However, a variety of food allergens, including cow's milk β-lactoglobulin [253], wheat gliadin [254], peanut [255] and hen's egg allergens [256], have all been detected in human milk. Moreover, a causal relationship between maternal allergen passage and infant allergic manifestation has been established [190][257].

Exposure to maternally-derived allergen in pregnancy has more recently been confirmed with the detection of dust mite allergen, Der p1, in both cord plasma and amniotic fluid [258] and hen's egg ovalbumin in amniotic fluid [86]. The implication of this exposure for infant allergic sensitisation and disease expression is not yet known.

1.4.5 'Other' environmental factors and allergy

A number of other postnatal environmental factors, including indoor [259] and outdoor air pollution [260][261] and environmental tobacco smoke (ETS), have been implicated

in the pathogenesis of allergic disease. Passive ETS exposure has been shown by many studies to adversely affect the child's respiratory health by decreasing lung growth and increasing the risk of respiratory infections and symptoms [262][263][264], though not the risk of allergic sensitisation [265][266]. *In utero* ETS exposure, through maternal smoking, may independently increase the risk of wheeze and doctor-diagnosed asthma [264]. Indeed, several studies have shown that maternal smoking during pregnancy is a stronger predictor of wheezing and asthma than postnatal ETS exposure [263][267][268].

1.5 Allergy Prevention Strategies

Since allergen exposure is integral to allergic sensitisation and disease expression, the corollary is that reduced exposure may be associated with a reduction in the incidence of atopic disease. The benefits of allergen avoidance in improving allergic symptomatology are well appreciated: living at high altitude, where house-dust mite allergen concentrations are low, improved asthma control in house-dust mite sensitive individuals [269] and house-dust mite avoidance measures, such as encasing mattresses with occlusive covers, are routinely used in the clinical setting.

Consequently many investigators have explored extending allergen avoidance into the pre-and postnatal periods as a means of primary prevention – namely the prevention of allergic sensitisation – in a high-risk population. The level of exposure to many environmental allergens is not open to manipulation. However, dietary allergens are more easily excluded and anti-house-dust mite measures are effective in lowering exposure [270]. Therefore, attention has focused on these allergens in evaluating a primary prevention strategy.

Maternal exclusion diets in pregnancy alone, both pregnancy and lactation, and lactation only have been investigated [reviewed in (271)]. In general, the allergic outcomes of the offspring have been disappointing. Dietary interventions beginning in pregnancy have elicited either little or no [272][273][274], or only temporary [275], benefit and concerns have been raised regarding potential maternal nutritional compromise and infant welfare [276]. Whilst interventions through lactation have been viewed with

more optimism [277], even so, the reduced prevalence of infant atopic dermatitis associated with maternal dietary prophylaxis [278] was not found to be maintained at later time points [279].

Several factors may have contributed to the negative outcome of these studies, including low subject numbers, lack of randomisation and blinding. Subject compliance may be questioned since multiple foods (ranging from 2-5) were excluded. Furthermore, the prophylactic measures in pregnancy were implemented in the third trimester, long after the fetus has demonstrated the immunological capacity for priming [95][200][201]. A lack of atopic benefit might then have resulted from the regimen missing the window of immunological susceptibility.

Rigorous anti-house-dust mite measures can significantly reduce HDM exposure [270] and these measures, taken through pregnancy and postnatally, have been associated with less respiratory symptoms through the first year of life in infants with an atopic genotype [280]. Furthermore, in an earlier study, dietary exclusion through lactation combined with stringent HDM avoidance measures during the infant's first year were associated with significantly less eczema and atopic sensitisation at 4 years of age [281]. Thus, environmental manipulation may indeed have a sustained influence over infant allergic phenotype. It is likely that clinical outcome is determined by many factors, none the least of which is the success of the intervention.

1.6 Hypothesis and Aims of Project

The evidence reviewed so far suggests that early life, including fetal life, is a critical time period during which the infant's ultimate atopic phenotype may be determined. Genetic susceptibility is the cornerstone of atopic programming, but clinical outcome may be modulated by various environmental factors acting, and interacting, during the window of immunological susceptibility.

Allergic sensitisitation is a multistep process that begins with T-cell priming. The observations of antigen-specific immunological memory at birth, and a capacity for immune reactivity from 22 weeks of gestation [95], suggest that priming may occur *in*

utero. These findings necessitate fetal antigen exposure to have taken place, but when, where and how does this occur?

Unravelling the mechanisms of allergic sensitisation has huge implications for the health of the nation. Evidence suggests that the nature of the immune response to an allergen, that is, whether sensitisation or tolerance ensues, is determined by the conditions under which the initial encounter takes place. While many factors influence this encounter, an important determinant of a sensitising response may be the characteristics of the antigen itself, in particular, the dose, timing of exposure and the manner of presentation.

High antigen doses may be tolerogenic by inducing T-cell anergy [282] or deletion [283], but atopic sensitisation is also commonly associated with exposure to high levels of antigen during infancy [244][245]. Low dose antigen exposure, as will occur *in utero*, may induce tolerance by immune deviation [284], but conversely has also been shown to promote a T_H2 cytokine profile and IgE production [57][285][286].

The timing of allergen exposure is critical as implied by month of birth studies [244][245]. Newborn mice were primed if fed ovalbumin within the first week of life, this response being enhanced if feeding began antenatally [287]. However, the situation is not clear-cut as other studies report tolerance from neonatal [288] or antenatal exposure [289]. Furthermore, outcome may be influenced by the timing of exposure *within* the antenatal period as has been suggested by the observation that fetal susceptibility to birch pollen priming varied according as to when in pregnancy the mother was exposed to the birch pollen season, the majority of responders being exposed between 20-28 weeks of gestation [200].

The manner in which an allergen is presented to the fetus is unknown. Since IgG is actively transported to the fetus in large quantities, particularly in the third trimester [114], it is the ideal vehicle for allergen transport. Furthermore, there is evidence to suggest that maternal IgG may regulate priming: in animal experiments, maternal IgG had a suppressive effect on neonatal IgE production [290], while in human studies, CBMC proliferative responses to house-dust mite inversely correlated with levels of cord, maternally-derived, HDM-specific IgG [206]. When infant atopic outcome has

been evaluated in relation to cord blood IgG, both beneficial [291] and adverse [292] effects have been reported.

These experimental, epidemiological and observational data thus lend themselves to the hypothesis that the nature of early life exposure to an allergen determines infant allergic sensitisation.

The mother-infant immunological interaction has a significant role in the allergic process, - a role that includes a source and route of transmission of allergenic peptides. However, unlike other maternal immunoregulatory factors, maternal allergen exposure is open to environmental manipulation.

The central role of egg allergen in atopic programming has been consistently recognised: not only is egg allergy common in infancy, incurring considerable morbidity, but it also has significant repercussions for later, and possibly lifelong, allergic respiratory disease [174]. Moreover, the dietary sources of egg protein are well known and therefore may be excluded with relative ease, and without causing any nutritional compromise. Dietary egg exclusion by pregnant women, initiated not later than 22 weeks gestation, may therefore also offer the opportunity to evaluate dietary allergen exclusion measures as a means of primary allergy prevention.

The hypothesis of this thesis was that,

'The characteristics of early life exposure to dietary egg allergen determine infant atopic phenotype'.

In order to address this hypothesis the following aims were defined:

1 To directly demonstrate fetal and newborn exposure to ovalbumin, the principal component and major allergen of egg white.

2 To characterise exposure in terms of dose, timing and mode of allergen presentation, and in the context of maternal diet through pregnancy and breast-feeding.

3 To ascertain the relationship between the characteristics of allergen exposure and allergic sensitisation and disease in a group at high genetic risk of atopy.

4 To evaluate the effects of dietary egg avoidance and egg sensitisation on ovalbumin specific humoral responses.

Chapter Two

General materials and methods

Chapter 2

General materials and methods

2.1 General materials

2.1.1 General reagents

General laboratory reagents used in the methods of this thesis are shown in *table 2.1*. All reagents were stored at room temperature, unless otherwise stated.

Table 2.1 General reagents.

Reagent	*Supplier*
Bovine serum albumin (BSA) (fraction V)	Sigma (stored at 4^0C)
Human serum albumin (HSA)	Sigma (stored at 4^0C)
Carbonate bicarbonate capsules	Sigma
Sodium chloride (NaCl)	Sigma
Trizma base	Sigma
Tween 20	Sigma
Ponceau S solution	Sigma
Concentrated (conc) HCl	Merck
conc H_2SO_4	Merck
conc NaOH	Merck
Methanol	Merck
Ethanol	Merck

2.1.2 General buffers

Buffers *(table 2.2)* were prepared in ultra high quality reverse osmosis water purified through a Ropure ST (Barnstead) system. All chemicals were stored at room temperature, unless otherwise stated.

Table 2.2 General buffers.

Buffer	*Composition*
Coating buffer	Carbonate bicarbonate buffer 0.05 M, pH 9.6
Assay buffer (10x concentrated)	Trizma base 100 mM; NaCl 9% w/vol, pH 7.4
Blocking buffer	1x assay buffer, BSA 3% w/vol (Stored at 4^0C)
Wash buffer	1x assay buffer, Tween 20 0.05% v/vol (Stored at 4^0C)
Antibody buffer	Wash buffer, BSA 1% w/vol (Stored at 4^0C)

2.1.3 Enzyme substrates and stopping solutions

Table 2.3 Commonly used substrates and corresponding stopping solutions.

Substrate	*Supplier*	*Stopping solution*
o-phenylenediamine dihydrochloride (OPD)	Sigma	3M HCl
p-nitrophenyl phosphate (pNPP)	Sigma	3M NaOH
Tetramethylbenzidine (TMB)	Pharmingen	1M H_2SO_4
Diaminobenzidine (DAB)	Sigma	*(running water)*

2.1.4 General apparatus

Table 2.4 General laboratory equipment.

Application	*Apparatus*	*Supplier*
ELISA	NUNC, Maxisorp microtitre plates	Life Technologies
	Microtitre plate reader	Titertek Multiskan Plus, EFLABor, Finland
Immunoblotting	Hybond nitrocellulose paper	Amersham
	Filter paper	Whatman International Ltd
	Universal tubes	Greiner
	Staining trays	Greiner
	Pipettes	Alpha Laboratories
Sample storage	Eppendorfs	Alpha Laboratories
	Glass ware	Merck

2.1.5 Allergens and skin prick test solutions

All allergens and skin prick test solutions were stored at 4^0C.

Table 2.5 Allergens used in laboratory methods.

Allergen	*Supplier*
Hen's egg ovalbumin (OVA) (grade VII)	Sigma
Ovomucoid (MUC)	Sigma
β-lactoglobulin (BLG)	Sigma
Lysozyme (LYS)	Sigma

Solutions for infant and adult skin prick testing were all supplied by ALK, Abelló *(table 2.6)*.

Table 2.6 Solutions for skin prick testing.

Skin Prick Test solution	*Concentration*
Histamine (positive) control	10 mg/ml
Saline (negative) control	
House-dust mite (Dermatophagoides pteronyssinus)	Soluprick standard quality (SQ)
Cat dander	Soluprick (SQ)
Timothy grass pollen	Soluprick (SQ)
Birch pollen	Soluprick (SQ)
Horse	Soluprick (SQ)
Dog dander	Soluprick (SQ)
Alternaria	1:20 w/v
Whole egg	1:100 w/v
Egg white	1:100 w/v
Egg yolk	1:100 w/v
Raw cow's milk	1:20 w/v
Peanut	1:20 w/v

2.2 Subjects and samples

2.2.1 Non-pregnant women

Healthy, non-pregnant, staff members or mothers of children attending the paediatric allergy clinic (n=30), aged 18 – 50 years, participated in a project investigating the lifestyle issues associated with an egg exclusion diet. The women were allocated to control (n=10) or intervention (n=20) groups. The intervention group women were asked to exclude egg and egg products from their diet for 5 weeks. Blood samples were taken at the beginning and end of the 5-week study period.

2.2.2 Unselected pregnant women

Plasma samples and matched amniotic fluid samples were available from randomly selected pregnant women (n=206) undergoing routine diagnostic amniocentesis at 16-17 weeks gestation *(kind permission of Dr CA Jones)*.

2.2.3 High-risk pregnant women

Pregnant women with a personal, or partner, history of atopy (n=190), whose infants were considered to be at high-risk of developing allergic disease, were randomised to egg exclusion or a normal healthy diet from 17-20 weeks gestation till the end of breast-feeding. Parental atopic status was established at study recruitment by allergic history, based on the *ISAAC* questionnaire [293], and at least one positive skin prick test (SPT). The panel for skin prick testing included a positive histamine control (10mg/ml), negative saline control and 8 common allergens - house-dust mite, cat dander, timothy grass pollen, birch pollen, horse, dog dander, alternaria and egg *(table 2.6)*. A wheal ≥ 2mm greater than the negative control, in the presence of an appropriate positive control, was considered to be a positive test.

Blood samples

Blood samples were collected from these women at study recruitment, at 24 weeks and 32 weeks gestation, and at the time of labour.

Breast milk samples

Breast milk samples were collected by expression from the women at 3 months post-partum (n=68).

2.2.4 High-risk intervention cohort babies

Blood samples were collected from the babies born to the dietary intervention study women *(2.2.3)*, at birth (umbilical cord), 6, 12 and 18 months of age.

2.2.5 High-risk infants (archive blood samples)

Plasma samples were available from an archive collection of blood specimens obtained from babies born to at least one atopic, asthmatic parent (n=157) *(kind permission of Dr JA Warner)*. Blood samples had been collected at birth (umbilical cord) and at 6 months, 1 year and 5 years of age.

2.2.6 Serum pool

Serum samples from healthy, egg-eating, staff volunteers (n=4) were pooled and stored in aliquots. The serum pool provided a reference curve for specific IgG concentration measurements.

2.2.7 Control blood samples

Serum was available from a healthy, non-pregnant, non-egg allergic staff member who had excluded eggs from her diet for more than a decade. Aliquots of the serum provided a negative control serum (NS) for ovalbumin detection assays. A plasma sample (from *2.2.2*) containing high levels of ovalbumin was used as a positive control for ovalbumin detection assays.

2.2.8 Sample preparation

2.2.8.1 Serum samples

Prospectively collected blood samples were left to clot by standing for at least one hour at room temperature. They were then centrifuged at 4000xg for 10 minutes. The serum was carefully removed and stored in aliquots at -80^0C until further assay.

2.2.8.2 Breast milk samples

Breast milk samples were defatted by centrifuging at 4000xg for 20 minutes at 4^0C. The supernatant fatty layer was then carefully removed and discarded. The remaining sample was aliqotted and stored at -80^0C until further assay.

2.3 Intervention cohort - dietetic monitoring

A dedicated dietitian advised and monitored the women participating in the dietary intervention study *(2.2.3)*. The intervention group received information and guidance on healthy eating and an egg-free diet, while the control group received healthy eating advice alone. Both groups were evaluated at 24 and 32 weeks gestation to ensure satisfactory nutritional intake by interview and analysis of a 7-day food diary completed during the preceding week. Between assessments the women were encouraged to ring and discuss any dietary difficulties with the dietitian.

Accidental ingestion of egg was recorded prospectively throughout the study by the intervention group subjects and discussed at the periodic reviews.

2.4 Intervention cohort - infant clinical assessment

Infants at high-risk of developing allergic disease *(2.2.4)* were assessed at 3, 6, 12 and 18 months of age by a dedicated paediatric doctor (GV) or nurse (RB) who were blind to randomisation category. Any allergic symptoms identified by the research nurse were also evaluated by the clinician. Between infant visits the mothers were encouraged to ring and discuss any concerns regarding their child with the study doctor or nurse. Additional clinical assessments were arranged accordingly.

Allergic assessment *(Appendix 1)* was based on the model for infant evaluation used in the ETAC (Early Treatment of the Atopic Child) study – a multi-centre study to investigate the potential for the anti-histamine cetirizine to prevent the development of asthma in infants with atopic dermatitis [294].

Atopic dermatitis (AD) was defined as an erythematous-papulo-vesicular chronic skin disease with dry skin, itching and typical distribution (cheeks, abdomen, extensor surface of limbs) [295].

Asthma was defined as 3 separate episodes of nocturnal cough causing sleep disturbance lasting for 3 consecutive nights and/or 3 separate episodes of wheezing,

separated by at least 7 days, in a clinical setting where asthma was likely and other conditions had been excluded.

2.4.1 Diary record

The parents were asked to record chest symptoms and/or skin rashes. The diary was completed once per week if there were no concerns, or on a daily basis if the infant was symptomatic. The diaries were reviewed at each infant assessment.

The diagnosis of AD and number of acute exacerbations, and the diagnosis of asthma, and/or number of validated coughing or wheezing episodes were recorded at each infant visit.

2.4.2 Clinical examination

The infants were examined for evidence of respiratory disease. Severity of eczema was scored using the SCORAD index, which accounts for extent and intensity of the skin lesions and subjective symptoms [296]. General health and growth parameters were also monitored at each infant visit.

2.4.3 Skin prick testing

In addition to symptom review and clinical examination, the infants' atopic phenotype was also assessed by skin prick testing. Skin prick tests were performed at 6, 12 and 18 months of age. The panel included a positive histamine control (10mg/ml), negative saline control, house-dust mite, cat dander, timothy grass pollen, raw cow's milk, whole egg, egg white, egg yolk and peanut *(table 2.6)*. A reaction was considered positive if the wheal diameter was ≥ 2mm greater than the negative control, in the presence of an appropriate positive (histamine) control.

2.5 Laboratory methods

All general reagents, buffers and apparatus used were as described previously *(2.1)*. Those materials specific to a method are described in detail in the relevant section.

2.5.1 Measurement of specific IgG concentration

Ovalbumin (OVA G), ovomucoid (MUC G) or β-lactoglobulin (BLG G) specific IgG concentrations were measured by a previously established, in-house, indirect enzyme linked immunosorbent assay *(ELISA)*.

2.5.1.1 Principles

Antigen is bound to a microtiter well and incubated with diluted samples or standards, after non-specific binding is blocked. Free IgG is washed away and the presence of bound IgG detected by the addition of enzyme-conjugated anti-human IgG antibody (primary detector) which binds to the antigen specific IgG. Free primary detector is washed away and chromogenic enzyme substrate added. The amount of coloured reaction product that forms is proportional to the concentration of bound specific IgG and this is measured by a spectrophotometric plate reader and expressed as an absorbance. The concentration of specific IgG in the sample can be calculated by comparing the sample absorbance to the reference curve produced by dilutions of the standard.

2.5.1.2 Method

A 96 well plate was coated with either ovalbumin, ovomucoid or β-lactoglobulin, at 100 μg/ml diluted in coating buffer, 100 μl per well, covered and left overnight at 4^0C. A bovine serum albumin (BSA) coated plate, 100 μg/ml diluted in coating buffer, 100 μl per well, was included in each experiment as a control for non-specific binding. The plates were washed once with assay buffer, 200 μl per well and then blocked for 1 hour with 200 μl blocking buffer. The plates were again washed once with assay buffer, 200 μl per well, and then 100 μl of standards and samples added in duplicate to both the allergen-coated and control plates and incubated for 1 hour.

Samples and serum pool standards were diluted in antibody buffer (1:100, and 1:100-1:6400, respectively). The plates were washed three times with 200 µl wash buffer and then 100 µl of peroxidase-conjugated rabbit anti-human IgG (Dako), diluted 1:6000 in antibody buffer, was added to each well and incubated for 1 hour. The plates were washed a further three times with wash buffer before adding 200 µl of OPD substrate buffer and incubating in the dark for 30 minutes. The reaction was stopped by adding 50 µl of 3M HCl and the absorbance read at A_{492nm}.

Samples with absorbances above the reference curve were repeated at higher dilutions such that the absorbance fell on the linear part of the reference curve.

2.5.1.3 Calculation of specific IgG concentration

The absorbances obtained for the standards and samples from the BSA-coated plate were subtracted from the absorbances obtained from the allergen-coated plate. A reference curve of standard absorbance versus dilution was then constructed, whereby a 1:100 dilution of the serum pool represented 0.01 arbitrary units (AU). Sample absorbance was compared to the reference curve and concentration of IgG expressed in AU, taking into account the dilution of the sample.

2.5.2 Measurement of serum ovalbumin IgG1 and IgG4 subclass concentrations

2.5.2.1 Development and optimisation

An indirect *ELISA* to measure ovalbumin IgG1 (OVA G1) and IgG4 (OVA G4) subclass concentrations was developed using commercially available enzyme-conjugated antibodies *(table 2.7)*. Optimal concentrations of primary detector were determined by preliminary experiments in which the concentration of antibody was titrated to achieve maximum sensitivity, with least background from control (antibody buffer) wells *(appendix 2)*.

Details and working concentrations of the primary detector antibodies are shown in *table 2.7.*

Table 2.7 Primary detector antibodies for ovalbumin IgG subclass measurement.

Type	Manufacturer	Concentration
HRP-conjugated mouse anti-human IgG1	Pharmingen	1:500
HRP-conjugated mouse anti-human IgG4	Binding Site	1:1600

2.5.2.2 Method

A 96 well plate was coated with ovalbumin, at 100 µg/ml diluted in coating buffer, 100 µl per well, covered and left overnight at 4^0C. A BSA coated plate, 100 µg/ml diluted in coating buffer, 100 µl per well, was included in each experiment as a control for non-specific binding. The plates were washed once with assay buffer, 200 µl per well, and then blocked for 1 hour with 200 µl blocking buffer. Again the plates were washed once with assay buffer, 200 µl per well, and then 100 µl of standards and samples *(table 2.8)*, diluted in antibody buffer, were added in duplicate to both the allergen-coated and control plates and incubated for 1 hour. The plates were washed 3 times with 200 µl wash buffer and then 100 µl of primary detector antibody *(table 2.7)*, diluted in antibody buffer, added to each well and incubated for 1 hour. The plates were washed a further 3 times with wash buffer before adding 200 µl of TMB substrate buffer and incubating in the dark for up to 30 minutes. The reaction was stopped by adding 50 µl of 1M H_2SO_4 and the absorbance read at $A_{450nm.}$

Table 2.8 Standards and sample dilutions for ovalbumin IgG subclass ELISAs.

OVA G subclass *ELISA* type	Concentrations of human IgG calibrator serum (Binding site)	Sample dilution
IgG1	0.39 µg/ml –100 µg/ml IgG1	1:100
IgG4	0.16 µg/ml –20 µg/ml IgG4	1:50

Samples with absorbances above the reference curve were repeated at higher dilutions such that the absorbance fell on the linear part of the reference curve.

2.5.2.3 Calculation of specific IgG subclass concentrations

The absorbances obtained for the standards and samples from the BSA-coated plate were subtracted from the absorbances obtained from the allergen-coated plate. A reference curve of standard absorbance versus concentration was then constructed, on which the standard serum concentrations of IgG subclasses were denoted as arbitrary units. Sample absorbance was compared to the reference curve and concentration of OVA IgG subclasses expressed in AU, taking into account the dilution of the sample.

2.5.3 Measurement of Total IgA concentration

2.5.3.1 Development and optimisation

A sandwich *ELISA* to measure total IgA concentration in breast milk was developed using commercially available monoclonal anti-human IgA antibodies *(table 2.9)*. Optimal concentrations of capture, and biotin-conjugated detector, antibodies were determined by preliminary chequer-board experiments (n=2) *(appendix 3)*.

Details and working concentrations of the sandwich antibodies are shown below:

Table 2.9 Sandwich ELISA antibodies for total IgA measurement.

	Type	**Source**	**Concentration**
Capture	Mouse anti-human IgA	Pharmingen	4 μg/ml
Detector	biotin-conjugated mouse anti-human IgA	Pharmingen	1 μg/ml
	Extravidin-HRP	Sigma	1:1000

2.5.3.2 Method

A 96 well plate was coated with capture antibody diluted in coating buffer, 100 µl per well, covered and left overnight at 4^0C. The plate was washed once with assay buffer, 200 µl per well and then blocked for 1 hour with 200 µl blocking buffer. Again the plate was washed once with assay buffer, 200 µl per well, and then 100 µl of standard (human myeloma IgA, courtesy of M.Power, Cancer Sciences, University of Southampton) and samples *(table 2.10)*, diluted in antibody buffer, were added in duplicate to the plate and incubated for 1 hour. The plate was washed 3 times with 200 µl wash buffer and then 100 µl of biotin-conjugated detector antibody, diluted in antibody buffer, was added to each well and incubated for 1 hour. The plates were washed a further 3 times with wash buffer before adding 100 µl of extravidin-HRP, diluted in antibody buffer, and incubating for 1 hour. The wash step was repeated again before adding 200 µl TMB substrate buffer and incubating in the dark for up to 30 minutes. The reaction was stopped by adding 50 µl of 1M H_2SO_4 and the absorbance read at A_{450nm}.

Table 2.10 Standards and sample dilutions for total IgA ELISA.

Concentration of human myeloma IgA standard	Sample dilution
1.5625 ng/ml –100 ng/ml IgA	1:1000

Samples with absorbances above the reference curve were repeated at higher dilutions such that the absorbance fell on the linear part of the reference curve.

2.5.3.3 Calculation of total IgA concentration

A reference curve of standard absorbance versus IgA concentration was plotted. Sample absorbance was compared to the reference curve and concentration of total IgA expressed in ng/ml, taking into account the dilution of the sample.

2.5.4 Measurement of total IgE concentration

Serum total IgE concentration was measured by a commercially available immunoassay (MagicLite Immunoassay, Ciba Corning).

2.5.4.1 Principle

Mouse anti-human IgE is covalently bonded to paramagnetic particles (solid phase) or labelled with acridinium ester. Incubation of the solid phase and labelled antibody with sera containing IgE will induce the formation of a 'sandwich'. Unbound labelled antibody is removed by magnetic separation and decantation of the supernatant. The amount of bound label is measured in the MagicLite analyser, which automatically injects the reagents necessary to initiate a chemiluminescent reaction and quantitates the subsequent photon output. The magnitude of the photon output is directly proportional to the quantity of IgE in the sample *(figure 2.1)*.

Figure 2.1 Principle of Total IgE measurement by MagicLite Immunoassay.

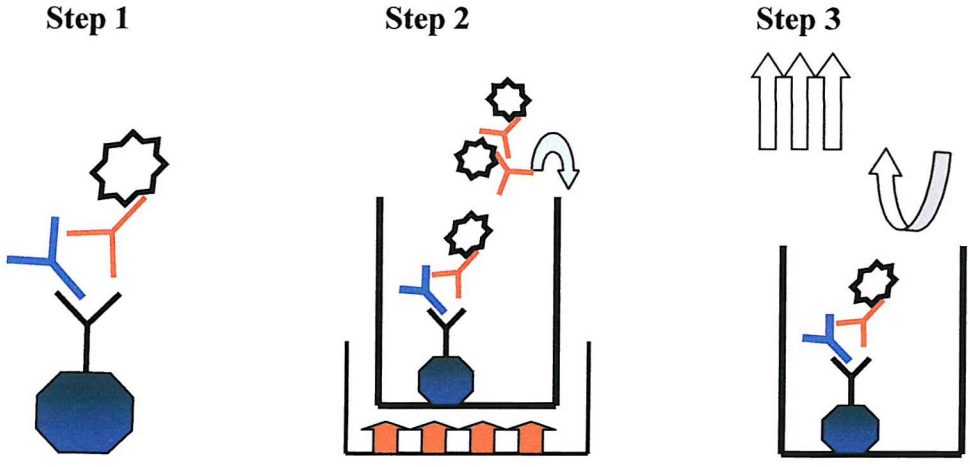


Figure 2.1 Total IgE was measured by a chemiluminescent assay. A sandwich is formed when patient sera containing IgE (blue Y) is incubated with mouse anti-human IgE coupled to paramagnetic beads (black Y) and acridium ester labelled anti-IgE (red Y) (step 1). Unbound label is removed by magnetic separation and decantation of the supernatant (step 2). The addition of chemiluminescent reagents provokes a light reaction. The magnitude of photon output is directly proportional to the quantity of IgE in the sample (step 3).

2.5.4.2 Method

Serum total IgE was measured according to the manufacturer's instructions.

Briefly, 50 µl of calibrator reagents or patient sera were added to polysytrene test tubes (Sarstedt LTD). These were mixed with 100 µl of Lite reagent (acridinium ester anti-IgE conjugate) by vortexing 3 times for 5 seconds using the Multi-Tube Vortexer. Then, 500 µl of solid phase was added to each tube, mixed, as above, and incubated for 30 minutes at room temperature. Separation of bound from unbound label was achieved by standing the tube rack in a magnetic separation unit for 3 minutes and then gently decanting the supernatant. The tube rack was removed from the separation unit and the tubes washed by adding 1 ml of distilled water to each, mixing and separating, as above. This wash step was repeated once more before adding 100 µl of distilled water to each tube, mixing and then reading the photon output for 2 seconds in the MagicLite analyser.

2.5.4.3 Calculation of total IgE concentration

Sample IgE concentration was expressed in International Units (IU)/ml, having been calculated from the high and low calibrator sera by the MagicLite Analyser.

2.5.5 Dot-blotting

2.5.5.1 Principles

Rapid immobilisation of proteins on a thin support membrane (dotting) offers a simple and convenient system for immunodetection (blotting).

2.5.5.2 Method

Proteins, diluted in assay buffer, were dotted onto nitrocellulose paper by adding 50 µl to individual wells of a dot-blotter (Bios Corporation) under suction. The membrane was washed with Ponceau S solution, thereby revealing the dots and

facilitating division of the membrane into strips for comparison of antibody binding. The nitrocellulose strips were washed with assay buffer and unoccupied sites were blocked by incubation overnight in blocking buffer. Immunoblotting was then performed as described in *2.5.7.2 (table 2.13),* with the exception that individual membrane strips were incubated with antibody and washed in separate universal tubes to allow for comparison of binding.

2.5.6 Separation of proteins using SDS polyacrylamide gel electrophoresis (SDS-PAGE)

2.5.6.1 Principles

By SDS-PAGE, proteins are separated according to their molecular weight. The proteins are coated in a negatively charged detergent, sodium dodecyl sulfate (SDS) and then exposed to an electric current, which pulls them through a matrix of polyacrylamide. The distance travelled by an individual protein is a function of its molecular weight, whereby a small protein will travel further than a larger one. The distance travelled by a sample protein is compared to the migratory profile of a set of reference proteins of known molecular weights, thereby permitting calculation of the molecular weight of the sample protein.

2.5.6.2 Method

The Novex NuPAGE electrophoresis sytem was used *(table 2.11).*

Sample buffer was diluted four-fold in the sample and the sample solution then heated to 70^0C for 10 minutes. For samples requiring electrophoresis under reducing conditions, NuPAGE reducing agent, containing dithiothreitol, as 10% final sample volume, was added just prior to heating. The standards were prepared by heating them to 100^0C for 3 minutes.

The gel cassette and gel wells were rinsed with working strength running buffer. The cassette was then placed in the Mini-Cell chamber so that the wells faced inwards and

the plastic dam secured opposite, so forming the inner buffer chamber. The inner buffer chamber was sealed tightly by placing plastic wedges behind the chamber and both inner and outer chambers filled with running buffer. In order to prevent reduced samples reoxidising during the electrophoresis, 500 µl of antioxidant was added to 200 ml of running buffer in the inner chamber immediately prior to the run.

A volume of 10 µl of samples and standards were added to individual wells; the power pack connected and the gel run at 200V for approximately 35 minutes.

Table 2.11 Materials required for SDS-PAGE using Novex NuPAGE electrophoresis system.

Apparatus	Xcell II Mini-Cell
Gel	4-12% NuPAGE Bis-Tris gel
Sample buffer	4x concentrated: diluted in sample
Sample reducing agent	*If reducing conditions* NuPAGE reducing agent (0.5M dithiothreitol) - as 10% final sample volume
Standards	SeeBlue Pre-Stained Standards containing proteins of molecular weights: 188 kDa, 62 kDa, 49 kDa, 38 kDa, 28 kDa, 18 kDa, 14 kDa, 6 kDa, 3 kDa.
Running buffer	20x concentrated MES SDS: diluted in distilled water (2-(N-morpholino) ethane sulfonic acid) - inner chamber containing 0.25% running buffer antioxidant, *if reducing conditions*

2.5.7 Western blotting

2.5.7.1 Principles

Western blotting is a process by which proteins, separated by SDS-PAGE, are electrophoretically transferred from the gel to a thin support membrane, most

commonly nitrocellulose paper, thereby allowing immunodetection of specific proteins with antibodies.

2.5.7.2 Method

Western blotting of the separated proteins was carried out using the Novex NuPAGE electrophoresis system according to the manufacturer's instructions *(table 2.12).*

Briefly, the gel cassette was opened leaving the gel resting on the larger plate and the wells removed with the gel knife. Pre-soaked filter paper, cut to the correct size, was placed on top of the gel ensuring it remained saturated with transfer buffer. The gel and filter paper were then removed from the cassette plate by inverting the plate and gently pushing the gel off the cassette through the slot in the plate. The pre-soaked nitrocellulose blotting membrane, cut to the correct size, was placed on the gel surface, followed by another layer of pre-soaked filter paper. The gel sandwich was then placed in the blot module between pre-soaked blotting pads in such an arrangement that the gel was closest to the negative electrode. The blot module was then placed in the Mini-Cell chamber and secured in place with plastic wedges. Transfer buffer (containing 0.1% antioxidant, if electrophoresis under reducing conditions) was added to the blot module and to the outer chamber. The power pack was connected and set to 25V for 1 hour.

Table 2.12 Materials for protein transfer using Novex NuPAGE electrophoresis system.

Apparatus	Xcell II Mini-Cell & blot module
Transfer buffer	20x concentrated transfer buffer: diluted in distilled water - containing 0.1% antioxidant, *if reducing conditions* 10% methanol

The nitrocellulose paper was carefully removed from the transfer apparatus, washed in assay buffer and the outline of the wells drawn around with pencil and each well

numbered. Protein staining was revealed by immersing the membrane in Ponceau S solution. The membrane was then divided into 2 pieces with the same profile of standards and samples on each, one to be probed with specific antibody, and the other with a matched isotype control antibody. The optimal concentrations of the detection antibodies had been determined previously in preliminary experiments *(table 2.13)*. The membranes were washed again in assay buffer before blocking overnight in blocking buffer.

The membranes were washed by gently shaking them in a clean plastic container containing assay buffer for 10 minutes. They were then placed in separate shallow plastic trays and covered with primary antibody, diluted in antibody buffer, and incubated for 2 hours. The membranes were washed again with shaking for 30 minutes in 2 separate washing containers containing wash buffer, with the wash buffer being changed every 10 minutes. They were then placed in a clean, shallow, plastic tray and covered with secondary antibody, diluted in antibody buffer, and incubated for 1 hour. The 30-minute wash step was repeated before covering the membranes with extravidin-HRP, diluted in antibody buffer and incubating for 1 hour. The 30-minute wash step was again repeated and binding revealed by covering the membranes with DAB for 2 minutes. The reaction was stopped by holding the nitrocellulose paper under running water.

Table 2.13 Optimal antibody concentrations for immunodetection.

Function	*Type*	*Source*	*Concentration*
Primary antibodies	Rabbit anti-ovalbumin	Sigma	10 µg/ml
	Rabbit gamma globulin (isotype control)	Pierce	10 µg/ml
	Goat anti-ovalbumin	Cappel, ICN	1.25 µg/ml
	Goat gamma globulin(isotype control)	R&D systems	1.25 µg/ml
Secondary antibodies	Goat anti-rabbit biotin	Dako	1:8000
	Rabbit anti-goat biotin	Jackson	1:20000
	Extravidin-HRP	Sigma	1:2000

2.5.8 Enhanced chemiluminescent Western blotting (ECL)

2.5.8.1 Principles

In order to improve the sensitivity of protein detection, the Western blotting protocol was modified to permit antigen detection by chemiluminescence.

2.5.8.2 Development and optimisation

In view of the extreme sensitivity of the method, repeat optimisation of the primary and secondary detector antibodies, by dot-blot chequer-board titration, was required *(appendix 4)*.

Details and working concentrations of the primary and secondary antibodies are shown in *table 2.14*.

Table 2.14 Optimal concentrations of primary and secondary antibodies for ovalbumin Western blotting using ECL.

Function	*Type*	*Source*	*Concentration*
Primary antibody	Rabbit anti-ovalbumin	Sigma	10 µg/ml
	Rabbit gamma globulin (isotype control)	Pierce	10 µg/ml
Secondary antibody	HRP- conjugated donkey anti-rabbit	Amersham	1:1000

2.5.8.3 Method

Dot-blot, or gel electrophoresis of sample and transfer of proteins to a nitrocellulose membrane, were performed as described previously *2.5.5 - 2.5.7.* Western blotting was carried out as in *2.5.7.2* with the exception that after incubation with the HRP-

conjugated secondary antibody, and subsequent wash step, the membranes were incubated with the ECL reagents and exposed to X-ray film.

In detail, this step required an equal volume of detection solution 1 to be mixed with detection solution 2 *(ECL detection reagents, Amersham)* to give a final volume of 0.125 ml/cm^2. Excess wash buffer was drained from the membranes, which were then placed on a piece of cling film, protein side up. The detection reagent mixture was then poured over the protein side of the membranes, ensuring complete coverage, and incubated for exactly one minute. Excess detection reagent was drained off and the membranes wrapped smoothly in cling film, placed protein side up in an X-ray cassette (Amersham) and exposed to film (Hyperfilm, ECL, Amersham) in the dark, for up to 15 seconds. The films were developed using an X-ray film processor (Fuji).

2.5.9 Protein A affinity chromatography

2.5.9.1 Principles

Affinity chromatography is a separation technique in which a molecule is specifically, and reversibly adsorbed by a complementary binding substance (ligand) immobilised on an insoluble support (matrix), most commonly sepharose 4B, a bead-formed agarose gel. The sample mixture is applied under conditions that favour ligand binding of the molecule. Unbound substances are washed away and the bound molecules recovered by changing the experimental conditions, for example, pH, to those that favour desorption.

The technique permits isolation, and hence purification, of a desired substance. A host of substances, for example, antibodies, antigens, receptors and DNA binding proteins, can be purified in this way by choosing an appropriate biospecific ligand. Protein A, a bacterial protein derived from *Staphylococcal aureus*, binds specifically to the F_C portion of human IgG. The immobilised ligand can thus be used to isolate IgG, thereby facilitating purification of monoclonal antibodies and IgG immune complexes.

2.5.9.2 Method

Buffer composition was as described in *table 2.15.*

Table 2.15 Buffer composition for protein A affinity chromatography.

Buffer	*Composition*
Running buffer (conc)	0.2M Tris, 1M NaCl, 1mM EDTA, 0.1M HCl, pH 8.0
Running buffer (working strength)	0.04M Tris, 0.2M NaCl, 0.2mM EDTA, 0.02M HCl, pH 8.0
Elution buffer	0.1M glycine, 0.1mM EDTA, 0.02M HCl, pH 3.0

A 250 µl aliquot of human serum was diluted 1:2 in working strength running buffer and added to the Sepharose 4B protein A column (Pharmacia). The gel was washed through with running buffer and the unbound - IgG depleted - fraction collected, using a Uvicord (LKB Bromma) to monitor the absorbance ($A_{280\ nm}$) of the eluent.

The buffer was changed to the elution buffer and the direction of flow reversed. The bound IgG containing fraction was then collected using the Uvicord to monitor the absorbance of the eluent. The pH of the IgG fraction was returned to pH 8.0 by adding a few drops of concentrated running buffer.

The 2 fractions were concentrated by centrifuging in a Millipore concentrator unit (MW cut-off 10 kDa) (Sigma) at 2700xg for 25 minutes at 10^0C. Any residual dilutional factor was accounted for in subsequent assays.

IgG depletion was confirmed by serum protein electrophoresis (SPE) (Beckman) (in assistance with M Power, Cancer Sciences, University of Southampton) and OVA IgG *ELISA (2.5.1).*

SPE is a technique whereby serum proteins are electrophoretically separated in a buffered agarose gel, immobilised in a fixative solution and visualised by staining the dried gel with a protein-specific stain. This demonstrated a significant depletion of the immunoglobulin band compared to the untreated sample. Furthermore, the OVA IgG *ELISA* produced a high absorbance from the native serum and the bound fraction, while no signal was detected from the unbound fraction.

2.5.10 Gel filtration

2.5.10.1 Principles

Gel filtration is a process by which solutes are separated according to differences in their molecular size as they pass through a chromatographic medium - the gel - packed in a column.

The test solution is added to the top of the gel column. The sample moves down the column as eluent is added to the top. Small molecules that can diffuse into the gel beads are delayed in their passage while large molecules, unable to diffuse into the gel, move continuously down the column in the flowing eluent. The large molecules thus leave the column first followed by the smaller molecules in order of their sizes.

2.5.10.2 Sample characterisation

Continuous detection of solute in the eluent by an ultraviolet monitor permits display of the sample size profile, called the elution diagram.

The distribution coefficient, Kd, characterises solute elution. It is the fraction of the stationary phase available for solute diffusion, the stationary phase being the volume of the liquid phase inside the gel available to very small molecules.

This may be mathematically represented as:

$Kd = V_e - V_o / V_t - V_o - V_{gel}$

OR

$Kav = V_e - V_o / V_t - V_o$

where V_e is the elution volume of the solute; V_o is the void volume, namely the elution volume of molecules confined to the mobile phase because they are too large to penetrate the gel pores and V_t is the total volume of the column. Kav (average) is used interchangeably with Kd since for a given gel there is a constant ratio of Kav:Kd which is independent of the nature of the solute or its concentration.

2.5.10.3 Molecular weight calculation

For a series of compounds of similar molecular shape and density, a sigmoidal relationship exists between Kav and log MW, known as the selectivity curve. A series of proteins of known MW *(table 2.16)* – calibration proteins – may therefore be used to construct the selectivity curve for a column from which an unknown MW of test solute may be calculated *(figure 2.2)*.

Table 2.16 Characteristics of calibration proteins for gel filtration.

Protein standard	MW	Elution time (min)	Elution volume, Ve (ml)	Kav = Ve-Vo / Vt-Vo
Thyroglobulin	670000	14.56	7.28	-0.045
Gamma Globulin	158000	16.32	8.16	0.0100
Ovalbumin	44000	20.54	10.27	0.1419
Myoglobin	17000	25.17	12.585	0.2866
Vit B12	1350	36.9	18.45	0.6531

where Vt = total volume of column = 24 mls & Vo = void volume = 8 mls

Figure 2.2 Selectivity curve of protein standards.

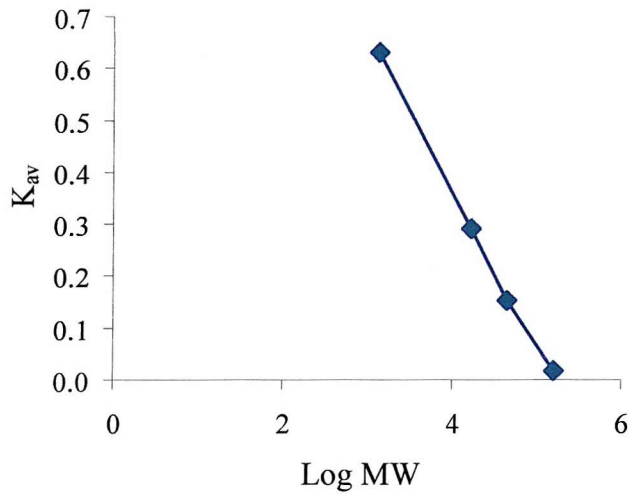


Figure 2.2 Elution of a standard mixture of proteins permitted calibration of the column and construction of a selectivity curve. Reference to this curve allows the MW of an unknown test protein to be calculated.

2.5.10.4 Method

Prior to the initial run, and if the column was adjusted or the time between run batches was prolonged, the column was calibrated with the protein standard mixture (Bio-Rad). The lyophilised standard mixture was reconstituted by adding 1 ml of ultrapure water and standing the vial on ice for 3 minutes. The mixture was then microfuged at 7500xg for 3 minutes to clear insoluble material before injecting 250 µl onto the column. Passage of the standards through the column induced protein separation and enabled generation of an elution diagram. The elution times of the individual peaks were used to calculate K_{av} for each protein and hence construct a selectivity curve for test sample reference.

Neat sample (serum or breast milk) aliquots were thawed and microfuged at 7500xg for 3 minutes before injecting 250 µl onto the column. The sample was then eluted with assay buffer at a flow rate of 0.5 ml/min.

Fraction collection of separated samples was facilitated using the Bio-Rad biologic system. This system permitted the programmed collection of 250 µl fractions between 2 selected time points – and hence a known MW range. Fractions were

collected in polystyrene tubes (Becton Dickinson) and stored in a rack at –80°C prior to assay.

After every run the column was washed with assay buffer. Between run batches it was washed and stored in 20% ethanol to prevent bacterial growth.

2.6 Ethics

All subjects were recruited to the studies with informed consent. They were reassured that they could refuse sample collection or withdraw at any time without giving a reason and without affecting subsequent treatment. Pregnant mothers participating in the dietary intervention study gave consent on behalf of their infants. They could decline infant skin prick testing or venepuncture if they had any reservations at the time of the infant assessment. Blood samples were taken by individuals experienced in phlebotomy. Paediatric venepuncture was performed by the study clinician or research nurse. Blood, breast milk and amniotic fluid samples were stored, processed and corresponding data analysed under a linked anonymous protocol.

All studies and sample collection were approved by the Southampton and SW Hants Local Research Ethics Committee. For the dietary intervention study, additional ethical approval was obtained from the North and Mid Hants Local Research Ethics Committee for the recruitment of subjects from Winchester and surrounding areas.

2.7 Statistics

Sample data

The data from the intervention cohort *(2.2.3 & 2.2.4)* presented in this thesis were derived from a larger cohort (n=251) of pregnant women participating in a randomised controlled trial of egg avoidance from the second trimester of pregnancy until the end of lactation as a means of primary allergy prevention. The primary outcome measures for that larger study were changes in the rates of infant egg IgE sensitisation (75% drop in incidence) and development of eczema (50% drop in

incidence) at 6 and 18 months of age. The expected prevalence of these parameters in the control group was determined from data collected from an earlier, comparable, Southampton birth cohort. Accordingly, the sample size was calculated using a statistical package (Epi Info) designed for use in public health, and which has been approved by the World Health Organisation and the Centres for Disease Control [297]. In order to achieve these primary outcome measures, it was calculated that a total study population of 480 women was required (allowing for a 10% drop-out rate). In an attempt to recruit this study number, more than 1300 potentially eligible women were contacted by the project coordinator with further details of the Maternal Egg Avoidance Diet (MEAD) study. The majority of these women did not meet the study recruitment criteria on further questioning, but in addition, recruitment was hampered by a wide variety of family and personal considerations, for example, full-time employment, difficult travelling arrangements, other children at home and school and personal dietary preferences. Thus, despite an enthusiastic recruitment drive, the MEAD cohort fell short of the projected sample size. Nevertheless, preliminary analyses of the complete MEAD clinical data indicate that statistically significant differences in atopic sensitisation, including egg sensitisation, are indeed apparent between the two study groups, despite the limited numbers. Work in this thesis aimed to examine immunological characteristics of the MEAD cohort and to evaluate the allergic outcome of particular subgroups of infants, for example, those with evidence of exposure to dietary egg allergen in early life.

Analyses

All statistical testing was carried out using non-parametric tests since initial analysis of the outcome data showed that they were not normally distributed. A significance level of $p<0.05$ was used for all the tests performed.

In order to compare data relating to the same subjects, the Wilcoxon test was applied, and for data derived from different subjects, the Mann-Whitney U test was performed. The Spearman correlation coefficient was used to test for an association between variables. For the analysis of ovalbumin specific IgG subclass concentration with regard to asthma outcome in childhood *(chapter 9)*, a receiver-operator characteristic (ROC) curve was constructed. This plot of sensitivity (true positive) against 1-

specificity (false positive) enabled selection of a cut-off value of subclass concentration for the diagnosis of asthma.

Associations between categorical (binary) variables were tested using Chi-square (Chi^2); Fisher's exact test was used when more than 20% of categories had an expected count of less than 5.

Statistical comparisons were calculated using SPSS for windows v10.1.

The data relating to ovalbumin exposure throughout pregnancy & lactation and infant atopic outcome at 6 months of age *(chapter 4)* necessitated multiple comparisons of the study group. Therefore, the need for statistical adjustment for multiple tests (the Bonferroni correction) was considered. This method deflates the type I error rate, α (set at 0.05), and thereby controls for an increased chance of measuring statistical significance that is actually consequent on repeated testing of the same study population. However, the best way in which to manage multiple testing is a contentious statistical issue, and indeed, many statisticians view the Bonferroni adjustment as having only a limited application in biomedical research [298]. A number of difficulties for clinical research have been cited, including the concern that whilst the Bonferroni adjustment reduces the chance of making a type I error - incorrectly measuring a difference in a particular test - it increases the chance of making a type II error – namely, that no effect or difference is measured, when the opposite is true. Thus, since type II errors are no less 'false' than type I errors, applying Bonferroni statistics there may be no guarantee of appropriate interpretation of results.

In the data to be presented *(chapter 4)*, several different 'critical' outcome measures have been compared within the same study group, as had been planned *a priori*. Furthermore, the comparisons have been made between subgroups (maternal dietary exclusion / maternal atopic predisposition), for which a variety of studies have indicated already that their characteristics might influence offspring atopic outcome. In these circumstances many statisticians would not view a Bonferroni adjustment to be justified. Furthermore, on reviewing the literature, Bonferroni statistics have been applied typically for analysis of variance and not for data presented in contingency

tables. However, the limitations incurred by multiple comparisons are appreciated in this thesis and it is to be emphasised at this juncture that further, repeated analysis with different, and larger, subject numbers will be necessary in order to confirm the findings and ensure statistically, and more importantly, clinical stringency of the results.

Chapter Three

Development of an ovalbumin detection *ELISA*

Chapter 3

Development of an ovalbumin detection ELISA

3.1 Aims

In order to ascertain if the fetus and newborn might be directly exposed to egg allergen and to evaluate the characteristics of this exposure, the development of a sensitive and specific assay for measurement of hen egg ovalbumin in pregnancy-associated biological fluids was needed.

Hen egg antigens were first detected in human breast milk in 1930 [299]. Since then the detection of egg ovalbumin and other dietary proteins, such as cow's milk β-lactoglobulin, wheat gliadin and peanut in breast milk and human sera, have been reported by several authors using *ELISA* and radioimmunoassay methods [254][255] [300][301]. Though samples have often been taken after purposeful ingestion of the related food [256][300][302][303], dietary antigens have also been detected in breast milk randomly collected from lactating women taking a normal diet without any compulsory doses of the foods: for example, in a Swedish study, β-lactoglobulin was found in 40% of breast milk samples taken throughout lactation from 25 healthy women and in 7 of 13 serum samples [253], while ovalbumin was demonstrated in 17% of breast milks from lactating Japanese women who were following their usual diet [304].

In utero exposure to food allergens via maternal sources has been implied by the demonstration of antigen-specific T-cell responses at birth [199][207] and the detection of food-specific IgE in cord blood [304]. However, detection of ovalbumin in pregnancy-associated fluids, such as umbilical cord serum and amniotic fluid, necessitated the development of an exquisitely sensitive *ELISA*, since only $10^{-4} - 10^{-7}$ of the amount of food ingested reaches the maternal circulation [301] and probably only a further fraction of this will reach the fetus. Furthermore, the prohibition of food challenge studies through pregnancy required the assay system to have the power to detect ovalbumin derived from a normal intake of egg. While high sensitivity was desirable, rigorous assay validation was essential before conclusions could be drawn

regarding the relationship between infant allergic disease development and early life exposure to ovalbumin as determined by *in vitro* measurements.

3.2 Ovalbumin detection ELISA

3.2.1 Optimisation

Preliminary experiments (n=30) were carried out using commercially available anti-ovalbumin antibodies to evaluate the optimal design and reagent concentrations for maximal detection sensitivity. In these experiments the optimal secondary antibody concentration was also titrated and a comparison made of enzyme-mediated reaction development with time-resolved fluoresence (DELFIA system – dissociation-enhanced lanthanide fluorescence immunoassay, Wallac OY, Turku, Finland). The DELFIA method is based on antibody labelling with a stable europium chelate. In this form the europium is only weakly fluorescent. Release of the europium from protein binding by the addition of a highly lipophilic chelator enhances fluorescence and the signal can be measured over 1 second using a fluorometer.

A multilayer sandwich *ELISA* with polyclonal goat anti-ovalbumin capture antibody, and polyclonal rabbit anti-ovalbumin detector antibody was found to be the most sensitive design *(figure 3.1)*. Reagent manufacturers and concentrations are shown in *table 3.1*.

Figure 3.1 Capture ELISA for Ovalbumin detection.

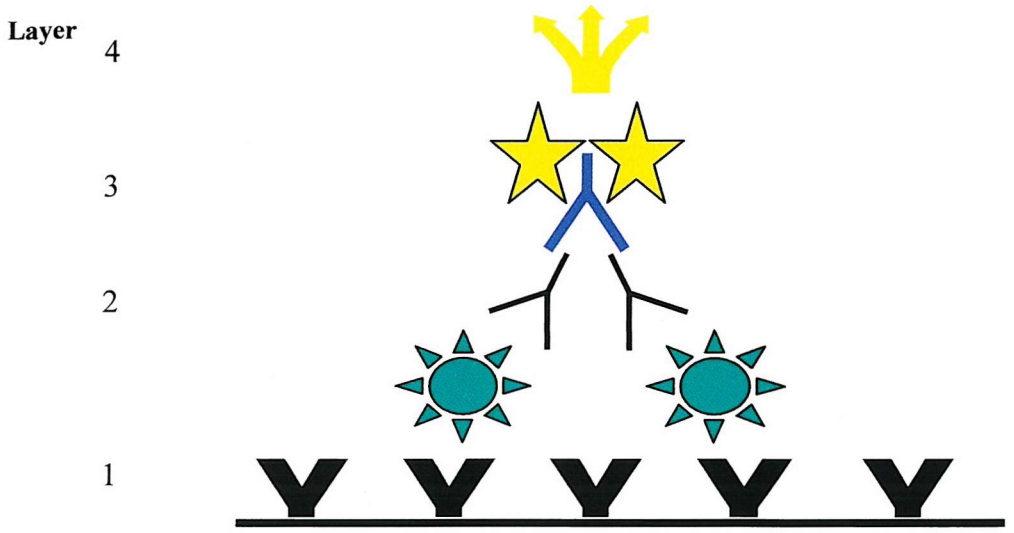


Figure 3.1 A multilayer sandwich ELISA was developed for the detection of OVA in pregnancy-associated fluids (for method see 3.2.2 and reagents table 3.1). Capture antibody (layer 1) was a goat anti-OVA and detector antibody (layer 2) a rabbit anti-OVA. The signal was amplified by a biotinylated goat anti-rabbit antibody (layer 3) and extravidin alkaline phosphatase (layer 4). A colour change was elicited on addition of enzyme substrate, p-nitrophenyl phosphate (pNPP).

Table 3.1 Reagent sources & optimal concentrations for ovalbumin ELISA.

Layer	***Function***	***Reagent***	***Optimal concentration***
1	Capture	Goat IgG anti-ovalbumin (ICN)	2.5 µg/ml in coating buffer
2	Detector	Rabbit anti-ovalbumin (Sigma)	0.5 µg/ml in antibody buffer
3	Secondary	Goat anti-rabbit biotin (Dako)	1:10 000 in antibody buffer
4	Avidin conjugate	Extravidin ALP (Sigma)	1:50 000 in antibody buffer
	Substrate	p-nitrophenyl phosphate (pNPP) (Sigma)	

3.2.2 *ELISA* method

All general materials, including reagents, buffers and equipment, used were those as described previously *(2.1)*.

A 96-well *ELISA* plate was coated with goat anti-ovalbumin antibody, 100 µl per well, covered and left overnight at 4^0C **(layer 1)**. The plate was washed once with assay buffer, 200 µl per well, and incubated with blocking buffer, 200 µl per well, at room temperature for one hour. The plate was washed again once with assay buffer, 200 µl per well, before adding 100 µl of the ovalbumin standards (range 3.9 pg/ml - 8 ng/ml) and samples, diluted in antibody buffer, in duplicate to the wells and incubating for two hours. The plate was washed three times with wash buffer, 200 µl per well, and then incubated for one hour with the detector antibody, rabbit anti-ovalbumin, 100 µl per well **(layer 2)**. The wash step was repeated and biotin-conjugated goat anti-rabbit added, 100 µl per well, and incubated for one hour **(layer 3)**. The wash step was repeated again and then the plate incubated for one hour with extravidin alkaline phosphatase (ALP), 100 µl per well **(layer 4)**. After a further wash step, 200 µl of pNPP substrate buffer was added to each well and the plate incubated in the dark for up to thirty minutes. The reaction was stopped by the addition of 50 µl 3M NaOH, and the absorbance read at A_{405nm}.

3.2.3 Assay sensitivity

A reference curve was constructed from standard concentrations of ovalbumin (range 3.9 pg/ml - 8 ng/ml) *(figure 3.2)*. The limit of detection, defined as three standard deviations above zero, was 15–30 pg/ml. Thus the developed *ELISA* was more sensitive than any of the assays described to date, for which the lower levels of detection have ranged between 100 pg/ml [256][300] and 1 ng/ml [303].

Figure 3.2 Ovalbumin detection ELISA standard curve.

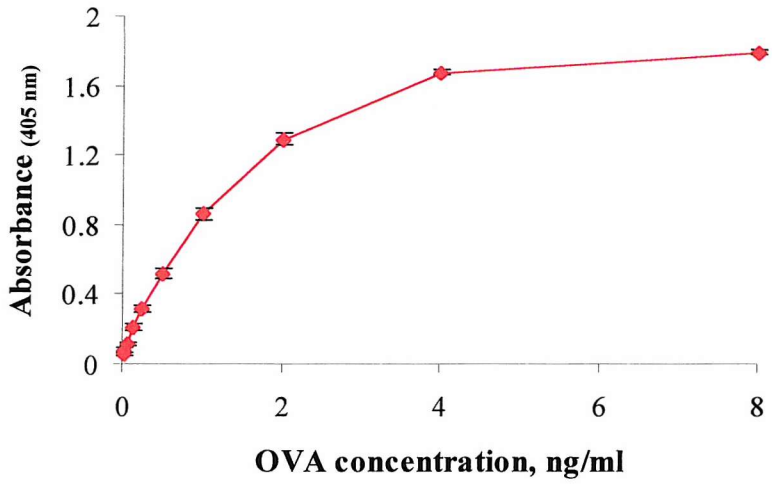


Figure 3.2 The figure shows a reference curve (± standard deviations) for ovalbumin standards from a set of ELISAs performed on the same day.

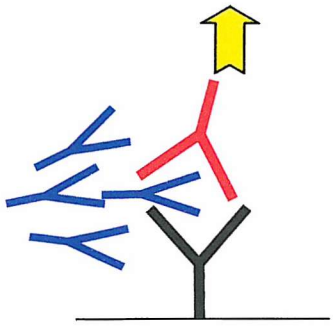
3.3 Assay validation

The validity of the *ELISA* was confirmed by experiments to assess assay specificity, recovery and parallelism. However, these parameters could only be evaluated after recognition and elimination of non-specific antibody interaction.

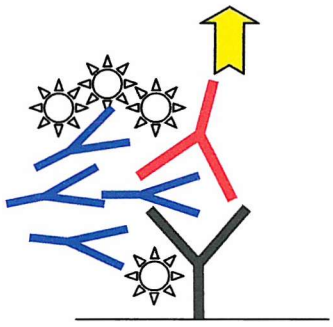
3.3.1 Non-specific antibody interactions

Preliminary experiments with volunteer sera *(2.2.1)* suggested non-specific antibody interaction *(figure 3.3)*: OVA *ELISA* of volunteer serum A (VOLA) gave a signal that intimated protein presence, but this signal was not augmented on spiking the sample with 1ng/ml ovalbumin. Pre-incubation of the sample with non-specific goat serum (Sigma), diluted 1:10 in antibody buffer, eliminated the signal, thereby confirming the non-specific nature of the finding.

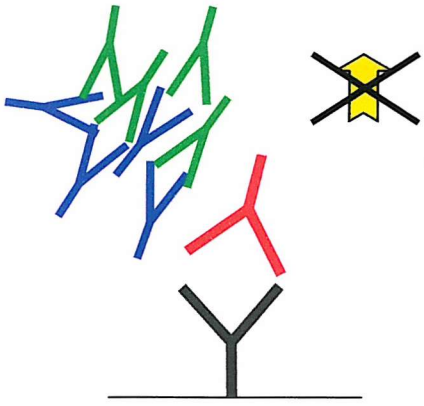
Figure 3.3 Identification of non-specific ELISA antibody interaction.



Serum VOLA (blue shapes) was incubated with the OVA ELISA sandwich antibody pairs – in this case the capture antibody (black shape) was rabbit anti-OVA and detector antibody (red shape) goat anti-OVA. The ELISA produced a signal, intimating that the native serum may contain ovalbumin.



When serum VOLA was spiked with 1 ng/ml OVA (spiked circles) the expected increase in ELISA OD was not observed. This suggested that the signal from the native serum might be as a result of non-specific antibody interaction.



When VOLA was incubated with non-specific goat antibody (goat serum, green shapes) before incubating with the anti-OVA antibodies, the positive signal was eliminated, thus confirming that the signal was related to non-specific antibody interactions.

In the absence of either the capture antibody or the detector antibody the positive signal from VOLA was eliminated *(figure 3.4)*, indicating that the presence of both antibodies was necessary to generate the non-specific interaction and suggesting that this interaction arose from reagent antibody cross-linking by the serum.

Figure 3.4 Non-specific antibody interaction – identification of site of interaction.

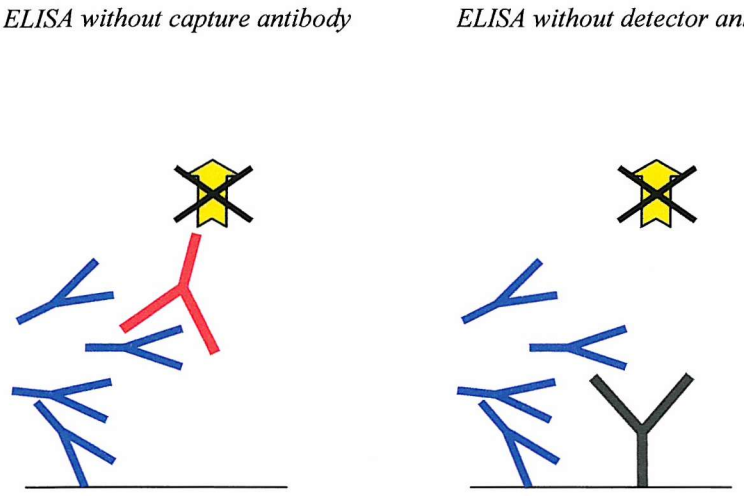


Figure 3.4 If the ELISA was repeated without either the capture antibody or the detector antibody then the positive signal from VOLA was eliminated. This indicated that the non-specific signal arose from an interaction between both antibodies and the serum, most likely due to antibody cross-linking by the serum.

Subsequently gel chromatography of the goat anti-ovalbumin antibody *(2.5.10)* (courtesy of M Power, Tenovus) revealed that the preparation was impure, containing high molecular weight, complexed material. Therefore, pure goat IgG anti-ovalbumin was produced by protein A affinity chromatography of the whole preparation *(2.5.9)* (courtesy of M Power, Tenovus).

When the purified goat anti-ovalbumin antibody was compared to the whole preparation it was found that VOLA gave a non-specific signal when the whole preparation coated the plate but not when the purified fraction was used as the capture antibody *(figure 3.5)*.

Figure 3.5 Affinity purification of the capture antibody eliminated the non-specific signal.

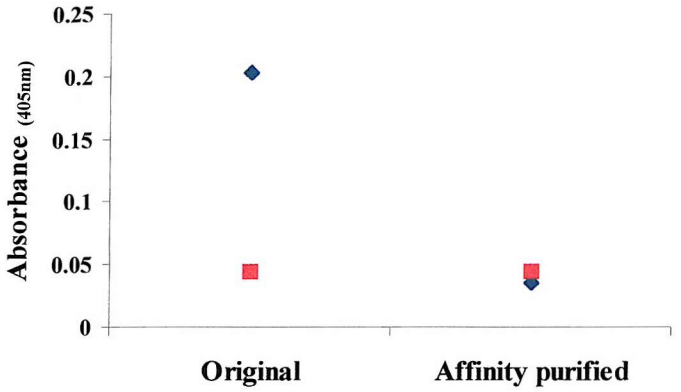


Figure 3.5 The capture antibody was affinity purified (2.5.9). As a result, the false positive signal (blue diamond) above the assay background (red square) of the original preparation was eliminated.

3.3.2 Specificity

Having eliminated non-specific antibody interaction, the specificity of the assay was confirmed by several different methods: by Dot-blot, inhibition *ELISA* and by Western blot.

3.3.2.1 Dot-blot

By Dot-blot, cross-reactivity of the anti-OVA antibodies with other proteins could be screened.

3.3.2.1.1 Method

The experiments were carried out as described previously *(2.5.5 & 2.5.7)*.

The antigens probed were hen's egg ovalbumin (OVA), human serum albumin (HSA), bovine serum albumin (BSA), cow's milk allergen β-lactoglobulin (BLG) and other egg allergens - ovomucoid (MUC) and lysozyme (LYS). Aliquots (50 µl) of protein concentration 100 µg/ml were dotted onto nitrocellulose paper. These

antigens were blotted by both rabbit and goat anti-OVA antibodies, with appropriate isotype controls.

3.3.2.1.2 Results

Both anti-OVA antibodies showed strong binding to ovalbumin but no binding to BSA or HSA. Neither displayed reactivity towards β-lactoglobulin or to lysozyme. Rabbit anti-OVA showed limited cross-reactivity with ovomucoid *(figure 3.6).*

Figure 3.6 Dot-blot of unrelated proteins to screen for sandwich ELISA antibody cross-reactivity.

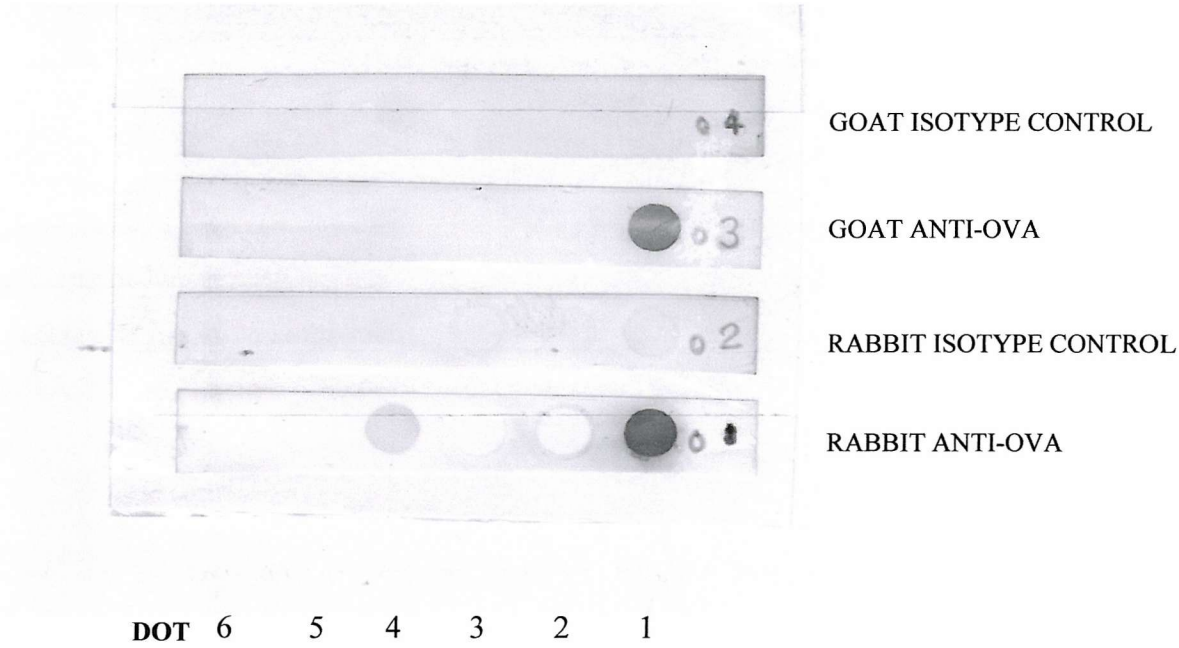


Figure 3.6 OVA (dot 1), HSA (dot 2), BSA (dot 3), MUC (dot 4), BLG (dot 5) & LYS (dot 6) were dotted onto a nitrocellulose membrane and probed with anti-OVA antibodies and appropriate isotype controls, as described in 2.5.5 & 2.5.7. Strong binding to OVA by the sandwich ELISA antibodies was apparent. Limited cross-reactivity of rabbit anti-OVA with MUC was noted.

In view of the limited reactivity toward ovomucoid displayed by rabbit anti-OVA, the experiment was repeated with decreasing doses of ovalbumin and ovomucoid. At a concentration of 100 ng/ml there was good antibody binding to ovalbumin, but none was observed to ovomucoid *(figure 3.7).*

Figure 3.7 Dot-blot of decreasing doses of Ovalbumin and Ovomucoid.

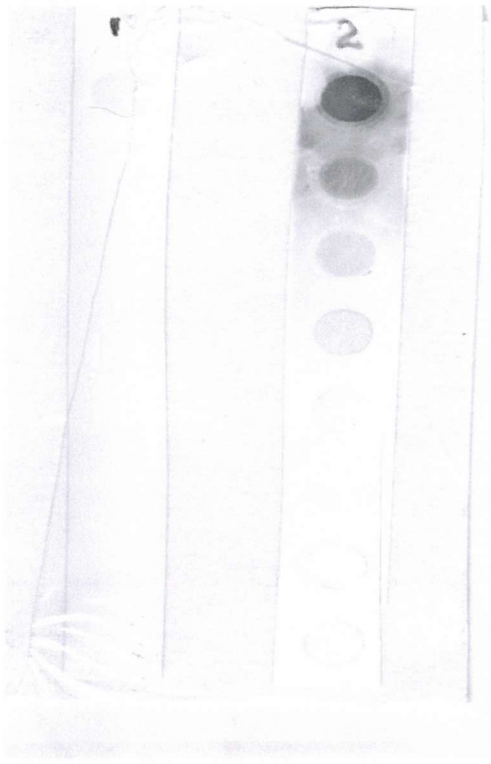


Figure 3.7 Rabbit anti-ovalbumin (OVA) showed limited cross-reactivity with ovomucoid (MUC) at a concentration of 100 µg/ml (figure 3.6). This cross-reactivity diminished at lesser concentrations and at a protein concentration of 100 ng/ml no binding to ovomucoid was visible, while good reactivity with ovalbumin persisted.

3.3.2.1.3 Dot-blot discussion

The antibodies were specific for ovalbumin. There was limited cross-reactivity of rabbit anti-OVA with ovomucoid, but this was only seen at high doses of the protein. Since ovalbumin has generally been detected in human samples in nanogram amounts, and the quantity of ovomucoid in egg white is 1/10 that of ovalbumin, the concentrations anticipated in the study test samples would not be expected to interfere with the *ELISA*. Furthermore, the clear-cut specificity of the capture antibody would compensate for any cross-reactivity displayed by the detector antibody.

3.3.2.2 Inhibition ELISA

An inhibition *ELISA* permitted assessment of the ability of an unrelated food antigen to compete with ovalbumin in the detection assay.

3.3.2.2.1 Method

A modified OVA *ELISA* was performed. All wells were incubated with 1 ng/ml ovalbumin, except for control wells, to which antibody buffer or 1 ng/ml β-lactoglobulin was added. The primary detector antibody was spiked by pre-incubating with BLG or OVA, in doses that ranged from 2.6 pg/ml to 5 μg/ml, increasing in five-fold steps. Unspiked detector antibody was added to the control wells and spiked detector to the remaining wells. The *ELISA* was then continued as previously described *(3.2.2)*.

3.3.2.2.2 Results (1)

No signal was observed from the control wells. Pre-incubation of the primary detector antibody with BLG caused no signal inhibition. In contrast, spiking the primary detector with OVA increased the *ELISA* signal in a bell-shaped dose-response curve, with the maximum signal observed at a spiking dose of 200 ng/ml OVA *(figure 3.8)*.

Figure 3.8 Inhibition ELISA.

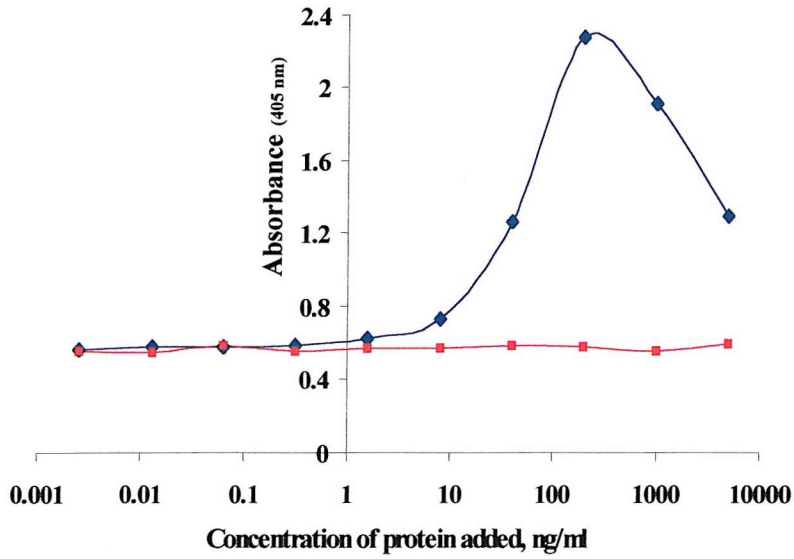


Figure 3.8 An inhibition ELISA was performed by modifying the developed OVA assay. Pre-incubation of the primary detector antibody with β-lactoglobulin did not inhibit detection of 1ng/ml ovalbumin (red squares), whereas spiking of the primary detector with ovalbumin resulted in an increase in the ELISA signal in a bell-shaped profile (blue diamonds).

These results suggested that the capture antibody might be saturated at an OVA concentration of 200 ng/ml. Therefore, the experiment was repeated and the wells incubated with 200 ng/ml OVA prior to adding the primary detector antibody. The primary detector was again pre-incubated with OVA, in spiking doses that ranged from 64 pg/ml–125 µg/ml, increasing in five-fold steps, before adding to the plate and continuing the *ELISA* protocol.

3.3.2.2.3 Results (2)

The signal was inhibited by pre-incubation of the primary detector with OVA in a dose-dependent manner *(figure 3.9)*.

Figure 3.9 Inhibition ELISA after saturation of capture antibody.

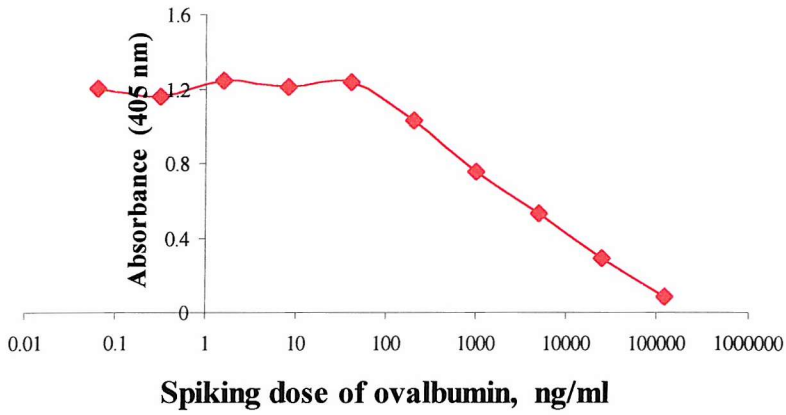


Figure 3.9 The inhibition ELISA was repeated to assess ovalbumin detection when the capture antibody was saturated. Increasing spiking concentrations of ovalbumin resulted in a dose-dependent inhibition of antigen detection.

3.3.2.2.4 Inhibition ELISA - discussion

The unrelated antigen, cow's milk β-lactoglobulin, a commonly encountered food allergen, did not inhibit OVA detection. However, inhibition increased in a dose-dependent manner on spiking the primary detector antibody with OVA. The findings support the specificity of the detection system for ovalbumin.

3.3.2.3 Western blots

Western blots allow the MW of the protein captured by *ELISA* to be determined. Therefore, this method may assess *ELISA* specificity.

Application of Western blotting will be reported in greater detail in subsequent chapters. However, *figure 3.10* gives an example of a Western blot of OVA positive and OVA negative test samples, as determined by *ELISA*. Aliquots (10 µl) of a breast milk sample containing OVA, a breast milk sample without OVA collected from the same subject but at a different time and an OVA positive cord serum were run in duplicate, with OVA standards (1 µg/ml) and a MW reference, under non-reducing conditions on SDS-PAGE *(2.5.6)*. The proteins were transferred to a nitrocellulose

membrane and probed with rabbit anti-OVA or a rabbit IgG isotype control. In order to improve detection sensitivity enhanced chemiluminescence was used to reveal protein binding *(2.5.8)*.

The blot shows that only the *ELISA* positive samples contained detectable protein of MW consistent with ovalbumin.

Figure 3.10 Western blot of ELISA positive and negative test samples.

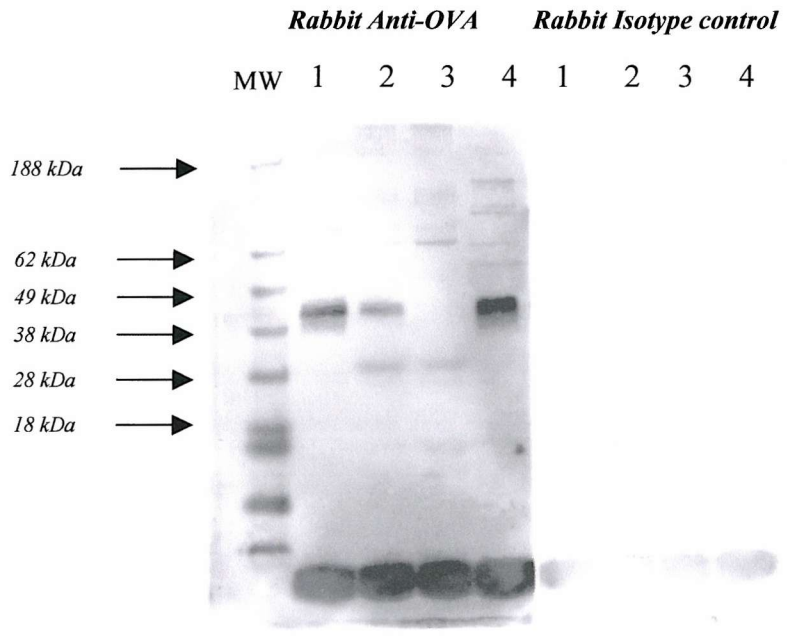


Figure 3.10 Breast milk samples and cord sera were Western blotted using enhanced chemiluminescence (for method see 2.5.6 & 2.5.8). In this experiment OVA positive controls (lanes 1) (concentration 1 µg/ml), breast milk with (lanes 2), and without (lanes 3), detectable OVA, as measured by ELISA, and an ELISA positive cord serum (lanes 4) were probed with rabbit anti-OVA or a rabbit IgG isotype control. The ELISA positive samples contained detectable protein of MW consistent with OVA. This protein was not detected in the ELISA negative sample.

3.3.3 Recovery

3.3.3.1 High and low serum ovalbumin IgG concentration

The ability of the *ELISA* to detect all the ovalbumin present in a biological fluid was assessed by recovery experiments.

3.3.3.1.1 Method

A 450 µl volume of negative control serum (NS) - with undetectable OVA and extremely low OVA specific IgG (OVA G) concentration (0.08AU) *(2.2.7)* - was spiked with ovalbumin in concentrations ranging from 15.6 pg/ml to 1 ng/ml. In order to assess whether OVA specific IgG concentration influenced recovery, a volunteer serum (VOLB) with high OVA G concentration (3.67 AU) but no detectable ovalbumin was spiked in the same manner. The spiked sera were assayed and the resultant optical densities compared with those of the OVA standard curve.

3.3.3.1.2 Results

Full recovery was obtained from NS (average 116%) *(figure 3.11)* but no recovery of ovalbumin was measured in VOLB *(figure 3.12)*.

Figure 3.11 Recovery of ovalbumin from serum with low ovalbumin IgG concentration (NS).

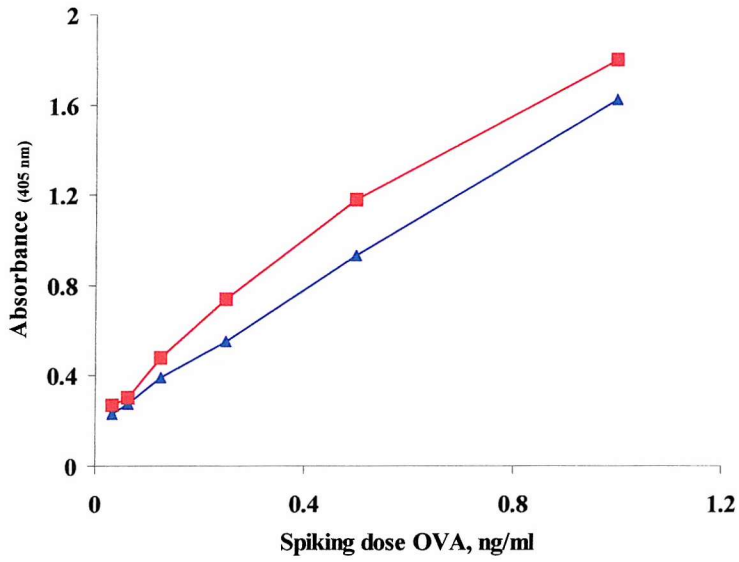


Figure 3.11 A serum with low OVA G concentration (NS) was spiked with increasing doses of OVA. OVA recovery was assessed by ELISA (3.2) where the absorbances of the spiked serum (red squares graph) were compared to those obtained from the same doses of OVA in antibody buffer (blue triangles graph). Full OVA recovery (average 116%) was obtained from this serum.

Figure 3.12 Recovery of ovalbumin from serum with high ovalbumin IgG concentration (VOLB).

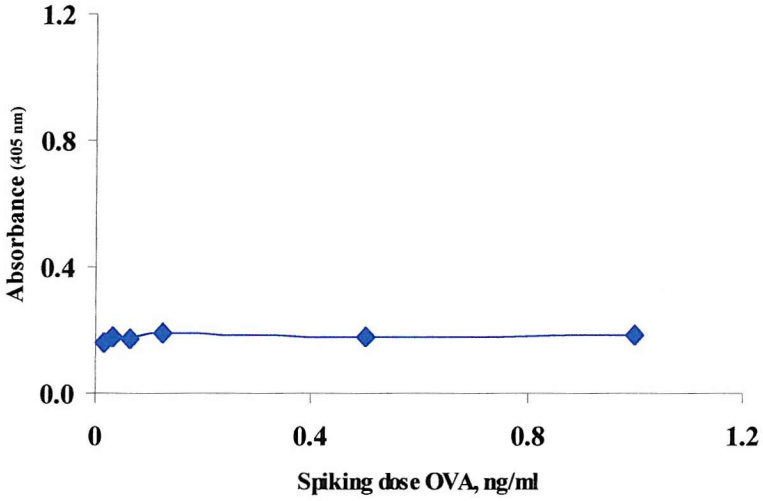


Figure 3.12 Serum with high OVA G concentration (VOLB) was spiked with increasing doses of OVA, as above. No ovalbumin recovery was observed in this serum.

3.3.3.1.3 Conclusion

These results suggested that ovalbumin recovery measurable by the *ELISA* might depend on the serum specific IgG concentration. It raised the possibility that a high IgG concentration could sequester added ovalbumin and block epitope recognition by the specific detector antibody.

3.3.4 Parallelism

Parallelism - namely, a comparative behaviour of standard OVA and test sample OVA in the *ELISA* - was established by comparing the slopes of the curves of serially diluted ovalbumin positive serum samples with the slope of the standard curve. *Figures 3.13 & 3.14* show the ODs for serum samples of two subjects *(2.2.3)* diluted 1:2 and 1:4 in antibody buffer and processed by the detection *ELISA*. One serum had known high OVA G concentration (M14, 5.03AU) and the other low OVA G concentration (M49, 0.1AU). The overlapping graphs of subject and standard OVA indicate close parallelism.

Figure 3.13 Parallelism: high serum ovalbumin IgG, high circulating ovalbumin concentration.

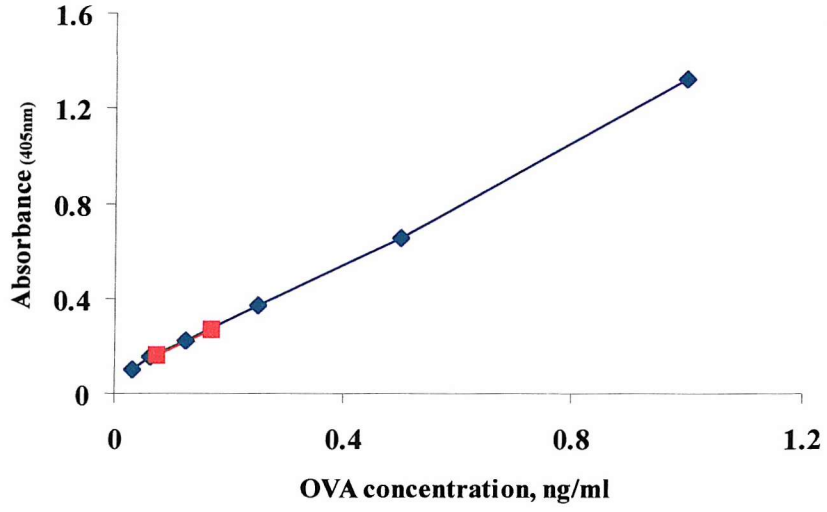


Figure 3.13 Serum with high OVA G concentration containing OVA was serially diluted in antibody buffer. The absorbances of this test sample OVA (red curve) overlapped that of the standard OVA (blue curve), confirming parallelism.

Figure 3.14 Parallelism: low serum OVA IgG, high circulating OVA concentration.

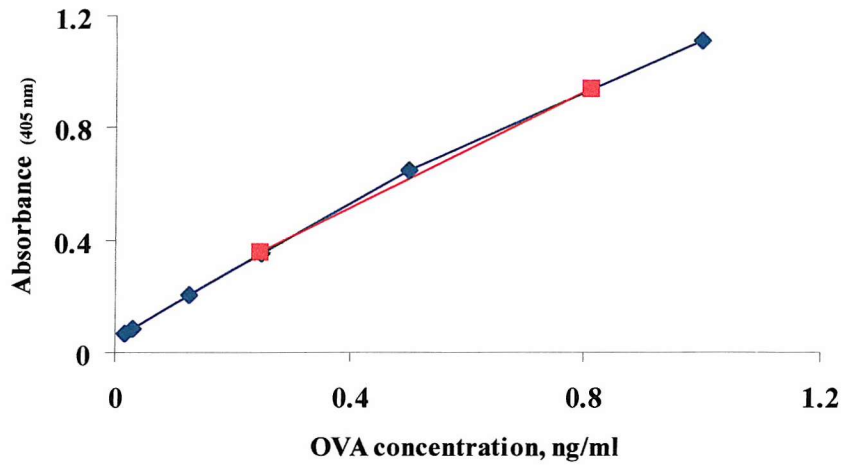


Figure 3.14 Serum with low OVA G concentration containing OVA was serially diluted in antibody buffer. The OVA ELISA absorbances for this test sample (red curve) overlapped that of the OVA standards (blue curve), confirming parallelism.

3.4 Spike–recovery experiments

The observation that ovalbumin recovery was dependent on serum specific IgG concentration *(3.3.3)* was evaluated by performing further spike-recovery experiments on a larger number of sera with varying OVA IgG concentrations.

3.4.1 Methods

Blood samples without detectable OVA were selected (OVA –). These samples were collected from healthy non-pregnant volunteers (n=19) *(2.2.1)*, unselected pregnant women at the time of routine amniocentesis (n=9) *(2.2.2)* and pregnant women with a family history of atopy randomised at 17-20 weeks gestation to egg avoidance or a normal diet from the second trimester of pregnancy till the end of breast-feeding (n=56) *(2.2.3)*.

A 110 µl aliquot from each of these samples was spiked with 2 ng/ml ovalbumin and further diluted 1:2 in antibody buffer. Unspiked and spiked samples were then

assayed for OVA by the detection *ELISA*. Ovalbumin recovery was expressed as a percentage of the spiking dose.

Ovalbumin specific IgG concentration of the samples (n=81) was measured by indirect *ELISA (2.5.1)*. In order to compare ovalbumin specific IgG concentration of blood samples containing ovalbumin (OVA +), OVA G concentration was also measured in OVA + blood samples collected from unselected pregnant women (n=18) *(2.2.2)*.

3.4.2 Results

After spiking, 32 of 84 (38.1%) OVA – blood samples had detectable OVA, with recovery ranging from 2.9 – 75.4% (median 44%). Samples with OVA recovery had significantly lower OVA G concentration than had the samples without recovery ($p<0.001$, Mann-Whitney) *(figure 3.15)*. The percentage of recovered protein showed a significant negative correlation with OVA G ($r=-0.551$, $p<0.001$, Spearman's) *(figure 3.16)*.

Figure 3.15 Ovalbumin IgG concentration in samples with, and without, OVA recovery.

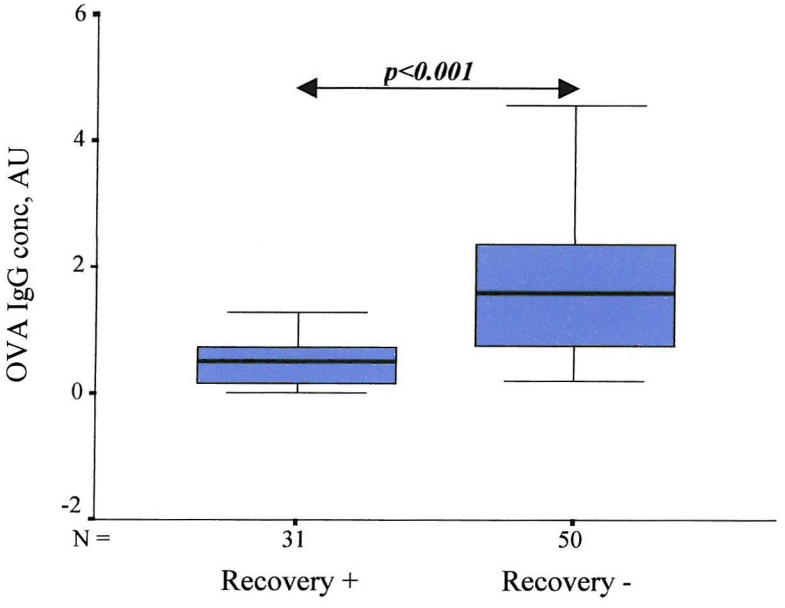


Figure 3.15 OVA – blood samples were spiked with ovalbumin and recovery of the protein determined by capture ELISA (3.2). Ovalbumin IgG concentration was measured by indirect ELISA (2.5.1). OVA G concentration was significantly lower in those samples permitting recovery.

Figure 3.16 Correlation of ovalbumin recovery with specific IgG concentration.

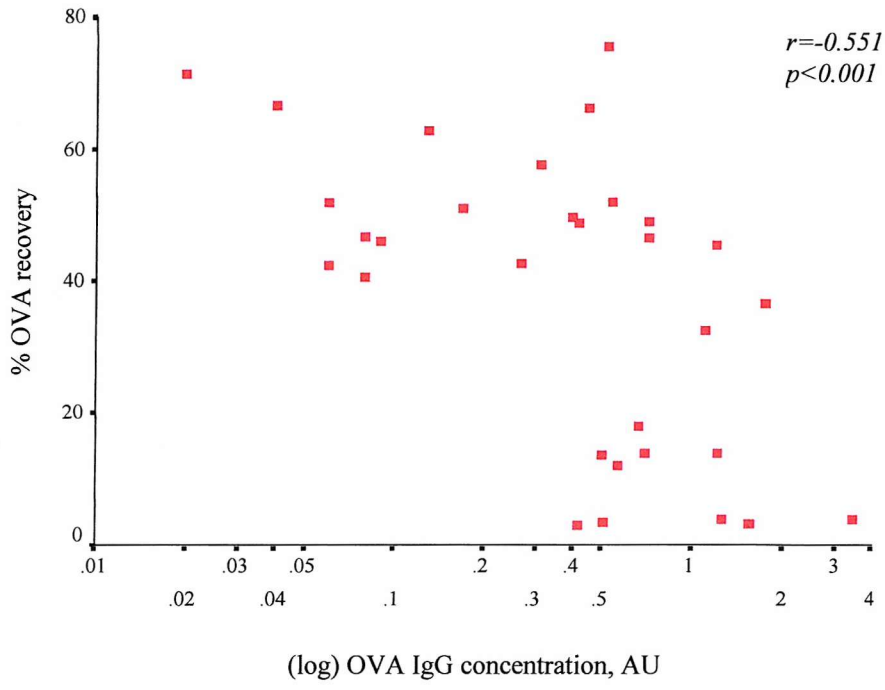


Figure 3.16 OVA negative blood samples were spiked with ovalbumin and recovery of the protein determined by capture ELISA. OVA recovery showed a significant negative association with sample OVA G concentration.

However, there was no significant difference in OVA G concentration of OVA + samples compared with OVA – samples of pregnant women (p=0.155, Mann-Whitney, *figure 3.17*) (p=0.211, Mann-Whitney, *figure 3.18*).

Figure 3.17 Ovalbumin specific IgG concentration in plasma samples with, and without, ovalbumin collected from unselected pregnant women at amniocentesis.

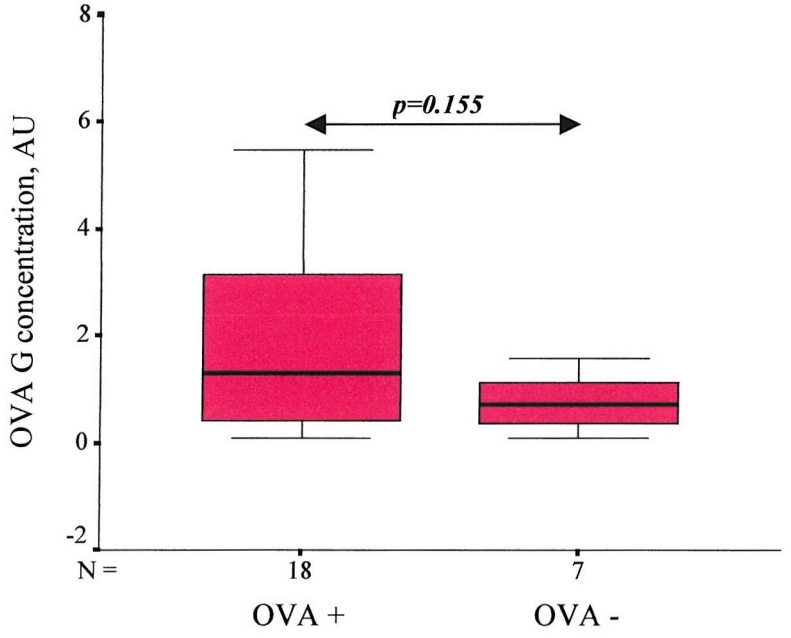


Figure 3.17 OVA G concentration was measured by indirect ELISA (2.5.1). There was no significant difference in concentration between samples with (OVA +) or without (OVA -) ovalbumin. These blood samples were collected from unselected pregnant women at the time of routine amniocentesis.

Figure 3.18 Ovalbumin specific IgG concentration in blood samples with, and without ovalbumin collected from pregnant women in the second trimester of pregnancy.

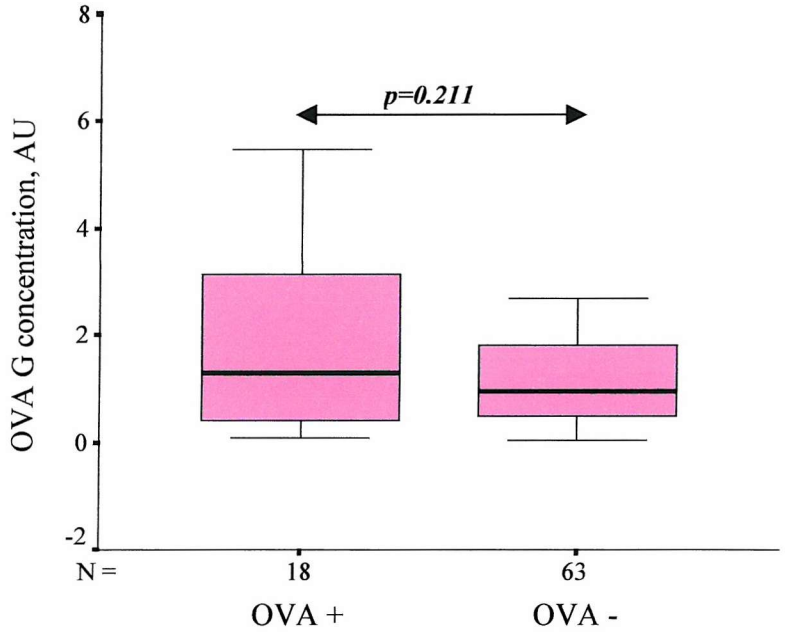


Figure 3.18 OVA G concentration was measured by indirect ELISA (2.5.1). There was no significant difference in concentration between samples with (OVA +), or without (OVA -) ovalbumin. These blood samples were collected from pregnant women in the second trimester of pregnancy. OVA + samples were those from unselected pregnant women at the time of routine amniocentesis. OVA – samples were collected from women in the same study cohort, but this analysis also included samples from women with a family history of atopy randomised to egg avoidance, or a normal healthy diet, through the remainder of pregnancy and lactation.

3.4.3 Spike–recovery discussion

Ovalbumin recovery by the detection *ELISA* was possible in only a proportion of the OVA - samples, and in these samples recovery was incomplete. Such serum related inhibitory effects on antigen detection have been noted by other authors. Kilshaw and Cant found that the addition of serum or breast milk to an ovalbumin or β-lactoglobulin assay reduced protein measurement by amounts differing with the donor of the sample [300]. They noted that this inhibition was immunologically specific, and therefore an antibody, as individual subjects showed different levels of inhibition depending on the antigen being measured.

IgG was implicated in this inhibitory effect by food challenge experiments in which high serum ovalbumin IgG concentration was associated with measurement of significantly lower quantities of serum ovalbumin compared to low serum specific IgG concentration after the challenge dose [303]. A blocking effect of IgG was clearly seen at the highest concentrations, but between the lowest and highest levels there was no correlation between antibody titre and detection of circulating antigen [300][302]. An inhibition of antigen uptake from the gut, in addition to a circulatory effect of high IgG, was postulated as the mechanism of inhibition [303].

In this current work protein recovery was also determined by ovalbumin IgG concentration, such that high concentrations inhibited ovalbumin detection, but unlike previous authors, a significant negative correlation of recovery with IgG was observed over the range of concentrations measured. The mechanism of this inhibition was independent of mucosal absorption and suggests that the IgG may bind antigen in a dose-related manner and so block epitope recognition by the assay detection antibodies. However, the assay power of detection was not limited by the IgG concentration, as shown by the lack of any significant difference in ovalbumin IgG concentration between those samples in which endogenous ovalbumin was detected and those in which none was detected. Therefore, blocking may not be just a function of concentration but may depend on other factors such as antibody functional affinity or avidity for the antigen.

3.5 Summary

An ovalbumin multilayer sandwich *ELISA,* with a sensitivity of 15-30 pg/ml, has been developed and extensively validated. This level of sensitivity surpasses that of any previously reported work and may allow for detection of a dietary allergen in gestation-associated fluids.

In the course of validation experiments serum specific IgG was found to have an inhibitory effect on antigen detection, the effect directly relating to IgG concentration. This implies that IgG may determine allergen form - specifically the presence of antigen in IgG immune complex - and raises the question of whether IgG may block

allergenic epitope recognition *in vivo,* with implications for early life sensitisation and infant atopic outcome. The observations in this chapter, which suggest that the characteristics of maternal specific IgG may modulate early life allergen exposure, form the basis for further work in this thesis: the consequence of specific IgG concentration and functional affinity for immunological form of antigen presentation and infant allergic outcome will be examined in subsequent chapters.

Chapter Four

Early life exposure to ovalbumin

Chapter 4

Early life exposure to ovalbumin

4.1 Aims

At the centre of the complex interaction of immunological processes that result in atopic disease manifestation is a requirement for allergen exposure, and priming, of the developing immune system. Neonatal exposure to allergens via a maternal route is evident from the detection of a variety of food antigens in breast milk, including cow's milk, egg and peanut proteins [253][300][305][306]. However, direct exposure to dietary allergen *in utero* remains to be confirmed by specific detection of the protein in amniotic fluid or cord blood. Therefore, a primary aim of this work was to establish and characterise early life exposure to the common dietary allergen, hen's egg ovalbumin, by employing a sensitive in-house developed detection *ELISA (chapter 3).*

A direct association between antigen exposure via breast milk and the expression of allergic disease in susceptible infants has been revealed by anecdotal reports [252][307][308] and double blind studies [190][257]. Furthermore, a relationship between dose of allergen and symptoms was suggested by Axelsson *et al* who reported that the presence of gastrointestinal symptoms and skin rashes in breast-fed infants was related to high levels of β-lactoglobulin in mothers' milk [253].

An association between early life allergen exposure and the development of atopic disease has implications for primary allergy prevention strategies. The observation of food-associated allergic disease in purely breast-fed infants initiated studies to investigate whether avoidance of allergenic foods in lactation by high-risk mothers prevented atopic sensitisation of their offspring [reviewed in (309)]. These avoidance regimes were associated with less eczema in early childhood [278][310][311], but not at 10-year follow-up [279]. Similar strategies implemented only during pregnancy failed to have any benefit [273], but as the dietetic measures began in the third trimester, well after fetal immunological capacity for priming has been established [95][200][201], so the benefit of such a regimen may have been missed.

In this study, a cohort of pregnant women, with personal or partner history of atopy, were randomised to either complete dietary egg exclusion or a normal healthy diet from 17-20 weeks gestation until the end of breast-feeding. This protocol allowed for clearer evaluation of infant atopic outcome in the context of maternal dietary intervention, whilst optimising subject compliance and without compromising nutrition.

There is no literature on what effect maternal dietary manipulation has on food allergen exposure in early life. House-dust mite avoidance measures have been shown to successfully reduce environmental mite levels [270] and these levels have been assumed to represent what the fetus actually encounters [203]. But is that necessarily the case? A second aim of this work was therefore to characterise fetal and neonatal exposure to ovalbumin with regard to maternal diet by direct measurement of the protein. This would allow objective analysis of how exposure relates to dietary intervention and, in turn, to later infant atopic phenotype.

4.2 Subjects, samples and methods

4.2.1 Subjects and samples

Samples were available from four groups of subjects *(table 4.1)*.

Table 4.1 Subjects and samples for ovalbumin detection.

Group	*Subject details*	*Sample details*
Group 1	Healthy, non-pregnant, women (n=26) *(2.2.1)*	sera, n = 26
Group 2	Unselected pregnant women undergoing routine amniocentesis at 16-17 weeks gestation (n=128) *(2.2.2)*	• maternal plasma, n = 128 • matched cord plasma, n = 17 • matched amniotic fluid of +ve maternal plasma, n = 19
Group 3	Pregnant women, with personal or partner history of atopy, randomised to egg avoidance, or normal diet, from 17-20 weeks gestation to the end of lactation (n=190) *(2.2.3)*.	• maternal sera, n = 350 • matched cord sera, n = 27 • defatted breast milk samples, collected at 3/12 postpartum, n = 125
Group 4	Infants at 6 months age, born to Group 3 women (n=110) *(2.2.4)*.	sera, n=110

4.2.2 Laboratory methods

All general materials, including reagents, buffers and apparatus, used were as those listed previously *(2.1)*.

4.2.2.1 Ovalbumin detection ELISA

Blood, amniotic fluid and breast milk samples were analysed for the presence and concentration of ovalbumin by an in-house capture *ELISA (3.2.)*.

Blood and breast milk samples were diluted 1:2 and 1:4 in antibody buffer. Samples producing an OD above the standard curve were repeated at higher dilutions (range 1:8 – 1:1000). Amniotic fluid samples were assayed neat.

OVA concentration was expressed in ng/ml, after taking into account the dilution of the sample, by comparison of the sample OD to the ovalbumin standard curve.

4.2.2.2 Measurement of breast milk ovalbumin IgG concentration

Breast milk samples (n= 24) were analysed for ovalbumin specific IgG (OVA G) concentration by indirect *ELISA (2.5.1)*. Samples were diluted 1:10 in antibody buffer. Since breast milk IgG concentration was likely to be low [312], the serum pool *(2.2.6)* was used to provide the assay standard curve.

Breast milk OVA G concentration was expressed in arbitrary units (AU), after taking into account the dilution of the sample, by comparison of the sample OD to the reference curve of the standards.

4.2.2.3 Measurement of breast milk ovalbumin specific IgA

Breast milk samples (n= 33) were analysed for ovalbumin specific IgA (OVA A) concentration by *ELISA* according to the manufacturer's instructions (Genesis Diagnostics, UK).

Briefly, breast milk samples were diluted 1:5 in sample diluent. Test samples (100 µl) were added singly, and a positive control, negative control, cut-off calibrator and sample diluent as background calibrator (all 100 µl) were added in duplicate, to the kit pre-ovalbumin-coated plate. Samples and standards were incubated for 1 hour in the dark. Then the well contents were discarded and the plate washed 3 times with wash

buffer. Peroxidase-conjugated species anti-IgA (100 µl) was added to each well and the plate incubated for a further 1 hour in the dark. The washing step was repeated before adding 100 µl of TMB enzyme substrate and incubating for 10 minutes in the dark. The reaction was stopped by the addition of 100 µl of stopping solution and the colour change read at $A_{450\ nm}$.

Ovalbumin specific IgA concentration was expressed as an index, calculated as follows:

$$\text{OVA A Index} = \frac{\text{OD of sample - background}}{\text{OD of cut-off calibrator - background}}$$

An Index greater than 1 indicated a positive sample.

4.2.2.4 Measurement of total IgE concentration

Total IgE concentration in infant sera collected at 6 months of age was measured by a commercially available chemiluminometric sandwich immunoassay (MagicLite, Ciba Corning), as described previously *(2.5.4)*.

Total IgE concentration was expressed in IU/ml.

4.2.3 Assessment of compliance to an egg exclusion diet

All women participating in the egg avoidance study (Group 3) were supported and monitored by a dedicated study dietitian. Those women randomised to an egg exclusion diet were asked to keep a diary record of known accidental ingestion of egg *(2.3)*. In addition, dietary compliance was objectively confirmed by the measurement of egg specific IgG concentration in serum samples collected through pregnancy. This will be discussed in detail in *chapter 5*.

4.2.4 Assessment of infant atopic phenotype at 6 months of age

Group 4 infants born to mothers participating in the egg avoidance study (Group 3) were clinically evaluated for allergic symptoms and signs at 3, 6, 12 and 18 months of age *(2.4)*. At six months of age, an atopic phenotype was defined as a history or presence of eczema and/or a positive SPT to one or more of a panel of common dietary and inhalant allergens *(table 2.6)*. A positive SPT was regarded as a wheal ≥ 2mm in the presence of appropriate negative and positive (histamine) controls.

4.3 Results

4.3.1 Antigen detection

4.3.1.1 Adult Blood

Ovalbumin detection rates in blood samples from the three adult study groups are summarised in *table 4.2*.

Group 1 Healthy, non-pregnant women

OVA was detected in 5 of 26 serum samples (19.2%), range 67 - 740 pg/ml (median 0.39 ng/ml).

Group 2 Unselected pregnant women at 16-17 weeks gestation

OVA was detected in 26 of 128 plasma samples (20.3%), range 15 pg/ml – 10.6 ng/ml (median 0.6 ng/ml).

Group 3 Pregnant women, with personal or partner history of allergy, participating in a dietary intervention study

Serum samples (n=350), available from 190 women through pregnancy, were analysed.

OVA was detected in 55 of 350 samples (15.7%), range 0.06 – 17.4 ng/ml (median 0.32 ng/ml). Ovalbumin was detected in one or more samples from 36 of the 190 subjects (18.9%) (OVA + group). Sample pairs from 17-20 weeks gestation (study recruitment) and the third trimester of pregnancy were available for 28 of the OVA + group. OVA was present at both time points in samples from 19 of these 28 subjects (67.9%).

Table 4.2 Ovalbumin detection in adult blood.

Source	Frequency of OVA detection	Concentration range (median), ng/ml
Group 1 Healthy, non-pregnant women (n=26)	**5/26 (19.2%)**	**0.067 – 0.74 ng/ml (0.39 ng/ml)**
Group 2 Unselected pregnant women (n=128)	**26/128 (20.3%)**	**0.015 – 10.6 ng/ml (0.6 ng/ml)**
Group 3 Pregnant women with family history of allergy (n=190)	**55/350 samples (15.7%)** from **36/190 subjects (18.9%)**	**0.06 – 17.4 ng/ml (0.32 ng/ml)**

4.3.1.2 Amniotic fluid

Matched amniotic fluid (AF) samples were available for 19 of the 26 Group 2 subjects who had detectable plasma OVA. Ovalbumin was detected in 3 of these 19 amniotic

fluids, range 20 – 37 pg/ml (median 33.5 pg/ml). The positive AFs matched the plasma samples with the highest quantities.

4.3.1.3 Breast milk

Breast milk samples (n=125) were collected by expression from 68 women at 3 months post-partum. One or more samples from 24 of these 68 subjects (35.3%) were found to contain ovalbumin. OVA was detected in 40 of 125 samples (32 %), range 0.12 – 1258 ng/ml (median 0.66 ng/ml). The milk with the highest concentration (1258 ng/ml) came from a woman who had ingested an egg-based meal 2 hours prior to giving the sample *(table 4.3)*.

Table 4.3 Ovalbumin detection – Breast milk.

Source	Frequency of OVA detection	Concentration range (median)
Women with family history of allergy (Group 3) *(2.2.3)*, at 3 months post-partum.	**40 / 125 (32%) samples** collected from **24 / 68 women (35.3%)**	**0.12 – 1258 ng/ml (0.66 ng/ml)**

4.3.1.4 Cord blood

Cord plasma was available for 9 of the 26 Group 2 women with detectable OVA at 16-17 weeks gestation: ovalbumin was present in 7 of these. Matched cord sera for OVA + Group 3 maternal samples at delivery were available for 12 infants: ovalbumin was present in 7 of these. The protein could not be detected in any cord sample of infants born to mothers with no detectable OVA in pregnancy (n=23).

Ovalbumin detection in maternal blood throughout pregnancy was significantly associated with the presence of OVA in infant blood at birth ($p=<0.01$, Chi^2) *(table 4.4)*.

Table 4.4 Ovalbumin detection in cord blood according to the presence or absence of ovalbumin in maternal blood throughout pregnancy.

		Infant OVA – cord blood		
		Present	Absent	Total
Maternal OVA in pregnancy	Present	**14**	**7**	**21**
	Absent	**0**	**23**	**23**
	Total	**14**	**30**	**44**

$p<0.01$

Ovalbumin concentration in the positive cord samples ranged between 0.1 and 5.7 ng/ml (median 0.39 ng/ml). There was a significant direct correlation between concentration in cord and maternal plasma or serum (r=0.549, p=0.01, Spearman's) *(figure 4.1)*.

Figure 4.1 Correlation between ovalbumin concentration in cord & maternal blood.

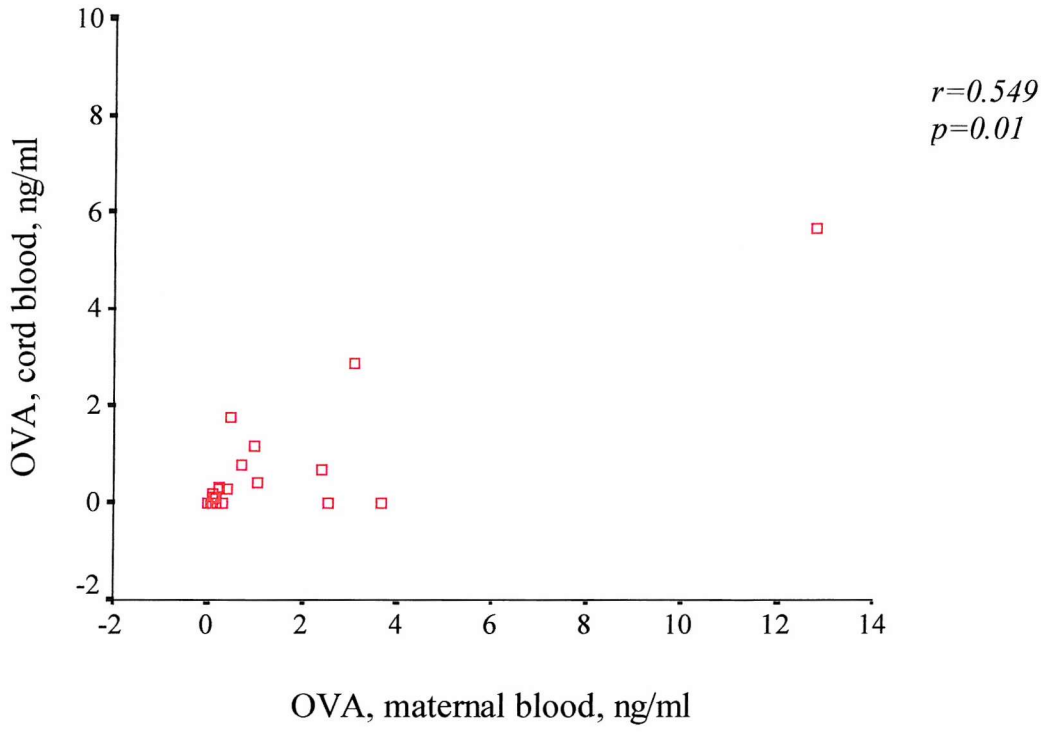


Figure 4.1 Ovalbumin was measured in cord blood and matching maternal blood collected throughout pregnancy (n=44) by capture ELISA (3.2). Cord blood ovalbumin concentration directly correlated with that in maternal blood through pregnancy.

4.3.1.5 Infant sera at 6 months of age

Circulating OVA was detectable at 6 months of age in 6 of 110 (5.5%) babies for whom a serum sample was available at that time point, range 94 pg/ml – 2.57 ng/ml (median 0.19 ng/ml) *(table 4.5)*.

Table 4.5 Ovalbumin detection – infant sera.

Source	Frequency of OVA detection	Concentration range (median)
Babies with atopic family history (Group 4) *(2.2.4)*, at 6 months age.	**6 / 110 *(5.5%)***	**94 pg/ml – 2.57 ng/ml *(0.19 ng/ml)***

4.3.2 Factors determining exposure

For Group 3 women the detection of ovalbumin was analysed with regard to maternal atopic status and study category – namely, avoidance of egg in pregnancy and breast-feeding (intervention group) or normal diet (control group).

OVA detection was equally common in breast milk collected from atopic women (18/48 women; 37.5%) as non-atopic women (6/20 women; 30%) ($p=0.555$, Chi^2). It was also as frequently present in breast milk collected from intervention women (11/36 women; 30.5%) as control women (13/32 women; 40.6%) ($p=0.386$, Chi^2). However, samples collected from women avoiding dietary egg, who were also atopic, were significantly more likely to contain ovalbumin than if the women were not atopic ($p=0.037$, Chi^2) *(table 4.6)*. Furthermore, breast milk samples collected from atopic women in the intervention group contained significantly greater quantities of the protein than samples from non-atopic subjects in the intervention group ($p=0.048$, Mann-Whitney) *(figure 4.2)*.

Table 4.6 Detection of ovalbumin in breast milk samples according to study category and maternal atopic status.

Maternal Study Group		**(Samples) OVA present**	**(Samples) OVA absent**	Total
Intervention	Atopic	16	26	42
	Non-atopic	2	16	18
	Total	18	42	60

p=0.037

Control	Atopic	15	31	46
	Non-atopic	7	12	19
	Total	22	43	65

p=0.743

Figure 4.2 Ovalbumin concentration in breast milk samples, according to maternal atopy and study category.

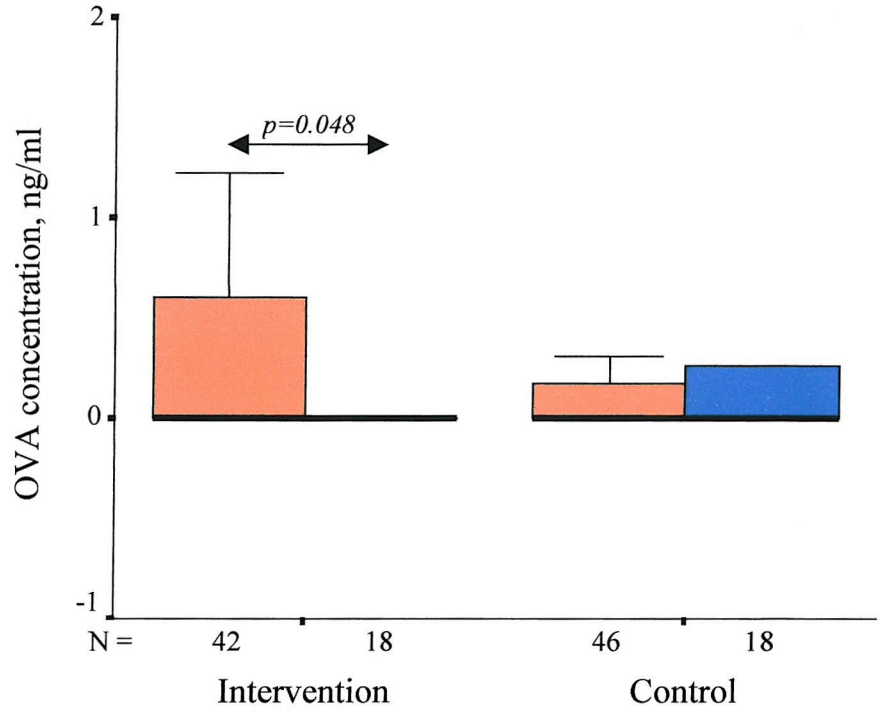


Figure 4.2 The presence and concentration of ovalbumin in breast milk samples collected from women with a family history of atopy (Group 3) was measured by in-house ELISA (3.2). Samples from atopic women (orange boxes) avoiding dietary egg had significantly higher ovalbumin concentration than had samples from non-atopic women (blue boxes) undertaking the same dietary intervention.

No intergroup differences were identified for rates of OVA detection in maternal serum samples through pregnancy. At least one positive serum sample was found in 23 of 136 atopic women (16.9%) as compared to 12 of 53 women whose partners alone were atopic (22.6%) (p=0.362, Chi^2). Similarly, 17 of 96 intervention women (17.7%) compared to 19 of 94 control women (20.2%) had one or more OVA + serum samples through pregnancy (p=0.660, Chi^2). However, the concentration of ovalbumin found in samples from atopic women was significantly higher than that found in samples from non-atopic subjects (p=0.022, Mann-Whitney) *(figure 4.3)*.

Figure 4.3 Quantity of ovalbumin in serum samples collected through pregnancy according to maternal atopic status.

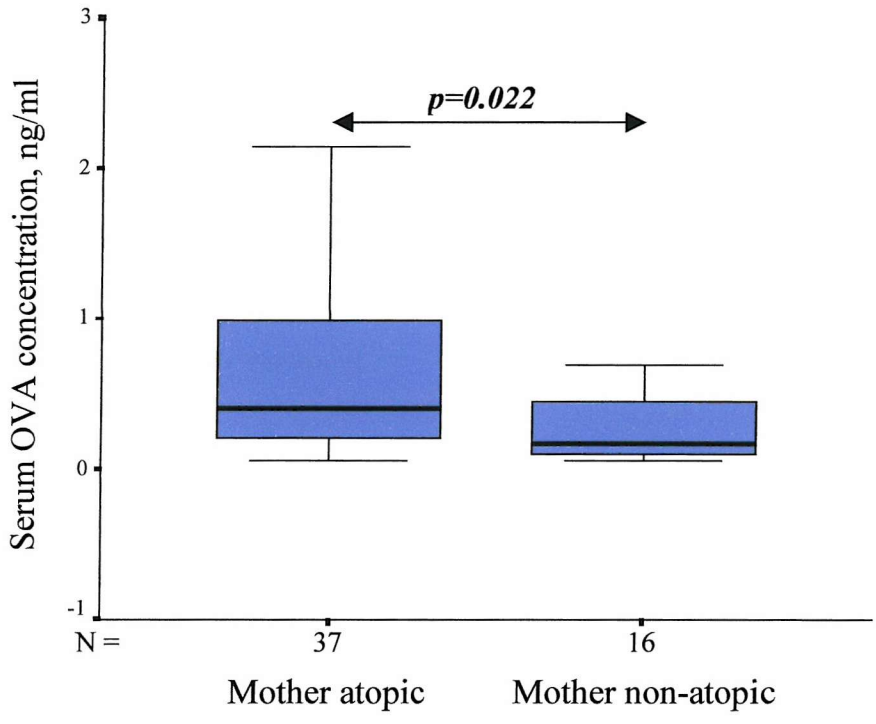


Figure 4.3 Ovalbumin concentration in 'positive' serum samples collected throughout pregnancy from Group 3 women was significantly higher in samples from atopic, than from non-atopic, pregnant women.

4.3.3 Relationship between ovalbumin exposure and infant atopic outcome

Ovalbumin presence in samples from Group 3 women was analysed with regard to infant atopic status at six months of age.

Infants with a history of eczema at 6 months of age had significantly higher total IgE concentration at this time point than had infants with no reported eczema (p=0.004, Mann-Whitney) *(figure 4.4)*. Furthermore, there was a significant relationship between a history of eczema and the presence of a positive SPT to any of the panel of allergens tested (p=0.003, Chi^2) *(table 4.8)*. Both eczema and/or skin prick test positivity were thus used as monitors of infant atopic phenotype at this time point.

Figure 4.4 Infant serum IgE levels at 6 months of age according to history of eczema.

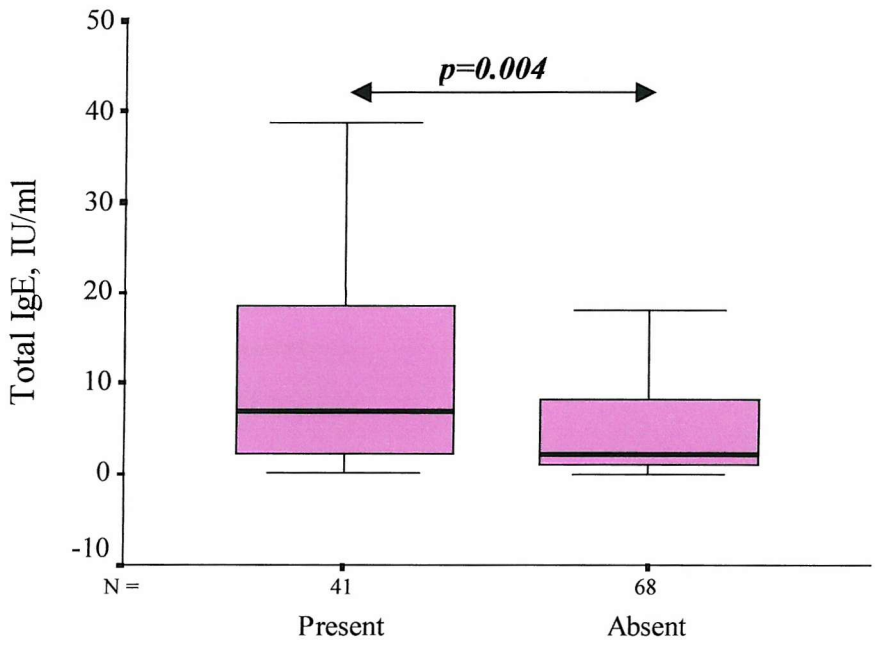


Figure 4.4 Serum IgE at 6 months of age, as measured by MagicLite (2.5.4), was significantly higher in infants with a history of eczema. Thus eczema was a marker of infant allergic sensitisation.

4.3.1.3 In utero exposure via maternal sources

Infant atopic phenotype at 6 months of age was available for 157 babies born to 155 of the women (2 sets of twins) for whom serum OVA presence through pregnancy had been ascertained (31 mothers with circulating ovalbumin).

As summarised in *table 4.7*, 72 of 157 babies (45.9%) had eczema and/or a positive SPT at 6 months of age. There was no significant difference in the number of babies with an atopic phenotype born to OVA + mothers - 13 of 32 (40.6%) - as born to OVA – mothers - 59 of 125 (47.2%) (p=0.505, Chi^2). Also, as many babies with an atopic phenotype were born to atopic mothers (52/112, 46.4%) as non-atopic mothers (19/44, 43.2%) (p=0.714, Chi^2), or to intervention mothers (33/73, 45.2%) as control women (39/84, 46.4%) (p=0.878, Chi^2).

Table 4.7 Infant atopic phenotype according to in utero exposure to ovalbumin.

		Atopic phenotype		
		Yes	**No**	**Total**
Serum OVA	**Yes**	13	19	32
	No	59	66	125
	Total	72	85	157

p=0.505

Eczema and SPT positivity were closely linked: of 39 babies with one or more positive skin tests, 21 (53.8%) also had eczema and of all babies with eczema, 21 of 54 (39%) were SPT positive (p=0.003, Chi^2) *(table 4.8)*.

Table 4.8 Atopic phenotype at 6 months of age of infants born to Group 3 mothers.

		History of eczema		
		Yes	**No**	**Total**
SPT positivity	**Yes**	21	18	39
	No	33	85	118
	Total	54	103	157

p=0.003

However, when infant allergic outcome was analysed with respect to exposure to dietary ovalbumin through pregnancy and the defining maternal characteristics of atopy and dietary intervention, a significant association of infant atopy with evidence of exposure, maternal atopy and dietary exclusion was revealed (p=0.031, Fisher's exact test) *(table 4.9)*. No such association was apparent for infants born to OVA – women. This suggested that presumed *in utero* exposure, if occurring in association with a maternal atopic milieu and dietary manipulation, might be associated with an increased infant risk of expression of an allergic phenotype.

Table 4.9 Atopic phenotype of infants born to mothers with detectable serum ovalbumin through pregnancy according to maternal atopy and dietary intervention group.

		Atopic phenotype at 6 months		
		Present	Absent	Total
Intervention	Mother atopic	6	3	9
	Non-atopic	0	5	5
	Total	6	8	14

p=0.031

Control	Mother atopic	5	8	13
	Non-atopic	1	3	4
	Total	6	11	17

p=1.0

4.3.1.3 Postnatal exposure via maternal sources

Infant atopic phenotype at 6 months of age was known for 65 babies born to the 68 women for whom the presence of OVA in breast milk (BM) had been ascertained (22 mothers with BM OVA). There was no significant difference in the rates of atopy amongst babies exposed (OVA + BM) (12/22, 54.5%), or not exposed (OVA – BM) (19/43, 44.2%) (p=0.429, Chi^2) postnatally via their mother's milk.

However, for the exposed infants, there was a strong trend for an increased prevalence of atopy amongst those exposed in the context of an atopic mother (p=0.056, Fisher's exact test) *(table 4.10)*, and a trend for an association with atopic mothers who were also egg avoiding (p=0.086, Fisher's exact test) *(table 4.11)*.

Table 4.10 Atopic phenotype of infants born to mothers with detectable breast milk ovalbumin according to maternal atopy.

		Atopic phenotype at 6 months		
		Present	Absent	Total
Maternal atopy	Atopic	11	5	16
	Non-atopic	1	5	6
	Total	12	10	22

p=0.056

Table 4.11 Atopic phenotype of infants born to mothers with detectable breast milk ovalbumin according to maternal atopy and dietary intervention category.

		Atopic phenotype at 6 months		
		Present	Absent	Total
Intervention	Mother atopic	6	1	7
	Non-atopic	0	2	2
	Total	6	3	9

p=0.083

Control	Mother atopic	5	4	9
	Non-atopic	1	3	4
	Total	6	11	13

p=0.559

4.3.4 Breast milk ovalbumin IgG concentration

Ovalbumin IgG was detected in 6 of 24 (25%) randomly selected breast milk samples (3 samples with detectable OVA), in extremely low concentrations (median 0.011 AU). There was no significant difference in OVA G concentration between milks with or without ovalbumin (p=0.398, Mann-Whitney) *(figure 4.5)*, or between milks from intervention or control mothers (p=0.106, Mann-Whitney).

Figure 4.5 Ovalbumin IgG concentration in breast milk according to the presence of absence of detectable ovalbumin.

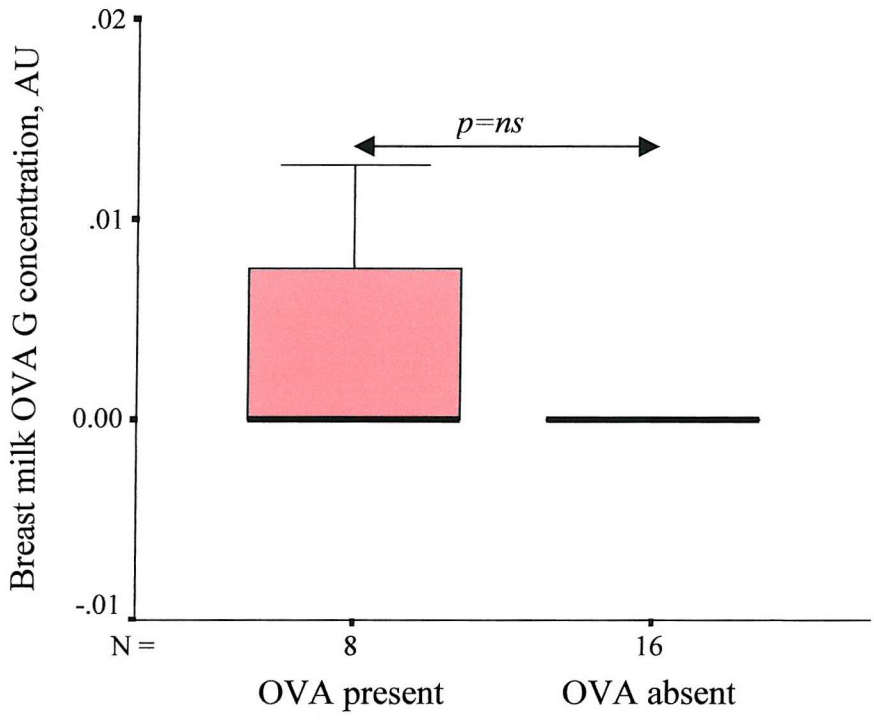


Figure 4.5 Breast milk ovalbumin IgG concentration was measured by indirect ELISA (2.5.1). Extremely low concentrations were found in 6 of 24 selected samples. There was no significant difference in concentration between samples with or without detectable ovalbumin.

4.3.5 Breast milk ovalbumin IgA

Ovalbumin specific IgA was detectable in 25 of 33 (75.7%) randomly selected breast milks (OVA A Index: range 1 – 25.5, median 3.1). Breast milk samples with OVA had significantly higher concentrations of OVA A than had breast milks without OVA (p=0.004, Mann-Whitney) *(figure 4.6)*.

Figure 4.6 Breast milk ovalbumin IgA concentration according to the presence or absence of detectable ovalbumin.

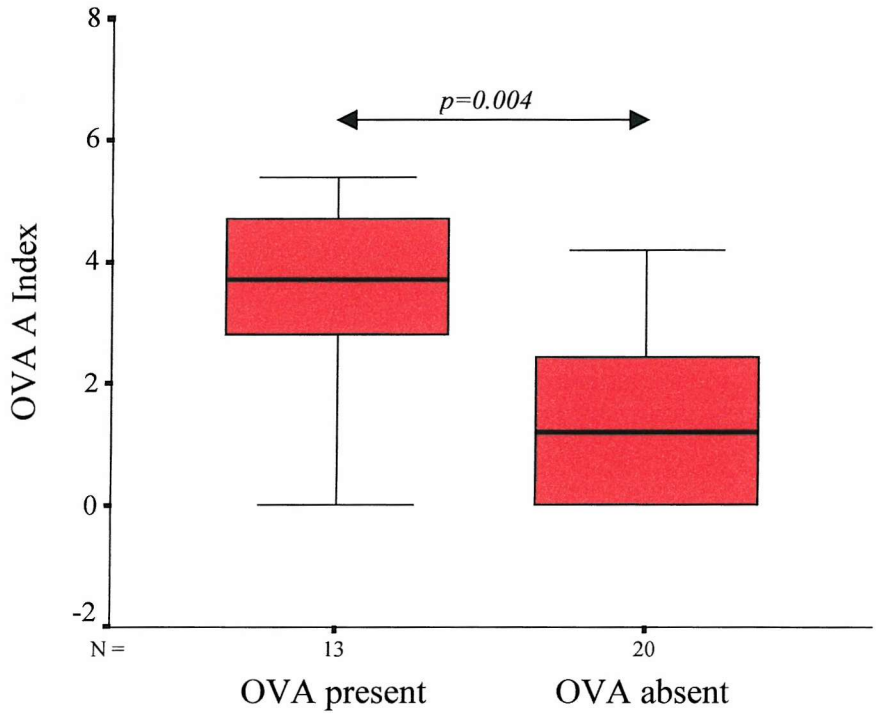


Figure 4.6 Ovalbumin IgA index (concentration) in breast milk was measured by ELISA (4.2.2.3). Those breast milk samples with ovalbumin had significantly higher concentrations of ovalbumin IgA than those milks without ovalbumin.

There was no significant difference in OVA A concentration between breast milks from intervention women as compared to control women, or between milks from atopic as compared to non-atopic subjects. However, for both intervention group women, and atopic women, the presence of ovalbumin in breast milk was associated with a significantly higher OVA A concentration (Intervention women, p=0.002, Mann-Whitney, *figure 4.7*), (Atopic women, p=0.008, Mann-Whitney, *figure 4.8*).

Similarly, there was no significant difference in breast milk OVA A concentration according to the atopic outcome of the infant at 6 months of age. However, for those infants with an atopic phenotype, the presence of OVA in mothers' milk was associated with a significantly higher OVA A concentration (Atopic phenotype, p= 0.008, Mann-Whitney, *figure 4.9)*.

Figure 4.7 Ovalbumin IgA Index in breast milk according to maternal study category and presence or absence of ovalbumin.

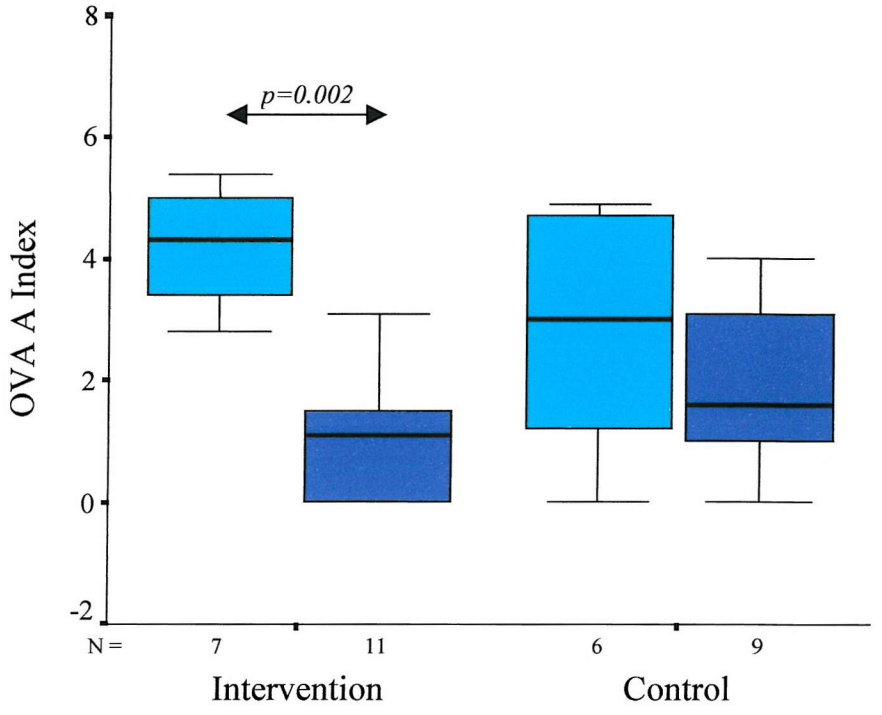
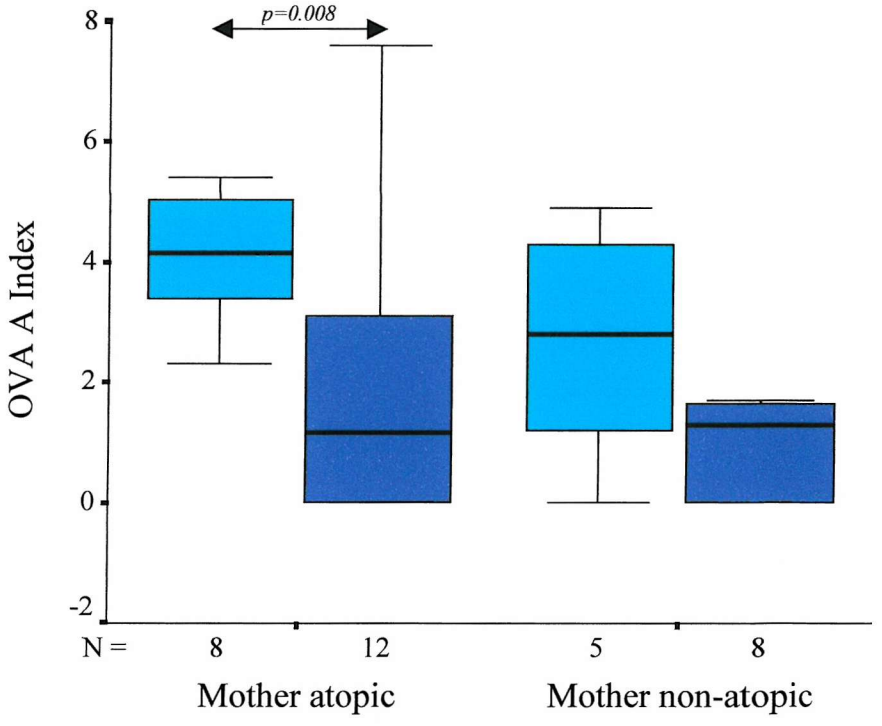


Figure 4.8 Ovalbumin IgA Index in breast milk according to maternal atopic status and presence or absence of ovalbumin



Figures 4.7 & 4.8 Breast milk samples with ovalbumin (pale blue boxes) had significantly higher ovalbumin IgA Index than had samples without ovalbumin (dark blue boxes), but only for dietary intervention and atopic women.

Figure 4.9 Ovalbumin IgA Index in breast milk according to infant atopic phenotype at 6 months age and presence or absence of ovalbumin in mothers' milk.

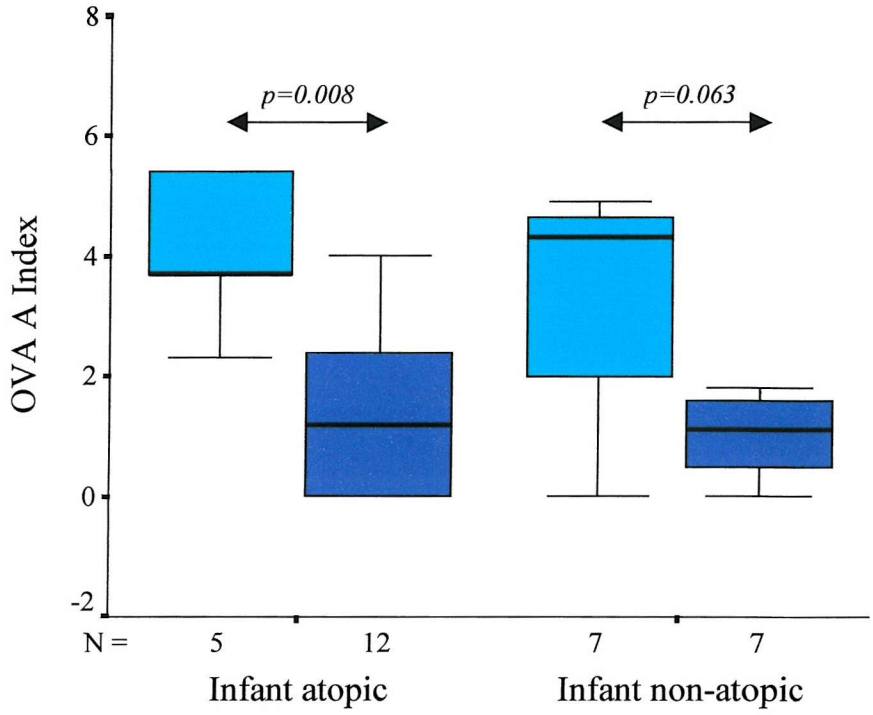


Figure 4.9 Maternal breast milk with ovalbumin (pale blue boxes) was associated with significantly higher specific IgA compared to milks without ovalbumin (dark blue boxes) where the infants subsequently had an atopic phenotype at 6 months of age.

Breast milk ovalbumin IgA concentration showed a significant, direct correlation with the quantity of OVA found in the same sample (r=0.513, p=0.002, Spearman's) *(figure 4.10)*.

Figure 4.10 Correlation between breast milk ovalbumin IgA Index and ovalbumin quantity.

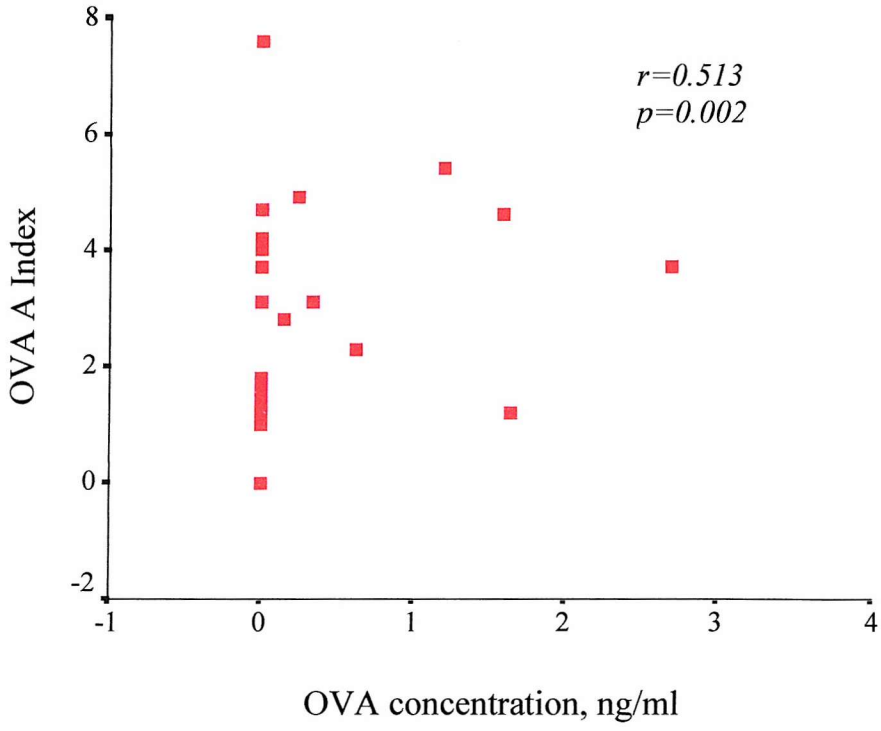


Figure 4.10 There was a significant, direct correlation between ovalbumin concentration and ovalbumin IgA index in the same breast milk sample.

4.4 Discussion

This work is the first to demonstrate *in vivo*, early life exposure to hen's egg ovalbumin in humans. Together with the recent publication reporting the presence of the house-dust mite allergen, Der p1, in cord plasma and amniotic fluid [(258)], these studies have confirmed *in utero* exposure to both dietary and inhalant allergens. In addition, these data have considered maternal factors which might affect exposure and have suggested that exposure, under certain conditions, may determine infant atopic phenotype.

The quantities of ovalbumin found in serum (up to 17.4 ng/ml) are in keeping with the ranges quoted by other authors [(302)(303)], although the lower median (Group 3 - 0.32 ng/ml) may be explained partly by the much lower limit of detection in this study, and partly by the fact that the subjects of these earlier studies had ingested challenge doses

of egg prior to laboratory measurement. This work is unique in detecting circulating ovalbumin under normal dietary conditions.

Similarly, the dose range of ovalbumin found in breast milk (0.15-1258 ng/ml) is consistent with that of other studies [300][304][305]. The one sample with a very high level (1258 ng/ml) came from a mother who had ingested an egg-containing meal prior to donating the sample. However, such an extreme value would not be unexpected as cow's milk β-lactoglobulin has been found in breast milk of women consuming a normal diet in quantities of 800 ng/ml [253].

The concentration of ovalbumin in breast milk (median - 0.66 ng/ml) was greater than that in maternal serum (median - 0.32 ng/ml), as also reported for β-lactoglobulin [253]. Other authors have found the converse to be true [300], but in that earlier work, purposeful ingestion of egg prior to laboratory measurement and a small number of sera (n=4) may have influenced the results.

Detectable OVA in maternal blood was significantly associated with the presence of OVA in infant blood at birth. Moreover, the concentration in cord blood correlated closely with that in the maternal circulation. This relationship between mother and fetal exposure was not just restricted to samples taken at delivery, but was also noted for measurements made of maternal levels at 16-17 weeks gestation *(4.3.1.4)*. The implications of such observations are two-fold: firstly, that fetal exposure via maternal sources may occur throughout gestation and thus the naïve immune system may indeed be susceptible to priming *in utero,* and secondly, that factors which modulate levels of circulating maternal protein may also have an impact on the fetus.

These data suggest three routes by which allergen exposure via the mother occurs in early life, namely, prenatally via transplacental and transamniotic routes, and postnatally via breast milk. In terms of quantity the breast milk route predominates (median 0.66 ng/ml), followed by transplacental (median 0.39 ng/ml) and then transamniotic (median 0.0335 ng/ml). The route(s) with most impact on IgE production and sensitisation remain to be established, although antenatal exposure is implicated in determining the atopic phenotype in view of the significant association of infant atopic outcome with ovalbumin exposure throughout pregnancy.

The work revealed that the maternal factors of atopic status and dietary intervention had a significant impact on exposure parameters. Thus, women who were atopic and actively avoiding dietary egg were more likely to have detectable OVA in their breast milk, and in greater quantities, than avoiding, non-atopic women *(table 4.6 & figure 4.2)*. Similarly, a higher concentration of circulating ovalbumin was found in the sera of atopic women compared to non-atopic subjects *(figure 4.3)*.

An association between atopy and antigen detection profile has been described by other authors. Ogura *et al* also found ovalbumin to be more readily detectable in breast milk of allergic, as compared to non-allergic, women [304] and serum ovalbumin after oral challenge was increased in atopic children, compared to non-atopic children, perhaps due to increased gut absorption [303].

The presence of detectable ovalbumin, despite subject avoidance of dietary egg, is more of an enigma. However, there are several factors that may contribute to this finding:

Firstly, the majority of women on the intervention arm of the trial reported at least one episode of accidental ingestion of egg, and whilst the cohort were a highly motivated group, clearly it is possible that the number of recorded accidents may have been underestimated.

Secondly, the home environment may be a source of adventitious exposure [313]. For example, the study protocol did not prohibit the women cooking eggs for other family members and nanogram quantities of β-lactoglobulin have been detected in washings from the fingers of laboratory personnel who only had normal domestic contact with milk [300].

The finding of serum OVA in only 5.5% babies at 6 months of age *(4.3.1.5)* would also suggest inadvertent environmental exposure to dietary antigen, since as a group at high genetic risk of atopy, all the mothers were advised to delay the introduction of egg into weaning foods until their infant was at least 8 months old. The lower frequency of detection compared with the adults would be consistent with this

explanation, since at this age, the infant's environmental contact would, for the most part, be controlled by the mother.

Thirdly, as will be presented in *chapter 5,* the intervention group women had a significant drop in serum ovalbumin specific IgG concentration through pregnancy. Since IgG has a role in antigen elimination [314][315], the lower levels may permit the persistence of circulating ovalbumin.

Therefore, exposure to egg through pregnancy, even though infrequent and in small quantities, occurring in the context of reduced antigen elimination, may explain the detection of ovalbumin in the intervention group as a whole, and the enhanced detection in samples from atopic, egg-avoiding women, who may, in addition, have increased antigen absorption. Fukushima *et al* found that it was the long term ingestion of milk that determined β-lactoglobulin concentration in breast milk [256] and that finding supports the concept that the history of ovalbumin exposure by these women over the prolonged study period might be revealed by the laboratory findings at this time point.

IgA is the major immunoglobulin in human milk [27]. An anti-allergic effect of IgA has been proposed since the antibodies, acting at the gut mucosal surface, provide a barrier to antigen penetration [316][317]. Indeed, differences in specific IgA levels depending on maternal atopy and infant allergic disease have been found by some [318][319], though not all [320][321], authors. In this study, breast milk specific IgA concentration was not influenced by dietary intervention (as also reported by Fälth-Magnusson [321]), maternal atopic status or infant atopic phenotype, unless the presence of ovalbumin in the milk was also considered *(figures 4.7-4.9).* Specific IgA concentration was also found to correlate with the quantity of ovalbumin present *(figure 4.10).* These observations raise the possibility that ovalbumin may be transported from blood to milk by dimeric IgA [322], although a likely alternative (to be addressed in *chapter 7*) is that the ovalbumin could stimulate local IgA production, as has been described for IL-8 induction of IgA secretory component in the gut [323]. IgG is unlikely to play a role in antigen transport since the concentrations in breast milk were universally extremely low.

Most startling of these results were the observations regarding infant atopic outcome and allergen exposure in early life. *In utero* exposure to ovalbumin, as inferred by the presence of circulating maternal ovalbumin was not, *per se*, associated with later infant atopy, but if the mother was atopic and on the intervention arm of the egg avoidance trial, then her infant was significantly more likely to have an atopic phenotype at 6 months of age *(table 4.9)*. The study population in this work have been subject to multiple statistical analyses by virtue of the number of different outcome measures under scrutiny. As discussed in *chapter 2*, the possibility of a type I error consequent on multiple testing is acknowledged. Nevertheless, most statisticians would view that each independent research question, within the larger study, should be considered in its own right and not be subjected to further statistical correction (Bonferroni method), particularly when the findings are biologically plausible and relate to the original hypothesis.

This association may contribute to the debate over whether allergen-specific proliferative responses at birth indicate *in utero* sensitisation or predict later infant allergic disease [(324)]. Antigen-induced T-cell proliferation is a common finding at birth and has been viewed as a marker of universal antenatal allergen exposure [(197)]. These current data, where OVA was detected in samples from, *at least,* 18.9% subjects would support this concept. The question of whether differential lymphoproliferation may represent sensitisation, or has any diagnostic potential for infant allergy, remains a controversial issue. However, these results would suggest that the newborn immune system could be directed towards an atopic phenotype by the time of birth, if consideration is made of the exposure characteristics in early life and the maternal atopic milieu in which the allergen is encountered.

What might the mechanism of this initial imprinting be? Dysregulation of tolerance, which underlies IgE-mediated disease is poorly understood, but animal models have identified the significance of antigen dose, timing, route of administration and mode of presentation in the phenomenon [(325)]. In the current cohort, infants of intervention mothers may have experienced *in utero* low dose and intermittent exposures, a pattern associated with priming in animal studies [(326)(327)]. Furthermore, the atopic mother presents a greater risk factor for infant allergy than the atopic father [(328)] and this may be due to immune differences between atopic and non-atopic women, as has been

intimated by breast-feeding data. Prolonged breast-feeding by asthmatic mothers was associated with an increased risk of childhood asthma [329], a finding that could be explained by differences in IgA levels [330], leucocyte [214], cytokine [213][331] or fatty acid [332] profiles. Indeed in this work there was a strong suggestion of an independent, adverse, association of maternal atopy with infant allergic phenotype, as a higher proportion of breast-fed infants exposed to ovalbumin through milk from atopic mothers had an allergic presentation themselves at 6 months of age *(table 4.10)*. Clearly it may be reasonable to postulate that a pro-sensitising pattern of allergen exposure combined with an 'adverse' maternal environment may permit early life programming of the atopic phenotype. Clinical data at this juncture were only available for the infants aged 6 months. It remains to be established if early life experiences have repercussions for allergic disease at later time points.

The point prevalence at 6 months of age of egg sensitisation in the dietary intervention study cohort *(Group 4)* was 10.5% (data not shown). Therefore, in view of these relatively small numbers SPT positivity to any of a panel of common allergens was used in the definition of infant atopy. It might be argued that only egg sensitisation was likely to be influenced by the pattern of ovalbumin exposure in early life. However, a bystander effect has been described in animal models whereby though tolerized T-cells secrete a suppressive cytokine (such as TGF-β) in an antigen specific fashion, its release into the local microenvironment also suppresses ongoing immune responses to unrelated, but anatomically colocalised, antigens [23][333][334]. If such a mechanism exists for tolerance and prevention of experimental colitis [23][333][334], then could a similar mechanism exist for antigen-specific priming? A bystander effect may then regulate the development of T_H2 memory to unrelated antigens, such as house-dust mite allergen Der p1, also shown to be present in the fetal environment [258].

In 1997 the government published a white paper advising that women, with atopic family history, might wish to avoid eating peanuts, or peanut-containing foods through pregnancy and breast-feeding. Whilst the inherent statistical dangers of multiple analyses are recognised and whilst it cannot be assumed that the mechanisms underlying, and characteristics of, egg allergen exposure also apply to peanut

allergens, nevertheless, the current results would imply that caution in this approach is needed. Firstly, the work indicates that maternal dietary avoidance measures are difficult, if not impossible, to achieve, and they do not eliminate allergen exposure completely. Secondly, the influence of the maternal atopic state cannot be overestimated. Together these factors may create an environment that predisposes to the genesis of allergy – at least in early infancy. The implication is therefore that the government's precautionary advice may actually result in an increase in the prevalence of peanut allergy in children, potentially adding to what is already a growing public health issue

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Chapter Five

Serum IgG responses to exclusion of dietary egg through pregnancy

Chapter 5

Serum IgG responses during exclusion of dietary egg through pregnancy

5.1 Aims

The assessment of the effect of exclusion of dietary egg through pregnancy and breast-feeding on early life egg allergen exposure and the consequences for infant atopic outcome is crucially dependent on maternal compliance to the elimination diet. Since self-reporting is prone to intentional, or non-intentional, error, compliance is best judged by an objective method. The aim of this work was to employ measurement of serum food specific IgG concentration, which reflects dietary intake [271][335][336], to monitor subject compliance.

The association of infant IgG at birth with subsequent atopic disease remains to be clarified. Some authors have reported high maternal and cord IgG to food [337][338], and inhalant [291] allergens to be associated with less infant atopy, while others have reported either no association [339], or even an increased risk of infant allergy [292]. A secondary aim of this work was therefore to evaluate the effect of maternal dietary manipulation on cord levels of egg specific IgG, and in turn, to examine the relationship between cord IgG concentration and later infant atopy in the study population.

5.2 Subjects, samples and methods

5.2.1 Subjects and samples

Serum samples were available from pregnant women, with a personal or partner history of atopy, randomised to egg exclusion or a normal diet from 17-20 weeks gestation until the end of breast-feeding. Blood samples were collected on study recruitment, at 24 weeks and 32 weeks gestation, and at delivery of the infant. Umbilical cord blood was obtained from these babies at birth *(2.2.3)*.

In order to examine the relationship between the dietary intervention and egg specific IgG responses, ovalbumin (OVA G) and ovomucoid IgG (MUC G) concentrations were measured in available maternal serum samples at recruitment (n=190) and delivery (n=137), and in matched umbilical cord samples (n=136). A further subset of sample pairs collected at study recruitment and 32 weeks gestation (n=22) were also analysed in order to investigate the time at which differences in humoral responses may be measured first.

As the dietary intervention was restricted to egg avoidance, it was important to establish that there was no difference between the study groups in specific IgG responses to an unrelated food product. Therefore, cow's milk β-lactoglobulin IgG (BLG G) concentration was measured in a randomly selected subset of recruitment and delivery serum sample pairs from the cohort (n=32).

5.2.2 Laboratory methods

Serum ovalbumin, ovomucoid and β-lactoglobulin IgG concentrations were measured by indirect *ELISA,* as outlined previously *(2.5.1)*.

Subject sample pairs were processed on the same *ELISA* plate. Samples from an equal number of intervention and control women were analysed on the same day, and on the same *ELISA* plate.

Serum samples were initially diluted 1:100 – 1:200 in antibody buffer. For those samples with absorbance above the standard curve the assay was repeated following further dilution in antibody buffer (range 1:500 - 1:8000).

OVA G, MUC G and BLG G concentrations were expressed in arbitrary units (AU), after taking into account the dilution of the sample, by comparison of the sample absorbance to the standard curve of the serum pool.

5.2.3 Assessment of infant atopic phenotype at 6 months of age.

Infants born to these mothers were clinically evaluated for allergic symptoms and signs at 3, 6, 12 and 18 months of age *(2.4)*. At six months of age, an atopic phenotype was defined as a history or presence of eczema and/or positive SPT to one or more of a panel of common dietary and inhalant allergens. A positive SPT was regarded as a weal ≥ 2mm in the presence of appropriate negative and positive (histamine) controls.

5.3 Results

5.3.1 Maternal serum egg specific IgG responses through pregnancy

5.3.1.1 Humoral responses according to dietary intervention

Ovalbumin specific IgG concentration at recruitment and delivery was analysed for 134 matched serum pairs. The intervention group (n=62) had a significant reduction in OVA G concentration through pregnancy ($p<0.001$, Wilcoxon) *(figure 5.1)*, a pattern that was established by 32 weeks gestation ($p=0.021$, Wilcoxon) *(figure 5.2)*. A fall in concentration (by 18.4 – 88.2% of initial levels) was measured in 58 of 62 (93.5%) matched serum pairs collected from egg-avoiding women. No significant change through gestation was observed in the control group ($p=0.195$, Wilcoxon). The intervention group had higher OVA G concentration at study recruitment compared to control women ($p=0.023$, Mann-Whitney), such that, despite the observed decline through pregnancy, there was no inter-group difference at delivery ($p=0.392$, Mann-Whitney).

Figure 5.1 Change in maternal serum ovalbumin IgG concentration, from recruitment till delivery, according to study group.

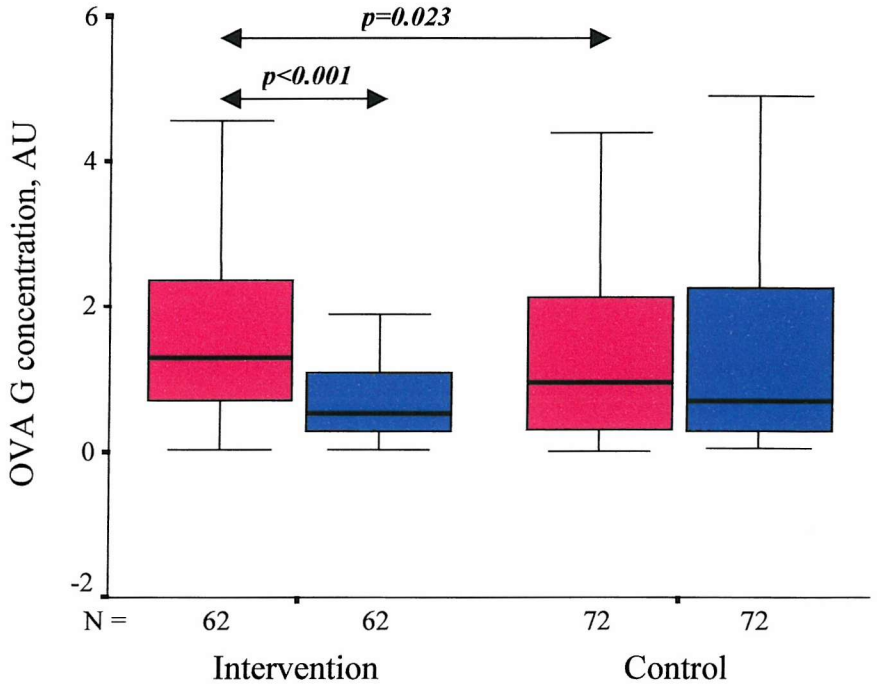


Figure 5.1 Ovalbumin IgG concentration was measured by indirect ELISA (2.5.1). Subjects in the intervention group had significantly higher OVA G concentration at study recruitment (pink boxes) compared to the control women. OVA G fell significantly from recruitment till delivery (blue boxes) in the intervention group only.

Figure 5.2 Change in maternal serum ovalbumin IgG concentration, from recruitment till 32 weeks gestation, according to study group.

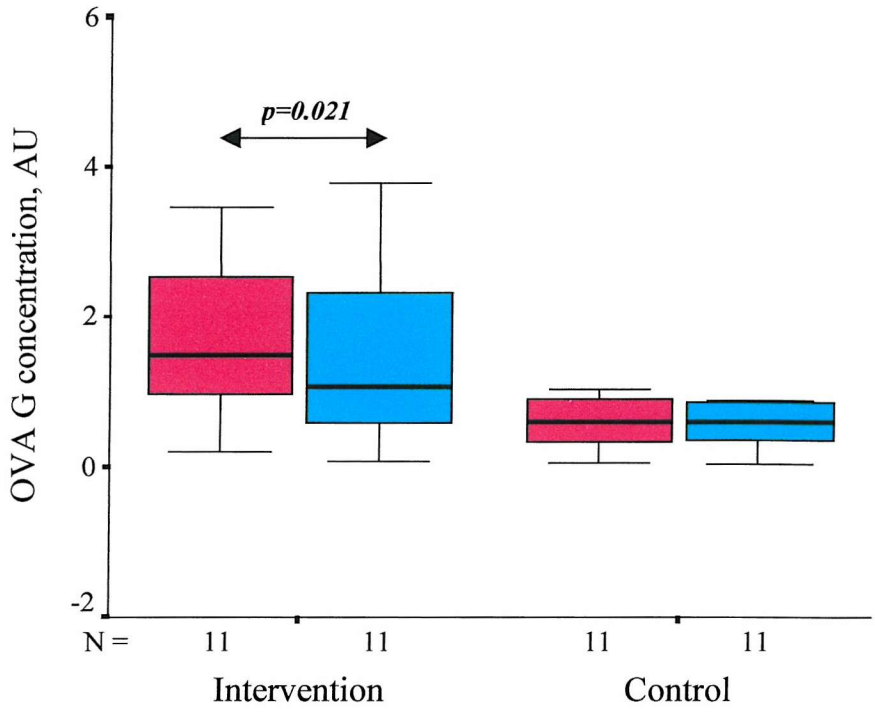


Figure 5.2 Ovalbumin IgG concentration showed a significant decline from study recruitment (pink boxes) to 32 weeks gestation (blue boxes) in a randomly selected subgroup of intervention group subjects. There was no significant change in a subgroup of control women.

A similar pattern was observed for changes in serum ovomucoid IgG concentration. Analysis of 131 matched maternal serum pairs collected at recruitment and delivery showed that the intervention group (n=61) had a significant fall in specific IgG concentration through pregnancy ($p<0.001$, Wilcoxon) *(figure 5.3)*. A fall in concentration (by 2.9 – 87.9% of initial levels) was measured in 54 of 61 (88.5%) matched serum pairs collected from egg-avoiding women. No significant change through pregnancy was observed for the control group ($p=0.286$, Wilcoxon).

Measurement of MUC G in recruitment samples (n=188) revealed that intervention group women had significantly higher levels at study onset compared to control women ($p=0.002$, Mann-Whitney) (not graphically represented). For the subgroup with available serum pairs (n=131), a strong trend for higher MUC G levels in intervention women at study recruitment was seen ($p=0.063$, Mann-Whitney) *(figure*

5.3). As a consequence, there was no significant inter-group difference in MUC G at delivery (p=0.281, Mann-Whitney).

Figure 5.3 Change in maternal serum ovomucoid IgG concentration, from recruitment till delivery, according to study group.

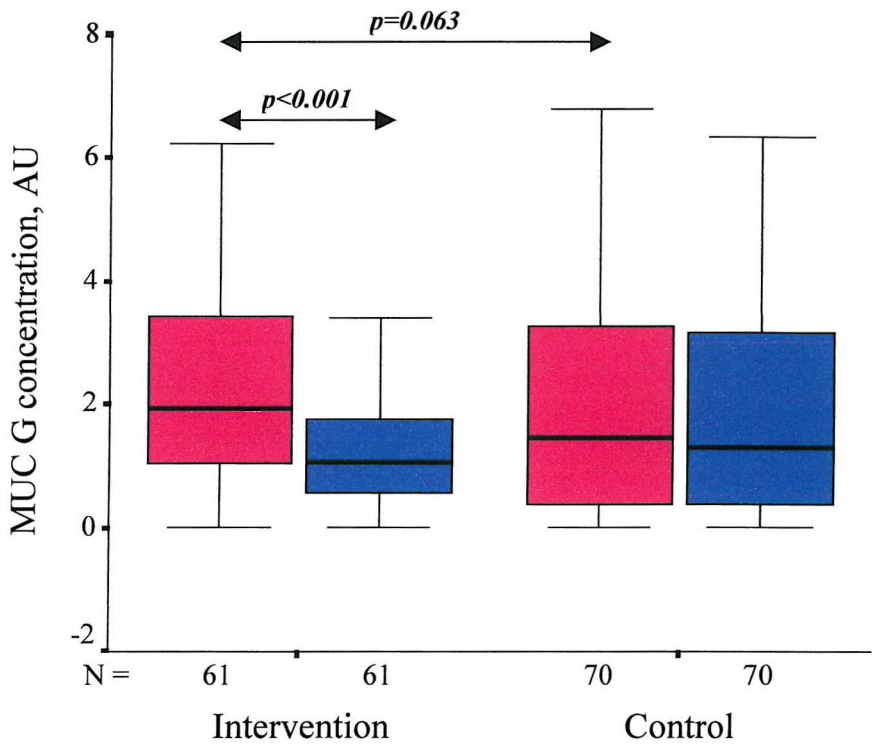


Figure 5.3 Ovomucoid IgG was measured by indirect ELISA (2.5.1). A significant fall through pregnancy from 17-20 weeks (pink boxes) till delivery (blue boxes) was associated with dietary egg exclusion.

5.3.1.2 Humoral responses according to maternal atopic status

No significant differences were observed in ovalbumin or ovomucoid IgG concentrations, either at recruitment, or at delivery, between atopic and non-atopic women. Also within the intervention group, who displayed a decline in specific IgG through pregnancy, the OVA and MUC G concentrations at delivery were not influenced by maternal atopic status.

5.3.2 Maternal serum cow's milk specific IgG responses through pregnancy

Change in concentration of a dietary protein not excluded by the elimination diet was analysed. Cow's milk β-lactoglobulin IgG concentration showed no significant change in the egg-avoiding group through pregnancy and rose significantly in the control group (p=0.038, Wilcoxon) *(figure 5.4)*.

Figure 5.4 Change in maternal serum β-lactoglobulin concentration, from recruitment till delivery, according to study group.

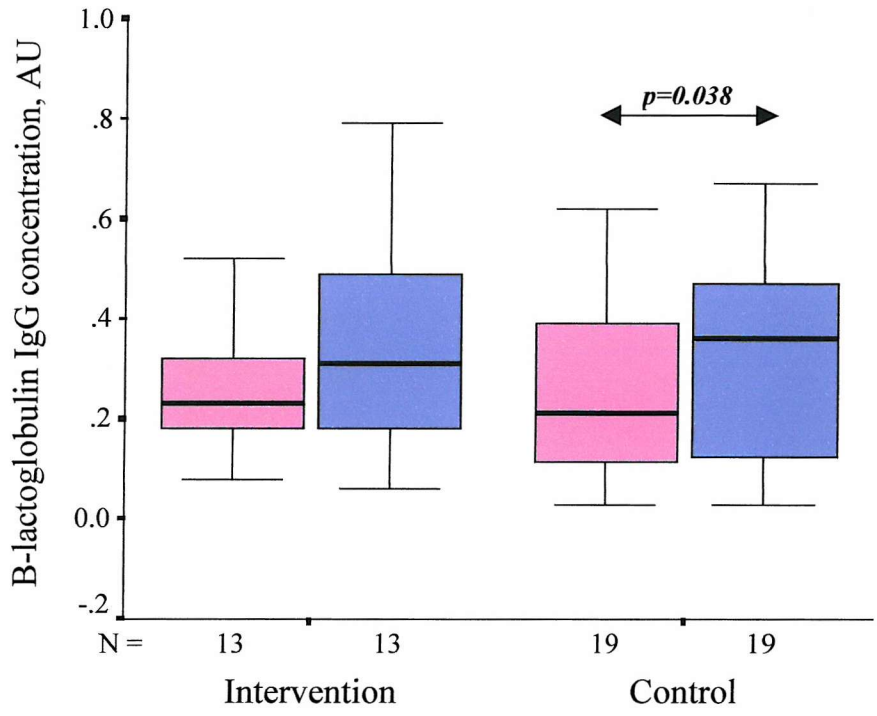


Figure 5.4 Serum β-lactoglobulin concentration was measured at recruitment (pink boxes) and at delivery (mauve boxes) by in house, indirect ELISA (2.5.1). There was no significant change in concentration through pregnancy in the intervention group. BLG concentration at delivery was significantly higher than at recruitment in the control group.

5.3.3 Infant serum egg specific IgG responses at birth

Cord OVA G directly correlated with maternal serum OVA G at delivery (r=0.944, p<0.001, Spearman's) *(figure 5.5)*. A similar positive correlation (r=0.919, p<0.001, Spearman's) between cord and maternal serum IgG concentrations was observed for ovomucoid (not graphically represented).

Figure 5.5 Correlation between maternal ovalbumin IgG at delivery and cord ovalbumin IgG.

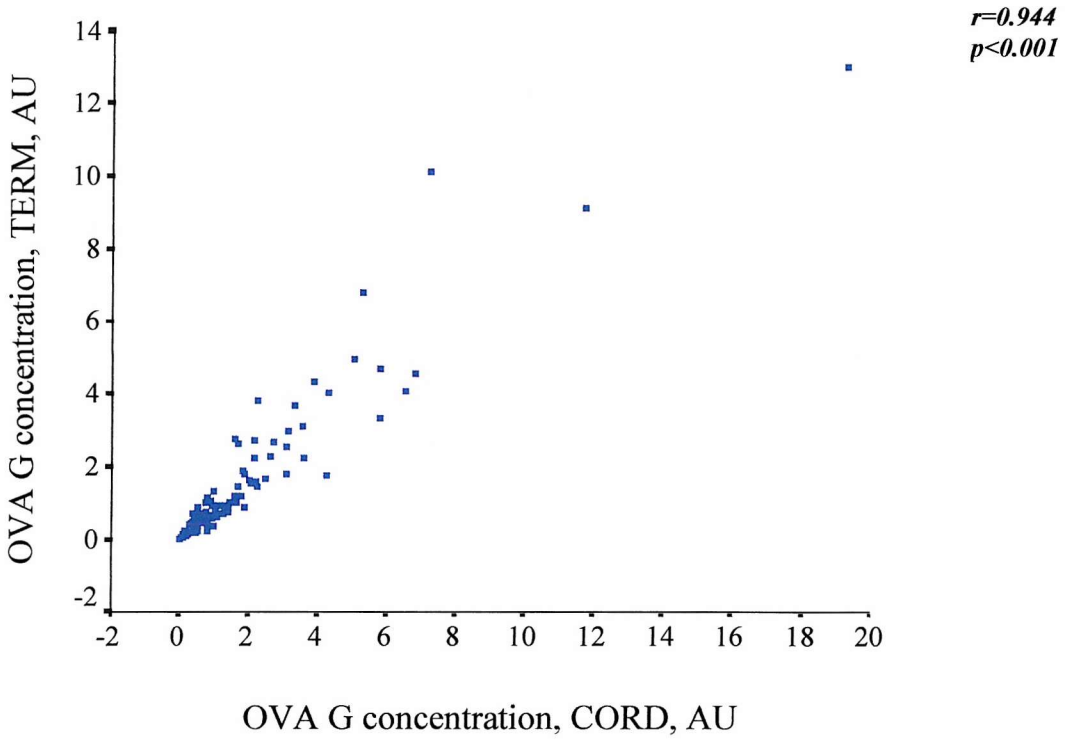


Figure 5.5 A significant direct correlation was observed between maternal serum ovalbumin IgG concentration at delivery and infant ovalbumin IgG concentration at birth (cord).

There was no significant difference in serum OVA G concentration at birth between infants born to intervention mothers as compared to control mothers (p=0.605, Mann-Whitney), or between infants born to atopic, as compared to non-atopic, mothers (p=0.837, Mann-Whitney).

However, infants born to atopic mothers had significantly higher serum OVA G concentration at birth than their mothers had at delivery (p<0.001, Wilcoxon). No such relationship was apparent for non-atopic mother-infant pairs (p=0.147, Wilcoxon) *(figure 5.6)*. Similarly, analysis of ovomucoid IgG responses showed a higher concentration of MUC G in infant serum at birth compared to matched maternal serum at delivery for babies born to atopic women (p<0.001, Wilcoxon), but not non-atopic women (p=0.102, Wilcoxon) (not graphically represented).

Figure 5.6 Cord ovalbumin IgG concentration in relation to maternal serum ovalbumin IgG concentration at delivery, according to maternal atopic status.

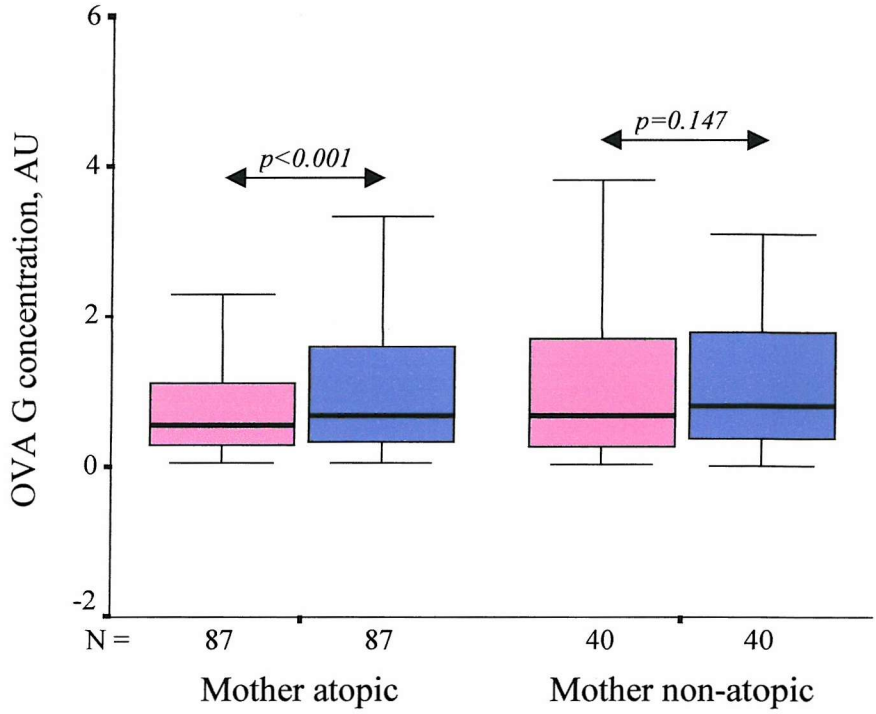


Figure 5.6 Cord ovalbumin IgG concentration (blue boxes) was significantly higher than matched maternal serum ovalbumin IgG concentration at delivery (pink boxes) for infants born to atopic women. No such association was apparent for infants of non-atopic women.

5.3.4 Infant atopic phenotype according to cord specific IgG responses

There was no significant difference in cord OVA G concentration between those infants who subsequently had an atopic phenotype and those who had not, irrespective of maternal study group or atopic status. However, if cord OVA G concentration was grouped into quartiles, where the first quartile represented the lowest IgG concentrations and the fourth quartile, the highest concentrations, then an association between infant atopic outcome and OVA G concentration emerged. For infants born to control women, there was a significant association for the lowest and highest IgG quartiles to have the least number of infants with an atopic phenotype and the middle quartiles to have the greatest number of atopic infants (p=0.02, Chi^2) *(figure 5.7)*. No such relationship was observed for infants of intervention mothers.

Figure 5.7 Atopic phenotype of infants born to control women according to quartile of ovalbumin IgG concentration.

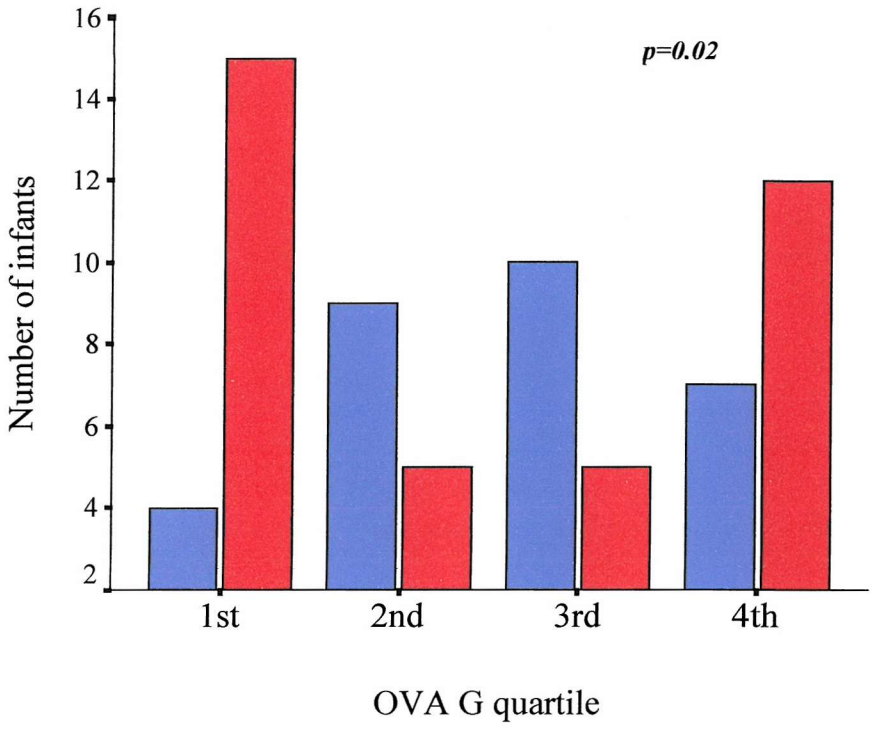


Figure 5.7 Significantly fewer infants with ovalbumin IgG in the lowest & highest quartiles had an atopic phenotype (purple bars) and significantly more were atopic at 6 months of age if cord ovalbumin IgG fell into the middle quartiles. Non-atopic infants are represented by red bars.

5.4 Discussion

This work set out primarily to ascertain the compliance of a cohort of women, whose infants were at genetic risk of allergic disease, to an egg exclusion diet through pregnancy and breast-feeding.

Avoidance diets, as a primary prevention strategy, have been criticised because of a lack of [274], or transient [340][341], allergic benefit for the infant, and possible nutritional compromise of mother and baby [274][336]. However, central to any potential benefit of dietary allergen exclusion is the question of subject compliance, particularly if multiple foods are excluded. In most maternal dietary avoidance studies compliance has been monitored by diary record [278][310], or dietary recall [342], both of which are

open to subject bias and error. This study attempted to gauge subject compliance by an *in vitro*, objective method, in addition to diary record of accidental exposures.

Serum food specific IgG concentration has been reported to reflect dietary intake [335][336] and these current data would support such a correlate. In this study dietary egg exclusion resulted in a significant fall in ovalbumin IgG concentration throughout pregnancy *(figure 5.1)*, a change occurring not later than 32 weeks gestation *(figure 5.2)*. No decline was measured in the control group. This observation, though expected, has not always been found in other studies [336]. Its importance is that the differential IgG responses may then reliably be taken to represent dietary differences between the groups and consequently, it suggests that the control women were not, inadvertently or otherwise, making dietary adjustments. Parallel responses were observed for ovomucoid, also a principal allergen of egg white, but present in smaller quantities than ovalbumin *(figure 5.3)*. Serum OVA G concentration fell through pregnancy in 93.5% of the intervention women. Serum pairs (n=3) in which a rise in OVA G was measured were all collected from intervention group women who had discontinued the exclusion diet through pregnancy. This change occurred in the face of the majority of intervention women reporting at least one - known - occasion of accidental ingestion of egg (number of accidental exposures over the intervention period ranging from 1 – 28, personal communication, Mrs KE Grimshaw, research dietitian). These data therefore confirmed that the intervention cohort avoided egg through pregnancy, but moreover, they have also suggested that even if dietary exclusion is incomplete, then the overall reduction in intake can still provoke a change in specific IgG levels.

This study not only reported an objective measure of patient compliance, but unlike other studies [335][336], also confirmed that the dietary restriction was limited to egg. Participation in a study of this type runs the risk of subjects, whether intervention or control, avoiding other well-known allergenic foods, which may well cause nutritional compromise if undertaken without dietetic supervision, in an attempt to improve their infant's allergic outcome. Cow's milk β-lactoglobulin is a common allergen, - but cow's milk is also a nutritious food product, particularly through pregnancy and breast-feeding. The β-lactoglobulin IgG concentration showed no decline in either study group *(figure 5.4)*, and indeed the significant rise in the control group was

further reassurance that these subjects were not taking prophylactic dietary measures into their own hands.

At study recruitment the intervention group women had significantly higher egg specific IgG concentration than the control women had. Since specific IgG reflected egg intake, it was supposed that this might be a consequence of an inter-group dietary difference. Indeed, analysis of the subjects' egg consumption during the month prior to study commencement showed that the intervention group women had ingested significantly greater quantities of egg compared to control women (personal communication, KE Grimshaw, research dietitian). However, neither the initial serum egg specific IgG concentrations, nor the decline in specific IgG in the intervention group, were influenced by maternal atopic status. This is contrary to the findings of Fälth-Magnusson *et al* who reported higher pre-diet IgG concentrations in atopic women [336]. No explanation for this finding was given by the Swedish authors, and whilst a relationship between high IgE and IgG has been described [343][344], in the absence of maternal egg allergy, one would not expect to measure disproportionately high levels of egg specific IgG in an adult cohort.

Maternal IgG responses in pregnancy were closely related to infant IgG responses at birth, as evidenced by the significant, direct correlation between matched maternal and cord specific IgG concentrations *(figure 5.5)*. Previous authors have found no significant difference in cord specific IgG concentrations between diet and non-diet groups [335][336], which might be an unexpected finding in view of the significant change in maternal serum concentrations measured and the close correlation between maternal IgG at delivery and cord IgG found in these previous studies [335] and the current work. In this study no significant difference was found between cord OVA G concentration of the 2 study groups, but this could be explained by the higher initial levels of the egg-avoiding women. When comparison was made of infant specific IgG at birth and matched maternal specific IgG at delivery, it was revealed that the infants had significantly higher levels, but only in the context of maternal atopy *(figure 5.6)*. Maternal atopy has been associated previously with high levels of IgG antibodies in cord blood, both against food and inhalant allergens [291][345] and this has been presumed to reflect differing immune responses between atopic and non-atopic individuals. This current work has offered a further unique insight on this association.

IgG is actively transported across the placenta by a receptor-mediated mechanism [346]. In the asthmatic lung, the high affinity receptor for another immunoglobulin (FcεRI for IgE) is present in increased numbers on dendritic cells [347] and this is thought to be a feature of the microenvironment of the atopic individual [348]. If a similar phenomenon, relating to placental IgG receptor expression, existed in the pregnancy-associated environment of the atopic mother, then might the discrepant cord levels represent a difference in the rate of transplacental IgG transport that is a function of maternal atopic status?

A beneficial [291][338], adverse [292], or no [339] effect of high cord blood allergen specific IgG concentration on the development of later infant allergic disease has been described. In these data no association of cord specific IgG with infant atopic phenotype was apparent until the cord measurements were ranked by grouping into quartiles. For infants born to control mothers, if their cord OVA G fell into the quartile containing the lowest concentrations (1st quartile) or the quartile containing the highest concentrations (4th quartile), then they were significantly less likely to have an atopic profile at 6 months of age *(figure 5.7)*.

In order to formulate an explanation for this observation, it is important to remember two facts that have been discussed previously. Firstly, egg specific IgG reflects dietary intake and secondly, allergic sensitisation is a complex process that is regulated by many factors, but central to the process is a requirement for allergen exposure. Therefore, the most simple, and crude, explanation of this profile may be that the infants with cord OVA G at either end of the spectrum do not encounter allergen. For those with cord OVA G in the 1st quartile, this may be because their mothers did not eat a sufficient quantity of egg through pregnancy, while for those with OVA G in the 4th quartile, this may be because allergen presentation was 'blocked' by the high IgG concentration, as has been suggested by animal studies [290] and immunotherapy data [349][350].

That allergic sensitisation is a complex process should be emphasised, as this may explain why the association was not observed for infants of intervention mothers. These women had inordinately high initial egg specific IgG and this may have distorted the profile. Moreover, more importantly, the intervention may have

consequences for the dose and pattern of allergen exposure, which, as discussed in *chapter 4*, may also have implications for infant allergic outcome. It may be that successful 'blocking' of sensitisation is dependent on a high serum specific IgG concentration being sustained throughout the 2nd and 3rd trimesters of pregnancy and these data have shown that OVA G fell relentlessly during egg exclusion, despite accidental ingestion, not later than 32 weeks gestation. It remains to be established if a fall in concentration has occurred by the 24 weeks gestation sample. It is not known whether the dietary manipulation has differential effects on the IgG subclasses. In this work only ovalbumin IgG was measured, but as a variety of data - presented later in this thesis *(chapter 9)*, and by previous authors [291][351][352][353] - has implicated specific IgG1 and IgG4 in the immunopathology of allergic disease, it will be important to reassess the humoral responses during the diet in terms of the OVA G1/OVA G4 balance. Finally, it might be that the fall in specific IgG concentration alters the range of ovalbumin epitopes recognised, with implications for immunoregulation, and this could be examined by tracking OVA G binding to a spectrum of OVA epitopes in serial bloods collected from intervention and control women. Thus, for the infants of intervention women many factors may act to determine atopic phenotype. The infants of control women have experienced no environmental manipulation and hence their humoral responses may be more easily correlated with clinical outcome.

To summarise: This work has shown that women on the intervention arm of a randomised controlled trial of egg avoidance through pregnancy and breast-feeding were compliant with the elimination diet, as evidenced by a significant fall in egg specific IgG concentration through pregnancy. The control group did not exclude egg, and neither group showed evidence of other dietary manipulation, such as avoidance of cow's milk. The maternal humoral changes induced by the elimination diet were also experienced by the fetus/newborn. Variations in infant specific IgG concentration may have consequences for later atopy, with a protective association of both the lowest and highest levels being seen. The implication of this observation is that the humoral changes consequent on dietary exclusion may potentially contribute to the complex process of allergic sensitisation.

Chapter Six

Investigation of ovalbumin form in the fetal environment

Chapter 6

Investigation of ovalbumin form in the fetal environment

6.1 Aims

The detection of ovalbumin in umbilical cord blood and amniotic fluid confirms fetal exposure to this dietary allergen, but raises the further question: how is the antigen transported to the fetus? Specifically, in what form is ovalbumin presented to the developing immune system – as free allergen or immune complex?

IgG is an ideal vehicle for allergen transport to the fetus. It is actively transported across the placenta by a mechanism dependent on receptor-mediated endocytosis and transcellular passage [reviewed in (24)]. Transport starts at 17 weeks gestation and gradually increases as pregnancy progresses, so that fetal IgG concentration approximates maternal levels by 33 weeks gestation. Levels continue to rise thereafter, sometimes reaching more than twice maternal concentration by the time of birth [(354)].

Carriage of antigen in immune complex form is well documented in autoimmune disease [(355)] and dietary antigen-IgG immune complexes have been reported in adult sera, mostly after food challenges [(356)(357)(358)]. The possibility of transplacental transport of antigen in IgG immune complexes was raised by Malek *et al* who reported a close correlation between fetal:maternal ratios of tetanus antigen and anti-tetanus IgG in placental perfusion studies [(359)]. The passage of the dietary allergen cow's milk β-lactoglobulin across the placenta was enhanced by the addition of human immunoglobulin, and in the case of the inhalant birch pollen allergen, Bet v1, was essential for transplacental transport [(360)]. Furthermore, the inhalant cat allergen, Fel d1, has been detected in complex with IgG in up to 40% of infant cord sera [(361)]. These observations therefore suggest that maternal ovalbumin specific IgG may also have a particular role in regulating transport of this allergen to the fetus.

The antigen detection data *(chapter 4)* suggested that *in utero* exposure to ovalbumin occurred more frequently, and in higher doses, through transplacental transfer than via a transamniotic route. For this reason maternal and cord serum samples were chosen

for the investigation of allergen form in the fetal environment. Gel filtration, a technique by which samples may be separated according to the molecular weights (MWs) of their components, was employed for this work. This permitted the MWs of the fractions containing OVA to be calculated, and from this, to deduce a profile of its form in selected serum samples of wide ranging specific IgG concentration.

6.2 Samples and methods

6.2.1 Samples

Maternal pregnant sera (n=3) and cord sera (n=9) with known ovalbumin specific IgG (OVA G) concentrations and ovalbumin quantity were selected *(table 6.1)*. OVA G concentrations ranged from values in the lowest maternal or cord IgG quartiles (M49, C49) to values in the highest quartiles (M44, C44, C105) *(chapter 5)*. Ovalbumin was either present (OVA +) (M44, C44, M49, C49, C14, C105, C122, C176, C195) or undetectable by *ELISA* (OVA -) (M2, C147, C167).

Sample M2 was characterised by an inhibition of antigen recovery when spiked with ovalbumin ('masking' serum) *(chapter 3)*.

Negative control serum (NS) was derived from a non-pregnant adult who had vigilantly excluded eggs from her diet for more than a decade *(2.2.7)*.

Table 6.1 Sample characteristics for gel filtration chromatography.

Sample Type	ID	OVA G (AU)	OVA G quartile	OVA (ng/ml)
Maternal serum	M49	0.1	1	1.013
Cord serum	C49	0.1	1	0.165
Maternal serum	M44	9.10	4	0.119
Cord serum	C44	11.77	4	0.307
Maternal serum	M2	0.97	2	0*
Cord serum	C14	5.61	4	0.236
Cord serum	C105	3.88	4	10.2
Cord serum	C122	1.39	3	0.81
Cord serum	C147	1.10	3	0*
Cord serum	C167	1.27	3	0*
Cord serum	C176	1.57	3	0.33
Cord serum	C195	0.52	2	0.31
Adult serum	NS	0.08	Control	0*

* below limit of *ELISA* detection

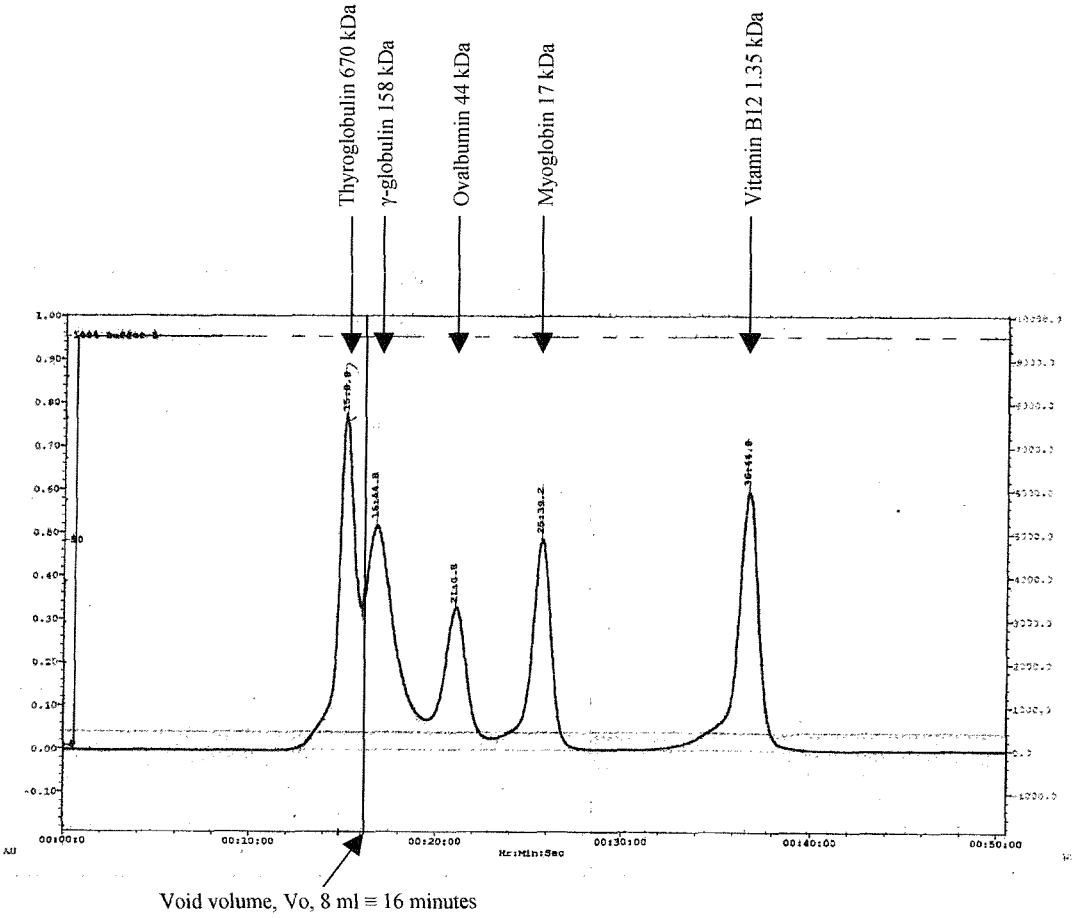
6.2.2 Laboratory methods

All general materials, including reagents, buffers and apparatus, used were as those listed previously *(2.1)*.

6.2.2.1 Gel filtration chromatography

The samples were fractionated by gel filtration chromatography using a Superdex 75 10/30 column (MW separation range 3-70 kDa) (Pharmacia) as described previously *(2.5.10)*. The column (volume 24 ml; void volume 8 ml) was eluted at a rate of 0.5 ml/minute. Fraction collection (each of volume 0.25 ml) started at 12 minutes run time (volume 6 ml) in order to facilitate the collection of protein of MW consistent with ovalbumin in IgG complex (≥ 190 kDa).

The molecular weight of individual fractions was calculated by reference to the selectivity curve of the calibrator proteins (typical elution profile, including standard OVA, shown below). Serum sample free OVA separated in the MW range of the column, whereas complexes of IgG-OVA (≥ 190 kDa) were detected in the void volume.



Typical calibration curve

The calibrator protein mixture comprised thyroglobulin (MW 670 kDa), γ-globulin (MW 158 kDa), ovalbumin (MW 44 kDa), myoglobulin (MW 17 kDa) and vitamin B12 (MW 1.35 kDa). The diagram shows the time ranges over which they were eluted. The incomplete separation of thyroglobulin and γ-globulin reflects the separation range of the column (3-70 kDa) and indicates that calculated MWs of proteins above 70 kDa can only be estimated. This calibration curve specifically applies to M44 *(figure 6.1)*, C44 *(figure 6.9)*, C14 *(figure 6.10)* and C49 *(figure 6.11)*. Calibration curves for the other samples differed only in minimal changes of the time of peak elution of the individual standard proteins, and any differences will be described in the text.

6.2.2.2 Ovalbumin detection

Ovalbumin concentration in sample fractions was measured by capture *ELISA (3.2).* The fractions were initially added neat to the microtitre wells. For those fractions with absorbance above the standard curve the assay was repeated following further fraction dilution in antibody buffer (range 1:10 - 1:4000). OVA concentration was expressed in ng/ml, after taking into account the dilution of the sample, by comparison of the sample absorbance to the ovalbumin standard curve.

6.2.2.3 Measurement of ovalbumin IgG concentration

The distribution and concentration of OVA G in the gel chromatography fractions was determined by indirect *ELISA (2.5.1).* The fractions were added neat to the microtitre wells. For a sample known to have high specific IgG concentration, the fractions were further diluted 1:10 in antibody buffer. OVA G concentration was expressed in arbitrary units (AU), after taking into account the dilution of the fraction, by comparison of the fraction absorbance to the standard curve of the serum pool.

6.2.2.4 Serum spiking

In order to validate the detection of OVA after fractionation, 400 µl of the negative serum was spiked with 200 ng/ml of ovalbumin and the sample incubated at room temperature for at least 30 minutes before fractionation.

In order to evaluate recovery of OVA following fractionation, 1 ml of a serum sample noted previously to mask recovery (M2) *(chapter 3)* was spiked with 1 ng/ml ovalbumin and incubated for 2-3 hours at room temperature prior to fractionation. In addition, a 600 µl aliquot of the spiked serum was depleted of IgG by protein A affinity chromatography before fractionation.

6.2.2.5 Protein A affinity chromatography

To establish whether circulating ovalbumin might be complexed with IgG, selected serum samples with high OVA G concentration (M44 and spiked M2) were depleted

of IgG by protein A affinity chromatography *(2.5.9)* prior to fractionation and the concentration of OVA in fractions before and after treatment compared. For each sample, the unretarded, IgG deplete fractions were pooled and concentrated toward the initial volume. Any residual dilutional factors were accounted for in subsequent *ELISAs*. IgG depletion was visualised by serum protein electrophoresis (Beckman, USA) (courtesy of M.Power, Tenovus) and confirmed on measurement of OVA G by indirect *ELISA (2.5.1)*.

6.2.2.6 Western blotting

The low molecular weight fractions with detectable OVA from maternal sample M44 were further evaluated by Western blotting. Aliquots of 10 µl volume from each of the positive fractions were pooled and the pool further diluted 10-fold and 100-fold. These dilutions, a MW standard and positive OVA controls (1 µg/ml) were run on SDS-PAGE, under non-reducing conditions and Western blotted as described previously *(2.5.6 & 2.5.7)*.

The form of OVA present in the low MW fractions of cord sample C49 was also further examined by Western blotting. Aliquots (9 µl volume) from each of the fractions comprising the OVA peak were pooled. The pooled OVA peak fractions, a 1 in 10 dilution of the pool and individual fractions were run on SDS-PAGE under reducing conditions, along with positive OVA controls (1 µg/ml) and a MW standard *(2.5.6)*. The proteins were Western blotted using the sensitive chemiluminescent technique *(2.5.8)*.

6.3 Results

6.3.1 Pregnant maternal sera and negative control serum

Ovalbumin *ELISA* of fractionated maternal serum M44 (high OVA G, OVA +) produced a biphasic pattern for the detection of ovalbumin. OVA was equally distributed between two peaks. The early peak constituted high MW ovalbumin. This peak was distributed between fractions 8, approximate MW 165 kDa, (total run

time 16 minutes, elution volume, Ve=8 ml) and 13, approximate MW 92 kDa, (total run time 18.5 minutes, Ve=9.25 ml). This was comparable to the elution time of calibrator γ-globulin, total run time 16-18 minutes, peak 16.7 minutes, and suggested that the ovalbumin in this peak might be in complexed form, or exist as multimers. The low MW ovalbumin peak was eluted between fractions 19 and 23 (total run time 21.5-23.5 minutes), which was comparable to the elution profile of calibrator ovalbumin. This later peak represented proteins in the MW range 29-45 kDa and therefore would be consistent with free ovalbumin, or fragments of ovalbumin *(figure 6.1)*. (For corresponding calibrator protein standard curve, see *6.2.2.1*).

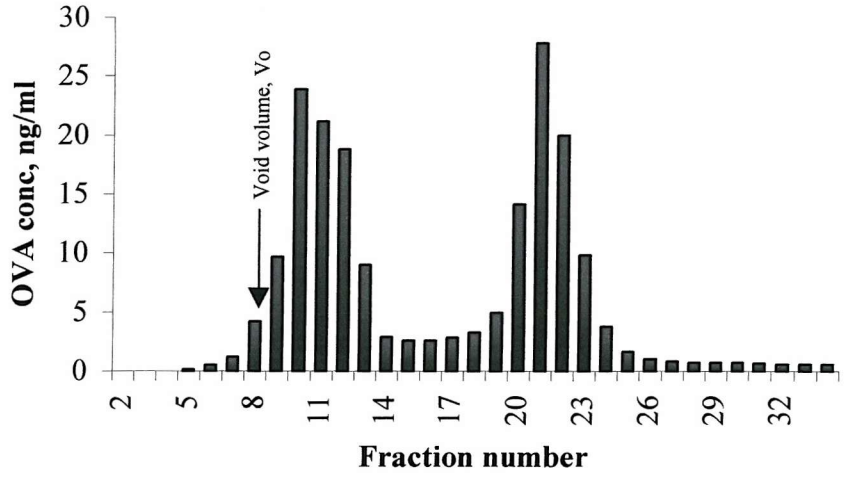


Figure 6.1 Ovalbumin detection – maternal serum, M44.

Maternal serum M44 had high specific IgG concentration and detectable ovalbumin. The sample was separated by gel filtration chromatography using a Superdex 75 10/30 column (Pharmacia). The column was eluted at 0.5 ml/minute and fraction collection started at 12 minutes (6 ml volume). A total of 35 fractions (each 0.25 ml volume) were collected (for method detail, see 2.5.10). Ovalbumin was detected in the fractions by ELISA (3.2). In this sample ovalbumin was found in both high MW and low MW peaks. The high MW ovalbumin was distributed through fractions 8–13 (total run time 16-18.5 minutes). This compared to an elution time of approximately 16-18 minutes (peak 16.7 minutes) for calibrator γ-globulin. Low MW ovalbumin was eluted between fractions 19 (run time 21.5 minutes) (MW 45 kDa, approximately) and 23 (23.5 minutes) (MW 29 kDa, approximately), which was comparable to the elution profile of calibrator ovalbumin (20-22 minutes, peak 21.7 minutes). (For the corresponding calibrator protein standard curve, see 6.2.2.1).

ELISA of fractionated maternal serum M49 (low OVA G, OVA +) also produced a biphasic pattern. However, in this sample there was a reduction in the proportion of ovalbumin found in the high MW peak compared with the low MW peak. This supported the suggestion that the OVA in these fractions might be in complex with IgG and that the reduction in the proportion of total OVA in high MW form was a consequence of the lower concentration of OVA G in this sample *(figure 6.2)*. Indeed, analysis of the negative control serum, NS, which had virtually undetectable OVA G, showed no ovalbumin peak in the high molecular weight fractions. However, in this sample OVA was detectable in fractions of MW compatible with free ovalbumin, even though the individual had no recorded overt egg ingestion (fraction 19: MW 41 kDa, approximately; fraction 23: MW 25 kDa, approximately) *(figure 6.3)*.

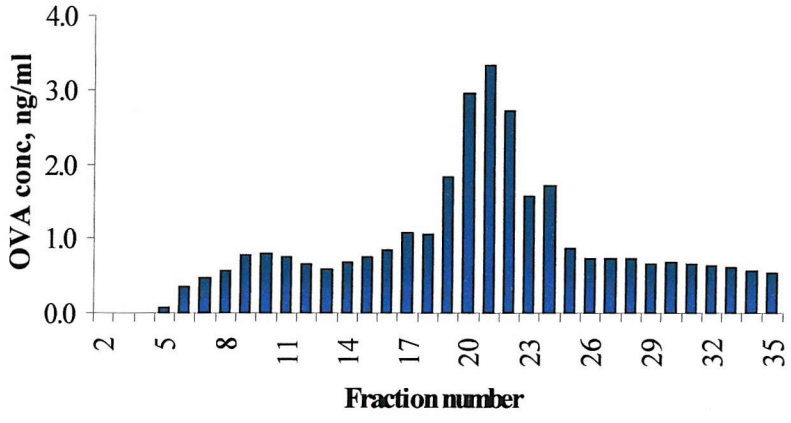


Figure 6.2 Ovalbumin detection – maternal serum, M49.

Maternal serum M49 had extremely low specific IgG, but detectable ovalbumin. The sample was separated by gel filtration chromatography and ovalbumin detected in the fractions by ELISA, as described in figure 6.1, and text. In contrast to sera with high specific IgG (for example, figure 6.1), in this sample ovalbumin was found predominantly as a low MW peak. This was distributed between fractions 19 (MW 41 kDa, approximately) and 24 (MW 22 kDa, approximately), total run time 21.5-24 minutes, which compared to the elution profile of the corresponding calibrator standard ovalbumin of 19–23 minutes (peak 20.9 minutes).

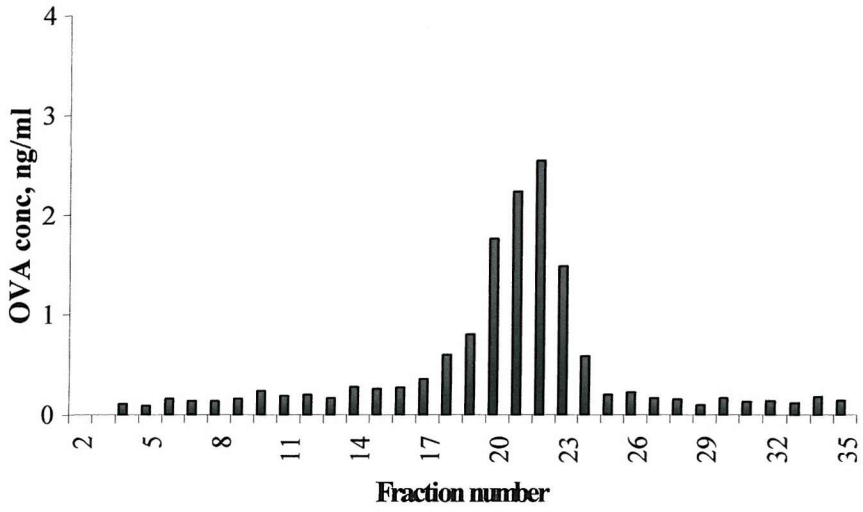


Figure 6.3 Ovalbumin detection – non pregnant adult negative serum, NS

Serum NS was derived from a healthy, non-pregnant woman who had avoided eggs for many years. Nevertheless, after fractionation (for method see figure 6.1, and text), a limited quantity of ovalbumin could be detected in low MW fractions between fraction 19 (MW 41 kDa, approximately) and 23 (MW 25 kDa, approximately), total run time 21.5-23.5 minutes, which compared to an elution profile of the calibrator standard ovalbumin of 19–23 minutes (peak 20.9 minutes).

6.3.2 Detection validation

When NS was spiked with OVA prior to fractionation, the ensuing pattern of OVA detection in the spiked fractions mirrored that of the native serum *(figure 6.4)*. This supported the conclusion that it was ovalbumin, rather than another cross-reacting protein, that was being measured. The presence of OVA in fractions was visually confirmed by Western blotting the low MW, OVA + fractions of M44. This revealed a band, the intensity of which diminished with sample dilution, at the molecular weight of the ovalbumin control *(figure 6.5)*.

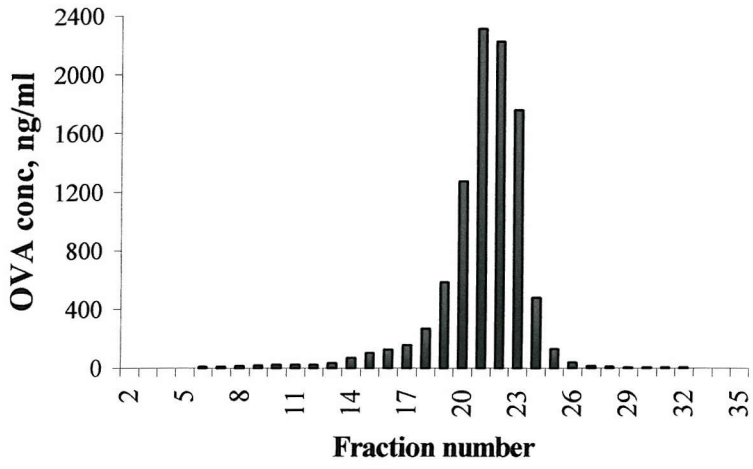


Figure 6.4 Fractionation of ovalbumin spiked negative control serum, NS.

The negative control serum, NS, was spiked with 200 ng/ml ovalbumin, separated by gel filtration chromatography and the ovalbumin detected by ELISA (for method see figure 6.1, and text). The corresponding profile of protein detection was superimposable on that obtained from the untreated serum, figure 6.3.

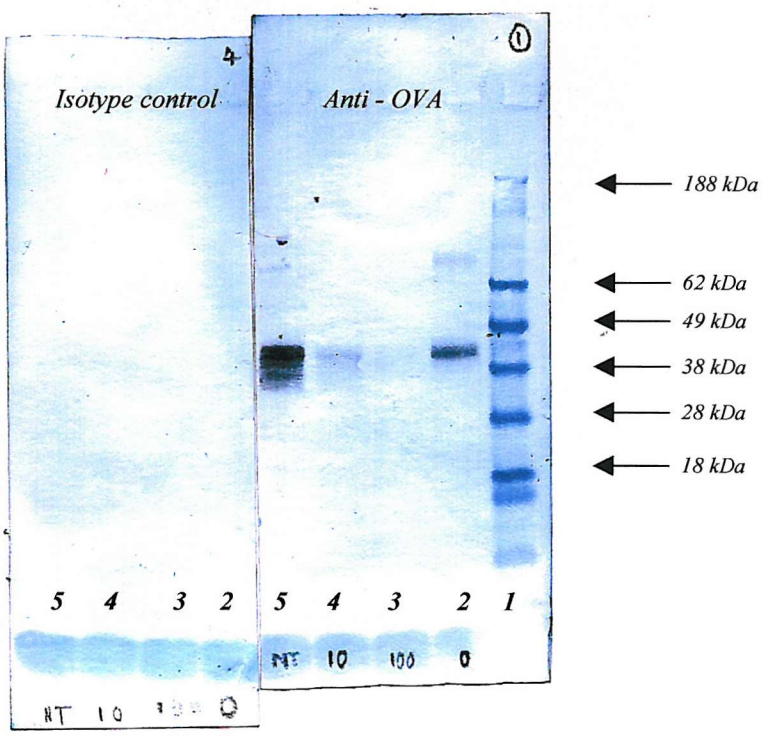


Figure 6.5 Western blot of fractionated maternal serum sample, M44.

Fractions of the low MW ovalbumin peak of maternal serum sample M44 were pooled and Western blotted. One membrane was probed with rabbit anti-ovalbumin and the other with a rabbit IgG isotype control. Lane 1 contained the MW standards and lanes 2 the positive ovalbumin control (1µg/ml). Lanes 3, 4 and 5 contained the pooled fractions diluted 1 in 100, 1 in 10 or neat, respectively. A distinct signal at the MW of the ovalbumin control was obtained from the samples probed with anti-ovalbumin.

6.3.3 Ovalbumin specific IgG measurement

In order to establish if the high MW ovalbumin peak could be attributed to OVA-IgG complexes, ovalbumin specific IgG concentration in each of the fractions was measured. Analysis of maternal serum samples M44 and M49 confirmed the presence of ovalbumin IgG in the OVA-containing high MW fractions *(figures 6.6 & 6.7)*. The molecular weight of proteins in the high MW OVA peak was greater than those in the IgG peak (by values of approximately 39 kDa & 51 kDa, respectively). This could suggest binding of ovalbumin to IgG. Moreover, following protein A serum IgG depletion of M44, the high MW ovalbumin peak was obliterated *(figure 6.8)*.

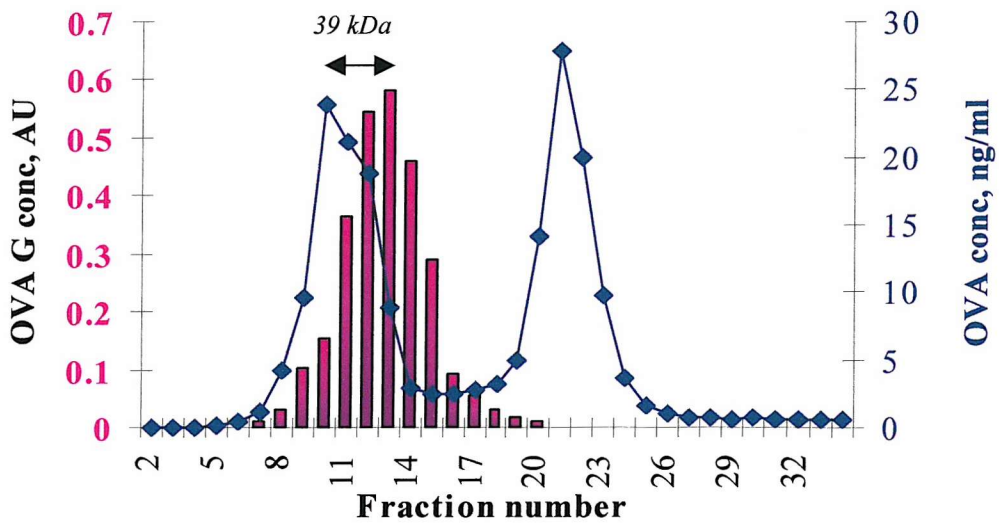


Figure 6.6 Distribution of ovalbumin IgG in fractionated maternal serum, M44.

Ovalbumin specific IgG concentration (shown in pink bars) was measured in fractions of maternal serum M44 (separated as described in figure 6.1, and text) by indirect ELISA (2.5.1). Ovalbumin IgG was found in the high MW fractions containing ovalbumin. The molecular weight of proteins in the heavy fractions could only be estimated since they fell outside the separation range of the column. However, the high MW OVA peak (blue line) was greater than those in the ovalbumin IgG peak by approximately 39 kDa.

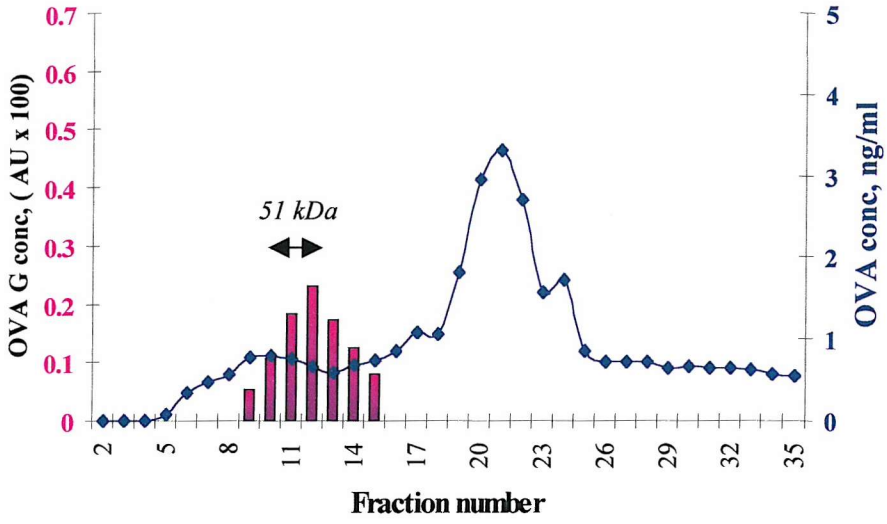


Figure 6.7 Distribution of ovalbumin IgG in fractionated maternal serum, M49.

Maternal serum M49 had been shown previously to have low ovalbumin specific IgG concentration (chapter 5). This was confirmed by the low concentrations found in high MW fractions of the sample (shown in pink bars). Ovalbumin specific IgG was found in the high MW ovalbumin fractions (blue line). As described in figure 6.6, MWs > 70 kDa could only be estimated, but the MW difference between the high MW ovalbumin peak and the ovalbumin specific IgG peak was approximately 51 kDa in this sample.

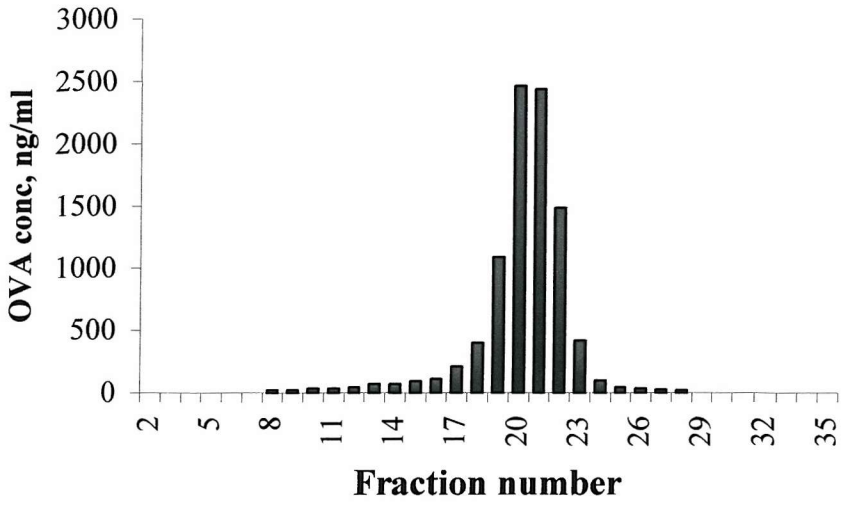


Figure 6.8 Ovalbumin detection in IgG deplete maternal serum, M44.

Maternal serum M44 was depleted of IgG by protein A affinity chromatography (method, 2.5.9). The IgG deplete sample was separated by gel filtration chromatography and ovalbumin detected in the fractions by ELISA, as described previously (figure 6.1, and text). Ovalbumin could only be found in low MW fractions. This profile should be compared to that of figure 6.1, where in untreated, IgG containing M44, ovalbumin was present in a biphasic, high and low MW, pattern.

6.3.4 Fractionation of ovalbumin positive cord sera

Cord samples with detectable ovalbumin had specific IgG concentrations that varied over a wide range. For the two samples with the highest OVA G concentrations (C14 – 5.61 AU; C44 – 11.77), ovalbumin was found predominantly *(C44, figure 6.9)* or entirely *(C14, figure 6.10)* in high MW fractions. By contrast, in samples C49 and C195, which had the lowest ovalbumin IgG concentrations, OVA was found almost entirely as a low MW peak *(figures 6.11 & 6.12)*. OVA + samples with specific IgG concentrations between these extremes (C122, C176, C105) showed the biphasic pattern of OVA seen previously *(figures 6.13, 6.14, & 6.15)*.

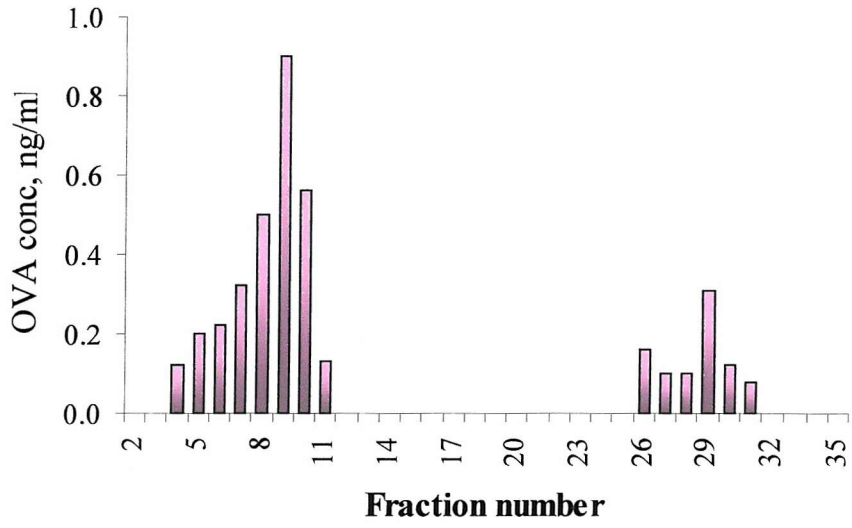


Figure 6.9 Ovalbumin detection - cord serum, C44.

Cord serum C44 had high ovalbumin IgG concentration (11.77 AU) & detectable ovalbumin. The sample was separated by gel filtration chromatography and fraction ovalbumin detected by ELISA, as described previously (figure 6.1, and text). Ovalbumin was found predominantly in high MW fractions: fractions 7-10 represent approximate MW range = 131-185 kDa, total run time 15.5-17 minutes. Very small quantities were present in fractions of low MW (fraction 29, 14 kDa). This might represent ovalbumin fragments – but that remains to be evaluated. (For corresponding calibrator protein standard curve, see 6.2.2.1)

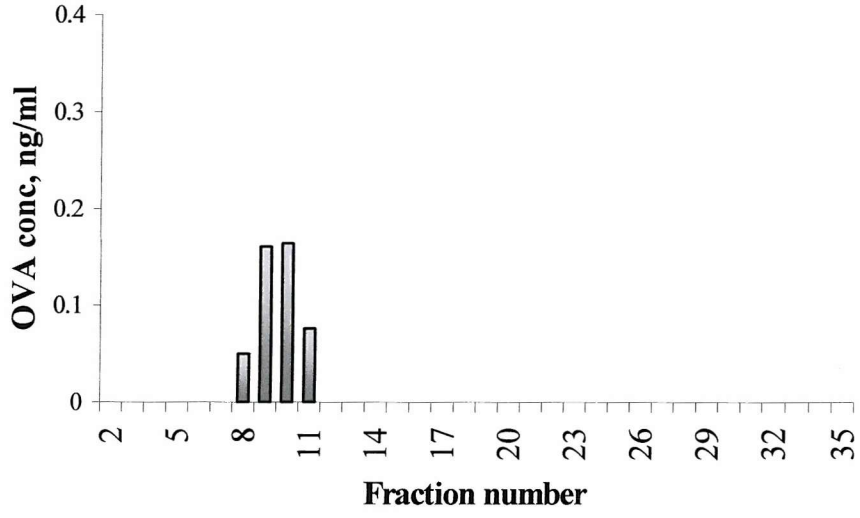


Figure 6.10 Ovalbumin detection – cord serum, C14.

Cord serum C14 had high ovalbumin IgG concentration (5.61 AU) & detectable ovalbumin. The sample was fractionated, as described previously (figure 6.1, and text). Ovalbumin was only detectable in high MW fractions 8-11, representing approximate MW range 116-165 kDa, total run time 16–17.5 minutes. (For corresponding calibrator protein standard curve, see 6.2.2.1)

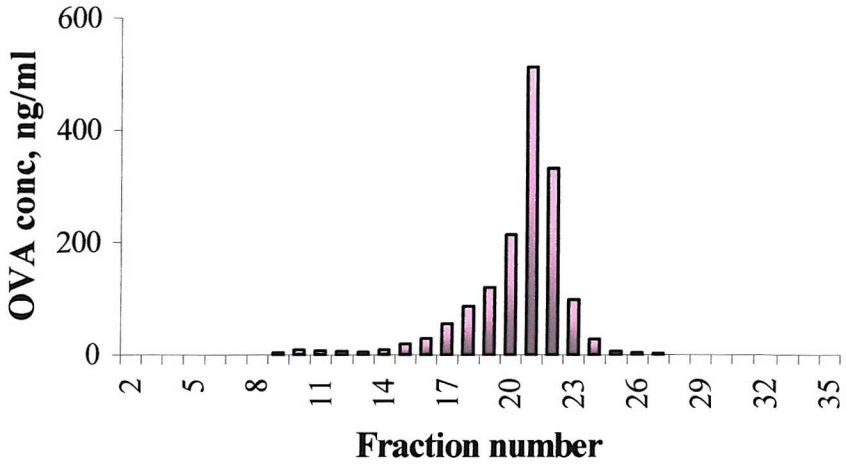


Figure 6.11 Ovalbumin detection - cord serum, C49.

Cord serum C49 had low ovalbumin specific IgG & detectable ovalbumin. The sample was processed as described previously (figure 6.1, and text). Ovalbumin was found only as a low MW peak, particularly fractions 19 (MW 45 kDa, approximately) through 23 (MW 29 kDa, approximately), total run time 21–23.5 minutes. (Corresponding calibrator protein standards shown in 6.2.2.1)

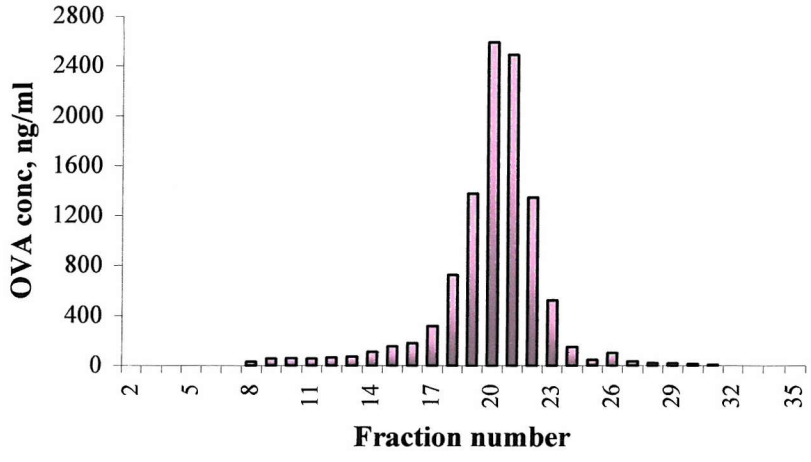


Figure 6.12 Ovalbumin detection – cord serum, C195.

Cord serum C195 had low ovalbumin specific IgG & detectable ovalbumin. The sample was processed in the same manner as described in figure 6.1, and text. Ovalbumin was found only as a low MW peak, particularly fractions 18 (MW 45 kDa, approximately) through 22 (MW 28 kDa approximately), run time 21–23minutes. (Corresponding ovalbumin standard – total run time 19–22 minutes, peak 20.4 minutes).

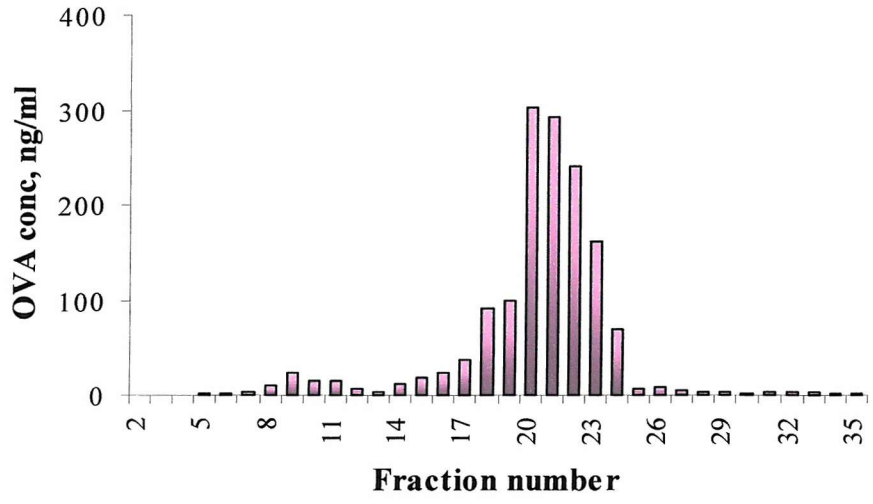


Figure 6.13 Ovalbumin detection – cord serum, C122.

Cord serum C122 had higher ovalbumin IgG concentration (1.39 AU) & detectable ovalbumin. The sample was processed in the same manner as described in figure 6.1, and text. The ovalbumin fraction profile was biphasic, though a low MW ovalbumin peak predominated. The high MW ovalbumin peak extended from fraction 8 through 11, total run time 16–17.5 minutes. (Corresponding γ-globulin standard, run time approximately 15–19 minutes, peak 16.4 minutes). The low MW ovalbumin peak extended from fraction 19 (MW 42 kDa, approximately) through 23 (MW 26 kDa, approximately), total run time 21–23.5 minutes. (Corresponding ovalbumin standard, run time approximately 19–22 minutes, peak 20.6 minutes)

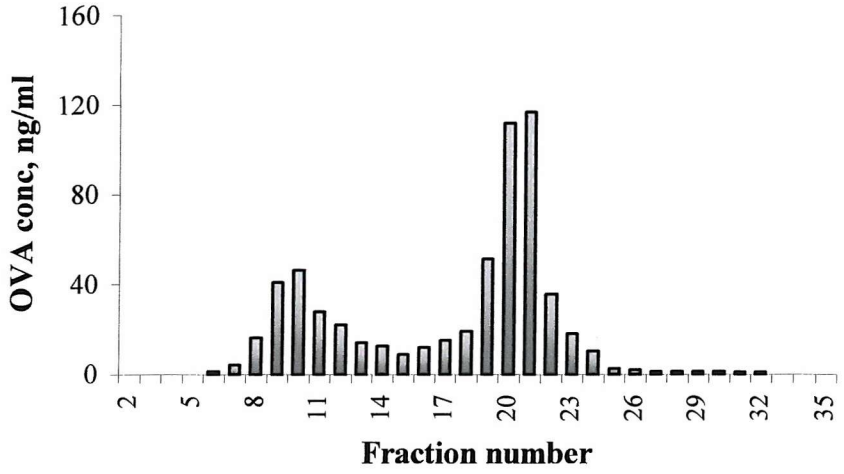


Figure 6.14 Ovalbumin detection – cord serum, C176.

Cord serum C176 had higher ovalbumin IgG concentration (1.57 AU) & detectable ovalbumin. The sample was processed in the same manner as described in figure 6.1, and text. The fraction ovalbumin profile was biphasic, though a low MW ovalbumin peak predominated. The high MW ovalbumin peak extended from fraction 8 through 12, total run time 16–18 minutes. (Corresponding γ-globulin standard, run time approximately 15.5–18 minutes, peak 16.4 minutes). The low MW ovalbumin peak extended from fraction 19 (MW 40 kDa, approximately) through 22 (MW 28 kDa, approximately), elution time 21–23 minutes. (Corresponding ovalbumin standard, elution time approximately 19–22 minutes, peak 20.4 minutes)

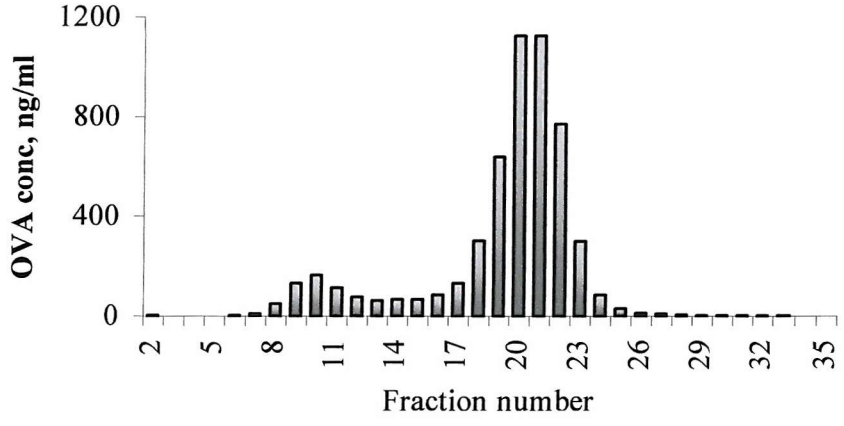


Figure 6.15 OVA detection - cord serum, C105.

Cord serum C105 had higher ovalbumin IgG concentration (3.88 AU) & detectable ovalbumin. The sample was processed in the same manner as described in figure 6.1, and text. The fraction ovalbumin profile was biphasic, though a low MW ovalbumin peak predominated. The high MW ovalbumin peak extended from fraction 8 through 12, total run time 16–18 minutes. (Corresponding γ-globulin standard as for figure 6.13). The low MW ovalbumin peak extended from fraction 18 (MW 47 kDa, approximately) through 23 (MW 26 kDa, approximately), elution time 21 – 23.5 minutes. (Corresponding ovalbumin standard as for figure 6.13).

6.3.5 Fractionation of ovalbumin negative cord sera

Unfractionated cord sera C147 and C167 did not have detectable ovalbumin. However, when processed by gel filtration chromatography low concentrations of OVA could be detected. In cord serum C147, ovalbumin was found equally in high and low MW fractions *(figure 6.16)*. However, in cord C167, ovalbumin existed almost entirely as a complex, even though this sample had an OVA G concentration comparable to that of cord C147 (C167 1.27 AU; C147 1.1 AU) *(figure 6.17)*.

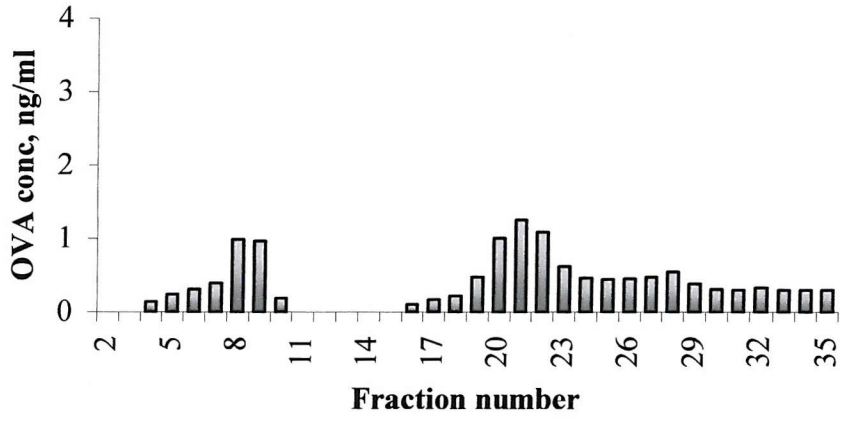


Figure 6.16 Ovalbumin detection – cord serum, C147.

Cord serum C147 did not have detectable ovalbumin. However, following fractionation in the manner described in figure 6.1 and text, ovalbumin could be detected in low quantities in both high MW (especially fractions 8, MW 150 kDa & 9, MW 131 kDa, approximately) and low MW (especially fractions 20-22, MW range 28-36 kDa, approximately) peaks. (Corresponding calibrator standards as described in figure 6.14)

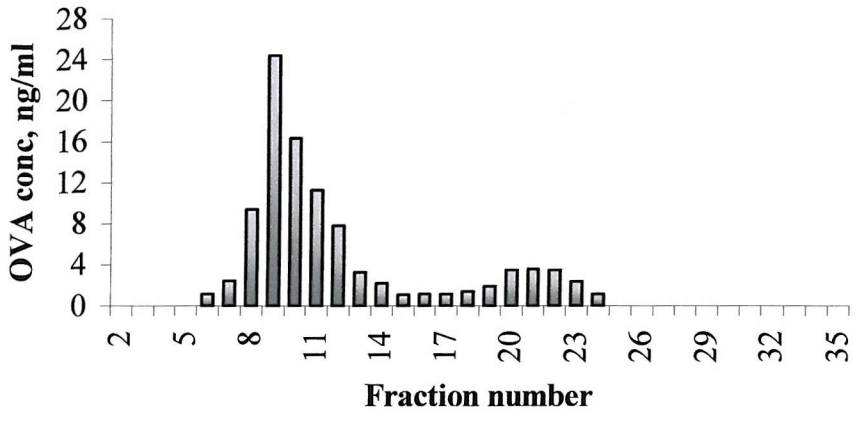


Figure 6.17 Ovalbumin detection – cord serum, C167.

Cord C167 did not have detectable ovalbumin, though the matched maternal sample at delivery was ovalbumin positive. Following fractionation in the manner described in figure 6.1, and text, ovalbumin could be detected predominantly in a high MW peak from fraction 8 (MW approximately 150 kDa) through 12 (MW approximately 92 kDa) (total run time 16–18 minutes). (Corresponding standards as described in figure 6.14)

6.3.6 Western blotting

Western blotting of cord serum C49 (low OVA G, OVA +) under reducing conditions showed the presence of at least two bands in the ovalbumin region *(figure 6.18, lane 2)*. Such a pattern suggested that 'clipped' OVA fragments were present in these fractions. Whilst the Western blot was not quantitative, the strongest signal was obtained from fraction 21, in keeping with this fraction having the highest quantity of ovalbumin *(figure 6.11)*.

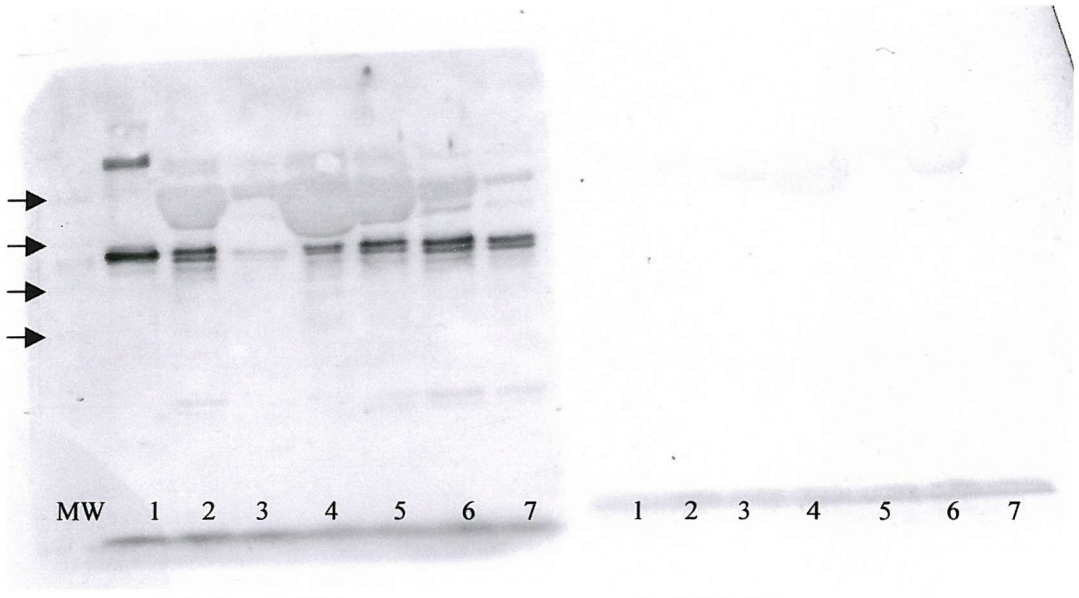


Figure 6.18 Western blot of cord C49 run under reducing conditions.

Fractionated cord serum C49 was run on SDS-PAGE under reducing conditions (for method see 2.5.6). The membranes were probed with rabbit anti-OVA or rabbit IgG isotype control (for method see 2.5.8). In lanes 1 was a positive OVA control (1μg/ml). In lanes 2 and 3 were pooled fractions 19-22 of the low MW peak, neat and 1 in 10 dilution, respectively. In lanes 4 – 7 the individual fractions were run (neat). The blot shows at least 2 bands in the OVA region indicating that the OVA existed as clipped fragments and suggesting that the protein may have been processed. The picture also shows arrows indicating MW bands 62 kDa (top), 49 kDa, 38 kDa & 28 kDa (bottom). Some non-specific binding to protein of heavier MW than OVA is apparent. This probably relates to protein overload in the lanes.

6.3.7 Fractionation of 'masking' serum

A paradox noted in developing the ovalbumin *ELISA* was that there was no measurable recovery of ovalbumin on spiking certain sera *(chapter 3)*. One of these samples, M2, was fractionated pre- and post- spiking with OVA (1 ng/ml) and after the spiked sample was IgG depleted by protein A affinity chromatography. After fractionation, OVA could be detected in the untreated serum, predominantly in low MW fractions. After spiking, the sample profile was unchanged. In the spiked sample there was no increase in protein detection, in fact the concentration of OVA detected in these fractions was less than in the untreated sample *(figure 6.19)*. After IgG depletion of the spiked sample there was a huge increase in the quantity of OVA detectable in the low MW fractions *(figure 6.20)*.

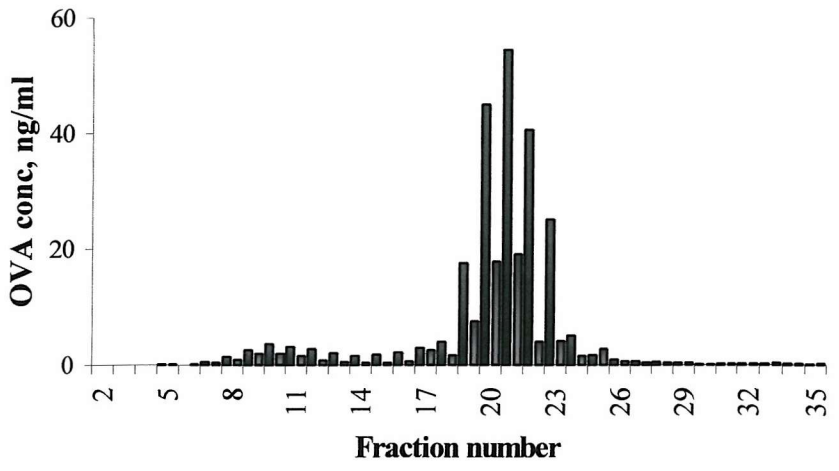


Figure 6.19 Ovalbumin detection in maternal serum M2 after fractionation, before and after spiking with ovalbumin.

Maternal serum M2 was known to 'mask' detection of a spiking dose of ovalbumin. After fractionation of the untreated serum (for method see figure 6.1, and text), ovalbumin could be detected in a low MW peak extending from fraction 19 through 23, MW range approximately 25-40 kDa, (run time 21.5–23.5 minutes) (shown in blue bars). The addition of a spiking dose of ovalbumin (1 ng/ml) did not further augment detection (shown in brown bars). (Corresponding ovalbumin standard eluted between 19.5 & 22 minutes, approximately, peak 20.75 minutes)

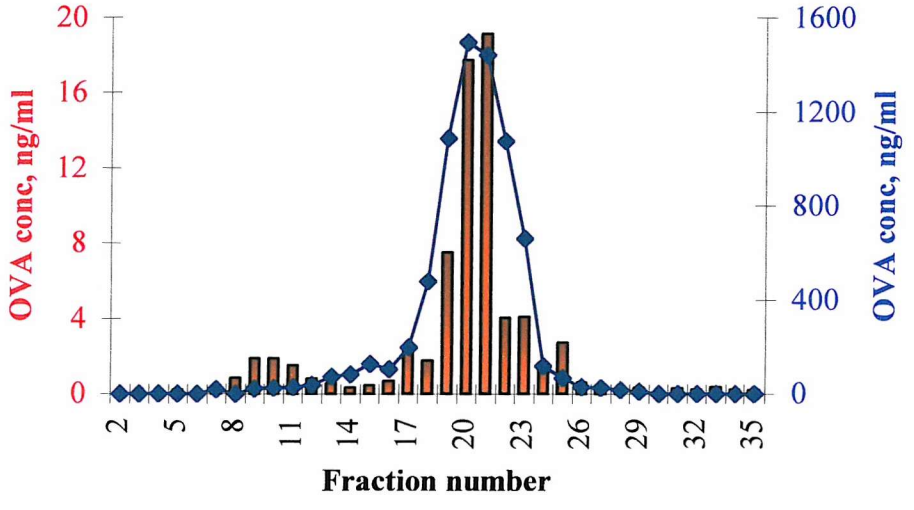


Figure 6.20 Ovalbumin detection in spiked maternal serum M2 after IgG depletion.

Spiked maternal serum M2 was IgG depleted by protein A chromatography (method detail in 2.5.9) and fractionated, as described previously (figure 6.1 and text). This resulted in a large increase in the quantity of ovalbumin detectable in the low MW peak (shown as a blue line) compared to the spiked, IgG replete sample (shown as brown bars).

6.4 Discussion

To date, little is known about the form - whether free or complexed - of allergens to which the developing immune system is exposed. Therefore, the principle aims of this work were to establish the mode of passage of dietary ovalbumin in the materno-fetal unit and the particular role of serum IgG in this process. The results suggest that the allergen circulates in *free* form and in a *complex* with IgG and that both these forms are passed via the placenta to the fetus.

The method employed gel filtration chromatography, which permits biological fluids to be separated according to the molecular weights of their components, in conjunction with a specific ovalbumin *ELISA (chapter 3)*. In several samples low concentrations of ovalbumin, above the individual assay level of sensitivity, were found in many low MW fractions (for example, *figure 6.16*). This may constitute degraded ovalbumin - perhaps a result of processing the serum samples by gel filtration at room temperature. Alternatively, it may reflect inefficient sample separation, which could have been improved by a slower elution speed (set at 0.5 ml/min). Nevertheless, these low 'background' levels do not detract from the distinctive ovalbumin profiles comprising high and low molecular weight peaks.

In vitro placental perfusion techniques have been used to directly demonstrate passage of free antigen into the fetal circulation. These include infectious agents, such as HIV p24 antigen [362], immunogens, such as tetanus toxoid [359], hormones, such as thyroid stimulating hormone, TSH [363] and allergens, such as cow's milk β-lactoglobulin and birch pollen [360]. Cotinine, the main metabolite of nicotine, has been found in fetal serum when women have been exposed to cigarette smoke through pregnancy [364] and inulin, an inert, non-metabolised substance, was detectable in first trimester fetal fluids after intravenous administration to the mother [365]. Furthermore, in animal studies, ovalbumin could be measured in the fetal environment after forced maternal ingestion [366]. The current detection of free ovalbumin in human cord serum, under normal physiological conditions, is therefore not an unexpected, but nevertheless, unique finding.

The low molecular weight ovalbumin profiles of the test sera overlapped that of the calibrator standard ovalbumin. The discrepancy between test and standard OVA profiles, whereby sample OVA was eluted slightly later (at lower MW) than standard OVA, might relate to a matrix effect whereby serum retarded the elution of endogenous OVA. Such an explanation is supported by the observation that the OVA profile of fractionated spiked negative serum *(figure 6.4)* could be superimposed on the detection profile of the untreated sample *(figure 6.3)*, suggesting that the free circulating OVA was intact. However, it should be noted that in Western blotting serum under reducing gel electrophoresis conditions (which breaks cross-linking interchain disulphide bonds and improves the clarity of the protein distribution) ovalbumin was found to exist as shorter peptides as well as the intact protein. Indeed, it might have been anticipated that a dietary allergen, taking the long entero-placental route, would have been degraded by the mother before reaching the fetus. Such alterations might have implications for fetal priming by, for example, revealing immunogenic epitopes or facilitating antigen uptake and presentation.

The mechanism by which free ovalbumin crosses the placental remains to be established. Inulin molecules are thought to cross the placental barrier by an extracellular porous route [367]. It is conceivable that the OVA peptides could take a similar course.

The negative control serum, from an individual who has excluded dietary egg for many years, consistently gave no signal in the ovalbumin *ELISA* but when assayed after column fractionation had free ovalbumin detectable *(figure 6.3)*. It is possible that dietary avoidance by this subject may not have been absolute, or that dietary egg in 'hidden' forms was not excluded and column fractionation, which often increases the level of sensitivity of protein detection by dispersing a complex fluid, revealed small quantities. The increased sensitivity afforded by the technique could also explain the detection of ovalbumin in the two 'negative' cord sera - C147 and C167 - particularly as the mothers of these infants were not on the dietary intervention arm of the study *(figures 6.16 & 6.17)*. Such a phenomenon may also go some way to explain the antigen-specific T-cell reactivity that is commonly found at birth [197][200][198].

This work has also provided supportive evidence for the transplacental passage of complexed allergen. The presence of the inhalant cat allergen, Fel d1, in complex with IgG has been reported recently in cord blood [361], though, surprisingly, in that study, maternal IgG concentration was not found to be related to the presence and/or concentration of the immune complexes in the fetal circulation. By contrast these current data have indicated a key role for IgG in determining allergen form. In samples with low IgG (M49, NS, C49, C195) serum ovalbumin was found wholly, or predominantly in free form, while in samples with high IgG (C14, C44) allergen was primarily complexed. A complex of IgG with ovalbumin is expected to have a MW of around 190 kDa, but in these maternal and cord serum samples ovalbumin was detected in a MW range of, for example, 92-165 kDa *(figure 6.1)*. However, since the separation range of the available gel column (3-70 kDa) necessitated collection of a proportion of the column void volume in order to collect high MW ovalbumin, that means that the MWs of these OVA-containing fractions cannot be calculated accurately, and, at best, only represent an estimation. Nevertheless, recognising this limitation, the calculated MWs of the heavy ovalbumin peak would still support the notion that the ovalbumin was bound to IgG, for example, *figure 6.9*. However, more definitive evidence in support of serum OVA in IgG complex derives from the observation that depletion of IgG by protein A affinity chromatography obliterated the high MW ovalbumin peak. This also suggests that other circulating carrier proteins have little or no significant role in allergen transport *(figure 6.8)*. Protein A treatment notably resulted in large quantities of free ovalbumin *(especially figure 6.20)*, a finding that could be explained by the process of affinity chromatography having disrupted immunoglobulin-antigen binding and thereby facilitating release of free ovalbumin into the serum.

What repercussions might antigen form have for the early programming of atopy? A number of clinical studies have suggested an allergy protective effect of high maternal IgG. For example, children of mothers who had rye grass immunotherapy through pregnancy, with consequent development of high maternal specific IgG levels, had fewer positive skin prick tests to rye grass compared to children of untreated mothers [349]. Similarly, high concentrations of cat and birch pollen specific IgG in cord sera were associated with less atopic symptoms and lower IgE in children during the first 8 years of life [291].

The mechanism by which IgG may be protective is not known. It may act by binding antigen and eliminating it through phagocytic pathways; alternatively the processing and presentation of low doses of antigen complexed with IgG may induce tolerance through immune deviation. IgG may directly confer inhibitory signals via an immunoreceptor tyrosine based inhibitory motif (ITIM) on the low affinity IgG receptor ($F_c\gamma$RIIB), CD32b. CD32b inhibits BCR-mediated B-cell activation [368] and TCR-mediated T-cell activation [369]. It may thus negatively regulate immune reactivity towards the initial encounters with allergen. If this is the case, then the reduction in maternal specific IgG through pregnancy induced by an exclusion diet *(chapter 5)* may have a significant impact on infant allergic outcome.

However, by direct contrast, high cord egg specific IgG has also been associated with an increased risk of developing allergic disease [292]. Fcγ receptors on the surface of dendritic cells have the potential to capture IgG-antigen complexes at low concentrations, a function that may facilitate antigen focusing and T-cell priming in the antenatal environment containing minute quantities of allergen [370].

Consideration of the subsequent atopic phenotype of a larger cohort of infants whose cord bloods could be analysed for antigen form may help to solve this quandary. In this initial study, while subject numbers are too small to draw any firm conclusions, two of three infants with cord blood OVA primarily in free form subsequently developed eczema and raised total IgE concentration at 6 months of age. Neither baby with OVA predominantly in complexed form had any atopic symptoms at 6 months of age, though infant M44 had raised total IgE concentration.

Infant 167 presented a particular enigma. The maternal sample at delivery contained high levels of OVA (1.81 ng/ml), but the matched cord serum was consistently negative. The infant subsequently developed moderately severe eczema with food and inhalant skin sensitivity. After fractionation OVA could be detected in the cord sample primarily in complexed form, even though samples with a comparable OVA G concentration produced a biphasic profile of detection *(figure 6.17)*. It could be hypothesised that the ovalbumin IgG in this sample was of a higher affinity and hence required the more sensitive immunoassay for protein detection [371][372]. However,

such higher antibody affinity may also enhance antigen focusing and potentially promote T-cell priming [373].

Certain serum samples had been noted to be resistant to ovalbumin spiking, with little or no measurable protein recovery *(chapter 3)*. On fractionating such a spiked sample, (M2), recovery was still not apparent *(figure 6.19)*. However, the huge increase in measured ovalbumin concentration following IgG depletion of the sample suggested that the protein was bound to IgG and had been released by the process of protein A affinity chromatography *(figure 6.20)*. Immunoglobulin affinity is a heterogeneous property, varying from clone to clone within an individual and between individuals [374]. It may be that this subject had particularly high affinity IgG that prevented epitope recognition by the detector antibody. Such an extreme blocking capacity may have a functional effect as high affinity/avidity maternal anti-gp120 antibody has been associated with reduced materno-fetal transmission of HIV infection [375]. Furthermore, since high affinity/avidity IgG may cross the placenta more easily [376], so it might be envisaged that inhibition of epitope presentation combined with ready passage to the fetus might reduce the likelihood of fetal sensitisation and have benefit for allergic outcome.

In conclusion, this work has shown for the first time that a dietary allergen may pass from the mother to fetus in both free and complexed forms and that the presence of complexed antigen is dependent on circulating specific IgG. The characteristics of serum IgG that might determine allergen form were identified. These included serum concentration, since samples with low OVA G contained predominantly free OVA. Immunogloblin affinity was also implicated, since samples were identified in which OVA was only present in complexed form despite relatively low specific IgG concentration and where allergen could only be recovered on IgG depletion. Ultimately how infant atopic phenotype relates to the form of allergen encountered in the fetal environment is the key issue. Antigen detection in 'negative' sera is reminiscent of the earlier report of OVA in serum and breast milk of some women actively avoiding egg *(chapter 4)*. These findings suggest a need for similar processing of a greater number of infant samples in order to fully characterise allergen form and thus to correlate form with subsequent infant allergic outcome.

Chapter Seven

Investigation of ovalbumin form in the postnatal environment

Chapter 7

Investigation of ovalbumin form in the postnatal environment

7.1 Aims

Human milk is considered to be the ideal food for newborns during the first few months of life. Not only does it have an optimal nutritional composition [115], breast milk also contains an array of specific and non-specific bioactive factors which protect the infant from infection by various micro-organisms [116]. Whilst the value of breast-feeding for infant growth and survival is indisputable, the benefit for primary allergy prevention is more controversial. Breast-feeding may both provoke the expression of food-related hypersensitivity reactions [252] and perpetuate infant allergic disease [377]. The presence of food allergens in breast milk has been reported by several authors [251][300] and indeed in this cohort ovalbumin was found most frequently, and in the greatest quantities, in the mothers' milk *(chapter 4)*. Despite the recognition of the contribution of breast-feeding to infant allergic sensitisation and/or disease expression, nothing is known about the mode of allergen passage via this route and the form in which allergens are presented to the neonatal gut. The aim of this work was therefore to evaluate the antigenic form of ovalbumin in breast milk. The previously established technique of gel filtration chromatography was modified in order to separate breast milk into fractions of different molecular weights, so permitting a size profile of OVA to be established.

7.2 Samples and methods

7.2.1 Samples

Breast milk (BM) samples collected by expression at 3 months post partum (n=7) were selected *(table 7.1)*. Samples had known ovalbumin IgA (OVA A) concentration and ovalbumin quantity *(chapter 4)*. Ovalbumin was either present (OVA +) (BM 3, BM 15, BM 44, BM 60, BM 86) or absent (OVA -) (BM 51, BM 92).

Table 7.1 Breast milk sample characteristics for gel filtration chromatography.

Sample ID	OVA (ng/ml)	OVA A Index
BM 3	170.9	4.3
BM 15	1.64	1.2
BM 44	1258	4.7
BM 51	0*	<1
BM 60	2.7	3.7
BM 86	0.33	3.1
BM 92	0*	4

* below limit of *ELISA* detection

7.2.2 Laboratory methods

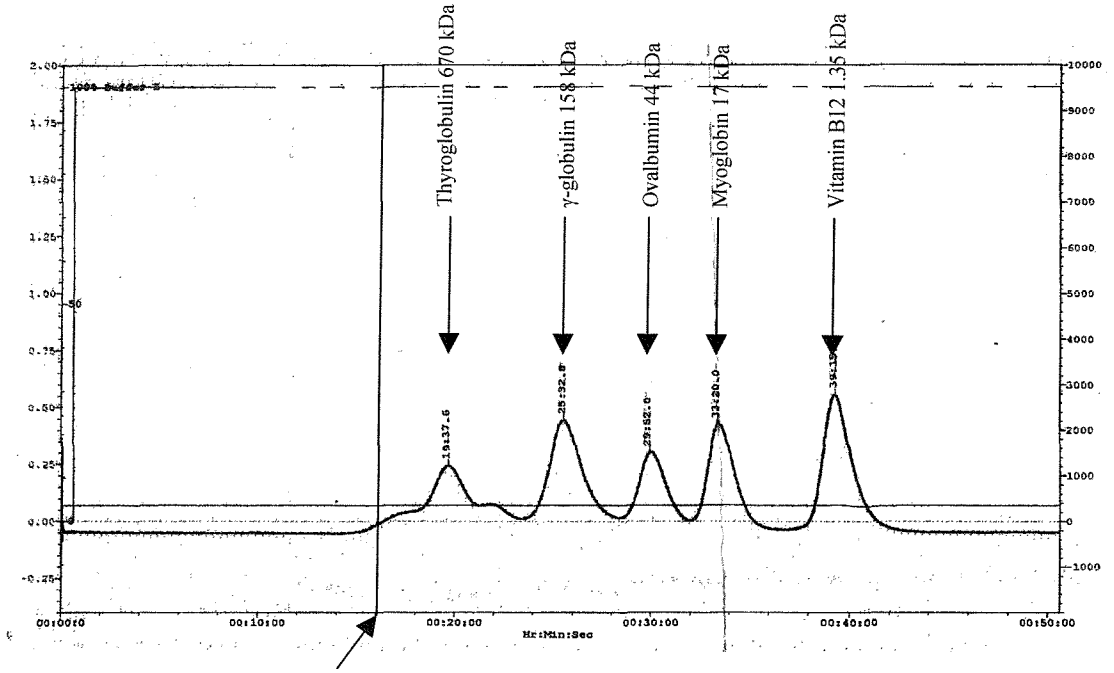
All general materials, including reagents, buffers and apparatus, used were as those listed previously *(2.1)*.

7.2.2.1 Sample preparation

Defatted, cell-free breast milk samples were prepared by centrifugation *(2.2.8)*. Further delipidation of milks BM 51 and BM 92 was performed according to the method of Cham & Knowles [378] after the gel filtration column was blocked by preceding samples. A 3 ml volume of 60:40 diisopropylether butanol (Merck) was added to 1 ml of milk in a pyrex screw cap culture tube (Corning) and mixed thoroughly by rotating the tube at room temperature for 6 hours in a rotator unit (Stuart Scientific). The tube was then centifuged at 3000xg for 15 minutes at 4^0C, after which the solvent supernatant containing the lipid was aspirated using a glass pipette and discarded. The lower breast milk layer was then transferred to an eppendorf tube and centrifuged once more (9000xg, 10 minutes, room temperature). The lower breast milk layer was carefully aspirated again using a glass pipette and transferred to an eppendorf. The sample was stored at -20^0C prior to fractionation.

7.2.2.2 Gel filtration chromatography

Samples were separated according to the molecular weights of their constituents by gel filtration chromatography using a Superdex 200 10/30 column (column - 24 ml; void volume - 8 ml) (MW separation range 10-600 kDa) (Pharmacia), as described previously *(2.5.10)*. The column was eluted at a rate of 0.5 ml/minute and fractions, each of 0.25 ml volume (n=55), were collected between 12 and 40 minutes run time. The MW of individual fractions was calculated by reference to the calibration curve prepared using standard calibrator proteins (typical curve shown below). The column was calibrated prior to running the samples. It was recalibrated if the interval between experimental runs was prolonged or if the column was adjusted. Sample fractions were stored at -80^0C prior to further assay.



Typical calibration curve for breast milk separation by gel filtration chromatography

The calibrator protein mixture comprised thyroglobulin (MW 670 kDa), γ-globulin (MW 158 kDa), ovalbumin (MW 44 kDa), myoglobulin (MW 17 kDa) and vitamin B12 (MW 1.35 kDa). The diagram shows the time ranges over which they were eluted on the Superdex 200 10/30 column, MW separation range 10-600 kDa. This calibration curve applies to BM 15 *(figure 7.1)*, BM 60 *(figure 7.2)*, BM 3 *(figure 7.3)*, BM 44 *(figure 7.4)* and BM 86 *(figure 7.5)*. The calibration curve for BM 51 & BM 92 *(figures 7.6 & 7.7)* differed only in minimal changes of the times of peak elution of the individual standard proteins.

7.2.2.3 Ovalbumin detection

Ovalbumin concentration in sample fractions was measured by capture *ELISA (3.2).* The fractions were added at a dilution of 1 in 10 in antibody buffer to the microtitre wells and in view of the number of fractions (n=55), they were run singly. For those fractions with absorbances above the standard curve the assay was repeated following further fraction dilution (range 1:100 - 1:4000). OVA concentration was expressed in ng/ml, after taking into account the dilution of the sample, by comparison of the sample absorbance to the ovalbumin standard curve.

7.2.2.4 Measurement of total IgA concentration

The distribution and concentration of total IgA in fractions of samples BM 15, BM 86 and BM 92 was measured by an in-house developed *ELISA (2.5.3).* Fractions were diluted 1 in 1000 in antibody buffer. Those fractions with absorbances above the standard curve were repeated at a dilution of 1 in 10 000 in antibody buffer. IgA concentration was expressed in ng/ml, after taking into account the dilution of the fraction, by comparison of the fraction absorbance to the IgA standard curve (human myeloma IgA, courtesy of M.Power, Tenovus).

7.2.2.5 Measurement of ovalbumin IgG concentration

The distribution and concentration of ovalbumin IgG (OVA G) in fractions of sample BM 92 was measured by indirect *ELISA (2.5.1).* Fractions were diluted 1 in 2 in antibody buffer. OVA G was expressed in arbitrary units (AU), after taking into account the dilution of the fraction, by comparison of the fraction absorbance to the standard curve of the serum pool.

7.2.2.6 Western blotting

In order to confirm the presence, and further evaluate the form, of ovalbumin in breast milk, positive and negative fractions of BM 86 were Western blotted. Aliquots (5 µl) from each of the positive fractions 38 – 42 and negative fractions 17 – 21 were pooled. Aliquots (10 µl) of these pools and a positive OVA control (1 µg/ml) were

run in duplicate on SDS-PAGE under reducing conditions, along with a molecular weight standard *(2.5.6)*. The proteins were transferred to a nitrocellulose membrane and Western blotted using enhanced chemiluminescence to improve detection sensitivity *(2.5.8)*.

7.3 Results

7.3.1 Positive breast milks

Ovalbumin *ELISA* of fractionated positive breast milks consistently produced a single peak for the detection of ovalbumin. This low MW peak extended predominantly through fractions 37–41, MW range approximately 20-39 kDa, (total run time 30.5–32.5 minutes) and suggested that the protein might be present in the samples in free or fragmented form. Regardless of whether the specific IgA concentration was low (BM 15, *figure 7.1*) or high (BM 60, *figure 7.2*), the profile was the same and no high MW peak – that might be consistent with ovalbumin in IgA complex ($\geq$ 460 kDa) – was detected.

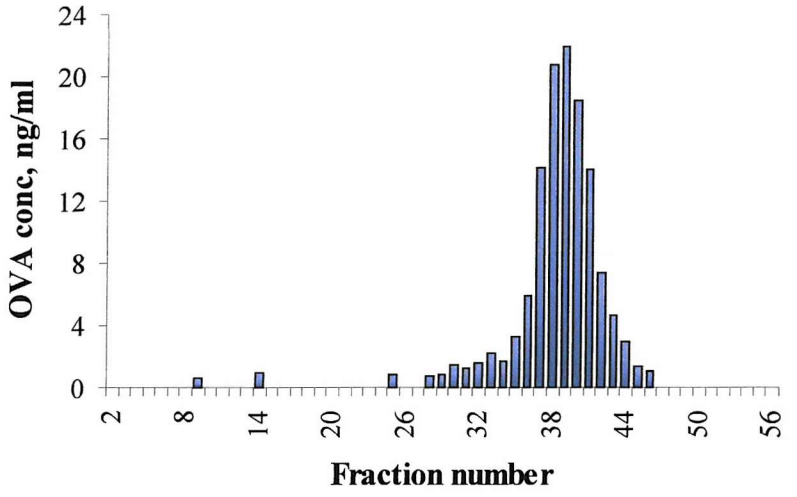


Figure 7.1 OVA detection – breast milk, BM 15 (OVA +, low OVA A concentration).

BM 15 was OVA + (1.64 ng/ml) and had a low OVA A concentration (OVA A Index 1.2). The sample was separated by gel filtration chromatography using a Superdex 200 10/30 column (Pharmacia). The column (volume 24 ml) was eluted at 0.5 ml/min and fraction collection started at 12 minutes run time (for method detail see 2.5.10). Ovalbumin was detected by ELISA (method 3.2). The protein was found in a low MW peak distributed through fractions 37 (MW 39 kDa, approximately) to 41 (MW 20kDa, approximately), run time 30.5–32.5, which compares to an elution time of 29–32 minutes (peak 29.9 minutes) for calibrator standard ovalbumin.

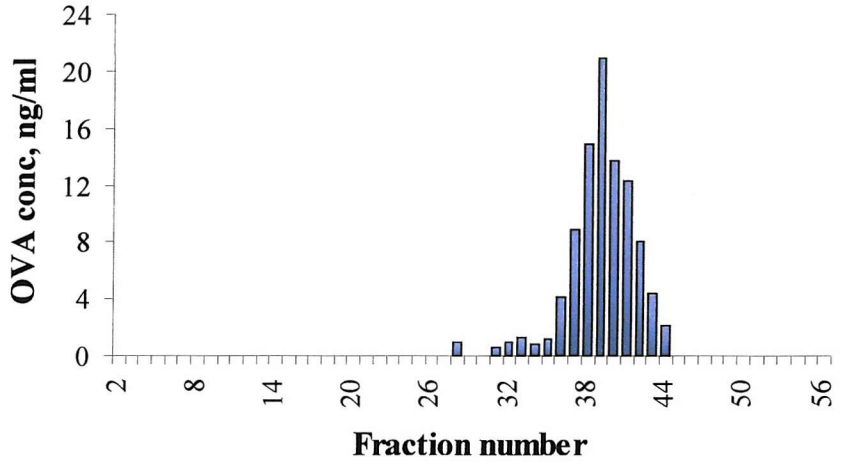


Figure 7.2 OVA detection – breast milk, BM 60 (OVA +, high OVA A concentration).

Breast milk BM 60 had a higher OVA A concentration (OVA A Index 3.7) than BM 15 (OVA A index 1.2) (chapter 4). The sample was separated by gel filtration fractionation and ovalbumin detected by ELISA, as described in figure 7.1 and text. Ovalbumin was present only as a single low MW peak. (For calibrator standard ovalbumin, see figure 7.1)

The quantity of ovalbumin detected in the intact sample was generally reflected in the concentrations that could be measured in the breast milk fractions. Thus breast milks BM 3 and BM 44, that had OVA concentrations of 170.9 ng/ml and 1.258 μg/ml respectively, contained the highest quantities of ovalbumin, up to 6 μg/ml, in low MW fractions *(figures 7.3 & 7.4)*.

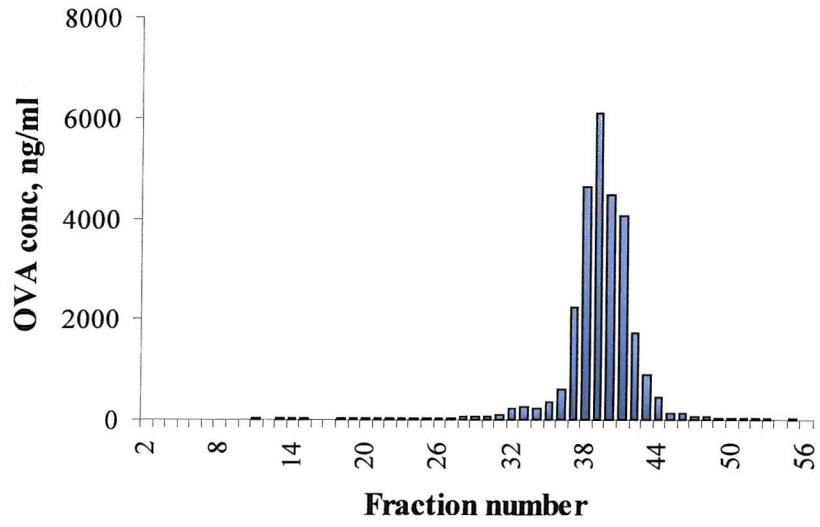


Figure 7.3 Ovalbumin detection – breast milk, BM 3.

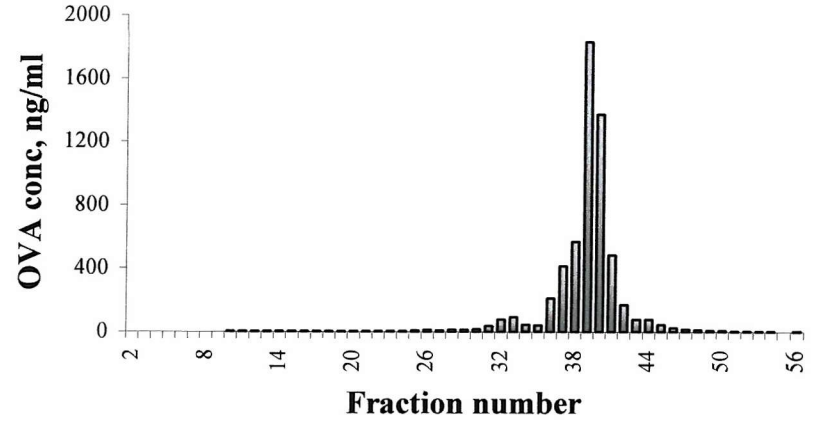


Figure 7.4 Ovalbumin detection – breast milk, BM 44.

Figures 7.3 & 7.4 Breast milks BM 3 & BM 44 had the highest ovalbumin contents (170.9 and 1258 ng/ml, respectively). Following gel filtration separation and ELISA of the fractions in the manner described previously (figure 7.1 and text), large quantities of ovalbumin - in excess of 6 μg/ml – could be detected in the fractions.

The OVA peak was a maximum in fractions 38-39, MW range approximately 28-33 kDa, which compares to a known MW of 44-45 kDa for ovalbumin. This raised the question of whether the free ovalbumin in breast milk might be in peptide fragments *(figure 7.5)*.

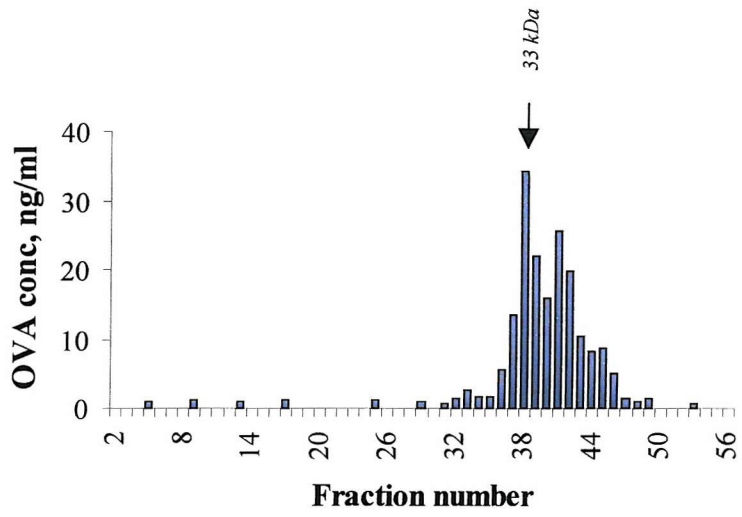


Figure 7.5 Ovalbumin detection – breast milk, BM 86 (OVA +).

Breast milk, BM 86, was processed by gel filtration & ELISA in the same manner as the previous samples (described in figure 7.1 and text). Ovalbumin was found in a low MW peak, especially fractions 38 – 42 (run time 31 – 33 minutes). Maximal ovalbumin quantity was detected in fraction 38, calculated MW 33 kDa, approximately, run time 31 minutes (Calibrator standard ovalbumin, as figure 7.1 - peak at 29.9 minutes).

7.3.2 Negative breast milks

Neither BM 51 nor BM 92 had detectable OVA. However, consistent with the observation made for serum samples, OVA could be detected in these 'negative' samples after fractionation *(figures 7.6 & 7.7)*. As seen in the positive breast milk samples, ovalbumin was found primarily in a low MW, single peak profile, suggesting the presence of free antigen. In BM 92 the occurrence of small quantities of OVA in a high MW peak (185 kDa) raised the possibility of a complex. OVA detection was independent of sample specific IgA concentration since BM 51, with OVA A Index less than the level of detection, contained measurable ovalbumin.

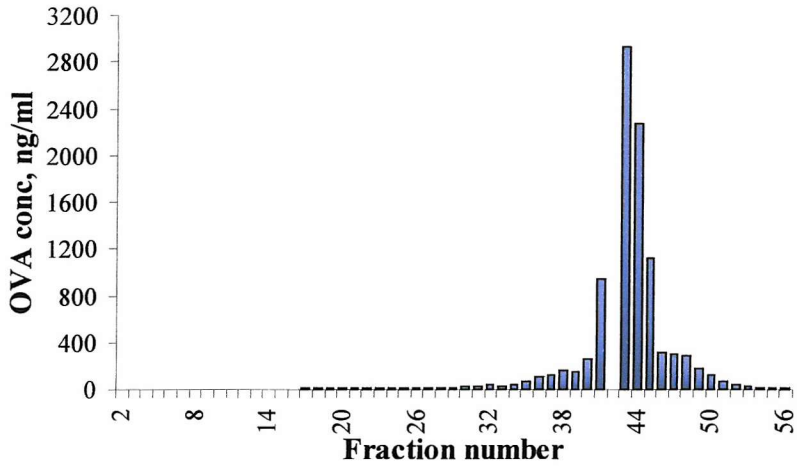


Figure 7.6 Ovalbumin detection – breast milk, BM 51 (OVA -).

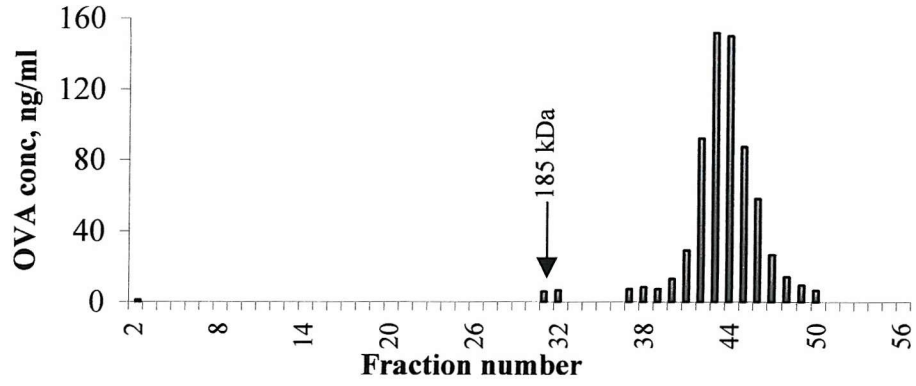


Figure 7.7 Ovalbumin detection – breast milk, BM 92 (OVA -).

Figures 7.6 & 7.7 Ovalbumin was undetectable in breast milks BM 51 and BM 92 by ELISA (chapter 4). These samples were delipidated by centrifugation and by diisopropylether butanol extraction (for method detail, see 7.2.2.1). They were then separated by gel filtration chromatography and ovalbumin detected in the fractions by ELISA, as for previous samples (figure 7.1 and text). After fractionation ovalbumin was detected in a low MW peak extending through fractions 41–47, estimated MW range 16-40 kDa, total run time 32.5–35.5 minutes. This compared to an elution time of 31–34 minutes, peak 32 minutes, for calibrator standard ovalbumin (curve not shown). Detection was independent of sample specific IgA concentration, since in BM 51 OVA A was undetectable. In BM 51 a small ovalbumin peak was found in fractions 31 (MW 185 kDa) & 32.

7.3.3 Distribution and concentration of IgA in breast milk

The question of whether ovalbumin in breast milk might be present in complex with IgA was further evaluated by the measurement total IgA concentration in a selection (n=3) of the samples. In all cases the OVA profile showed no overlap with the distribution of IgA (BM 92, IgA concentration peaked in fractions 22-24, MW 430-530 kDa), supporting the interpretation that ovalbumin in the positive fractions was most likely present as free antigen.

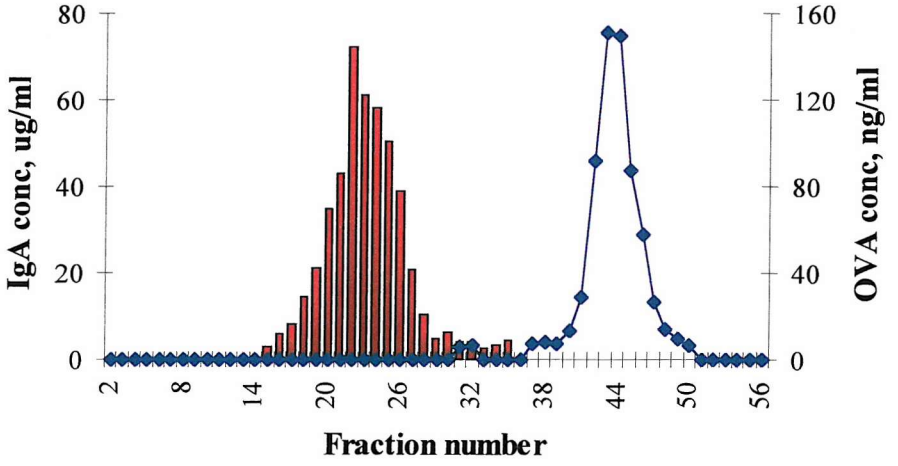


Figure 7.8 Total IgA concentration and distribution – breast milk, BM 92.

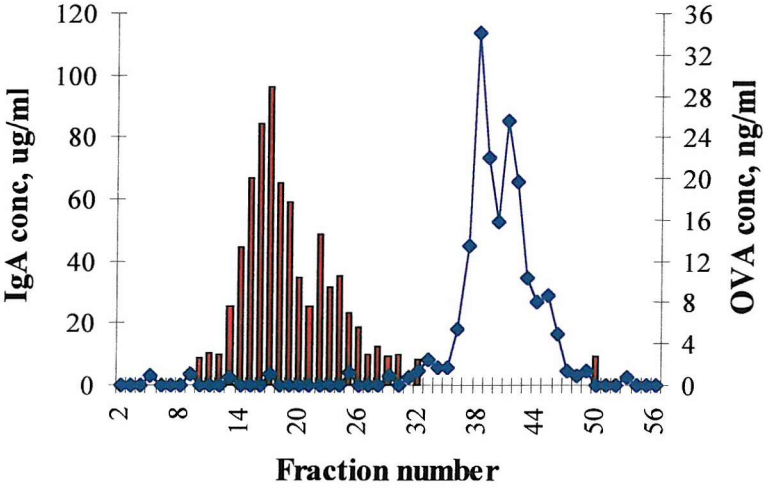


Figure 7.9 Total IgA concentration and distribution – breast milk, BM 86.

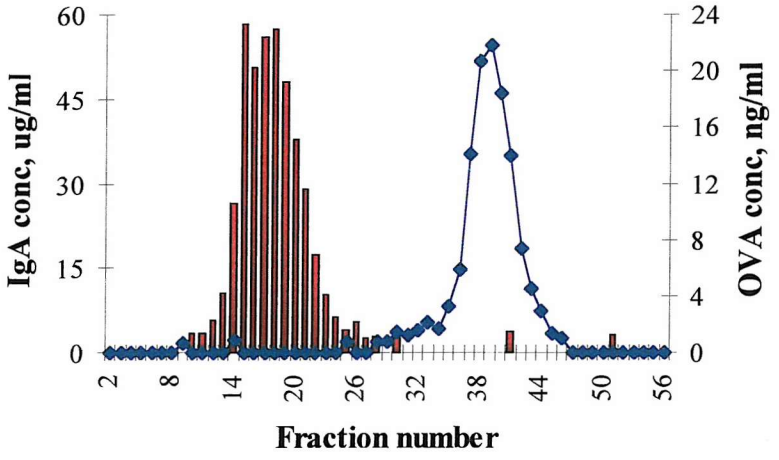


Figure 7.10 Total IgA concentration and distribution – breast milk, BM 15.

Figures 7.8 – 7.10 Total IgA (red bars) was measured by ELISA in each of the fractions of breast milks, BMs 92, 86 & 15 (for method detail, see 2.5.3). IgA was not found in the fractions containing ovalbumin (blue line). The two profiles did not overlap.

7.3.4 Distribution and concentration of ovalbumin IgG in breast milk

BM 92 had a small OVA peak in high MW fractions (185 kDa) raising the possibility of allergen in complex. Since these fractions did not contain IgA *(figure 7.8),* the distribution and concentration of specific IgG were also evaluated. OVA G was found in a low concentration peak adjacent to the high MW OVA peak *(figure 7.11)*. This raised the possibility that a very small proportion of breast milk ovalbumin may be complexed with IgG. In comparison to the proportion of free antigen, the complexed form was of secondary, and likely limited functional, importance.

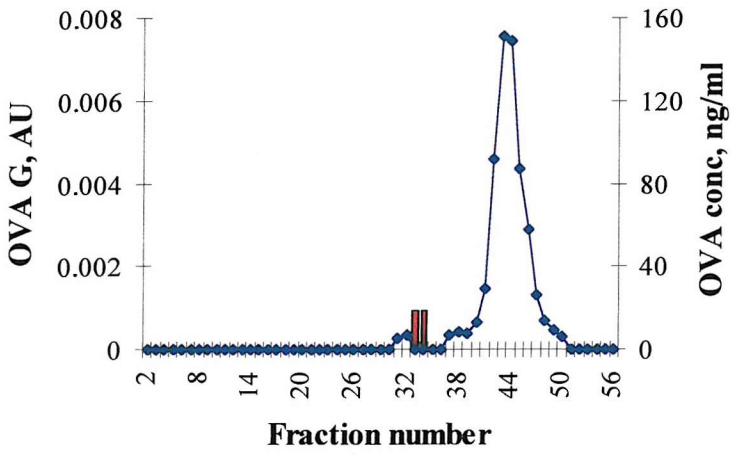


Figure 7.11 Ovalbumin IgG distribution and concentration – breast milk, BM 92.

Ovalbumin IgG concentration (red bars) was measured in fractions of BM 92 by ELISA (for method detail, see 2.5.1). Ovalbumin specific IgG was found in a low concentration peak next to the high MW OVA peak (blue line) in fractions 31(MW 185 kDa) & 32.

7.3.5 Western blotting of breast milk

Western blotting of breast milk, BM 86, after being run on SDS-PAGE under reducing conditions, showed the presence of ovalbumin in the positive fractions only, supporting assay validity and the data interpretation. In this 'positive' lane *(figure 7.12)* there were at least 2 bands in the ovalbumin region, both of MW lighter than the intact protein, further suggesting that the protein had been altered in the course of entero-mammeric transport.

Figure 7.12 Western blot breast milk, BM 86 (run under reducing conditions).

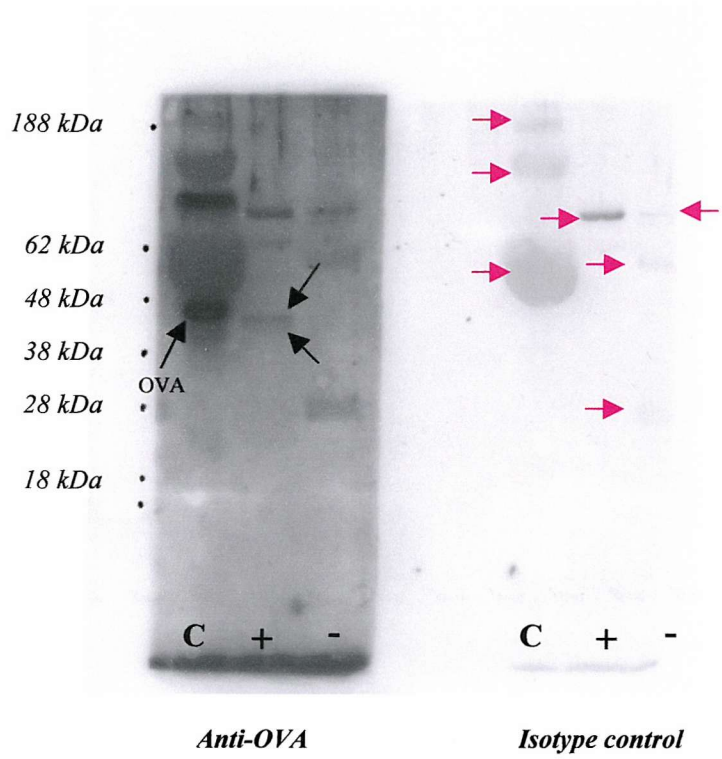


Figure 7.12 Breast milk, BM 86 was Western blotted (for method see 2.5.6 & 2.5.8). Positive fractions (+) were run with negative fractions (-) and an OVA control (C). They were probed with either rabbit anti-ovalbumin (Anti-OVA) or a rabbit IgG isotype control. Only the positive fractions gave a signal in the OVA region. This signal was from at least 2 bands with lighter MW than intact OVA indicating a degree of processing of the breast milk protein. A number of other bands are apparent. These are due to non-specific binding, as evidenced by their presence on the isotype control blot (pink arrows), and are most likely due to protein overload in the well.

7.4 Discussion

Allergen exposure, particularly in the postnatal period, has been implicated as a major determinant of whether a baby at high risk of atopy actually develops disease [244][245][247]. As regards dietary allergens, breast milk provides an rich source for exposure in early life [190][253][257]. However, despite the recognition that breast milk is an important interface between mother and baby, little is known about the mechanism, or control of antigen passage between the two. Therefore, this work set out to further characterise postnatal ovalbumin exposure via breast milk. Breast milk samples were separated according to the molecular weights of their components. Detection of

ovalbumin in these fractions then permitted the construction of a size profile, from which ovalbumin form might be inferred.

The results of this work were clear-cut. In all samples OVA was present in fractions that formed a uniphasic low MW peak, suggesting that the dietary ovalbumin encountered in postnatal life via breast milk was a free protein. This is in contrast to the transplacental route. In cord blood OVA was found in both free and complexed forms, the form being regulated by the presence of specific IgG *(chapter 6)*. IgA is the immunoglobulin that predominates in breast milk [379)(& this work)]. The plasma cells that produce IgA home to the interstitium underlying the mammary secretory cells after migrating from the lymph tissue in the intestine (Peyer's patches) and bronchial tree. This secretory IgA provides protection at the mucosal surface likely to be the first site of microbial encounter. IgA mainly exists as dimers in breast milk (dimeric secretory IgA, MW 415 kDa). No ovalbumin was found in fractions of appropriate corresponding MW: moreover, the lack of overlap between the IgA and ovalbumin profiles *(figures 7.8-7.10)* supported the conclusion that ovalbumin did not exist as OVA–IgA complexes. It might be argued that complexed OVA was present in high MW fractions but that the polymeric secretory IgA 'blocked' antigen recognition by the detector antibody, as was postulated for specific IgG in some serum samples *(chapter 3)*. However, since serum samples with such an inhibitory capacity were 'unmasked' by fractionation, revealing the presence of antigen in free and complexed forms, so this was unlikely to be the case.

Breast milk BM 92 was the only sample that produced a second small OVA peak in the region of 185 kDa *(figure 7.7)*. When the distribution of ovalbumin IgG was plotted this revealed a low concentration of OVA G in the same fractions *(figure 7.11)*. The presence of OVA-IgG complexes in this milk is therefore a possibility, but in the context of an overwhelming quantity of free protein, the finding is unlikely to have a functional significance.

As was seen for OVA negative serum samples, intact breast milks without OVA were noted to have detectable allergen after fractionation. The fractionation process is recognised to be able to improve assay sensitivity by dispersing a complex fluid. This methodological feature may also explain why the negative breast milks, which had

undergone a further processing step prior to fractionation, were found to contain higher quantities of ovalbumin. What can be concluded from these cord and breast milk data is that allergen exposure through maternal routes may be a universal finding. How extremely low dose antigen exposure, below the limit of the conventional detection method, relates to infant allergic sequelae is likely to be a complex issue.

The breast milk ovalbumin peak overlapped that of the calibrator ovalbumin but was a maximum at MW 29-33 kDa (approximately)(for example, *figure 7.5*), which compares to a known MW of 44-45 kDa for the protein. This finding is reminiscent of the data presented in *chapter 6*, where serum sample ovalbumin was found to elute later (at lower MW) than calibrator ovalbumin, but at the same time as a spiking dose of ovalbumin. This raised the question of whether a 'matrix effect' of serum might retard the progress of the protein down the column gel. It is possible that breast milk may have a similar property, though in this work the profile of a spiking dose of ovalbumin was not evaluated. Clearly, it is also possible that the protein may be presented to the neonatal immune system in fragmented form. Indeed, Western blotting showed the presence of at least 2 bands, which might represent ovalbumin peptides *(figure 7.12)*. Alteration of the protein as the ingested allergen traverses the maternal gut, circulation and mammary epithelium might be an obvious expectation, but hitherto has not been described.

The exact mechanism by which a dietary allergen such as ovalbumin is transported into milk remains uncertain. IgA is transferred into breast milk by a mechanism that involves receptor-mediated endocytosis, transcellular passage and secretion into the lumen [119]. The significant direct correlation between OVA quantity and specific IgA concentration noted previously *(chapter 4)* had raised the possibility that IgA might carry OVA, as a 'bystander', in complex, into the milk. These current data would argue against such a mechanism. However, as a number of other intact proteins, such as insulin and prolactin, are thought to cross the mammary epithelium by transcytosis [380][381], it is entirely possible that a similar process could exist for ovalbumin.

Again the ultimate question is what consequence might antigen form in breast milk have for allergic sensitisation of infants at genetic risk of atopy? Since secretory IgA

protects the gut mucosal surface by binding large foreign antigens, preventing epithelial attachment and so promoting elimination through gut peristalsis, it might be reasonable to hypothesise that allergen in free form could exert a greater influence on the neonatal immune system than could complexed allergen.

The gut mucosa is a highly developed immunological organ [317]. T-cells are found between epithelial cells at certain parts of the small intestine, or are grouped in lymphoid aggregates or follicles. Peyer's patches are well-organised structures, comprising 30 – 40 lymphoid follicles, found in the distal jejunum and ileum. They are capped by microfold or 'M' cells that lack a brush border and are specialised for the capture and transport of antigens. Thin extensions of these cells surround lymphoid cells, macrophages and dendritic cells in the underlying dome region of the patch and beneath the dome region are B-cell zones surrounded by T-cells, most of which are CD4+. Accordingly, these structures are well adapted for antigen processing and presentation and furthermore they are well developed by the time of birth [86]. Thus, exposure of these cells to breast milk-derived ovalbumin might result in the induction of an immune response.

This work has continued to contribute to our knowledge regarding the role of breast-feeding in the aetiology of allergic disease. The association of allergy with breast-feeding is a contentious issue and is likely influenced by inter-individual variation in composition, often genetically determined [213][214][382]. However, underlying disease expression is the prerequisite for allergen exposure. These current data have further characterised this exposure in terms of antigen form, which, in turn, has added to our understanding of the mechanism of breast milk induced antigen-specific immune reactivity.

Chapter Eight

Investigation of the functional affinity of serum ovalbumin IgG

Chapter 8

Assessment of the functional affinity of serum ovalbumin IgG

8.1 Aims

Whilst it is generally accepted that an IgE-dependent mechanism underlies Type I hypersensitivity reactions, the possible role of IgG in the allergic process is not so well defined. IgG antibodies to airborne allergens have been noted to correlate closely with specific IgE in atopic individuals [383] and both protective [291][350] and anaphylactic functions [384][385][386], in inhalant [291] and food allergy [387], have been ascribed to them.

The work presented so far in this thesis has supported the concept of a significant role of serum specific IgG in the allergic mechanism and has also suggested that a modulatory function may be in place in the antenatal period. IgG has been shown to play a unique role in allergen carriage to the fetus, since ovalbumin in complexed form was entirely dependent on the presence of circulating ovalbumin specific IgG *(chapter 6)*. This suggested that serum concentration was a fundamental property of immunomodulatory IgG. Concentration was also implicated in a further possible regulatory role of IgG, namely epitope recognition. The OVA-specific antibodies used in the antigen detection system were able to recover ovalbumin from serum in amounts that were inversely proportional to the concentration of ovalbumin IgG in the same sample *(chapter 3)*. If such a phenomenon also occurred *in vivo*, then this could have implications for priming, since presentation of immunogenic epitopes to the cells of the immune system is a prerequisite for immune reactivity. This is supported by the observation that non-atopic individuals exposed chronically to airborne occupational allergens frequently have elevated IgE but no allergic symptoms [388], which has been attributed to high titre specific IgG inhibiting, or 'blocking', epitope recognition by sensitised mast cells or basophils.

The appreciation that a second property of IgG, namely antibody avidity/functional affinity, might be an additional regulatory factor has also evolved in the preceding chapters: maternal serum samples from different individuals could have detectable, or

conversely, non-detectable, non-recoverable ovalbumin, despite having comparable concentrations of ovalbumin IgG *(chapter 3)*. Therefore, antigen detection was not solely dependent on specific IgG levels. Samples without ovalbumin were observed to have a 'blocking' capacity of a degree that ranged between the need for more sensitive detection, to requiring total IgG depletion, in order to reveal the allergen *(chapter 6)*.

Antibody avidity or functional affinity describes the strength of antigen–antibody binding. Strong, multiple interactions between ovalbumin and specific IgG afforded by high affinity/avidity immunoglobulin could inhibit recognition of allergenic epitopes. Therefore, the hypothesis of the current study was that those samples without demonstrable allergen recovery were of a higher affinity than those with recoverable protein.

Just as the contribution of IgG concentration in allergic mechanisms is a contentious issue, so there are opposing reports of the functional consequences of high affinity immunoglobulin. High affinity antibodies more efficiently clear microbes, perhaps by enhanced opsonisation, than low affinity antibodies [376]. Indeed excessive production of low affinity antibodies has been considered an expression of immunodeficiency [389]. On the other hand, high affinity IgE to Der p2 promoted basophil histamine release suggesting that in the atopic individual the enhanced biological activity of higher avidity immunoglobulin may lower the threshold of symptom manifestation [390].

A variety of methods have been proposed to measure antibody avidity, including ammonium sulphate precipitation [391] and solid phase immunoassay [392]. This work employed a modified *ELISA* using the chaotropic agent, ammonium thiocyanate, to disrupt antigen-antibody complexes. The method, first described for rubella virus [393] and E Coli polysaccharide [394], was adapted and validated for the current application.

8.2 Samples and methods

8.2.1 Samples

8.2.1.1 Maternal sera

Maternal serum samples (n=16) from pregnant women *(2.2.3)* were selected for this analysis. No sample had detectable ovalbumin *(chapter 4)*. For each sample ovalbumin specific IgG (OVA G) concentration (expressed as Arbitrary Units, AU) was known *(chapter 5)*. Sera were grouped according to whether they permitted (n=7) *(recovery +)* or inhibited (n=9) *(recovery -)* the recovery of a spiking dose of ovalbumin *(chapter 3)*. Sample characteristics are outlined in *table 8.1*.

Table 8.1 Sample characteristics for functional affinity – maternal sera.

Recovery +		*Recovery -*	
ID	OVA G (AU)	ID	OVA G (AU)
M104	0.54	M107	0.45
M123	1.23	M117	0.59
M126	0.57	M118	0.31
M128	0.27	M127	2.68
M141	1.75	M131	1.78
M143	0.7	M132	2.12
M146	1.21	M134	0.96
		M140	2.37
		M145	2.05

8.2.1.2 Matched maternal and cord sera

In order to evaluate the relationship between maternal and infant IgG functional affinity, maternal sera at delivery and matched cord sera (n = 6), selected from this same study cohort *(2.2.3)*, were also analysed. Sample characteristics are shown in *table 8.2*.

Table 8.2 Sample characteristics for measurement of functional affinity – matched maternal and cord sera.

	Maternal	Cord
ID	**OVA G (AU)**	**OVA G (AU)**
100	0.45	0.61
106	0.28	0.27
107	0.17	0.37
108	1.45	2.23
123	0.76	0.75
128	0.29	0.27
131	0.94	0.93

8.2.2 Infant atopic evaluation

The infants born to the women analysed in this investigation were evaluated for atopic disease as described previously *(2.4)*. In this analysis, skin prick test (SPT) positivity to one or more of a panel of common dietary and inhalant allergens at 6 months of age was used as a measure of atopy. A weal diameter $\geq$ 2 mm, in the presence of appropriate negative and positive (histamine) controls, was considered to be a positive reaction.

8.2.3 Method

8.2.3.1 Principle

The technique is based on the susceptibility of antigen-antibody complexes to dissociation by the chaotropic ion, thiocyanate. The indirect *ELISA* for measurement of ovalbumin specific IgG concentration *(2.5.1)* was modified to include an additional incubation step of antigen–immunoglobulin with increasing concentrations of ammonium thiocyanate (AT) before detecting the remaining bound IgG. The molarity of ammonium thiocyanate required to reduce the initial absorbance by 50%

represented the affinity index. Thus the higher the functional affinity of specific IgG, the higher the affinity index.

8.2.3.2 Optimisation

8.2.3.2.1 Evaluation of elution of bound antigen

In order to ensure that changes in absorbance were due to thiocyanate dissociation of IgG-OVA complexes, and not to disruption of the coating antigen, the elutional effect of the chaotrope on the antigen bound to the microtitre wells was evaluated.

The indirect *ELISA* was performed as in *2.5.1.* All general materials, including reagents, buffers and apparatus, used were as described previously *(2.1).*

Briefly, microtitre plates were coated with 100 µl OVA or BSA at 100 µg/ml. The wells were washed once with 200 µl assay buffer and then blocked for 1 hour with 200 µl blocking buffer. The wells were again washed once with assay buffer before incubating with 100 µl of antibody buffer, 0.1 M sodium phosphate buffer pH 6.0 or ammonium thiocyanate (Promega) in 0.1M phosphate buffer, concentration 1- 4 M, for 30 minutes. The plates were washed 3 times with 200 µl wash buffer and then the serum pool standards *(2.2.6)*, serially diluted in antibody buffer, were added in duplicate to the wells (100 µl per well). The wells were incubated for 1 hour, then washed 3 times with wash buffer and incubated with 100 µl HRP-conjugated rabbit anti-human IgG (Dako), diluted 1:6000 in antibody buffer. After 1 hour the plates were washed a further 3 times with wash buffer. Bound IgG was detected by incubating 100 µl TMB in each of the wells for up to 30 minutes. The reaction was stopped by the addition of 50 µl 1M sulphuric acid and the colour change read at 450 nm. Absorbances from the untreated and treated wells were plotted against serum pool dilution after subtracting the absorbance from the matched wells on the control BSA plate from the absorbance obtained from the OVA coated wells.

As shown in *Figure 8.1,* ammonium thiocyanate, but not the phosphate buffer, eluted the bound antigen in amounts that increased in proportion with the molarity of the chaotrope.

Figure 8.1 Ammonium thiocyanate elution of coating antigen.

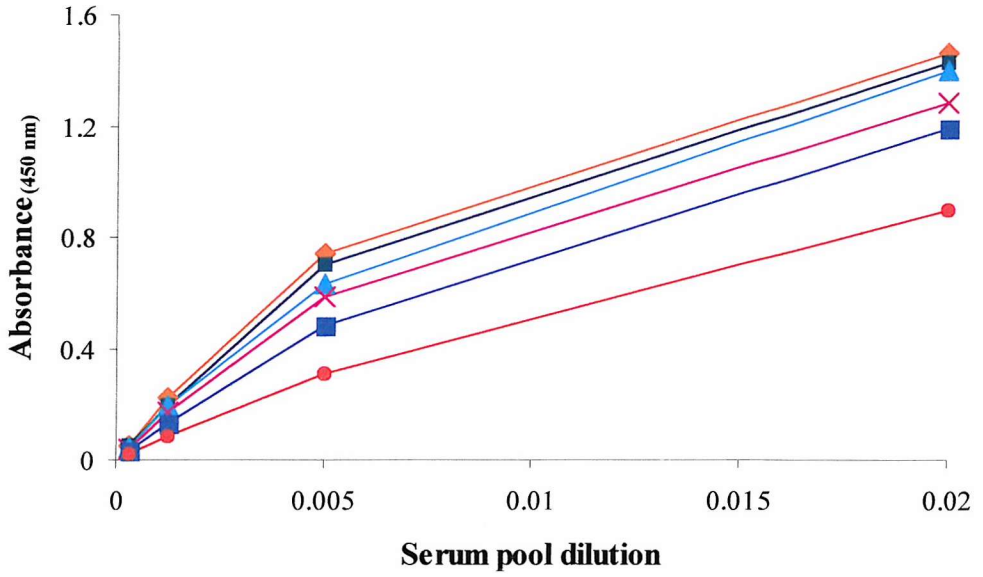


Figure 8.1 A modified ELISA for measurement of OVA G concentration was performed (8.2.3). The absorbances produced by the serum pool (orange diamonds) were diminished at each dilution by prior incubation of the antigen coated plates with ammonium thiocyanate, indicating antigen elution. The reduction was directly related to the concentration of AT: thus the greatest reduction was with 4M AT (red circles) and the least with 1M AT (turquoise triangles). 2M AT is shown in pink crosses and 3M in light blue squares. There was no elution with 0.1M phosphate buffer pH 6.0 alone (dark blue squares).

8.2.3.2.2 Increasing concentration of coating antigen concentration

In order to evaluate if an increased concentration of coating antigen might compensate for the elution associated with the treatment process, the experiment was repeated as in *8.2.3.2.1* except that a coating antigen concentration of 200 µg/ml was compared to 100 µg/ml.

As shown in *figure 8.2,* the elutional effect was neither diminished, nor eliminated by increasing the coating antigen concentration.

Figure 8.2 Coating concentration of 100 µg/ml v 200 µg/ml.

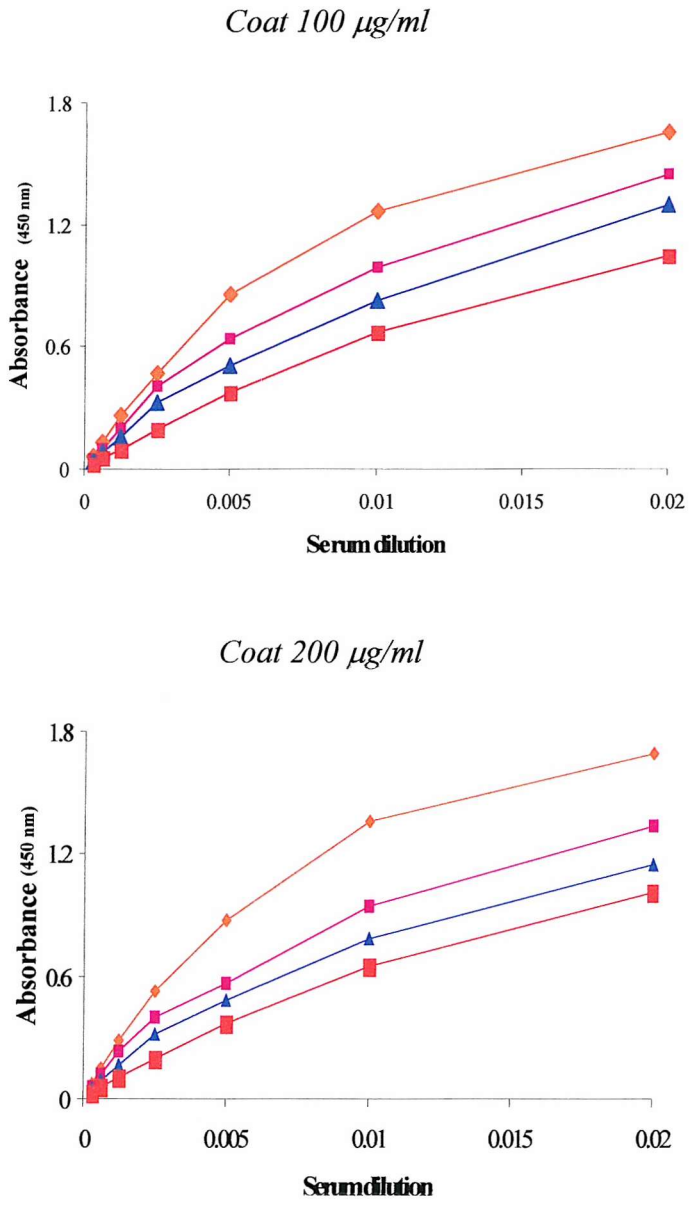


Figure 8.2 Increasing the coating antigen concentration from 100 to 200 µg/ml made no difference to the elution effect. The absorbances of the serum pool (orange diamonds) were diminished on prior AT treatment of the ELISA plate, the effect increasing in proportion with the molarity of AT – shown in red squares for 4M, blue triangles for 3M and pink squares for 2M.

8.2.3.2.3 Reducing volume and incubation time of ammonium thiocyanate

In order to assess if a reduced volume and/or incubation time of AT prevented the elutional effect, the experiment was repeated as in *8.2.3.2.1* except that the wells were

incubated with either 50 µl or 100 µl of antibody buffer, 0.1 M phosphate buffer or AT in various concentrations, for either 15 or 30 minutes.

In order to maximise binding of coating antigen, all plates were incubated at 37^0C for 2 hours before storing at 4^0C overnight. Also, wash steps were performed with assay buffer (no tween) in order to limit antigen disruption.

As shown in *figure 8.3,* a smaller volume of AT diminished the elutional effect. With 50 µl of chaotrope, antigen limitation, as reflected by a diminution of serum pool absorbance, was restricted to the highest concentration of standard. There was no further benefit of reducing the incubation time from 30 minutes to 15 minutes.

Figure 8.3 Coating antigen elution – varying volume and incubation time of AT.

Figure 8.3(a) A 50 µl volume of AT & 15 minute incubation time.

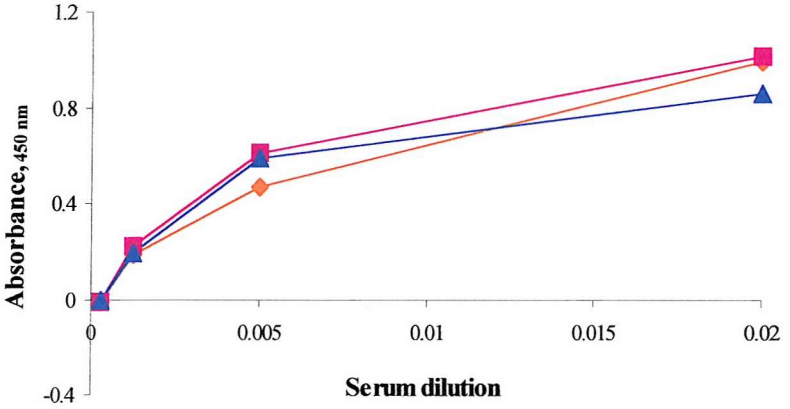


Figure 8.3(a) A 50 µl volume of AT was incubated with the plates for 15 minutes. With this regimen there was no evidence of antigen limitation (elution) by 2M AT (pink squares) at any serum pool dilution. For 3M AT (blue triangles), there was only evidence of antigen limitation at the most concentrated serum pool (orange diamonds) dilution (1:50).

Figure 8.3(b) 50 μl AT & 30 minutes incubation.

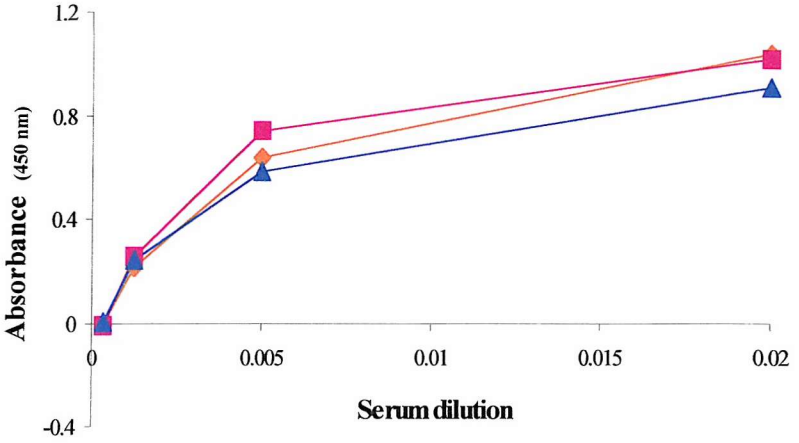


Figure 8.3(b) A 50 μl volume of AT was incubated with the plates for 30 minutes. With this regimen there was no evidence of antigen limitation by 2M AT (pink squares). The difference between untreated (orange diamonds), and 3M treated (blue triangles), serum standard absorbance was <10% at 1:200 dilution and 12 % at 1:50 dilution, indicating very mild antigen elution and limitation, particularly at high serum concentration.

Figure 8.3(c) 100 μl AT & 15 minutes incubation.

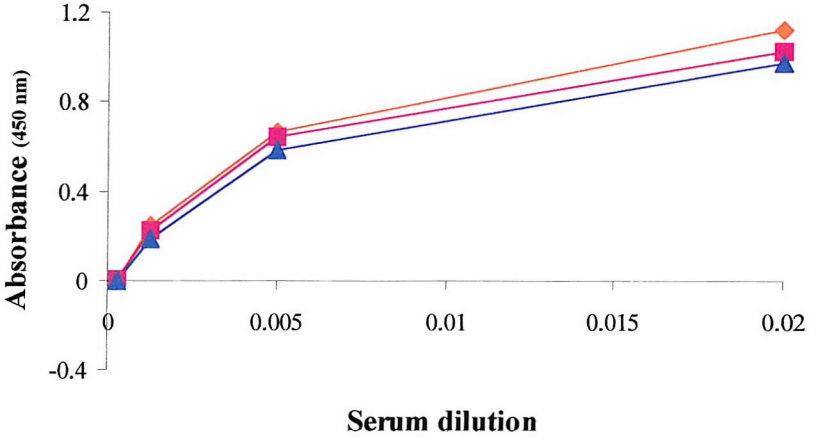


Figure 8.3(c) A 100 μl volume of AT was incubated with the plates for 15 minutes. There was mild antigen limitation, reflected in a reduced standard pool absorbance (orange diamonds) at each serum dilution (by 12.5 – 24%), for both 2M (pink squares) and 3M (blue triangle) AT.

Figure 8.3(d) 100 µl AT & 30 minutes incubation.

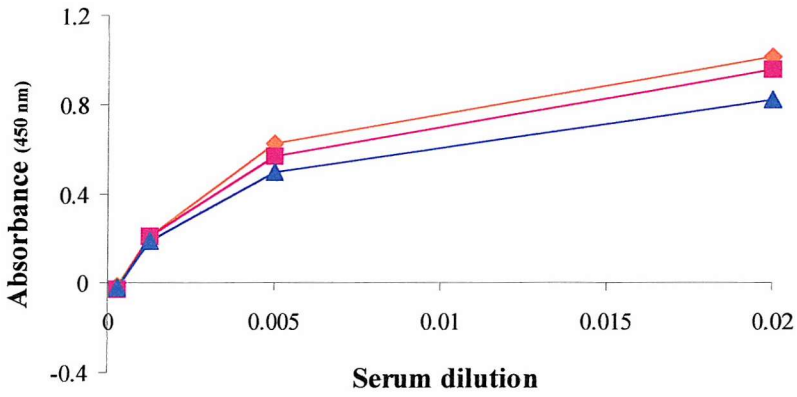


Figure 8.3(d) The elutional effect of AT on coating antigen, reflected in a reduction of serum pool absorbance (orange diamonds), was more marked for 2M (pink squares) and 3M (blue triangles) AT at 100 ul and 30 minute incubation.

8.2.3.2.4 Reducing volume of serum

A reduced volume of chaotrope was seen to alleviate antigen limitation. The experiment was therefore repeated using this regimen (50 µl AT, 30 minute incubation) but with 50 µl of serum in order to assess if this might completely eliminate the elutional effect by further promoting antigen excess.

As shown in *figure 8.4*, with this protocol there was very little antigen limitation, even with 4M AT and the most concentrated serum. The standard curves of the serum pool incubated with untreated and treated plates now ran in parallel.

Figure 8.4 Antigen elution – effect of reducing serum volume.

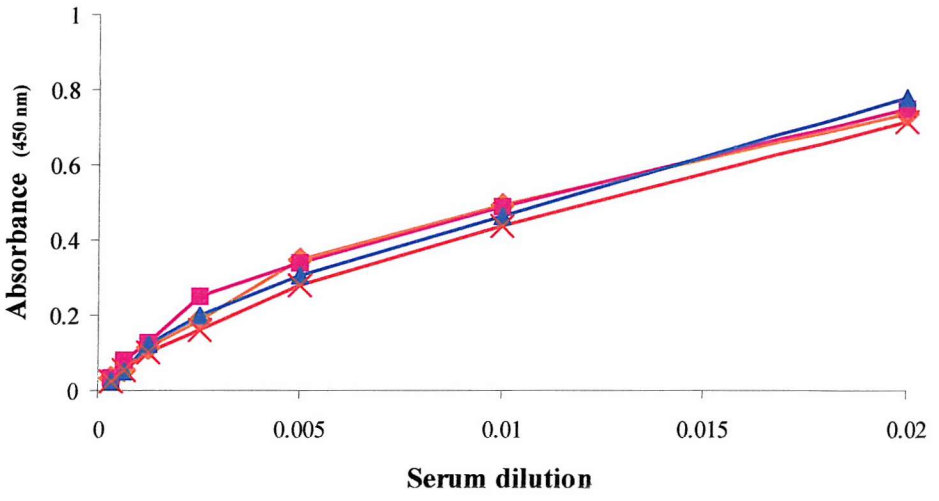


Figure 8.4 When 50 µl serum was incubated with the wells the resultant standard curves showed no evidence of antigen limitation from plates treated with 2M (pink squares), 3M (blue triangles) or 4M (red crosses) AT compared to untreated plates (orange diamonds).

8.2.3.3 Method

The optimisation experiments clarified the regimen of ammonium thiocyanate treatment that minimised elution of coating antigen. Serum IgG affinity index could thus be measured by the modified *ELISA*.

The method was as described in *2.5.1*, but with the following modifications.

Coated microtitre *ELISA* plates were incubated at 37^0C for 2 hours and then at 4^0C overnight. The plates were washed once with assay buffer, blocked for 1 hour and again washed once. Serum pool standards and test samples were diluted in antibody buffer. The test sera dilutions were those known by previous ovalbumin IgG assay to produce a sample absorbance that fell on the linear part of the reference curve.

A 50 µl volume of standard or test sample was added in duplicate to the microtitre wells and incubated for 1 hour. After 3 washes with assay buffer, the wells were then incubated with either 50 µl of phosphate buffer or AT in phosphate buffer,

concentration 1 - 4 M, for 30 minutes. After a further 3 washes, bound IgG was detected by incubation of the wells with HRP-conjugated antihuman IgG and measurement of the enzyme-mediated TMB substrate colour change *(8.2.3.2.1)*.

8.2.3.3.1 Calculation of Affinity Index

For each sample a graph of $\log_{10}$ (% of initial absorbance) versus molarity of AT was plotted. Initial absorbance was that obtained from wells with no chaotrope present. From this graph the molarity of AT inducing a 50% fall in initial absorbance was calculated. This represented the affinity index of the sample.

8.3 Results

8.3.1 Maternal sera

The affinity indices of the maternal serum samples with known OVA recovery capacity are shown in *tables 8.3 & 8.4*. Affinity index ranged between 0.94 and 2.21, median 1.735. Atopic outcome at 6 months of age of infants born to these women, as measured by skin prick test positivity, is shown alongside.

Table 8.3 Affinity Indices of serum samples permitting detection of a spiking dose of OVA (recovery +).

Recovery +			
ID	OVA G (AU)	Affinity Index	Infant SPT
M104	0.54	1.75	negative
M123	1.23	2.21	negative
M126	0.57	1.52	N/A
M128	0.27	1.82	negative
M141	1.75	1.73	N/A
M143	0.7	2.04	negative
M146	1.21	2.02	negative

Table 8.4 Affinity Indices of serum samples inhibiting detection of a spiking dose of OVA (recovery -).

Recovery -			
ID	OVA G (AU)	Affinity Index	Infant SPT
M107	0.45	1.54	negative
M117	0.59	1.45	negative
M118	0.31	2.20	negative
M127	2.68	0.94	positive
M131	1.78	1.74	N/A
M132	2.12	1.42	negative
M134	0.96	1.45	positive
M140	2.37	1.24	negative
M145	2.05	1.78	positive

There was no significant correlation between ovalbumin specific IgG concentration and affinity index. However, there was a trend for an inverse relationship whereby sera with the highest OVA G concentrations had the lowest affinity indices ($r = -0.465$, $p=0.07$, Spearman's) *(figure 8.5)*.

Figure 8.5 Correlation between ovalbumin specific IgG concentration and Affinity Index.

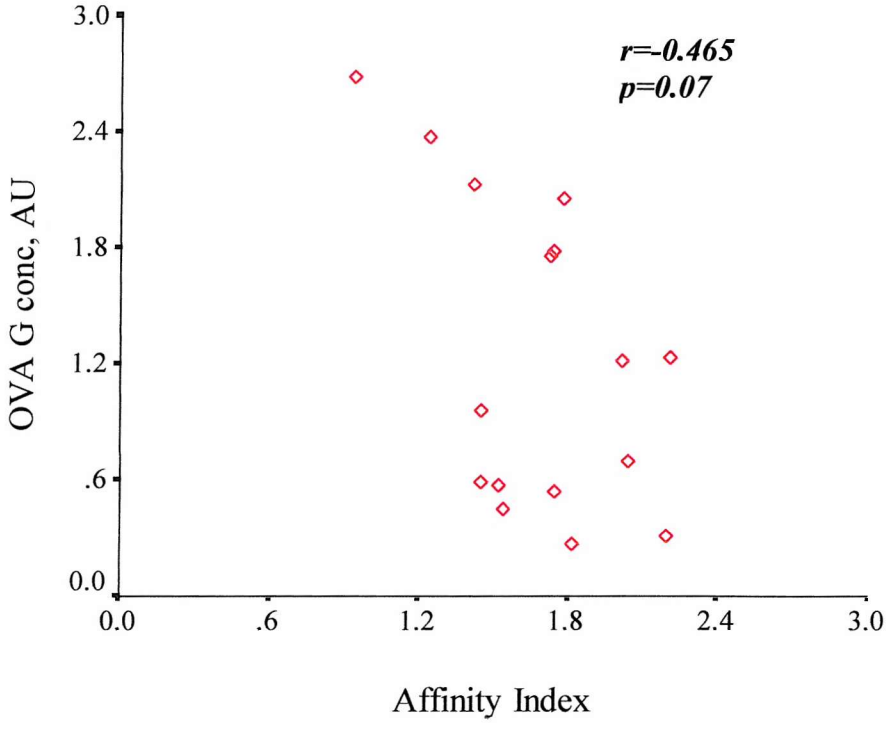


Figure 8.5 Serum relative avidity was measured by a modified ELISA using ammonium thiocyanate elution (for method, see 8.2.3.3). Sample relative avidity was expressed as an affinity index. No significant relationship between affinity index and ovalbumin specific IgG concentration was identified.

Affinity index was found to be significantly higher in *recovery* + samples compared to *recovery* – sera (p=0.039, Mann-Whitney) *(figure 8.6)*. There was no significant difference in affinity index according to maternal atopic status (p=0.212, Mann-Whitney) or dietary intervention (p=0.958, Mann-Whitney) randomisation.

Figure 8.6 Affinity Index according to recovery characteristics.

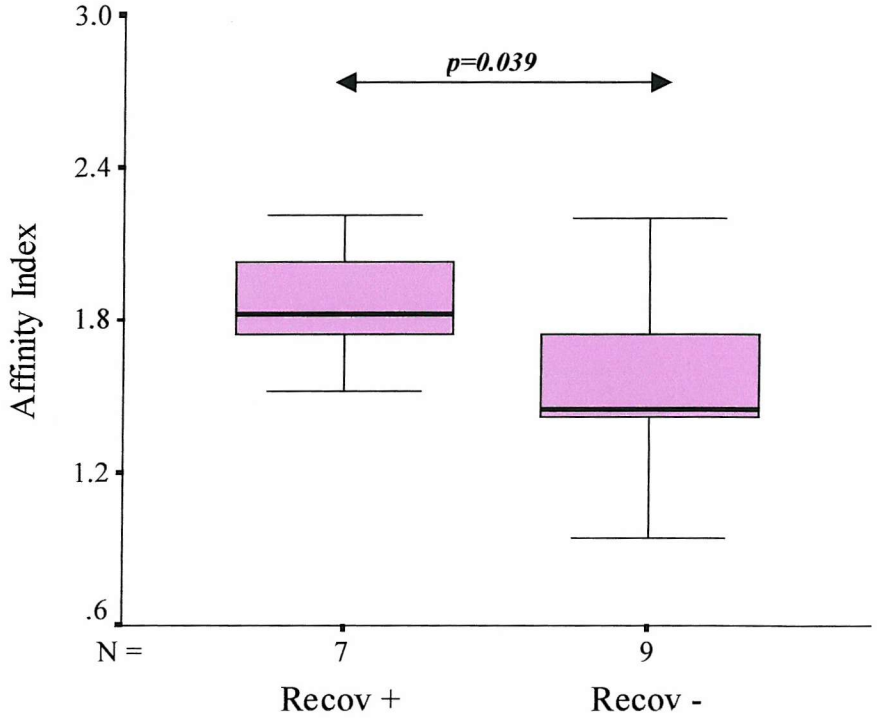


Figure 8.6 Serum relative avidity was measured by a modified ELISA using ammonium thiocyanate elution (for method, see 8.2.3.3). Sample relative avidity was expressed as an affinity index. Serum samples previously shown to permit the recovery of a spiking dose of ovalbumin (Recov +) had significantly higher affinity indices than samples which prevented ovalbumin recovery (Recov –).

8.3.2 Matched maternal and cord sera

Affinity indices of maternal sera at delivery and matched cord sera were directly correlated, though failed to reach statistical significance using non-parametric analysis (r=0.714, p=0.071, Spearman's) *(figure 8.7)*.

Figure 8.7 Correlation between maternal, and matched cord sera Affinity Indices.

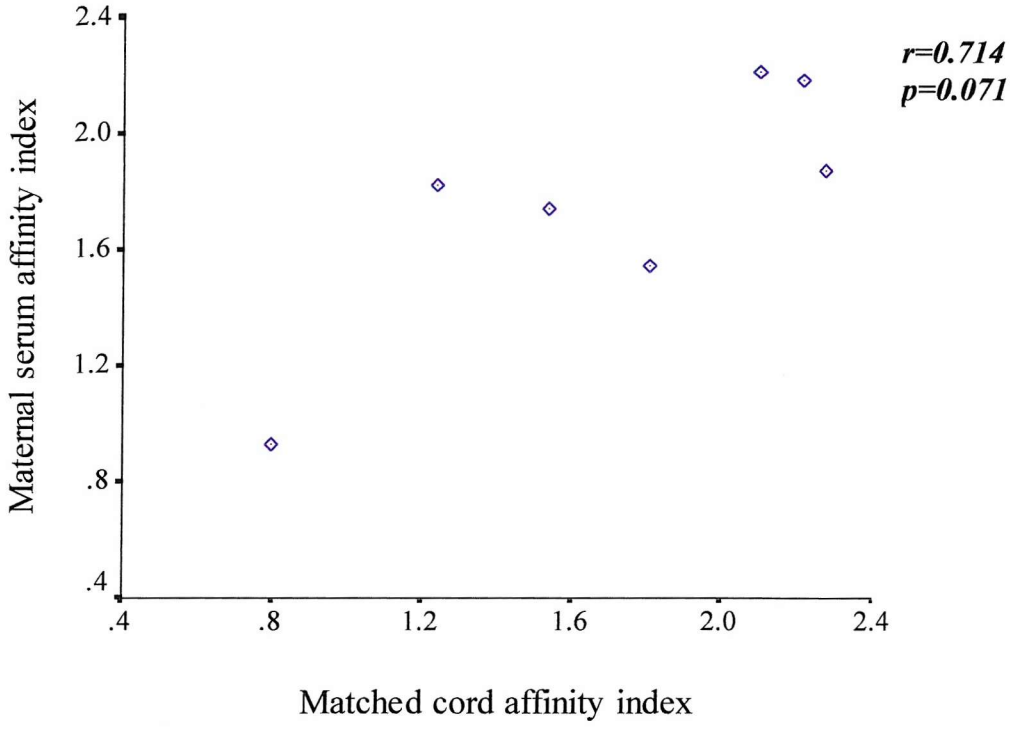


Figure 8.7 Serum relative avidity was measured by a modified ELISA using ammonium thiocyante elution (for method, see 8.2.3.3). Maternal and matched cord serum affinity indices were directly related, though failed to reach statistical significance.

8.4 Discussion

It is becoming increasingly clear that the allergic phenotype results from a complex interaction between many different processes. One of these processes is likely to be a regulatory effect of serum IgG. Earlier work in this thesis has identified a relationship between IgG concentration and epitope recognition (at least by the specific anti-ovalbumin antibodies of the detection *ELISA*) *(chapter 3)*. This study aimed to further unravel the immunomodulatory role of IgG by identifying if there were interindividual differences in functional affinity of circulating IgG and how these might relate to epitope recognition, and by inference, immune reactivity.

Serum samples without detectable ovalbumin were selected. A proportion of these inhibited antigen recovery when spiked with the protein. This observation provoked

the hypothesis that such sera might have a higher functional affinity for ovalbumin and hence were able to block epitope recognition.

The functional affinity of serum IgG for ovalbumin was estimated using a modified *ELISA* incorporating thiocyanate elution. This validated technique [395] has advantages over other methods by permitting measurement of the affinity of polyclonal antibodies - which recognise soluble antigens. However, it has not been used before in relation to food antigens. Interpretation relies on the chaotropic ion disrupting only the antigen-IgG complex and the optimisation experiments identified the importance of careful evaluation of this elution process. Non-specific elution of the coating antigen was found to depend on the volume of ammonium thiocyanate. The preliminary experiments thus permitted the development of a protocol whereby changes in absorbance could confidently be related to strength of antigen-antibody binding.

No direct correlation between serum OVA G concentration and functional affinity was observed, as has been reported for circulating rubella IgG [393] and for E Coli antibodies in serum and breast milk [394]. Indeed, there was a trend for an inverse relationship between the two, whereby sera with the highest OVA G concentrations had the lowest affinity indices *(figure 8.5)*.

In murine systems the mechanisms controlling antibody affinity are independent of those governing antibody levels [396]. These current data would support the existence of a similar mechanism in man. If there was a reciprocal control mechanism, which allowed for low antibody production to be compensated for by high functional affinity, then this could be postulated as conferring a survival advantage for the species by optimising pathogen clearance. Further work is needed to clarify this relationship.

The hypothesis was that sera which were able to block antigen recovery would have a higher functional affinity than would those that permitted recovery. In fact the reverse was found *(figure 8.6)*. Significantly higher functional affinity was associated with recovery positive sera. How might this be explained?

Serum IgG produced in response to an antigen is polyclonal in nature: - a number of clones produce and secrete antibodies of varying affinity for the antigen. The current method allows for estimation of the *average* avidity of the sample. Therefore, *recovery* – samples, though of lower average avidity, might well have clones of high affinity IgG in excess of the spiking dose, and so still be capable of binding to the exogenous OVA and inhibiting detection. A better indicator of avidity might have been the measurement of the affinity distribution, since this will take into account the heterogeneous nature of IgG affinity [390]. Of course, an alternative explanation might be that higher avidity IgG, under certain circumstances, promotes antigen detection. Recovery is dependent on the *in vitro* detection system encountering antigen as well as being able to bind to it. It could be argued that complexing the entire spiking OVA dose with specific IgG might increase the chances of the detector antibodies encountering antigen.

These data provide further evidence for a close mother-baby immunological dyad. Just as infant specific IgG at birth significantly correlated with maternal IgG concentration *(figure 5.5)*, so the evidence from this work was that the infants' IgG functional affinity correlated with that of their mother's *(figure 8.7)*. Thus, variations in the maternal immune system may modulate the developing immune response, with implications for immune reactivity - including antigen tolerance or sensitisation. In this study there are insufficient numbers to draw any conclusions regarding circulating IgG avidity and infant allergic outcome, though it is tempting to speculate that higher avidity promotes immune tolerance. At 6 months of age, no infant born to a *recovery* + mother (higher functional affinity) was atopic, as measured by positive skin testing, whereas 3/8 (37.5%) infants born to *recovery* – mothers were sensitised.

To conclude: in this work a rapid and simple method for measurement of antibody functional affinity has been developed. Significant differences between individuals were identified indicating that antibody avidity, as well as titre, should be considered when investigating the immunological role of IgG. These preliminary data were derived from a small number of subjects. It will be essential to increase the sample size in order to characterise fully how inter-maternal differences in specific IgG functional affinity, particularly in relation to a change in concentration induced by dietary intervention, relate to subsequent infant allergic outcome. The close

immunological interaction between mother and baby underlines the importance of research aimed at understanding the maternal factors regulating the immune response and how these may direct infant atopic phenotype.

Chapter Nine

IgG and subclass responses to egg sensitisation in the first 5 years of life

Chapter 9

IgG and subclass responses to egg sensitisation in the first 5 years of life

9.1 Aims

A role for serum IgG antenatally in the pathophysiology of infant atopy in at-risk pregnancies has been suggested by earlier work in this thesis. Maternal IgG may dictate the form in which allergen is presented to the fetus *(chapter 6)*, and may even determine whether the fetus is exposed to allergen at all *(chapters 3 & 8)*.

Postnatally, serum IgG continues to be implicated in the allergic process. High levels of specific IgG have been found in food allergic children [343] and adults [397], while raised egg specific IgG in infancy has been associated with an increased risk of developing elevated IgE to food [398] or inhalant [344] allergens in both high-risk [399] and unselected populations [344]. Such observations raise the possibility of a predictive value of IgG measurement for later IgE-mediated disease.

Measurement of IgG subclasses has given further information on the relationship between IgG and atopy. Significantly higher levels of IgG1 and IgG4 were measured in infants with raised IgE [400] and allergic disease [401][402], while Jenmalm *et al* identified differences in concentrations of specific G subclasses to ovalbumin, birch and cat allergen between atopic and non-atopic children [353].

Several studies have reported an association of raised egg IgG with later atopic disease [348][392], a relationship already noted for egg IgE sensitisation [403][404]. The early appearance, at 6 months age, of a dichotomous egg specific IgG and subclass pattern, between atopic and non-atopic children [353] is therefore encouraging of using IgG and IgG subclass measurement to predict later onset IgE-mediated disease.

The finding that infants with persistent sensitisation to foods had a 3-5 fold greater risk of developing allergic airways disease than transient food sensitisation was central to the work in this chapter [405]. Previous studies of humoral responses have not distinguished between these 2 categories. The aim of this investigation was

therefore to evaluate the pattern of ovalbumin IgG and subclass responses over the first 5 years of life according to egg sensitisation status and subsequent allergic phenotype.

9.2 Subjects, samples and methods

9.2.1 Subjects & samples

Plasma samples were available from an archive collection of blood specimens obtained from babies born to at least one atopic, asthmatic parent *(kind permission of Dr JA Warner) (2.2.5)*. Plasma samples were prepared from blood collected at birth (umbilical cord) and at 6 months, 1 year and 5 years of age.

Clinical outcomes of the babies, assessed at 6 months age, and annually between 1 and 5 years of age, were known. Children were classified as asthmatic if they had episodic (frequent or infrequent) or chronic wheeze. Non-asthmatic children were those with no respiratory symptoms, only cough or transient wheeze. Skin prick testing (SPT) to a panel of common dietary and inhalant allergens had been carried out at these assessments from 1 year of age. A weal diameter $\geq$ 2 mm, in the presence of appropriate negative and positive (histamine) controls, was considered to be a positive reaction.

A subset of the cohort was used for this analysis. Blood samples from children who were egg sensitive (ES) (n=21), and control samples from non-egg sensitive children (NES) (n=25) were selected. The egg sensitive group was further categorised into those with transient egg sensitivity (positive egg SPT at 1 year age only) (n=9) and those with persistent egg sensitivity (positive egg SPT for at least 2 years) (n=12). The control group was randomly selected based on subjects with a complete set of blood samples at the 4 time points. They comprised children who had at least one positive SPT to an allergen other than egg at any time point (n=14) (atopic, non-egg sensitive) and a group who never had a positive SPT (n=11) (non-atopic, non-egg sensitive).

9.2.2 Laboratory methods

Plasma ovalbumin IgG (OVA G), IgG1 (OVA G1) and IgG4 (OVA G4) concentrations were measured by in-house indirect *ELISAs*, developed and optimised as previously described *(2.5.1 & 2.5.2)*.

Sample dilutions varied according to the *ELISA* being performed *(table 9.1)*.

Table 9.1 Standard & sample dilutions for ovalbumin IgG & G subclass ELISAs.

OVA *ELISA*	**Concentrations of human IgG calibrator serum (Binding site)**	**Sample dilution**
IgG1	0.39 μg/ml –100 μg/ml IgG1	1:100
IgG4	0.16 μg/ml –20 μg/ml IgG4	1:50
IgG	0.73 μg/ml –188 μg/ml IgG	1:100

Samples with absorbances above the reference curve were repeated at higher dilution such that the absorbance fell on the linear part of the reference curve. Samples from an equal number of egg-sensitive and non-egg sensitive subjects were run on the same day. Where possible, all measurements for an individual sample were carried out at the same analysis.

9.2.3 Calculation of specific IgG and IgG subclass concentrations

The mean absorbance for the standards and samples obtained from the BSA-coated control plate was subtracted from the mean absorbance obtained from the allergen-coated plate. A reference curve of standard absorbance versus concentration was then constructed, on which the standard serum concentrations of ovalbumin (total) IgG and IgG subclasses were denoted as arbitrary units (AU). Sample absorbance was compared to the reference curve and concentration of OVA G and subclasses expressed in AU, after taking into account the dilution of the sample.

9.3 Results

Ovalbumin IgG concentration of the whole group fell between birth and 6 months age (p=0.036, Wilcoxon), rose again by 1 year age (p=<0.001, Wilcoxon) and remained elevated at 5 years age *(figure 9.1)*.

Figure 9.1 Change in ovalbumin IgG concentration over the first 5 years of life.

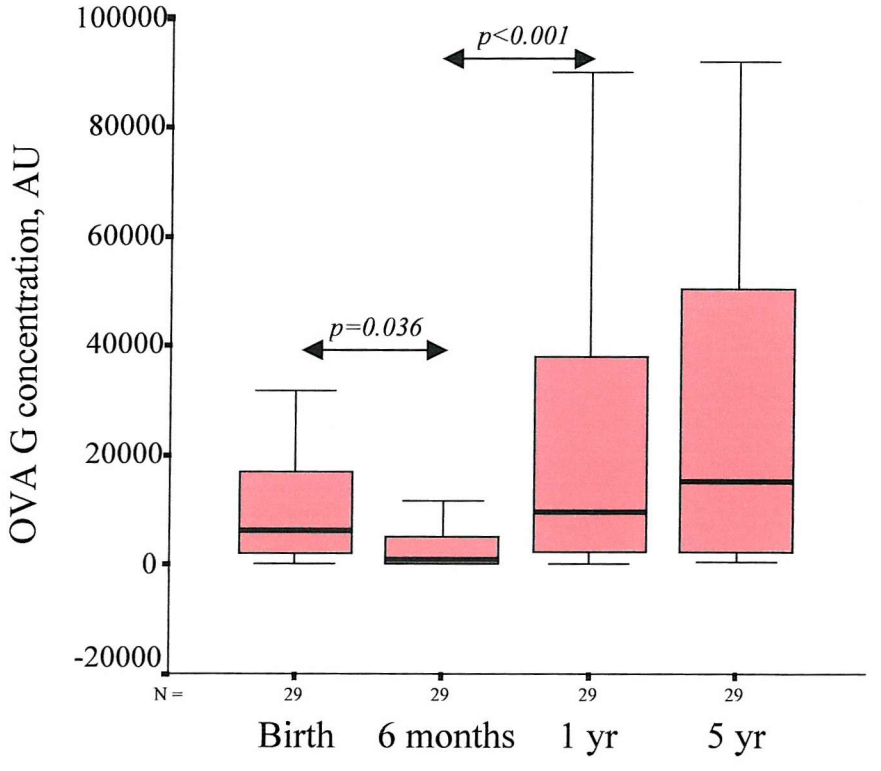


Figure 9.1 Plasma OVA G concentration was measured by indirect ELISA (2.5.1) at 4 time points through the first 5 years of life in selected samples from a birth cohort at genetic risk of atopy. The measurements revealed that the concentration fell to a nadir at 6 months, rose by 1 year and remained elevated at 5 years of age.

However, on analysing OVA G kinetics according to egg sensitivity status, two different patterns emerged depending on whether the children were egg SPT positive or not. Only the non-egg sensitive group displayed the previous pattern of fall in concentration from birth to 6 months (p=0.015, Wilcoxon), rise by 1 year (p=<0.001, Wilcoxon), continuing to 5 years (p=0.048, Wilcoxon). The egg sensitive group had no significant change in OVA G concentration from birth to 6 months, and concentration peaked at 1 year of age (p=0.003, Wilcoxon) *(figure 9.2)*.

Figure 9.2 Change in ovalbumin IgG concentration from birth to 5 years according to egg sensitivity status.

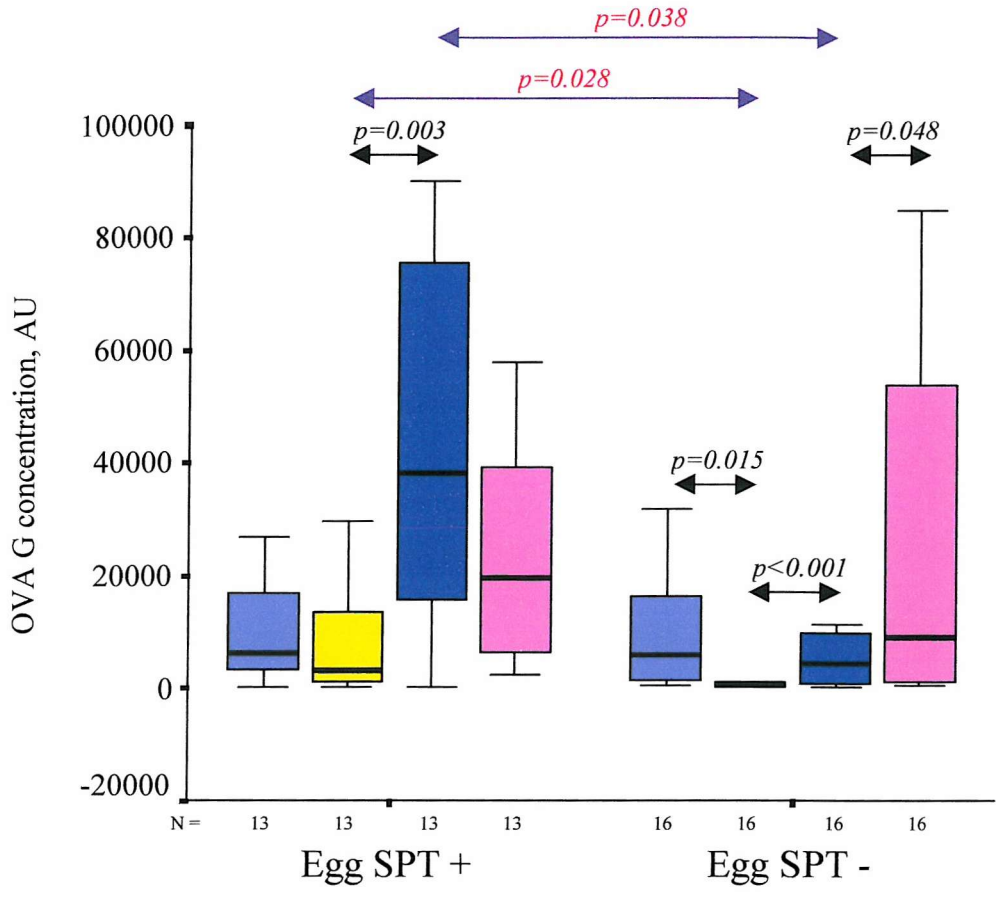


Figure 9.2 Ovalbumin IgG concentration was measured at birth (mauve boxes), 6 months (yellow boxes), 1 year (blue) and 5 years (pink) of age in selected samples collected from egg sensitive (ES) (Egg SPT +) and non-egg sensitive (NES) (Egg SPT -) children. A difference between the categories was observed: only Egg SPT - children had a drop in concentration from birth to 6 months, OVA G rising by 1 year and again at 5 years of age. Egg SPT + children had no decline in OVA G in the first 6 months and concentration reached a peak at 1 year of age. Egg SPT + children had significantly higher OVA G concentration at 6 months and 1 year than had Egg SPT - children.
The chart colour coding above for ovalbumin IgG and subclass concentration measurements at the 4 time points through the 1st 5 years of life is continued in all subsequent figures.

Measurement of ovalbumin IgG1 concentration at the 4 time points revealed a similar pattern to OVA G, though the fall in OVA G1 concentration from birth to 6 months of age in the non-egg sensitive group failed to reach significance in this analysis *(figure 9.3)*. Ovalbumin IgG4 concentration, for both ES and NES children, was significantly lower than OVA G1 at all time points (ES & NES: $p<0.001$, Wilcoxon). Both groups showed a significant fall in OVA G4 concentration from birth to 6 months (ES & NES: $p=< 0.001$, Wilcoxon) and rise by 1 year (ES: $p=0.005$, NES: $p=0.05$,

Wilcoxon), which reached a maximum at 5 years of age (ES & NES: p=0.001, Wilcoxon) *(figure 9.4)*.

Figure 9.3 Change in ovalbumin IgG1 concentration from birth to 5 years according to egg sensitivity status.

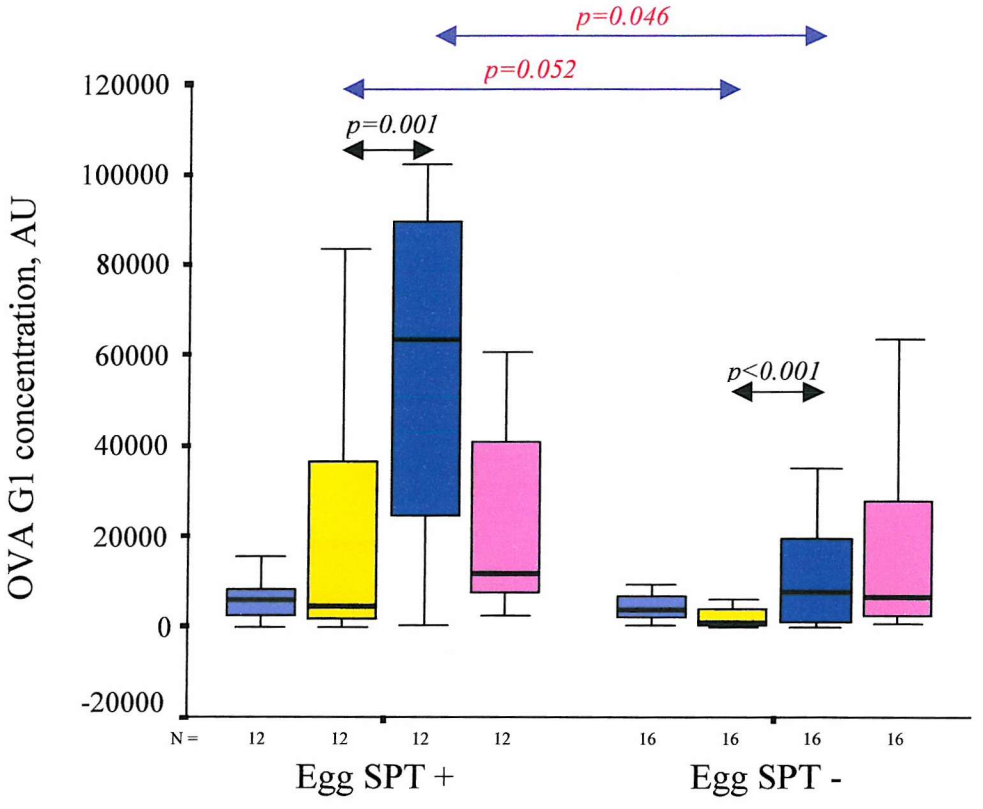


Figure 9.3 Ovalbumin IgG1 concentration was measured by indirect ELISA (2.5.2) at 4 time points through the 1st 5 years of life in selected samples from egg sensitive (ES) (Egg SPT +) and non-egg sensitive (NES) (Egg SPT -) children. The measurements revealed that the profile differences between ES and NES children mirrored those of OVA G (figure 9.2). ES children had significantly higher OVA G1 concentration at 6 months and 1 year than had NES children.

Figure 9.4 Change in ovalbumin IgG4 concentration from birth to 5 years according to egg sensitivity status.

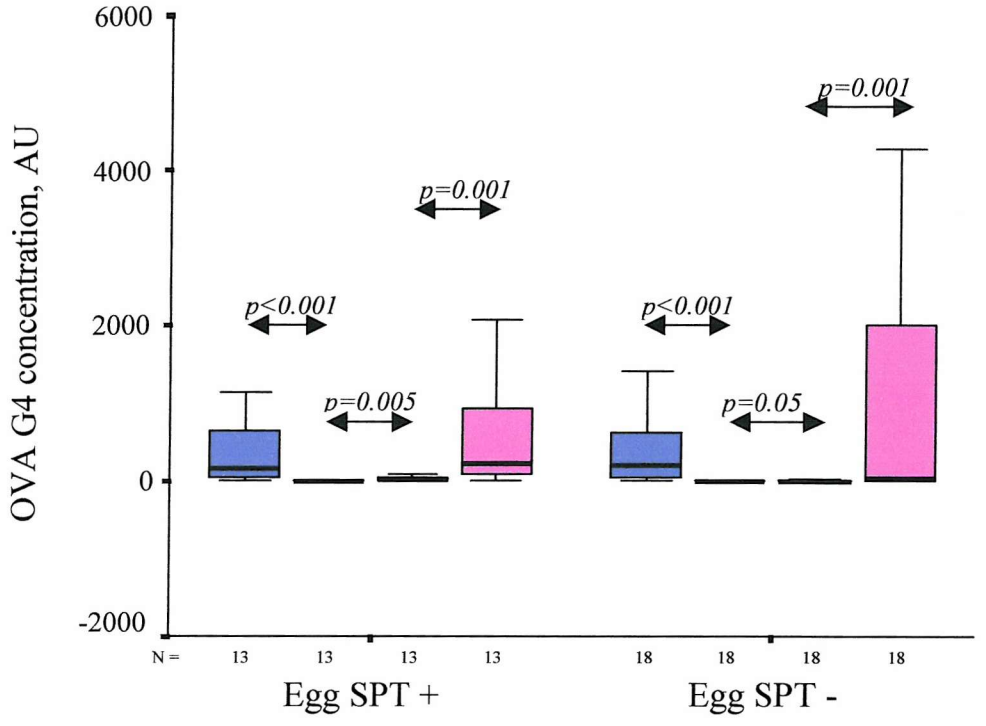


Figure 9.4 Plasma ovalbumin IgG4 concentration was measured by indirect ELISA (2.5.2) at 4 time points from birth till 5 years of age in samples selected from egg sensitive (ES) (Egg SPT +) and non-egg sensitive (NES) (Egg SPT -) children at genetic risk of atopy. In contrast to OVA G and G1, the measurements revealed that the OVA G4 profile was the same for both groups – namely, there was a fall in concentration over the first 6 months of life followed by a continued rise to 5 years of age.

OVA G concentration was significantly higher at 6 months (p=0.028, Mann-Whitney) and at 1 year of age (p=0.038, Mann-Whitney) in egg sensitive children compared to non-egg sensitive children *(figure 9.2)*. Similarly, OVA G1 concentration was significantly higher in this group at 1 year (p=0.046, Mann-Whitney), with a strong trend for a higher concentration at 6 months age (p=0.052, Mann-Whitney) *(figure 9.3)*. In contrast, OVA G4 concentration showed no significant differences between the two groups at any of the 4 time points.

Humoral responses were further evaluated according to the four categories of ES and NES subjects, namely, transiently egg sensitised (transient ES), persistently egg sensitised (persistent ES), atopic but not egg sensitised (atopic, NES) and non-atopic & not egg sensitised (non-atopic, NES).

The profiles for OVA G and G1 were similar *(figure 9.5)*.

Persistent ES children: from birth to 6 months there was no significant change in OVA G concentration, whilst OVA G1 rose significantly (p=0.028, Wilcoxon). At 6 months age this group had significantly higher OVA G and G1 concentrations than the non-atopic, NES category (p=0.001 & p=0.005, Mann-Whitney, respectively). Concentrations continued to rise from 6 months to 1 year of age (OVA G, p=0.017; OVA G1, p=0.008, Wilcoxon), and at 1 year were higher than the concentrations measured in all the other categories (Transient ES: OVA G, p=0.021; OVA G1, p=0.051. Atopic NES: OVA G, p=0.043; OVA G1, p= 0.026. Non-atopic NES: OVA G, p=0.001; OVA G1, p=<0.001. All Mann-Whitney). OVA G and G1 concentrations of the persistent ES group peaked at 1 year of age.

Transient ES and atopic NES children: these categories showed similar patterns of ovalbumin IgG and G1 responses. There was no significant change between birth and 6 months, while concentrations rose at 1 year (OVA G: transient ES: p=0.091; atopic NES: p=0.001, Wilcoxon. OVA G1: transient ES: p=0.043; atopic NES: p=0.004, Wilcoxon), remaining elevated at 5 years of age. There were no significant differences in OVA G or G1 concentrations between these categories at any time point. Transient ES had higher OVA G concentration at 6 months of age compared to non-atopic NES children (p=0.023, Mann-Whitney) but had no significant differences in OVA G1 concentration. Atopic NES children had significantly higher ovalbumin IgG and IgG1 concentrations than non-atopic NES children at 6 months (OVA G, p=0.001; OVA G1, p=0.014, Mann-Whitney) and 1 year of age (OVA G, p=0.014; OVA G1, p=0.021, Mann-Whitney).

Non-atopic NES children: by contrast this group had a significant fall in OVA G concentration (p=0.005, Wilcoxon), not observed in the other categories, and a trend for a fall in OVA G1 (p=0.091, Wilcoxon), from birth to 6 months. As was consistently observed, these specific immunoglobulins rose by 1 year (OVA G & G1 p=0.028, Wilcoxon) and remained elevated at 5 years of age.

Figure 9.5 Change in ovalbumin IgG & IgG1 concentrations according to sensitisation category.

Figure 9.5(a) Ovalbumin IgG concentration.

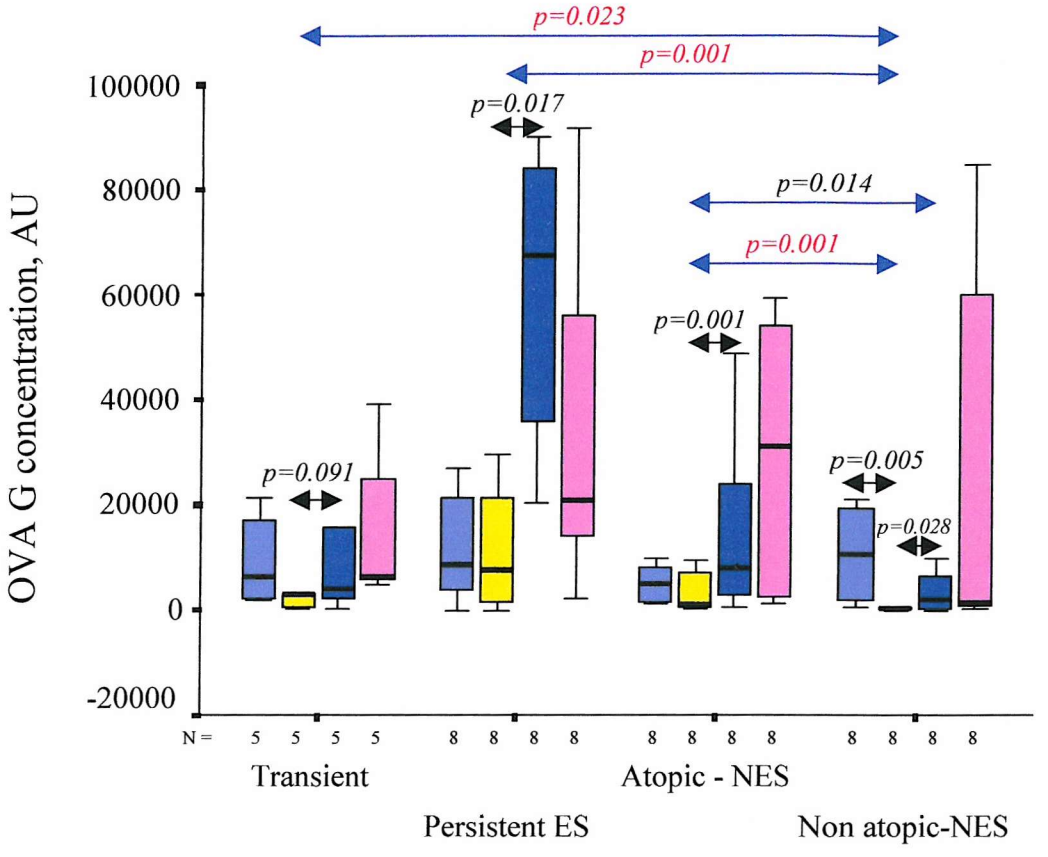


Figure 9.5(b) Ovalbumin IgG1 concentration.

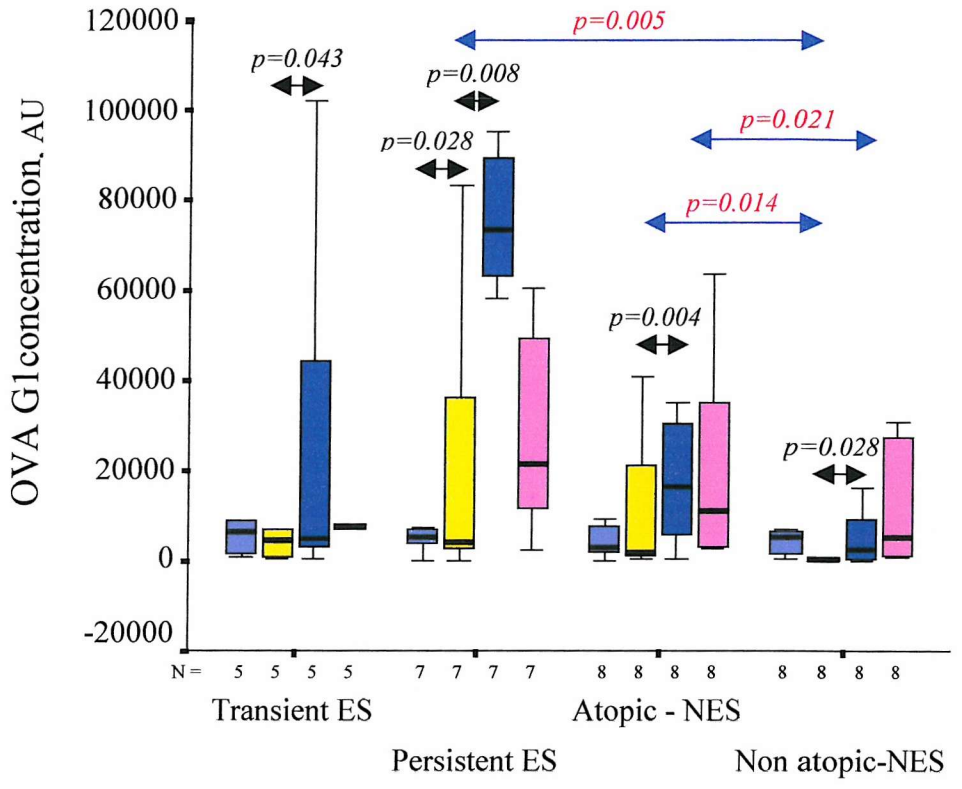


Figure 9.5(a) &(b) Ovalbumin IgG & IgG1 measurements (method 2.5.2) across the 1st 5 years of life were compared in the 4 atopic categories. OVA G and OVA G1 had similar profiles within each category. A particular difference noted was a fall in concentration from birth to 6 months age in the non-atopic NES group compared to a rise in the persistent ES group. Also, the persistent category had peaks in OVA G & G1 concentrations at 1 year of age and had significantly higher levels than the other categories at this time point.

The change in OVA G4 concentration across the 5-year period was similar irrespective of atopic category: there was a significant drop from birth to 6 months (p=0.002 – p=0.018, Wilcoxon), and significant rise between 1 and 5 years of age (p=0.012 – p=0.043, Wilcoxon). In addition, the persistent ES subjects showed a significant rise in concentration between 6 months and 1 year of age (p=0.015, Wilcoxon). No significant differences in OVA G4 concentration were observed between the study categories, except at 1 year of age when persistent ES subjects had a higher ovalbumin IgG4 concentration than non-atopic NES subjects (p=0.022, Mann-Whitney) *(figure 9.6)*.

Figure 9.6 Change in ovalbumin IgG4 concentration according to sensitisation category.

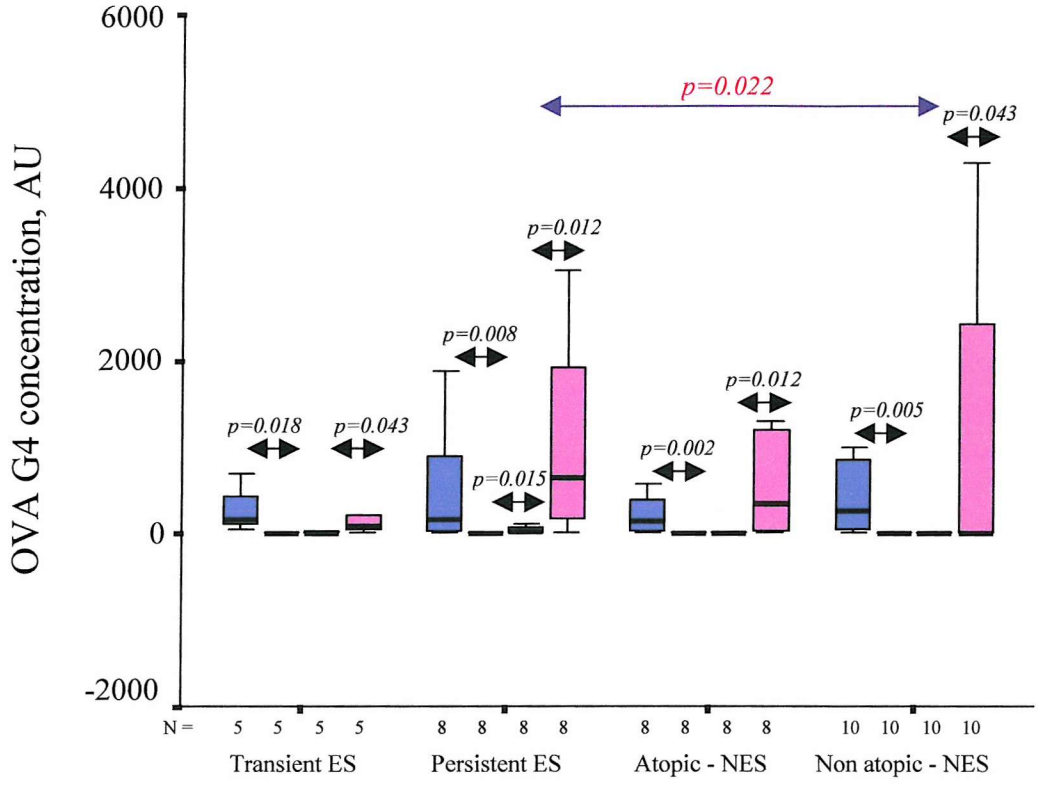


Figure 9.6 Ovalbumin IgG4 measurements (method 2.5.2) were compared in the 4 atopic categories. The OVA G4 profile was the same in all groups: OVA G4 concentration fell from birth to 6 months, rose by 1 year and again by 5 years of age. Only the persistent ES group had a significantly higher concentration than the non atopic–NES group at 1 year of age.

Asthmatic outcome at 5 years of age was analysed with respect to ovalbumin IgG and subclass concentrations. No association between OVA G or OVA G4 concentrations and asthma was observed, but significantly higher concentrations of plasma ovalbumin IgG1 were found at birth (p=0.049, Mann-Whitney), 1 year (p=0.026, Mann-Whitney) and 5 years of age (p=0.036, Mann-Whitney) in children who were subsequently diagnosed with asthma *(figure 9.7)*. At these time points no significant difference in OVA G1 concentration between asthmatic and non-asthmatic children of the four individual atopic categories was observed.

Figure 9.7 Ovalbumin IgG1 concentration according to asthmatic outcome.

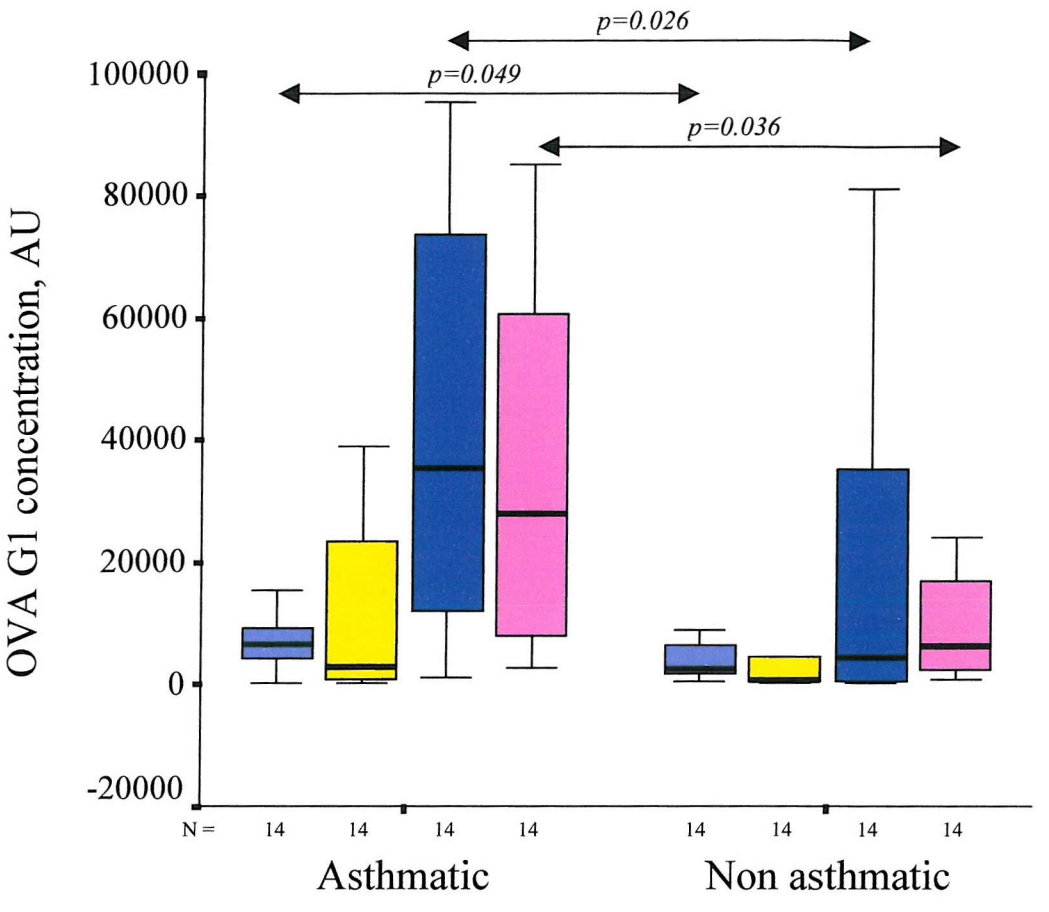


Figure 9.7 Ovalbumin IgG1 concentration (method 2.5.2), measured through the 1st 5 years of life in samples selected from children at genetic risk of atopy, was analysed with regard to asthma outcome (defined in 9.2.1). Those children who developed asthma had a significantly higher OVA G1concentration at birth, 1 year & 5 years of age compared to those who did not develop asthma.

A logistic regression model was constructed for the maximum likelihood estimation of the presence or absence of asthma (dichotomous variable) with OVA G1 concentration (continuous linear effect). Measurements at 1 year of age were chosen since at this time point inter-category differences were most commonly seen.

The model showed that an OVA G1 concentration of 14,500 AU predicted asthma ($p=0.017$, Chi^2) *(table 9.2)* with a sensitivity of 64% and specificity of 74%, and an area under the receiver operator characteristics (ROC) curve of 70.3% *(figure 9.8)*.

Figure 9.8 ROC curve: asthma outcome by ovalbumin IgG1 concentration at 1 year of age.

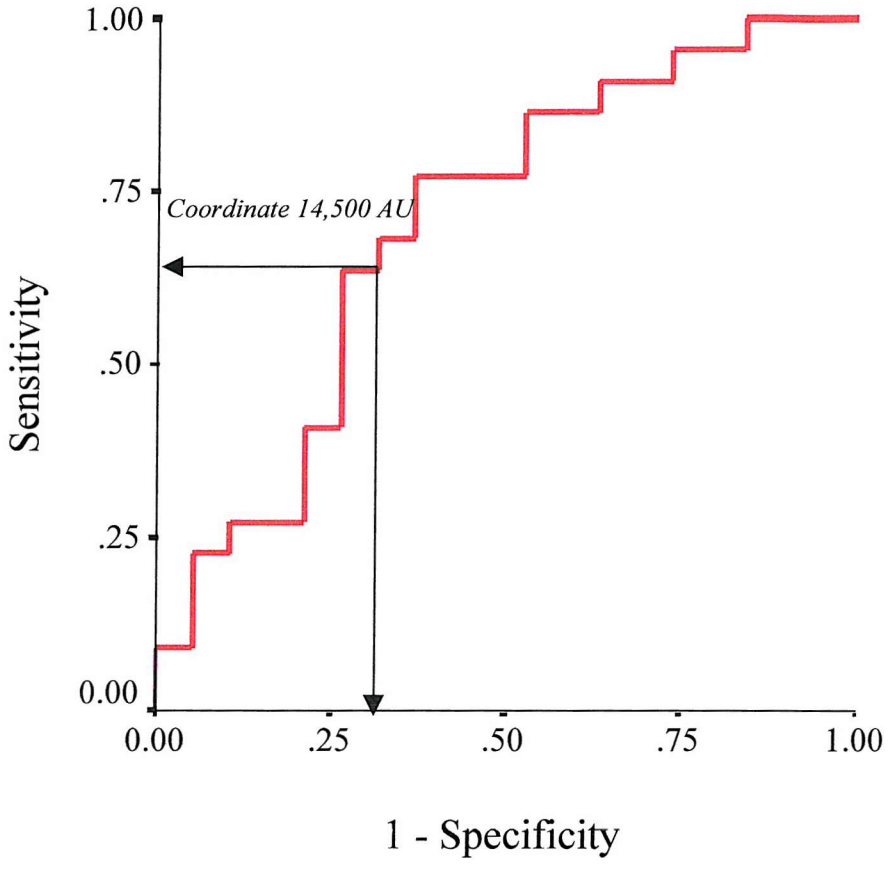


Figure 9.8 An ROC curve was plotted for asthma outcome as a dichotomous variable and ovalbumin IgG1 at 1 year of age as a continuous linear effect. This plot of sensitivity (true positive) against 1-specificity (false positive) enabled selection of an arbitrary cut-off value of subclass concentration for the diagnosis of asthma. A value of at least 14,500 AU allowed for the prediction of asthma with a sensitivity of 64% and specificity of 74%, and area under the curve of 70.3%.

Table 9.2 Asthma outcome according to OVA G1 at 1 year of age.

		Asthma		
		YES	NO	Total
OVA G1	>14500 AU	14	5	19
	<14500 AU	8	14	22
	Total	22	19	41

p=0.017

9.4 Discussion

This study has evaluated retrospectively IgG and IgG subclass responses to the common dietary allergen, hen's egg ovalbumin, over the first 5 years of life in a group of egg sensitive and non-egg sensitive children followed up to monitor the development of asthma.

Consistent with other studies [339][398], OVA G concentration of the cohort fell from birth to 6 months age, reflecting the natural attrition of transplacentally acquired maternal antibody. Concentrations rose again by 1 year, most likely due to the introduction of egg into the infants' diet. However, on analysing the data according to egg skin sensitivity status, this predictable pattern was only observed for non-egg sensitive subjects, in particular, non-atopic, non-egg sensitive children. For the remaining categories OVA G concentration remained static over the first 6 months of life, an observation that can only be accounted for by the endogenous production of ovalbumin IgG. The infant weaning practices of this cohort were not monitored, but as a group at high-risk of allergy, delayed introduction of egg into the infants' diet was advised. In the absence of ingested egg, exposure to ovalbumin, with B-cell activation, could have occurred through maternal sources, either antenatally or via breast milk *(chapter 4)*, or by adventitious contact with the antigen, for example, during food preparation [300].

Ovalbumin IgG1 and IgG4 subclass concentrations were also measured. Evidence suggests that IgG1 is T_H1, and IgG4 T_H2 - dependent: *in vitro* studies on mitogen-stimulated human lymphocytes have shown that IL-4 stimulated, and IFN-γ inhibited IgE and IgG4 synthesis [406][407], while birch allergen induced expression of IL-4 correlated with serum specific IgG4, and IFN-γ secretion with IgG1 responses [408]. Measurement of these 2 subclasses therefore facilitated assessment of IgG responses in terms of T_H1 / T_H2 deviation.

The kinetics of IgG1, the predominant IgG subclass, followed IgG closely with concentrations reaching a maximum by 1 year. Ovalbumin IgG4, present in significantly smaller quantities than specific IgG1, fell to a nadir at 6 months and rose

to maximum concentrations much later - at 5 years of age - a pattern also observed by Jenmalm et al [353], and which may be explained by delayed maturation of IgG4 production [409].

Differences in IgG responses between egg sensitive and non-egg sensitive children have been reported in earlier studies [348][392], but none of these have distinguished between transient and persistent egg sensitisation. In this study clear differences in the pattern, and quantity, of IgG subclass responses were observed between these categories.

The most striking differences were for ovalbumin IgG1. A significant rise in OVA G1 concentration from birth to 6 months age was unique to the persistently egg sensitised group. At 6 months age, these subjects had higher OVA G1 concentration than the non-atopic, non-egg sensitive children, and at 1 year of age had higher levels than all the other categories. In contrast, the transiently egg sensitised group showed no difference in OVA G1 kinetics from the atopic, NES group and no difference in quantities at any time point from non-egg sensitive children.

Somewhat surprisingly ovalbumin IgG4 showed little difference between the atopic categories, but other authors have also found food-specific IgG1 subclass antibodies to discriminate better between IgE-sensitised and non-sensitised children than IgG4 antibodies [399]. Only the persistently egg sensitive children had higher levels compared with the non-atopic, non-egg sensitive children at 1 year of age.

Taken together, these data have shown that persistently egg sensitised children have a distinctive pattern of ovalbumin IgG responses, with enhanced production of both IgG1 and IgG4 subclasses, particularly at 1 year of age. This enhanced humoral responsiveness could be caused by the increased promotion of immunoglobulin synthesis by T_H2 cells [410], which characterise the atopic phenotype. Such a relationship of atopy with antibody production was also suggested by the current observation that atopic children, even though non-egg sensitive, had higher ovalbumin IgG1 concentrations than had non-atopic children.

The clinical relevance of this finding is that egg skin sensitisation could be classified as transient or persistent, with major implications for stratification of risk of allergic disease [405], by concurrent consideration of IgG humoral responses. The numbers in this study were limited and therefore clinical extrapolation must be guarded. However, the benefit for asthma prevention implied by these observations demands further work with increased subject numbers.

Children diagnosed with asthma had significantly higher levels of ovalbumin IgG1 than non-asthmatics at birth, 1 year and 5 years of age. This concurs with a Swedish study [353] which reported atopic symptoms in the first 8 years of life to be associated with raised ovalbumin IgG1 (also IgG and IgG3) concentration at 6 months of age. Since the majority of childhood asthma is atopic in nature [411][412], and these data have shown atopy to be associated with a raised ovalbumin IgG1 concentration in the first year of life, the observation was not unexpected.

Since raised cord blood IgE is not a sensitive predictor of later atopy [100][98][407], finding a more reliable marker of atopy would be of considerable benefit for implementation of secondary allergy prevention strategies. The association of high OVA G1 concentration and asthma is therefore of particular interest as a potential early marker for the later onset of respiratory allergic disease. Logistic regression analysis revealed that OVA G1 measurement (in excess of 14,500 AU) may predict the subsequent development of asthma with a sensitivity of 64%, a predictive performance that outranks that of (total) IgE measurement, either at birth reviewed in [413] or in infancy [414]. These data did not include measurement of specific IgE concentrations, though many studies have reported circulating IgE to be associated with high levels of IgG subclasses [343][400][415]. Since raised egg specific IgE is itself a risk factor for later atopic disease, it is possible that measurement of specific IgG and subclasses in conjunction with specific IgE may offer a useful serological adjunct to secondary allergy prevention strategies.

Chapter Ten

General discussion and plans for further work

Chapter 10

General discussion and plans for further work

10.1 Overview

In an era when the prevalence of atopic disease has been steadily increasing, related research has been directed towards an understanding of the events that regulate the allergic cascade. Current treatments are aimed primarily at alleviating the symptoms of established disease and even the successful prevention of asthma with the use of the histamine receptor antagonist cetirizine was described only for a subgroup of children who already had moderately severe atopic dermatitis [416]. The ultimate therapeutic targets for allergy must therefore be the events resulting in the initiation and propagation of the allergic cascade. Much evidence points to early life as a critical time period during which allergic sensitisation occurs, to be followed months or years later by organ-specific disease. As allergic sensitisation is a major risk factor for the development of atopic disorders, probing the mechanism by which allergic sensitisation occurs will be critical to the search for new therapeutic options.

The fundamental aim of the work presented in this thesis was to broaden our limited understanding of how the maternally-derived antenatal and postnatal environments might programme the atopic phenotype. The recognition that fetal T-cells have the capacity for antigen-specific priming by 22 weeks of gestation in a cytokine milieu that is biased towards T_H2 immune responsiveness raised the question of whether *in utero* allergic sensitisation might occur. And while debate reigns as to the relation of immune responses at birth with later clinical outcome, there is still little known about the mechanism of immunological priming. An essential requirement for T-cell priming is antigen exposure. Maternally-derived food allergens have been detected in breast milk, but until recently, demonstration of antigen in the pregnancy-associated environment has been elusive. Dietary egg is a common allergenic food in infancy and notably egg sensitisation and allergy have implications for later, and possibly lifelong, allergic disease. Therefore, this work sought to fully characterise exposure to the egg allergen ovalbumin in early life and to examine how the nature of allergen exposure, in the context of maternal dietary intake through pregnancy and lactation,

might modulate the development of infant atopy. These data therefore also offer the opportunity to rationalise dietary intervention strategies as a means of primary allergy prevention.

10.2 Ovalbumin detection - methodology

A study of early life ovalbumin exposure and its relation to subsequent infant atopic phenotype required unequivocal measurement of the protein. Enzyme-linked immunosorbent assays have the benefit of being able to quantify specific antigen in a sample, and previous authors have reported successful detection of ovalbumin by this method [256][417][418]. However in this study antigen detection was complicated by the need for maximum sensitivity as the samples were derived from the pregnancy-associated environment and reflected only a normal maternal dietary intake. After painstaking assay development, including extensive validation experiments *(chapter 3)*, an *ELISA* with a threshold for OVA detection of 15 pg/ml was established, making this method almost 10 times more sensitive than other published *ELISA* systems [256][417][418].

Future directions

The developed assay offers a simple, cheap and reliable method for ovalbumin detection in pregnancy-associated biological fluids. However, while the sensitivity of this *ELISA* surpasses that of any previously reported assays, additional valuable information could be obtained by a further lowering of the detection threshold. The sensitivity of a standard *ELISA* can be improved by the inclusion of immuno-polymerase chain reaction (Immuno-PCR). Immuno-PCR employs PCR from a secondary antibody complexed to a PCR-target DNA to detect the specific binding of a primary antibody to its antigen immobilised in a well. Immuno-PCR thus combines the amplification power of PCR with the antigen specificity of standard *ELISA*, allowing the detection of antigens down to a single molecule level [419]. In our department a project is underway to develop an *ELISA*-PCR technique for the detection of peanut allergen in the same samples used in this study. The impact of

such minute allergen exposures in early life on atopic sensitisation and disease expression is awaited with interest.

10.3 Early life exposure to ovalbumin and its relation to allergic disease

The *ELISA* developed for ovalbumin detection was employed to address the question of whether the fetus/newborn is directly exposed to this egg allergen and to characterise when, where and how this exposure may occur.

The primary encounters of the immune system with allergen are crucial in determining sensitivity to that allergen, especially in those with an atopic genotype [420]. For most people the immune system is tolerant of soluble protein antigens, such as ovalbumin, while pathogenic stimuli, such as invasive microorganisms, elicit protective active immunity. Dysregulation of tolerance underlies IgE-mediated allergic disease: the allergic phenotype is characterised by the expression of T_H2-skewed immunity towards antigens present in the normal environment, whereas non-allergic individuals display low level T_H1-skewed immunity to the same antigens [421], a response perceived as conferring protection against T_H2-dependent allergic sensitisation. The questions pertinent to the aetiology of allergy therefore relate to how these alternate forms of T-Helper memory are imprinted, and in particular, how the potentially pathogenic T_H2-skewed memory is selected in individuals with a genetic predisposition to atopy [422]. The fetal immune response to antigens is dominated by T_H2 cytokines [197], consistent with the T_H2 milieu of the intrauterine environment [68][71][423]. This means that newborns [97], and in particular infants with an atopic genotype [205][345][424], already favour T_H2 immune responses, which, as described for animal models [425], may be capable of deviating subsequent immune responses towards the selection of potentially polarised T_H2 memory. Therefore factors determining tolerance to allergens may operate in early life and consequently it is possible that the route, dose, form and timing of allergen exposure during this time may predispose to T_H2 imprinting with the development of long term T_H2-skewed allergen-specific immunological memory.

In this study ovalbumin was found in umbilical cord blood, amniotic fluid and breast milk of pregnant and breast-feeding women, confirming for the first time direct fetal exposure to a dietary allergen in humans *(chapter 4)*. Thus, these data suggest three routes of transmission of maternally-derived antigen to her offspring – antenatally via transplacental and transamniotic pathways and postnatally via breast milk. A fourth route of passive antigen transfer was suggested by the measurement of ovalbumin in a small number of infant sera at 6 months of age. While this observation may have been due to inadvertent egg ingestion, the finding could otherwise have occurred by transcutaneous antigen transfer, since in animal experiments primary sensitisation to both inhalant and food allergens has been shown to occur through the skin [426].

Ovalbumin was found in blood samples from around 20% of pregnant women. These blood samples had been collected at time points through the day that were convenient to the subjects and which were unrelated to the timing, or nature, of their last meal. Since food antigen detection has been shown to be optimal 2-3 hours after a test meal [302], it is therefore likely that this observation underestimates the true prevalence. Moreover, the significant association of circulating maternal OVA throughout pregnancy with circulating infant OVA at birth consequently suggests that early life antigen exposure may be a common experience, as has been intimated previously by the ubiquitous nature of allergen-specific cord T-cell proliferative responses [225].

An association of allergen-specific immune responsiveness at birth with later atopic disease has been reported by a number of authors [198][207][208], though most recently the significance of proliferative responses for the development of allergy has been contested [203][324]. Likewise, antenatal dietary exclusion of common allergenic foods by women with a family history of atopy has been regarded previously as an unsuccessful primary allergy prevention strategy [309]. The implication of these latter conclusions is that fetal allergen exposure has limited relevance for the atopic phenotype. However, by analysis of the current results with regard to two important immune determinants, namely maternal atopy and the pattern of early life allergen exposure as modulated by maternal dietary egg elimination, the data presented in this thesis challenge such an assumption.

For the infants of the dietary intervention study, the prevalence of atopy at 6 months of age was not significantly different amongst babies born to egg avoiding mothers as control mothers. However, babies with evidence of exposure to ovalbumin, either antenatally via the placenta or postnatally via breast milk, were significantly more likely to express an atopic phenotype if allergen exposure occurred in the context of dietary exclusion and/or maternal atopy.

These observations have 3 main implications: firstly, that maternal atopy may create an environment in pregnancy that promotes infant allergy; secondly, that dietary exclusion does not eliminate completely antigen exposure and thirdly, that the alterations in environmental antigen exposure consequent on dietary manipulation may interact with maternal atopy to direct allergic programming.

The atopic mother has been shown to have a particular role in the processes that regulate the allergic cascade: for example, the genetic inheritance of IgE responsiveness was reported to be transmitted through the mother only [427] and infants born to an allergic mother had a higher IgE at birth [428] and a higher risk of developing atopic dermatitis [328], persistent wheeze [429], asthma and hayfever [430] than those born to an allergic father. In these data exposure to ovalbumin via breast milk from an atopic mother was a risk factor for expression of an atopic phenotype at 6 months of age. Likewise, antenatal OVA exposure in the context of maternal atopy and dietary manipulation was significantly associated with later infant allergy. The relationship of maternal atopy with infant outcome might relate to an increased chance of antigen exposure – atopic mothers avoiding dietary egg were more likely to have detectable OVA, and in greater quantities, in their breast milk than their non-atopic counterparts. Similarly, atopic women had a higher concentration of serum OVA throughout pregnancy than did non-atopic subjects, and this was not the result of a higher egg intake during the study period (personal communication, KE Grimshaw). However, there are many 'environmental' factors of the atopic mother that might interact with allergen exposure in early life to promote allergic sensitisation. Among these is the prevailing cytokine milieu. Amniotic fluid and breast milk are both rich sources of cytokines, which serve an essential and formative role in the development of the immune system [431][432][433]. Since the cytokine profile may fundamentally determine whether pregnancy succeeds [70], it is easy to suppose

that subtle perturbations of the cytokine balance may mediate altered reactivity of the developing immune system towards allergens encountered in early life.

Mechanisms of tolerance - namely, the state of unresponsiveness towards non-pathogenic antigens, such as food antigens - are not well understood, but have been proposed to include functional, or actual, elimination of the T-cell (anergy or deletion respectively), or alternatively immune deviation by the induction of regulatory cells or mediators that modify the immune response [434]. Animal models have suggested that the mechanisms are dose-related, such that exposure to single high doses induces suppression of virtually all responses by clonal anergy [282][435], whereas multiple low doses are more likely to generate regulatory cells with consequently more potential for inter-individual variation of response [282]. Of note, very low doses of antigen prime the animal for subsequent systemic and local immune responses [326]. A further factor is the frequency of antigen exposure - continuous exposure of mice to ovalbumin via drinking water induced more profound tolerance, irrespective of the total dose administered [327]. Application of these factors to the current study could therefore explain the observed paradox of atopic programming despite rigorous prophylactic egg avoidance. A dietary exclusion regime is difficult to sustain, particularly for a prolonged period. The women actively excluding egg in this study were a highly motivated group, but even so recorded unintentional accidental ingestion of egg. It is therefore likely that the infants of these women experienced very low dose, intermittent exposures to ovalbumin in early life compared to consistent and/or higher dose exposures of their control counterparts, a pattern predisposing to a sensitising immune response.

Not all babies with an atopic genotype develop allergic disease and this observation is supported by the current work. The babies of this cohort who developed an atopic phenotype were those who had experienced an immunological triad comprising the maternal atopic influence, antigen exposure and a pro-sensitising pattern of antigen contact.

These data have not only increased our understanding of the mechanisms underlying allergic sensitisation, but they also have implications for national health policy. Previous trials of dietary allergen exclusion in pregnancy and lactation have reported

either no allergic benefit [271][272], or only a temporary benefit of the intervention for the infant [273][336]. However, in these earlier studies the primary outcome measure was simply the prevalence of infant atopy according to study group. The more detailed examination of the current results has suggested that maternal dietary manipulation may actually have an *adverse* consequence for infant allergy, an outcome that would not have been revealed by simple 2x2 contingency analysis. Whilst the subgroup of this cohort at increased risk of atopy was relatively small, currently a Department of Health (UK) programme actively advises peanut avoidance by pregnant and breast-feeding women with a family history of atopy. With the comprehensive antenatal care offered today, few pregnant women will not be aware of these guidelines and it is likely that many are making attempts to comply. As a result, it is possible that a significant number of infants are at an increased risk of allergic sensitisation, with huge implications for individual quality of life and health economics.

Future directions

In this thesis, the clinical outcome data for the egg avoidance study cohort relate to infant atopic phenotype at 6 months of age. It will be essential to clarify if the association of early life environmental characteristics with allergic programming extends beyond infancy and whether there are implications for the development of respiratory allergic disease. The infants of this cohort are being followed up at 12 and 18 months of age and plans are in place to review them again at 3 years of age. Analysis of the data at these later time points will allow a clearer understanding of the evolution of atopy in relation to early life experiences.

It is also imperative to characterise the relationship of peanut exclusion in pregnancy and breast-feeding with infant allergic outcome. Peanut allergy is a growing health care problem [436], inhibiting many aspects of daily life as well as carrying the risk of life-threatening anaphylaxis. It cannot be assumed that the immunological parameters of ovalbumin exposure or the association of ovalbumin exposure in early life with later infant atopy can be extrapolated to peanut allergen. Therefore, ongoing work in our department using this study cohort to examine peanut allergen exposure and its

association with infant sensitisation and disease will be essential to defuse a potential 'allergic minefield'.

10.4 Characterisation of passage of ovalbumin via the placenta and breast milk

The demonstration of ovalbumin in amniotic fluid, cord blood and breast milk indicated that early life priming might occur in response to *direct* antigen exposure, rather than as the result of transfer of maternal anti-idiotype antibodies or presentation of allergenic peptide by maternal antigen presenting cells [437][438]. This observation then raised the question of *how* the antigen could be transferred from maternal to fetal/newborn compartments.

Carriage of antigen in complex with immunoglobulin was the most likely mode of antigen passage. Maternal IgG is actively transported across the placenta and this transfer is maximal in the 3rd trimester of pregnancy [114]. Similarly, breast milk is rich in secretory IgA [27]. *In vitro* placental perfusion studies have suggested that IgG may provide a vehicle for antigen transport [360][439] and indeed circulating cat allergen, Fel d1, has been demonstrated in complex with IgG at birth [361].

The current investigation has led to an increased understanding of the mechanism of antigen transfer. Gel filtration separation of sera and breast milk permitted determination of the MW of sample ovalbumin, from which its form could be inferred. An additional advantage of the technique was an increase in detection sensitivity consequent on sample separation. The lower threshold for detection enabled Western blotting of fraction samples and consequently further clarification of antigen form.

These data have shown that ovalbumin may be transported across the placenta in free form or in complex with IgG and that specific IgG concentration has a key role in determining antigen form *(chapter 6)*. Therefore, these observations support the previous conclusion that maternal antigen exposure via the placental route may influence fetal atopic programming *(10.3)*. Evidence to date would suggest that the susceptible time period for fetal priming/sensitisation is early in gestation, before the

IgG transport mechanism has been activated. Therefore, demonstration of free antigen in fetal blood, indicating IgG-independent passage, implies that *in utero* antigen exposure via the placenta may indeed occur at the gestational time points when exposure characteristics might have consequences for the responses of the developing immune system.

Since IgG concentration was a major determinant of antigen form, it is likely that factors altering IgG concentration might have repercussions for the nature of the antigen encountered by the fetus. In this study dietary egg exclusion resulted in a significant fall in ovalbumin IgG concentration. Therefore, it is possible that dietary elimination could result in a reduction in the proportion of antigen presented to the fetus in complexed form, without eliminating exposure.

In contrast to cord blood, ovalbumin in breast milk was found uniformly in free form, irrespective of specific IgA concentration *(chapter 7)*. On first consideration this seemed to be a surprising finding: an enteromammeric IgA route, whereby antigen encountered at the maternal gut mucosa drives the specificity of sIgA secreted into breast milk, is recognised and the mechanism of transcellular IgA passage has been characterised [119]. Indeed, in this study the presence and concentration of breast milk ovalbumin was found to have a direct relationship with the concentration of specific IgA in the same sample *(chapter 4)*. However, on further consideration the finding may have relevance for the induction of oral tolerance. The gut mucosal immune system is central to the mechanism of tolerance, which is essential for health. Antigen presentation to the mucosal immune system may occur following uptake by absorptive epithelium [440][441], Microfold, or M, cells – specialised enterocytes overlying Peyer's patches [442] – or by subepithelial dendritic cells that extend processes into the gut lumen [443]. Since soluble antigens induce tolerance more easily than particulate antigens [444][445], and since ovalbumin in complex with IgA is most likely eliminated from the body, the presence of free allergen in breast milk may relate to an evolutionary necessity to establish tolerance. However, the corollary of this immune activation is that environmental modulation of antigen exposure, or the interaction of antigen with the immune system, may have repercussions for allergic sensitisation.

Future directions

This chapter has identified a number of areas for further work. This includes the need for a great deal more basic knowledge in order to fully understand the mechanism of passage of antigen from mother to baby. It is possible that free ovalbumin may pass into cord blood by passive diffusion, as has been suggested for human chorionic gonadotrophin (hCG) (MW 42 kDa) [359], and also demonstrated for the transfer of ovalbumin across human gut epithelium [446][447]. In an attempt to further clarify the mechanism of transplacental antigen passage, the development of an *in vitro* placental syncytiotrophoblast monolayer was undertaken. However, this investigation was hampered by an inability to achieve a confluent cell layer. Further optimisation of the method may yet produce a suitable model to study the factors modulating the kinetics of antigen passage across the placenta.

The form in which allergen is presented to the developing immune system may direct whether allergic sensitisation or tolerance develops. Antigen-immunoglobulin complex may enhance antigen presenting cell function by allowing focusing, and efficient selection, concentration and presentation of allergen, with consequent activation of allergen-specific T-cells at 100-1000-fold lower dose than required for activation by native antigen [146]. However, this activity may not correlate with atopic disease: *in vitro* research has demonstrated that the low affinity IgG receptor CD32b (FcγRIIB) may inhibit IgE-induced mast cell activation via an immunoreceptor tyrosine based inhibitory motif (ITIM) [448]. CD32b has also been shown to negatively regulate BCR-mediated B cell activation [368] and TCR-mediated T cell activation [369]. This issue could be explored by further application of these chapters' methodology. Using the egg avoidance study cohort, comparison of allergen form in selected subgroups with and without an atopic phenotype may allow clarification of relation between form of allergen exposure in early life and subsequent allergic outcome.

10.5 The role of maternal IgG in atopic programming and allergic disease

Serum IgG plays an essential part in host defence, as attested by the serious clinical sequelae of immunoglobulin deficiency syndromes. Whilst the prototypic immunoglobulin of atopic disease is IgE, the work in this thesis has shown that IgG may also have a key role in the allergic process.

Egg specific IgG was found to reflect dietary intake, as shown by the significant fall in concentration in sera of egg avoiding women *(chapter 5)*. Measurement of serum specific IgG, in addition to offering an objective monitor of dietary compliance, may thus be a marker of *overall* egg allergen exposure in early life – though as discussed above, this gives no indication of the pattern of exposure.

Also, as discussed above, ovalbumin specific IgG likely facilitated materno-fetal allergen transfer in complexed form. An implication of this finding is then that IgG might have an immunoregulatory function deriving from differences in allergen form experienced *in utero*. An immunomodulatory function of IgG has been suggested in previous studies. For example, passive immunisation with immunoglobulins from beekeepers (predominantly IgG4) offered protection from venom-induced anaphylaxis [449], suggesting that IgG could compete with IgE for allergen and in so doing dampen IgE-mediated immune reactivity. An ability of IgG to prevent IgE sensitisation has also been inferred by a number of studies. Passive administration of IgG to rabbits had a suppressive effect on IgE antibody formation [450] and maternal rye grass immunotherapy in pregnancy, with consequent rise in specific IgG concentration, was associated with a reduced risk of infant rye grass sensitisation [349], while children with the highest IgG titres to cat allergen Fel d1 were the least likely to be sensitised to cat [451].

The mechanism by which IgG may 'block' sensitisation has not been addressed previously. However, this work has offered some insight into the process. Experiments revealed that antigen detection was inhibited in certain sera, a property that was dependent on serum IgG concentration *(chapter 3)* and functional affinity *(chapter 8)*. If variations in these IgG characteristics result in sequestration of allergenic epitopes then this phenomenon might have implications for T-cell

activation and hence initiation of the allergic cascade in early life. Such a postulate is supported by an earlier observation from our department that cord blood mononuclear cell proliferative responses to house-dust mite negatively correlated with the concentration of maternal specific antibody in autologous plasma [206].

The atopic outcome of babies in this cohort was found to vary according to the concentration of OVA IgG at birth *(chapter 5)*. Infants born to mothers with an unmodified diet were less likely to express an atopic phenotype at 6 months of age if their cord OVA IgG concentration fell into the lowest or highest quartiles, a similar bell-shaped association of atopy and specific IgG also being described for the inhalant allergen, cat Fel d1 [451]. Could measurement of cord specific IgG therefore provide a marker for babies with an atopic genotype who have an increased risk of developing allergy? Certainly measurement of OVA IgG subclasses through the 1st 5 years of life showed a distinctive response pattern in persistently egg sensitised children and in those children who later developed asthma *(chapter 9)*. Thus, measurement of humoral responses in infancy, and possibly at birth, may facilitate introduction of secondary prevention measures to reduce the risk, or severity, of asthma.

Final summation

This work has identified 3 main factors that may regulate atopic programming: firstly, maternal atopy in pregnancy; secondly, the pattern of antigenic challenges and thirdly, the characteristics of circulating maternal IgG. The babies at the greatest risk of atopy were the ones whose early life environment was modulated by all 3 factors, namely, the babies born to atopic, egg-avoiding mothers. A unifying hypothesis to explain this association might then be that dietary avoidance results in low dose, intermittent exposures, which in the presence of lower levels of circulating maternal specific IgG consequent on the dietary intervention together create a sensitising pattern and form of early life allergen exposure, which in the environment created by the atopic mother predisposes to the genesis of allergy.

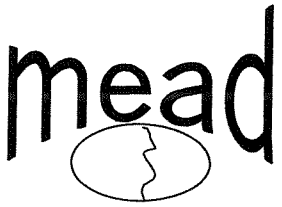
Certainly these early life events must interact with later environmental experiences, such as viral infections and non-maternally derived allergen exposure, to ensure the

expression of the atopic phenotype. However, the maternal factors identified in this thesis may direct the initiation of a cascade which if prevented, rather than halted, might result in a better outcome for the infant. Consideration of maternal serum IgG characteristics and molecular determinants of immune responsiveness and immune phenotype, such as chemokines and cytokines, before the women becomes pregnant, may thus permit stratification of infant allergic risk and open a whole new chapter of intervention strategies.

Appendices

Appendix 1 Infant Evaluation

INFANT ASSESSMENT



Visit Details:

Date: ☐☐.☐☐.☐☐ →

Infant Age (wks): ☐☐ ☐☐

Growth:

Weight: ☐☐.☐☐☐ ☐☐.☐☐☐

Weight centile: (please circle)

<0.4 th [1]	0.4 th [2]	0.4-2 [3]	2 [4]	2-9 [5]
9 [6]	9-25 [7]	25 [8]	25-50 [9]	50 [10]
50-75 [11]	75 [12]	75-91 [13]	91 [14]	91-98 [15]
98 [16]	>98 [17]			

☐☐

☐☐☐.☐

Length (cms): _____

Length centile: (please circle)

<0.4 th [1]	0.4 th [2]	0.4-2 [3]	2 [4]	2-9 [5]
9 [6]	9-25 [7]	25 [8]	25-50 [9]	50 [10]
50-75 [11]	75 [12]	75-91 [13]	91 [14]	91-98 [15]
98 [16]	>98 [17]			

☐☐

☐☐.☐

Head circumference (cms): _____

Head circumference centile: (please circle)

<0.4 th [1]	0.4 th [2]	0.4-2 [3]	2 [4]	2-9 [5]
9 [6]	9-25 [7]	25 [8]	25-50 [9]	50 [10]
50-75 [11]	75 [12]	75-91 [13]	91 [14]	91-98 [15]
98 [16]	>98 [17]			

☐☐

Health:

Any ongoing medical problems? Yes[1] / No[2] ☐

Current medication? Yes[1] / No[2] ☐

Vaccinations: All to date Yes[1] / No[2] ☐

Eczema Yes[1] / No[2] ☐

Eczema episodes Total Number/flare ups: ☐☐

Date	Site	Treatment	Duration

Wheezing episodes Total Number:

Max Severity:

Date	Treatment	Duration

Coughing episodes Total Number:

Max severity:

Date	Treatment	Duration

Asthma Yes[1] / No[2]

Other allergic feature? Yes[1] / No[2]

Examination

Clubbing: Yes[1] No[2]

Chest Deformity: Yes[1] No[2]

Wheeze: Yes[1] No[2]

Eczema: Yes[1] No[2]

Scorad: .....

Bloods Taken: Yes[1] No[2]

Skin Prick Test Results (mm):

-ve	
Egg	
Egg White	
Egg Yolk	
Cows Milk	
House Dust Mite	
Cat	
Timothy Grass Pollen	
Peanut	
+ve	

Appendix 2 Optimising Ovalbumin IgG1 and IgG4 ELISAs

Figure 1 Optimising OVA G1 assay.

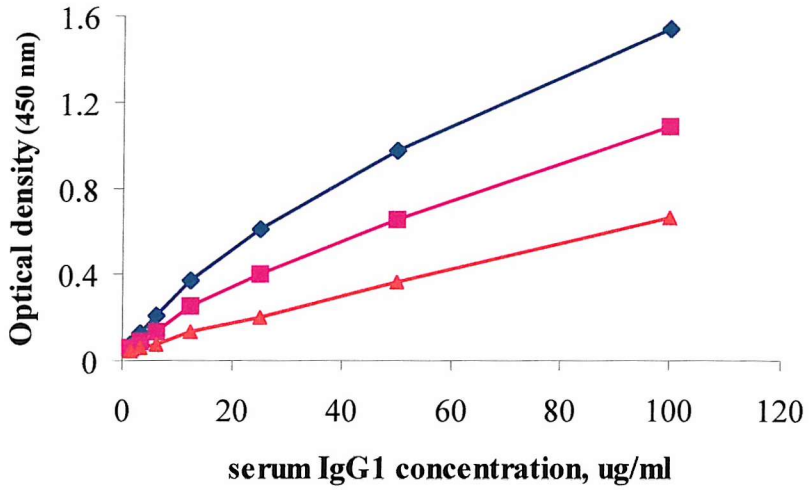


Figure 1 The plates were coated with OVA or BSA (as a control for non-specific binding) at 100 µl, 100 µg/ml. The ELISA was carried out as outlined in 2.5.2 with the exception that 3 different concentrations of mouse monoclonal anti-human IgG1 were tested: 1:2000 (red graph), 1:1000 (pink graph) & 1:500 (blue graph). OVA G1 detection was clearly limited by the concentration of the detector. Therefore in the subsequent assays the detector antibody was used at a concentration of 1:500.

Figure 2 Optimising OVA G4 assay.

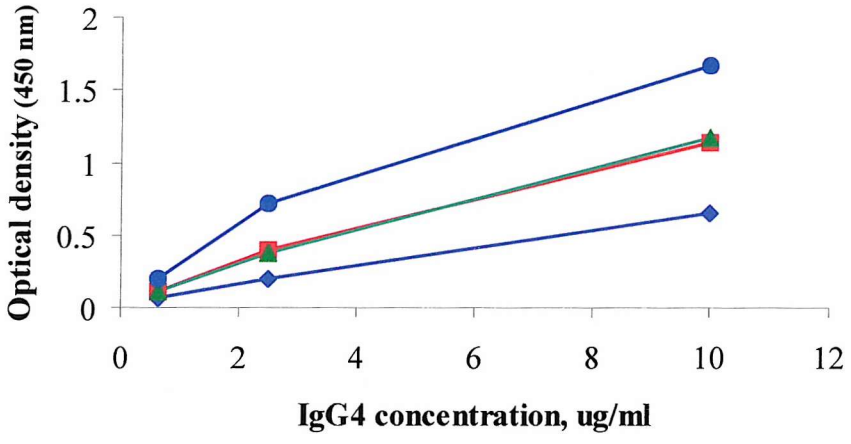


Figure 2 The plates were coated with OVA or BSA (as a control for non-specific binding) at 100 µl, 100 µg/ml. The ELISA was carried out as outlined in 2.5.2 with the exception that 4 different concentrations of mouse monoclonal anti-human IgG4 were tested: 1:1600 (red graph), 1:3200 (blue graph), 1:6400 (yellow graph) & 1:12800 (purple graph). OVA G4 detection was clearly limited by the concentration of the detector. Therefore in the subsequent assays the detector antibody was used at a concentration of 1:1600

Appendix 3 Optimising Total IgA ELISA

Figure 1 Chequer-board titration of coating and detector antibody concentrations.

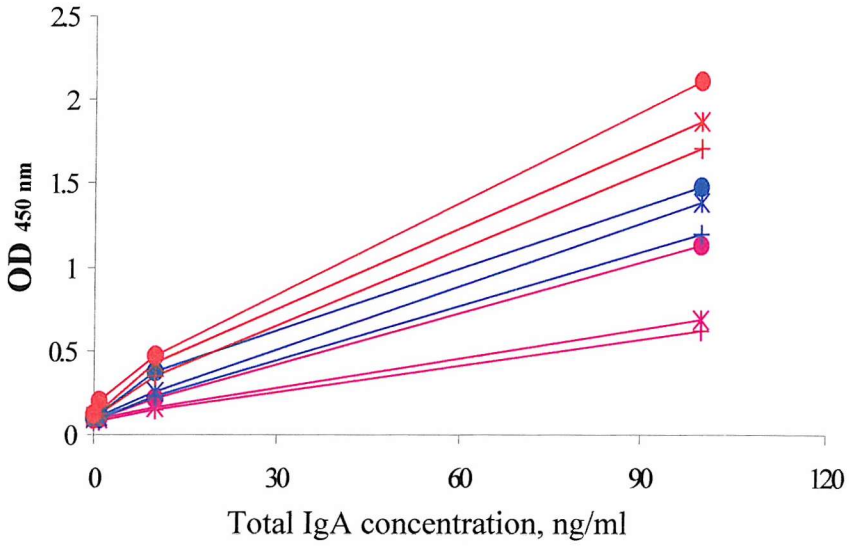


Figure 1 A chequer-board titration of antibody concentrations was performed using 'high', 'medium' and 'low' concentrations of human myeloma IgA. Capture antibody concentrations were 1 µg/ml (pink lines), 2 µg/ml (blue lines) or 4 µg/ml (red lines). Detector antibody concentrations were also 1 µg/ml (cross), 2 µg/ml (star) or 4 µg/ml (circle). OD was limited by antibody concentration. A coating concentration of 4 µg/ml allowed for higher signals.

Figure 2 Titrating the concentration of detector antibody.

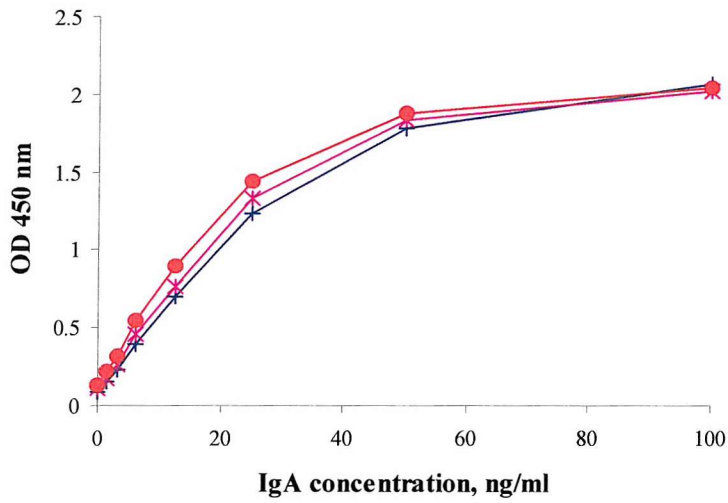


Figure 2 The optimal concentration of detector antibody was further evaluated over a wider range of standard IgA concentrations. For a capture concentration of 4 µg/ml, previously shown to enhance assay OD, there was little difference between detector concentrations of 1 µg/ml (blue line), 2 µg/ml (pink line) and 4 µg/ml (red line). A concentration of 1 µg/ml was therefore used in subsequent experiments.

Appendix 4 Optimising Western blotting using enhanced chemiluminescence development (ECL)

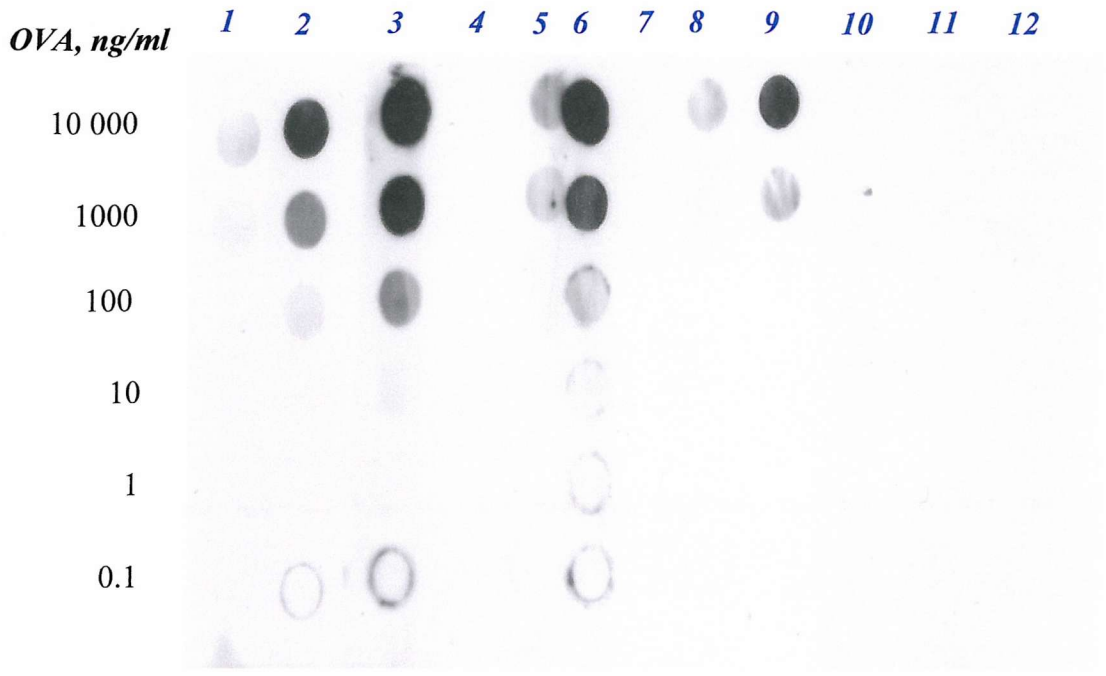


Figure 1 The concentrations of the primary and secondary antibodies used for protein detection by Western blotting with ECL were optimised by dot-blot chequer-board titration (for method see 2.5.5 & 2.5.8). The concentrations of the antibodies used in the titration are summarised in table 1. The figure shows that maximal sensitivity (5ng protein), without any detectable background (lanes 10-12), was achieved with a primary concentration of 10 µg/ml and secondary concentration of 1:1000 (lane 3).

Table 1 Antibody concentrations used in chequer-board titration.

Lane	*Primary antibody concentration*	*Primary antibody*	*Secondary antibody concentration*	*Secondary antibody*
1	10 *μg/ml*	Rabbit anti-OVA (Sigma)	1:25000	Donkey anti-rabbit (Amersham)
2	10 *μg/ml*		1:5000	
3	10 *μg/ml*		1:1000	
4	2.5 *μg/ml*		1:25000	
5	2.5 *μg/ml*		1:5000	
6	2.5 *μg/ml*		1:1000	
7	0.625 *μg/ml*		1:25000	
8	0.625 *μg/ml*		1:5000	
9	0.625 *μg/ml*		1:1000	
10	10 *μg/ml*	Rabbit gamma globulin (Pierce)	1:25000	
11	10 *μg/ml*		1:5000	
12	10 *μg/ml*		1:1000	

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