Immunobiology Unit
Institute of Child Health
University College London Medical School
30 Guilford Street
London WC1N 1EH

Immunoregulation after intestinal and cutaneous exposure to food proteins

- tolerance versus sensitisation -

Karin Jessica Strid

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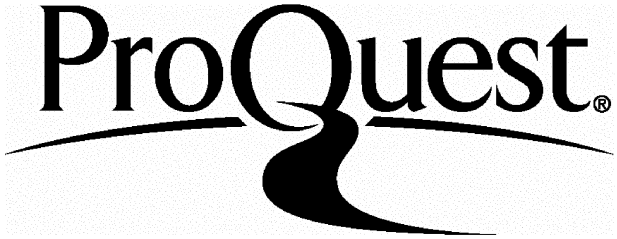

Abstract

The prevalence and severity of allergic diseases is rising and poses an increasing clinical problem. Food allergies are a major course of life-threatening hypersensitivity reactions, but the immunological mechanisms underlying the induction of sensitisation or tolerance to food proteins are poorly understood. This study examined the immunological outcome of exposure to peanut protein and chicken egg ovalbumin (OVA) at the intestinal mucosae and skin surfaces. The aim was to determine whether skin exposure to allergens may interfere with oral tolerance and promote food allergies.

A murine BALB/c model of oral tolerance or sensitisation to peanut protein was developed and compared to a model of oral tolerance to OVA. Tolerance to peanut required a significantly higher oral dose than tolerance to OVA and low doses of peanut protein were more likely to induce sensitisation. Oral tolerance mediated suppression of both Th1 and Th2 responses. A high dose feed of peanut protein induced a population of $CD4^+CD25^+CTLA\text{-}4^+$ T cells with regulatory properties, suggesting that a population of T_{reg} cells similar to the naturally occurring thymic derived $CD4^+CD25^+$ T_{reg} can be induced in the periphery.

Epicutaneous exposure to peanut protein and OVA on abraded skin (epicutaneous immunisation) induced potent Th2-type immunity with high levels of antigen-specific IL-4, IgE and IgG1 with no IgG2a. Abrasion of the skin increased expression of activation markers on Langerhans cells (LCs), but rapid migration from epidermis to draining lymph nodes was observed only after both skin abrasion and exposure to antigen, suggesting that maturation and migration of LCs are independently regulated events. Primary skin exposure prevented the induction of oral tolerance. Upon oral challenge mice were further sensitised and developed strong specific IL-4 and IgE responses as well as clinical signs of anaphylaxis. This suggests that epicutaneous exposure to protein allergens specifically drives Th2 responses and may promote food allergy.

Primary epicutaneous immunisation changed responses elicited by subcutaneous antigen in complete Freund's adjuvant from Th1 to Th2. Additionally, epicutaneous immunisation converted an existing antigen-specific Th1 response to a Th2 response, indicating dominance of the skin-induced immunity. Together these results suggest that epicutaneous immunisation may be a useful non-invasive, non-adjuvant-dependent method of vaccination and therapy.

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This Thesis is dedicated to Gideon
and to the loving memory of Gustav

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Abbreviations

Ab	Antibody
AchR	Acetylcholine receptor
AD	Atopic dermatitis
Ag	Antigen
AO	Abraded skin, painted with OVA
AOOOO	Skin abraded, painted with OVA, fed OVA, immunised with OVA in CFA, recall immunised with OVA
AP	Abraded skin, painted with peanut
APC	Antigen presenting cell
APP	Skin on ears abraded, painted with peanut, immunised with peanut in the footpad
APPP	Skin abraded, painted with peanut, immunised with peanut in CFA, recall immunised with peanut
APPPP	Skin abraded, painted with peanut, fed peanut, immunised with peanut in CFA, recall immunised with peanut
AS	Abraded skin, painted with saline
ASOOO	Skin abraded, painted with saline, fed OVA, immunised with OVA in CFA, recall immunised with OVA
ASP	Skin on ears abraded, painted with saline, immunised with peanut in the footpad
ASPP	Skin abraded, painted with saline, immunised with peanut in CFA, recall immunised with peanut
ASPPP	Skin abraded, painted with saline, fed peanut, immunised with peanut in CFA, recall immunised with peanut
BM	Basement membrane
BSA	Bovine serum albumin
CBB	Carbonate bicarbonate buffer
CCR	CC chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary deoxy-ribonucleic acid
CFA	Carboxyfluorescein diacetate succinimidyl ester

CFA	Complete Freund's adjuvant
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CLA	Cutaneous lymphocyte-associated antigen
CLN	Cervical lymph nodes
ConA	Concanavalin A
CpG motifs	Cytosine and guanine dinucleotide repeat motifs
cpm	Counts per minute
CT	Cholera toxin
CTACK	Cutaneous T cell-attracting chemokine
CTB	Cholera toxin B subunit
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
DC	Dendritic cell
DIS	Dermal immune system
DMSO	Dimethylsulphoxide
DNA	Deoxy-ribonucleic acid
DTH	Delayed type hypersensitivity
EAE	Experimental autoimmune encephalomyelitis
EAU	Experimental autoimmune uveitis
EDTA	Ethenediaminetetraacetic acid, di-sodium salt
ELISA	Enzyme-linked immunosorbent assay
EPI	Epicutaneous immunisation
FACS	Fluorescence activated cell sorter
FAE	Follicle-associated epithelium
Fc	Crystallisable fraction of the immunoglobulin molecule
FcR	Receptor for the Fc region of immunoglobulin
FCS	Foetal calf serum
FITC	Fluorescein 5-isothiocyanate
GAD	Glutamate decarboxylase
GALT	Gut-associated lymphoid tissue
GM-CSF	Granulocyte-macrophage colony stimulating factor
H&E	Haematoxylin and eosin
HBSS	Hanks buffered saline solution

HEV	High endothelial venule
hr	Hour(s)
HSP	Heat shock protein
IBD	Inflammatory bowel disease
IDDM	Insulin dependent diabetes mellitus
IEC	Intestinal epithelial cell
IEL	Intraepithelial lymphocyte
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRBP	Interphotoreceptor retinoid-binding protein
kDa	KiloDalton
KGF	Keratinocyte growth factor
KLH	Keyhole limpet haemocyanin
KO or $^{-/-}$	Gene knock-out
L	Ligand
LC	Langerhans cell
LN	Lymph node
LPL	Lamina propria lymphocyte
LPS	Lipopolysaccharide
MACS	Magnetic cell sorting
MALT	Mucosa-associated lymphoid tissue
MBP	Myelin basic protein
M-CSF	Macrophage colony stimulating factor
2-ME	2-Mercaptoethanol
mH_2O	MILLIQ ($18\Omega/cm^3$) water
MHC	Major histocompatibility complex
min	Minutes
MIP	Macrophage inflammatory protein
MLN	Mesenteric lymph node
MMP	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis

NGS	Normal goat serum
NK	Natural killer
NMS	Normal mouse serum
NOD	Non-obese diabetic
NP	Naïve skin, painted with peanut
NRS	Normal rat serum
NS	Naïve skin, painted with saline
OO	Immunised with OVA in CFA, recall immunised with OVA
OOO	Fed OVA, immunised with OVA in CFA, recall immunised with OVA
OVA	Ovalbumin
P	No skin procedure, immunised with peanut in the footpad
PAP	Fed 100mg peanut, skin abraded, painted with peanut
PAPP	Immunised with peanut in CFA, skin abraded, painted with peanut, recall immunised with peanut
PAS	Fed 100mg peanut, skin abraded, painted with saline
PASP	Immunised with peanut in CFA, skin abraded, painted with saline, recall immunised with peanut
PBS	Phosphate buffered saline
PBST	PBS supplemented with 0.05% v/v Tween-20
PFA	Paraformaldehyde
PGE_2	Prostaglandin E_2
PLN	Para-aortic lymph nodes
PLP	Proteolipid protein
PopLN	Popiteal lymph nodes
PP	Immunised with peanut in CFA, recall immunised with peanut (Chapter 5 and 7)
PP	Peyer's patch
PPP	Peanut fed, immunised with peanut in CFA, recall immunised with peanut
RAST	Radio-allergo-sorbent test
RT-PCR	Reverse transcriptase-polymerase chain reaction
SALT	Skin-associated lymphoid tissue
SAP	Fed saline, skin abraded, painted with peanut
SCID	Severe combined immunodeficiency

SDF	Stromal cell-derived factor
SDS	Sodium dodecyl sulphate [Lauryl sulphate]
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
SED	Subepithelial dome
SEM	Standard error of the mean
SIS	Skin immune system
SLC	Secondary lymphoid-tissue chemokine
SOO	Saline fed, immunised with OVA in CFA, recall immunised with OVA
SPP	Saline fed, immunised with peanut in CFA, recall immunised with peanut
SPT	Skin prick test
SUB	Subcutaneous immunisation
TCR	T cell receptor
TDA	Thymus-dependent area
TGF	Transforming growth factor
Th	T helper cell
TNF	Tumour necrosis factor
T_{reg}	Regulatory T cell
TRITC	Tetramethyl rhodamine isothiocyanate

Chapter 1

Introduction

1. Introduction

The discipline of immunology grew out of the observation that individuals who had recovered from certain infectious diseases were thereafter protected from the disease. Although this concept of immunity existed in folklore, it was almost two thousand years before the concept was converted into a medically effective practice.

We know now that the major mission of the immune system is to recognise molecules foreign to self and direct an appropriate response against them. This task is formidable considering the wide variety of pathogenic organisms and their ability to rapidly mutate under evolutionary selection as well as the many and ever changing innocuous food and environmental molecules encountered. Two general systems of immunity to infectious agents have been selected during evolution: Innate or natural immunity, and acquired (adaptive) or specific immunity. Functionally, an immune response can be divided into two interrelated activities – recognition and response. The essential difference between the innate and the acquired immune system is the means by which they recognise microorganisms and foreign molecules [Fearon and Locksley, 1996]. Innate immunity refers to the basic resistance to disease that a species possesses and the target of recognition represents molecular patterns rather than particular structures [Janeway and Medzhitov, 2002]. The acquired immune system displays specificity, diversity, memory and self/non-self recognition. The specificity of the system is a result of its ability to generate a tremendous diversity of recognition molecules allowing the immune system to distinguish subtle differences among antigens. This is an important feature, as induction of an immune response is only appropriate if the antigen recognised is derived from, or belongs to, a pathogen. Activation of lymphocytes specific for self antigens, or benign persistent environmental antigens, may result in autoimmune disorders and deleterious hypersensitivity reactions, respectively. This is why the immune systems ability to distinguish self from non-self and harmless form harmful antigens is crucial [Janeway and Medzhitov, 2002].

The driving force for the immune system is antigen, but the number of antigens that gain access to the systemic immune system is limited by both physical and chemical barriers. Physical and anatomical barriers that tend to prevent the entry of pathogens are the first line of defence against infection. The skin and the surface of mucous membranes provide a physical barrier to the entry of most microorganisms. Despite this, the amount of antigen in contact with, inside or transversing the body surfaces is immense. The membrane barriers

have thus evolved features that make the lining epithelium an active immunological as well as anatomical barrier [Shao et al., 2001]. The enormous exposure of antigens at the body surfaces and efforts to understand the outcome of such exposure has become a paradigm for understanding systemic immune function. The mucosal- and body surface immune system must thus be regarded as an important local starting point for the immune response as a whole.

1.1 The mucosal immune system

The immune system at body surfaces is constantly exposed to a vast number and variety of bacterial-, food- and environmental antigens. An essential feature of the immune system at the body surfaces is the ability to discriminate between potentially harmful and harmless foreign proteins. All mucosal surfaces with their associated lymphoid structures are part of a common mucosal immune system or mucosa-associated lymphoid tissue (MALT) [Brandtzaeg et al., 1998; Mowat and Viney, 1997]. Each mucosal surface is covered by a layer of epithelium (in some sites just one cell layer thick and in others multiple cell layers thick) directly overlying the loosely organised lymphoid tissue in the lamina propria. The gastrointestinal tract and the gut-associated lymphoid tissue (GALT) are the best defined and most studied, but distinct epithelium-associated lymphoid tissue also exists in the skin, the reproductive tract and the lung.

The intestinal epithelium is composed of one layer of columnar epithelial cells with non-epithelial cells present in large numbers within in it [Collan, 1972; Ferguson, 1977; Otto, 1973]. The cellular infiltrate consists of granular and non-granular lymphocytes, dendritic cells, macrophages, eosinophils, mast cells and the occasional neutrophil (Fig. 1.1). These cell types can be demonstrated both in the epithelium and the lamina propria; B cells and plasma cells are normally restricted to the latter. Intestinal T cells occur primarily in three major compartments: a) organised lymphoid aggregates present in the wall of the small and large intestine termed Peyer's patches (PP); b) the mucosal lamina propria, which contains mature lamina propria T lymphocytes (LPLs), and B cells as well as other cells necessary for an adaptive immune response and c) the surface epithelium, which contains a unique population of T cells called intraepithelial lymphocytes (IELs). The phenotypic T cell distribution in the gut differs among the intestinal compartments and is strikingly different from that of peripheral blood [Abreu-Martin and Targan, 1996].

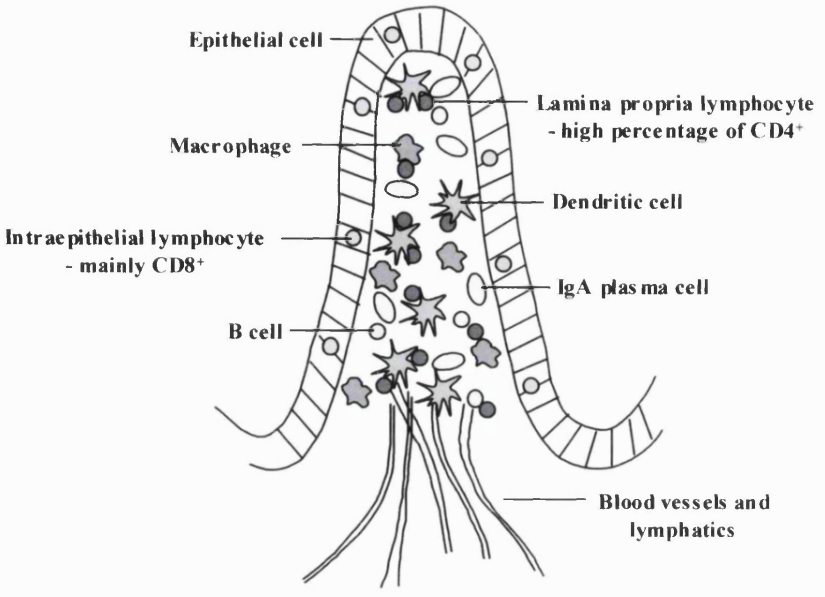


Fig. 1.1 Intestinal villus and associated immune cells
Diagram showing the general structure of the epithelium and the major cell populations present

The epithelium maintains constant cell to cell contact with the IELs and lymphoid cells of the lamina propria thereby enabling constant immune surveillance of the antigens encountered in the gut lumen. As a result of this close relationship between the different cell types it is possible to regard the mucosal immune system as comprising two parts: 1) The 'non-professional' gut epithelial cells or enterocytes and 2) The 'professional' lymphoid tissues of IELs, lamina propria and Peyer's patches. These integrated mucosal compartments are discussed separately below.

1.1.1 Intestinal epithelium and M cells

The intestinal epithelium consists of large columnar epithelial cells (enterocytes), which are derived from rapidly dividing precursors situated in the crypts of the villus. The intestinal epithelial cells (IECs) have a rapid turnover rate of normally 2-3 days. The turnover of IECs is controlled by apoptosis and is necessary to maintain homeostasis and integrity of the epithelial barrier [Watson, 1995]. The IECs are polarised cells which have a specially adapted brush border of microvilli facing into the lumen of the gut, thereby dramatically enhancing the surface area of each IEC [Mooseker, 1985]. Adjacent enterocytes are joined together by adhesion proteins forming tight junctions that restrict transepithelial movement of particulates and even hydrophilic molecules of molecular weight higher than ~ 2kDa [Madara, 1998].

During inflammatory responses in the gut, these IEC tight junctions can be disrupted, allowing free passage of lumenal antigens into the lamina propria.

Interspersed between the IECs are goblet cells, which provide the glycocalyx and mucus that coats the surface of the epithelial microvilli and acts as an additional barrier against pathogen penetration into the underlying tissues [Maury et al., 1995; Mayer, 2000a]. Mucosal IgA is recognised as the first line of immunological defence against invading agents in the gut lumen. IEC exposure to luminal antigens induces the production of IgA from lamina propria- and particularly PP plasma cells [Kawanishi et al., 1983]. Class switching from IgM to IgA in B cells occur under influence of the IgA switch factor TGF-β [Lebman et al., 1990; Sonoda et al., 1989]. Dimeric IgA is taken up via a specific polymeric immunoglobulin receptor expressed on the basolateral surface of the IECs. IgA is then transcytosed through the IEC and secreted into the lumen, where it may prevent viral and bacterial binding to the epithelium by forming complexes with the antigens [Mayer, 2000a; Mostov, 1994]. Healthy intestinal epithelium thus forms a highly selective barrier giving primary protection from the vast commensal microbial ecosystem and from pathogenic bacteria entering the intestine.

A major function of IECs is to take up and transport food nutrients and solutes from the gut lumen to underlying tissues for distribution around the body. The intestinal epithelium thus represents a potential entry point for both dietary and non-dietary lumenal antigens and IECs may have a physiological role in the uptake and processing of lumenal antigens and pathogens. Indeed, antigen processing and presentation by IECs to both $CD4^+$ and $CD8^+$ T cells within the mucosa has been demonstrated (reviewed in [Hershberg and Mayer, 2000]). Major histocompatibility complex (MHC) class II are expressed on IECs from both humans [Mayer et al., 1991], mice [Kaiserlian et al., 1989], rats [Bland and Warren, 1986; Bland and Whiting, 1992] and guinea pigs [Wiman et al., 1978] with elevated levels consistently seen in the presence of intestinal inflammation. Likewise MHC class I is expressed on IECs, although some data points towards a role for 'non-classical' interactions between $CD8^+$ T cells and IECs via class Ib molecules such as CD1 [Balk et al., 1994; Bleicher et al., 1990]. However, the IECs ability to function as professional antigen presenting cells (APC) is hampered by the fact that under normal conditions they do no not express the costimulatory molecules CD80 or CD86 [Hershberg and Mayer, 2000]. The expression of CD86 has however been demonstrated on IECs under inflammatory conditions such as inflammatory bowel disease

(IBD) [Nakazawa et al., 1999]. Further, constitutive expression of the costimulatory molecule CD58 which binds CD2 expressed on both IELs and LPLs (see below) has been described on IECs [Framson et al., 1999].

The polarised phenotype, the expression of 'non-classical' antigen-presenting molecules and unusual costimulatory molecules suggests that antigen processing and presentation by IECs differs from that of 'professional' APCs in the GALT. The ability of IECs to stimulate a T cell response is likely to be dependent on the underlying level of inflammation in the intestinal mucosa. IECs additionally secrete cytokines and chemokines which can have direct effects on proximate mucosal T cells [Kagnoff et al., 1996]. The intimate contact and bi-directional regulation between IECs, IELs and LPLs makes a complex, efficient but far from fully understood network. (The contact between IECs and lymphocytes of the intestinal mucosa is outlined in Fig. 1.2). Clearly, the delineation of the role of IEC-T cell interactions in health and disease awaits further developments.

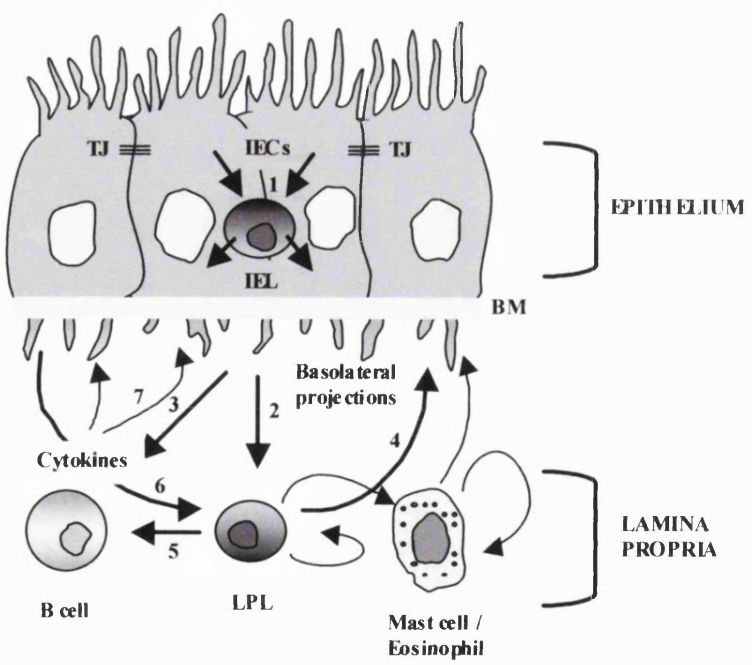


Fig. 1.2 Communication network between intestinal epithelial cells and mucosal lymphocytes
IECs are in intimate contact with two distinct populations of T cells – intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs). IELs migrate through the basement membrane (BM) and lodge between adjacent intestinal epithelial cells (IECs). IELs produce cytokines and other factors that influence IEC growth and differentiation (1) as well as the function of underlying cells in the lamina propria (2). Cytokines produced by IELs also interact with B cells in the lamina propria (3). LPLs locate below the BM. When activated by IELs and IECs they release mediators which act on IECs (4), B cells (5) as well as other cell populations within the lamina propria. In turn, cytokines produced by IECs can stimulate IELs (1) and LPLs (6) as well as having autocrine and paracrine effects on other epithelial cells (7). Additionally, 'pores' exist within the BM that allow IECs to extend 'basolateral projections' facilitating cognate interaction between IECs and immune cells in the underlying lamina propria. [TJ: Tight junction]

M cells

An additional type of specialised epithelial cells are called M cells. The M cells are scattered throughout a specialised follicle-associated epithelium (FAE) or dome epithelium, overlaying the only truly organised lymphoid structure in the GALT, the PP [Owen and Jones, 1974]. M cells, unlike the IECs, have a poorly developed brush border without microvilli or glycocalyx. Instead they are covered in microfolds over the luminal surface which allow sampling of a broad range of microorganisms and macromolecules [Neutra et al., 1996; Owen, 1994; Owen and Jones, 1974]. M cells have unique elongated cytoplasm extending into the lamina propria forming a 'pocket' which surrounds the underlying lymphoid cells. The 'pocket' contains T- and B cells as well as macrophages, suggesting that T cells can interact with APCs already in this intraepithelial 'pocket'. Below the epithelium of the dome lies an extensive network of dendritic cells and macrophages intermingled with $CD4^+$ T cells and B cells [Farstad et al., 1994; Neutra et al., 1996]. The APCs situated directly below M cells are thus ideally located to sample transported antigens and carry them into the underlying PP where an active immune response can be generated.

1.1.2 Intraepithelial lymphocytes

The intestinal epithelium harbours a unique population of constitutive resident lymphocytes – the intraepithelial lymphocytes (IELs) - of which more than 98% are T cells [Mayer, 2000a]. By virtue of the large surface area of epithelia (not only the gut), such IELs may be among the most abundant T cell subsets [Girardi et al., 2002; Hayday et al., 2001; Trepel, 1974].

Origin of IELs

The origin and fate of IELs are still a matter of controversy, and lymphoid as well as non-lymphoid origins have been proposed [Ferguson and Parrott, 1972; Guy-Grand et al., 1974; Guy-Grand et al., 1978; Guy-Grand et al., 1991; Hayday et al., 2001; Mayrhofer, 1980]. Most IELs seem to originate from the bone marrow and develop into T cells in the intestinal epithelium. Others develop in the thymus and migrate to the epithelium directly or via the PP. Thirtyfive years ago, Fichtelius proposed that gut IELs, then called theliolymphocytes, were not thymus-derived cells, and suggested that the intestinal epithelium was itself a first level lymphoid organ [Fichtelius, 1968]. Subsequently, a number of reports have supported that conclusion [Bandeira et al., 1991; Ferguson and Parrott, 1972; Klein, 1986]. The presence of T cells within the intestinal epithelium but not in the periphery of athymic nude mice has been

documented [Bandeira et al., 1991] and suggests that the intestinal epithelium can support some T cell development in the absence of a functional thymus. Likewise, neonatal thymectomy prevents the production of peripheral T cells, but the number of IELs recovered from the small intestine is only slightly reduced compared with normal control animals [Ferguson and Parrott, 1972]. Despite strong evidence that the intestinal epithelium can support extrathymic T cell development, it has been suggested that it is not entirely thymus-independent [Lefrancois et al., 1996; Lefrancois and Olson, 1994; Lin et al., 1993].
Once the IELs are mature they do not seem to recirculate in a manner similar to conventional T cells but rather show a distinct tissue-specific migration behaviour [Poussier et al., 1992]. Adoptively transferred labelled IELs appear to selectively 'home' to the intestinal epithelium, whereas other adoptively transferred labelled cells from other compartments do not [Sydora et al., 1993].

Phenotype of IELs

Phenotypically IELs are heterogeneous but have a number of characteristics that distinguish them from the majority of blood lymphocytes, thymocytes and LPLs. Over 80% of IELs express CD3 and are negative for intracytoplasmic and cell surface immunoglobulin. On the basis of TCR and co-receptor expression IELs can be separated into five main subsets: TCR$\gamma\delta$ CD8$\alpha\alpha$, TCR$\alpha\beta$ CD8$\alpha\alpha$, TCR$\alpha\beta$ CD8$\alpha\beta$, TCR$\alpha\beta$ CD4 and TCR$\alpha\beta$ CD4 CD8$\alpha\alpha$. There are also two small intraepithelial populations of TCR positive, double negative cells ($CD8^-$, $CD4^-$) and cells without T cell receptors [Lefrancois, 1991; Mosley et al., 1990]. Human [Deusch et al., 1991; Lundqvist et al., 1996] and mouse [Bonneville et al., 1990; Goodman and Lefrancois, 1988] small and large intestine IELs contain a much larger percentage of $\gamma\delta$ T cells than any other lymphoid sites. Analysis of IELs in the small intestine has revealed an approximately equal number of cells using the TCR$\alpha\beta$ and the TCR$\gamma\delta$ receptors – although considerable variation in the ratio of TCR$\alpha\beta$: TCR$\gamma\delta$ has been found between studies [Bonneville et al., 1990; Goodman and Lefrancois, 1988; Lefrancois, 1991].

An interesting characteristic of IELs is their predominant expression of the CD8 molecule (~80% of IELs express CD8 compared to ~35% of LPLs and blood lymphocytes). Within the IEL compartment CD8 can be expressed as a CD8$\alpha\beta$ heterodimer or a CD8$\alpha\alpha$ homodimer. The CD8$\alpha\alpha$ homodimer is unique to IELs and evidence is accumulating that this molecule is

a phenotypic marker for thymus-independent development of at least one IEL subpopulation [Guy-Grand et al., 1991; Rocha et al., 1994]. Coexpression of accessory (CD2, CD5, Thy1, CD45RO or RA), costimulatory (CD28), adhesion and homing ($\alpha_E\beta_7$, CD62L) activation (CD25, CD69) and NK associated molecules (CD16, NK2.1, Ly49) has allowed further characterisation of IELs into distinct subpopulations with different activities (reviewed in [Beagley and Husband, 1998; Hayday et al., 2001; Lefrancois, 1991; Strid and Strobel, 2002]). Most human IELs express CD2, and indeed signalling by binding CD58 on IECs may be of importance within the intestinal epithelium [Ebert, 1989; Hershberg and Mayer, 2000]. In contrast to blood lymphocytes, most IELs are memory and/or activated cells with the majority (~85%) expressing CD45RO. Only a minority show the truly naïve (CD45RA) phenotype. Nearly all IELs are $\alpha_E\beta_7^+$ (binds E-cadherin expressed on IECs) which enables them to 'home' to the GALT and pass through the basement membrane and interdigitate between IECs. Blood lymphocytes do not express this specific intergrin [Lefrancois, 1991]. The expression of many surface molecules varies among IEL subsets and the composition of IELs within the mucosa is affected by their location along the small and large intestine. In general, IELs from the large intestine are more similar in phenotype to splenic and lymph node T cells than small intestine IELs [Camerini et al., 1993; Ibraghimov and Lynch, 1994].

Function of IELs

The precise effector function of mucosal T lymphocytes is controversial and remains essentially unresolved. In accordance with their anatomical location, the following roles have been attributed to IELs:

- Surveillance of the epithelial layer for detection of microbial pathogens
- Removal of damaged or transformed epithelial cells
- Maintenance of epithelial integrity via secretion of trophic factors important for epithelial cell growth and differentiation
- Regulation of local cell-mediated or humoral immune responses

Most IELs are $CD8^+$ and can mediate multiple forms of cytotoxity [Beagley and Husband, 1998]. Cytotoxic activities can be mediated by at least two distinct mechanisms: One involves the release of perforin and granzyme molecules, the other involves the expression of Fas ligand, which then delivers an apoptotic death signal via the Fas signalling pathway [Gelfanov et al., 1996]. IELs also have the potential to secrete a wide array of cytokines, which are

determined by the type of activation signal provided. Cytokine mRNA levels measured by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) has revealed expression of mRNA for IL-1, IL-2, IL-4, IL-5, IL-10, IL-15, IFN-γ, TNF-α and TGF-β in freshly isolated IELs from the small and large intestine of mice [Beagley et al., 1995]. It is probable that cytokines produced by IELs are intimately involved in immune responses within the epithelium and/or can affect immune responses in the underlying lamina propria (Fig. 1.2).

Increasing evidence implies a role of IELs in immuneregulation and homeostasis in the MALT. TCRγδ IELs are capable of producing keratinocyte growth factor (KGF), which may be important for IEC growth or repair of damaged IECs [Boismenu and Havran, 1994]. IELs might also directly remove damaged or transformed IECs through cytotoxic mechanisms. Further data has suggested an immunoregulatory role for TCRγδ$^+$ IELs in regulating responses to soluble antigens encountered via the oral route [Ke et al., 1997; Mengel et al., 1995] (see section 1.2.3.1). And recently, it was shown that IELs in the mouse skin actively suppress local infiltration of systemic, antigen-specific TCRαβ$^+$ T cells and thus may play a role in regulation of a normal cutaneous microenvironment [Girardi et al., 2002].

1.1.3 Lamina propria

The intestinal lamina propria is situated between the epithelium and the muscularis mucosa in both the small and large intestine of all mammals. The lamina propria is infiltrated by lymphoid/myeloid cells of all types. IgA plasma cells make up 30-40% of the mononuclear cells in human intestinal lamina propria. In the jejunum around 80% of the total plasma cells secrete IgA, around 18% secrete IgM and only 3% secrete IgG [Brandtzaeg et al., 1989; Crago et al., 1984; Strober and Harriman, 1991]. Large numbers of macrophages, dendritic cells and T cells are also present in the lamina propria, making it likely that antigen crossing the epithelium may be processed and presented to lamina propria T cells directly. These T cells could then provide B cell help as well as functioning in mucosal cell-mediated immunity.

Phenotype of LPLs

Scattered throughout the lamina propria are large numbers of thymus derived T cells. Approximately 40-90% of lamina propria lymphocytes (LPLs) are T cells expressing the CD3 surface marker. The vast majority of LPLs (~95%) bear an αβTCR complex [Fujihashi et al.,

1994; Ullrich et al., 1990]. Compared with the 50% of IELs expressing the γδ TCR, only around 3% of $CD3^+$ LPLs express this form of TCR [Ullrich et al., 1990] – a situation similar to that in blood. Likewise, the ratio between cells being positive for the coreceptors CD4 or CD8 in the lamina propria is similar to that found in blood, with a high percentage of $CD4^+$ cells (35-65%) [James et al., 1986; Senju et al., 1991]. Although blood lymphocytes and LPLs share several common phenotypic features, LPLs differ in their maturational state. $CD4^+$ LPLs are predominantly of a memory phenotype with most cells (~80%) expressing CD45RO. LPLs frequently express surface markers associated with activation, presumably the result of continuous antigenic and mitogenic challenge encountered in the gut. 15-30% of freshly isolated intestinal LPLs has been shown to be $CD25^+$ (IL-2 receptor α-chain) [Schieferdecker et al., 1992; Zeitz et al., 1988]. In addition, a putative marker of recent activation, CD69, is expressed by 80-90% of $CD4^+$, 90-100% $CD8^+$ and on all TCRγδ LPLs, while it is virtually absent on the circulating counterparts [De-Maria et al., 1993]. Other activation markers found on LPLs include MHCII [Zeitz et al., 1988] and a frequent expression (~40%) of the $\alpha_E\beta_7$ integrin, which is expressed during activation [Cerf-Bensussan N. et al., 1987; Schieferdecker et al., 1990].

Function of LPLs

Although LPLs display an increased expression of activation markers, dividing T lymphocytes within the normal lamina propria are rare [Fell et al., 1996]. Freshly isolated LPLs however contain a high frequency of cytokine-secreting cells compared with blood T cells from the same individual [Carol et al., 1998; Hauer et al., 1997]. As with IELs, LPLs do not respond well to CD3 ligation but stimulation with anti-CD2 elicits a strong cytokine response including production of IFN-γ, IL-2, IL-4 and IL-5 [Carol et al., 1998; Hauer et al., 1997]. Even if a strong IFN-γ response has been demonstrated from LPLs it has also been shown that LPLs are very high producers of IL-10 [Braunstein et al., 1997] and has the highest proportion of cells spontaneously secreting IL-5 compared with IELs and $CD4^+$ cells isolated from PP [Taguchi et al., 1990]. Functionally, it seems, that although both Th1- and Th2-type cytokines are produced by LPLs, the overall effect is Th2-predominant.

LPLs and their derived cytokines provide marked help during IgA production [Smart et al., 1988] and are crucial during two major steps: The induction of μ to α isotype switching in the

PP and the subsequent differentiation of surface IgA^+ B cells to IgA-secreting plasma cells in the lamina propria [Mega et al., 1991]. The generation of secretory IgA in the gut is driven by Th2 cytokines: both IL-5 [Beagley et al., 1988; Sonoda et al., 1989] and TGF-β [Lebman et al., 1990; Sonoda et al., 1989] are involved in the isotype switch from IgM to IgA. In general, LPLs functional capacity is shifted towards helper function. LPLs exert a regulatory influence (help/suppression) on immunoglobulin synthesis and also play a role in regulating other lymphoid and non-lymphoid cells within the GALT. Cytotoxic activities are infrequent in the lamina propria [James and Graeff, 1985; Pang et al., 1993] and many of the immunoregulatory functions carried out by LPLs are thought to be mediated at least in part by their secreted cytokines. The importance of appropriate immune regulation and homeostasis of IELs and LPLs in the GALT is highlighted by the increased number of IELs and LPLs in the intestinal epithelium and lamina propria of patients with Crohn's disease, ulcerative colitis and food-sensitive enteropathies (reviewed in [Strid and Strobel, 2002]). The specialised functions of LPLs (and IELs) are likely to be critical in maintaining normal host defence and homeostasis as well as to downregulate immune responses to ubiquitous lumenal antigens.

1.1.4 Peyer's patches

Peyer's patch lymphoid nodules, first described by Peyer in 1667, are the main organised lymphoid tissue of the intestinal mucosa. The PP together with the mesenteric lymph nodes (MLN – see section below) constitute the afferent limb of the gastrointestinal immune system. PP appear as lymphoid aggregates arrayed along the small intestine with the majority in the distal small intestine [Cornes, 1965a; Cornes, 1965b]. Each PP consists of multiple lymphoid follicles which are arranged into T and B cell dependent areas in a similar way to conventional peripheral lymph nodes [Henry et al., 1970]. PP B cell-follicles consist of a mantle of IgM^+/IgD^+ B cells surrounding a germinal centre made up of B cell centrocytes and centroblasts. The germinal centres of the PP are unique in that they contain high numbers of B cells expressing surface IgA rather than IgG, found in germinal centres in peripheral lymphoid tissues [Kelsall and Strober, 1999]. T cells in the PP, of which the majority are $CD4^+$, are present in the greatest density in areas surrounding the high endothelial venules (HEV) and in the intrafollicular areas. T cells are also present surrounding the follicle, in the mixed cell zone of the dome and occasionally in the follicle centre [Spencer et al., 1986].

Lymphocyte migration to PP

It has been established for many years that PP are on the major route of lymphocyte recirculation. However, PP are unique among lymphoid tissues in that they contain no afferent lymphatics, which means that all lymphoid cell traffic into the PP is via migration from the bloodstream across the PP HEV. The main ligand involved in cell entry into PP is MadCAM-1, a molecule expressed on the HEV of PP, as well as on venules in the lamina propria and on other cell populations of the PP including some DCs [Butcher and Picker, 1996; Szabo et al., 1997]. This addressin is a ligand for two receptors on lymphocytes and appears to have a dual function in mediating cell traffic to the intestine. Firstly, it binds L-selectin and thus is important for the initiation of lymphocyte binding to the endothelial surface and lymphocyte rolling. Secondly, and perhaps more importantly, it binds the $\alpha_4\beta_7$ integrin, which is presumed to result in the passage of lymphocytes into the mucosal tissue [Kelsall and Strober, 1999]. T cells activated in mucosal tissues such as the PP (and MLN), will preferentially recirculate back to mucosal surfaces, whereas lymphocytes activated in peripheral lymphoid tissues will not enter the mucosa [Hamann et al., 1994]. The $\alpha_4\beta_7$ integrin receptor has been shown to be important for directing this lymphocyte migration to the intestine (reviewed in [Viney and Fong, 1998].

DCs in PP

Lumenal antigens are transported into the PP via specialised M cells situated in the FAE (discussed in section 1.2.1). The region of the PP between the FAE and the B cell follicle is the subepithelial dome (SED). DCs are present in high concentrations in the SED and are thus likely to be the main APCs in this region. Three phenotypically distinct subsets with proposed specific roles in tolerance and priming have been characterised in the PP. $CD11c^+$ $CD11b^+$ myeloid DCs are found in the SED, $CD11c^+$ $CD8\alpha\alpha^+$ lymphoid DCs are located in the T cell-rich intrafollicular areas whereas $CD11c^+$ DC lacking expression of both CD11b and $CD8\alpha\alpha$ are present both in the SED and intrafollicular region [Iwasaki and Kelsall, 1999a; Iwasaki and Kelsall, 1999b; Kelsall and Strober, 1996]. DCs in the SED appear to have a less mature phenotype than those in the intrafollicular region, suggesting that they process antigen from the lumen as relatively immature cells and then migrate to T cell-rich regions where they present antigen to T cells [Kelsall and Strober, 1996]. Myeloid PP DCs have been shown to produce IL-10 and to induce naïve T cells to do the same, while lymphoid PP DCs produce IL-12 [Iwasaki and Kelsall, 1999a; Iwasaki and Kelsall, 2001]. Kelsall et al. has

proposed that SED DCs upon encountering antigen transported via M cells may undergo selective differentiation pathways dependent upon the nature of the antigen. Innocuous food antigens would promote $CD11c^+$ $CD11b^+$ DCs to generate $Th3/T_{reg}$ responses via secretion of TGF-β and IL-10 in the SED. Antigens encountered together with some inflammatory or microbial stimuli ('danger'), would in contrast mature the DCs and promote migration to the intrafollicular region, where they would secrete IL-12 and prime naïve T cells to produce IFN-γ [Iwasaki and Kelsall, 1999a; Iwasaki and Kelsall, 1999b; Iwasaki and Kelsall, 2001; Kelsall and Strober, 1999; Kelsall and Strober, 1996].

1.1.5 Mesenteric lymph nodes

The mesenteric lymph nodes (MLN) represent a bridge between the mucosal and systemic immune system. There is a constant cell migration from the PP and lamina propria to the MLN. DCs from the PP and intestinal wall have been shown to continually emigrate to the MLN [Huang et al., 2000; Liu and MacPherson, 1993] and also activated T and B cells rapidly migrate from the PP to the MLN [Kelsall and Strober, 1999; Mayer, 2000]. MLN, like the PP, lack an organised afferent lymphatic system. As a consequence, antigen entering the lamina propria through the normal intestinal epithelium, and not via the M cell into the SED of the PP, is likely to pass directly to the MLN. In the MLN, the antigen may induce not only a mucosal immune response, but also a systemic immune response as antigen specific immune cells from the MLN follow the thoracic duct lymph into the peripheral circulation (see discussion below and Fig. 1.4).

1.1.6 Antigen sampling in the GALT and mucosal immunity

Gastrointestinal exposure to antigen has several possible outcomes, including: 1) induction of systemic immunological hyporesponsiveness (oral tolerance), 2) systemic priming and/or 3) induction of local secretory IgA responses in the absence of a measurable systemic immune response [Strobel and Mowat, 1998]. The normal healthy gut exhibits tolerance to both beneficial food antigens and to antigens derived from commensal flora, but will mount active and protective immune responses against detrimental and damaging antigens from gut pathogens [Bilsborough and Viney, 2002]. The multiple cell types present and functions composing the GALT are increasingly seen as the key to how antigens delivered to body

surfaces can elicit either immunogenic or tolerogenic responses. In some instances, these responses occur purely within the local tissue, but in other cases both local and systemic responses are elicited [Hayday and Viney, 2000]. What happens at the mucosal surfaces can thus determine the systemic response and local and systemic immune responses are probably closely connected via an 'information network' (Fig. 1.3). The choice of action at the MALTs can dramatically change the systemic immunological outcome and this 'information network' provides a starting point for elucidating how differential immune reactivity is decided.

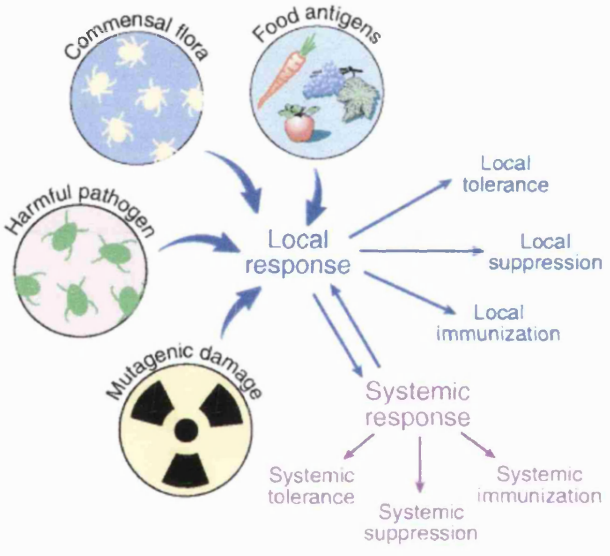


Fig. 1.3 The 'information network' in the gut
The mucosal surfaces of the gut are exposed to a myriad of antigens that require very different types of responses, ranging from tolerance to active immunity. The nature of the response is determined by whether the antigen is likely to be beneficial or detrimental to the body. When initiated, antigen responses can either be limited to the local tissues or be extended to elicit systemic responses. The systemically activated cells may then be required to feed back into the locally challenged area. Figure reproduced from [Hayday and Viney, 2000]

As most mucosal immune responses are antigen-specific, the journey of orally delivered antigens is of importance for the outcome of the immune response. An antigen delivered into the intestine encounters a very complex environment with many cell types (as described above) as well as ubiquitous digestive enzymes. The route of antigen entry determines the

cellular environment a specific antigen will encounter and is likely to be of pivotal importance in determining the resulting active immunity or tolerance. The first cells in the GALT that orally administered antigen is likely to contact are the epithelial cells lining the small intestine. These cells could play a number of roles in the regulation of mucosal immune responses including the exclusion, filtration or presentation of antigen [Garside and Mowat, 2001]. Filtration may exclude potentially immunogenic aggregates permitting only soluble antigens to gain access to the immune system. Particulate materials and microorganisms such as bacteria and viruses can be taken up via M cells and pass intact to the M cell pocket, from where they are transported to the PP by DCs. Particulate material gaining access to the GALT in this way appear to promote active immunity [Neutra et al., 1996; Neutra, 1998]. Soluble antigens may not use this route of entry to a great extent but have two other options by which it can access the GALT: the paracellular route between the IECs or the transcellular route through the IECs. It is assumed that whether the primary activation of intestinal T cells takes place in the PP or in the lamina propria, the cells will rapidly migrate to the MLN, from where they will follow the thoracic duct lymph into peripheral circulation. Thereafter, many of the memory/effector cells are dispersed back to mucosal tissues by site-specific homing mechanisms and thus complete the circuit of the 'information network' in the gut. Fig. 1.4 shows a general schema for antigen sampling in the gut and the basis for relaying information to the systemic immune system. Although the GALT is the only MALT to have PP, the remaining structures are common to all body surfaces.

The possible routes of soluble antigen entry via the intestinal lumen and the outcome of such antigen encounter is discussed further below with an emphasis on tolerance induction/maintenance (section 1.2.1).

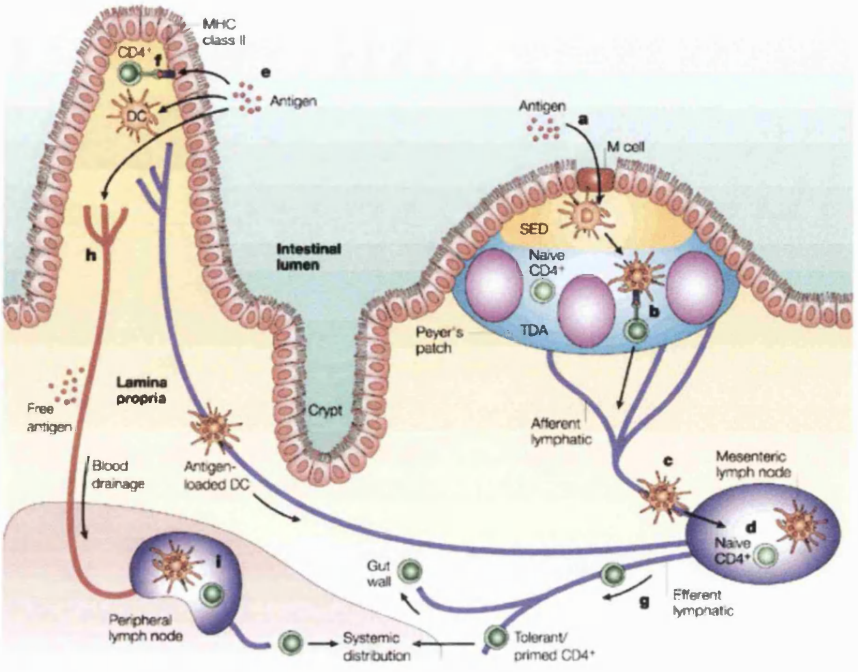


Fig. 1.4 Schematic diagram of the integrated mucosal immune system and antigen sampling in the gastrointestinal tract
Antigen can enter the intestine through the M cells (**a**) into the M cell pocket, where it will be taken up by DCs and carried to the PP (**b**). Antigen or antigen-loaded DCs from the PP may also gain access to the draining lymph (**c**), with subsequent T cell recognition in the MLN (**d**). Alternatively, antigen can be taken up by the intestinal epithelial cells (**e**) and presented to T cells in the lamina propria (**f**). Antigen entering though the epithelium may also disseminate directly to the MLN alone or loaded on APCs. The M cell pathway favours particulate antigen while only soluble antigens will enter through the epithelium. Antigen-primed lymphoid cells emigrate via the efferent lymph (**g**) and reach the peripheral blood through the thoracic duct. Antigen may also gain direct access to the bloodstream from the gut (**h**) and interact with T cells in the peripheral lymphoid tissues (**i**) [SED: Subepithelial dome; TDA: Thymus-dependent area]. Figure from [Mowat, 2003]

1.2 Oral tolerance

The best understood mechanism of immunological tolerance is central tolerance, which is established in the primary lymphoid organs of the thymus and bone marrow by the physical deletion of self-reactive T and B cells . However, not all self-antigens are represented in the primary lymphoid organs and central tolerance must therefore be complemented by additional mechanisms of peripheral tolerance. Oral tolerance can be considered a special case of peripheral tolerance.

Oral tolerance is defined as a state of antigen-specific systemic hyporesponsiveness induced by oral exposure to a specific antigen. It was first reported by the American physician R Dakin in 1829, when he described the belief of South American Indians to eat poison ivy leaves to prevent subsequent contact sensitivity reactions [Dakin, 1829]. The first experimental report came from the Russian-French scientist Alexandre Besredka, who in 1909 showed that guinea pigs fed with milk protein became refractory to anaphylaxtic reactions to milk [Besredka, 1909]. This work was confirmed and expanded by H.G. Wells in 1911 when he demonstrated that feeding of hen's eggs protein and vegetable proteins to guinea pigs could prevent anaphylaxis upon re-challenge [Wells, 1911]. The immunological basis of these observations was investigated by Chase in 1946 using contact sensitising agents, and became known as the Sulzberger-Chase phenomenon. Systemic tolerance after feeding re-emerged as a subject for study 20 years later and was subsequently termed oral tolerance [Tomasi et al., 1980].

Orally induced systemic hyporesponsiveness is an active process and not simply the absence of a response. The context in which an antigen is encountered usually determines the resulting immune response. Oral tolerance is thus just one end of a spectrum of responses to exogenous antigens. Immunological unresposiveness is probably more common than immune responsiveness and the scope of oral tolerance is highlighted by the fact that most people have lifelong clinical and immunological tolerance both to food antigens and to their gut flora. A number of diseases however may owe their aetiology to a failure of oral tolerance induction or breakdown of its maintenance. The limitation of a response to innocuous antigens at mucosal surfaces is thus as important as the stimulation of immune responses to antigens of pathogenic microbes.

1.2.1 Sites of oral tolerance induction

The exact site of tolerance induction to an orally administered antigen has not been clearly established. Antigen present in the gut can gain access to the MALT either through M cells into PP, between epithelial cells (paracellular) or through the epithelium (transcellular). The candidate sites for oral tolerance induction are therefore PP or lymphoid follicles, the epithelial layer with the lamina propria or the systemic lymphoid compartment.

M cells and Peyer's patches

The pathway involving M cells has been the best characterised and there is clear data that lymphoid follicles or PP of the gut are a site of tolerance induction [Richman et al., 1981; Santos et al., 1994]. This does not exclude other sites of tolerance induction, as highlighted by recent studies showing the induction of oral tolerance to cellular immune responses in the absence of PP [Spahn et al., 2001; Spahn et al., 2002]. Spahn et al. demonstrated that PP are not an absolute requirement for the induction of oral tolerance, although tolerance could not be induced in mice devoid of both PP and MLN. In addition, it has long been known that M cells are not very efficient in taking up soluble protein antigens, which are the classical tolerogens. Furthermore, M cells are a very small part of the cells within the intestinal epithelium, which also raises questions regarding the M cell/PP pathway's importance in tolerance induction.

Lamina propria via paracellular antigen entry

The paracellular route of antigen entry is less likely to play a role in tolerance induction under normal physiological conditions where tight junctions joining the IECs makes the extracellular epithelial space impermeable to peptides. In fact, some observations suggest that mucosally induced systemic tolerance depends on an intact epithelial barrier [Strobel et al., 1983]. The importance of cellular junctions is also evident by the observation of permeability defects in disease. In diseases such as Crohn's disease, ulcerative colitis and inflammatory bowel disease (IBD) there is an increased flux of antigen across both inflamed and uninflamed areas of the mucosa. The results of disrupted barrier function are clear, as is seen in patients with IBD where there is abrogation of tolerance to luminal flora [Shao et al., 2001]. However, were antigen to utilise the paracellular pathway it could gain direct access to the lamina propria where it would be taken up by APCs such as DCs. Antigen presentation by DCs is normally associated with immunological priming, but DCs in the lamina propria usually reside in an immature state lacking a full range of costimulatory molecules such as CD80/CD86, CD40 and CD54 and a number of studies have suggested that these cells may be tolerogenic if presenting antigen in the absence of inflammation [Finkelman et al., 1996; Viney et al., 1998]. Indeed, it has been shown that expanding but not maturing DCs *in vivo* with Flt3L enhances the induction of oral tolerance [Viney et al., 1998] while expanding DCs *in vivo* with Flt3L and simultaneously maturing them with IL-1α to express high levels of MHCII and costimulatory molecules abrogates the induction of oral tolerance and induces active immunity [Williamson

et al., 1999b]. An intriguing novel way for antigens to gain paracellular entry to the GALT has been suggested to occur by DCs penetrating IEC tight junctions and sampling bacteria directly from the gut lumen [Rescigno et al., 2001]. DCs were shown to express tight junction proteins such as occludin, claudin 1 and zonula occludens 1 and were able to extend processes through IEC tight junctions both *in vitro* and *in vivo* while preserving the integrity of the epithelial barrier. The lumenal bacteria were internalised, transported below the epithelial surface and subsequently the DCs migrated out of the intestine [Rescigno et al., 2001]. Although many unanswered questions remain, these lamina propria DCs are numerous and this newly identified mechanism of immunosurveillance could, if confirmed, play an important role in adaptive immunity. Whether these DCs also sample soluble antigens and participate in tolerance induction remains to be seen.

Lamina propria and MLN via transcellular antigen entry

The transcellular route of antigen entry implies that IECs might take up, process and present antigen to T cells next to them in the epithelium or in the lamina propria. Indeed there has been increasing recognition that the intestinal epithelial layer is an active component of the mucosal immune system and IECs have been demonstrated as potential APCs based on their ability to process and present antigen to primed T cells (see section 1.1.1). IECs express both classical and non-classical MHC molecules, and antigen presentation by gut IECs *in vitro* seems to preferentially induce $CD8^+$ suppressor T cells [Allez et al., 2002; Bland and Warren, 1986; Mayer and Shlien, 1987]. This possibly relates to the expression of unconventional costimulatory molecules such as gp180 and CD58 as well as a lack of conventional costimulatory molecules on IECs, which could promote suppressive or anergic T cell responses respectively [Hershberg and Mayer, 2000; Sanderson et al., 1993]. There is, as yet, no *in vivo* data to support the concept that IECs actually present antigen to the local specialised T cell populations and that these T cell if activated by IECs could travel to systemic lymphoid tissues. The current hypothesis is that antigen sampled by IECs and presented to local T cells may be responsible for the control of mucosal inflammatory responses but not oral tolerance [Mayer, 2000a]. There are other ways than direct antigen presentation in which IECs could contribute to the induction of oral tolerance. It has been shown that local DCs take up apoptotic IECs and transport them to T cell areas of the MLN. Here they can cross-present the content of the IECs and thus may induce and/or maintain peripheral tolerance to lumenal and/or self-antigens [Huang et al., 2000]. Additionally, IECs may signal directly to underlying

cells in the lamina propria whether an antigen is 'dangerous' or not. Upon contact with invasive bacteria IECs are capable of activating a pro-inflammatory cascade through the transcription factor NF-κB [Elewaut et al., 1999], while encounter with non-invasive bacteria seem to induce an anti-inflammatory state through inhibition of IκB-α ubiquitination and thus inhibition of the NF-κB signal transduction pathway [Neish et al., 2000].

Systemic lymphoid tissue

Another possible site for tolerance induction is the systemic lymphoid compartment. In addition to stimulating the GALT, some oral antigen is absorbed into the systemic circulation. Although dietary antigens are degraded by the time they reach the small intestine, studies have shown that the degradation is partial and that some intact antigen as well as antigen fragments are absorbed and can be detected in serum within minutes of ingestion [Bruce and Ferguson, 1986; Peng et al., 1990; Swarbrick et al., 1979]. Absorbed antigen can then be distributed to the spleen, peripheral LN and the liver, where presentation can take place. Oral tolerance has been demonstrated to be transferable by serum obtained one hour after feeding antigen to mice [Bruce and Ferguson, 1986; Strobel et al., 1983]. Intriguingly, serum from mice injected with serum spiked *in vitro* with the antigen did not induce subsequent hyporesponsiveness, suggesting that absorbed antigen itself can not tolerise but that there may be some unique tolerogenic properties of antigen that passes through the gut. It has been proposed that IECs are involved in this 'serum transferred tolerance' by processing lumenal antigens and then budding off small $MHCII^+$ particles. These tolerosomes or exosome-like structures may be released into the serum, from where they can be acquired by APCs, or perhaps recognised directly by T cells [Karlsson et al., 2001].

It is likely that multiple pathways are involved in the generation of tolerance and that the extent of contribution at each site is influenced by the nature of the antigen and the ingested dose. The importance of tolerance and gut homeostasis makes it all the more probable that immuneregulation of intestinal immune responses is controlled at many levels.

1.2.2 Features of oral tolerance

A number of important features of oral tolerance have been defined:

Tolerance is specific for the antigen that is fed in both cellular and humoral responses [Miller and Hanson, 1979]. The majority of tolerogens are soluble proteins; larger particulate antigens,

globular antigens and carbohydrates are more likely to induce an active immune response [MacDonald, 1998; Mayer, 2000b]. Furthermore, aggregated or heat-treated soluble proteins loose their capacity to induce oral tolerance [Peng et al., 1995]. Another common feature of tolerogenic antigens is that most induce T cell dependent immune responses when given parenterally and most are good immunogens. Oral tolerance does not appear to be induced by T cell independent antigens [Titus and Chiller, 1981]. Achieved tolerance is fairly long lasting but does wane with time. Suppression of specific immunoglobulin responses in mice lasts up to three month after the initial feed [Strobel and Ferguson, 1987], while the cellular immune response to a second antigen challenge remains profoundly suppressed for up to 17 months after the initial antigen exposure [Strobel and Ferguson, 1987]. Although tolerance has been reproducibly demonstrated after the feeding of many different proteins, the total number of antigens that have been fed to rodents to experimentally induce oral tolerance is fairly limited compared to the large number of antigens that must be encountered at mucosal surfaces. Furthermore, most experimental systems use animals that are naïve to the protein when it is administered. A major feature of oral tolerance is that feeding of antigen prevents the induction of an immune response more effectively than it reduces an established one [Elson and Zivny, 1996].

The level and the nature of the suppression achieved by oral administration of antigen is dependent on the parameters assessed. In general cellular immune responses are more profoundly inhibited than humoral responses. There also appears to be a gradient of sensitivity of different cell types to the induction of oral tolerance with Th1 $CD4^+$ T cells the most susceptible followed by Th2 $CD4^+$ T cells [Burstein et al., 1992; Melamed and Friedman, 1994]. Moreover, it seems that tolerance is T cell-mediated and the reduction in antibody responses after antigen feeding is more likely to be due to a reduction in T helper activity than to direct tolerisation of B cells [Elson and Zivny, 1996; Titus and Chiller, 1981].

The sequence of events immediately following antigen feeding has been explored by several scientific groups and it has been demonstrated that there is a stage of T cell activation, cell cycling and cytokine production prior to suppression of the response [Gutgemann et al., 1998; Smith et al., 2002; Sun et al., 1999; Williamson et al., 1999a]. Moreover, the activation events found in the PP and MLN of fed animals have been found to be paralleled by simultaneous T cell activation in peripheral lymphoid organs [Gutgemann et al., 1998; Smith et al., 2002; Sun

et al., 1999]. T cell activation and accumulation were detected within 24 hr of feeding in both PP, MLN and peripheral LN, but only 3 days post feeding in the lamina propria. Interestingly, the kinetics of the T cell responses after oral antigen administration did not differ from those observed after antigen was given in an immunogenic form with adjuvant and administered subcutaneously [Smith et al., 2002]. Only in the MLN did tolerised T cells undergo fewer cell divisions than primed T cells, which may suggest that in spite of the simultaneous T cell activation in mucosal and peripheral tissues, (irrespective of whether a tolerogenic or immunogenic stimuli is given), the ultimate decision of tolerance lies within the mucosal tissues.

1.2.3 Factors affecting oral tolerance

Many different antigens, doses and feeding regimes have been shown to induce tolerance in different experimental models. However, a variety of both external- and host factors have been demonstrated to be important for successful induction of oral tolerance (Table 1.1).

Table 1.1
Factors important for the induction of oral tolerance

Nature of antigen
Immunogenicity of the antigen
Dose
Frequency of feeding
Mucosal route
Species
Genetic background
Age
Intestinal flora
Delivery system/adjuvant

It has become clear that different feeding regimes result in different pathways to tolerance. It is likely that the administered dose of antigen will determine the site of tolerance induction, which could in turn influence the mechanism of tolerance. The effect of varying the dose of fed antigen in tolerogenic protocols is highlighted by the fact that very small doses may prime the animal for subsequent systemic and local immune responses [Mowat, 1987]. However, if the smaller priming dose is fed repeatedly, priming does not occur and a strong tolerogenic

response is initiated [Peng et al., 1989]. Indeed, increasing the frequency of oral antigen exposure results in a more marked and longer lasting tolerance [Strobel and Mowat, 1998].

Host factors such as the genetic background of the animal appear to play a large but ill-defined role in the induction of tolerance. BALB/b mice are more difficult to tolerise than their congenic counterpart BALB/c – but no clear linkage between the ease of tolerance induction and MHC haplotype has been found [Lafont et al., 1982; Stokes et al., 1983; Tomasi et al., 1983]. The genetics regulating these differences have not been carefully studied even if such studies might aid in understanding differences between human and murine models.

Age of the animal also plays a role. Intragastric antigen administration to neonatal rodents does not induce tolerance and may prime for later systemic immunity and autoimmune responses [Miller et al., 1994; Strobel, 1996; Strobel and Ferguson, 1984]. An analogous deviation from tolerance induction occurs during weaning. This may not be a consequence of age though, but due to intestinal changes characteristic of this period [Strobel, 1992; Strobel, 1996]. Indeed, the extent and nature of intestinal colonisation by bacterial flora can effect the outcome of oral administration of antigen. It has been observed that tolerance to fed antigens is relatively short-lived in germ-free animals [Moreau et al., 1995] and a further indirect role for the digestive flora comes from experiments demonstrating enhanced tolerance induction in normal mice if lipopolysaccharide (LPS) is given orally at the same time as antigen feeding. Conversely, if LPS is administered subcutaneously at the time of feeding, oral tolerance is abrogated [Khoury et al., 1990].

Few studies have addressed whether oral tolerance can be enhanced by using mucosal adjuvants or varying the delivery of antigen. However, it has been demonstrated that recombinant cholera toxin B subunit coupled to antigen reduces the amount of antigen needed for tolerance induction [Sun et al., 2000; Sun et al., 1994]. As a natural adjuvant, it has been shown that expanding lamina propria DCs with Flt3L likewise enhances the induction of oral tolerance [Viney et al., 1998]. Little is known about the mechanism of enhancement of tolerance in these systems, but they represent evidence that oral tolerance can be enhanced and further development of the area may be crucial for successful application of oral tolerance to the treatment of human disease.

1.2.3.1 Modulation of oral tolerance

There is considerable data on the influence of different agents on induction and modulation of oral tolerance. Table 1.2 summarises some of the tested agents and their effect on oral tolerance.

Table 1.2
Agents modulating oral tolerance

Enhances	Decreases
IL-2	IFN-γ
IL-4	IFN-γ KO
IL-10	IL-12
Anti-IL-12 Ab	CT
IFN-β	Anti-CD86 Ab
LPS	CD80 expression
CTB	Anti-CTLA-4 Ab
Flt3L	CD40L KO
	γδ KO
	Anti-γδ Ab

IL = interleukin; Ab = antibody; IFN = interferon; LPS = lipopolysaccharide; CTB = cholera toxin B subunit; Flt3L = Flt3 Ligand; CT = cholera toxin; KO = gene knock-out; CTLA-4 = cytotoxic T-lymphocyte antigen-4

Effect of cytokines on oral tolerance

Oral tolerance has often been defined in terms of Th1/Th2 responses. A number of reports suggest that inhibition of Th1 and/or enhancement of Th2 or Th3 cell development promotes oral tolerance. Intraperitoneal administration of IL-4 enhances low-dose tolerance to myelin basic protein (MBP) in the experimental autoimmune encephalomyelitis (EAE) model [Weiner, 1997]. IL-4 and IL-10 given orally can enhance tolerance when co-administered with antigen [Inobe et al., 1998]. On the other hand, mice with a disrupted IL-4 gene ($IL\text{-}4^{-/-}$) exhibit normal oral tolerance to OVA [Garside et al., 1995b]. IFN-γ and IL-12 have been shown to abrogate oral tolerance induction [Marinaro et al., 1997; Zhang et al., 1990] and treatment with anti-IL-12 has independently shown to enhance oral tolerance and to increase TGF-β production and T cell apoptosis [Marth et al., 1996]. However, oral tolerance can only be partially induced in IFN-γ deficient mice [Kweon et al., 1998], suggesting that IFN-γ has an

important role in at least the inductive phase of oral tolerance. IFN-γ may contribute to suppression of cytokine production (e.g. IL-4) leading to the induction of B cell tolerance and reduced production of antigen-specific antibodies. In the effector phase of oral tolerance, IFN-γ production is strongly down-regulated. Overall, it seems to be an oversimplification to describe oral tolerance purely in terms of Th1/Th2 balance.

In the murine uveitis model, IL-2 potentiates oral tolerance and is associated with increased production of TGF-β, IL-10 and IL-4 [Rizzo et al., 1994]. Oral IFN-β synergises with the induction of oral tolerance in SJL/PLJ mice fed low doses of MBP [Nelson et al., 1996]. Cholera toxin (CT) is a potent mucosal adjuvant and feeding CT together with an otherwise tolerogenic protein promotes active immunity as opposed to tolerance [Elson and Ealding, 1984; Iijima et al., 1998]. However, peripheral tolerance is enhanced when a protein is coupled to CTB and given orally [Sun et al., 2000; Sun et al., 1994].

Effect of costimulatory molecules on oral tolerance

In the EAE model, anti-CD86 antibody treatment but not anti-CD80 antibody treatment abolished the tolerance induced by MBP feeding [Liu and Weiner, 1998]. In an intra-PP gene transfer model, CD80 expression was shown to partly abrogate oral tolerance, while the co-transfer of CD80 and IL-12 totally abrogated oral tolerance [Chen et al., 2000]. These experiments suggests some differential activity of the CD80 and CD86 molecule in activation and tolerance induction [Freeman et al., 1995] (and reviewed in [Sharpe and Freeman, 2002]). Reports from several laboratories indicate that CD80/86 may be required for the induction of tolerance and that the CD80/86: CTLA-4 interaction is essential for maintaining T cell tolerance [Chen et al., 2002; Perez et al., 1997; Samoilova et al., 1998]. The role of CD40L-CD40 interactions in oral tolerance has also been investigated. CD40L deficient mice can not be orally tolerised to OVA, suggesting that CD40L-CD40 engagement may be essential for induction of oral tolerance to proteins [Kweon et al., 1999].

Role of TCRγδ T cells in oral tolerance

Oral tolerance can not be induced in mice lacking γδ T cells [Ke et al., 1997] or if signalling through γδTCR is blocked by anti-γδ T cell antibody [Mengel et al., 1995]. Another report however finds that γδ T cells play a pivotal role in low-dose oral tolerance but that tolerance

to large oral doses of antigen can still be induced in δ-chain deficient mice [Fujihashi et al., 1999]. This data may suggest that the dose of the antigen determines the importance of each distinct immunological site in tolerance induction. In addition, it has been shown that transfer of as little as 500 antigen-specific γδ T cells in a model of inhaled antigen in mice could suppress $CD4^+$ responses and IgE production [McMenamin et al., 1994]. The role of these specialised mucosal T cells and their mechanism of action in oral tolerance and tolerance at other body surfaces is as yet poorly understood.

1.2.4 Mechanisms of oral tolerance induction and maintenance

Whether tolerance or immunity is generated to an antigen depends mainly on two factors: how the antigen is presented to lymphocytes (antigen concentration, tissue location, persistence, and the nature of the cells that present the antigen), and how the responses of the antigen-specific lymphocytes are regulated. Unresponsiveness of T cells in the periphery may be achieved either by a process of functional inactivation (anergy), or by actual elimination of the cell by apoptotic death as a result of antigen stimulation (deletion). Alternatively, regulatory cells or mediators may be induced that actively modify the immune response. Multiple mechanisms of oral tolerance have been identified consistent with its importance to the functioning of the mucosal immune system. The most commonly described mechanisms are clonal deletion, anergy or the induction of suppressor or regulatory cells. Which of these mechanisms operate in the induction of oral tolerance is not clear and seems to depend on the tolerising regimen employed. Thus it has been proposed that clonal deletion may result from feeding high doses of antigen while some form of active regulation may be a feature of low-dose and/or repeated feeding regimes [Chen et al., 1995; Melamed and Friedman, 1993; Thorstenson and Khoruts, 2001]. The suggested mechanisms are not mutually exclusive though, and it may well be that more than one of them operates simultaneously.

1.2.4.1 Clonal deletion

Clonal deletion by cell death via apoptosis is well documented as a procedure for inducing central tolerance in the thymus [Sprent and Kishimoto, 2001]. If clonal deletion of antigen-specific T cells in the periphery is induced by feeding a high dose of antigen, it would be expected to result in a stable, long-lasting tolerance, but presumably with a risk of repopulation with naïve T cells from the thymus that could be activated at a later stage.

Various studies have shown evidence of clonal deletion as a mechanism of oral tolerance induction in mice. Relatively large doses of antigen were given orally in those studies, suggesting that apoptosis might occur as a result of activation-induced cell death. Deletion of $CD4^+$ T cells via apoptosis has been clearly shown *in vivo* after high dose oral feeds of OVA in OVA T cell receptor (TCR) transgenic mice [Chen et al., 1995; Marth et al., 1996; Marth et al., 1998]. In normal mice the results are more conflicting, and under physiological conditions gastrointestinal exposure to such large amounts of protein is unlikely. The fact that oral tolerance can be induced normally in Fas deficient lpr mice argues against a role for Fas-dependent apoptosis in an intact animal [Mowat et al., 1998]. However, there is some indication that tumour necrosis factor (TNF)-receptor mediated apoptosis could possibly play a role in oral tolerance [Garside and Mowat, 2001; Mowat et al., 1998].

1.2.4.2 T cell anergy

T cell anergy is defined as a state of T lymphocyte unresponsiveness characterised by absence of proliferation, IL-2 production and diminished expression of the IL-2 receptor [Schwartz, 1990]. The anergic state can be reversed by addition of exogenous IL-2. The proposed mechanism for the induction of T cell anergy has been the recognition of antigen on APCs providing TCR ligands (signal 1) in the absence of costimulatory molecules (signal 2). It is becoming increasingly clear however that it is not the expression of costimulatory molecules alone that determines whether APCs induce tolerance or immunity [Banchereau et al., 2000; Blankenstein and Schuler, 2002; Jonuleit et al., 2001b]. Anergy as a mechanism for oral tolerance has been shown both directly and indirectly in several different models [Melamed and Friedman, 1993; Van Houten and Blake, 1996; Whitacre et al., 1991]. Hyporesposiveness of antigen-specific T cells following a single oral administration of antigen can be reversed *in vitro* by addition of IL-2, indicating the presence of antigen-reactive cells [Melamed and Friedman, 1993; Whitacre et al., 1991]. Adoptively transferred antigen-specific TCR transgenic T cells persists *in vivo* following oral tolerance induction but are hyporesponsive to antigen when re-activated *in vitro* [Van Houten and Blake, 1996], suggesting functional inactivation of specific T cells without deletion.

1.2.4.3 Regulatory T lymphocytes

It has long been known that tolerance can be induced in naïve animals by adoptive transfer of T cells from orally tolerised animals. The concepts of anergy and clonal deletion as

mechanisms for oral tolerance induction cannot explain this 'transferable tolerance' and the existence of a subpopulation of T cells that specialises in the suppression of immune responses was originally postulated already in the early 1970s [Gershon and Kondo, 1971]. However, the cellular and molecular mechanisms responsible for the suppressive phenomenon were never characterised and the interest in the field declined. The suppressor T cell has now been reborn as the regulatory T cell (T_{reg}) and in recent years many new reports on the biology, development and function of these T_{reg} cells have been published (reviewed in [Maloy and Powrie, 2001; Shevach, 2000; Shevach, 2002]).

$CD8^+$ versus $CD4^+$ T_{reg}

Many early studies found that tolerance could be transferred by cells that were predominantly $CD8^+$ and it was proposed that oral tolerance was a result of the activation of antigen specific $CD8^+$ suppressor T cells [Mowat, 1987]. In recent years, the existence of a discrete population of regulatory $CD8^+$ T cells has been questioned. The fact that oral tolerance can be induced in CD8 or β_2-microglobulin knockout mice and not in severe combined immunodeficiency (SCID) mice reconstituted only with $CD8^+$ T cells, indicates that there is no absolute requirement for $CD8^+$ T cells in the induction or maintenance of systemic tolerance [Lycke et al., 1995; Vistica et al., 1996; Yoshida et al., 1998]. In contrast to the more controversial role of $CD8^+$ T cells, there is overwhelming evidence for an essential role of $CD4^+$ T cells in the induction and maintenance of oral tolerance. Oral tolerance is abrogated if $CD4^+$ T cells are depleted *in vivo* [Barone et al., 1995; Desvignes et al., 1996; Garside et al., 1995a], MHCII-deficient mice with a total or partial defect in class II-restricted activation of $CD4^+$ T cells can not be orally tolerised [Desvignes et al., 1996], SCID mice can only be tolerised if reconstituted with both $CD4^+$ and $CD8^+$ T cells and not if reconstituted with $CD8^+$ T cells alone [Yoshida et al., 1998], and it has repeatedly been reported that $CD4^+$ T cells can transfer oral tolerance *in vivo* [Chen et al., 1994; Hirahara et al., 1995]. Originally these results were thought to reflect a preferential downregulation of Th1 $CD4^+$ cells by Th2 cells in oral tolerance. However, oral tolerance can suppress both Th1 and Th2 responses adequately [Garside et al., 1995b], which seems reasonable as Th2 mediated responses may be equally pathogenic as Th1 responses. In recent years the role of $CD4^+$ T cells as active mediators of oral tolerance has been explained by the presence of a population of T_{reg} cells within the CD4 population.

Generation of $CD4^+CD25^+$ T_{reg} cells

Studies in a number of experimental models of organ-specific autoimmune diseases provide evidence that specialised T_{reg} cells capable of controlling autoimmunity are an integral part of the T cell repertoire in normal animals. The spectrum of T cell populations to which regulatory functions have been attributed has been thoroughly reviewed [Roncarolo and Levings, 2000; Thornton and Shevach, 2000]. Naturally occurring T_{reg} cells are predominantly present in the small $CD4^+CD25^+$ T cell population [Maloy and Powrie, 2001]. There is accumulating evidence that $CD4^+CD25^+$ T_{reg} cells are part of a unique lineage of T cells that are selected during the normal process of T cell differentiation in the thymus [Seddon and Mason, 2000; Shevach, 2002]. It is not entirely clear at what stage in the selection process or where in the thymus this occurs. It has been suggested to occur as an altered negative selection in the thymic medulla [Itoh et al., 1999; Shevach, 2000], or at an earlier stage during positive selection in the thymic cortex [Bensinger et al., 2001]. After leaving the thymus, little is known of how T_{reg} cells are maintained within the peripheral pool. They are long lived cells and have been shown to persist for long periods after thymectomy [Papiernik et al., 1998]. Cytokines and costimulatory molecules are important in the homeostasis and/or generation of $CD4^+CD25^+$ T_{reg} cells, as these cells are absent from the periphery and thymocyte pool of IL-2 KO mice [Papiernik et al., 1998] and severely diminished in CD28 KO or B7 KO nonobese diabetic mice (NOD) [Salomon et al., 2000]. In addition to thymus generated and peripherally maintained $CD4^+CD25^+$ T_{reg} cells, evidence is emerging that $CD4^+CD25^+$ cells with similar regulatory functions may be acquired by naïve T cells in the periphery. T cell tolerance induced by oral administration of antigen would support the existence of peripheral pathways of T_{reg} cell induction. Indeed, the emergence of $CD4^+CD25^+$ T cells with anergic regulatory properties has been demonstrated *in vivo* in the OVA-specific transgenic $CD4^+$ T cell transfer model [Thorstenson and Khoruts, 2001]. A recent study suggests that human $CD4^+CD25^+$ T_{reg} cells may be generated from highly differentiated primed $CD4^+$ memory T cells in the periphery as a consequence of repeated antigen encounter on non-professional APCs [Taams et al., 2002] (and reviewed in [Akbar et al., 2003]). Thus, pathways additional to the thymus may exist whereby naïve or memory $CD4^+$ T cells in the periphery can be induced to differentiate into T_{reg} cells. 'Education' of peripheral $CD4^+$ T cells could provide a potential mechanism to extend the functional repertoire of T_{reg} cells to antigens that may not be encountered in the thymus.

Phenotype and function of $CD4^+CD25^+$ T_{reg} cells

$CD4^+CD25^+$ T_{reg} cells were originally discovered in mice, but a population with identical phenotypic and functional properties has now been identified in humans. $CD4^+CD25^+$ T cells constitute about 10% of both peripheral murine- and human $CD4^+$ T cells [Jonuleit et al., 2001a; Ng et al., 2001; Taams et al., 2001]. They have also been found in human cord blood [Wing et al., 2002], thymus [Stephens et al., 2001; Wing et al., 2002] and lymphoid organs like tonsil and spleen [Taams et al., 2001]. The $CD4^+CD25^+$ T_{reg} cell population possesses potent regulatory and suppressor properties both *in vitro* [Takahashi et al., 1998; Thornton and Shevach, 2000] and *in vivo* [Read et al., 2000; Suri-Payer et al., 1998]. They have been shown to inhibit pathogenesis in a range of different models. They can inhibit autoimmune diabetes, EAE, autoimmune gastritis and IBD. They can also induce tolerance to alloantigens, provide anti-tumour immunity and regulate the expansion of other peripheral $CD4^+$ T cells. Transfer of $CD25^-$ T cells into mice lacking $CD25^+$ T cells such as lymphopoenic *nu/nu* or SCID mice promotes development of various autoimmune disorders such as autoimmune gastritis (Reviewed in [Maloy and Powrie, 2001; Shevach, 2002]). The suppressor function of $CD4^+CD25^+$ T_{reg} cells is dependent upon prior stimulation through their TCR, but once activated they are able to inhibit both $CD4^+$ and $CD8^+$ T cells in an apparently antigen-nonspecific manner [Thornton and Shevach, 1998; Thornton and Shevach, 2000]. $CD4^+CD25^+$ T_{reg} cells display a memory phenotype ($CD45RA^-$, $CD45RO^+$) and express little CD45RB. In addition to the memory/activation antigens a significant proportion constitutively express CTLA-4 [Read et al., 2000; Takahashi et al., 2000], which distinguishes them from other lymphocyte subpopulations in mice and humans [Shevach, 2002]. They proliferate poorly upon TCR stimulation *in vitro* and their growth is dependent on exogenous IL-2 [Papiernik et al., 1998]. The use of CD25, a commonly expressed activation molecule on all lymphocytes, as a marker for T_{reg} cells may be misleading. The $CD4^+CD25^+$ T population is most certainly a heterogeneous population and even if a high proportion of these cells may be T_{reg} cells, this may not be true for the whole population and it might be that not all T_{reg} cells express CD25 either.

Mechanisms of function of $CD4^+CD25^+$ T_{reg}

It is becoming clear that $CD4^+CD25^+$ T_{reg} cells may use multiple mechanisms to suppress immune responses and the relative importance of these mechanisms seem to depend on the

experimental model. Regulatory cytokines and/or regulation through cell-cell contact have provided the strongest evidence of T_{reg} effector function (Fig. 1.5).

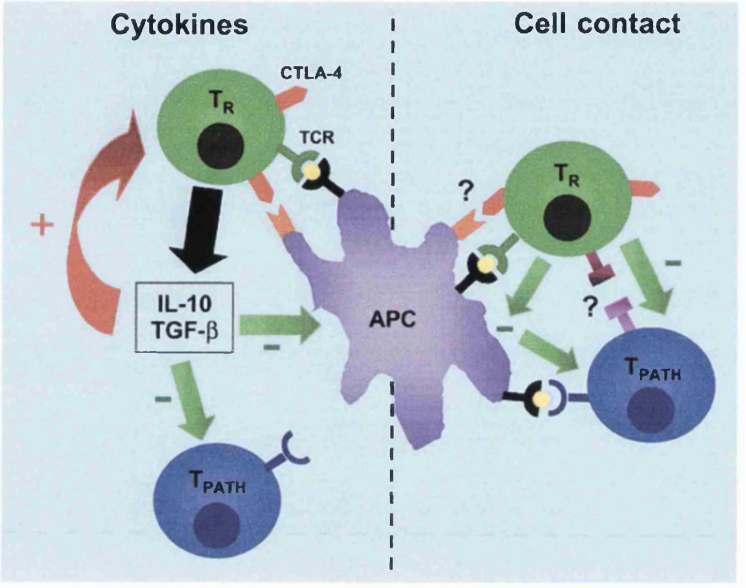


Fig. 1.5 T_{reg} cells can mediate their function via multiple mechanisms
The inhibitory function of T_{reg} cells on potentially pathogenic T cells (T_{PATH}) may be mediated through the actions of cytokines and/or via a cell contact-dependent mechanism. Although triggering through the TCR is required to induce T_{reg} function, the precise role of CTLA-4 is not clear. Similarly, the existence of putative molecules which may be involved in direct T cell-T cell interactions also remains to be established. Figure reproduced from [Maloy and Powrie, 2001]

Regulatory cytokines

The role of cytokines in T_{reg} function has been receiving much attention. The cytokines that have received most interest are IL-4, IL-10 and TGF-β depending on the model under investigation. *In vivo* studies have provided the strongest evidence of a role for cytokines in the effector function of T_{reg}. In the SCID IBD model, protection from colitis does not require IL-4, but is crucially dependent on TGF-β and IL-10 production by T cells [Asseman et al., 1999; Powrie et al., 1996]. IL-10 KO mice spontaneously develops severe IBD [Asseman et al., 1999]. The suppression of autoimmune thyroditis by T_{reg} cells in the rat model is reversed by anti-IL-4 or anti-TGF-β, while the role of IL-10 is yet to be determined [Seddon and Mason, 1999]. IL-4, IL-10 and especially high amounts of TGF-β have also been associated with the active suppression component of oral tolerance [Chen et al., 1994; Chen et al., 1995].

However, marked suppression of IL-10 and IL-4 have been reported in mice fed OVA [Garside et al., 1995b] and normal oral tolerance occurs in mice depleted of IL-10 [Aroeira et al., 1995]. TGF-β plays an important role in normal immune homeostasis in the gut tissue, and as such may be essential for maintaining tolerance at mucosal surfaces. However, enhanced TGF-β production is not a universal finding in models of mucosal tolerance [Smith et al., 2000], and the role of TGF-β in oral tolerance requires further investigation. *In vitro* studies have mostly failed to identify cytokines on which the effector function of $CD4^+CD25^+$ T_{reg} depend and $CD25^+$ T cells from IL-4 KO and IL-10 KO are fully competent suppressors [Thornton and Shevach, 1998]. Addition of neutralising antibodies specific for IL-4 or IL-10 does not reverse suppression, while addition of anti-TGF-β may [Nakamura et al., 2001] or may not reverse suppression [Takahashi et al., 1998; Thornton and Shevach, 1998]. Studies of TGF-β KO mice are difficult as they die of severe and widespread inflammation soon after loosing access to TGF-β in mothers milk.

Cognate interactions

While most *in vitro* studies of $CD4^+CD25^+$ T_{reg} cells do not support a role for cytokines, T_{reg} cells in both mice and humans appear to inhibit the proliferation of other T cell population *in vitro* by a cell-cell dependent mechanism which is independent on IL-4, IL-10 and TGF-β [Dieckmann et al., 2001; Taams et al., 2001; Takahashi et al., 1998; Thornton and Shevach, 1998]. There is conflicting data on whether the cell-cell contact-dependent inhibitory effects of T_{reg} cells are mediated via APCs (possibly DCs) or can act directly on other T cells. $CD4^+CD25^+$ T_{reg} cells have been shown to reduce amounts of costimulatory molecules on DCs and their ability to induce active immunity [Cederbom et al., 2000]. In another study however, upregulation of several costimulatory molecules on APCs occurred normally in the presence of $CD25^+$ T cells [Thornton and Shevach, 2000]. Cognate interactions between a T_{reg} and APC may 'prime' the APC to deliver a tolerogenic signal to the next T cell. In support of this hypothesis, $CD4^+CD25^+$ T_{reg} cells have been shown to modify APCs such that they can induce tolerance in subsequently encountered naïve T cells [Taams et al., 1998], and normal $CD4^+$ T cells have been shown to activate DCs in a contact-dependent manner to subsequently deliver help to $CD8^+$ cells of different specificity [Ridge et al., 1998]. Conversely, it has been found that $CD4^+CD25^+$ T_{reg} can inhibit responses even when the antigens recognised by the T_{reg} cells and the responder T cell are presented by separate APC

populations [Takahashi et al., 1998; Thornton and Shevach, 2000]. Additionally, $CD8^+$ T cells that can be activated directly by peptide-MHC tetramers in the absence of APCs can be readily suppressed by $CD4^+CD25^+$ T cells [Piccirillo and Shevach, 2001], suggesting the involvement of a direct T cell-T cell interaction that is independent of APCs.

The mechanisms by which these cognate interactions mediate tolerance remain unclear. A possible important role for binding of CTLA-4 to its ligand CD80 and CD86 on APCs has been demonstrated in several studies [Read et al., 2000; Takahashi et al., 2000]. The regulatory control of $CD4^+CD25^+$ T cells on IBD and colitis is dependent upon signalling through CTLA-4 [Read et al., 2000]. Maintenance of tolerance to self by $CD4^+CD25^+$ T_{reg} cells requires CTLA-4, as blocking this signal leads to spontaneous development of autoimmune diseases such as gastritis [Takahashi et al., 2000] and peripheral tolerance to OVA peptides is abrogated by administration of anti-CTLA-4 Ab *in vivo* [Perez et al., 1997]. *In vivo* blocking of CTLA-4 signalling has also been shown to prevent oral tolerance induction in the OVA transgenic T cell transfer model [Chen et al., 2002]. CTLA-4-deficient mice develop a fatal lympho-proliferative disease and multi-organ immune pathology, highlighting the essential role of CTLA-4 in normal homeostasis of the immune system [Tivol et al., 1995; Waterhouse et al., 1995]. Exactly how CTLA-4 may be involved in tolerance and the function of T_{reg} is yet to be defined. One possibility is that CTLA-4 signalling is required for the activation and mediation of the effector function of $CD4^+CD25^+$ T_{reg} cells. Signalling through CTLA-4 has been shown to enhance proliferation and TGF-β secretion of $CD4^+CD25^+$ T cells [Chen et al., 1998; Nakamura et al., 2001]. Nakamura et al. [Nakamura et al., 2001] have raised the possibility that this enhanced TGF-β production by $CD4^+CD25^+$ T_{reg} cells can mediate the cell-cell contact–dependent immunosupression by binding TGF-β to an as yet undefined molecule on their cell surface. They found large amounts of surface bound TGF-β by flow cytometry and blocking TGF-β was able to abrogate $CD25^+$ T cell mediated suppression completely. Hypothetically, the TGF-β bound to the surface of the activated $CD4^+CD25^+$ T_{reg} cells is delivered directly to the responder $CD25^-$ T cells by a cell contact-dependent mechanism [Nakamura et al., 2001]. This intriguing possibility remains to be confirmed by other laboratories. Finally, it has been revealed that the Notch-Serrate receptor-ligand pair may play a role in cognate regulation of tolerance. Overexpression of the Notch ligand Serrate-1 by DCs induce naïve $CD4^+$ T cells to become regulatory cells, which have immunosuppressive functions on primary and secondary

immune responses as well as being able to transfer antigen-specific tolerance to recipient mice [Hoyne et al., 2000].

Together these findings show that T_{reg} cells may use multiple mechanisms to inhibit immune activation. The relative roles of different suppressive mechanisms *in vivo* may be highly dependent on the local environment of the target organ and on the nature of the pathological immune response that is to be inhibited. There is little doubt that T_{reg} cells play an important role in regulating immune responses in health and disease. However, many questions of their development, how many types of T_{reg} that exist, how they are distinguished from other T cell populations and how they function remain partially unanswered.

1.2.5 The concept of bystander suppression

Bystander- or linked suppression is the concept that T_{reg} cells induced to a specific antigen can suppress immune responses stimulated by a different antigen as long as the specific antigen is present in the anatomical vicinity (reviewed in [Cobbold and Waldmann, 1998]). It was demonstrated experimentally, when it was found that cells from MBP-fed animals suppressed proliferation of an OVA-specific cell line *in vitro*, but only when triggered by the fed antigen. *In vivo,* rats tolerised by oral OVA administration were also tolerant to MBP induced EAE when MBP was administered together with OVA but not when administered without OVA [Miller et al., 1991]. Bystander suppression associated with oral tolerance has been demonstrated *in vivo* in several different autoimmune and organ-specific inflammatory disease models [von Herrath, 1997; Yoshino et al., 1995]. It has also been reported *in vitro* in humans [Zivny et al., 2001]. However, little is known about whether bystander suppression occurs *in vivo* in humans or how it can be triggered.

The ability of T_{reg} cells to inhibit both Th1 and Th2 responses makes them excellent targets for immune therapy in a diverse number of diseases, including allergy, transplantation and autoimmune diseases. Traditionally, antigen-specific approaches to immune therapy have been hampered because the precise antigen which triggers the disease in many cases is unknown. The ability of T_{reg} cells to mediate antigen-driven bystander suppression may circumvent this problem if T_{reg} cells could be generated to antigens known to be present in the target organ or in ill tolerated food. As the molecular and functional events associated with the generation and modulation of oral tolerance and of T_{reg} cells are better understood, the ability to apply oral

tolerance and/or T_{reg} cells successfully for the treatment of human disease is a distinct possibility.

1.2.6 Consequences of breakdown of (oral) tolerance

The same effector mechanisms that have evolved to protect the host from invading microorganisms may also induce immune pathology. A number of diseases owe their aetiologies to the induction of aberrant immune responses or loss of tolerance and a sizeable minority of the population suffer from autoimmunity or adverse reactions to foods.

Food-allergic diseases (Section 1.3.1) are mainly a feature of childhood and may represent a breakdown or failure of oral tolerance induction or maintenance. Failure to induce tolerance could result in systemic immune responses and a local destructive DTH reaction at the site of subsequent antigen challenge [Ferguson et al., 1983]. Several clinical disorders are compatible with this pattern, producing intestinal damage and malabsorption. The commonest of these enteropathies is cow's milk allergy which starts in infancy. The most studied permanent form of food-sensitive gastroenteropaty is coeliac disease with its persistent intolerance to gluten. An abnormal primary immune response of the small intestinal mucosa to gluten (gliadin being identified as the antigenic trigger) has been suggested to result in an allergic response and subsequent inflammation of the small intestine. In both cow's milk enteropathy and coeliac disease, the intestinal epithelium and lamina propria is densely infiltrated with IELs and LPLs [Halstensen et al., 1993; Phillips et al., 1979; Spencer et al., 1989]. It has likewise become apparent that IBDs such as Crohn's disease and ulcerative colitis may reflect a breakdown of tolerance to commensal bacteria [Duchmann et al., 1995; Elson et al., 1995]. This is consistent with the frequent usefulness of antibiotics in the treatment of human IBD and with the fact that all models of experimental IBD can be prevented by antibiotics or a germ-free environment [Garside et al., 1999]. Studies of most other food-related hypersensitivities and that of IBD has been hampered however by the lack of clearly defined antigenic triggers.

Much of our current knowledge of tolerance has come from models of autoimmunity. These studies have led to potential therapeutic strategies involving oral tolerance and a number of human clinical trials have been undertaken (Section 1.2.7 and Table 1.3).

1.2.7 Treatment of autoimmune disease by oral tolerance

Table 1.3
Suppression of autoimmunity by oral tolerance

Animal models		
Model	**Protein fed**	**Outcome**
EAE	MBP, PLP	Protection
Arthritis	Type II collagen, HSP	Suppression depend on antigen dose
Diabetes (NOD mouse)	Insulin, GAD	Delayed onset, possible prevention
Uveitis	S-Ag, IRBP	Suppression
Myasthenia gravis	AchR	Delayed onset
Thyroiditis	Thyroglobulin	Delayed onset
Colitis	Haptenised colonic proteins	Protection
Transplantation	Alloantigen, MHC peptide	Suppression of allograft rejection
Human disease trials		
Disease trial	**Protein fed**	**Outcome**
Multiple sclerosis	Bovine myelin	No significant effect
Rheumatoid arthritis	Chicken type II collagen	Improvement, but differences between studies and oral doses
Type I diabetes	Human insulin	Ongoing
Uveitis	Bovine S-Ag	No effect

EAE = Experimental autoimmune encephalomyelitis; NOD = Non-obese diabetic; MBP = Myelin basic protein; PLP = Proteolipid protein; HSP = Heat-shock protein; GAD = Glutamate decarboxylase; S-Ag = S antigen; IRBP = Interphotoreceptor retinoid-binding protein; AchR = Acetylcholine receptor; MHC = Major histocompatibility complex

1.2.7.1 Treatment of autoimmunity in animal models

Orally administered autoantigens have been successful in preventing, treating or suppressing autoimmunity in several animal models (Table 1.3) (reviewed in [Garside et al., 1999; Liu and Weiner, 1998; Weiner, 1997]).

Various models of multiple sclerosis (MS) have demonstrated suppression of **EAE** after feeding of myelin antigens. In the Lewis rat, oral MBP can suppress EAE by different mechanisms, which are dependent on the dose [Weiner, 1997]. Oral administration of myelin to sensitised animals in a guinea pig model or to mice in the murine EAE model is protective and importantly does not exacerbate disease [Brod et al., 1991; Meyer et al., 1996]. The mechanism of orally-induced suppression of EAE is not entirely clear. In low-dose-fed Lewis rats, suppression is transferable to naïve animals and mediates bystander suppression [Miller et

al., 1993] as well as downregulation of IFN-γ and TNF-α and upregulation of TGF-β and IL-4 in the brain [Khoury et al., 1992]. Intriguingly, feeding of LPS together with MBP has a synergistic effect and enhances the suppressive effect on EAE disease [Khoury et al., 1990].
Oral administration of the autoantigen implicated in the inflammation of ocular tissue, bovine S-antigen (S-Ag), has been shown to prevent or markedly diminish the clinical appearance of experimental autoimmune uveitis (**EAU**) [Gregerson et al., 1993; Vrabec et al., 1992]. Clonal anergy and active suppression have both been implicated as mechanisms for the suppression of active EAU disease depending on the dose [Gregerson et al., 1993; Vrabec et al., 1992].
Soluble type II collagen has been shown to suppress disease in a number of experimental models of autoimmune **arthritis** such as rat collagen-induced arthritis [Thompson and Staines, 1986]. Suppression in these models is observed typically with collagen doses of 3μg and 30μg, but not at 300μg or 1000μg, suggesting that the suppressive mechanism involves T_{reg} cells rather than clonal anergy or deletion [Zhang et al., 1990].
Oral insulin has been shown to delay and in some instances prevent **diabetes** in the NOD mouse model. Insulitis in the pancreatic islets of Langerhans is decreased and associated with a decrease in IFN-γ, while expression of TNF-α, IL-4, IL-10, TGF-β and prostaglandin E2 (PGE_2) is increased [Hancock et al., 1995]. Adoptive transfer of T cells from tolerised animals confer protection against the onset of diabetes, pointing toward a mechanism of active suppression. Oral administration of just the β-chain of insulin also slowed the development of diabetes and in some cases prevented disease entirely. The suppression of diabetes was shown to be associated with a switch from Th1 to Th2 cytokines [Polanski et al., 1997]. Glutamate decarboxylase (GAD) from a transgenic plant source has also proven effective in suppressing the development of diabetes when given orally [Ma et al., 1997].
Oral administration of autoantigens has also proved effective in delaying the onset of **myasthenia gravis**, although large doses were required. As well as delaying or preventing **thyroiditis** and **colitis** in mouse models. It has also been possible to suppress transplantation **allograft rejection** by feeding allogenic cells or MHC peptides (for review refer to [Garside et al., 1999; Liu and Weiner, 1998; Weiner, 1997]).

The above studies highlight a number of features important for possible clinical application. None of the studies observed exacerbation of disease, at worst the oral administration was ineffective. In some cases antigen given orally inhibited established disease, although quite large and repeated doses are necessary. The induction of bystander-suppression has been

shown frequently, allowing the use of antigens other than the particular autoantigen triggering disease. Finally, the use of small specific epitopes or peptides has proven efficient and these could be specifically engineered to induce bystander-suppression or direct disease suppression.

1.2.7.2 Humans clinical trials

Oral tolerance was demonstrated experimentally in humans volunteers fed repeated doses of keyhole limpet haemocyanin (KLH) and was reported to decrease subsequent cell-mediated immune responses to parenteral challenge, although humoral responses were not affected [Husby et al., 1994]. On the basis of the long history of oral tolerance, the encouraging results from animal models and the safety of the approach; formal clinical trials have been initiated and carried out in patients with MS, rheumatoid arthritis, uveitis and insulin dependent diabetes mellitus (IDDM).

In a double blind pilot trial, **MS** patients with relapsing remitting disease were fed 300mg bovine myelin/day. No toxicity was observed and there did appear to be some reduction in the number of major attacks in patients fed myelin. It was however only a subset of male HLA-DR2$^+$ patients that responded to the oral antigen [Weiner et al., 1993]. In another study MBP-specific TGF-β-secreting T_{reg} type cells were found in the blood of MS patients treated with oral bovine myelin but not in the control group; resembling findings reported in rodent experiments [Fukaura et al., 1996]. A subsequent larger double-blind phase III trial of single-oral-dose bovine myelin in MS patients, failed to show any difference between treated and placebo groups and a large placebo effect was observed [Weiner, 1997].

Several clinical trials involving feeding type II collagen to treat **rheumatoid arthritis** have taken place. Early reports described a significant clinical improvement in patients with severe active adult and juvenile arthritis fed between 0.1mg and 0.5mg of type II collagen per day for three months [Trentham et al., 1993]. A subsequent phase III trial using larger doses of type II collagen (1-10mg) did not show a significant difference between experimental and placebo groups [Sieper et al., 1996], but a double blind phase II trial feeding a range of doses of type II collagen showed statistically significant positive effects in the group treated with the lowest dose of 20μg [Barnett et al., 1996]. Furthermore, a recent clinical trial with 55 patients fed either 0.05mg, 0.5mg or 5mg type II collagen tablets daily for 6 months, showed that treatment is well tolerated and produces small but significant disease improvements although this was only in the group fed 0.5mg [Choy et al., 2001]. The therapeutic window appears to

be narrow and the discrepancy between studies may thus reflect dose-dependent effects. The smaller oral doses seem to be the more efficacious, which is consistent with animal models of orally administered type II collagen for protection against induced arthritis. Bystander-suppression was also observed only at the lower doses [Yoshino et al., 1995; Zhang et al., 1990].
Clinical trials feeding S-Ag for **uveitis** have shown positive trends but no significant effect. Other trials are ongoing in **diabetes** where recombinant human insulin is administered orally to newly diagnosed diabetic patients and in other trials to patients at risk of developing IDDM.

Although it is clear that oral administration of antigen can suppress autoimmunity in animals, much remains to be learned before this approach can be used to treat human disease. The nature and the origin of the autoantigen fed, the dose and frequency of their administration and the clinical status of the patient at onset of treatment are all likely to influence the outcome and may explain the discrepancies between clinical trials. Given the notable dependency on dose and nature of antigen in animal experiments, it is perhaps not surprising that clearer results were not obtained in humans where more arbitrary doses had to be used. As the mechanisms underlying the induction and modulation of oral tolerance becomes better understood, the prospects of applying oral tolerance for the control of autoimmune and allergic diseases in humans will be enhanced.

1.3 Allergy

The term *allergie*, meaning altered reactivity, was first introduced in 1906. Arthur Coca and Robert Cooke tempted a classification system of allergy in 1922 under the broad heading of hypersensitivity, which they defined as 'a susceptibility in man and animal'. At this time they also coined the term *atopy* (meaning without place), to denote the sense of a strange disease. Much controversy was to follow the introduction of the hypersensitivity classification and its strict distinction between anaphylaxis (meaning contrary to protection) and atopy. Indeed, considerable confusion about the definitions of allergy and atopy has continued to persist through the years. In 1963 Coombs and Gell grouped anaphylaxis and atopic allergies in one class (Type I hypersensitivity) and divided hypersensitivity reactions into four types: Three immediate type hypersensitivity- and one delayed type hypersensitivity reaction [Cohen et al., 2003]. The distinguishing features between the hypersensitivity reactions is reflected in the

differences in effector molecules generated during the course of the reaction. Only Type I IgE-mediated hypersensitivity will be discussed in this section.

Type I hypersensitivity reaction

A Type I hypersensitivity reaction is induced by certain antigens, referred to as allergens, and has all the hallmarks of a normal humoral response. What distinguishes a Type I hypersensitivity response from a normal humoral response is that the plasma cells secrete large amounts of antigen-specific IgE antibodies. IgE was identified in 1967 as the antibody responsible for early allergen-induced wheal-and-flare reactions in the skin [Ishizaka and Ishizaka, 1967] and it is now widely accepted that IgE mediates immediate Type I allergies. IgE binds with high affinity to Fc receptors (FcεRI) on mast cells throughout the tissues and to basophils in the circulation. Allergen binds to the Fab region of cell-associated IgE and crosslinks the IgE molecules on the cell-membrane. Crosslinking of several IgE molecules results in an intra-cellular signalling cascade causing degranulation of mast cells and basophils and release of chemical mediators such as histamines, leukotrienes, prostaglandins, platelet-activating factor and cytokines, which cause the allergic symptoms. The symptoms may occur within minutes to days after contact with the offending allergen and can involve the gastrointestinal tract, skin, respiratory tract and circulation. In the worst case, symptoms can result in an (fatal) anaphylactic shock (Reviewed in [Knippels and Penninks, 1999; Oettgen and Geha, 1999]).

The pathogenesis of hypersensitivity and allergic reactions has been described primarily as a polarised Th2 $CD4^+$ response. Several studies have shown that $CD4^+$ T cell clones from atopic donors have a Th2 phenotype with high production of IL-4, IL-5 and IL-13 and little or no IFN-γ, whereas T cell clones from non-atopic donors produce IFN-γ and no or little IL-4 upon antigen stimulation [de Jong et al., 1996; Wierenga et al., 1990]. A major effect of IL-4 and IL-13 is to induce class switching in B cells to IgE [Oettgen, 2000]. In contrast, IgE synthesis is inhibited by IFN-γ. As a consequence of this, atopic individuals have elevated levels of IgE. However, cytokines are not the only force driving the IgE switch. Additional signals through CD40-CD40L have also proved necessary [Oettgen, 2000]. IgE can in turn regulate the expression of its own receptors, the high affinity FcεRI receptor and the low affinity IgE receptor (CD23) on mast cells and basophils [Oettgen and Geha, 1999; Yamaguchi et al., 1997; Yamaguchi et al., 1999]. IgE-dependent upregulation of FceRI and

CD23 thus serves as an amplification mechanism in IgE-reactions, and mast cell and basophil mediators can be released at lower concentrations of the specific antigen. IL-5 regulates eosinophil growth, differentiation and survival and eosinophil accumulation at body surface tissues has been well documented especially in chronic allergic disorders [Helm and Burks, 2000].

Causes of allergy

The cause of allergic responses is difficult to determine. It is well established that genetic and environmental factors contribute to the onset and maintenance of allergic diseases such as bronchial asthma and atopic dermatitis. Genetic factors can predispose individuals to mount Th2-type responses and subsequent IgE synthesis upon antigen stimulation. Polymorphisms in genes which are likely to contribute to the allergic pathogenesis have been identified. Non-specific tendencies to produce high IgE levels are linked to chromosome 5q31-33. This region of chromosome 5 codes for multiple Th2 cytokines (e.g. IL-4, IL-5, IL-13 and GM-CSF) [Marsh et al., 1994]. Clinical atopy and the production of allergen-specific IgE has also been linked to 11q12-13 and may be related to polymorphisms of the high-affinity IgE receptor β chain [Shirakawa et al., 1994]. Mutations in the SPINK5 gene on chromosome 5q32 has been shown to cause Netherton syndrome and be critically important in epidermal barrier-function. Polymorphisms in SPINK5 show significant association with atopy and AD, suggesting a novel pathway responsible for high serum IgE and atopic disease [Chavanas et al., 2000; Walley et al., 2001]. As with other antigens, responses to allergens are additionally modulated by the inheritance of particular MHCII alleles. However, environmental factors also undoubtedly play an important role in atopy and IgE production. Monozygotic twins have been shown to have asthma concordance rates of less than 20% [Oettgen and Geha, 1999], and as the natural gene mutation rate in the population is low, altered environmental and/or lifestyle conditions are ultimately thought to be responsible for the fast increasing prevalence and incidence of allergic disease.

The hygiene hypothesis

The frequency and the severity of atopic disorders are steadily increasing, particularly in the developed world [Holgate, 1999; ISAAC Steering Committee, 1998; von Mutius, 1998]. The impact of allergic diseases on mortality, morbidity and on the economy is staggering. About 20% of the population in Western countries suffer from allergic diseases such as asthma,

allergic rhinitis, atopic dermatitis, eczema and food allergy [Geha, 2000]. The theory that has attracted most attention to explain this continuous rise in allergy is 'the hygiene hypothesis' first proposed by Strachan and coworkers [Strachan, 1989] and more recently reviewed in [Erb, 1999; Folkerts et al., 2000; Herz et al., 2000; Rook and Stanford, 1998]. This hypothesis proposes that a cleaner environment and frequent use of antibiotics resulting in fewer childhood infections promoting Th1 responses predisposes to later allergic Th2 dominated responses. In particular, prenatal and early childhood are considered to be critical intervals for viral and bacterial infections to prevent allergic Th2 responses and for establishment of the Th1/Th2 balance. A number of epidemiological and clinical studies support this hypothesis. Recovery from natural measles infection significantly reduces the incidence of atopy and of allergic reactions to house dust mite compared to the incidence in vaccinated children [Shaheen et al., 1996]. Other childhood infections may be protective against allergic diseases as allergies are less frequent in children from large families [Strachan, 1989] and those attending day care [Kramer et al., 1999], suggesting that a frequent exchange of viral and/or bacterial infections may be beneficial. There are also clear differences in the prevalence of allergies between rural and urban areas within one country. Asthma for example is more common in children from cities than in children of farmers living in rural areas [von Ehrenstein et al., 2000]. In adults, an inverse relationship between hepatitis A seropositivity and atopy as well as enteric infections and atopy has been shown in retrospective studies of Italian Air Force cadets [Matricardi et al., 2000; Matricardi et al., 1997]. There is thus compelling evidence to suggest a link between the relative lack of infectious disease and the increase in allergic disorders. However, other studies suggest that viral and/or bacterial infections exacerbate allergic diseases such as bronchial asthma, airway hyperresponsiveness and atopic dermatitis [Herz et al., 2000]. Additionally, an immunological switch to a Th2 response does not *per se* give rise to an increased frequency of allergy. Pregnancies [Raghupathy, 1997] and chronic helminth infections [Yazdanbakhsh et al., 2001; Yazdanbakhsh et al., 2002] are examples in which a Th2 dominance does not give rise to enhanced incidence of allergy or atopy. An immunological framework where the imbalance between Th1 and Th2 responses is pivotal to avoid allergies may thus not be sufficient to explain disease. The dramatic increase of allergic and atopic diseases in the developed world also show a striking correlation with Th1-mediated autoimmune diseases [Stene and Nafstad, 2001] and diseases of unknown aetiology such as inflammatory bowel disease [Sawczenko et al., 2001]. A recent population study found a significant positive association between physician-diagnosed common allergic disorders and

physician-diagnosed autoimmune disorders [Sheikh et al., 2003]. Such data indicates that the potential root cause of the increase in allergic disease might be the same as that responsible for the escalation in autoimmune Th1 disorders and implies a general increase in immunodysregulatory diseases. It may be that persistent immune challenges in childhood does not specifically dampen Th2 allergic type responses but induces the development of a robust anti-inflammatory regulatory network, which protects against excessive Th2 as well as Th1 diseases. This could explain the inverse association between infections and allergic disorders as well as the positive correlation between autoimmune and allergic diseases.

1.3.1 Food allergy

Food allergy is a major cause of life-threatening hypersensitivity reactions and food-induced anaphylaxis is the most common cause of anaphylactic reactions presented to hospital emergency departments [Burks et al., 2001]. Adverse reactions to foods are a particular problem for infants and young children, with a reported incidence up to 8% compared to 1-2% in adults [Helm and Burks, 2000]. The prevalence of food allergy, as with other types of allergies, is steadily rising. Immunologically, only IgE-mediated Type I allergic reactions are known for certain to play a major role in food allergy [Bruijnzeel-Koomen et al., 1995]. Eight food items: milk, egg, peanut, soy, wheat, tree nuts, fish and shellfish account for more than 90% of cases of food-related IgE mediated allergic reactions in the USA [Bock et al., 1988; Burks et al., 1998]. Food allergy in children usually appears to be a transient phenomenon and in 75% of cases the children have outgrown their allergic reaction within 5 to 9 years after the onset of clinical symptoms [Knippels and Penninks, 1999]. However, some food allergies, like allergic reactions to peanuts, are more persistent and do not often resolve with age. The reasons for this persistence are at present unknown.

An accurate diagnosis of food allergy or intolerance can be difficult. IgE-mediated food allergies often occur as part of the so called atopic syndrome, where people with atopy are considered to have a hereditary trait associated with greater risk of developing IgE-mediated diseases. As such a family history can give an indication of an atopic constitution. Laboratory tests to demonstrate sensitisation to specific foods include skin prick test (SPT) as well as radio-allergo-sorbent test (RAST) or enzyme-linked immunosorbent assay (ELISA) to detect allergen-specific IgE and/or IgG. Most often a combination of SPT and RAST is used for diagnosis [Knippels and Penninks, 1999]. The sensitivity and specificity of these tests are

however controversial and no laboratory test can actually prove (or disprove) food intolerance (reviewed in [Freed and Waickman, 2002]). A double-blind, placebo-controlled food challenge has been named as the gold standard for diagnosis of food allergy [Bruijnzeel-Koomen et al., 1995]. However, several problems and practical difficulties are associated with these challenges and the proportion of apparently food-sensitive patients confirmed by double-blind challenge is low, suggesting either that patients and clinicians are easily fooled or that false-negative results are high [Freed and Waickman, 2002]. A reliable laboratory test for food intolerance would be immensely valuable.

Inflammatory disorders of the human intestine, including food allergy, celiac disease and Crohn's disease, are associated with epithelial barrier dysfunction [Helm and Burks, 2000]. It is however not clear if the increased epithelial permeability is a consequence of inflammation or a predisposing factor. When the mucosal barrier is disrupted, various agents including food antigens and bacteria can directly stimulate the local DCs and lymphocytes thereby breaking the induction and/or maintenance of tolerance. However, the mechanisms involved in food-allergy remain essentially unresolved. Very few animal models of food-allergy have been developed, and none are completely satisfactory for studying the clinical features and the mechanisms of immunopathogenesis of IgE-mediated food-allergy [Helm and Burks, 2000; Knippels and Penninks, 1999].

1.3.1.1 Food allergens

In theory, every food protein is a potential food allergen. Most food allergens are glycoproteins with a molecular weight between 10 and 60 kDa. Food allergens are normally naturally occurring proteins, but new allergenic epitopes may result from technological processing, heat treatment or digestion. Although food contains numerous individual proteins, comparatively few have been documented as allergens. Some foods contain multiple allergenic proteins (e.g. peanuts, cow's milk and egg), while in other foods (e.g. Brazil nuts and codfish) one specific protein seems to be responsible for the allergic reaction [Bush and Hefle, 1996]. Even among the foods containing multiple allergens, not all of the proteins are capable of eliciting IgE production. The immunochemical and physiochemical properties that account for a protein's unique allergenicity are poorly understood. Important factors, although not universal to all food protein allergens, may be: resistance to proteolysis (stability in the gastrointestinal environment), enzymatic activity (which could non-specifically activate the

immune cells) and post-translational glycosylation (that would increase receptor-mediated uptake) [Heyman, 1999]. Although there are criteria to guide our determination of allergenicity, it is unlikely that they will clearly identify all potential allergenic molecules and the future will most likely demonstrate clinical symptoms caused by a wide variety of foods and other antigens.

1.3.1.2 Peanut allergy

Peanut is an oil-legume containing about 44-56% oil and 22-30% protein [Fries, 1982]. Many of the proteins in peanut are allergenic and have been categorised into major and minor peanut allergens. Although not all peanut-allergic individuals react to the same peanut proteins, peanut contains several major allergens that bind IgE from the majority of peanut-allergic patients. Two major peanut proteins have been identified, Ara h1 and Ara h2, which are recognised by IgE from more than 95% of patients with peanut allergy. A third protein, Ara h3, is recognised by about 45% of patients [Burks et al., 1991; Burks et al., 1992; Rabjohn et al., 1999]. Ara h1 is a 63kDa glycoprotein, Ara h2 a seed storage protein with a molecular weight of 17kDa and Ara h3 a glycinin-like protein of 14kDa. All three proteins contain multiple IgE binding epitopes [Burks et al., 1997; Rabjohn et al., 1999; Stanley et al., 1997].

Peanuts are highly allergenic and may cause severe allergic reactions in sensitised children and adults. Peanut allergy is one of the most common food allergies, with a prevalence in the UK and US adult population of 0.5% and 0.6% respectively [Emmett et al., 1999; Sicherer et al., 1999] and affecting up to 1.5% of British pre-school children [Grundy et al., 2002]. The clinical features are frequently expressed as an acute IgE-mediated reaction after ingestion of peanuts. Peanuts are the most common cause of fatal and near-fatal food-related anaphylaxis in all age groups [Bock et al., 2001] and the threshold dose needed to elicit a reaction in peanut allergic individuals is often very low. 95% of people with peanut allergy suffer from other atopic diseases with eczema being particularly prevalent in infants although high incidences of asthma and hay fever have also been reported [Hourihane et al., 1996]. 80% of subjects react to their first known oral exposure to peanuts, implying some prior occult sensitisation [Hourihane et al., 1996]. Babies may have been sensitised *in utero* or by oral exposure to peanut in infancy from breast milk, formula milks made with peanut oil, vitamin supplements and weaning foods that contain peanut or peanut oil with small amounts of peanut protein. Eczema is often controlled with topically applied creams and lotions. Even children without

eczema have lotions applied to their skin, particular after bathing, when skin permeability is increased. Peanut oil has been a common ingredient in skin ointments and in nipple creams, used by breast-feeding mothers. Whether primary sensitisation to peanut allergens can occur through the skin is unknown (compare section 1.4.4), but it has been suggested that use of peanut-containing creams may be more widespread in children who become allergic to peanut than in control infants [Lack et al., 2003].

There has been an increasing amount of research into the allergenicity of peanuts and the immunopathogenic mechanisms of peanut allergy. However, the probability of a life-threatening reaction after exposure to even minute amounts of peanut limits human testing and reinforces the need for animal models. One murine model of peanut allergy has been described showing T- and B cell responses to Ara h2 similar to those found in human peanut allergic individuals [Li et al., 2000]. In this murine model, IgE antibody responses to peanut depends on repeated concomitant feeding of cholera toxin with peanut. Indeed, allergic sensitisation via the gastrointestinal tract might be facilitated by injury in an as yet undefined way.

1.3.2 Routes of allergic sensitisation

The primary sensitisation to protein antigens is a key step in the pathogenesis of allergic diseases. Clearly both genetic and environmental factors as well as the nature of the sensitising antigen and the frequency of exposure contributes to the ontogeny of allergic sensitisation. Additionally, the route of allergen exposure and the microenvironmental milieu in the specific tissue at the time of exposure may play a pivotal role in determining the immunological outcome of an allergen encounter. The immune system is most likely to have primary contact with allergens at the body surface, i.e. the gut, the nose, the lung or the skin. Multiple cell types and specific microenvironmental factors at these tissue sites can thus determine the local and systemic response to a particular antigen/allergen (see 'information network' in Fig. 1.3 and section 1.1.6). The nature and maturation state of the APC taking up and processing antigen and the secretion of cytokines can all influence the phenotype of the resultant T cell response. The route of antigen exposure has been shown to have a direct impact on the IgE response and the severity of anaphylaxis in mice sensitised to OVA. The most efficient IgE-production in this study was achieved by long-term antigen exposure to the airways or the skin, while intraperitoneal sensitisation led to significantly lower IgE levels [Nelde et al., 2001].

Intraperitoneal sensitisation appears however to lead to greater IgE responses than exposure via the gut in healthy animals [Dearman et al., 2001]. Independent on the route of exposure, IgE synthesis is induced more efficiently by increasing the frequency of antigen application than by increasing the dose of antigen [Nelde et al., 2001].

1.3.3 Immunotherapy for food allergy

Atopic diseases mediated by IgE pose a considerable public health problem in westernised societies because of the potential severity of allergic hypersensitivity. The socio-economic burden of the increasing prevalence of allergic and atopic diseases is highlighted by the cost of treating asthma alone in the United States, which has been estimated at $8 billion per year [Moffatt and Cookson, 1999]. Any possibility of preventing or treating allergies should in these circumstances be greatly welcomed.

For food allergic individuals the only accepted 'treatment' is strict avoidance of the allergenic food. Complete avoidance of all possible sources of the offending food is difficult however and accidental exposure frequently occurs. Because of the ubiquitous use of peanut protein in a variety of food products and medication, accidental ingestion has been estimated to occur in up to 50% of patients per year [Lycke et al., 1990; Snider et al., 1994]. Given the large number of individuals with potentially fatal food-allergy and the extreme difficulty in total avoidance of all food allergen exposure, effective prophylactic and therapeutic strategies are much needed. Indeed, several different forms of immunomodulatory therapies for food allergy are currently under investigation (reviewed in [Burks et al., 2001; Wohlleben and Erb, 2001]).

Traditional immunotherapy. Re-induction of tolerance for IgE-mediated diseases by subcutaneous injection of increasing amounts of aqueous purified allergen has proven successful with insect-sting allergy and inhaled allergies but has not shown much success in the treatment of food allergy. Clinical trials of immunotherapy for the treatment of anaphylactic hypersensitivity to peanuts induced a high rate of severe adverse systemic reactions, which made the treatment practically impossible to perform on a routine basis [Nelson et al., 1997; Oppenheimer et al., 1992]. In general, contradictory results of the efficacy of injection immunotherapy for food allergy has been reported and the method is currently not recommended [Burks et al., 2001; Helm and Burks, 2000].

Peptide- and mutated protein immunotherapy. Peptide immunotherapy requires subcutaneous injection of peptide fragments containing the T cell reactive epitopes of the allergen instead of the complete protein molecules. The peptide fragments are unable to crosslink IgE on mast cells and do not activate the effector arm of the allergic response. They appear to induce T cell hyporesponsiveness upon subsequent allergen exposure and could theoretically re-introduce tolerance [Burks et al., 2001]. Pepsin-digested peanut allergen has been shown to contain T cell epitopes but no IgE-binding epitopes and on ingestion induces immune deviation from Th2 to Th1 with production of IFN-γ [Hong et al., 1999]. Similarly, mutated protein immunotherapy is achieved by altering the IgE-binding epitopes of the major allergens in foods, without affecting the T cell epitopes. The technique is developed through alteration of the primary amino-acid sequences of IgE-binding epitopes by cDNA mutations. It has been demonstrated that mutations of Ara h1 and Ara h2 (the major allergens in peanut) produces less-allergenic proteins and that peanut hypersensitive patients have reduced IgE binding to the mutated peanut proteins compared to the wild type proteins [Stanley et al., 1997]. Although encouraging results have been reported for both peptide- and mutated protein immunotherapy for treating food allergy, these techniques have not yet reached human clinical trials.

Anti-IgE immunotherapy. This is a promising allergen-independent therapeutic option effective for multiple atopic allergies. IgE depletion can be achieved by repeated injections of humanised monoclonal antibody against IgE. The anti-IgE antibody binds freely circulating IgE and hinders its binding to FcεRI on mast cells and basophils. Several clinical trials have demonstrated that anti-IgE treatment substantially reduces free serum IgE, downregulates FcεRI expression and has beneficial effects on allergic symptoms and medication use in patients with allergic rhinitis and asthma [Milgrom et al., 1999] as well as increasing the threshold of sensitivity to peanut [Leung et al., 2003]. However, treatment with monoclonal antibodies is expensive and requires infusions every 2-3 weeks to maintain the IgE-deficient state. Alternatively, an active immunisation strategy against IgE has been shown to induce therapeutic antibody responses in animals. This vaccination against IgE decreased circulating serum IgE by 90% and reduced mast cell reactivity in the skin of OVA-sensitised rats [Vernersson et al., 2002].

DNA vaccination. This novel approach for the treatment of food allergies employs the subcutaneous injection of a plasmid DNA vector encoding a specific allergenic protein. The plasmid DNA is taken up by APCs, incorporated into the cell and the DNA encoding the allergen transcribed and translated. The endogenously produced allergenic protein is then presumably presented to T cells on the surface of APCs in the context of MHC [Burks et al., 2001]. Such plasmid DNA vaccinations have shown to induce Th1-type responses with increased IFN-γ secretion and the generation of IgG2a but almost no IgG1 or IgE antibodies in BALB/c mice upon re-exposure [Spiegelberg et al., 1997]. In a later study however a strain-dependent induction of allergic sensitisation and anaphylaxis was caused by similar plasmid DNA immunisation [Li et al., 1999]. These results suggest that the type of immune response initiated by plasmid DNA immunisations in mice is strain dependent, and suggests that a similar interindividual variation is likely to be seen in humans. Another immunoprophylactic strategy using oral gene-immunisation to modulate peanut-induced anaphylactic responses in AKR/J mice has been reported. Plasmid DNA encoding Ara h2 were complexed to chitosan (a natural polysaccharide) and the DNA nanoparticles administered orally. This resulted in elevated secretory IgA, immune deviation toward Th1 responses with IgG2a production and suppression of Th2 responses with reduced IgE levels as well as a substantial reduction in allergen-induced anaphylaxis and plasma histamine levels [Roy et al., 1999]. Indeed, it may be ideal to utilise the oral route of immunisation to pre-modify the mucosal immune system, which in turn can protect the host from systemic food-allergen induced anaphylaxis.

Adjuvants. Immunisation with immunostimulatory sequences containing cytosine and guanine dinucleotide repeat motifs (CpG motifs) have shown to induce immune deviation from Th2 to Th1 responses [Chu et al., 1997]. These sequences can be administered alone, with DNA encoding the allergen or conjugated with the allergen and has been reported to reduce allergenicity and inhibit IgE synthesis [Bohle et al., 1999].

1.4 Skin immunology

The skin consists of several distinct layers with specialised cell types (Fig. 1.6). Resistance to microbes, environmental antigens, chemicals and ultraviolet light resides almost entirely in the outermost layer of the skin – the stratum corneum. The stratum corneum is a horny layer of dead cells on the skin surface, which arises from exocytosis of the lipid contents of epidermal lamellar bodies [Elias and Menon, 1991]. Beneath the stratum corneum is an epithelial layer of

keratinocytes, which comprise around 95% of the cells of the epidermis. Interspersed in the keratinocyte layer are specialised APCs known as Langerhans cells (LCs), which constitute approximately 2-4% of all epidermal cells [Maurer and Stingl, 2001], and a population of intraepithelial T lymphocytes (IELs). Among the IELs are the dendritic epidermal T cells, which exclusively express the γδTCR and populate the skin of all normal mice [Girardi et al., 2002]. Human epidermal T cells, however, are not dendritic and the proportion of αβ and γδ T cells is not substantially different from that observed in normal human peripheral blood [Foster et al., 1990]. The epidermis is separated from the thicker dermis by a thin basement membrane. The basement membrane is a complex barrier where the collagen fibrils are tightly packed to form a very dense meshwork [Stoitzner et al., 2002]. The underlying dermis also has a dense collagen meshwork but it is less compact than the basement membrane.

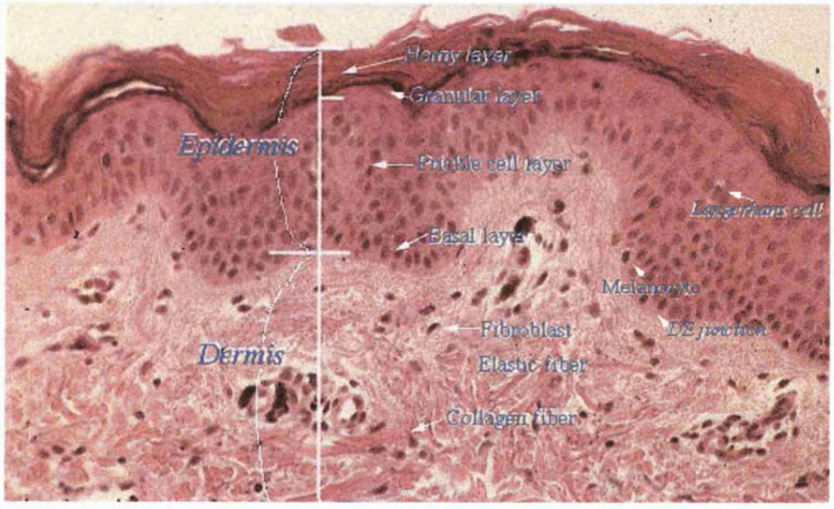


Fig. 1.6 Structure of normal skin
H&E stained section of normal human skin showing the major distinct layers of the skin and the cell types present within. Figure from [King, 2003]

The dermis and especially the perivascular area contain the highest concentration of immune response-reactive cells of the skin. Dermal dendritic cells, mast cells, monocytes, macrophages and T cells are present in large numbers close to the vascular endothelial cells. Many of the substantial numbers of skin T cells (around 4 billion in an adult) are activated and have a

memory phenotype [Bos and Kapsenberg, 1993] – similar to that in the lamina propria of the gut. A subset of peripheral blood T cells express cutaneous lymphocyte-associated antigen (CLA) representing a specific population of skin-homing memory T cells [Mackay, 1991]. The immune-related functions of the skin are sometimes referred to as skin-associated lymphoid tissue (SALT) [Streilein, 1983], skin immune system (SIS) [Bos et al., 1987; Bos and Kapsenberg, 1993] or dermal immune system (DIS) [Nickoloff, 1993].

Keratinocytes are capable of producing and secreting a large array of mediators including IL-1, IL-3, IL-6, IL-7, IL-8, IL-10, IL-12, IL-15, IL-18, IFN-α, β and γ, TNF-α, TGF-α and β, GM-CSF, M-CSF, a steadily increasing number of chemokines as well as various growth factors. With the exception of IL-1, IL-7 and TGF-β plus the chemokines macrophage inflammatory protein (MIP)-3α, cutaneous T cell-attracting chemokine (CTACK) and stromal cell-derived factor (SDF)-1α most of these biological response modifiers produced by keratinocytes are not constitutively expressed *in vivo*, but can be induced after pertubation of the epidermal homeostasis [Bos and Kapsenberg, 1993; Maurer and Stingl, 2001; Nickoloff and Naidu, 1994; Nishijima et al., 1997; Steinhoff et al., 2001; Wood et al., 1992]. Upon any epithelial injury, keratinocyte derived factors act on immune-response cells and stimulate expression of endothelial adhesion molecules necessary for leucocyte immigration from the circulation. The skin lacks any organised lymphoid tissue within the dermal stroma and induction of immune responses does not normally occur within the skin itself [Bos and Kapsenberg, 1993]. The migratory capacity of epidermal LCs and dermal DCs thus becomes pivotal in bridging the sites of antigen uptake and clonal T cell activation in draining lymph nodes.

1.4.1 Langerhans cells

LCs are dendritic leukocytes and members of the wider family of highly specialised APCs, the DCs. Skin LCs were first described in the suprabasal region of the epidermis by Paul Langerhans in 1868 who (because of their dendritic morphology) suggested they might be a new type of intraepithelial neurons. Different origins and functions were postulated for LCs over the following years. It was not until the early 1970s that DCs were identified and characterised as specialised bone marrow derived APCs [Steinman and Cohn, 1973]. It is now established that epidermal LCs reside as a contiguous network above the basal layer of

proliferating keratinocytes in the epidermis, where they serve as sentinels of the immune system, sampling antigens and responding to changes in the external environment. They have potent antigen processing and presenting capabilities and can relay antigenic information to the adaptive immune system by migrating from the epidermis to the draining lymph nodes where they present processed antigens to naïve or memory T cells [Banchereau and Steinman, 1998]. The density of LCs vary between approximately 200 and 1000 cells/mm^2 of the epidermal surface depending on the anatomical area. They are more abundant on the face and neck skin and least abundant on the palms and soles [Berman et al., 1983]. LCs attach to neighbouring keratinocytes by an E-cadherin- and Ca^{++}-dependent mechanism [Tang et al., 1993], and contrast with most DC populations in that they have much longer lifespans in the periphery [Kamath et al., 2002]. LCs are difficult to visualise in sections routinely stained with H&E and their visualisation *in situ* requires the use of appropriate histochemical and/or immuno-labeling techniques. The hallmark of LCs are the Birbeck granules seen on electron microscopy as a rod-shaped trilaminar membrane structure initially identified by Birbeck in 1961 [Maurer and Stingl, 2001].

Ontogeny

The ontogeny of LCs has been much studied, but the results remain conflicting. Current opinion generally accepts that LCs arise from bone marrow-derived blood-borne precursors, although these have not yet been clearly identified. Human cord blood CD34$^+$ progenitors can give rise to CD1$^+$, E-cadherin$^+$, Birbeck granule containing cells which resemble LCs in phenotype and function when exposed to GM-CSF and TNF-α *in vitro* [Caux et al., 1992]. LCs can also be obtained from CD34$^+$ progenitors in the presence of TGF-β under serum-free conditions [Strobl et al., 1997] and from human peripheral blood monocytes with a combination of TGF-β, GM-CSF and IL-4 [Geissmann et al., 1998]. The presence of TGF-β has been shown to be crucial for the development and/or homing of LCs, as TGFβ$^{-/-}$ mice are totally devoid of epidermal LCs but have normal bone marrow morphology and proper differentiation of the major myeloid cell lineages [Borkowski et al., 1996]. Circulating MHCII$^-$ (I-A^b) CD11c$^+$ cells [del Hoyo et al., 2002] and dermal-resident CD14$^+$ cells have also been shown to differentiate into LCs [Larregina et al., 2001]. The origin of repopulating LCs in the epidermis *in vivo* has been found to differ under steady-state- and inflammatory conditions [Merad et al., 2002]. Merad et al. analysed the recruitment of circulating LC precursors to the skin using lethally irradiated mice transplanted with congenic bone marrow and congenic

parabiotic mice. Under normal conditions, host LCs were continually expressed up to 18 months, while >90% of spleen, liver and kidney DCs, peritoneal macrophages and blood monocytes were of donor origin by 8 weeks after transplantation. In contrast, when the skin was exposed to ultraviolet light, LC replacement was almost entirely from donor cells. This suggests, that the seeded pool of LCs (as early as 1 day after birth) become autonomous in the skin and are able to compensate for the low LC loss that occurs during steady-state turn-over and migration through replacement from a local precursor pool - possibly through LC division. However, once an inflammatory threshold is reached, multiple changes occur in the skin, resulting in marked loss of LCs and the recruitment of blood-borne bone marrow derived LC precursors [Merad et al., 2002]. These experiments suggest that the origin of LCs is determined by local conditions within the skin.

Phenotype and function

LCs usually reside in the epidermis in a resting state expressing low levels of surface MHCII and MHCI (but high levels of intracellular MHCII) and low or no costimulatory and activation markers such as CD25, CD40, CD54, CD58 and CD80/86. They are well equipped to capture antigens and express both Fcγ- and Fcε-receptors as well as CD1, Birbeck granules and the lectin Langerin, a marker that is relatively Langerhans cell-restricted (reviewed in [Larregina and Falo, 2001; Maurer and Stingl, 2001]). Langerin has been hypothesised to be an antigen-capturing molecule which channels the antigen into Birbeck granules and thus possibly provides access to a non-classical, MHCII-independent antigen-processing pathway [Valladeau et al., 2000]. The high expression of FcεR (both the high-affinity and low-affinity receptor for IgE) on LC surfaces may serve as allergen-focusing molecules, which upon binding of IgE and ligation by allergen would facilitate processing and presentation of allergens and serve as an amplifying factor for allergic reactions [Nagaoka et al., 2002; Shibaki, 1998]. Resident LCs also express several adhesion molecules notably E-cadherin and CLA [Maurer and Stingl, 2001]. As a consequence of their resting phenotype, LCs *in situ* only have a limited potency for stimulating naïve T cells. They are however extremely efficient in taking up antigens and particles by phagocytosis, macropinocytosis and receptor-mediated uptake [Banchereau and Steinman, 1998].

Upon receipt of stimuli by a variety of factors such as whole bacteria, bacterial DNA or LPS, cytokines such as IL-1, GM-CSF and TNF-α, binding to CD40L, ultraviolet light or chemical haptens, LCs undergo a complex process of activation and differentiation usually termed LC

maturation [Banchereau and Steinman, 1998; Schuler and Steinman, 1985]. The maturation of LCs is crucial for the initiation of immunity. The process is characterised by morphological changes which renders LCs highly motile with delicate processes extending in many directions from the cell body, bending, retracting and re-extending in a non-polarised fashion. Antigen capturing devices disappear and T cell stimulatory functions increase. The essential features of the maturation process are: 1) redistribution of MHCII from intracellular vesicles to the cell surface, where peptide-MHC complexes can be displayed for prolonged periods [Pierre et al., 1997; Turley et al., 2000], 2) downregulation of antigen-uptake moieties such as FcεRI, FcγR, Birbeck granules and Langerin, 3) upregulation of costimulatory molecules of T cell activation such as CD24, CD40, CD54, CD58 and CD80/86 and certain cytokines, 4) rearrangement of chemokine receptors as well as a marked rearrangement of adhesion molecules such as downregulation of E-cadherin and upregulation of β2-integrins (e.g. CD11c) and adhesion molecules of the immunoglobulin family. Mature LCs (and DCs in general) are extremely efficient in stimulating T cell responses (reviewed in [Banchereau and Steinman, 1998; Larregina and Falo, 2001; Maurer and Stingl, 2001; Winzler et al., 1997]). LCs have been shown to induce strong primary immune responses when presenting alloantigens [Inaba et al., 1986; Lenz et al., 1993], microorganisms [Konecny et al., 1999], soluble protein antigens and haptens which *in vivo* initiates hypersensitivity reactions [Hauser, 1990; Hauser and Katz, 1988; Kurimoto and Streilein, 1993], as well as tumour antigens where they can induce antigen-specific CTL responses [Celluzzi and Falo, 1997].

Migration

The prime function of DCs is to take up, process and transport antigens to lymphoid organs. LCs must thus migrate from the epidermis to the draining lymph nodes if they are to fulfil their function in immune regulation. A plethora of factors have been shown to trigger and guide the migration of LCs from the epidermis to the draining lymphoid tissue (reviewed in [Kimber et al., 2000; Randolph, 2001; Sallusto, 2001]). The proinflammatory cytokines IL-1β and TNF-α play a central role in the induction of LC migration and it has been proposed that LCs require both signals for mobilisation [Cumberbatch et al., 1997]. In addition IL-18 induces LC migration by a TNF-α and IL-1β-dependent mechanism [Cumberbatch et al., 2001]. The main source of IL-1α and TNF-α is the epidermal keratinocyte, while IL-1β is mainly produced by LCs [Heufler et al., 1992]. IL-1β is the first cytokine secreted in response

to topical allergens and is required for the initiation of primary immune responses in the skin [Enk et al., 1993]. TNF-α, IL-1α, IL-1β and LC maturation induces downregulation of E-cadherin and as a consequence LCs can release their attachment to neighbouring keratinocytes and become responsive to chemotactic stimuli [Schwarzenberger and Udey, 1996]. When LCs detach from the surrounding keratinocytes gaps and holes form in the keratinocyte layer where the emigrating LCs 'squeeze' through (Fig. 1.7A). This may happen in an active way by extention of long thin pseudopodia, attachment to keratinocytes and pulling themselves out of the epidermis (Fig. 1.7B) [Stoitzner et al., 2002]. After leaving the epidermis, LCs encounter the complex barrier of the basement membrane (Fig. 1.7C), which they have to pass through on their way to the dermis. Scanning electron micrographs show that LCs extend a pseudopod through the basement membrane and a gap in the membrane can be seen in a focused area of cell-membrane contact [Stoitzner et al., 2002]. This suggests that LCs may be equipped with enzymatic tools to transit through the membrane. Indeed, migrating LCs have been shown to produce special proteases such as matrix metalloproteinases (MMPs), which are able to digest collagen IV and cell-adhesion molecules [Kobayashi, 1997; Ratzinger et al., 2002]. MMP-2 and MMP-9 are expressed on the surface of LCs, and may allow for precise localised proteolysis, which would create a path for migrating cells. These MMPs have been shown to be necessary for the migration of both LCs and dermal DCs from human and murine skin [Ratzinger et al., 2002]. Such enzymatic tools may be needed for migration through the epidermal keratinocytes as well as the basement membrane and the dense collagen meshwork of the dermis. In the dermis, LCs attach to bundles of collagen fibrils with their pseudopodia and may actively pull themselves along the fibrils (Fig. 1.7D) [Stoitzner et al., 2002]. This suggests that the ability of LCs to flexibly change their shape is essential for successful movement through the dermis until they encounter a lymphatic vessel. Migrating LCs express CCR7 and are chemotactically attracted towards the dermal lymphatic vessels by the chemokine MIP-3β and secondary lymphoid-tissue chemokine (SLC), which is expressed by lymph endothelial cells *in situ* [Saeki et al., 1999; Sallusto and Lanzavecchia, 1999]. CCR2 and its ligands have also been implicated in active recruitment of LCs into the lymphatics [Sato et al., 2000]. After entering the lumen of a lymphatic vessel, LCs will travel with the lymph to the local lymph node where they can present antigen to T cells.

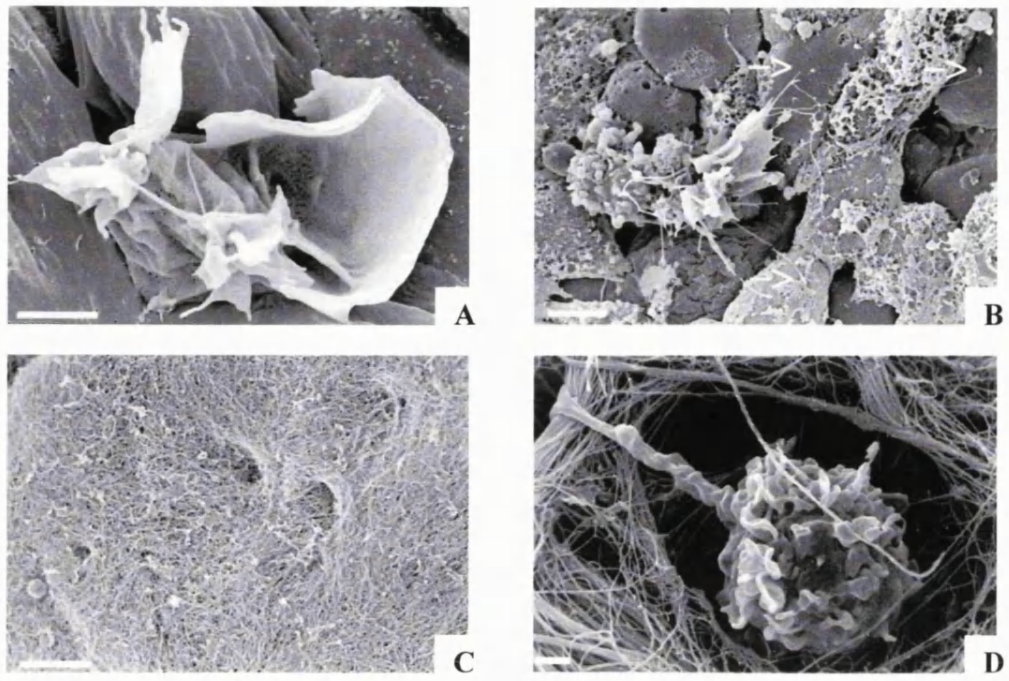


Fig. 1.7 Langerhans cells migration
Scanning electron microscopy of skin tissue and LCs migrating from the epidermis towards the draining skin lymph node. (A) LC leaving the epidermis through developing gaps between keratinocytes. (B) The LCs attach to neighboring keratinocytes with the help of thin cytoplasmic extensions (arrows) and pull themselves out of the epidermis. (C) The dense collagen meshwork of the basement membrane that LCs have to pass through to reach the dermis. (D) Migration of LC through the collagen meshwork of the dermis after passage through the basement membrane and before entry into lymphatic vessels. LCs cling to bundles of collagen fibrils with their veils and pseudopodia and guide their movement towards the lymphatic tissue. Scale bars: (A), (B) and (D) 2.5μm, (C) 10μm. Electron micrographs reproduced from [Stoitzner et al., 2002]

It is a widely accepted view that migration of LCs in response to inflammatory stimuli is coupled to and follows from their maturation. *In vivo* and *in vitro*, the inflammatory mediators that can promote LC maturation have also been shown to be able to mobilise LCs [Randolph, 2001; Randolph, 2002]. That migration is linked to maturation is also argued by evidence that molecules associated with migration, such as CCR7, are induced during LC maturation [Yanagihara et al., 1998]. However, cells containing Birbeck granules are found in lymph nodes draining the skin and cells with LC characteristics are present in normal skin lymph [Brand et al., 1999; Howard et al., 1996]. Melanin granules have also been shown to be transported from the epidermis to the regional lymph nodes during steady-state conditions [Hemmi et al., 2001] and accumulation of immature LCs expressing Langerin, CD1a and low

levels of E-cadherin occur in the draining lymph nodes of patients with chronically inflamed skin [Geissmann et al., 2002]. This suggests that LCs can migrate to the lymph nodes as immature DCs, and that LC migration and maturation can be independently regulated events. A similar conclusion was made in two recent *in vitro* studies showing that maturation of human monocyte derived DCs did not induce migration, and that a second signal which could be delivered through PGE_2 was required [Luft et al., 2002; Scandella et al., 2002]. Observations of DCs from the intestine suggest that immature DCs carry self antigens to the lymph nodes and thereby maintain tolerance [Huang et al., 2000]. It has also been shown that LCs transport melanosomes and apoptotic bodies, presumably collected from dying keratinocytes, through the dermal lymphatics to regional LN [Hemmi et al., 2001; Stoitzner et al., 2002]. The described efficient pathway of cross-presentation using apoptotic cells may thus also operate in the skin [Blankenstein and Schuler, 2002; Sauter et al., 2000; Steinman et al., 2000], although whether this promotes active immunity or tolerance has not been assessed.

Downregulation of immune responses

The immune-stimulatory function of skin LCs and DCs has been intensely investigated, but less is known of their role in mechanisms leading to the downregulation of immune responses. TGF-β released constitutively by keratinocytes has been shown to profoundly affect LC development and function and prevent the maturation of human LCs induced by LPS, TNF-α and IL-1β [Geissmann et al., 1999]. Low amounts of IL-10 are spontaneously expressed in normal skin and its secretion is increased upon injury or UV irradiation and can abrogate the immunostimulatory properties of LCs [Niizeki and Streilein, 1997]. IL-10 is produced by some progressive melanomas, and it has been hypothesised that this could be a mechanism of immune evasion [Enk et al., 1997]. IL-10 is known to interfere with the maturation process of DCs and is also involved in limiting inflammatory responses, as is seen in IL-$10^{-/-}$ mice that tend to have larger inflammatory reactions than wild type mice [Banchereau and Steinman, 1998]. Exaggerated contact hypersensitivity responses and enhanced LC migration were observed from the epidermis of IL-$10^{-/-}$ mice [Wang et al., 1999], suggesting that IL-10 may be involved in keeping LCs in the epidermis and minimising inflammation.

1.4.2 Diseases of the skin

Diseases of the skin are multiple and common. In this section only atopic dermatitis and atopic eczema will be discussed. The nominations atopic dermatitis and eczema are essentially synonymous and the definition fairly broad, meaning inflammation of the skin or skin rash with the 'atopy' denoting an underlying allergic constitution.

1.4.2.1 Atopic dermatitis and eczema

Atopic dermatitis (AD) is a common, chronic, relapsing inflammatory skin disease characterised by typically distributed eczematous skin lesions with lichenification, pruritic excoriations, severely dry skin and a susceptibility to cutaneous infection. The incidence of AD, as other atopic diseases, has increased steadily over the past 20-30 years and is now estimated to affect between 10-25% of the paediatric population (reviewed in [Akdis et al., 2000; Kapur and Rustin, 2002; Leung, 2000; Wollenberg et al., 2000]). There is believed to be a significant genetic contribution to the pathogenesis of AD, based on family occurrence and twin studies. Several candidate genes have been identified, most of which are associated with the atopic phenotype or to increased levels of IgE. However, concordance rates for AD in monozygotic and dizygotic twins, as well as the wide variation in prevalence between similar ethnic groups, suggests that environmental factors have considerable influence (compare section 1.3). Two distinct forms of AD have been identified: Extrinsic AD, accounting for 70-90% of cases and associated with sensitisation to environmental antigens and elevated serum IgE; and instrinsic AD, accounting for the remaining cases and not associated with a classical atopic phenotype [Wollenberg et al., 2000]. This section will concentrate on the classical extrinsic form of AD.

Pathogenesis of AD

Clinically normal-appearing skin of AD patients contains a sparse perivascular T cell infiltrate. Acute lesions are characterised by marked oedema of the epidermis, increased numbers of LCs with surface-bound IgE, sparse epidermal T cell infiltrate and marked perivenular T cell infiltrate in the dermis. The T cell infiltrate consists mainly of activated memory cells expressing CD3, CD4 and CD45RO. In chronic AD lesions, significantly increased amounts of IgE-bearing LCs persist and a large infiltrate of mast cells and eosinophils are observed in the dermis [Leung, 2000]. Increased expression of the high-affinity receptor for IgE (FcεRI) has been demonstrated on epidermal LCs from both non-lesional and lesional skin of patients

with AD, and the expression of FcεRI on LC correlates with the serum level of IgE [Wollenberg et al., 1996]. The important immunopathogenic role of IgE in skin disease was recently shown in antigen-specific IgE transgenic mice that developed chronic inflammation of the skin after a single subcutaneous antigen injection [Sato et al., 2003]. Most patients with AD also have elevated numbers of circulating eosinophils and increased serum IgE levels reflecting an increased expression of Th2 cytokines. T cells isolated from peripheral blood of infants with AD have shown increased frequency of allergen-specific T cells producing high levels of IL-4, IL-5 and IL-13, but little IFN-γ [Kimura et al., 1998a; Kimura et al., 1998b]. Likewise, T cells isolated from AD skin lesions have been shown to proliferate and secrete Th2 cytokines such as IL-4, IL-5 and IL-13 in response to food antigens and dust-mite antigen [Akdis et al., 2000; Leung, 2000]. GM-CSF is overproduced in the skin of AD patients, possibly contributing to the activation and proliferation of DCs [Pastore et al., 1997]. Moreover, patients with AD have an expanded subset of activated CLA^+ T cells in circulation, which homes to the skin and spontaneously secretes IL-5 and IL-13 thereby prolonging eosinophil survival and IgE synthesis [Akdis et al., 1997; Akdis et al., 1999].

Approximately 80% of patients with AD have elevated levels of serum IgE, evidence of IgE antibodies specific to a variety of foods and inhaled antigens and will eventually develop other IgE-mediated allergies such as allergic rhinitis or asthma. A number of pathogenic mechanisms including aeroallergens, food allergens and superantigens have been highlighted as being involved in AD. Numerous investigations have established that food allergy plays a pathogenic role early in AD, whereas inhalant allergens may be important in older children. Specific aeroallergen T cell clones can be derived from the skin of AD patients and aeroallergens can promote both immediate-type and delayed-type responses in the skin [van Reijsen et al., 1992; Varney et al., 1993]. Patients with AD additionally have an increased tendency to develop bacterial and fungal skin infections, and microbes have been suggested to be involved in the pathogenic mechanism of AD. *Staphylococcal aureus* is found in more than 90% of AD skin lesions compared to 5% in skin of healthy subjects [Leung, 2000]. Some studies suggest that superantigens (or toxins) from *S. aureus* can directly activate T cells and lead to their expansion [Akdis et al., 2000]. In addition to superantigens from *S. aureus*, lipoteichoic acid from the cell wall has been shown to induce Th2 type responses and cellular infiltrates in the dermis in a mouse model of AD [Matsui and Nishikawa, 2002].

Skin barrier disruption in AD

Impaired function of the epidermal barrier is characteristic of AD, and is seen even in areas of the skin without lesions [Wollenberg et al., 2000]. Whether this is an instrinsic defect in keratinocyte biology and the *primum movens* of the disease in these patients or a result of the underlying atopic disease is not known. Nethertons disease which is characterised by severe atopic dermatitis as well as other atopic manifestations is caused by mutations in the SPINK5 gene encoding a serine protease inhibitor, LEKTI, expressed by keratinocytes. It has been suggested that SPINK5 mutations cause a distinctive barrier dysfunction with defective cornification [Chavanas et al., 2000]. It is possible that an impaired epidermal barrier function will induce recurrent infections as well as easy access of allergens through the skin and induction of atopic diseases.

The events occurring when the permeability barrier is disrupted have been studied experimentally by either extracting the lipids from the stratum corneum with organic solvents or removal of the stratum corneum by tape stripping. These experiments have shown that there is a marked increase in permeation of antigens through barrier disrupted skin and alterations in immune function [Nishijima et al., 1997; Wood et al., 1992]. TNF-α, IL-1α, IL-1β and GM-CSF are quickly elevated several fold in the epidermis [Nickoloff and Naidu, 1994; Nishijima et al., 1997; Wood et al., 1992] and expression of MHCII and costimulatory molecules such as CD54 and CD86 are increased on LCs [Nishijima et al., 1997]. The chain of events that is initiated after an acute perturbation of the barrier ultimately leads to the return of lipids to the stratum corneum and a fast restoration of barrier function, but the abrogation and subsequent restoration of the epidermal barrier may well alter skin immune function, and thus profoundly affect the outcome of cutaneous inflammatory reactions.

1.4.3 Food allergy and atopic dermatitis

Skin manifestations represent the most frequently observed clinical symptoms in food allergy. These can present as immediate symptoms of urticara, angioedema or erythema or as delayed symptoms, which most often involve exacerbation of atopic dermatitis. There is little doubt that food allergy and AD are interlinked in as yet undefined ways [Werfel, 2001]. Most food allergic children also suffer from eczema or AD [Hourihane et al., 1996] and conversely, many patients with AD (or their parents) report that certain foods trigger skin abnormalities and are worsening their AD. Food allergy has been established to play a pathogenic role in AD,

although AD usually predates the development of food allergy [Sampson, 1999; Sicherer and Sampson, 1999]. It has been demonstrated that increased severity of AD is directly correlated with the presence of food allergy [Guillet and Guillet, 1992], and removal of the food allergen from the patients diet can lead to significant clinical improvement of AD [Kapur and Rustin, 2002; Leung, 2000]. Food-allergen specific T cells have been cloned from lesional and non-lesional skin of patients with AD [van Reijsen et al., 1998] and the expansion of peripheral blood CLA^+ T cells in response to casein has been described for children with milk-induced eczema [Abernathy-Carver et al., 1995], supporting a role for food-specific T cells in AD.

Patients with AD have a defective epidermal barrier function as well as an impaired intestinal barrier function and increased protein absorption across the gut mucosae [Majamaa and Isolauri, 1996]. In health, antigen transfer is well controlled, but during mucosal dysfunction impaired antigen handling may evoke aberrant immune responses and lead to sensitisation and possibly food allergy. Absorption of intact antigens is a feature of normal gastrointestinal function [Bruce and Ferguson, 1986; Peng et al., 1990; Swarbrick et al., 1979] and circulating food antigens are safely cleared by formation of antigen-antibody complexes with specific circulating antibodies, probably serum IgA [Kapur and Rustin, 2002]. It is hypothesised that the increase of intact food antigens absorbed into the circulation of AD patients may not be cleared and can form complexes with IgG and IgE, which may deposit in the skin and cause or exacerbate AD [Kapur and Rustin, 2002].

1.4.4 Allergic sensitisation through the skin

It is well established that systemic immune responses can affect the skin, as seen in many diseases and for example after clinical food challenges in allergic individuals [Sicherer and Sampson, 1999]. However, much less is known about the potential of the skin to influence systemic immunity. Several observations point to the possibility that the skin can affect the course of systemic disease. AD predates the development of other atopic diseases such as asthma and allergic rhinitis by several years. Children with AD frequently have more severe asthma than asthmatic children without AD, suggesting that allergen sensitisation through the skin may predispose to more severe and persistent respiratory disease [Beck and Leung, 2000]. In children there is strong exposure of the skin to foodstuffs, even when breastfed, and there often appears to be a striking association of the preferentially affected areas in AD at this age and the cutaneous exposure to food [Saloga and Knop, 2000]. Most peanut allergic individuals

additionally suffer from eczema and as 80% of these subjects react to their first known oral exposure to peanut [Hourihane et al., 1996], it has been speculated that the primary sensitisation could be through the skin. Eczema is often controlled with topically applied creams and lotions where peanut and vegetable oil containing small amounts of protein is a common ingredient (section 1.3.1.2).

Immune responses to chemical haptens after epicutaneous exposure are well characterised and manifest clinically as contact dermatitis [Enk and Katz, 1995; Kapsenberg et al., 1992]. However, whether large hydrophilic molecules such as protein antigens can enter through the hydrophobic skin has been a matter of controversy, and whether a primary systemic immune response can be elicited through cutaneous exposure has been little investigated. The fact that protein allergens that are relevant to atopic disease can elicit eczematous inflammatory reactions in the skin, as in the atopy patch reaction, proves that protein antigens can enter the skin in sufficient amounts and in immunogenic form [Darsow et al., 1996]. Prolonged epicutaneous exposure to OVA has been shown to systemically sensitise mice and induce a predominant Th2-type response [Wang et al., 1996]. Prolonged epicutaneous sensitisation to OVA followed by inhalation of OVA additionally induces localised allergic dermatitis, elevated serum IgE, airway eosinophilia and hyperresponsiveness to methacholine, suggesting that epicutaneous exposure to antigen in AD may enhance the development of allergic asthma [Spergel et al., 1998]. Epicutaneous sensitisation to haptens through shaved and barrier-disrupted skin, as a model of AD, has been demonstrated to occur with a Th2-dominant profile in mice, which is clearly distinct from the response induced through intact skin [Kondo et al., 1998]. However, the contribution made by cutaneous allergen exposure to the primary sensitisation, induction, continuation and outcome of atopic diseases remains elusive and requires further investigation.

1.5 Aims of Thesis

The overarching aim of this study was to investigate the immunological outcome of exposure to common food proteins at the body surface. In particular, it explored whether immune tolerance or sensitisation is induced to peanut proteins and OVA following exposure at the intestinal mucosae and skin surfaces. The study assessed the influence of antigen exposure at the skin surface on the response to antigen exposure in the gut, and *vice versa.* It also investigated the direct influence of the route of primary exposure to antigens on the subsequent systemic immune response.

Gastrointestinal exposure to peanut proteins was investigated by developing a novel BALB/c mouse model. The aim of this first part of the study was to gain an understanding of the factors involved in inducing oral tolerance or sensitisation to a mixture of allergenic protein antigens - such as in peanut - and to further the understanding of the mechanisms involved in inducing systemic tolerance through feeding of soluble protein antigens.

Another newly developed BALB/c mouse model was used to investigate epicutaneous exposure to peanut proteins or OVA. Specifically, whether common protein antigens can enter through the skin, cause immune activation and induce primary systemic sensitisation; and how this may influence the normal induction of oral tolerance. This addresses the question of whether exposure to allergens through the skin may promote food allergy. In addition, the local immune regulation in the skin following antigen exposure on intact or inflamed skin was explored.

Overall, this Thesis aims to further the understanding of the immunoregulation following intestinal and cutaneous exposure to common protein antigens. This will contribute to understanding the immunological balance between tolerance and sensitisation to food proteins and allergens.

Chapter 2

Reagents, materials and methods

2. Reagents, materials and methods

All reagents and materials used for the experimental research are listed in this chapter as well as generally used methods. Methods specific for particular sections will be described in the appropriate chapters.

2.1 General reagents

Table 2.1 General reagents

Substance	Purchased from
Acetic acid	Sigma, UK
Acetone	Sigma, UK
Acrylamide	Bio-Rad, UK
Ammonium bicarbonate (NH_4HCO_3)	BDH, UK
Ammonium persulphate (APS)	Bio-Rad, UK
Ammonium sulphate $(NH_4)_2SO_4$	BDH, UK
Aquamount	Citifluor Ltd, UK
Bicinchoninic acid protein assay kit	Sigma, UK
Biotin succinimide ester	Sigma, UK
Bovine serum albumin (BSA)	Sigma, UK
5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma fast BCIP/NBT)	Sigma, UK
Bromophenol blue	Sigma, UK
Carboxyfluorescein diacetate succinimidyl ester (CFSE)	Molecular Probes, USA
Complete Freund's adjuvant (CFA)	Sigma, UK
Concanavalin A (ConA)	Sigma, UK
Coomassie brilliant blue R	Sigma, UK
Cytofix/Cytoperm™	PharMingen, USA
Dimethylsulphoxide (DMSO)	Sigma, UK
DPX mount	BDH, UK
'ECL plus' detection reagents kit	Amersham, UK
ECL western blotting molecular weight marker	Amersham, UK
Eosin	BDH, UK
Ethenediaminetetraacetic acid, di-sodium salt (EDTA)	Sigma, UK
Fluorescein 5-isothiocyanate (FITC)	Sigma, UK
Foetal calf serum (FCS)	Helena Bioscience, UK
Gentamycin	Gibco, UK
Glycerol	Sigma, UK
Glycine	Bio-Rad, UK
GolgiStop™	PharMingen, USA
Halothane BP liquid 100% v/v	Concord Pharmaceuticals Ltd
Hanks buffered saline solution (HBSS)	Gibco, UK
Hematoxylin	BDH, UK

Substance	**Purchased from**
Hexane	Sigma, UK
Histoclear	Lamb Limited, UK
Hydrochloric acid (HCl)	BDH, UK
Isopropanol	BDH, UK
Liberase™	Boehringer Mannheim, UK
Lympho-Sep separation media for murine lymphocytes	Harlan, UK
Magnesium chloride (MgCl)	BDH, UK
Meltilex™	Wallac, UK
2-Mercaptoethanol (2-ME)	Gibco, UK
Methanol	BDH, UK
[methyl-^{3}H]-thymidine deoxyribonucleotide	Amersham, UK
Milk powder (Marvel)	Premier Brands Ltd, UK
N, N, N', N'-tetramethylenediamine (TEMED)	Bio-Rad, UK
N, N-methylene bisacrylamide (BIS)	Bio-Rad, UK
Normal goat serum (NGS)	Sigma, UK
Normal rat serum (NRS)	Sigma, UK
Ovalbumin Grade V	Sigma, UK
Paraformaldehyde	Sigma, UK
Peanut flour	The Golden Peanut Company, USA
Perm/Wash™	ParMingen, USA
Phosphate buffered saline tablets (PBS)	Oxoid, UK
PBS, Calcium and Magnesium free	Gibco, UK
p-nitrophenyl phosphate tablets (pNPP)	Sigma, UK
Polyoxyethylene(20)sorbitan monolaurate (Tween 20)	Sigma, UK
Rhodamine Phalloidin	Sigma, UK
Roswell Park Memorial Institute media (RPMI-1640)	Gibco, UK
SigmaMarker™ (Wide molecular weight)	Sigma, UK
Sodium azide	Sigma, UK
Sodium carbonate (Na_2CO_3)	BDH, UK
Sodium Chloride (NaCl)	BDH, UK
Sodium dodecyl sulphate [Lauryl sulphate] (SDS)	Bio-Rad, UK
Sodium hydrogen carbonate ($NaHCO_3$)	BDH, UK
Sodium hydroxide (NaOH)	BDH, UK
Streptavidin-alkaline phosphatase conjugate	Amersham, UK
Streptavidin-horseradish peroxidase conjugate	Amersham, UK
Streptavidin-Quantum Red™ conjugate	Sigma, UK
Tissue-Tek® O.C.T.™ compound	Sakura, UK
TO-PRO®-3 iodide (642/661)	Molecular Probes, USA
Tris (hydroxymethyl) aminomethane	Sigma, UK
Trypan blue	Sigma, UK

2.2 Buffers and Solutions

MILLIQ ($18\Omega/cm^3$) water (mH_2O) was used to prepare all buffers and solutions. Solutions were sterilised by autoclaving at 121°C for 20min.

- 0.1M Magnesium Chloride (MgCl)
- 0.1M Sodium Chloride (NaCl)
- 4% Paraformaldehyde (PFA): PFA dissolved in PBS using a few drops of 1N NaOH
- Blocking buffer for ELISA: The following was used as indicated:
  - ELISA wash buffer supplemented with 1% v/v Normal Goat Serum
  - ELISA wash buffer supplemented with 1% v/v Normal Rat serum
  - ELISA wash buffer supplemented with 3% w/v Milk Powder (Marvel)
- Blocking buffer for immunohistochemical staining: 2% w/v BSA in PBS
- Carbonate bicarbonate buffer (CBB): 35mM $NaHCO_3$ and 15mM Na_2CO_3. pH adjusted to 9.6 with NaOH or HCl
- ELISA wash buffer (PBST): PBS supplemented with 0.05% v/v Tween-20
- FACS wash buffer: PBS, 0.5% w/v BSA, 0.1% w/v sodium azide
- FACS wash buffer after enzymatic digestion: 5mM EDTA, 0.5% w/v BSA in PBS
- FACS wash for intracellular staining: 1/10 dilution of Perm/Wash™ (PharMingen, USA) in mH_2O
- FITC conjugation buffer: 1 part of 5.8ml 5.3% w/v Na_2CO_3 (anhydrous) and 10ml 4.2% w/v $NaHCO_3$ plus 9 parts 0.1M NaCl. pH adjusted to 9.5 with NaOH or HCl
- Liberase digestion buffer: 0.4mg/ml Liberase in Ca^{++} and Mg^{++} free sterile PBS
- Phosphate buffered saline (PBS): 1 PBS (Dulbecco 'A') tablet dissolved in 100ml mH_2O and the solution autoclaved to sterilise
- pNPP substrate for ELISA: One 20mg p-nitrophenyl phosphate tablet dissolved in 20ml CBB and 20µl 0.1M MgCl (0.1mM). Substrate used immediately
- SDS-PAGE running buffer: 0.025M Tris, 0.192M glycine, 0.1% w/v SDS, pH 8.3
- SDS-PAGE sample buffer: 0.06M Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 0.025% bromophenol blue; 5% 2-ME
- Transfer buffer for immunoblot: 48mM Tris, pH 9.2; 39mM glycine in 20% methanol

2.3 Tissue culture media

All *in vitro* lymphocyte preparations were prepared, held and washed in HBSS supplemented with 2% FCS and 5μg/ml gentamycin.

RPMI-1640 containing L-glutamine and supplemented with 10% FCS, 50μM 2-ME and 5μg/ml gentamycin was used for all cell cultures.

2.4 Antibodies

All antibodies were stored at 4°C in stock concentrations as purchased. The antibody reagents are listed in tables according to application:

Table 2.2 Antibodies for serum antibody assays

Antibody	Dilution used	Specificity	Source
Polyclonal goat anti-mouse IgG (Fc specific); alkaline phosphatase conjugated	1:3000	Mouse IgG subclasses G1, G2a, G2b and G3, but not IgA or IgM. No reaction with mouse FAB fragment or human IgG	Sigma, UK
Monoclonal rat anti-mouse IgG1; alkaline phosphatase conjugated	1:1000	Mouse IgG1	Zymed, USA
Monoclonal rat anti-mouse IgG2a; alkaline phosphatase conjugated	1:1000	Recognises an epitope in the CH3 domain of mouse IgG2a	PharMingen, USA
Monoclonal rat anti-mouse IgE; purified and biotin conjugated	1:500	Reacts specifically with mouse IgE of Igh-C^a and Igh-C^b haplotypes	PharMingen, USA
Polyclonal goat anti-human IgG (Fc specific); alkaline phosphatase conjugated	1:3000	Human IgG subclasses G1, G2 and G3, but not IgA or IgM. Affinity absorbed to remove reaction to human FAB	Sigma, UK

Table 2.3 Antibodies for cytokine assays

Antibody	Conc. used	Clone	Isotype	Specificity	Source
Monoclonal rat anti-mouse IFN-γ	4 μg/ml	R4-6A2	Rat IgG1	Mouse IFN-γ	PharMingen, USA
Monoclonal rat anti-mouse IFN-γ; biotin conjugated	0.5 μg/ml	XMG1.2	Rat IgG1	Mouse IFN-γ	PharMingen, USA
Monoclonal rat anti-mouse, human, pig TGF-β1	3.3 μg/ml	A75-2.1	Rat IgG2a, κ	Reacts with mouse, human and pig TGF-β1	PharMingen, USA
Monoclonal rat anti-mouse, human, pig TGF-β1; biotin conjugated	1 μg/ml	A75-3.1	Rat IgG2a, κ	Reacts with mouse, human and pig TGF-β1	PharMingen, USA
Monoclonal rat anti-mouse IL-4	1 μg/ml	BVD4-1D11	Rat IgG2b	Mouse IL-4	PharMingen, USA
Monoclonal rat anti-mouse IL-4; biotin conjugated	1 μg/ml	BVD6-24G2	Rat IgG1	Mouse IL-4	PharMingen, USA
Monoclonal rat anti-mouse IL-10	5 μg/ml	JES5-2A5	Rat IgG1, κ	Mouse IL-10	PharMingen, USA
Monoclonal rat anti-mouse IL-10, biotin conjugated	0.5 μg/ml	JES5-16E3	Rat IgG2b	Mouse IL-10	PharMingen, USA

Table 2.4 Antibodies for FACS staining

Antibody	Conc. used	Clone	Isotype	Specificity	Source
Purified monoclonal rat anti-mouse CD16/CD32 (FcγIII/II Receptor) (Fc Block)	0.25μg per 100μl	2.4G2	Rat IgG2b,κ	Reacts specifically with a common non-polymorphorphic epitope on the extracellular domains of the mouse FcγIII and FcγII receptors	PharMingen, USA
Monoclonal Cy-Chrome™-conjugated rat anti-mouse CD4	0.2 - 1μg per 100μl	RM4-5	Rat IgG2a, κ	Reacts with the CD4 (L3T4) differentiation antigen	PharMingen, USA
Monoclonal Cy-Chrome™-conjugated rat anti-mouse CD8	0.5 - 2μg per 100μl	53-6.7	Rat IgG2a, κ	Reacts with the 38-kDa α and 34-kDa α chains of the CD8 differentiation antigen (Ly-2 or Lyt-2)	PharMingen, USA
Monoclonal rat anti-mouse CD25	0.5 - 2μg per 100μl	7D4	Rat IgM, κ	Reacts with CD25, the low affinity IL-2 receptor α chain (IL-2Rα, p55)	PharMingen, USA

Antibody	Conc. used	Clone	Isotype	Specificity	Source
Monoclonal hamster anti-mouse CD152; purified and RPE-conjugated	0.5 - 2μg per 100μl	UC10-4F10-11	Armenian hamster IgG, Group 1, κ	Reacts with CD152 (CTLA-4) on activated T lymphocytes	PharMingen, USA
Purified monoclonal hamster anti-mouse CD11c	0.5 – 1μg per 100μl	N418	Hamster IgG purified from mouse ascites fluid	Specific for mouse p150,95 (CD11c)	Endogen, USA
Monoclonal mouse anti-TGF-β1, -β2 and -β3	1 –5μg per 100μl	1D11	Mouse IgG1	Recognises bovine, mouse and human TGF-β1 and TGF-β2	R&D Systems, UK
Monoclonal RPE-conjugated mouse anti-rat IgM	0.5μg per 100μl	G53-238	Mouse IgG1, κ	Reacts specifically with rat IgM monomers and pentamers	PharMingen, USA
RPE-conjugated Goat F(ab')$_2$ anti-rat IgG (H+L)	0.5μg per 100μl		F(ab')2 fragment of affinity purified goat anti-rat IgG	Reacts with all rat Ig	Caltag, USA
RPE-conjugated Goat F(ab')$_2$ anti-hamster IgG (H+L)	0.5μg per 100μl		F(ab')2 fragment of affinity purified goat anti-hamster IgG	Reacts with all hamster Ig	Caltag, USA
FITC-conjugated rabbit anti-rat immunoglobulin	10μg per 100μl		Purified Ig fraction of rabbit anti-serum	Reacts with rat Ig of all classes	DAKO, DK
RPE-conjugated F(ab')$_2$ fragment of rabbit anti-mouse Ig	2.5μg per 100μl		F(ab')$_2$ fragment of affinity-isolated rabbit anti-mouse Ig	Reacts with all mouse IgG subclasses (IgG1, IgG2a, IgG2b, IgG3), mouse IgA and mouse IgM	DAKO, DK

Table 2.5 Antibodies used for *in vitro* blocking studies

Antibody	Conc. used	Clone	Isotype	Specificity	Source
Monoclonal mouse anti-TGF-β1, -β2 and -β3 (No azide/low endotoxin)	50μg/ml	1D11	Mouse IgG1	Recognises bovine, mouse and human TGF-β1 and TGF-β2	R&D Systems, UK
Purified monoclonal hamster anti-mouse CD152 (No azide/low endotoxin)	10μg/ml	UC10-4F10-11	Armenian hamster IgG1, κ	Reacts with CD152 (CTLA-4) expressed on activated T lymphocytes	PharMingen, USA

Table 2.6: Antibodies for Immunohistological staining

Antibody	Conc. used	Clone	Isotype	Specificity	Source
Purified monoclonal mouse anti-mouse I-A^d	5μg/ml	AMS-32.1	Mouse IgG2b, κ	Reacts with the I-A^d MHC class II alloantigen. Cross-reacts with H-2^f, H-2^j and H-2^v haplotypes	PharMingen, USA
Purified monoclonal and FITC-conjugated rat anti-mouse I-A/I-E	5μg/ml	2G9	Rat IgG2a, κ	Reacts with the I-A^d and I-E^d MHC class II alloantigens. It has comparable reactivity on I-A^b, I-A^d, I-A^q, I-E^d and I-E^k alloantigens	PharMingen, USA
Purified monoclonal rat anti-mouse CD86 (B7.2)	10μg/ml	GL1	Rat IgG2a, κ	React with the CD86 (B7.2) costimulatory molecule expressed on a broad spectrum of leukocytes	PharMingen, USA
Purified monoclonal rat anti-mouse CD40	10μg/ml	3/23	Rat IgG2a, κ	Reacts with the 40-50kDa CD40 glycoprotein	PharMingen, USA
Purified monoclonal hamster anti-mouse CD54 (ICAM-1)	10μg/ml	3E2	Armenian hamster IgG, group 1, κ	Reacts with the 95kDa ICAM-1 Ig superfamily member	PharMingen, USA
Purified monoclonal hamster anti-mouse CD11c	10μg/ml	N418	Hamster IgG purified from mouse ascites fluid	Specific for mouse p150,95 (CD11c)	Endogen, USA
Rabbit anti-peanut protein immunoglobulin	10μg/ml		Rabbit antiserum. Total IgG fraction		BioGenes GmbH, D
FITC-conjugated rabbit anti-mouse immunoglobulin	10μg/ml		Purified Ig fraction of rabbit anti-serum	Reacts with mouse Ig of all classes	DAKO, DK
FITC-conjugated rabbit anti-rat immunoglobulin	10μg/ml		Purified Ig fraction of rabbit anti-serum	Reacts with rat Ig of all classes	DAKO, DK
TRITC-conjugated donkey anti-rat IgG (H+L)	15μg/ml		Affinity isolated from donkey anti-serum	Reacts with the heavy chains on rat IgG and with light chains common to most rat Ig	Jackson Immuno-Research, USA
TRITC-conjugated goat anti-Armenian hamster IgG (H+L)	15μg/ml		Affinity isolated from goat anti-serum	Reacts with heavy chains on Armenian hamster IgG and with light chains common to most Armenian hamster Ig	Jackson Immuno-Research, USA

Table 2.7 Antibodies used as isotype standards

Antibody*	Clone	Specificity	Source
Purified monoclonal mouse IgG2b, κ	MPC-11	Unknown specificity	PharMingen, USA
Purified monoclonal and Cy-Chrome™-conjugated rat IgG2a, κ	R35-95	Unknown specificity	PharMingen, USA
Purified monoclonal Armenian hamster IgG, Group 1, λ	G235-2356	Specific for the hapten TNP and belongs to hamster IgG group 1, λ	PharMingen, USA
Monoclonal RPE-conjugated Armenian hamster IgG, Group 1, λ	A19-3	Specific for the hapten TNP	PharMingen, USA
Purified monoclonal rat IgM, κ	R4-22	Unknown specificity	PharMingen, USA
Purified monoclonal Armenian hamster IgG1, κ (No azide/low endotoxin)	A19-3	Specific for the hapten TNP	PharMingen, USA
Purified monoclonal mouse IgG1 (No azide/low endotoxin)	11711.11	Specific for KLH	R&D Systems, UK

* All antibodies used as immunoglobulin isotype standards were used in the same concentration as the antibody of interest.

2.5 Cytokine and antibody standards

Recombinant cytokines, purified TGF-β and monoclonal IgE antibody were used to obtain standard curves for the relevant ELISA assays. TGF-β was additionally used for *in vitro* cell cultures. The cytokine standards were diluted in sterile PBS with 2% w/v BSA and stored in alliquotes at –70°C. Stock solution of standard IgE antibody was stored at 4°C.

Table 2.8 Cytokines and antibodies used as standards in ELISA

Standard	Clone/Protein source	Specific activity	Source
Purified monoclonal mouse IgE, κ	Clone C38-2		PharMingen, USA
Recombinant mouse IFN-γ	Produced using mouse IFN-γ cDNA cloned into a baculovirus expression vector and expressed in cultured T. *ni* insect cells	0.2 - $1x10^8$ Units/mg	PharMingen, USA
Human TGF-β	Purified from human platelets. Consists predominantly of TGF-β1		PharMingen, USA
Recombinant mouse IL-4	Produced using mouse IL-4 cDNA cloned into a baculovirus expression vector and expressed in insect cells	0.4 – $1.6x10^8$ Units/mg	PharMingen, USA
Recombinant mouse IL-10	Produced using mouse IL-10 cDNA cloned into a baculovirus expression vector and expressed in T. *ni* insect cells	0.5 – $2.0x10^7$ Units/mg	PharMingen, USA

2.6 Antigens

Peanut and OVA were used as the main protein antigens in all *in vivo* and *in vitro* experiments. Peanut was used as a mixture of antigens containing all the proteins in peanut while OVA was used as a single purified protein. The peanut flour was kindly given by the Golden Peanut Company, Ca, USA and the proteins concentrated using the method detailed below. OVA grade V was purchased from Sigma, UK.

2.6.1 Peanut proteins

Several kilograms of part-defatted peanut flour from light-roasted peanuts, containing approximately 50% protein and 12% lipid (determined by the Golden Peanut Company, USA) were obtained. A concentrated peanut protein extract was made using a modification of the extraction method described by de Jong et al. [de Jong et al., 1996]. In brief; 500g flour was defatted 5 times with 200ml hexane and the proteins then extracted by stirring the flour in 1.5litres 0.1M NH_4HCO_3 for 4 hr at 4°C. After centrifugation for 1 hr at 15000g, the supernatant was removed and saturated with 60% ammonium sulphate for 2 hr at 4°C. The precipitate was centrifuged for 30min. at 15000g and the pellet dissolved in a minimum volume of PBS. The preparation was then dialysed extensively against at least 3 changes of sterile PBS at 4°C for 24 hr. The dialysed peanut extract was filtered through 0.2μm filters, frozen at –70°C and lyophilised. The finished peanut powder was quality assessed by confirming sterility (culturing on blood agar plates), by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) (section 2.7) and by measuring LPS contamination. LPS content was determined using E-toxate® endotoxin reagent limulus polyphemus (Sigma, UK) according to manufacturers instruction. The peanut powder routinely contained < 0.05 - 0.1 EU/ml of LPS, which was the detection limit of the assay. The protein content of the peanut powder was then analysed using a standard bicinchoninic acid protein assay (section 2.8).

By this method large quantities of a uniform quality peanut extract containing ~ 90% pure peanut protein was obtained. This peanut extract was used for all experiments. Fig. 2.1 shows an example of a SDS-PAGE gel of whole peanut flour and the peanut extract demonstrating that none of the major proteins in peanut were lost during the concentration process.

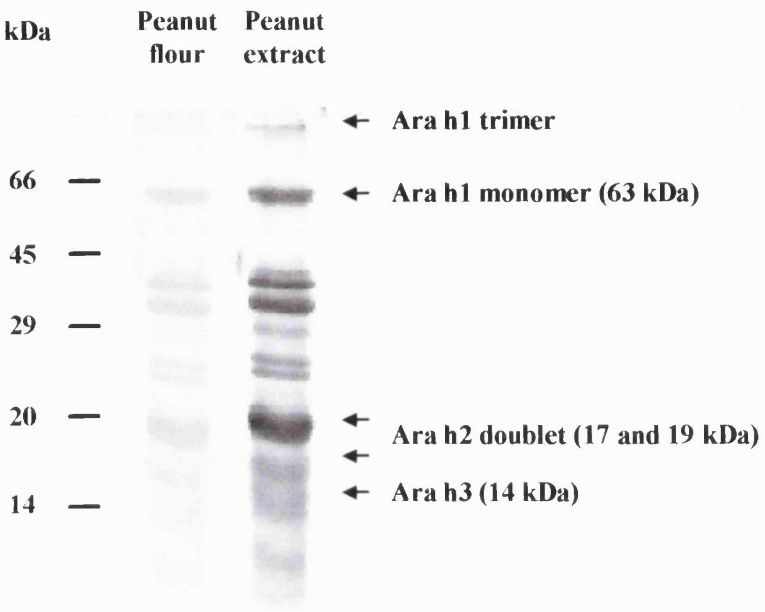


Fig. 2.1 SDS-PAGE gel of peanut proteins
Coomassie brilliant blue staining of peanut proteins in whole peanut flour and in the crude peanut extract after protein concentration. The molecular weight marker in the left lane is shown as kDa weight bands, from where the main allergenic proteins in peanut Ara h1, Ara h2 and Ara h3 were identified according to size

2.7 SDS-PAGE

12% polyacrylamide gels were prepared using the following stock solutions:

- Acylamide concentration: 30% acrylamide/N,N-methylene bisacrylamide (BIS) solution, 29:1
- Stacking buffer: 0.5M Tris-HCl, pH 6.8
- Resolving buffer: 1.5M Tris-HCl, pH 8.8
- 10% w/v sodium dodecyl sulphate (SDS)
- Sample buffer: 0.06M Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 0.025% bromophenol blue; 5% 2-ME
- Catalyst: 10% ammonium persulphate (APS); TEMED (N, N, N', N'-tetramethylenediamine)
- Electrode buffer: 0.025M Tris, 0.192M glycine, 0.1% w/v SDS, pH 8.3

1.0mm and 1.5mm thick SDS-PAGE resolving gels containing 12% acrylamide solution with 0.1% SDS in TRIS-HCl buffer were polymerised with 2.5% APS and 0.1% TEMED and cast in mini-Protean II cell-Biorad or Protean II cell-Biorad apparatus respectively. Stacking gels

containing 5% acrylamide and 0.1% SDS in TRIS-HCl buffer, pH 6.8 with 5% APS and 0.5% TEMED were cast on top of the resolving gel and allowed to polymerise.

Protein samples were diluted in an equal volume of sample buffer and the mixture boiled for 5min. Protein samples were loaded into the wells of the stacking gel alongside a range of molecular weight standards. Wide range colour marker from Sigma, UK (6.5 – 205 kDa) were used as molecular weight standard on gels that were to be stained with Coomassie Brilliant Blue. ECL western blotting molecular weight marker from Amersham, UK (14 – 97 kDa) were used as standard on gels which were to be immunoblotted.

The gels were run in the Biorad Protean II apparatus at 200V using the electrode buffer. Following the electrophoresis the gels were stained with Coomassie Blue or Western blotted.

2.7.1 Coomassie brilliant blue staining

Solutions used for staining were:

- Staining solution: 0.1% w/v Coomassie brilliant blue in 40% v/v methanol, 7% v/v acetic acid. The staining solution was filtered after the dye had dissolved
- Destain solution I: 40% methanol, 7% acetic acid
- Destain solution II: 5% methanol, 7% acetic acid

Gels were soaked in the staining solution for at least 30min. and then destained in excess volume of destain solution I, changing the destain solution several times until the background on the gel was satisfactory removed. The gel was then transferred to destain solution II for permanent fixation.

2.7.2 Western blotting

Solution used for immunoblotting:

- Blotting buffer: 48mM Tris, pH 9.2; 39mM glycine in 20% methanol

The SDS-PAGE gel was placed in the blotting buffer for 15min. and the transfer stack then built in a Biorad semi dry blotting system. On the pre-wetted cathode side of the unit were placed; one sheet of filter paper (Sigma, UK) soaked in blotting buffer, the gel, a nitrocellulose

membrane (Amersham, UK) soaked in blotting buffer and one additional sheet of filter paper (Sigma, UK) soaked in blotting buffer. The pre-wetted anode side of the unit was then placed on top to create a 'sandwich'. Any air trapped in the 'sandwich' was removed and it was ensured that the layers were flat before the transblotter was sealed. Transfer of proteins to the nitrocellulose membrane were conducted for 20min. at 12V.
The transfer of all proteins in the colour marker indicated a correct blotting.

2.7.2.1 Detection of immunoblots

Peanut protein extract was run on SDS-PAGE gels and the proteins transferred to nitrocellulose membranes as described above. The membranes were then incubated in 5% milk powder (Marvel) in PBS with 0.05% Tween-20 for 1 hr at room temperature to reduce non-specific binding. The blocking solution was discarded and replaced with 1:10000 dilution of individual mouse test sera or 1:1000 dilution of individual human test sera in the milk powder blocking solution and the sera was incubated with the nitrocellulose membranes overnight at 4°C on a roller/rocker. After incubation the membranes were washed five times with PBS, 0.05% Tween-20 and antibody binding was detected with alkaline phosphatase conjugated polyclonal goat anti-mouse or human IgG (Fc specific) diluted 1:3000 in the blocking buffer. After 2 hr incubation at room temperature on a roller, the membranes were washed five times in PBS, 0.05% Tween-20 and the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma fast BCIP/NBT) (Sigma, UK) was added. The membranes were incubated with the substrate at room temperature on a roller until a satisfactory signal appeared. The reaction was stopped by rinsing the membranes in distilled water. The membranes were then air dried and analysed.

2.8 Protein concentration determination

Protein concentration was determined using bicinchoninic acid protein assay kit from Sigma, UK. Solutions used for the protein assay were:

- Bicinchoninic acid solution (Sigma, UK): Containing bicinchoninic acid, sodium carbonate, sodium tartrate and sodium bicarbonate in 0.1M NaOH at pH 11.25
- Copper(II)sulphate pentahydrate 4% solution (Sigma, UK): Containing 4% w/v cupric sulphate $5H_20$

- Protein standard solution (Sigma, UK): Flame sealed glass ampoules containing 1ml of 1.0mg/ml bovine serum albumin (BSA) in 0.15M NaCl supplemented with 0.05% sodium azide as a preservative

The standard curve was prepared in a series of test tubes by mixing 0 - 100µg protein standard with 100µl mH_2O. 2ml protein determination reagent (1 part 4% solution of CopperIIsulphate pentahydrate and 50 parts bicinchoninic acid solution) was then added, the tubes vortexed and incubated at 37°C for 30min. Sample test tubes with a known weight of lyophilised sample powder per 100µl were treated identically. The test tubes were allowed to cool to room temperature and the absorbance determined at 562nm. From the standard curve (Fig. 2.2) the amount of protein in the test sample could be calculated as long as the net absorbance of the test sample was within the range of the standard curve.

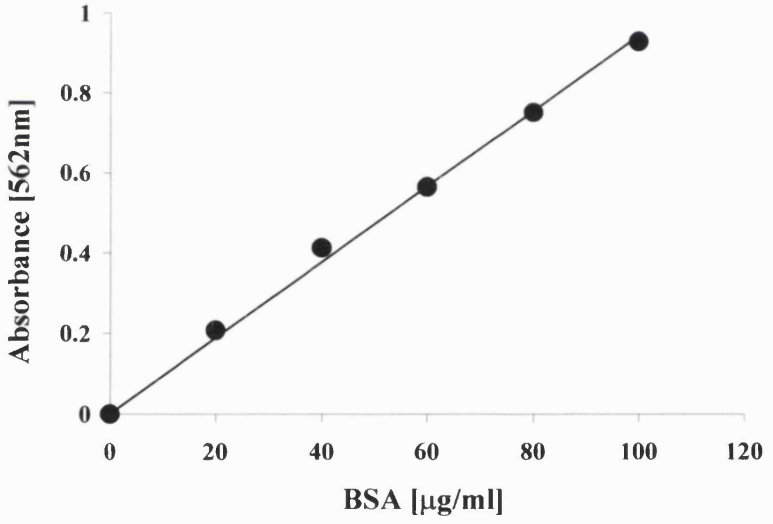


Fig. 2.2 Protein assay calibration curve
Example of BSA standard curve used to determine the protein concentration in test samples. Standard curves routinely yielded lines with R^2-values > 0.995

2.9 FITC/Biotin conjugation

FITC conjugation of antibody/peanut protein:

Antibody (usually 500µg/ml) or peanut protein at 10mg/ml were spun down at low speed to a minimal volume in Millipore filter Eppendorfs (Amicon, UK). The antibody or peanut protein

was washed three times with FITC conjugation buffer (section 2.2) and resuspended in 500µl conjugation buffer. FITC was dissolved to approximately 1mg/ml in conjugation buffer using a whirlmixer and was then filtered through a 0.2µm filter. 150µl FITC solution was added to the antibody/protein. The mixtures were incubated at room temperature in the dark for 3-3.5 hr. After incubation, unbound FITC was thoroughly washed away with PBS by low speed centrifugation until running buffer was clear. Antibody was made back to original concentration (usually 500µg/ml) in FACS wash buffer (section 2.2) and stored dark at 4°C. The peanut-FITC conjugation was diluted to 4mg/ml in sterile PBS and stored at –20°C.

Biotin conjugation of peanut/OVA:

24mg of peanut protein or OVA was dissolved in 6ml 0.1M $NaHCO_3$ at pH 8.6 and 200µl of biotin succinimide ester at 1 mg/ml in dimethylsulphoxide (DMSO) were added. The mixture were incubated in the dark on a roller for 4 hr at room temperature. After incubation, the biotin-conjugated mixture was dialysed against more than 4 changes of PBS at 4°C during 24 hr. The biotin-conjugated peanut and OVA was then diluted to a final concentration of 4mg/ml and stored in alliquotes of 500µl at –20°C.

2.10 Animals

BALB/c mice were bred and maintained on a special diet free of peanut, OVA, soy and cows-milk at the Western Laboratory facilities at the Institute of Child Health, UK. Additional female C57/BL6 and CBA/C9 mice were purchased from Harlan, UK Ltd. All mice were kept under specific pathogen-free conditions and provided the special diet and water ad libitum. 6-8 week old female mice were used.

2.10.1 Anaesthesia

All *in vivo* animal work was done as regulated procedures under the Animals (Scientific Procedures) Act 1986 and with emphasis on causing minimal stress and discomfort to the animals. All procedures – apart from gavage feeding – were carried out on mice under respiratory anaesthesia. A flow-chamber with 2-4% Halothane for induction concentration and 2% Halothane as maintenance concentration was used. An oxygen flow-rate of 2 l/min. was routinely used and the chamber was attached to a fluosorber scavenger system, which actively scavenged excess gases using charcoal. For some procedures a mouse face mask was

utilised to deliver maintenance anaesthetic. The depth of the anaesthesia was monitored at all time by assessing the rate, depth and pattern of breathing. Body temperature was monitored by feeling the animals paws and ears. Animals were protected from hypothermia with a heating pad during recovery.
The mice would routinely be on their feet within a minute of removing them from the Halothane and were watched till they recovered fully.

2.10.2 Immunisation procedures

A range of different immunisation procedures were utilised throughout this study; including oral, epicutaneous and subcutaneous immunisation with and without adjuvant:

Oral: Animals were fed by gavage using a 20-gauge, 30mm cannula. Each mouse received a single intra-gastric feed of antigen at doses varying from 0.02 – 100mg per mouse dissolved in 100µl sterile PBS. At the highest dose of peanut protein feed, i.e. 100mg, the peanut protein was dissolved in 200µl PBS and controls were accordingly sham-fed 200µl PBS. Otherwise controls were sham-fed 100µl PBS.

Epicutaneous: The stratum corneum was removed from both sides of the earlobe by application and removal of cellophane tape (Scotch^{TM}) 6-8 times (this procedure referred to as abrasion). Twenty-four hours later, OVA or peanut protein in PBS (4 mg/ml) were applied to both sides of the earlobe with a cotton bud. The application of protein to the skin was repeated on the next two consecutive days. An estimated maximum of 25µl, equalling 100µg of protein, was deposited on the ear by this technique. Antigen applied to intact skin that had not been tape-stripped, and PBS without antigen applied to stripped skin and intact skin were included as controls.

Subcutaneous with Complete Freund's adjuvant: Antigen were emulsified 1:1 in Complete Freund's adjuvant (CFA). 50µl of this mixture, containing 100µg antigen, were then injected subcutaneously at the base of the tail.

Subcutaneous without adjuvant: 100µg antigen in sterile PBS were injected subcutaneously into the left hind footpad or at the base of the ears.

Details of the specific immunisation protocol used for individual experiments and of timing between immunisation will be provided in the chapter wherein the protocol is used.

2.10.3 Delayed type hypersensitivity

To assess delayed type hypersensitivity (DTH) reactions 100μg peanut protein or OVA in 50μl PBS was injected subcutaneously into the left hind footpad using a 25G needle. Footpad thickness was measured with a microcalliper (Mitutoyo, Japan) just before and 24 hr after the injection. The Δ footpad swelling was calculated [mean footpad thickness at 24 hr – mean footpad thickness at 0 hr] and the results plotted as experimental groups ($n \geq 6$) against the mean Δ footpad swelling [$mmx10^{-1}$] ± 1 SEM.

2.11 Lymphocyte preparation

Mice were killed by cervical dislocation according to Schedule 1 of the Animals (Scientific Procedures) Act 1986. The abdomen and limbs were immediately swabbed with 70% IMS to reduce contamination by bacteria or hair. Using forceps and scissors, lymph nodes draining the immunisation site i.e. para-aortic lymph nodes (PLN), cervical lymph nodes (CLN), popliteal lymph nodes (PopLN), mesenteric lymph nodes (MLN) and spleens were collected. The lymphoid organs were placed in cold HBSS containing 2% FCS and 5μg/ml gentamycin and held on ice.

Single cell suspensions were prepared by mechanical disaggregation of the lymphoid tissue and gently pushing them through a 70μM Nylon mesh cell strainer (Falcon BD, UK). The LN cell-suspensions were washed twice in HBSS supplemented with 5% FCS and 5μg/ml gentamycin by centrifugation at 470g for 10min. at 4°C. After the last wash, cells were resuspended in RPMI-1640 medium supplemented with 10% FCS, 50μM 2-ME and 5μg/ml gentamycin. The viability of the cells was analysed using Trypan blue and viable cells were counted using a Neubauer haemocytometer (Weber, UK). More than 98% of cells in the cell-suspension would routinely be viable using the Trypan blue exclusion test. Cell-suspensions were made to $2x10^6$ cells/ml in culture medium.

Spleen cell-suspensions were carefully layered onto mouse Lympho-Sep separation media and centrifuged for 20min. at 1130g at room temperature. Mononuclear cells were removed from the interface and washed in HBSS, containing 2% FCS and 5μg/ml gentamycin at 4°C. Red blood cells in the resulting preparations were removed by giving cells a brief (~ 15sec.) osmotic shock with sterile mH_2O. Cell-suspensions were then washed twice, resuspended and viability tested by Trypan blue exclusion.

2.12 T cell proliferation measured by [^{3}H]-thymidine incorporation

$2x10^5$ lymphocytes per well were plated in flat-bottomed 96-microwell tissue culture plates (Nunc, DK) and cultured with peanut or OVA in serial 1:3 dilutions from 450μg/ml to 5.6μg/ml in a total volume of 200μl. Cells cultured without antigen and with an irrelevant control antigen (OVA, peanut or BSA) were used as controls. For positive controls, cells were stimulated with 1μg/ml ConA. All experimental samples and positive/negative controls were cultured in triplicate. Cells were incubated at 37°C with 5% CO_2 in the air for 90 hr and pulsed with 1μCi [^{3}H]-thymidine per well for the last 16 hr of culture. Cells were harvested onto glass fibre filter mats (Wallac, UK) with a Tomtec cell harvester. The mats were then dried and Meltilex™ scintillin sheets were melted onto the filter mats for counting in a Trilux 1450 MicroBeta Liquid scintillation and luminescence counter (Wallac, UK).

Results were expressed as mean counts per minute (cpm) of triplicate cultures ± 1 SEM and plotted against antigen concentration.

2.12.1 T cell proliferation in lymphocyte co-cultures

Lymphocytes from different experimental groups were co-cultured by varying the ratio between the cells in the culture, while keeping the overall density of cells constant at $2x10^5$ cells/well. Lymphocytes from two experimental groups were cultured in ratios of 10:0, 10:1, 10:5, 1:1, 5:10, 1:10 and 0:10. The cultures were then stimulated with 150μg or 50μg peanut/ml, 50μg OVA/ml, 1μg ConA/ml or left unstimulated for 90 hr at 37°C with 5% CO_2 in the air. The cells were pulsed with [^{3}H]-thymidine, harvested and [cpm] counted as above.

The lymphocyte co-cultures were additionally cultured with 50μg/ml neutralising monoclonal mouse anti-TGF-β antibody, 50μg/ml mouse IgG1 isotype control, 10μg/ml monoclonal purified hamster anti-mouse CTLA-4 (CD152) or 10μg/ml monoclonal hamster IgG1 isotype control.

2.13 T cell proliferation measured by CFSE staining

The dye carboxyfluorescein diacetate succinimidyl ester (CFSE) is an amine-reactive fluorescent dye that conjugates to proteins, amine-modified oligonucleotides or other amine-containing compounds. It is a useful tool in the study of live cells as it can permeate cell membranes and label cells. When cells are labelled with CFSE it allows the identification of cells in different cell cycles as the intensity of the dye on the cell surface decreases by a factor of two with each cell division. CFSE staining can thus be used as a measurement of proliferation when analysed on a fluorescence activated cell sorter (FACS). Non-divided cells will retain a high level of fluorescence and each division cycle can be detected as distinct peaks of fluorescent intensity reduced by half for each cell cycle.

Cell-suspensions at $2x10^6$ cells/ml RPMI were incubated with CFSE at a final concentration of between 1 - 2.5μM for 10min. at 37°C. The reaction was stopped by diluting the cell/CFSE mixture with 40ml of RPMI supplemented with 10% FCS. The cells were then washed a further three times with RPMI containing 10% FCS and resuspended to $2x10^6$ cells/ml in RPMI-1640 containing 10% FCS, 50μM 2-ME and 5μg/ml gentamycin. One ml of cell suspension per well was plated in 24-well tissue culture plates (Nunc, DK) and stimulated with 150μg peanut or OVA/ml or with 1μg ConA/ml. Cultures were incubated at 37°C with 5% CO_2 in the air for 90 hr when the cells were collected for FACS analysis.

2.14 FACS staining

Lymphocytes for FACS staining were collected, washed twice with HBSS and a cell-suspension of $1x10^6$ cells/ml made. 100μl, i.e. 10^5 cells, were plated per well in 96-well U-bottomed plates (Greiner, D) and the cells pelleted by centrifuging at 470g at 4°C for 5 min. The supernatant was discarded and the cells agitated by moving the plate over a whirlmixer. 0.25μg Fc Block (purified monoclonal rat anti-mouse CD16/CD32 antibody) diluted in FACS

buffer (PBS, 0.5% w/v BSA, 0.1% w/v sodium azide) was added to the cells in a total volume of 100µl and the cells incubated on ice for 15min. 100µl optimally diluted primary antibody (purified or fluorochrome conjugated) was then added and the mixture incubated in the dark on ice for 45min. (the optimal dilution for each antibody was determined in preliminary titrating experiments and is listed in Table 2.4). The cells were then spun down, the antibodies discarded, the cells agitated and then washed twice at 4°C in 200µl FACS buffer per well. Following the wash, 100µl optimally diluted secondary antibody (Table 2.4) was added, where the primary antibody was purified, and the cells incubated on ice, in the dark for 30min. After two further washes in 200µl FACS buffer the cells were fixed by adding 50µl HBSS and 100µl 4% PFA per well. The fixed cells were kept dark at 4°C for maximum 5 days before subjected to flow cytometric analysis on a FACScalibur using Cell Quest software (Becton Dickinson, UK).

For CTLA-4 staining the cells were permeabilised by adding 100µl Cytofix/Cytoperm™ (PharMingen, USA) and incubated on ice for 20min. The cells were washed twice in 1x Perm/Wash™ (PharMingen, USA) and 50µl optimally diluted monoclonal PE-conjugated hamster anti-mouse CTLA-4 antibody or PE-conjugated isotype control was then added and the mixture incubated for 30min. in the dark on ice. At the end of incubation the cells were washed twice with 1x Perm/Wash™ (PharMingen, USA), fixed and analysed on a FACScalibur as described above.

On FACS analysis dead and unwanted cells were excluded by gating on the lymphocyte cell population, which was determined by size (forward scatter) and granularity (side scatter).

2.15 MACS microbead sorting

Magnetic cell sorting (MACS) microbeads were used for negative selection of $CD25^+$ cells in lymphocyte cell-suspensions. Lymphocytes were collected and prepared as described (section 2.11) and then washed in calcium and magnesium free PBS containing 0.5% BSA and 2mM EDTA (wash buffer). Cells were stained with 2µg purified monoclonal rat anti-mouse CD25 in a 100µl volume on ice for 30min. After 2 washes in PBS, 0.5% BSA, 2mM EDTA the cell-pellet was resuspended in 80µl of the wash buffer and 20µl MACS mouse anti-rat kappa microbeads (Miltenyi Biotec, D) were added. The suspension was mixed well and incubated in

the fridge (6-12°C) for 15min, being thoroughly mixed every 5min. At the end of the incubation period the cell/bead suspension was washed twice and resuspended in 500µl wash buffer. Cells were then added to a MACS mini column, which had been pre-washed with two times 500µl wash buffer and secured to a magnet. The cell suspension was left to gently pass through the mesh of the column and drip into a sterile tube. The $CD25^-$ cells were passing through the column, while the $CD25^+$ cells were attached to the MACS beads and would thus be retained in the column on the magnet. When the cell suspension had passed through the MACS column, the column was washed twice with 500µl wash buffer and the buffer collected in the same sterile tube. The $CD25^-$ cell fraction was then washed and counted.

Cell samples were collected before and after the MACS selection, and the MACS separation efficiency checked by staining the CD25 marker. 0.5µg monoclonal RPE-conjugated mouse anti-rat IgM antibody was added in a total volume of 100µl to each cell fraction. The cell suspensions were incubated with the RPE-conjugated antibody for 30min. on ice and then washed twice in FACS buffer. The $CD25^-$ selection efficiency was checked by flow cytometric analysis on a FACScalibur using Cell Quest software (Becton Dickinson, UK). An example of CD25 staining on PLN cells before and after CD25 MACS negative selection is shown in Fig. 2.3.

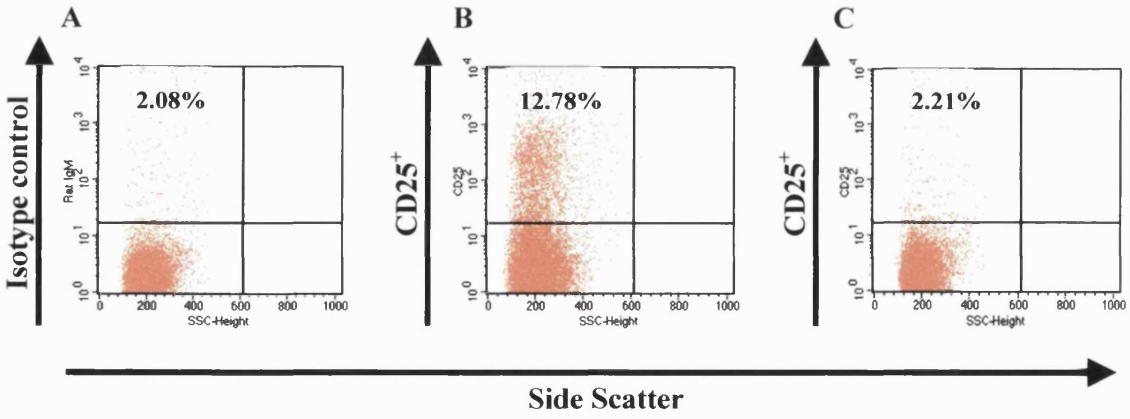


Fig. 2.3 MACS microbead negative selection of $CD25^+$ cells
PLN cells were stained for the CD25 marker and selected with MACS microbeads on a MACS minicoloumn. Before and after MACS selection cells were analysed for $CD25^+$ staining with PE-conjugated second layer antibody and analysed in the FL2 channel on a FACScalibur. (A) CD25 isotype control (rat IgM), (B) $CD25^+$ PLN cells before MACS negative selection, (C) $CD25^+$ PLN cells after MACS negative selection

2.16 Histological analysis

Experimental mice were killed by cervical dislocation and the ears collected for histological analysis of the skin. Skin from the ears was embedded in Tissue-Tek O.C.T. compound and snap frozen in cold isopropanol (BDH, UK) on dry ice. The samples were kept at –70°C at all times until they were cut into sections. Cryostat cross-sections, 7.5µm thick, were cut from the skin tissue and laid onto silane coated slides (BDH, UK). When the sections were dry, they were racked and stored at –70°C until assayed.

Haematoxylin and eosin (H&E) staining was performed on frozen skin sections by standard methods. In brief, slides were placed in tap water for 1min to rehydrate the tissue and then placed in full strength Ehrlich's haematoxylin for 45sec. After gently rinsing the slides in tap water they were dipped three times in acid alcohol and again gently rinsed in tap water for 5min. The slides were then placed in aqueous eosin for 15min., where after the excess eosin was gently rinsed away with tap water for 5min. Finally, the tissue was dehydrated by dipping the slides twice in 70% alcohol, twice in absolute alcohol and twice in histoclear and the slides were then mounted with DPX mount.

Microscopy was performed on a Leitz dialux 20 microscope, photographs taken with a Zeiss AxioCam camera and the images analysed using AxioVision software.

2.17 Immunohistological analysis

Immunohistology was performed on fresh epidermis from the ear skin. Epidermal sheets were prepared by mechanically splitting ears into dorsal and ventral sides and floating them in 20mM EDTA for 4 hr at 37°C on a shaker. Epidermal sheets were gently lifted from the dermis and subcutaneous fat using fine forceps and a dissecting microscope. Single epidermal sheets were placed in PBS in Eppendorf tubes and rotated slowly in a Dynal sample mixer. After one further wash in PBS the epidermal sheets were fixed in acetone for 20min. at –20°C. After two washes in PBS the tissue was blocked by incubating with 2% BSA in PBS for 1 hr at room temperature while rotating the tubes slowly on a Dynal sample mixer. Optimal amount of primary staining antibody (Table 2.6) or equal amount of isotype control antibody was then added to the block solution and the epidermal sheets incubated with the antibodies at 4°C overnight. The tissue was washed four times in PBS as before and counter-stained with

appropriate fluorochrome conjugated secondary antibodies. The epidermal sheets were incubated with optimally diluted secondary antibodies (Table 2.6) in 1% BSA in PBS for 90min., with rhodamine phalloidin (Sigma, UK) to stain F-actin for 60min and/or with TO-PRO® (Molecular Probes, USA) to stain the cell nucleus for 30min. at 37°C. At the end of the incubation the epidermal sheets were washed extensively with PBS (> 6x), carefully spread out on silane coated slides (BDH, UK) and mounted with aquamount.

Epidermal sheets were examined using a Leica SP2 Confocal laser scanning microscope system (Leica NT, Switzerland). In some experiments stained cells were quantified by microscopy. Positive cells were counted in at least 20 visual fields using a grid of known area per epidermal sheet. No background staining with the isotype control antibodies were detected in any of the experiments (not shown).

2.18 Tracking of Langerhans cell migration

Migration of LCs from the epidermis to the draining lymph node was monitored using FITC-labelled peanut protein. Skin on ears of experimental mice were abraded as described in section 2.10.2. Twenty-four hr after abrasion of the skin 100μg peanut-FITC in PBS or PBS alone was painted onto both sides of the earlobe. Mice with intact skin were painted identically. The topical application was done just once or repeated for three consecutive days. 18 hr after the last skin painting CLN were collected into Ca^{++} and Mg^{++} free PBS. Cell-suspensions were prepared by injecting 4mg/ml Liberase™ into the CLN and leaving them to gently digest for 25min. at 37°C. The cells were then passed through a 70μM nylon mesh and washed twice with PBS (Ca^{++}, Mg^{++} free), 5mM EDTA, 0.5% BSA (the same wash buffer was used as FACS wash buffer). Single cell-suspensions were stained for surface expression of CD11c according to the standard protocol in section 2.14 and subjected to flow cytometric analysis on a FACScalibur (Becton Dickinson, UK). Cells were gated on forward and side scatter to exclude dead cells and lymphocytes and staining of gated cells were then analysed using Cell Quest software.

2.19 Preparation of sera

Individual mice were bled by cardiac puncture under deep Halothane anaesthesia using a 25G needle. The blood was allowed to settle for 2 hr at room temperature and then spun in a microcentrifuge at 3800g for 10min. The serum was removed and stored at –20°C until assayed.

2.20 Antibody measurement

Indirect enzyme-linked immunosorbent assays (ELISA) were developed for detection of antigen specific IgG and the IgG subclasses IgG1 and IgG2a in serum. IgE levels in the serum of experimental animals were measured as both total IgE and as antigen specific IgE. Total IgE levels were measured by sandwich ELISA and a modification of the standard sandwich ELISA was developed for the measure of antigen specific IgE.

2.20.1 Antigen-specific IgG, IgG1 and IgG2a

96-well flat-bottomed Maxisorb microtiter plates (Nunc, DK) were coated with 25µg of peanut protein or OVA in CBB at pH 9.6 and incubated at 4°C overnight. The plates were then washed twice with 400µl PBS supplemented with 0.05% Tween 20 (PBST). IgG plates were blocked with 200µl of 1% normal goat serum (NGS) in PBST, IgG1 and IgG2a plates were blocked with 200µl of 1% normal rat serum (NRS) in PBST at 37°C for 1 hr. After three washes in PBST, 100µl of optimally diluted test serum was added. Sera from individual mice were assayed in triplicate. 100µl PBST, 100µl blocking buffer and sera from naïve mice were assayed along with the test samples as controls. The plates were incubated at 37°C for 90min. and then washed three times with PBST. Bound IgG antibody was detected with 100µl alkaline phosphatase conjugated polyclonal goat anti-mouse IgG Fc diluted 1:3000 in blocking buffer. Bound IgG1 and IgG2a were detected with 100µl alkaline phosphatase conjugated monoclonal rat anti-mouse IgG1 or IgG2a diluted 1:1000 in blocking buffer. The plates incubated at 37°C for 1 hr and then washes four times in PBST. 100µl of pNPP substrate (20mg p-nitrophenyl phosphate (pNPP) tablet in 20ml CBB plus 20µl 0.1M MgCl) was added per well and the plates were left to develop with the substrate at room temperature in the dark. Absorbance was measured at 405nm after 10-30min. using a Dynatech MRX microplate

ELISA reader. Results were expressed as the mean absorbance of individual samples within a group ± 1 SEM.

Neither the inter- or the intra-assay coefficient of variance of the developed ELISA techniques would exceed 5% at any given time.

2.20.2 Total and antigen-specific IgE

96-well flat-bottomed Maxisorb microtiter plates (Nunc, DK) were coated with 100µl of monoclonal rat anti-mouse IgE at 1µg/ml in CBB at 4°C overnight. The plates were washed twice in PBST and 200µl of 1% NRS blocking buffer or 3% filtered milk powder blocking buffer was added per well to block non-specific binding to the total IgE and to the antigen-specific IgE plate respectively. The plates were incubated at 37°C for 90min. After three washes in PBST, triplicate test samples, controls and standards were added in a total volume of 100µl. For total IgE assays, monoclonal purified mouse IgE, κ immunoglobulin isotype serially diluted from 500ng/ml to 4ng/ml was used as standard. Optimally diluted experimental sera were added in a volume of 100µl for both total and antigen-specific IgE. The plates were incubated at 4°C overnight, washed three times in PBST and 1µg/ml biotin conjugated monoclonal rat anti-mouse IgE for total IgE or 100µg/ml biotin conjugated peanut or OVA diluted in blocking buffer for antigen-specific IgE were added in a volume of 100µl. The plates were incubated at 37°C for 2 hr, washed four times in PBST and 100µl of streptavidin-alkaline phosphatase conjugate added in a 1:1000 dilution in PBST. After incubation at 37°C for 1 hr, the plates were washed five times with PBST and 100µl of freshly prepared pNPP substrate added. The plates were left to develop at room temperature in the dark and the absorbance read at 405nm after 20-40min using a Dynatech MRX microplate ELISA reader.

Total IgE concentration in the serum samples was calculated from the standard curve using Revelation™ software and expressed as ng/ml. An example of an IgE standard curve is shown in Fig. 2.4 The limit of detection for the total IgE ELISA was 4ng/ml.
Antigen-specific IgE titers were expressed as the mean absorbance of individual samples within a group ± 1 SEM.

The inter-assay coefficient of variance and the intra-assay coefficient of variance were <4% for total and <6% for specific IgE measurements respectively.

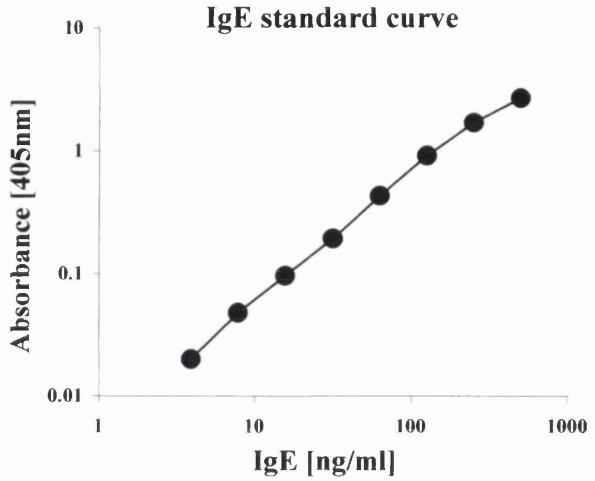


Fig. 2.4 IgE standard curve
Example of IgE isotype standard curve used to determine total serum IgE concentration in test samples by ELISA. IgE standard curves routinely gave sigmoid curves with R^2-values of ≥ 0.998

2.21 Cytokine measurement

Antigen-specific cytokine responses to the *in vivo* immunisation regimes used in this study was investigated by culture of LN and spleen cells with antigen. Two million lymphocytes were cultured in 1 ml RPMI-1640 supplemented with 10% FCS, 50μM 2-ME and 5μg/ml gentamycin in 24-well tissue culture plates (Nunc, DK). Cells were stimulated with 200μg peanut, 200μg OVA, 200μg irrelevant antigen, 1μg ConA or left unstimulated at 37°C in 5% CO_2 for between 48 and 168 hr. Culture supernatants were collected at 24 hr intervals between 48 and 168 hr of incubation and stored at –70°C until assayed.

IFN-γ, TGF-β, IL-4 and IL-10 in supernatants were determined by sandwich ELISA as follows:

96 well flat-bottomed Maxisorb microtiter plates (Nunc, DK) were coated with 50μl of either 4μg/ml of monoclonal rat anti-mouse IFN-γ, 3.3μg/ml of monoclonal rat anti-mouse TGF-

β1, 1μg/ml of monoclonal rat anti-mouse IL-4 or 5μg/ml of monoclonal rat anti-mouse IL-10 diluted in CBB at pH 9.6. Plates were incubated overnight at 4°C and then washed twice in PBST. The IFN-γ, TGF-β and IL-4 plates were blocked with 200μl of 1% NRS blocking buffer and the IL-10 plates were blocked with 200μl of 3% filtered milk powder blocking buffer for 2 hr at 37°C. After three washes with PBST, 100μl of standards, test supernatants and controls were added in duplicate wells. Serial 1:2 dilutions of recombinant mouse IFN-γ from 5000pg/ml to 39.06pg/ml, human TGF-β1 from 5000pg/ml to 39.06pg/ml, recombinant mouse IL-4 from 750pg/ml to 5.86pg/ml or recombinant mouse IL-10 from 5000pg/ml to 39.06pg/ml were used as standards. All samples were tested undiluted - except supernatants from ConA stimulated cells tested on IFN-γ plates, which were diluted 1:20. PBST, CBB and culture medium were used as negative controls. For the TGF-β assay, samples were acidified before testing by adding 8μl 1N HCl (40mM) to 200μl supernatant at 4°C for 1 hr. The samples were then neutralised by adding 8μl 1N NaOH (40mM) and assayed immediately for biologically active TGF-β. All cytokine plates were incubated overnight at 4°C, then washed four times and 100μl secondary antibody added. These were 0.5μg/ml of biotinylated monoclonal rat anti-mouse IFN-γ, 1μg/ml of biotinylated monoclonal rat anti-mouse TGF-β1, 1μg/ml of biotinylated monoclonal rat anti-mouse IL-4 and 0.5μg/ml of biotinylated monoclonal rat anti-mouse IL-10. IFN-γ, TGF-β and IL-4 antibodies were diluted in 1% NRS blocking buffer and the IL-10 antibody was diluted in 1% filtered milk powder. The plates were incubated for 2 hr at 37°C, washed five times in PBST and 100μl of streptavidin-alkaline phosphatase conjugate diluted 1:1000 in PBST added and incubated for 1 hr at 37°C. Finally, the plates were washed six times in PBST and 100μl of pNPP substrate was added. Plates were incubated at room temperature in the dark and the absorbance read at 405 nm on a Dynatech MRX microplate reader.

IFN-γ, TGF-β, IL-4 and IL-10 concentration in the test supernatants were determined from the standard curve using Revelation™ software and expressed as pg/ml. An example of the cytokine standard curves is shown in Fig. 2.5. The limit of detection was 40 pg/ml for IFN-γ, TGF-β and IL-10 and 5 pg/ml for IL-4. The inter-assay coefficient of variance and the intra-assay coefficient of variance were <4 % for IFN-γ and IL-4, <6% for IL-10 and <8% for TGF-β.

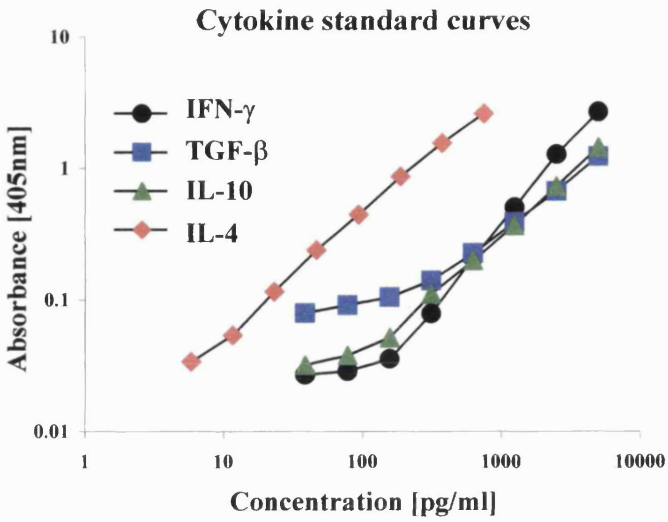


Fig. 2.5 Recombinant cytokine standard curves
Example of cytokine standard curves used to determine cytokine concentration in test samples by ELISA. The titration of the recombinant cytokines routinely yielded sigmoid curves with R^2-values between 0.992 – 0.999

2.22 Definition of animal groups

Peanut-sensitive animals: Animals displaying strong DTH responses to peanut protein and whose isolated lymphocytes show marked proliferation and cytokine secretion when cultured with peanut protein *in vitro*. Animals also produce high levels of peanut-specific antibodies.

Peanut-tolerant animals: Animals that after immunisation with peanut protein do not show DTH responses to peanut protein and whose isolated lymphocytes proliferate poorly and secrete minimal amounts of cytokines when cultured with peanut protein *in vitro*. Animals also have low levels of peanut-specific antibodies.

2.23 Statistical evaluation

The statistical significance of difference between experimental groups in regard to DTH responses, serum antibody levels and cytokine secretion was determined using a two-tailed Student's t-test for unpaired data. Statistical significance in T cell proliferation between experimental groups over the entire dose-response curve was determined by a two-tailed t-test on the slopes of straight-line fits to the data, equivalent to a one-way ANOVA on two groups. If the slopes were not significantly different, the intercepts were compared also using a two-tailed t-test and equivalent to an analysis of covariance on two groups [Armitage, 1971]. Differences were regarded as significant when $p < 0.05$.

Chapter 3

A novel model of oral tolerance and sensitisation to peanut protein

3.1 Introduction

Oral tolerance can be considered to be the physiological or default immune response to food antigens. An increasing incidence of adverse reactions to ingested foods is however being reported, which may reflect a failure of oral tolerance induction or breakdown of its maintenance. Peanuts appear to consist of particular potent allergens and the prevalence of peanut allergy is rising in both children and adults [Emmett et al., 1999; Grundy et al., 2002; Sicherer et al., 1999].

Several experimental animal models of oral tolerance have been developed, but the total number of antigens used experimentally is fairly limited, and no models of oral tolerance to peanut proteins have been published. Most studies of oral tolerance use single, highly purified proteins and few have studied the effects of feeding a mixture of proteins. Requirements for tolerance induction may differ for individual purified antigens and for the more physiologically relevant exposure to antigen mixtures.

In the present chapter, the development of a novel murine model of oral tolerance or sensitisation to whole peanut protein extract is described. Mucosal and systemic immune responses to oral and systemic administration of peanut protein were analysed and both the cellular and humoral arms of the immune system explored. Emphasis in the current study was directed to the down-stream effects of gastrointestinal exposure to food-allergens, i.e. the response to secondary antigen challenge. The peanut protein model was based on previously described OVA models and OVA was used as a control antigen throughout. The developed model of oral tolerance or sensitisation to peanut protein is the basis for this Thesis and aimed at gaining an understanding of the factors involved- and the mechanisms underlying peanut sensitisation and tolerance. This may in the long run facilitate the development of clinical strategies for immunotherapy, or at least to a better characterisation of the hazards involved.

3.2 Immunisation protocol

Each mouse received a single intra-gastric feed of antigen dissolved in sterile PBS at doses varying from 0.02 – 100mg per mouse. Control mice were sham-fed with PBS. Seven days after the feeding, animals were immunised subcutaneously at the base of the tail with 100µg of antigen emulsified 1:1 in CFA. To measure DTH, the animals were given a recall immunisation with 100µg antigen in sterile PBS in the left hind footpad three weeks later. Just before and 24h after this immunisation the thickness of the footpad was measured and used as a measure of the DTH response. For T cell-, cytokine- and antibody responses to the immunising protocol, animals were sacrificed after one further week. A schematic diagram of the experimental protocol is shown in Fig. 3.1. Abbreviations used for the particular immunisations in this chapter are listed in Table 3.1

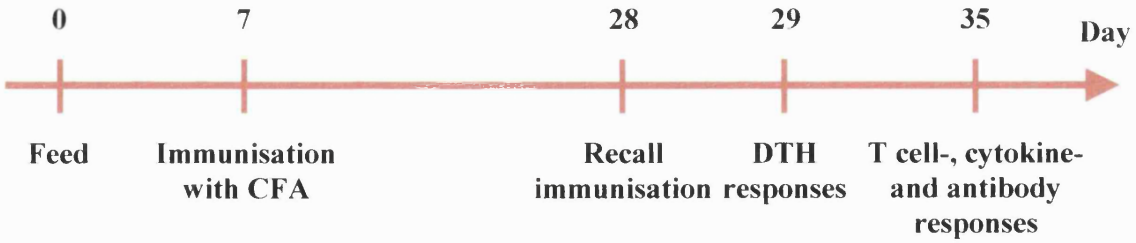


Fig. 3.1 Diagram of oral tolerance/sensitisation protocol
Schematic representation of the experimental protocol used for studying the influence of gastrointestinal exposure to antigen on subsequent systemic immunisation

Table 3.1 Abbreviations used for the oral immunisation protocol

Procedure / Abbreviation	Feed	Immunisation with CFA	Recall immunisation
SPP	Saline	Peanut	Peanut
PPP	Peanut	Peanut	Peanut
SOO	Saline	OVA	OVA
OOO	OVA	OVA	OVA

3.3 Results

3.3.1 Effect of oral antigen doses on delayed type hypersensitivity

As an index of cellular immunity, specific DTH responses to peanut protein and OVA were measured in mice fed various doses of antigen followed by systemic immunisation with antigen in CFA and recall immunisation in the footpad as shown in Fig. 3.1.

Mean DTH responses were significantly enhanced in animals fed either 0.02mg peanut protein or 0.02mg OVA compared to their saline fed controls (Fig. 3.2A). Animals fed a 10 times higher dose of peanut protein, 0.2mg, also elicited enhanced footpad increments upon peanut challenge that were significantly different to sham-fed animals (Fig. 3.2B). When a similar dose of 0.2mg OVA was fed, no difference in DTH responses were observed between the OVA-fed and the saline-fed control group (Fig. 3.2B). At a dose of 2mg peanut protein fed per animal there was no difference in footpad swelling compared to controls, while feeding a dose of 2mg OVA induced a significant reduction in the DTH response (Fig. 3.2C). Increasing the fed dose to 20mg per animal gave similar results; there was no difference in the DTH response between peanut fed animals and saline fed controls but a significant reduction in the mean footpad increment was induced in animals fed 20mg OVA (Fig. 3.2D). At the largest dose of 100mg antigen, both orally fed peanut protein and OVA significantly reduced the DTH response compared to the saline fed controls (Fig. 3.2E).

Throughout the study, DTH responses after peanut protein immunisations were larger than after OVA immunisations, perhaps indicating some differences in immunogenicity or allergenicity between the two proteins (Fig. 3.2). Nevertheless, when animals were fed 100mg peanut protein the reduction in the DTH response was of a similar magnitude as the OVA-induced reduction in footpad increments.

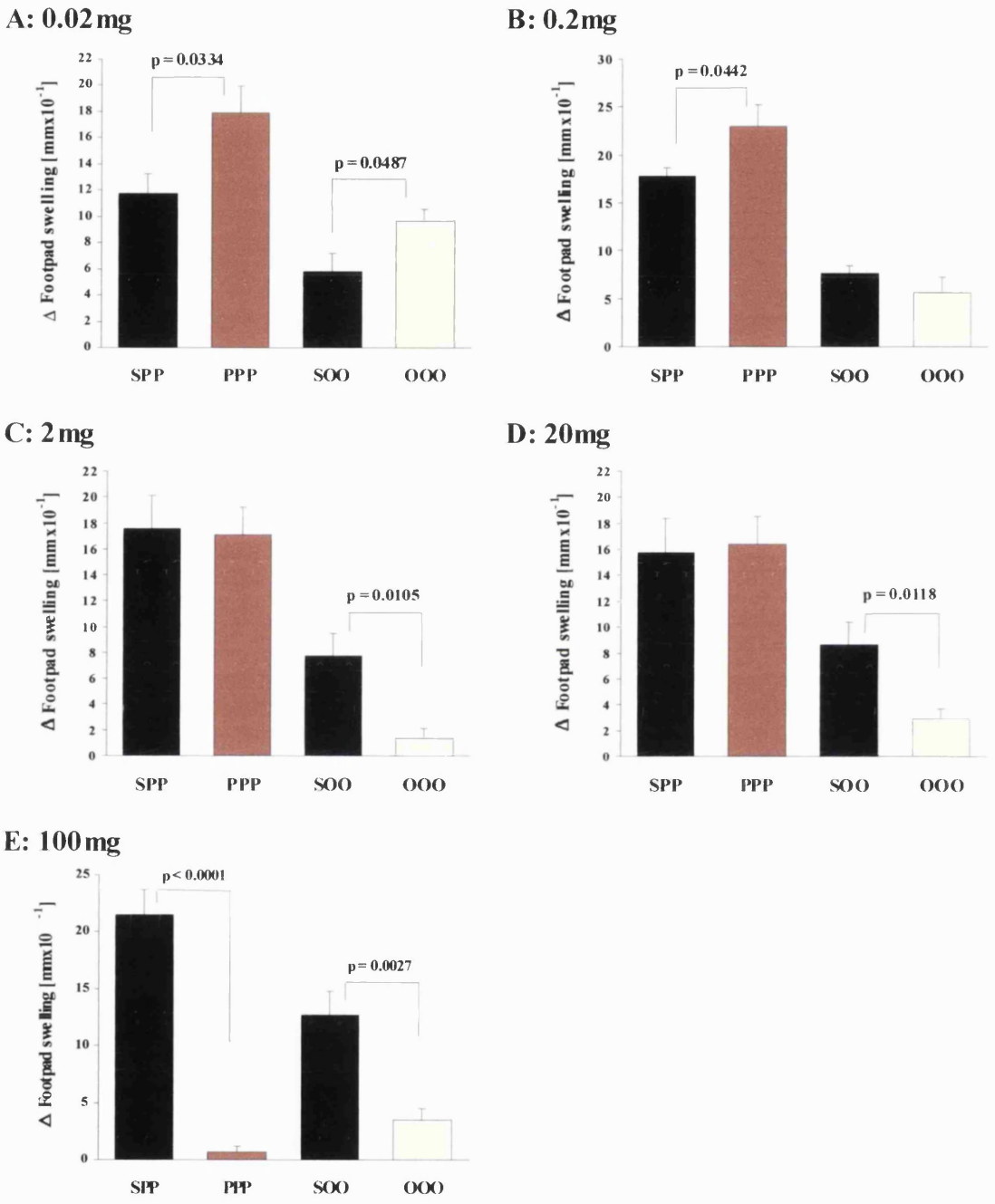


Fig. 3.2 DTH response to peanut protein and OVA

Animals were fed peanut protein or OVA at doses of 0.02mg (A), 0.2mg (B), 2mg (C), 20mg (D) or 100mg (E) and DTH responses were measured following immunisations. Results are expressed as mean footpad increment + 1 SEM ($n \geq 6$). Individual experiments were repeated between 2 and 10 times with similar results. [SPP: Saline fed, immunised with peanut in CFA, recall immunised with peanut; PPP: Peanut fed, immunised with peanut in CFA, recall immunised with peanut; SOO: Saline fed, immunised with OVA in CFA, recall immunised with OVA; OOO: Saline fed, immunised with OVA in CFA, recall immunised with OVA]

3.3.2 Effect of oral antigen doses on T cell proliferation

Antigen-specific T cell proliferation of T cells from PLN draining the tailbase immunisation site, MLN draining the gut and from the spleen were measured by incorporation of [^{3}H]-thymidine and by CFSE staining.

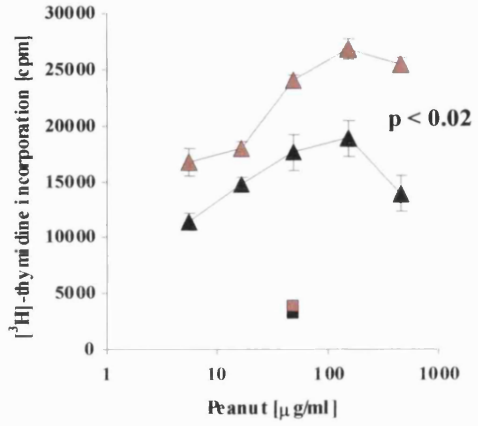
3.3.2.1 T cell proliferation measured by [^{3}H]-thymidine incorporation

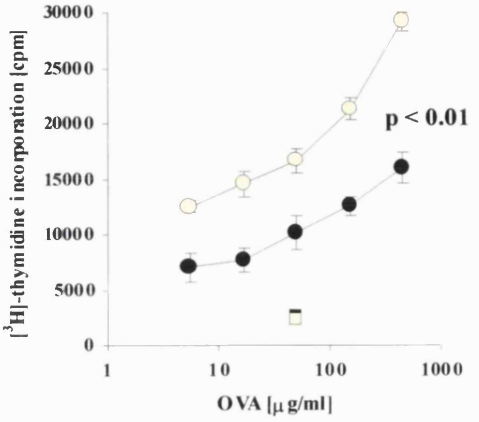
High levels of antigen-specific proliferation were seen in T cells from PLN after a saline feed and peanut protein or OVA immunisation with CFA and recall immunisation. A feed of 0.02mg peanut protein or OVA prior to this immunisation significantly enhanced the antigen-specific T cell proliferation compared to the saline fed controls (Fig. 3.3A). Proliferation was similarly significantly elevated after a feed of 0.2mg of peanut protein per animal, but proliferation of T cells from animals fed 0.2mg OVA was equivalent to saline fed controls (Fig. 3.3B). Proliferation by PLN T cells from animals fed 2mg peanut protein was no different from control animals, whereas proliferation of T cells from animals fed 2mg of OVA was significantly reduced compared to the saline fed animals (Fig. 3.3C). A very similar pattern was seen at the next dose of 20mg peanut protein or OVA fed per animal. PLN cells from peanut fed animals proliferated strongly and equally to the saline fed controls, while OVA fed animals displayed a highly significant reduction in antigen-specific T cell proliferation (Fig. 3.3D). Feeding 100mg peanut protein per animal resulted in a significant reduction of the proliferative response to peanut and a near total abrogation of [^{3}H]-thymidine incorporation compared to saline fed controls (Fig. 3.3E). Feeding 100mg OVA per animal also resulted in a profound reduction of the specific T cell proliferation (Fig. 3.3E), in the same way to that observed after feeding 2mg or 20mg of OVA.

Similar proliferative patterns as shown by T cells from PLN in Fig. 3.3 was observed with T cells from MLN and from the spleen, although the response by MLN T cells was routinely lower (results not shown). This suggests that the enhanced or reduced level of antigen-specific T cell proliferation observed following feeding of specific doses of antigen was a systemic phenomenon. No significant proliferation was observed to an irrelevant control antigen in any group, but all groups proliferated strongly and equally to the mitogen ConA (Fig. 3.4).

Peanut protein **OVA**

A: 0.02 mg





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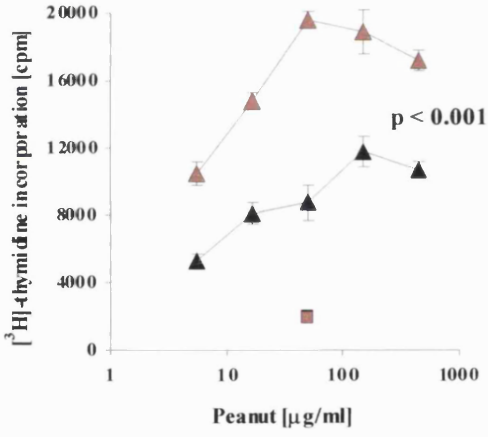

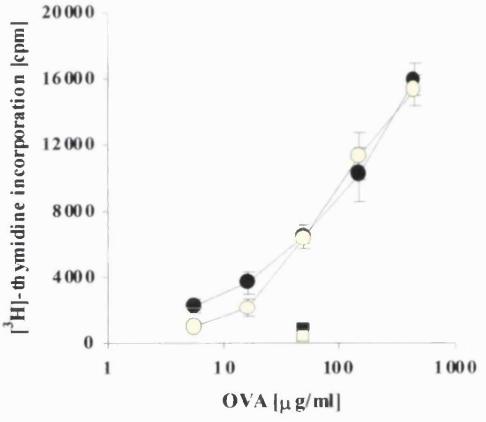

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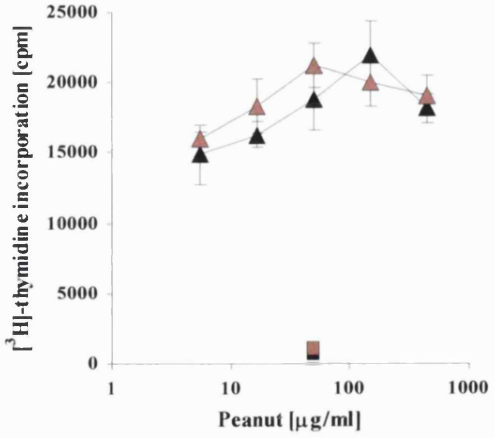

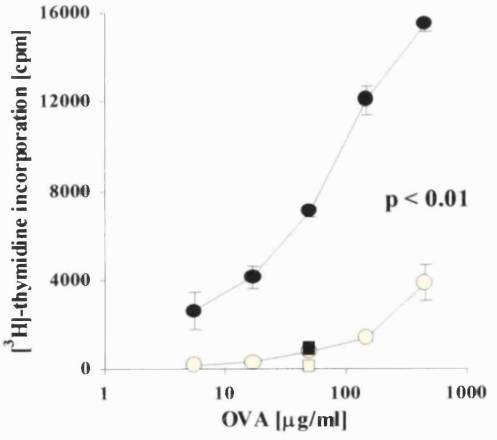

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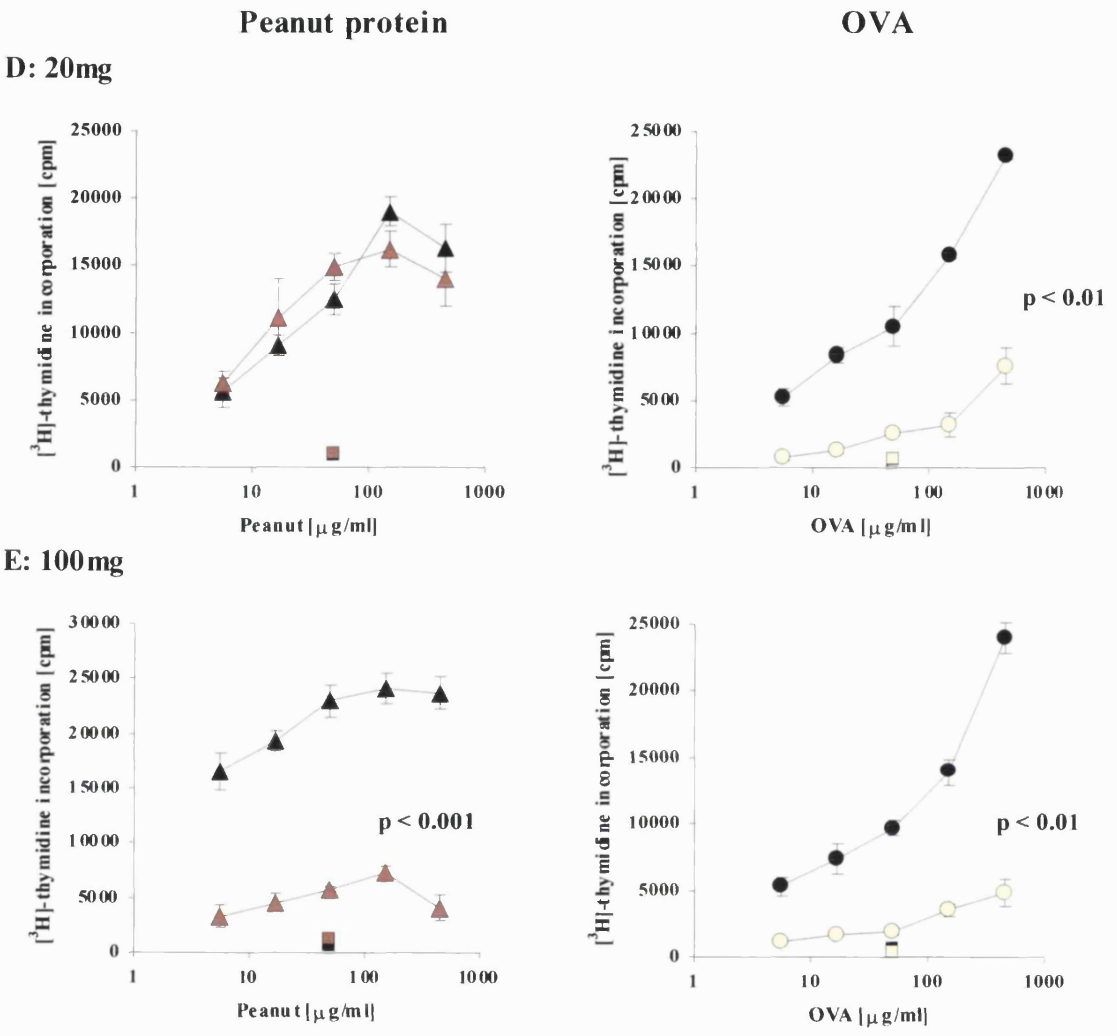

Fig. 3.3 Antigen-specific T cell proliferation

Peanut-specific (left column) and OVA-specific (right column) proliferation by PLN T cells were measured from animals fed peanut protein or OVA at doses of 0.02mg (A), 0.2mg (B), 2mg (C), 20mg (D) or 100mg (E) prior to tailbase immunisation with peanut protein or OVA in CFA and recall immunisation. Specific T cell proliferation was measured after 90 hr *in vitro* reactivation with peanut or OVA. Cells were pooled from ≥ 6 mice per group and results are expressed as the mean cpm of triplicate cultures ± 1 SEM. Background proliferation when no antigen was present has been subtracted. Squares represent proliferation to a control antigen. All groups proliferated with similar CPM to the mitogen ConA. Individual experiments were repeated between 2 and 10 times with near identical results. [▲: Saline fed, immunised with peanut in CFA, recall immunised with peanut; ▲: Peanut fed, immunised with peanut in CFA, recall immunised with peanut; ●: Saline fed, immunised with OVA in CFA, recall immunised with OVA; ○: OVA fed, immunised with OVA in CFA, recall immunised with OVA]

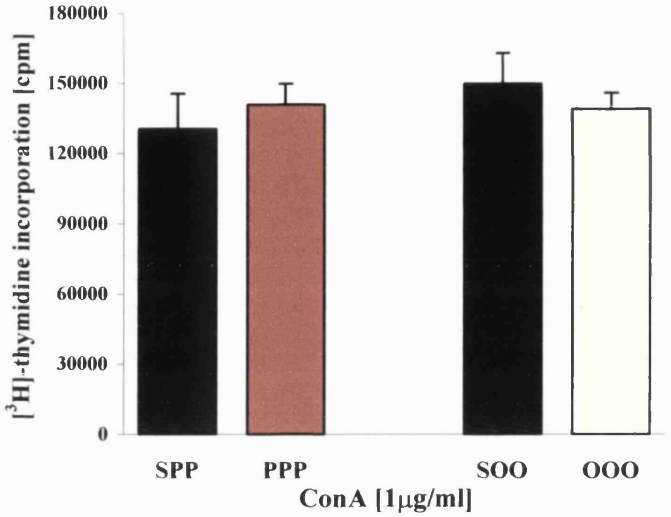

Fig. 3.4 T cell proliferation in response to ConA stimulation
Proliferation to ConA by PLN T cells from animals fed 100mg peanut protein, 100mg OVA or saline prior to peanut protein or OVA immunisations. Cells were pooled from≥ 6 mice per group and results are expressed as the mean cpm of triplicate culture + 1 SEM. ConA was used as a positive control in all experiments and consistently gave similar results, independent of prior antigen exposure. [SPP: Saline fed, immunised with peanut in CFA, recall immunised with peanut; PPP: Peanut fed, immunised with peanut in CFA, recall immunised with peanut; SOO: Saline fed, immunised with OVA in CFA, recall immunised with OVA; OOO: OVA fed, immunised with OVA in CFA, recall immunised with OVA]

3.2.2.2 T cell proliferation measured by CFSE staining

The T cell proliferation measured by CFSE staining of cultured PLN cells generally matched the results from the [^{3}H]-thymidine incorporation studies. An oral dose of 0.2mg peanut protein prior to the standard immunisation protocol induced a slightly higher percentage of $CFSE^{low}$ cells than the saline fed controls upon reactivation with peanut, while an oral dose of 100mg peanut protein reduced the level of $CFSE^{low}$ cells compared to the controls (Fig. 3.5B). All groups proliferated strongly and equally following ConA stimulation (Fig. 3.5C).

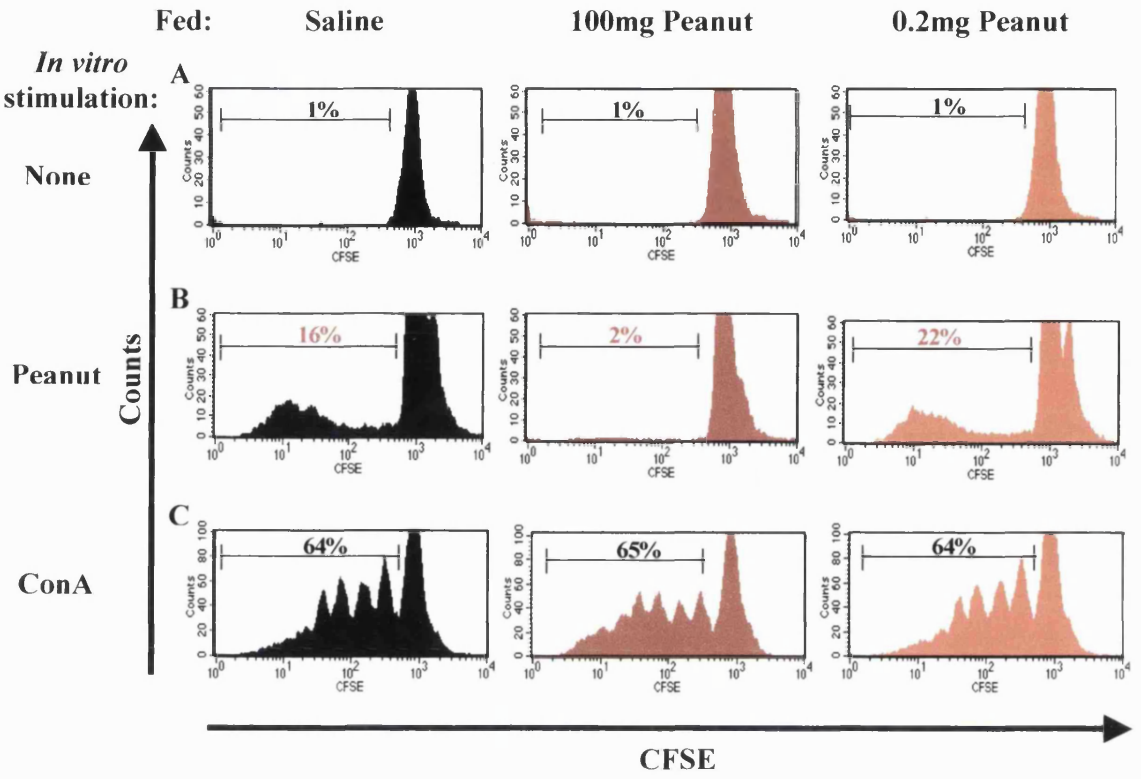

Fig. 3.5 Peanut-specific T cell proliferation following a 0.2mg or 100mg peanut feed
Peanut-specific proliferation by PLN T cells from animals fed saline, 100mg peanut protein or 0.2mg peanut protein followed by immunisation with peanut protein in CFA and recall immunisation with peanut. Proliferation was visualised by CFSE staining and analysed on the FL1 channel of a FACS flow cytometer. Cells were left un-stimulated (A), stimulated with 200μg peanut protein (B) or with 1μg ConA (C) for 90 hr *in vitro*. No proliferation was observed when the cells were stimulated with OVA. The experiment was repeated 3 times with similar results.

The CFSE staining showed that the majority of proliferating antigen-specific T cells went into cell cycle at the same time and divided approximately the same number of times when stimulated with peanut protein, creating a single $CFSE^{low}$ peak, which was distinct from the undividing $CFSE^{high}$ cells. This was a very different pattern from that observed when cells were stimulated with the mitogen ConA, where several peaks of varying CFSE intensity were seen, each indicating cells having undergone a defined number of cell divisions (Fig. 3.5). When culturing the cells for 90 hr with ConA, 6-7 peaks were observed indicating that the cells had undergone between 1 - 6 cell divisions. In contrast, when stimulating the cells with peanut for 90 hr, nearly all the proliferating cells from animals fed 0.2mg peanut protein or saline underwent 6 cell divisions.

3.3.3 Effect of oral antigen doses on cytokines secretion

To measure cytokine production by T cells from PLN, MLN and spleen, $2x10^6$ cells were reactivated with either peanut protein, OVA, control antigen (OVA or peanut protein respectively) or ConA *in vitro* for 24 hr – 168 hr. The cell supernatants were then collected and tested for amount of secreted IFN-γ, TGF-β, IL-4 and IL-10. A strong IFN-γ response was interpreted as being indicative of a Th1-type immune response, IL-4 and IL-10 of a Th2-type response and TGF-β of an involvement of Th3/T_{reg}-type immunity.

PLN T cells from animals fed 0.2mg peanut protein or sham-fed saline prior to immunisation with peanut protein in CFA and recall immunisation with peanut protein produced and secreted large amounts of IFN-γ, IL-4 and IL-10 upon reactivation with peanut protein *in vitro*. PLN T cells from the animals fed 0.2mg peanut protein consistently secreted slightly higher amounts of both IFN-γ, IL-4 and IL-10 than the saline fed controls, but this did not reach statistical significance (Fig. 3.6 and 3.7A). In contrast, when animals were initially fed 100mg of peanut protein both IFN-γ, IL-4 and IL-10 production by PLN T cells were significantly downregulated compared to the saline fed controls and to the animals fed 0.2mg peanut protein (Fig. 3.6 and 3.7A). Analysing TGF-β production by PLN T cells in relation to the initial oral dose of peanut protein showed a reverse pattern from that of the other cytokines. PLN T cells from animals fed saline or 0.2mg peanut protein secreted approximately 800-900pg TGF-β/ml, while a feed of 100mg peanut protein resulted in TGF-β levels of around 1500pg/ml after 96 hr stimulation with peanut protein *in vitro* (Fig. 3.7B). The increase in TGF-β secretion following a 100mg peanut feed protein was however not statistically significant ($p = 0.071$), but this tendency of increased TGF-β production was observed in every experiment (n=4).

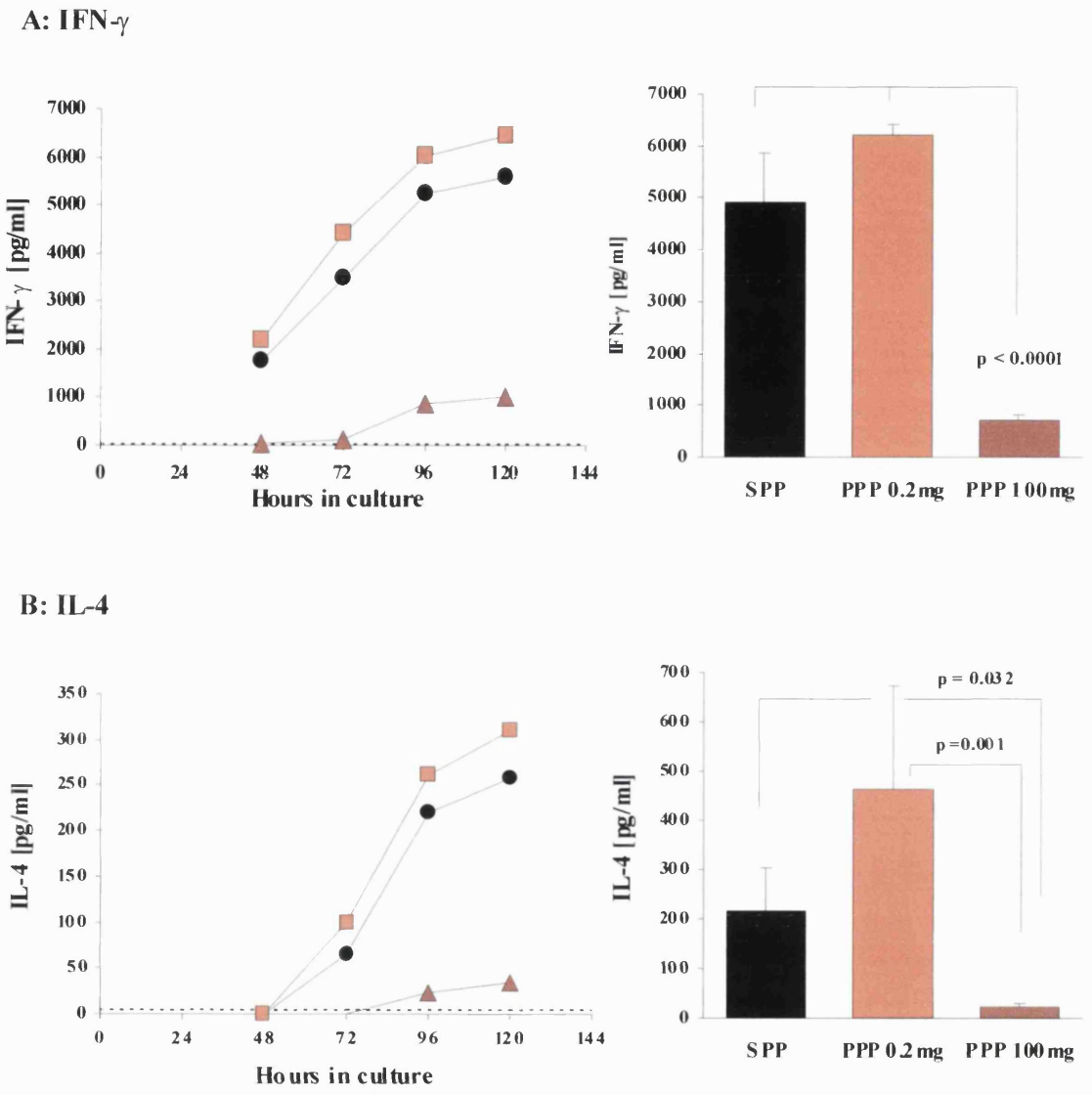

Fig. 3.6 Peanut-specific IFN-γ and IL-4 secretion

IFN-γ (A) and IL-4 (B) production by PLN T cells from animals fed saline, 0.2mg peanut protein or 100mg peanut protein followed by immunisation with peanut protein in CFA and recall immunisation with peanut. IFN-γ and IL-4 production was analysed following reactivation with peanut protein *in vitro* between 24 and 120 hr (left column) or routinely for 96 hr (right column). Cells were pooled≥ 6 mice per group and analysed in triplicate. Results are expressed as the mean cytokine secretion + 1 SEM of 2 (PPP 0.2mg) to 7 (SPP and PPP 100mg) individual experiments. The vertical dashed line indicates the limit of detection for the cytokine assays. No IFN-γ or IL-4 were released in response to a control antigen or when cells were not reactivated. [SPP (●): Saline fed immunised with peanut in CFA, recall immunised with peanut; PPP 0.2mg (■): Fed 0.2mg peanut, immunised with peanut in CFA, recall immunised with peanut; PPP 100mg (▲): Fed 100mg peanut, immunised with peanut in CFA, recall immunised with peanut]

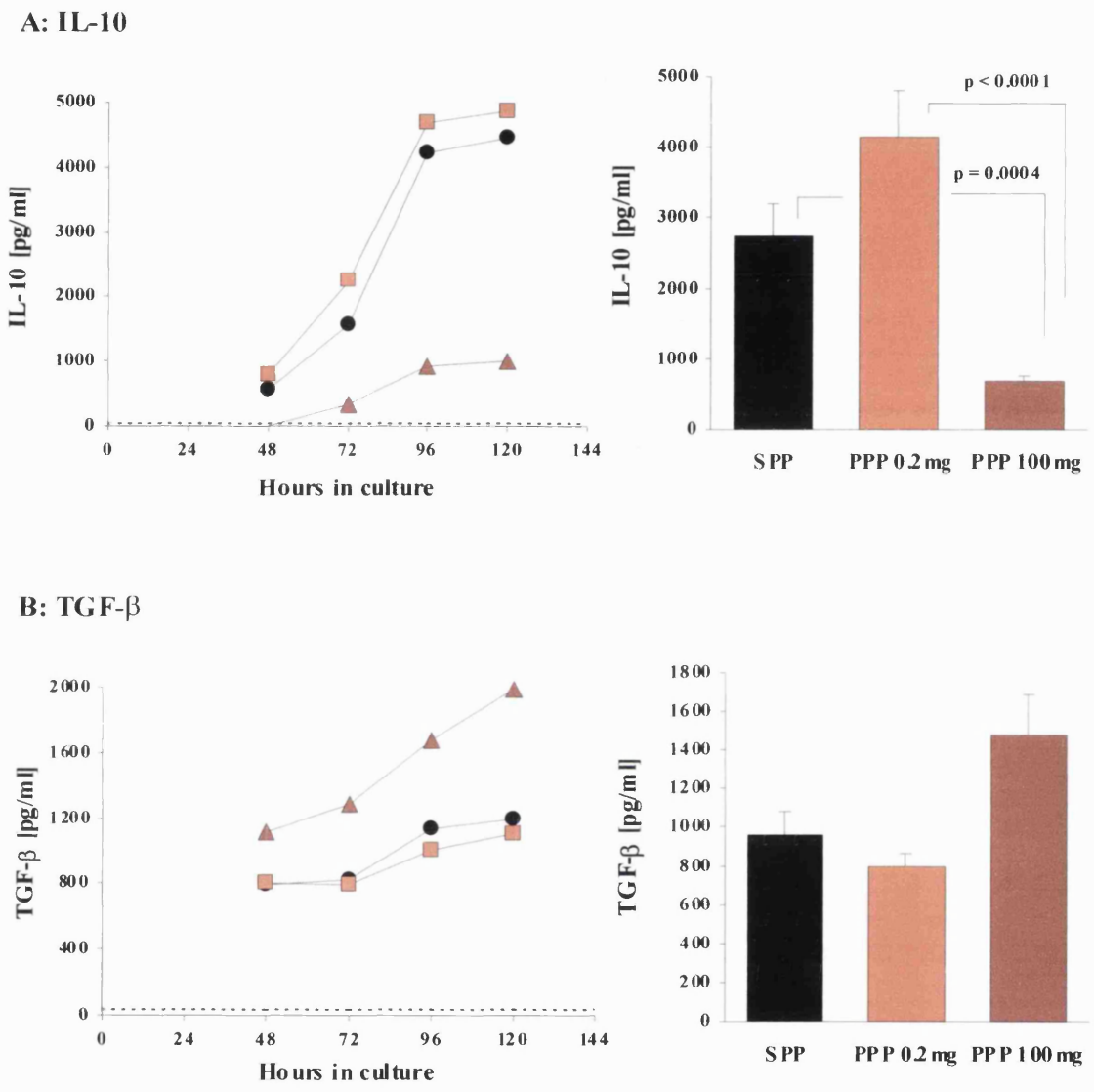

Fig. 3.7 Peanut-specific IL-10 and TGF-β secretion

IL-10 (A) and TGF-β (B) production by PLN T cells from animals fed saline, 0.2mg peanut protein or 100mg peanut protein followed by immunisation with peanut protein in CFA and recall immunisation with peanut. IL-10 and TGF-β production was analysed following reactivation with peanut protein *in vitro* between 24 and 120 hr (left column) or routinely for 96 hr (right column). Cells were pooled≥ 6 mice per group and analysed in triplicate. Results are expressed as the mean cytokine secretion + 1 SEM of 2 (PPP 0.2mg) to 7 (4 for TGF-β) (SPP and PPP 100mg) individual experiments. The vertical dashed line indicates the limit of detection for the cytokine assays. No IL-10 or TGF-β were released in response to a control antigen or when cells were not reactivated. [SPP (●): Saline fed, immunised with peanut in CFA, recall immunised with peanut; PPP 0.2mg (■): Fed 0.2mg peanut, immunised with peanut in CFA, recall immunised with peanut; PPP 100mg (▲): Fed 100mg peanut, immunised with peanut in CFA, recall immunised with peanut]

The kinetics of the cytokine production and release were similar for the 0.2mg peanut protein fed groups, the saline fed groups and the 100mg peanut protein fed groups; only the amount of secreted cytokine seemed to differ between the groups (Fig. 3.6 and 3.7 left column). None of the tested cytokines could be detected in the supernatant at 24 hr of culture with peanut protein. IFN-γ, TGF-β and IL-10 were detectable after 48 hr of culture, while IL-4 was only detectable after 72 hr of culture. All cytokines increased in the supernatant over time and plateaued between 96 – 120 hr. The cytokine levels were routinely measured after 96 hr reactivation of the cells. Very limited amounts of any cytokines were measured when the cells were not stimulated or stimulated with a control antigen. Cells from all animal groups produced large and similar amounts of IFN-γ when stimulated with ConA, which was routinely used as a positive control (results not shown).

Similar results to those shown in Fig. 3.6 and 3.7 were obtained from splenocytes. The patterns of cytokine secretion between the 0.2mg peanut fed groups, the saline fed groups and the 100mg peanut fed groups were identical, but the overall amounts of cytokines were slightly lower. MLN cells produced very low levels of all the measured cytokines (results not shown).

3.3.4 Effect of oral antigen doses on antibody production

Antibody responses to peanut protein and OVA were measured in serum from mice fed various doses of antigen followed by systemic immunisation with antigen in CFA and recall immunisation in the footpad. The level of antigen-specific IgG and the IgG subclasses, IgG1 and IgG2a, as well as both total and antigen-specific IgE were analysed in serum of all experimental animals. Presence of the IL-4-inducible immunoglobulins IgG1 and IgE was interpreted as indicative of a Th2-type immune response and the IFN-γ-inducible IgG2a subclass as induction of Th1-type immunity.

Experimental animals fed saline prior to immunisation with peanut protein or OVA in CFA and recall immunisation with peanut protein or OVA respectively gave a strong antigen-specific antibody response of IgG, IgG1, IgG2a and high levels of total IgE. A feed of 0.02mg peanut protein per animal prior to an identical immunisation protocol enhanced the antibody response with a significant increase of peanut-specific IgG, IgG1, IgG2a and of total IgE (Fig. 3.8A). An oral dose of 0.02mg OVA significantly enhanced the OVA-specific IgG, IgG1 and total IgE level, but no difference in the level of OVA-specific IgG2a was seen compared to

the saline-fed controls (Fig. 3.9A). Feeding a larger dose of 0.2 mg peanut protein prior to the immunisation regime resulted in similar antibody levels as observed after the 0.02mg dose. The levels of peanut-specific IgG immunoglobulins were significantly enhanced compared to the saline-fed controls and the level of total IgE in the serum was also significantly elevated (Fig. 3.8B). A feed of 0.2mg OVA made no difference to the level of OVA-specific IgG, IgG1, IgG2a or total IgE (Fig. 3.9B).

When 2mg or 20mg of peanut protein were given orally prior to immunisations with peanut protein there was no difference in levels of either peanut-specific IgG, IgG1, IgG2a or total IgE between the peanut-fed group and the saline-fed control group (Fig. 3.8C and D). Specific antibody levels after feeding of 2mg of OVA were also indistinguishable from controls. In contrast, feeding 20mg of OVA per animal significantly inhibited both OVA-specific IgG, IgG1, IgG2a and total IgE production (Fig. 3.9C and D). Feeding the highest oral dose of 100mg peanut protein per animal resulted in a highly statistical significant reduction in the level of both peanut-specific IgG, IgG1 and IgG2a as well as a reduction of total IgE levels compared to the saline-fed control animals (Fig. 3.8E). Similarly, a feed of 100mg OVA per animal resulted in an inhibited OVA-specific IgG antibody response as well as a reduction in total IgE (Fig. 3.9E).

Naïve mouse serum from BALB/c mice of the same colony tested negative for peanut- or OVA-specific IgG, IgG1 and IgG2a immunoglobulins as well as peanut- or OVA-specific IgE in all assays (data not shown). The levels of total IgE in serum of naïve mice were below the 4ng/ml detection limit of the total IgE assay. For a discussion of total versus antigen-specific IgE see page 117.

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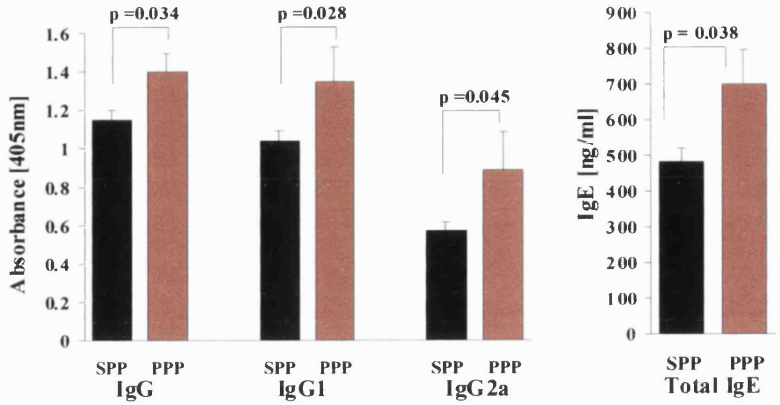

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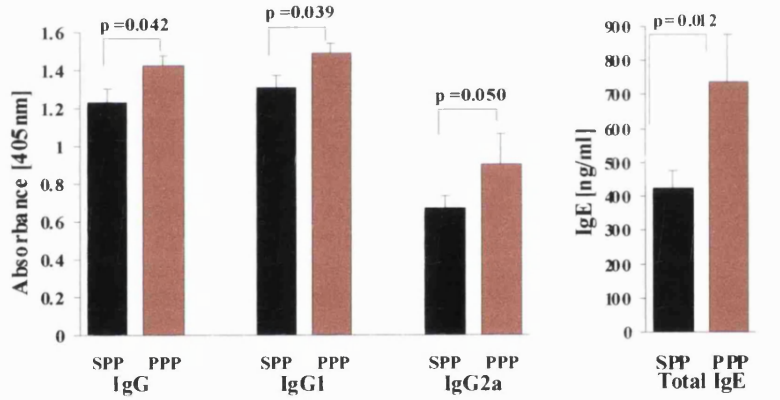

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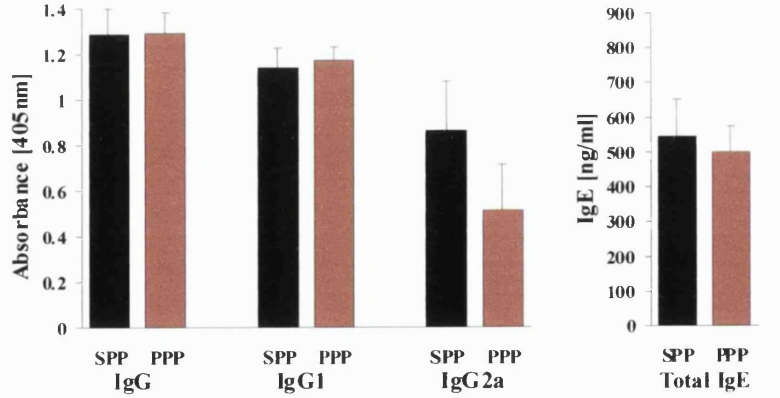

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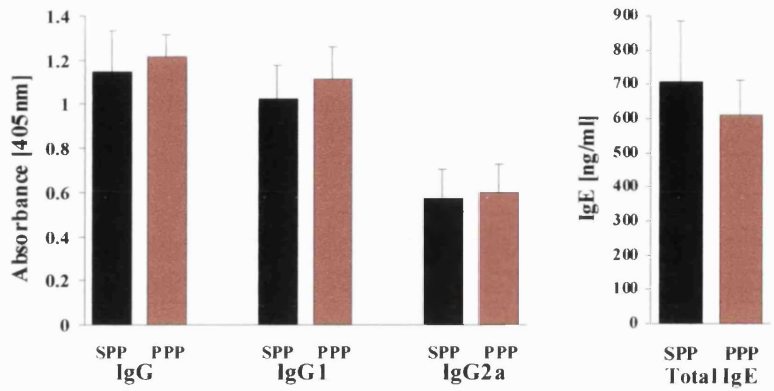

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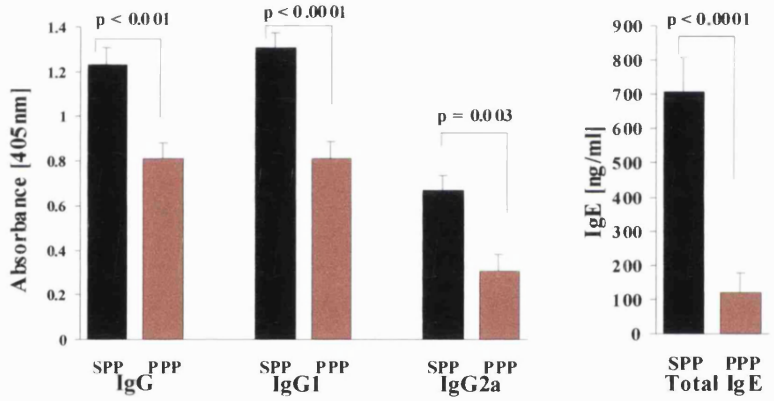

Fig. 3.8 Antibody response to peanut

Peanut-specific IgG, IgG1 and IgG2a as well as total IgE were measured in serum from animals fed peanut protein at doses of 0.02mg (A), 0.2mg (B), 2mg (C), 20mg (D) and 100mg (E) prior to immunisations with peanut protein. Sera were diluted 1000 times for peanut-specific IgG and IgG1, 200 times for IgG2a and 10 times for total IgE measurements. Results are expressed as mean antibody levels + 1 SEM of $\geq$ 6 animals per group. The individual experiments were repeated between 2 and 10 times with identical antibody patterns between the saline- and peanut-fed groups. [SPP: saline fed, immunised with peanut in CFA, recall immunised with peanut; PPP: peanut fed, immunised with peanut in CFA, recall immunised with peanut]

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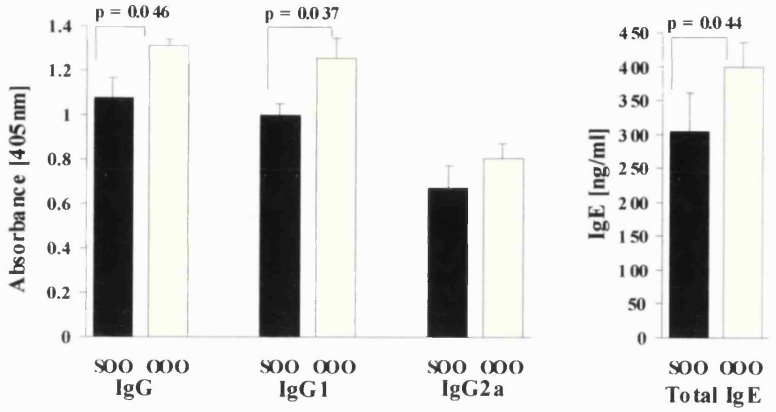

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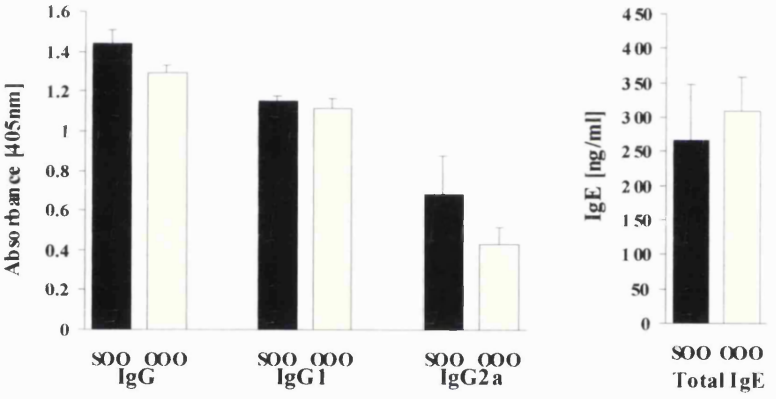

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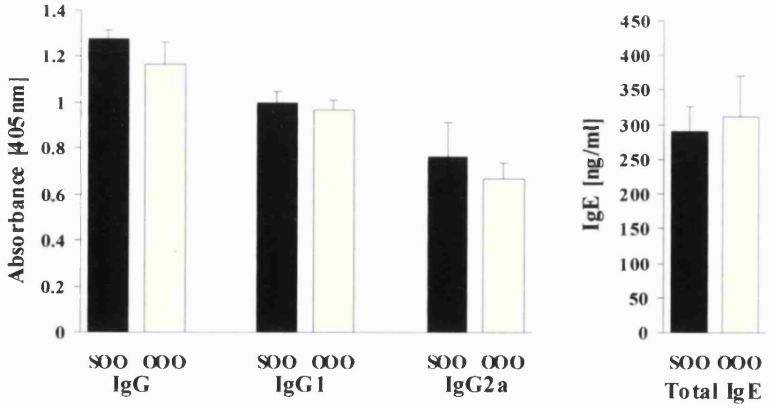

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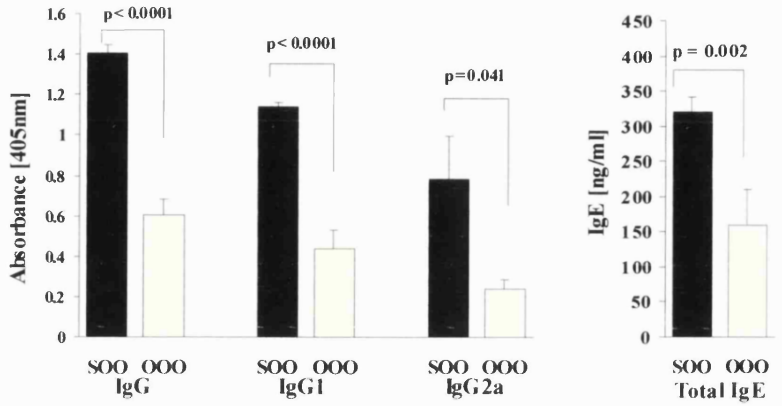

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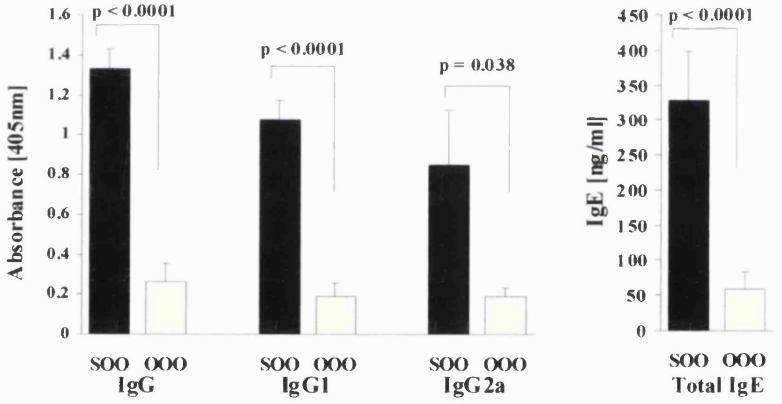


Fig. 3.9 Antibody response to OVA

OVA-specific IgG, IgG1 and IgG2a as well as total IgE were measured in serum from animals fed OVA at doses of 0.02mg (A), 0.2mg (B), 2mg (C), 20mg (D) and 100mg (E) prior to immunisations with OVA. Sera were diluted 1000 times for OVA-specific IgG and IgG1, 200 times for IgG2a and 10 times for total IgE measurements. Results are expressed as the mean antibody levels + 1 SEM of $\geq$ 6 animals per group. The individual experiments were repeated between 1 and 3 times with similar results. [SOO: saline fed, immunised with OVA in CFA, recall immunised with OVA; OOO: OVA fed, immunised with OVA in CFA, recall immunised with OVA]

3.3.4.1 Total serum IgE versus antigen-specific IgE

In contrast to antigen-specific IgG, IgG1 and IgG2a, antigen-specific IgE could not be detected by a simple direct ELISA technique, possibly because of competition from the much larger amount of antigen-specific IgG antibodies present in the sera. Consequently, only total levels of serum IgE measured by sandwich ELISA are given in the above section. However, during the course of these experiments an antigen-specific IgE ELISA technique was developed after which both total and specific IgE were routinely measured in all experiments. Fig. 3.10A demonstrates the results of both total IgE and peanut-specific IgE measurements in the sera of individual animals after a saline or 100mg peanut protein feed followed by immunisation with peanut protein in CFA and recall immunisation with peanut protein. The patterns of specific IgE in the serum from individual animals were identical to that of total IgE. The statistically significant difference between the saline-fed and the peanut-fed group were $p = 0.0026$ for specific IgE and $p = 0.0020$ for total IgE levels (Fig. 3.10A). Similarly, almost identical patterns of total IgE and OVA-specific IgE were routinely found in the OVA experiments. An example is shown in Fig. 3.10B, where p-values between the saline-fed and the OVA-fed group were < 0.0001 for both total and OVA specific IgE.

These data, together with the non-detectable total IgE in naïve mouse sera, may suggest that most of the detectable IgE in the serum of experimental animals was antigen-specific.

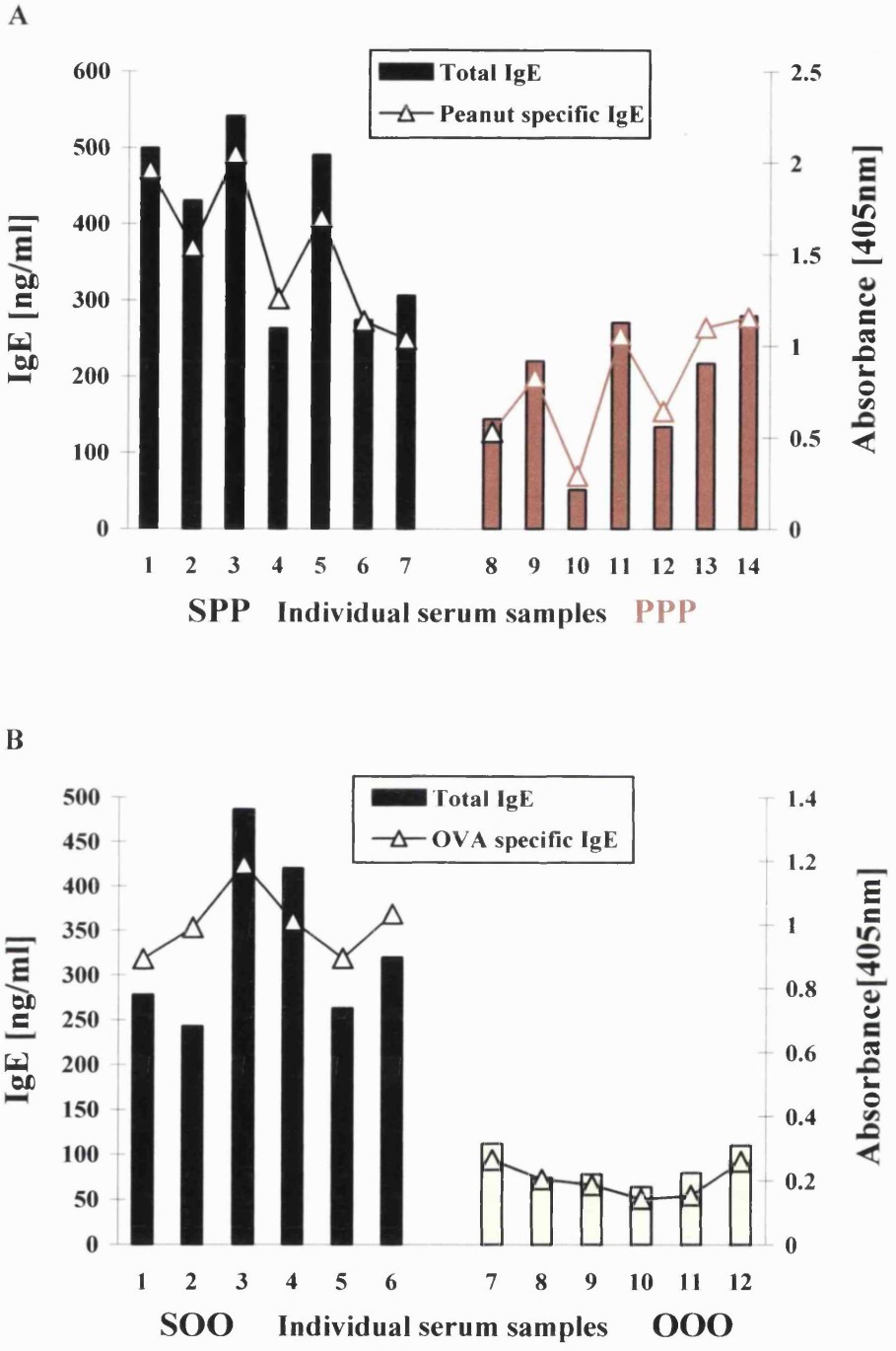


Fig. 3.10 Comparison between total and antigen-specific IgE in serum
Representative example of total IgE and peanut- (A) or OVA- (B) specific IgE in serum from the same individual animals fed 100mg peanut protein or saline (A) or 100mg OVA or saline (B) prior to peanut protein or OVA immunisations respectively. Total IgE is expressed as ng/ml and relative levels of antigen-specific IgE are given on the second y-axis as absorbance at 405nm. [SPP: Saline fed, immunised with peanut in CFA, recall immunised with peanut; PPP: Peanut fed, immunised with peanut in CFA, recall immunised with peanut; SOO: Saline fed, immunised with OVA in CFA, recall immunised with OVA; OOO: OVA fed, immunised with OVA in CFA, recall immunised with OVA]

3.3.4.2 Immunoblotting

To further analyse the differences in serology between the peanut-sensitive and the peanut-tolerant animals, gel electrophoresis and immunoblotting were performed. Proteins in the crude peanut extract were separated by SDS electrophoresis and transferred to nitrocellulose membranes, which were then incubated with serum from animals fed saline or 100mg peanut protein prior to immunisation with peanut in CFA and recall immunisation with peanut protein. The major peanut allergens in the peanut extract are outlined in Fig. 3.11 according to molecular weight.

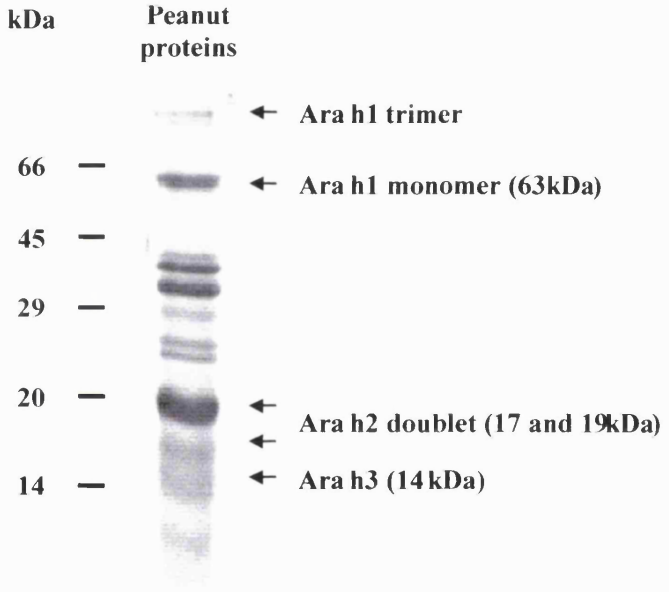


Fig. 3.11 SDS-PAGE gel of crude peanut extract
Coomassie brilliant blue staining of proteins in crude peanut protein extract. The molecular weight markers are shown as the kDa weight bands and the major allergenic proteins in peanut are identified by size on the right hand side. Similar protein separating gels were used for immunobloting

Immunoblotting showed a similar protein pattern as Coomassie brilliant blue gel staining demonstrating that the peanut extract contains multiple IgG-binding sites. Some protein bands however did not appear on the immunoblots, indicating the absence of an antibody response to these proteins (Fig. 3.12). Serum from peanut-sensitive animals fed saline prior to systemic peanut protein immunisations bound strongly to the peanut extract with especially strong bands corresponding to the Ara h1 and Ara h2 proteins (Fig. 3.12, lanes 2-5). Much lower levels of IgG-binding to peanut proteins were obtained with sera from mice fed 100mg peanut protein prior to similar peanut immunisations (Fig. 3.12, lane 6-8). There was however

some IgG-binding to a restricted number of proteins compared to IgG from peanut-sensitive animals (Fig. 3.12). This suggests that inducing oral tolerance to peanut by feeding 100mg peanut protein not only reduced the overall IgG-binding to peanut protein but additionally may have shifted the IgG-binding pattern or epitope recognition of peanut protein. Tolerance may have been induced to some proteins but not to others. As only a small study was carried out within the present Thesis, this interesting possibility requires further investigation.

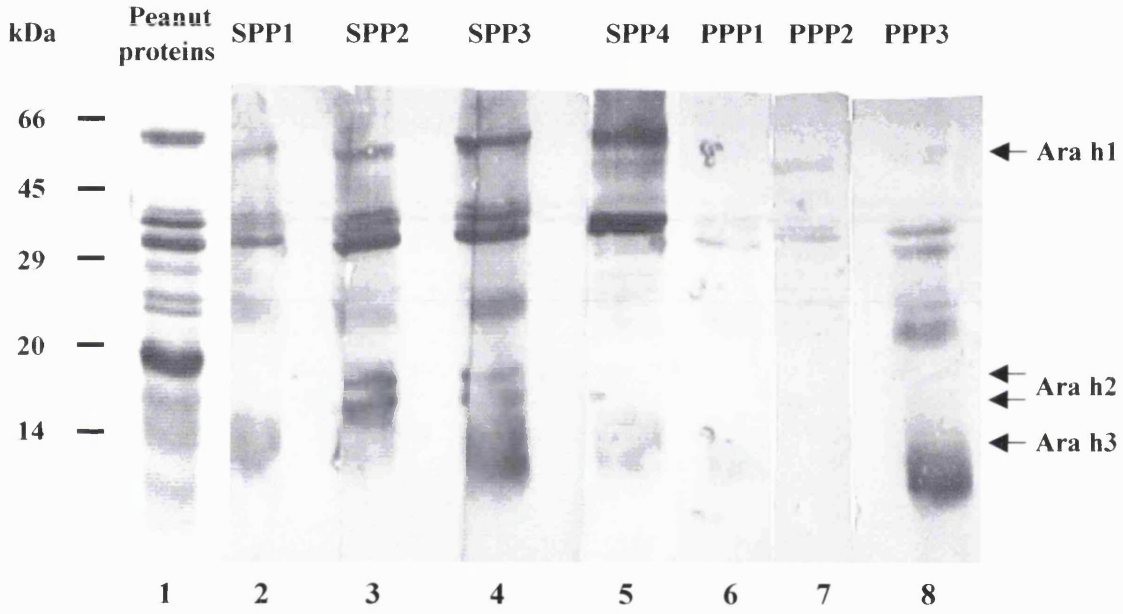


Fig. 3.12 Immunoblot detection of peanut-specific IgG-binding in sera from peanut-sensitive and peanut-tolerant animals

Immunoblots showing IgG-binding to multiple proteins in crude peanut extract. Lane 1: Coomassie brilliant blue staining of SDS gel separation of crude peanut extract. Lane 2-5: Immunoblots of peanut-specific IgG in serum of four individual peanut-sensitive animals. Lane 6-8: Immunoblots of peanut-specific IgG in three individual peanut-tolerant animals. All sera were diluted 1:10000 prior to analysis. Serum from naïve animals showed no IgG binding to any peanut protein. No bands were visible on control immunoblots which were not incubated with serum, but otherwise treated the same. [SPP: Saline fed, immunised with peanut in CFA, recall immunised with peanut; PPP: Fed 100mg peanut, immunised with peanut in CFA, recall immunised with peanut]

Ara h1 and Ara h2 are the major allergenic proteins in peanut, which are recognised by more than 95% of human peanut allergic patients [Burks et al., 1991; Burks et al., 1992]. After the detection of anti-Ara h1 and anti-Ara h2 specific IgG antibodies in sera from the peanut-

sensitive mice, a direct comparison of IgG antibody binding in mice and human subjects was carried out. Fig. 3.13 shows immunoblots of four peanut-sensitive mice (lanes 2-5) and three severely peanut allergic children, aged 3-6 years (lanes 6-8). Human allergic subjects were found to display a much more diverse binding pattern from subject to subject than the peanut-sensitive mice. The peanut allergic children examined in this study displayed only weak IgG-binding to Ara h2, which in the adult population has been reported to be one of the major allergenic proteins in peanut. It was repeatedly demonstrated however that IgG from human peanut allergic children and IgG from peanut-sensitive mice similarly recognised Ara h1 and other major proteins in peanut (Fig. 3.13 – and results not shown). Peanut IgG responses in the murine model of peanut sensitivity thus appeared to mimic that of human peanut allergic subjects, although further studies are required to confirm this.

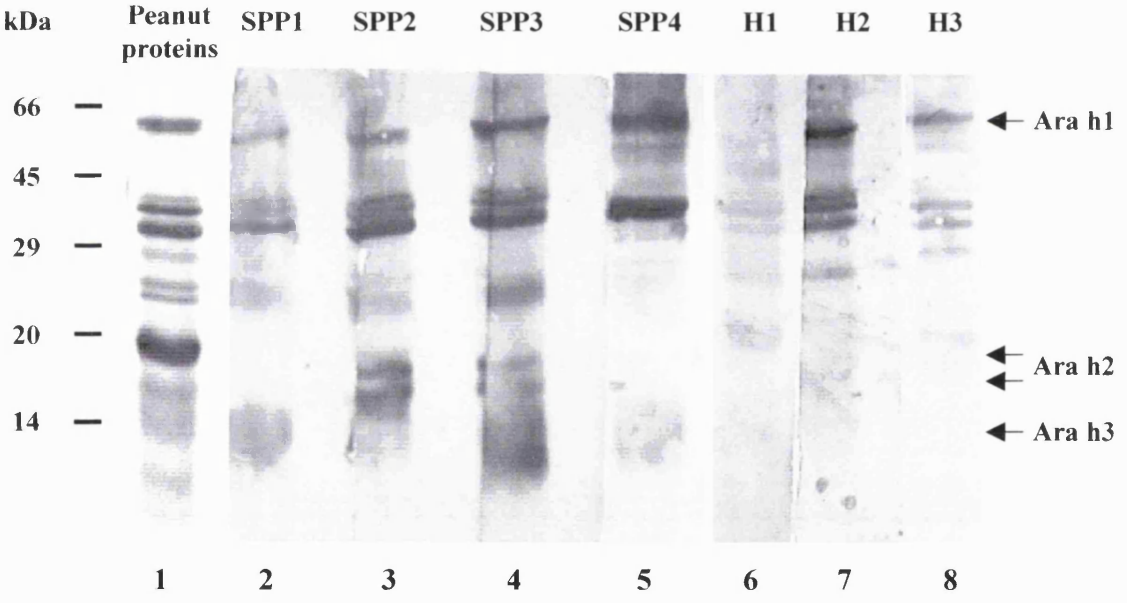


Fig. 3.13 Comparison of peanut-specific IgG-binding in sera from peanut-sensitive mice and peanut allergic children

Immunoblots showing IgG-binding to multiple proteins in crude peanut extract of immunised mice and allergic children. Lane 1: Coomassie brilliant blue staining of SDS gel separation of crude peanut extract. Lane 2-5: Immunoblots of peanut-specific IgG in serum of four individual peanut-sensitive mice. Lane 6-8: Immunoblots of peanut-specific IgG in three severely peanut allergic children. Mice sera were diluted 1:10000, and human sera were diluted 1:1000 prior to analysis. Serum separated from pooled cord blood showed no IgG binding to any peanut protein; nor did serum from naïve animals. No bands were visible on control immunoblots which were not incubated with serum, but otherwise treated the same. [SPP: Mice fed saline, immunised with peanut in CFA, recall immunised with peanut; H: Human peanut allergic subject]

3.3.5 Dose-response comparison of peanut and OVA

To summarise the oral dose-response studies for peanut protein and OVA with respect to both the cellular and the humoral arm of the immune system, graphs indicating the percentage antigen-specific T cell proliferation and the percentage antigen-specific IgG of the saline-fed controls were created as a function of the fed dose. The responses of matched saline-fed control animals were taken as 100%.

Results showing percentage T cell proliferation, measured as amount of [^{3}H]-thymidine incorporation, of the saline-fed controls demonstrated different dose-response relationships for peanut protein and OVA, with higher oral doses needed to reduce the peanut-specific T cell proliferation than the OVA-specific T cell proliferation (Fig. 3.14). Small oral doses of peanut protein were also shown to enhance the specific proliferative response more readily than small doses of OVA (Fig. 3.14). Graphs showing the percentage DTH response of the saline-fed controls as a function of the fed oral dose demonstrated identical dose-response patterns for peanut protein and for OVA as shown for the percentage antigen-specific T cell proliferation in Fig. 3.14 (results not shown).

The percentage antigen-specific IgG of the saline-fed controls plotted as a function of the fed oral dose showed a similar dose-response pattern between peanut protein and OVA as the proliferative data above. Higher oral doses of peanut protein were needed to reduce the peanut-specific IgG levels than oral doses of OVA to reduce the OVA-specific IgG levels. Moreover, oral peanut protein enhanced specific IgG levels at a higher dose than OVA (Fig. 3.15). Summarising antigen-specific IgG1, IgG2a or IgE in a similar manner as shown for specific IgG produced identical results (not shown).

By comparing the oral dose-response relationship for the cellular and the humoral responses in Fig. 3.14 and Fig. 3.15 it could be noted that at an intermediate dose of 2mg OVA fed per animal, the proliferative cellular response was inhibited but the level of OVA-specific IgG was not. These data suggest that a higher oral dose is needed to inhibit antibody synthesis than to inhibit specific T cell proliferation.

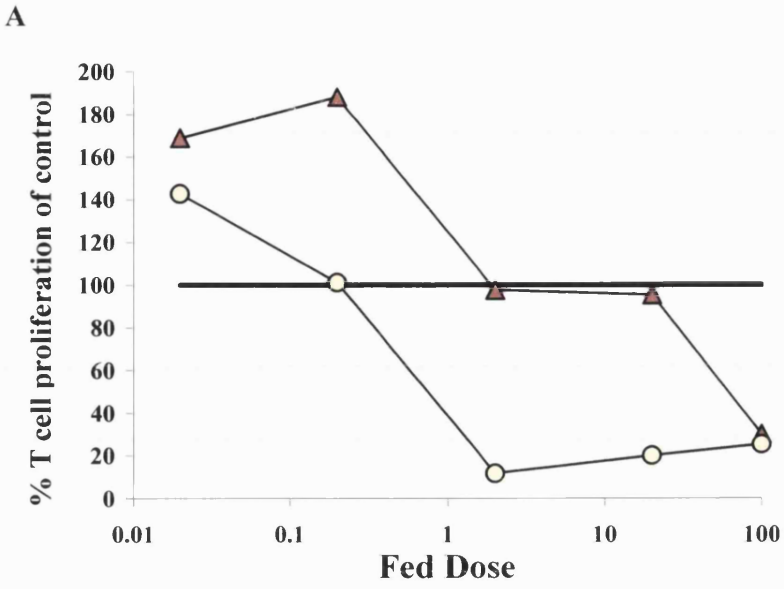


Fig. 3.14 Specific T cell proliferation as a function of the oral dose
Peanut- (▲) or OVA- ( ) specific proliferation by PLN T cell following feeding of varying doses of peanut protein or OVA prior to systemic immunisation with peanut protein or OVA in CFA and recall immunisation with peanut protein or OVA. Saline-fed and otherwise identically treated animals are indicated at a 100% proliferation (—). The peanut- and OVA-specific proliferative responses are indicated as % of saline-fed controls

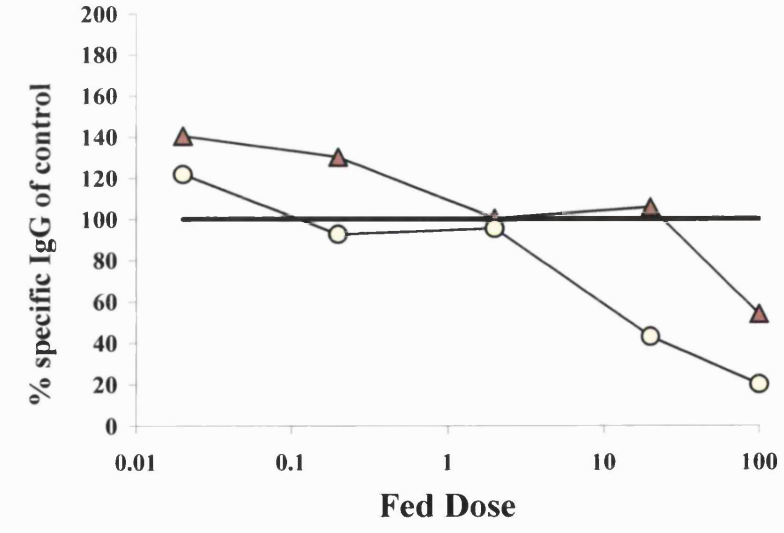


Fig. 3.15 Specific IgG as a function of the oral dose
Peanut- (▲) or OVA- ( ) specific IgG levels in serum following feeding of varying doses of peanut protein or OVA prior to systemic immunisation with peanut protein or OVA in CFA and recall immunisation with peanut protein or OVA. Saline-fed and otherwise identically treated animals are indicated at a 100% antigen-specific IgG level (—). The peanut- and OVA-specific IgG responses are calculated as % of saline-fed controls

3.3.6 Antigen specificity at induction of oral tolerance

In the present model of oral tolerance to peanut protein it was observed that in the effector phase, no T cell proliferation or cytokine production occurred when cells were reactivated with a control antigen (OVA). This indicated antigen specificity at the effector phase, but whether the induction of oral tolerance to peanut protein could be induced by feeding high doses of *any* protein remained unanswered. To address this question animals were fed either saline, 100mg peanut protein or 100mg OVA by intra-gastric gavage prior to identical immunisations with peanut in CFA and recall immunisation with peanut as shown in Fig. 3.1.

Animals fed either saline or OVA prior to peanut protein immunisation mounted a strong DTH response to peanut protein as demonstrated by mean footpad increments of 24.4 and 22.2 [mmx10^{-1}] respectively. In contrast, animals fed peanut protein did not mount a DTH response and mean footpad swellings were significantly reduced compared to both the saline-fed and the OVA-fed group (Fig. 3.16A). A similar pattern was demonstrated when peanut-specific T cell proliferation was measured in PLN and spleen cells. PLN T cells from saline-fed and OVA-fed animals proliferated vigorously to reactivation with peanut protein *in vitro* while the response by T cells from peanut-fed animals was significantly lower than both the saline-fed and the OVA-fed animals ($p < 0.001$) (Fig. 3.16B). Similar results were obtained with splenocytes (results not shown).

Cytokine production by PLN and spleen cells was additionally investigated. PLN and spleen cells were cultured *in vitro* for 96 hr with peanut protein or OVA at 200μg/ml, with ConA at 1μg/ml or without stimulation. PLN cells from animals fed saline or OVA and immunised with peanut protein produced large and equal amounts of IFN-γ, IL-4 and IL-10 but little TGF-β on *in vitro* stimulation with peanut. Conversely, PLN cells from animals fed 100mg peanut protein prior to peanut immunisations produced much lower levels of both IFN-γ, IL-4 and IL-10 compared to the saline- or OVA-fed groups but they produced higher levels of TGF-β (Fig. 3.17). Similar cytokine responses although with lower overall levels of all cytokines were observed with splenocytes (results not shown). PLN and spleen cells from all groups produced large and approximately equal amounts of IFN-γ in response to ConA and no cytokines were obtained when PLN or spleen cells were not stimulated or stimulated with OVA (results not shown).

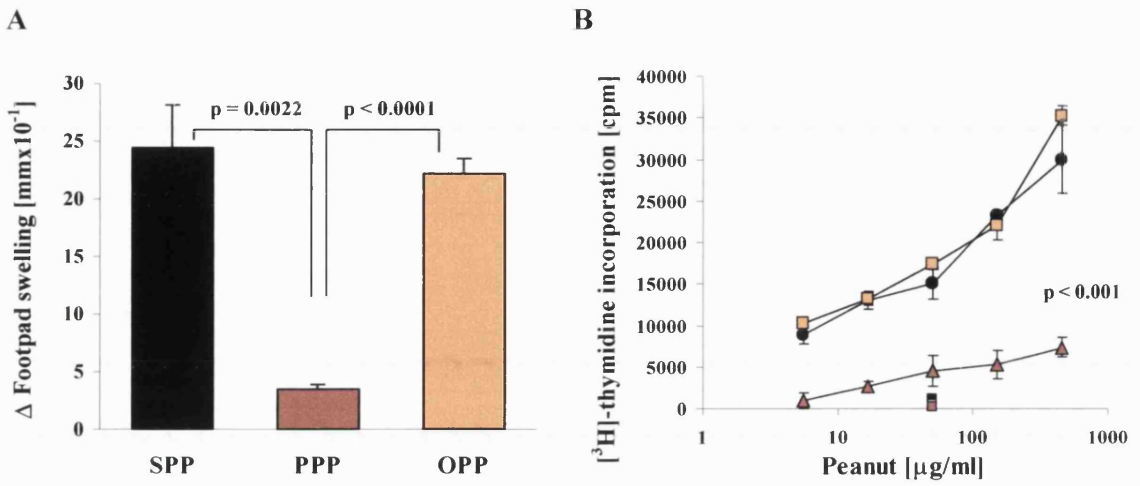


Fig. 3.16 Effect of feeding peanut protein or OVA on subsequent peanut-specific DTH and T cell proliferation

Animals (n=6/group) were fed saline, 100mg peanut protein or 100mg OVA prior to immunisation with peanut protein in CFA and recall immunisation with peanut. (A) DTH responses 24 hr after recall immunisation in the footpad. Results are expressed as the mean footpad increments + 1 SEM. (B) Peanut-specific T cell proliferation in PLN cell cultures after 90 hr *in vitro* reactivation with peanut protein. Cells were pooled from 6 mice per group and results are expressed as the mean cpm of triplicate culture ± 1 SEM. Background proliferation without antigen has been subtracted. Squares show proliferation to a control antigen. All groups proliferated with similar [cpm] to ConA stimulation. [SPP (●): Saline fed, peanut immunised and peanut recall immunised; PPP (▲): Peanut fed, peanut immunised and peanut recall immunised; OPP (■): OVA fed, peanut immunised and peanut recall immunised]

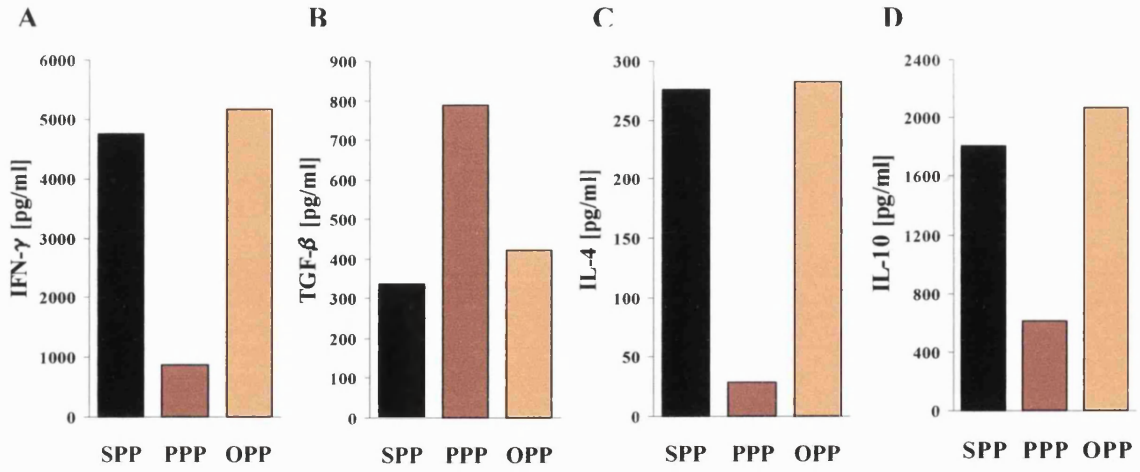


Fig. 3.17 Effect of feeding peanut protein or OVA on subsequent cytokine production in response to *in vitro* peanut priming

IFN-γ (A), TGF-β (B), IL-4 (C) and IL-10 (D) secretion from $2x10^6$ PLN cells after 96 hr culture with peanut protein *in vitro*. Cells were pooled from 6 mice per group and cytokine levels in the supernatant were analysed by ELISA in triplicate. Results are expressed as the mean cytokine level from triplicate analysis. No cytokines were produced in response to a control antigen or when the cells were not activated. [SPP: Saline fed, peanut immunised and peanut recall immunised; PPP: Peanut fed, peanut immunised and peanut recall immunised; OPP: OVA fed, peanut immunised and peanut recall immunised]

High levels of peanut-specific IgG, IgG1, IgG2a and IgE were detected in serum from animals fed either saline or OVA prior to peanut protein immunisations, but as expected feeding 100mg peanut protein prior to similar immunisation inhibited the antibody response to peanut (Fig. 3.18). Feeding OVA had no effect on the peanut antibody response and there were no significant differences in the levels of peanut-specific IgG, IgG1, IgG2a or IgE between the saline- and OVA-fed groups. In contrast, levels of peanut-specific IgG, IgG1, IgG2a and IgE were all significantly reduced in animals fed peanut protein compared to both saline- and OVA-fed animals (all $p < 0.01$ and in most cases $p < 0.0001$). In all experimental groups, total IgE levels in serum closely matched levels of peanut-specific IgE (results not shown).

Taken together, the above experiments (Figs. 3.16 – 3.18) show that feeding 100mg OVA prior to immunisation with peanut protein has no effect on T cell or B cell responses to peanut. Only when animals were fed 100mg peanut protein was tolerance to peanut observed. This suggests that the induction of oral tolerance is antigen-specific.

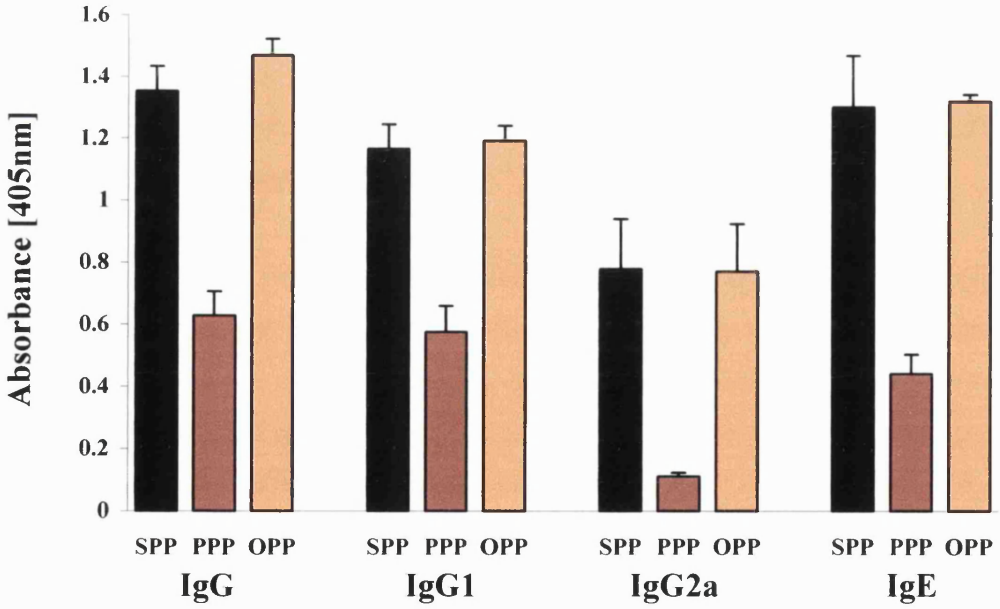


Fig. 3.18 Effect of feeding peanut protein or OVA on subsequent peanut-specific antibody responses

Animals were fed saline, 100mg peanut protein or 100mg OVA prior to immunisation with peanut protein in CFA and recall immunisation with peanut. Peanut-specific IgG, IgG1, IgG2a and IgE were measured in serum 7 days after recall immunisation. Sera were diluted 1000 times for IgG and IgG1, 200 times for IgG2a and 10 times for IgE measurements. Results are expressed as the mean antibody levels + 1 SEM of 6 individual animals per group. [SPP: Saline fed, peanut immunised and peanut recall immunised; PPP: Peanut fed, peanut immunised and peanut recall immunised; OPP: OVA fed, peanut immunised and peanut recall immunised]

3.4 Discussion

A novel model of oral tolerance or sensitisation to peanut protein has been developed and the cellular and humoral responses characterised. The induction of antigen-specific oral tolerance with single feeds was shown to be highly dose-dependent. Orally-induced systemic hyporesponsiveness to peanut protein was only successfully induced by feeding a high dose of 100mg peanut protein per animal. Oral administration of 20mg or 2mg peanut protein did not induce tolerance, while gastrointestinal exposure to 0.2mg or 0.02mg peanut protein induced significant priming of the animals upon secondary peanut challenge. Both the orally-induced hypo- and hyper-responsiveness was antigen-specific in the effector phase as T cell proliferation and cytokine production was only observed in response to stimulation with peanut protein and not to a control antigen such as OVA. The induction of oral tolerance to peanut protein was additionally shown to be antigen-specific as feeding of an equal amount of 100mg OVA prior to peanut protein immunisation did not attenuate the response to a subsequent challenge with peanut. This demonstrated that induction of oral tolerance to peanut required oral exposure to *peanut.* Only following feeding of peanut protein were specific DTH responses, T cell proliferation, cytokine responses and immunoglobulin synthesis suppressed. The model of oral tolerance to peanut protein thus appear to be antigen-specific, which confirms findings from other models of oral tolerance [Miller and Hanson, 1979] and the general consensus that oral tolerance is specific to the fed antigen [Mayer, 2000b; Strobel and Mowat, 1998].

Oral administration of 100mg peanut protein extract significantly downregulated responses of both the cellular and the humoral arm of the immune response to subsequent peanut protein immunisations, but did not completely abolish it. Antigen-specific T cell proliferation upon stimulation with peanut protein was significantly reduced, while the overall T cell proliferative capacity remained unaffected as demonstrated by vigorous responses to ConA. Peanut-induced secretion of IFN-γ, IL-4 and IL-10 was also significantly reduced, while the secretion of TGF-β was enhanced. TGF-β production was however not significantly increased compared to controls ($p=0.071$). This may be due to limited repeats of the TGF-β analysis ($n=4$) or it may be that TGF-β does not play a significant role in the maintenance of tolerance to peanut. The role of TGF-β in the induction and/or maintenance of oral tolerance has been much debated, with many reports of an important role for TGF-β [Chen et al., 1994; Chen et

al., 1995] whereas others find no evidence of TGF-β involvement in oral tolerance [Smith et al., 2000]. IL-10 is additionally an important immuoregulatory cytokine, which has been implicated in peripheral tolerance to the normal intestinal flora in the SCID IBD model [Asseman et al., 1999; Powrie et al., 1996] and the induction of low dose oral tolerance to autoantigens [Chen et al., 1995]. IL-10 does not appear to be directly involved in the maintenance- or effector phase of tolerance to peanut protein, which supports findings in other models of oral tolerance to OVA [Garside et al., 1995b]. The importance of IL-10 in the induction of oral tolerance is also questionable as oral tolerance can be induced in IL-$10^{-/-}$ mice [Aroeira et al., 1995].

Oral administration of 100mg peanut protein had a significant effect on antibody production, reducing the level of peanut-specific IgG and IgE, as well as the IgG subclasses IgG1 and IgG2a. Levels of all peanut-specific antibodies were shown to be decreased with no Th1- or Th2 bias, which was consistent with the decrease in the production of both IFN-γ and IL-4 and IL-10. When oral tolerance to peanut protein was successfully induced it thus suppressed both Th1 and Th2 responses, and does therefore not support the notion that feeding antigen preferentially suppresses Th1 responses. Similar conclusions have been reached in models of oral tolerance to OVA where both Th1 and Th2 type responses can also be suppressed [Garside et al., 1995b]. In control animals, most of the proteins in peanut evoked specific-IgG binding, although the highest level of specific IgG-binding to known allergenic peanut proteins was shown for Ara h1 and Ara h2, as demonstrated by immunoblotting techniques (Fig. 3.12). Oral administration of 100mg peanut protein not only significantly weakened the peanut-specific IgG-binding, but interestingly appeared to shift the epitope-recognition of the remaining peanut-specific IgG. There was no binding to Ara h2 and only limited binding to Ara h1 in the tolerant animals, while binding to other proteins partially remained. The relevance of this finding remains unknown. Antibody responses to the major peanut proteins in peanut-sensitive mice were additionally compared with human peanut allergic children. Peanut-sensitive mice all showed antibody binding to the major allergenic proteins Ara h1 and Ara h2, which have been reported to be recognised by over 95% of human peanut allergic subjects [Burks et al., 1991; Burks et al., 1992]. The peanut-allergic children analysed in this study, did not appear to recognise Ara h2 as readily as reported for adults in the literature, but all demonstrated strong IgG-binding to Ara h1. Food-specific IgG antibodies are also found in healthy human subjects [Freed and Waickman, 2002; Kolopp-Sarda et al., 2001], where they

may participate in the safe clearance of circulating food antigens. The relevance of food-specific IgG antibodies in allergy and whether a very high level of specific IgG or specific IgG with an abnormal epitope-recognition pattern can induce immune pathology remains unknown. IgE-mediated reactions are the only mechanism known for certain to play a major role in food allergy [Bruijnzeel-Koomen et al., 1995], and so it was of interest that induction of oral tolerance to peanut in the present mouse model significantly reduced the induction of peanut-specific IgE. Preliminary data (not shown) from analysis of peanut-specific IgE-binding additionally showed similar binding patterns in sensitised mice as those observed in peanut-allergic children, which suggests that B cell responses to the major peanut allergens in this model are similar to those found in human patients with peanut allergy. It would be of additional interest to study the effect of oral tolerance on IgE epitope-recognition patterns in mice, which was not assessed in this study.

Allergic diseases are associated with increased and sustained allergen-specific IgE production, and clearly the primary sensitisation to protein allergens is a key step in their pathogenesis. It is unknown whether primary systemic sensitisation to food allergen occurs through gastrointestinal exposure or via some other route. Gastrointestinal exposure to soluble protein antigens is a powerful way to induce systemic hyporesponsiveness, but as shown here primary oral exposure to protein antigens does not always lead to tolerance. Oral administration of peanut protein in doses of 0.2mg or 0.02mg induced systemic hyperresponsiveness upon re-exposure to peanut protein. This supports the notion that small doses of orally administered antigen can prime an animal for subsequent systemic and local responses, as shown for oral administration of OVA [Mowat, 1987]. Oral doses of either 0.2mg or 0.02mg peanut protein significantly enhanced peanut-specific T cell proliferation, without affecting the proliferation to mitogenic stimuli such as ConA. The kinetics of the T cell expansion when T cells from sensitised animals were stimulated with peanut protein or with ConA were remarkably different. Stimulation of antigen-specific T cells routinely induced cells to divide approximately the same number of times, while a mitogenic stimulus induced a varying number of T cell divisions, as demonstrated by a CFSE pattern of numerous peaks of lessening CFSE intensity. Double-staining with CFSE and CD4 or CD8, showed that proliferating peanut-specific, as well as non-specific ConA-stimulated, cells were primarily $CD4^+$ (results not shown). Enhanced secretion of the cytokines IFN-γ, IL-4 and IL-10, but reduced production of TGF-β, was shown from PLN and spleen cells from animals fed

sensitising doses of peanut protein, although this was not significantly different from controls. Analysing the antibody response in individual animals fed either 0.2mg or 0.02mg peanut protein demonstrated significantly elevated levels of peanut-specific IgG and IgE antibodies. The observation that oral exposure to small amounts of peanut protein enhances IL-4 secretion and significantly raises levels of peanut-specific IgE upon re-exposure to peanut may be of clinical relevance in explaining primary allergic sensitisation. Control animals used for comparison in this Chapter were made hypersensitive to peanut protein by subcutaneous immunisation with peanut protein in CFA and recall immunisation with peanut. The additional priming of animals through feeding of small doses of peanut protein highlights the powerful effect gastrointestinal exposure to protein antigens can have on systemic immunity.

The immunochemical and physiochemical properties that account for a proteins allergenicity are poorly understood, but the nature of the antigen is clearly important for the induction of tolerance [MacDonald, 1998; Mayer, 2000] and in directing the Th1/Th2 deviation [Racioppi et al., 1993]. This study permitted a direct comparison between responses following oral administration of peanut protein and OVA. Results showed that approximately 50 fold larger doses of peanut protein than OVA were required to induce suppression of specific T cell responses. This may not solely be explained by the peanut extract being a mixture of many proteins and the concentration of any given protein thus lower than that of the single OVA protein when administered. Immunoblots demonstrated that sensitised mice in the present model mainly reacted to Ara h1 and Ara h2, which in a quantitative analysis of different peanut varieties around the world were found to account for between 12 and 16% and 5.9 to 9.3% of the total protein content respectively [Koppelman et al., 2001]. The different dose requirements for peanut and OVA in the induction of oral tolerance may reflect differential immune mechanisms for the induction of tolerance to individual antigens and to antigenic mixtures, or may reflect differences in immunogenicity of OVA and peanut. The present study demonstrates that peanut protein has a potent sensitising effect and primes animals for a subsequent exposure more readily and at higher doses than OVA. Peanut protein reproducibly caused larger DTH responses than OVA and sensitised animals showed vigorous specific T cell proliferation at lower doses of peanut protein and with a steeper dose-response slope than animals sensitised to OVA. This suggests that peanut proteins may be more immunogenic (or allergenic) than OVA and that peanut protein may induce hypersensitivity more effectively. The sensitising effect of peanut proteins was similarly shown in a murine model of cholera

toxin-induced peanut anaphylaxis, where sensitisation to peanut protein required fewer doses and a shorter sensitising period than sensitisation to cows milk [Li et al., 2000]. These conclusions reflect findings in human food-allergic subjects and strongly suggests that the nature of the antigen plays an important role in determining the immunological outcome of an allergen encounter.

The model of oral tolerance or sensitisation to peanut protein developed here demonstrates that tolerance to peanut can be successfully induced experimentally, but that gastrointestinal exposure to peanut protein can also initiate potent sensitisation with high levels of IL-4 and specific IgE. Animals were naïve with regard to peanut protein and OVA prior to oral feeding and the strength of the oral tolerance induction or sensitisation to peanut protein has not yet been tested in already sensitised animals. The model can now be used to further define the regulation of specific immune responses following antigen feeding of allergenic proteins. As well as exploring the effect of non-oral routes of peanut exposure on the development of oral tolerance or allergy to peanut.

Chapter 4

Mechanisms involved in oral tolerance

4.1 Introduction

Peripheral tolerance is the mechanism which maintains unresponsiveness to antigens that either are not present in the generative lymphoid organs or to self antigen-specific lymphocytes that escape central tolerance. Induction and maintenance of peripheral tolerance is an important phenomenon for the control of homeostasis in the immune system. Peripheral tolerance to self antigens and to food antigens are distinct phenomena but the underlying mechanisms may be similar. Unresponsiveness of specific T cells in the periphery can be achieved in a number of ways. Clonal deletion, clonal anergy and active suppression have all been shown to be mechanisms of oral tolerance following gastrointestinal antigen exposure. The specific mechanism involved is likely to be dependent on the tolerising regime employed. High doses of antigen may result in clonal deletion and/or anergy [Chen et al., 1995; Melamed and Friedman, 1993], while small and/or repeated doses of antigen may result in active cellular suppression of subsequent responses [Friedman and Weiner, 1994; Thorstenson and Khoruts, 2001].

It has been known for many years that oral tolerance can be adoptively transferred *in vivo* with T cells to naïve animals. There is good evidence for an essential role of $CD4^+$ T cells in such 'infectious tolerance' [Chen et al., 1994; Hirahara et al., 1995] as well as for the induction and maintenance of oral tolerance as a whole [Desvignes et al., 1996; Garside et al., 1995a]. Recently, T cells with a suppressor or regulatory (T_{reg}) function have been shown to reside predominantly in the $CD4^+CD25^+$ T cell population, which is selected during T cell differentiation in the thymus [Bensinger et al., 2001; Itoh et al., 1999]. In addition to the $CD4^+CD25^+$ T_{reg} cells produced directly in the thymus, evidence is emerging to suggest that $CD4^+CD25^+$ T cells with similar immunoregulatory function may be induced in the periphery. Continuous exposure to oral OVA induces an anergic population of $CD4^+CD25^+$ T cells with immunoregulatory properties [Thorstenson and Khoruts, 2001]. These $CD4^+CD25^+$ T_{reg} cells may use multiple mechanisms to suppress immune responses, but most studies report that T_{reg} cell function is mediated by cytokines and/or through cell-cell contact. IL-4, IL-10 and TGF-β are the cytokines most often implicated with T_{reg} function, while suppression through cognate interactions may involve the engagement of CTLA-4 [Maloy and Powrie, 2001; Shevach, 2002].

In the current Chapter, possible mechanisms involved in the maintenance of oral tolerance to peanut protein are explored. This study addresses whether a population of T cells with regulatory properties can be induced by feeding of peanut protein. In particular, whether $CD4^+CD25^+$ T_{reg} cells can be induced in the periphery by single feeds of peanut protein and if these cells can actively regulate the function of cells from peanut-sensitive animals. Additionally, putative mechanisms by which these T_{reg} cells may mediate their suppressive function are elucidated. The study aims at gaining a beginning of an understanding of the mechanisms underlying peanut sensitisation and tolerance, but does not exclude that multiple other mechanisms may be operative simultaneously.

4.2 Results

Throughout this study the same *in vivo* feeding and immunisation protocol was followed as described in section 3.2.1 and shown in Fig. 3.1. Animals were made sensitive to peanut by feeding 0.2mg peanut protein or saline prior to immunisation with peanut protein in CFA and recall immunisation, while peanut tolerance was induced by feeding 100mg peanut protein prior to similar immunisations. The effector phase or maintenance of oral tolerance was examined by analysing cells one week after the second (recall) peanut protein immunisation.

4.2.1 Phenotypic analysis of cells from peanut-sensitive and peanut-tolerant animals

To investigate possible differences between T cells from peanut-sensitive and peanut-tolerant animals a phenotypical analysis of PLN cells were carried out. Freshly isolated $CD4^+$ LN cells from animals fed saline, 0.2mg peanut protein or 100mg peanut protein prior to immunisations all contained about 10% $CD4^+CD25^+$ cells (Fig. 4.1A-C). Upon reactivation with peanut protein through the TCR *in vitro* the $CD4^+CD25^+$ cell population rose to between 16 – 29% for all experimental groups, while the level of CD25 expression per cell (mean fluorescence intensity) remained approximately the same (Fig. 4.1D-F and results not shown). This showed that the % of T cells in PLN expressing the IL-2 receptor molecule, CD25, were similar in peanut-sensitive and in peanut-tolerant animals. There were however consistently slightly higher proportions of $CD4^+CD25^+$ cells in PLN from hyper-sensitised animals fed 0.2mg peanut protein than in PLN from animals fed saline prior to the peanut protein immunisations. More surprisingly, the percentage of $CD4^+CD25^+$ cells in PLN from peanut-

tolerant animals fed 100mg peanut protein were as high or higher than in PLN from the sensitised animals fed saline or 0.2mg peanut protein.

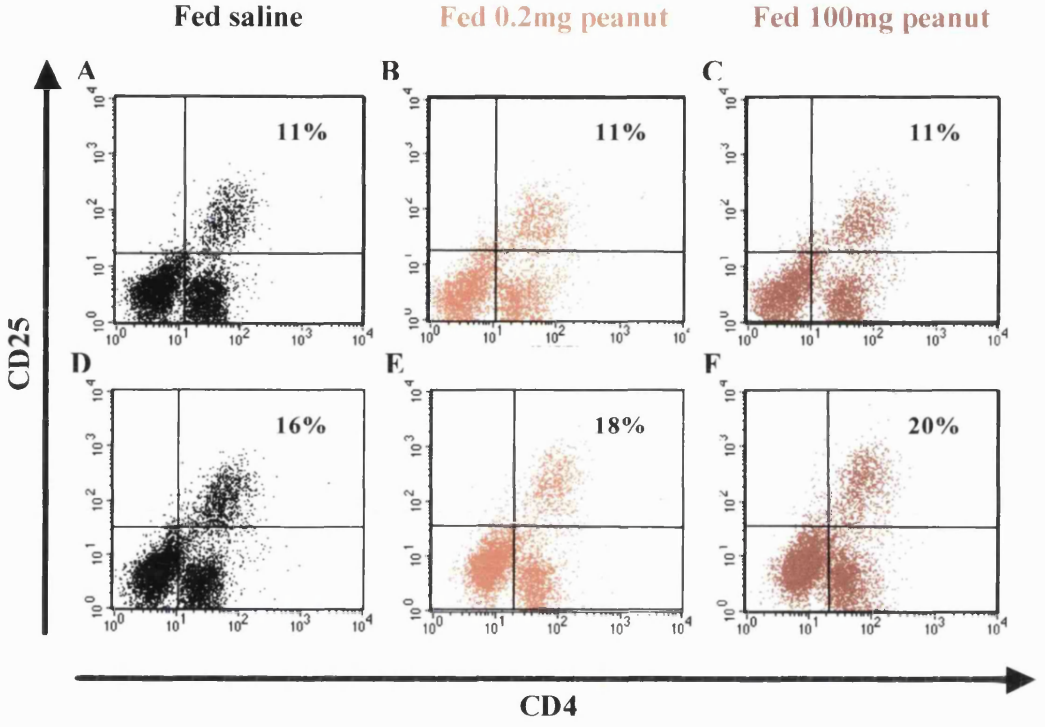


Fig. 4.1 CD4/CD25 analysis of PLN cells from mice fed a single oral dose of saline, 0.2mg peanut or 100mg peanut

Animals were fed either saline, 0.2mg peanut protein or 100mg peanut protein by gavage prior to immunisation with peanut protein in CFA and recall immunisation with peanut protein. PLN cells were collected 7 days after recall immunisation and stained for CD4 and CD25 either immediately (A-C) or after 90 hr *in vitro* reactivation with 150µg peanut protein/ml (D-F). CD4/CD25 expression was analysed on a FACSscan and are represented as dotplots of CD4/CD25 double staining. Cells were pooled from a minimum of 8 animals per group. The experiment was repeated 4 times with similar differences between experimental groups

As noted previously (Chapter 3) PLN cells from peanut-tolerant animals did not proliferate well when reactivated with peanut protein *in vitro*. The high expression of CD25 on these cells following culture with peanut protein was therefore puzzling. To investigate whether there was a difference between the $CD25^+$ cells in peanut-sensitive and in peanut-tolerant animals and whether the $CD25^+$ cells were involved in active proliferation, PLN cells were stained

with CFSE and reactivated *in vitro* with peanut protein for 90 hr. The cells were then stained for CD25 on the cell surface and analysed by flow cytometry. PLN cells from both saline-fed and peanut-fed animals contained a large percentage of $CD25^+$ cells (Fig. 4.2A). The $CD25^+$ cells from the saline-fed animals proliferated vigorously with the $CFSE^{low}$ peak constituting 44% of the $CD25^+$ cells (Fig. 4.2B). In contrast, only 10% of the $CD25^+$ cells from animals fed 100mg peanut were proliferating, creating a much smaller $CFSE^{low}$ peak (Fig. 4.2B).

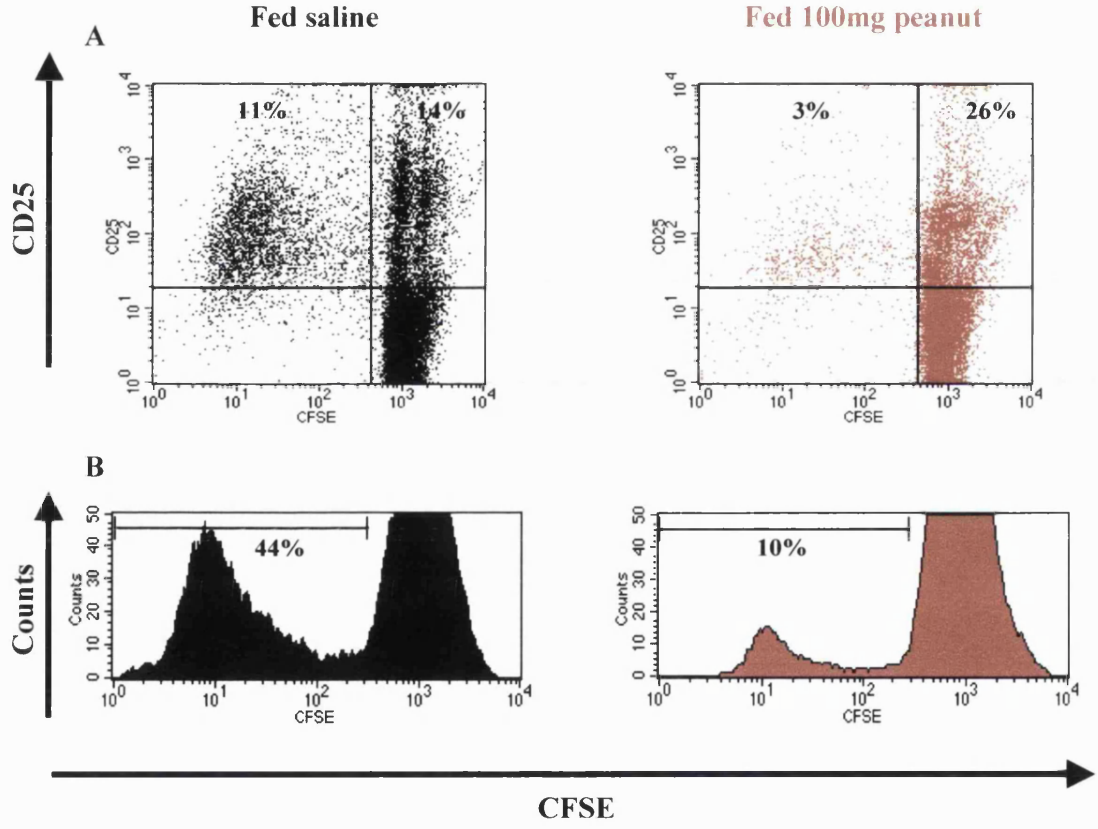


Fig. 4.2 CFSE/CD25 analysis of PLN cells from mice fed saline or 100mg peanut
PLN cells from animals fed a single oral dose of either saline or 100mg peanut protein prior to immunisation with peanut protein in CFA and recall immunisation with peanut protein were stained with CFSE 7 days after recall immunisation. Cells were reactivated with peanut protein *in vitro* for 90 hr and analysed for CFSE intensity and CD25 expression on a FACSscan. (A) Example of CFSE/CD25 double staining showing 25% $CD25^+$ cells in PLN from the saline fed animals and 29% $CD25^+$ cells in PLN from animals fed 100mg peanut protein. (B) Cells were gated on $CD25^+$ cells and analysed for cell division by the intensity of the CFSE staining in the FL1 channel on the FACS. The shown data is representative of three independent experiments

The data showed that the $CD25^+$ cells from both peanut-sensitive and peanut-tolerant animals were mainly $CD4^+$. Results from the CFSE staining experiments however indicated that there was a differences between the $CD25^+$ cell population from the peanut-sensitive and the peanut-tolerant animals. This hypothesis was explored further by investigating the expression of CTLA-4 on $CD4^+$ and $CD25^+$ PLN cells from animals fed saline or 100mg peanut protein prior to peanut immunisations. CTLA-4 staining of freshly isolated $CD4^+$ PLN from either saline- or peanut-fed animals showed very little or no CTLA-4 expression (Fig. 4.3A,C). Nor was any CTLA-4 expression detected on or in PLN from saline fed animals following 90 hr *in vitro* reactivation with peanut protein (Fig. 4.3B). In contrast, a small but distinct $CD4^+$CTLA-4^+ cell population (routinely around 7%) was demonstrated in PLN from peanut-fed animals following reactivation with peanut protein *in vitro* (Fig. 4.3D).

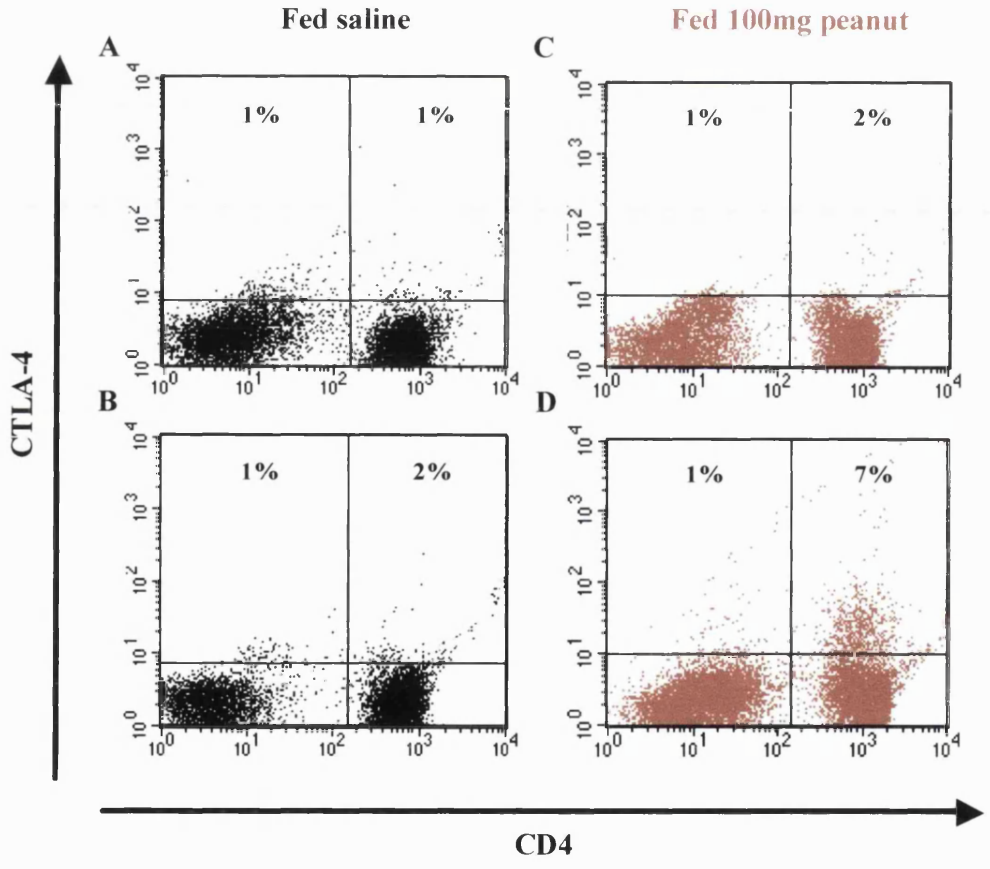


Fig. 4.3 CD4/CTLA-4 analysis of PLN cells from mice fed saline or 100mg peanut
Animals were fed either saline (A & B) or 100mg peanut protein (C & D) prior to immunisation with peanut protein in CFA and recall immunisation with peanut protein. PLN lymphocytes were analysed for CD4 and CTLA-4 expression immediately following collection 7 days after the recall immunisation (A & C) or following 90 hr reactivation with peanut protein *in vitro* (B & D). CTLA-4 expression was analysed by flow cytometry both on the cell surface and intracellular as described in section 2.14 The experiment was repeated three times with similar results

The difference in CTLA-4 expression on cells from saline- and 100mg peanut-fed animals became clearer when analysing the $CD25^+$ cell populations of the two experimental groups. Following reactivation with peanut protein *in vitro* about 3% of the $CD25^+$ cell population from saline-fed animals expressed CTLA-4 compared to around 24% of the $CD25^+$ cell population from the 100mg peanut-fed animals (Fig. 4.4). The $CD25^+$ cell populations from the saline-fed and the peanut-fed animals were additionally stained for surface TGF-β expression. Some $CD25^+TGF\text{-}\beta^+$ cells were detected in PLN from the peanut-fed group but not in PLN from the saline-fed group following reactivation with peanut protein *in vitro*. However, reproducible TGF-β surface staining was not achieved, and the issue of TGF-β surface expression on $CD25^+$ cells thus remains uncertain in this context.

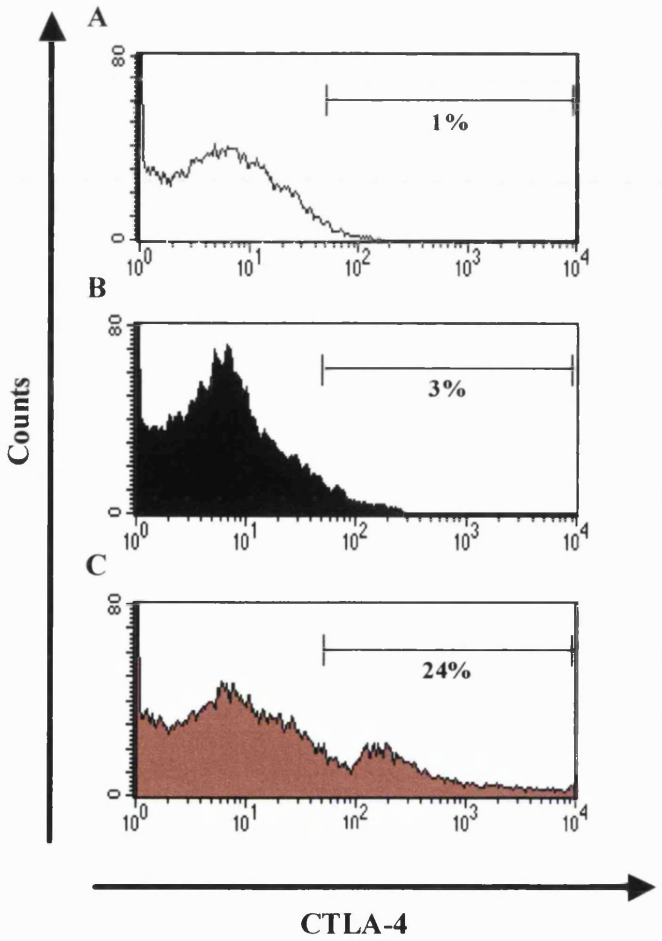


Fig. 4.4 CTLA-4 expression on $CD25^+$ lymphocytes
PLN cells from animals fed either saline (B) or 100mg peanut protein (C) prior to immunisation with peanut protein in CFA and recall immunisation with peanut protein were collected 7 days after the recall immunisation. PLN cells were reactivated with peanut protein *in vitro* for 90 hr and expression of CD25/CTLA-4 was analysed. $CD25^+$ cell fractions were gated and expression of CTLA-4 (intracellular and surface) on each cell population shown as a histogram. The open histogram (A) represents the CTLA-4 isotype control. The shown histograms are representative examples of three independent experiments

4.2.2 Co-culture of cells from peanut-sensitive and peanut-tolerant animals

The presence of $CD4^+CD25^+CTLA\text{-}4^+$ cells in PLN from animals fed 100mg peanut protein suggests generation of a population of T_{reg} in response to the peanut protein feed. To investigate whether cells from peanut-tolerant animals could actively regulate or inhibit the activity of cells from peanut-sensitive animals, an *in vitro* co-culture technique was used. PLN cells from peanut-sensitive animals fed saline prior to immunisation with peanut protein in CFA and recall immunisation were combined at different ratios with PLN cells from peanut-tolerant animals that had been fed 100mg peanut protein prior to immunisations. The overall cell density in the co-cultures was kept the same throughout. All cell cultures were stimulated with 150µg peanut protein/ml, with 150µg OVA/ml as a control antigen, 1µg ConA/ml as a positive proliferative control or left unstimulated.

It was clearly demonstrated in three independent experiments that the specific T cell proliferation of PLN from peanut-sensitive animals was markedly reduced when co-cultured with PLN from peanut-tolerant animals in the presence of peanut protein (Fig. 4.5). The proliferation expected in the co-cultures if there were no suppressive interactions between cells from peanut-tolerant and peanut-sensitive animals was calculated as follows:
Let a = [cpm] with 100% sensitive cells, b= [cpm] with 100% tolerant cells, f = fraction of cells in the culture that are sensitive. Then the expected [cpm] = a f + b(1-f).

The suppressive effect of the peanut-tolerant PLN on the peanut-sensitive PLN showed some dose-response correlation, with the proliferation of sensitive cells being more effectively reduced with higher ratios of tolerant : sensitive PLN cells. Remarkably though, by introducing just 10% peanut-tolerant PLN cells to the peanut-sensitive cells the incorporation of [^{3}H]-thymidine would be reduced to 50% of the expected (Fig. 4.5 and Fig. 4.6). Neither the peanut-sensitive nor the peanut-tolerant cells showed any proliferation to a control antigen, but both groups proliferated strongly and equally to mitogenic stimuli with ConA.

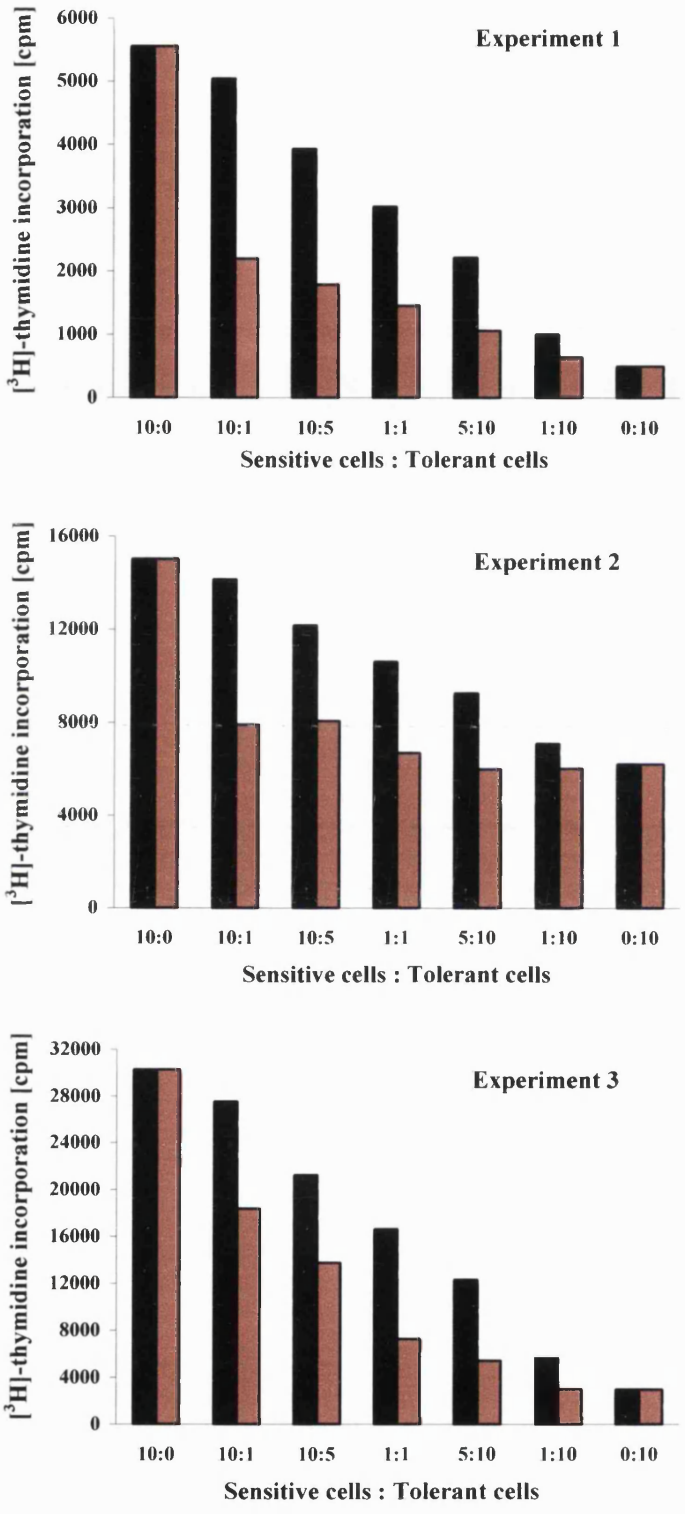


Fig. 4.5 Co-cultures of cells from peanut-sensitive and peanut-tolerant animals

Animals were fed either saline or 100mg peanut protein prior to immunisation with peanut protein in CFA and recall immunisation with peanut protein. PLN cells were collected from both groups 7 days after recall immunisation and cultured for 90 hr *in vitro* with 150µg peanut protein/ml. PLN cells from saline-fed animals (sensitive cells) were cultured on their own (10:0) or with varying ratios of PLN cells from animals fed 100mg peanut protein (tolerant cells). Total number of cells in the culture was kept constant at $2x10^5$ cells. The black bars (■) demonstrate the expected proliferation [cpm], calculated by the formula: [cpm] = a f + b(1-f), where a = [cpm] with only sensitive cells, b = [cpm] with only tolerant cells and f = fraction of sensitive cells in the culture. The actual [^{3}H]-thymidine incorporation [cpm] is shown in brown bars (■) and represents the mean of triplicate cultures. Three individual experiments are shown with cells pooled from a minimum of 10 animals per group. Background proliferation when no antigen was present has been subtracted. There was no significant proliferation to a control antigen in any group, but both the saline-fed and the peanut-fed group proliferated strongly and equally to ConA

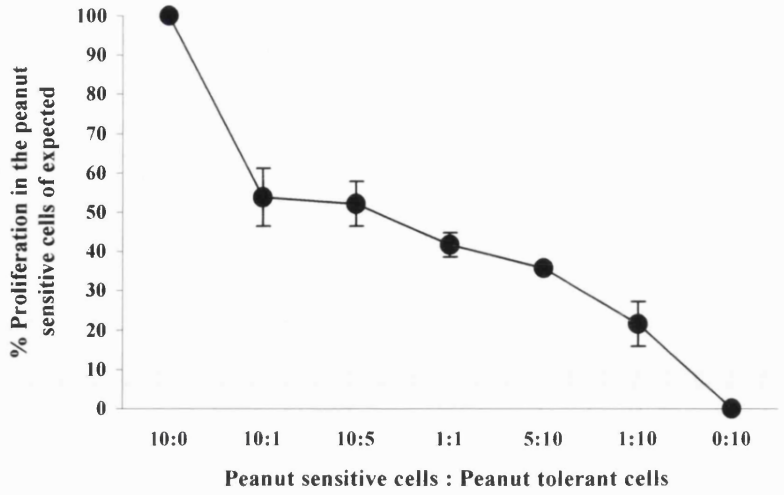


Fig. 4.6 Percentage reduction in peanut-specific proliferation of sensitive cells when cultured with tolerant cells

PLN cells from animals fed saline prior to immunisation with peanut protein in CFA and recall immunisation with peanut protein (peanut-sensitive cells) were co-cultured with PLN cells from animals fed 100mg peanut protein prior to a similar immunising regime (peanut-tolerant cells). Co-cultures were set-up and analysed as in Fig. 4.5. The reduced proliferation of the peanut-sensitive cells was calculated as percentage proliferation of the expected proliferation. The expected proliferation was calculated as the reduction in [cpm] due only to dilution of the sensitive cells. Each data point represents the mean ± 1 SEM of three individual experiments

Cytokine secretion from PLN co-cultured in a ratio of 1:1 between saline-fed and 100mg peanut-fed animals was also analysed and compared to cytokine secretion from PLN of saline-fed animals only and to PLN from peanut-fed animals only. Cells from the peanut-sensitive, saline-fed animals produced large amount of IFN-γ, IL-4 and IL-10 but little TGF-β upon re-activation with peanut protein (see also Chapter 3). In contrast, cells from peanut-tolerant, peanut-fed animals secreted little if any IFN-γ, IL-10 and IL-4, but larger amounts of TGF-β. Expected levels of IFN-γ, IL-4 and IL-10 secretion were markedly reduced when the cells were co-cultured, while TGF-β was up-regulated (Fig. 4.7). The levels of IFN-γ, IL-4 and IL-10 produced in the co-cultures were reduced to 20% or less of the amount produced by cells from peanut-sensitive animals only. Significantly lower levels of cytokines were thus secreted from peanut-sensitive cells when co-cultured with peanut-tolerant cells than was expected from the reduced number of cytokine-producing cells in the culture (Fig. 4.7).

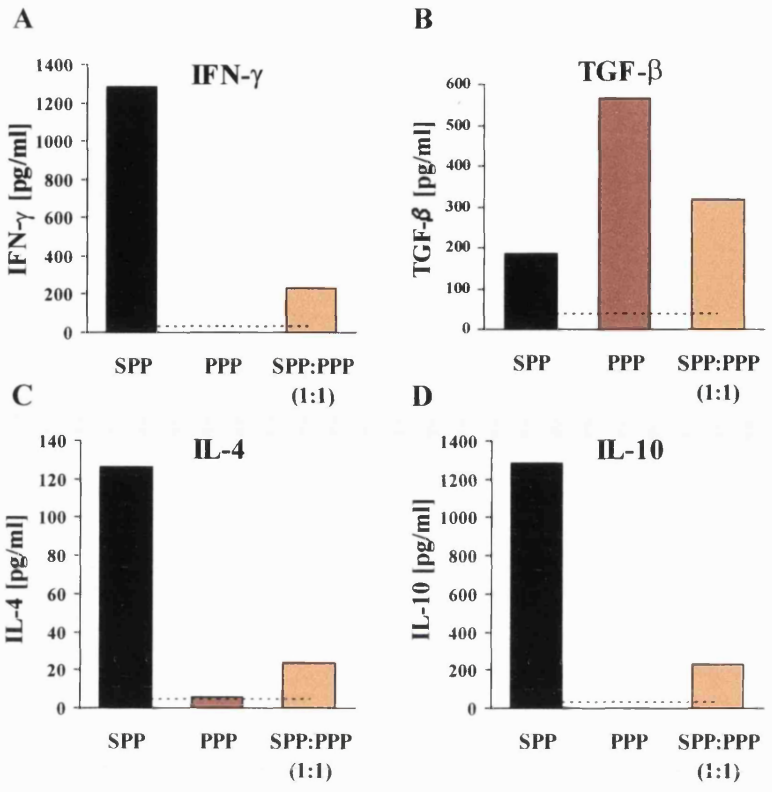


Fig. 4.7 Cytokine secretion in 1:1 co-cultures of peanut-sensitive and peanut-tolerant cells

Animals were fed either saline or 100mg peanut protein prior to immunisation with peanut protein in CFA and recall immunisation. PLN cells were cultured in a ratio of 1:1 between cells from saline- and peanut-fed animals with the total cell number at $2x10^6$ cells. IFN-γ (A), TGF-β (B), IL-4 (C) and IL-10 (D) production in the co-cultures were analysed following 96 hr re-activation with 150μg peanut protein/ml *in vitro*. Cells were pooled from minimum 10 mice per group and cytokine levels were analysed in triplicate from the supernatants. Results are expressed as the mean cytokine levels from triplicate analysis. PLN cells from saline-fed animals were cultured either alone (SPP) or 1:1 with PLN cells from tolerant, peanut-fed animals (PPP). No cytokines were produced in response to a control antigen or when the cells were not re-activated. [SPP: Saline fed, immunised with peanut in CFA and recall immunised with peanut; PPP: Peanut fed, immunised with peanut in CFA and recall immunised with peanut]

The $CD4^+CD25^+CTLA4^+$ T cells observed in PLN from peanut-tolerant animals were hypothesised to be responsible for the active reduction in proliferation of peanut-sensitive cells when co-cultured with peanut-tolerant cells. To explore this, PLN cells from animals fed 100mg of peanut protein prior to immunisation with peanut protein in CFA and recall immunisation with peanut protein were stained and negatively selected for $CD25^+$ cells with MACS microbeads. The $CD25^-$ cell fraction was then co-cultured with PLN from peanut-sensitive animals fed saline prior to peanut protein immunisation with CFA and recall

immunisation. The cell ratios between PLN cells from peanut-sensitive and peanut-tolerant animals were kept as in the initial experiments (Fig. 4.5) and the overall cell density per well was kept the same throughout. This experiment demonstrated that when the $CD25^+$ cell population was removed from the tolerant cells in the co-cultures, the proliferation of the peanut-sensitive cells was completely restored (Fig. 4.8). Removal of $CD25^+$ cells however did not restore the response from the tolerant animals (Fig. 4.8 at ratio 0:10). Analysis of cytokine secretion from co-cultures of peanut-sensitive cells and the $CD25^-$ cell fraction of peanut-tolerant cells additionally showed that without the $CD25^+$ cells the peanut-tolerant cells could no longer reduce the amount of secreted IFN-γ, IL-4 or IL-10 (Fig. 4.9). Levels of IFN-γ, IL-4 and IL-10 produced in the co-cultures were fully restored to the level produced from peanut-sensitive cells alone (taking into account the 50% reduction in sensitive cell numbers in the 1:1 co-cultures), while TGF-β was as low as in cultures of sensitive cells alone (Fig. 4.9).

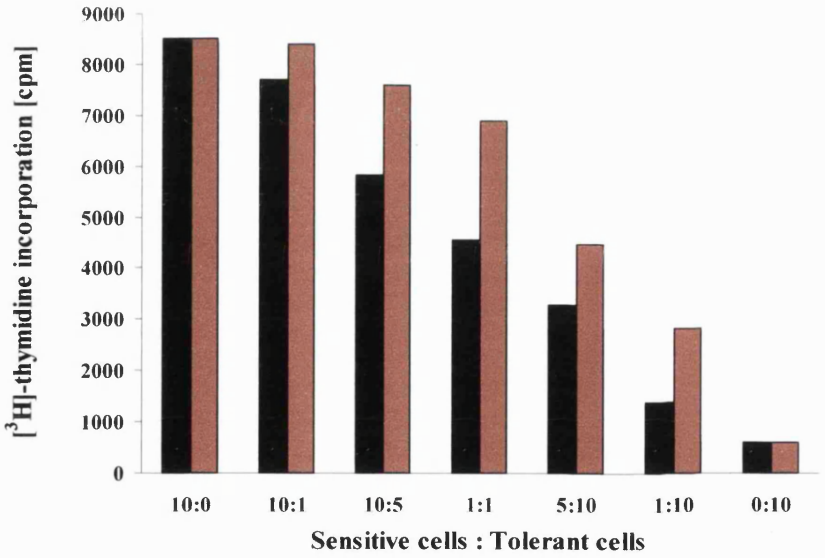


Fig. 4.8 Co-cultures of peanut-sensitive and the $CD25^-$ fraction of peanut-tolerant cells
PLN cells from animals fed 100mg peanut protein (tolerant cells) prior to immunisation with peanut protein in CFA and recall immunisation with peanut protein were negatively selected for $CD25^+$ cells 7 days after the recall immunisation. The $CD25^-$ cell fraction was co-cultured with PLN cells from animals fed saline (sensitive cells) prior to an identical immunising regime. The co-cultures were incubated for 90 hr *in vitro* in the presence of 150μg peanut protein/ml and proliferation then analysed as [^{3}H]-thymidine incorporation [cpm]. The black bars (■) demonstrate the expected proliferation [cpm], which was calculated by the formula: [cpm] = a f + b(1-f), where a = [cpm] with only sensitive cells, b = [cpm] with only tolerant cells and f = fraction of sensitive cells in the culture. The actual [^{3}H]-thymidine incorporation [cpm] is shown in brown bars (■) and represents the mean of triplicate culture

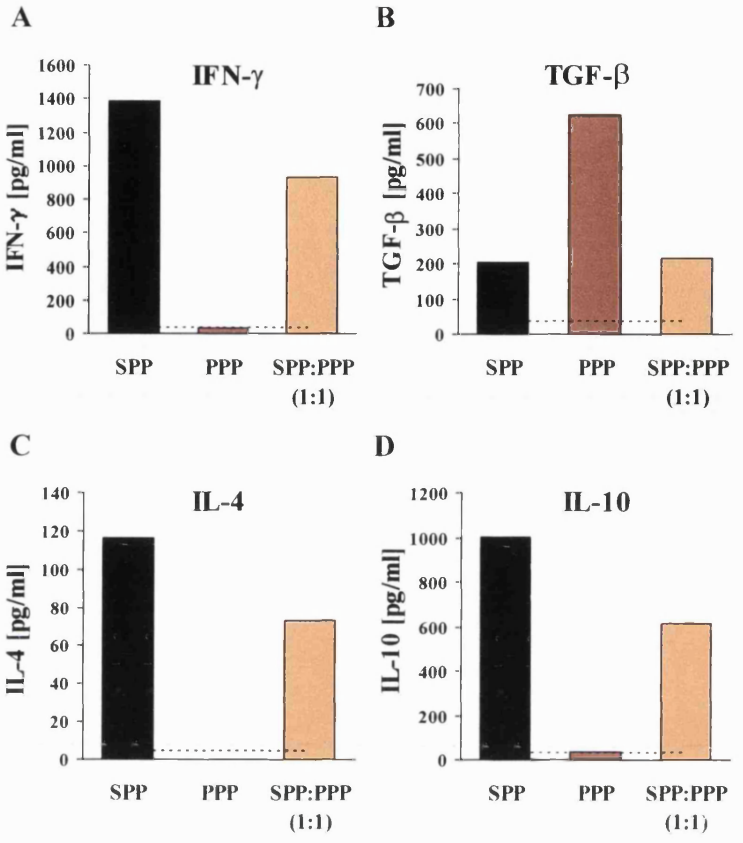


Fig. 4.9 Cytokine secretion in 1:1 co-cultures of peanut-sensitive and the CD25⁻ fraction of peanut-tolerant cells

IFN-γ (A), TGF-β (B), IL-4 (C) and IL-10 (D) production from $2x10^6$ PLN cells were analysed following 96 hr stimulation with 150μg peanut protein/ml *in vitro*. Cells were pooled from minimum 10 mice per group and cytokine levels were analysed in triplicate from the supernatants. Each bar show the mean cytokine level. PLN cells from saline fed (sensitive) animals were cultured either alone (SPP) or 1:1 with CD25⁻ PLN cells from peanut protein fed (tolerant) animals (PPP). No cytokines were produced from either experimental group in response to a control antigen or when the cells were not stimulated

To investigate and attempt to identify putative molecules and mechanisms important for the suppressive effect of the peanut-tolerant cells on the peanut-sensitive cells, additional co-cultures were analysed. Co-cultures between PLN from peanut-sensitive animals and PLN from peanut-tolerant animals were set up as shown in Fig. 4.5, and were additionally incubated in the presence of neutralising anti-CTLA-4 antibodies. Neutralising the CTLA-4 molecule completely reversed the suppressive effect of the tolerant cells, and the proliferation of the peanut-sensitive cells was restored to the expected level (Fig. 4.10). Similarly, when co-cultures of peanut-sensitive cells and peanut-tolerant cells were incubated with neutralising anti-TGF-β antibodies, the suppression of the sensitive cell proliferation was lifted and a normal full proliferative response was seen (Fig. 4.11). This inhibition of the suppressive effect of tolerant cells on sensitive cells was not seen when the co-cultures were incubated with the isotype control of either the anti-CTLA-4 or the anti-TGF-β antibodies.

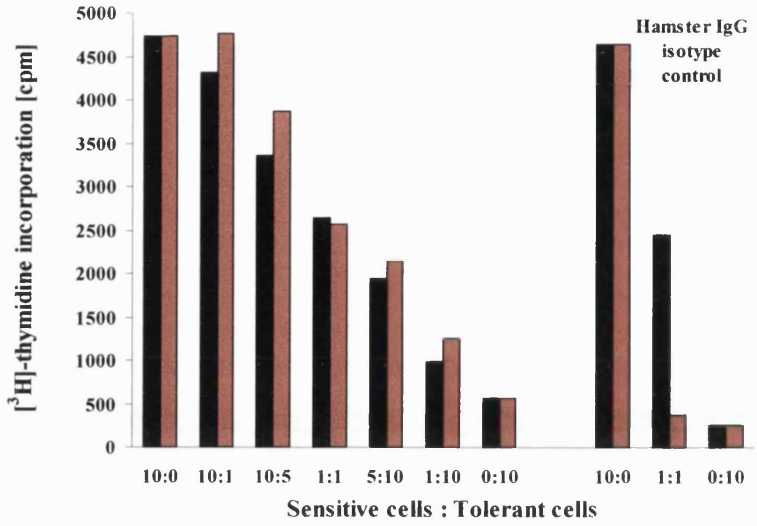


Fig. 4.10 Co-cultures of peanut-sensitive and peanut-tolerant cells with neutralising anti-CTLA-4 antibodies

Co-cultures of peanut-sensitive and peanut-tolerant cells were set-up as described for Fig. 4.5, but were additionally cultured with 10μg/ml neutralising anti-CTLA-4 antibodies or 10μg/ml isotype control. Cells were cultured *in vitro* for 90 hr in the presence of 150μg peanut protein/ml. The black bars (■) represents the expected proliferation [cpm], which was calculated by the formula: [cpm] = a f + b(1-f), where a = [cpm] with only sensitive cells, b = [cpm] with only tolerant cells and f = fraction of sensitive cells in the culture. The actual [^{3}H]-thymidine incorporation [cpm] is shown in brown bars (■) and represents the mean of triplicate culture

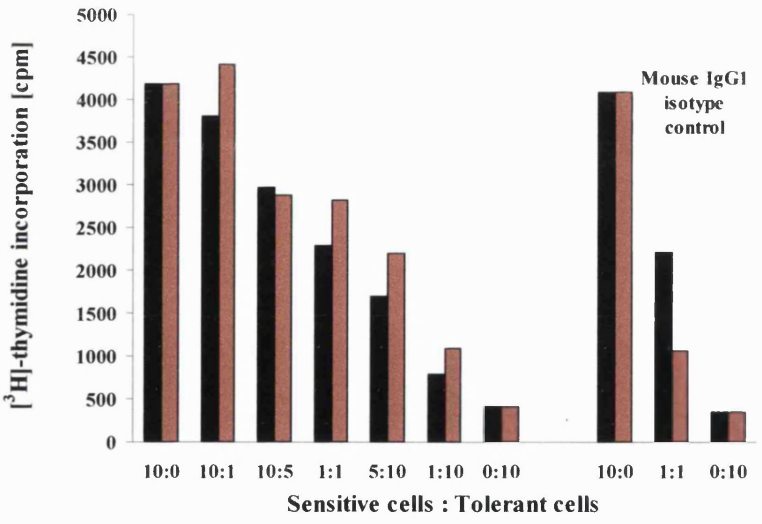


Fig. 4.11 Co-cultures of peanut-sensitive and peanut-tolerant cells with neutralising anti-TGF-β antibodies

Co-cultures of peanut-sensitive and peanut-tolerant cells were set-up as described for Fig. 4.5, but were additionally cultured with 50μg/ml neutralising anti-TGF-β antibodies or 50μg/ml isotype control. Cells were cultured *in vitro* for 90 hr in the presence of 150μg peanut protein/ml. The black bars (■) represents the expected proliferation [cpm], which was calculated by the formula: [cpm] = a f + b(1-f), where a = [cpm] with only sensitive cells, b = [cpm] with only tolerant cells and f = fraction of sensitive cells in the culture. The actual [^{3}H]-thymidine incorporation [cpm] is shown in brown bars (■) and represents the mean of triplicate culture

Moreover, when peanut-sensitive PLN cells from animals fed saline prior to immunisation with peanut protein in CFA and recall immunisation with peanut protein were incubated *in vitro* with peanut protein and a concentration range of active TGF-β cytokine, the proliferative response was markedly reduced. Peanut-sensitive cells cultured with peanut protein and active TGF-β showed a dose-dependent suppression of peanut-specific T cell proliferation. High doses of TGF-β abolished proliferation completely while even relatively small amounts of active TGF-β (30 - 75pg/ml) significantly suppressed the specific proliferation of the peanut-sensitive cells (Fig. 4.12). This suggests that TGF-β has the capacity to be a mediator of the suppressive function of T_{reg}.

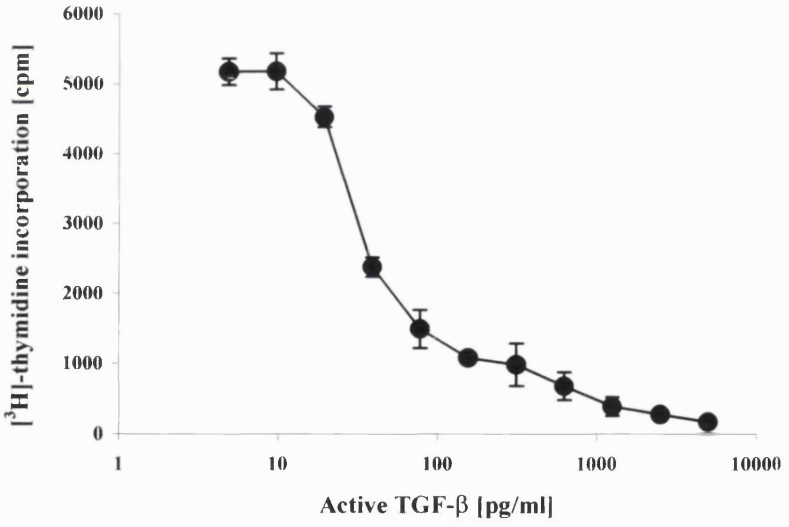


Fig. 4.12 PLN cells from peanut-sensitive animals cultured with active TGF-β
PLN cells from animals fed saline prior to immunisation with peanut protein in CFA and recall immunisation with peanut protein (peanut-sensitive cells) were collected 7 days after recall immunisation. The peanut-sensitive cells were cultured *in vitro* for 90 hr with 150μg peanut protein/ml and a concentration range of active human TGF-β. Cells were pooled from 10 animals and results are expressed as the mean cpm of triplicate culture ± 1 SEM. Background proliferation without antigen has been subtracted

4.3 Discussion

Since the first discoveries that anaphylaxis to OVA could be inhibited by prior oral administration of OVA [Wells, 1911] much has been learnt about the phenomenon of oral tolerance. A great deal of work has demonstrated that oral tolerance to antigens is an active process mediated by antigen-specific lymphocytes. Several mechanisms underlying oral tolerance have been proposed in different systems, and it is likely that multiple mechanisms have evolved to maintain this crucial physiological phenomenon. The principle immunological mechanisms that have been implicated in oral tolerance are clonal deletion, clonal anergy and antigen-driven suppression. These immunological mechanisms of peripheral tolerance are not mutually exclusive and it may be that more than one operate simultaneously.

It has been shown that oral administration of high doses of antigen can induce peripheral mechanisms resulting in functional anergy or extrathymic deletion of antigen-reactive cells [Chen et al., 1995; Melamed and Friedman, 1993]. Deletion as a mechanism of tolerance induction was not formally investigated in this study. However, clonal deletion as a response to oral antigen administration has generally been reported in TCR transgenic mice with a high frequency of antigen-specific T cells and is probably less likely to be the main mechanism of oral tolerance induction in conventional mice.

Hyporesponsiveness following a feed of 100mg peanut protein could be due to a functional inactivation of specific lymphocytes as seen in anergic T cell populations. T cells from peanut-tolerant animals were anergic and hyporesponsive with regard to both proliferation and cytokine secretion upon re-activation, which supports a mechanistic role for anergy in oral tolerance to peanut. However, the high expression of CD25 (the α-chain of the IL-2 receptor) on LN cells from tolerant animals activated with peanut protein argues against a role for anergy, as anergic cells are reported to not express the IL-2 receptor [Schwartz, 1990]. Expression of CD25 was shown primarily on $CD4^+$ cells and was expressed equally on around 10% of unstimulated $CD4^+$ lymphocytes from both peanut-sensitive and peanut-tolerant animals, which confirms figures of CD25 expression on resting $CD4^+$ T cells from other adult mice [Read et al., 2000; Takahashi et al., 1998]. The population of $CD4^+CD25^+$ cells nearly doubled following 96 hr stimulation with peanut protein. This could perhaps be expected in peanut-sensitive animals, as CD25 expression is induced on activated lymphocytes and needed for IL-2 responsiveness and proliferation. More surprisingly, it was shown that stimulation of

cells from peanut-tolerant animals induced CD25 expression in an equal or even higher number of $CD4^+$ cells. As cell death was minimal in the cultures of both peanut-sensitive and peanut-tolerant cells, it is unlikely that the increased proportion of peanut-specific $CD25^+$ cells observed following oral tolerance induction can merely be explained by preferential death of $CD25^-$ cells. Other experiments have demonstrated that immunogenic protocols (injecting peptide with an adjuvant) yields early and transient expression of CD25 on T cells, while in tolerogenic protocols (injecting low dose peptide without adjuvant) the emergence of antigen-specific $CD25^+$ T cells is later but the total number of specific $CD25^+$ cells remain constant for a longer period [Thorstenson and Khoruts, 2001]. Similarly, it may be that the peanut-sensitive $CD25^+$ cells would retract following the initial expansion, while the peanut-tolerant $CD25^+$ cells numbers would remain constant for longer. However, kinetic analysis of CD25 expression remains to be done in the current peanut protein model.

There is ample evidence that a subpopulation of immunoregulatory $CD4^+$ T cells contributes to immunological tolerance [Shevach, 2000] and oral administration of antigen is probably the 'oldest' approach to induce such suppressor or regulatory cells [Chen et al., 1994; Gershon and Kondo, 1971]. Recent work suggests that coexpression of CD25 defines at least a subset of this immunoregulatory $CD4^+$ T cell population (T_{reg}). Several studies have shown that $CD4^+CD25^+$ T cells are both anergic and suppressive *in vitro* [Nakamura et al., 2001; Takahashi et al., 1998; Thornton and Shevach, 1998] and *in vivo* [Read et al., 2000; Suri-Payer et al., 1998]. CD25 is however not an ideal marker to identify a T_{reg} population, as CD25 is transiently expressed upon activation of naïve $CD4^+$ T cells and its expression is highly dynamic *in vivo* [Annacker et al., 2001]. In the current model of oral tolerance to peanut protein, CD25 was highly expressed on activated $CD4^+$ cells from both sensitive and tolerant animals. However, double-staining with CFSE revealed functional differences between the $CD25^+$ cells from peanut-sensitive and peanut-tolerant animals. Peanut-tolerant $CD25^+$ cells were partially anergic and only minimal dilution of CFSE was observed, while $CD25^+$ cells from peanut-sensitive animals showed marked proliferation. In addition, the $CD4^+$ cells, and especially the $CD25^+$ cells, from peanut-tolerant animals expressed large amounts of CTLA-4 following stimulation with peanut protein, while very little or no CTLA-4 was expressed on $CD4^+$ or $CD25^+$ cells from peanut-sensitive animals upon stimulation.

Co-culture of peanut-sensitive LN cells with peanut-tolerant LN cells demonstrated marked suppression of the peanut-reactive cells from sensitive animals. Upon stimulation with peanut protein, proliferation as well as secretion of IFN-γ, IL-4 and IL-10 was significantly reduced when compared with cultures of stimulated peanut-sensitive cells only. In contrast, the secretion of TGF-β was enhanced. The peanut-reactive cells from sensitive animals thus appeared to become hyporesponsive to peanut stimulation when co-cultured with cells from peanut-tolerant animals. This strongly argues against a primary mechanistic role for deletion or anergy in oral tolerance and suggests that a mechanism of active suppression was responsible for the induction of oral tolerance to peanut protein. The regulatory or suppressive properties of the LN cells from animals orally-tolerised to peanut appeared to reside in the $CD25^+$ population. When peanut-sensitive cells were co-cultured with peanut-tolerant cells depleted of $CD25^+$ cells suppression was completely abolished. T cell proliferation as well as IFN-γ, IL-4 and IL-10 secretion was restored in the peanut-sensitised cells when only co-cultured with the $CD25^-$ LN fraction from peanut-tolerant animals. Several studies have previously shown that $CD4^+CD25^+$ T cells can suppress the proliferation of $CD4^+CD25^-$ T cells [Nakamura et al., 2001; Takahashi et al., 1998; Thornton and Shevach, 1998]. All these studies have however used purified $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell populations and co-cultures in a 1:1 ratio between $CD25^-$ and $CD25^+$ cells (or even higher ratios of $CD25^+$ T cells). In addition, naïve polyclonally activated $CD4^+CD25^-$ T cells have routinely been used as responder cells. In that light, the suppressive effect of whole LN cells from orally tolerised animals in this study is remarkable. Despite the dilution of $CD25^+$ cells by co-culture of whole LN cell suspensions, significant suppression was achieved in a 10:1 ratio between cells from peanut-sensitive and peanut-tolerant animals. Contrary to other studies, the demonstrated active suppression was on an established, memory immune response and not on the induction of a response from naïve cells.

The mechanisms mediating the suppressive function of $CD4^+CD25^+$ T_{reg} cells are probably multiple. The relative importance of individual factors in the function of T_{reg} depends on the experimental model. $CD4^+CD25^+$ T_{reg} cells ability to inhibit the proliferation of other T cell populations *in vitro*, has repeatedly been shown to be mediated through a cell contact-dependent mechanism independent of IL-4, IL-10 and TGF-β [Stephens et al., 2001; Takahashi et al., 1998; Thornton and Shevach, 1998]. Ligation of CTLA-4 on the surface of activated T cells, by its ligands CD80 and CD86, strongly inhibits T cell activation [Chambers

et al., 2001], and suppression through CTLA-4 has been demonstrated to be an important cell-contact dependent mechanism of $CD4^+CD25^+$ T_{reg} cells [Read et al., 2000; Takahashi et al., 2000]. Whether the inhibitory effect of peanut-tolerant cells on peanut-sensitive cells in this study was dependent on cell-cell contact or could be mediated through soluble factors alone was not directly addressed. However the fact, that CTLA-4 was solely expressed on peanut-tolerant $CD4^+$ cells and predominantly on the $CD25^+$ population, indicates that CTLA-4 may be functionally important for the inhibitory function of these cells. Indeed, the active suppression on peanut-sensitive cells by peanut-tolerant cells was shown to be critically dependent on CTLA-4 ligation, as suppression was completely reversed by blockade of CTLA-4. An important role for CTLA-4 has also been shown in the inductive phase of oral tolerance, where *in vivo* blockade of CTLA-4 induces enhanced T cell expansion and production of OVA-specific immunoglobulins following oral administration of OVA [Chen et al., 2002]. How CTLA-4 may be involved in oral tolerance and the function of T_{reg} remains to be defined. CTLA-4 binds B7 molecules with higher affinity than CD28, and it has been suggested that the preferential binding of CTLA-4 prevents CD28-mediated signals, which otherwise would abrogate the suppressive function of T_{reg} [Takahashi et al., 2000]. Additionally, CTLA-4 signalling may be required for the activation and induction of the effector function of $CD4^+CD25^+$ T_{reg} cells. It has been shown that signalling through CTLA-4 enhances proliferation of $CD4^+CD25^+$ T cells and induces secretion of TGF-β [Chen et al., 1998; Nakamura et al., 2001].

In contrast to a number of *in vitro* studies, *in vivo* experiments with animal models provide strong evidence of a role for cytokines in the effector function of $CD4^+CD25^+$ T_{reg} cells. The cytokines reported to be involved vary depending on the model. The cytokines receiving most attention as being implicated in oral tolerance are IL-10 and TGF-β. Following induction of oral tolerance to peanut protein in the current model, IL-10 secretion was significantly downregulated while TGF-β secretion was enhanced from peanut-tolerant cells (Chapters 3 & 4). The actual TGF-β production was not significantly higher ($p=0.071$) in cells from peanut-tolerant animals compared to peanut-sensitive animals. TGF-β was, however, demonstrated to play an important role in mediating the suppressive effect of peanut-tolerant cells on peanut-sensitive cells. Blocking TGF-β, with neutralising anti-TGF-β antibodies, in co-cultures of peanut-sensitive and peanut-tolerant cells, reversed the suppression and the proliferation of peanut-sensitive cells was completely restored. Exogenous addition of recombinant active

TGF-β to peanut-sensitive cells stimulated with peanut protein provided further confirmation that TGF-β was capable of significantly suppressing T cell proliferation under these conditions. In contrast, the addition of anti-IL-10 neutralising antibodies to the co-cultures had no effect on the suppressive mechanisms of peanut-tolerant cells (data not shown). High concentrations (50μg/ml) of high affinity anti-TGF-β antibodies were needed for inhibition of the suppressive mechanisms in this study, which were in a similar range as reported to reverse the suppressive function of purified $CD4^+CD25^+$ T_{reg} cells [Nakamura et al., 2001]. This may be due to the fact that TGF-β is normally present in its latent form, and anti-TGF-β antibodies can therefore only bind and neutralise TGF-β in the short period between its conversion to an active form and its interaction with a relevant TGF-β receptor. The importance of TGF-β on the suppressive effect of peanut-tolerant cells on peanut-sensitive cells reported here is contrary to most *in vitro* studies using purified $CD4^+CD25^+$ T cells, which suggest that the suppressive mechanisms are independent of regulatory cytokines, but critically dependent on cell-cell contact [Stephens et al., 2001; Takahashi et al., 1998; Thornton and Shevach, 1998]. This discrepancy could possibly be explained by the finding that $CD4^+CD25^+$ T cells express TGF-β bound to the cell-surface and thus can mediate cell contact-dependent suppression, which is simultaneously critically dependent on TGF-β [Nakamura et al., 2001]. Surface expression of TGF-β on peanut-tolerant cells was examined in this study using two different antibodies. No reproducible surface-bound TGF-β was detected, and this possible mechanism of T_{reg} mediated suppression could thus not be confirmed.

The pivotal roles of CTLA-4 and TGF-β in actively suppressing the response to peanut protein in this study additionally support the suggestion that T_{reg} cells require activation through their TCR for *in vitro* suppression. CTLA-4 was only expressed on peanut-tolerant $CD4^+CD25^+$ cells following stimulation with peanut protein, and TGF-β was only secreted in response to peanut protein. The induction of T_{reg} cells and their suppressive function thus appear to be antigen-specific. It has been suggested that although dependent on initial TCR activation the $CD4^+CD25^+$ T_{reg} cells inhibit T cell responses in an antigen-nonspecific manner [Thornton and Shevach, 2000]. As a consequence of the experimental set up, this could not be directly tested with the T_{reg} cells induced after oral administration of 100mg peanut protein in the present study.

Existing evidence suggests that $CD4^+CD25^+$ T cells are naturally occurring regulatory cells, which arise directly from the thymus following a unique lineage of $CD4^+$ T cell development [Bensinger et al., 2001; Itoh et al., 1999]. Whether *de novo* development of T_{reg} cells can occur in the periphery has remained elusive. Oral administration of antigens has since long been shown to induce cells with a suppressive function. The relationship between these induced suppressive cells and the naturally occurring $CD4^+CD25^+$ T_{reg} population is unclear. This study shows that naïve $CD4^+$ T cells in the normal peripheral lymphoid environment may develop into T_{reg} cells of similar phenotype as the immunoregulatory thymic $CD4^+CD25^+$ T cells. The specific factors that promote the differentiation of such peripheral T_{reg} are unknown. It may be that TGF-β and/or CTLA-4 are important as both suppress T cell proliferation, and the number of cell divisions following antigen stimulation has been shown to be a major variable controlling $CD4^+$ T cell differentiation [Bird et al., 1998]. The results presented here, suggest that antigen encounter accompanied by minimal or no cell division are optimal conditions for peripheral induction of T_{reg} from naïve precursors. The ease of oral tolerance induction in general may reflect that epithelial-associated lymphoid tissue such as the GALT could be ideal for *de novo* development of T_{reg}. In the gut microenvironment secretion of TGF-β, immature DCs without surface expression of CD80/CD86 and specialised subsets of DCs are abundant and could possibly participate in the education of naïve $CD4^+$ T cells into T_{reg}. However, many questions remain unanswered about how and where T_{reg} cells can be best induced, what their precise mechanisms of action are, what their target cells are, how they are controlled and how APCs such as DCs are involved. In addition it will be clinically important to determine whether differentiated effector or memory $CD4^+$ cells can also be driven to acquire an anergic and possibly immunoregulatory phenotype *in vivo*.

Results presented here demonstrate that a single high dose feed of peanut protein induced a population of regulatory T cells with suppressive properties. Other studies have shown that single high dose antigen feeds (mostly OVA) results in clonal deletion or anergy. This discrepancy may reflect physiological differences in handling high doses of single proteins and antigenic mixtures such as peanut. In addition, it highlights the importance of studying not only the dose required for oral tolerance to individual antigens (or antigenic mixtures), but also the underlying mechanisms. Oral administration of self antigens has been successfully applied as a therapeutic option in numerous animal models of autoimmune diseases. The encouraging results from these experimental models have unfortunately not been reproduced in human

disease trials. Clinical application of oral tolerance as a therapeutic option in food allergic diseases has also proven unsatisfactory. The application of oral tolerance in human disease trials has demonstrated difficulties such as dose-dependent effects in groups of patients, and has highlighted that the mechanisms of induction and maintenance of oral tolerance are not yet fully understood. Oral administration of soluble peanut protein has here been shown to induce a population of specific T_{reg} cells, which could suppress both Th1- and Th2-type responses. Importantly the induced T_{reg} cells were also able to down-regulate an established memory T cell response from peanut-sensitive animals. The ability to induce and manipulate such T_{reg} cells *in vivo* and *in vitro* for optimal induction of antigen-specific immunological tolerance may have important therapeutic benefits. An understanding of the milieu that is conducive for optimal expansion and function of T_{reg} cells would be of extreme clinical relevance and possibly have implications for immune therapy in a diverse number of diseases, including allergy, transplantation and autoimmune diseases.

Chapter 5

Epicutaneous exposure to protein antigens induces systemic Th2-biased immunity

5.1 Introduction

The skin is a unique immunological organ which is naturally exposed to a variety of environmental agents including allergens. The outer horny layer, the stratum corneum, normally presents a barrier between the skin-associated lymphoid tissue and environmental antigens. Resistance to biological and chemical permeants as well as ultraviolet light resides almost entirely in the stratum corneum [Elias and Menon, 1991]. Underneath, a multiple of cell types and functions compose the skin-associated lymphoid tissue, which holds the key to how antigens delivered to the skin can elicit either immunogenic or tolerogenic responses. Whether the responses remain local to the skin or extend to the systemic immune system is controlled by the resident antigen-presenting cells (APCs) and the resident T cells. The specialised APCs within the epidermis that perform this function are Langerhans cells (LC). LCs are members of the wider family of dendritic cells (DCs), but are functionally and spatially distinct from the dermal DCs that reside in the dermis [Maurer and Stingl, 2001]. LCs are interspersed in the keratinocyte layer and form a contiguous network in the epidermis where they serve as sentinels of the immune system, sampling antigens and responding to changes in the external environment. They can then relay antigenic information to the adaptive immune system by migrating from the epidermis to the draining skin lymph nodes [Banchereau and Steinman, 1998; Kimber et al., 2000].

In inflammatory diseases of the skin such as atopic dermatitis, eczema and psoriasis the cutaneous barrier properties are diminished and otherwise harmless environmental agents may cause irritation and inflammation. Disrupting the cutaneous permeability barrier experimentally, by extracting the lipids from the stratum corneum with organic solvents or removal of the stratum corneum by mechanical tape-stripping, initiates a chain of biological events in the epidermis very similar to that seen in inflammatory skin diseases. In both mice [Nishijima et al., 1997; Wood et al., 1992] and men [Nickoloff and Naidu, 1994] levels of TNF-α, IL-1α, IL-1β and GM-CSF are rapidly elevated several-fold in the disrupted epidermis, and LCs are induced to express high levels of MHCII and costimulatory molecules such as CD86 and CD54 [Nishijima et al., 1997]. Considerable evidence suggests that IL-1 and TNF-α play an important role in promoting LC migration [Cumberbatch and Kimber, 1992; Roake et al., 1995] and may also functionally mature the LCs into potent T cell stimulators. Such augmentation of immune reactivity may be critical to the elimination of microbes or environmental noxious agents that easily penetrate through the barrier-disrupted epidermis,

but may also potentially cause unwanted immune and/or inflammatory responses to innocuous agents and antigens.

Food allergy and skin diseases such as atopic dermatitis (AD) are interlinked, although the mechanisms involved remain unknown [Werfel, 2001]. Most food allergic children also suffer from eczema or AD, which usually predates development of the food allergy [Sampson, 1999; Sicherer and Sampson, 1999]. Ninety five percent of peanut-allergic individuals have been shown to suffer from other atopic diseases, and eczema is particularly prevalent. Eighty percent of peanut-allergic individuals react to their first known oral exposure to peanut, implying some prior occult sensitisation of which skin is a likely possibility [Hourihane et al., 1996]. Primary sensitisation through the skin could occur from the use of topically applied creams and lotions, where peanut oil has been a common ingredient, to control eczema. Protein allergens can elicit eczematous inflammatory reactions in the skin of sensitised individuals as demonstrated in the atopy patch reaction [Darsow et al., 1996] but it is less clear whether exposure of skin to protein antigens can stimulate a primary immune response. Prolonged cutaneous exposure to OVA in an occlusive patch on shaved skin has been shown to sensitise mice and induce a typical Th2-type response [Wang et al., 1996]. Epicutaneous sensitisation to the hapten picryl chloride through shaved and barrier-disrupted skin has also been shown to elicit a Th2-dominant response that is clearly distinct from the response induced through intact skin [Kondo et al., 1998].

In this Chapter, the capacity of peanut allergens and the commonly used protein antigen, OVA, to induce primary sensitisation through the skin is explored. Evidence is presented that exposure to protein allergens on abraded skin induces primary antigen-specific systemic sensitisation without adjuvants. This sensitisation was strongly Th2-biased as shown by IL-4 production and high levels of antigen-specific IgG1 and IgE but undetectable IgG2a. Removal of the stratum corneum by abrasion was found to induce maturation of LCs in the epidermis, but rapid migration to the draining lymph nodes only occurred following additional antigen exposure. Subcutaneous injections gave rise to less potent sensitisation and to immune responses showing Th1-bias. These results suggest that the epidermis and the LCs play a pivotal role in enhancing the protein immunisation and promoting Th2 responses.

5.2 Immunisation protocol

Epicutaneous immunisation

The skin on the ears of the animals was used for epicutaneous sensitisation and no shaving procedure was necessary. The corneum permeability barrier of the skin was disrupted by abrasion with cellophane tape. Twenty-four hr after tape-stripping, 100μg OVA or peanut protein in PBS were applied to both sides of the earlobe and again on the next two consecutive days. Mice with non-abraded intact skin were immunised in an identical manner. PBS without antigen was applied to abraded and intact skin for controls (for further details on the epicutaneous immunisation procedure see Chapter 2, section 2.10.2). Nineteen days after the initial skin exposure animals were injected with 100μg OVA or peanut in the footpad and the DTH responses were measured. Six, 10 or 20 days after the initial skin exposure, mice were killed and draining cervical lymph nodes (CLN), mesenteric lymph nodes (MLN), spleen and serum were collected and analysed. Fig. 5.1 below shows a schematic representation of the experimental protocol.

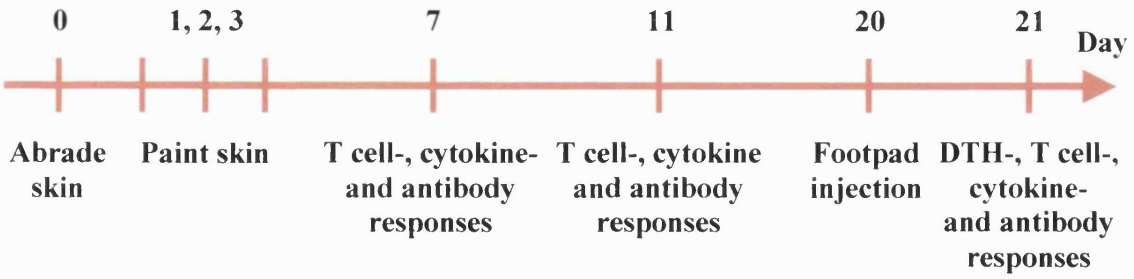


Fig. 5.1 Schematic diagram of the epicutaneous immunisation protocol
Diagram of the experimental protocol used to study the effect of epicutaneous exposure to protein antigen. Analysis of responses to epicutaneous protein antigen was carried out at 6, 10 and 20 days following the primary antigen exposure (on day 7, 11 and 21 respectively as shown on the diagram)

Subcutaneous immunisation with antigen in CFA

The effect of epicutaneous immunisation was compared to responses obtained after subcutaneous immunisation with antigen in CFA (the immunisation method employed in Chapter 3 & 4). Animals were tail-base immunised with 100μg peanut protein or OVA emulsified 1:1 in CFA. Three weeks later they were given a recall immunisation with 100μg peanut protein or OVA in the footpad and the DTH responses were measured. One week after the footpad injection the mice were killed and levels of specific serum antibodies determined.

Epicutaneous versus Subcutaneous immunisation

To assess the direct impact of the route of antigen exposure on the nature of the immune response, the epicutaneous immunisation was compared to a more conventional subcutaneous immunisation protocol. Epicutaneous immunisation was performed as described above and compared to subcutaneous injection of 100μg protein antigen in sterile PBS (no adjuvant) at the base of each ear for three consecutive days. Twenty days after the initial epi/subcutaneous exposure mice were killed and CLN, spleens and serum collected and analysed.

Abbreviations used for the particular immunisations in this Chapter are listed in Table 5.1.

Table 5.1 Abbreviations used for cutaneous immunisation protocols

Procedure / Abbreviation	Skin abrasion	Epicutaneous application	Subcutaneous without adjuvant	Subcutaneous with CFA and recall immunisation
NS	-	Saline	-	-
NP	-	Peanut	-	-
AS	Abrasion	Saline	-	-
AP	Abrasion	Peanut	-	-
AO	Abrasion	OVA	-	-
PP	-	-	-	Peanut
OO	-	-	-	OVA
EPI	Abrasion	Peanut	-	-
SUB	-	-	Peanut	-

5.3 Results

5.3.1 Primary sensitisation to protein antigens through the skin

Acute cutaneous barrier disruption as seen in patients with AD was mimicked experimentally by stripping the surface of the skin with cellophane tape (abrasion). Histological examination of H&E stained sections of ear skin following abrasion by tape-stripping showed that the stratum corneum was removed but the underlying living epidermal tissue remained apparently intact (Fig. 5.2). Ear swelling occurred transiently 1-4 hr after the skin abrasion. At 24 hr after abrasion the stratum corneum was partially restored. Some ear swelling was still evident 24 hr after the abrasion and a minor cellular infiltrate was observed in the dermis (Fig. 5.2). By 48 hr after the abrasion, the stratum corneum was fully restored and the skin had visually returned to normal.

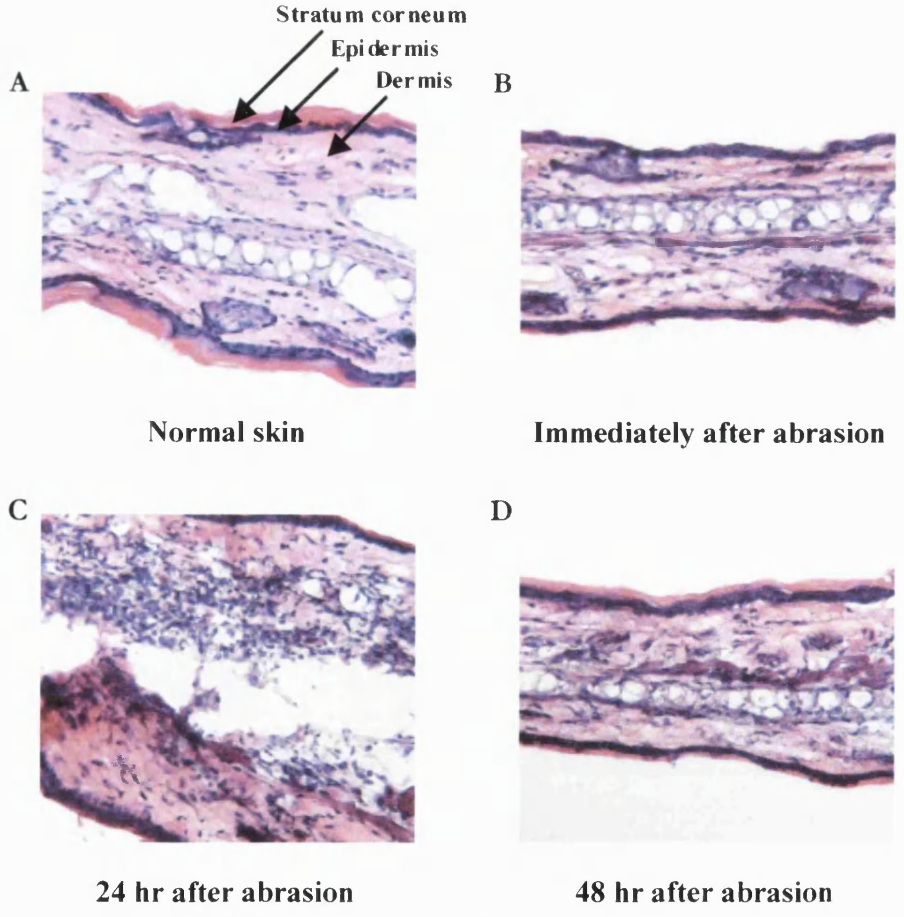


Fig. 5.2 H&E stained cryostat sections of ear skin

H&E stained cryostat sections (7.5µm thick) of normal ear skin (A), skin immediately (B), 24 hr (C) and 48 hr (D) after abrasion. Abrasion of the skin was performed by mechanical tape-stripping. Original magnification x20

Antibody and DTH responses obtained after epicutaneous exposure to crude peanut protein extract or highly purified commercially available OVA were compared to responses obtained after subcutaneous immunisation with CFA and recall immunisation. Epicutaneous exposure to antigen on abraded skin induced a definite antigen-specific immune response, involving both the humoral and cellular arms of the immune system. Levels of peanut- and OVA-specific IgG antibodies in serum were significantly elevated compared to controls exposed to saline (Fig. 5.3A). The levels of specific IgG induced by the epicutaneous route with no adjuvant were however significantly lower than in animals immunised subcutaneously with CFA (Fig. 3.5A). *In vivo* cellular responses were also evident following epicutaneous immunisation, as significant DTH responses were induced after footpad injection of peanut protein or OVA (Fig. 5.3B). DTH-responses obtained by epicutaneous immunisation were similar in magnitude to responses after subcutaneous immunisation with CFA (Fig. 5.3B).

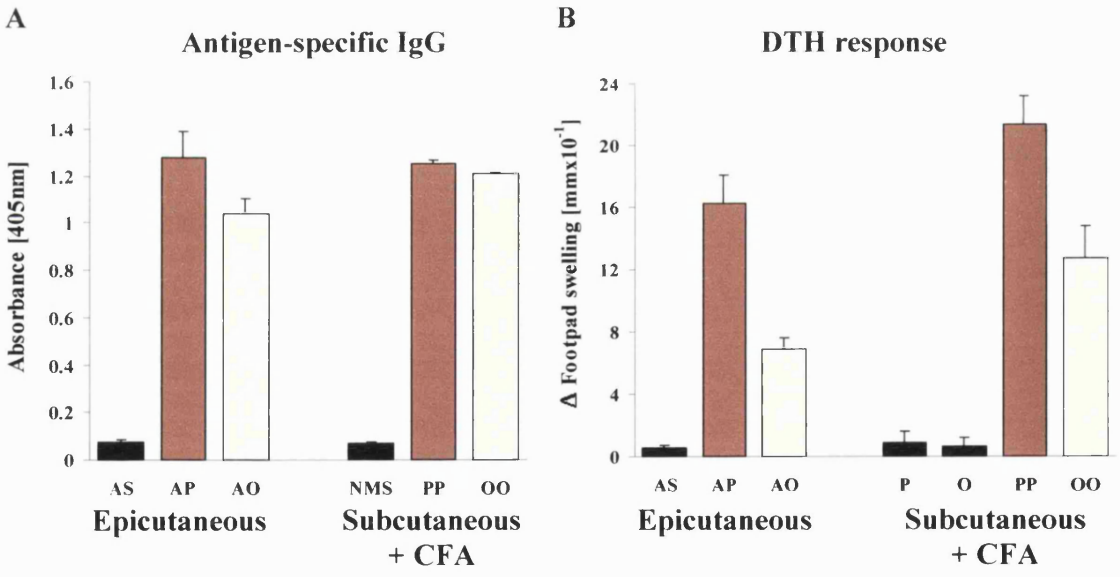


Fig. 5.3 Antigen-specific humoral and cellular responses following epicutaneous immunisation or subcutaneous immunisation with CFA

(A) Peanut- or OVA-specific IgG 20 days after epicutaneous immunisation or 4 weeks after subcutaneous immunisation with CFA. Sera were diluted 1:10 following epicutaneous immunisation and 1:1000 following subcutaneous immunisation with CFA. (B) DTH responses 24 hr after antigen challenge in the footpad. Challenge was performed 20 days after epicutaneous- or subcutaneous with CFA immunisation. Results are expressed as mean + 1 SEM (n = 6-10). Similar results were obtained from ≥ 8 individual experiments. [AS: Abraded skin painted with saline; AP: Abraded skin painted with peanut; AO: Abraded skin painted with OVA; NMS: Naïve mouse serum; PP: Immunisation with peanut in CFA and recall immunisation with peanut; OO: Immunisation with OVA in CFA and recall immunisation with OVA; P/O: Recall immunisation with peanut or OVA only]

5.3.2 Epicutaneous immunisation through abraded skin induces Th2-biased immunity

To characterise the nature of the immune response induced by epicutaneous immunisation to the protein antigens, levels of antigen-specific IgG subclasses and IgE antibodies were measured in serum 20 days after epicutaneous immunisation. A distinct humoral response with high levels of IgG1 and IgE but little or no IgG2a was obtained, indicating a potent Th2 bias. No specific antibodies could be detected in animals epicutaneously exposed to antigen without prior abrasion of the skin (Fig. 5.4A-D). Animals immunised subcutaneously with antigen in CFA showed a different antibody profile, with high levels of IgG2a and IgG1, but low levels of IgE (Fig. 5.4E-H). The very potent immunisation with CFA and recall immunisation, yielded higher levels of antigen-specific IgG than the epicutaneous route, but it gave rise to a markedly smaller IgE response. Levels of total IgE after epicutaneous immunisation with peanut protein reached 1609 ± 170 ng/ml which was significantly higher than the 704 ± 175 ng/ml IgE following subcutaneous immunisation with CFA ($p < 0.005$, $n = 6$). For OVA sensitisation the total IgE levels were 589 ± 49 ng/ml and 345 ± 42 ng/ml for epicutaneous immunisation and subcutaneous with CFA and recall immunisation respectively ($p < 0.005$, $n = 6$). Levels of total IgE corresponded completely with levels of antigen-specific IgE (results not shown).

It should be noted that following epicutaneous immunisation the primary immune responses to the antigens were analysed while following subcutaneous immunisation with CFA and recall immunisation the secondary immune responses were analysed.

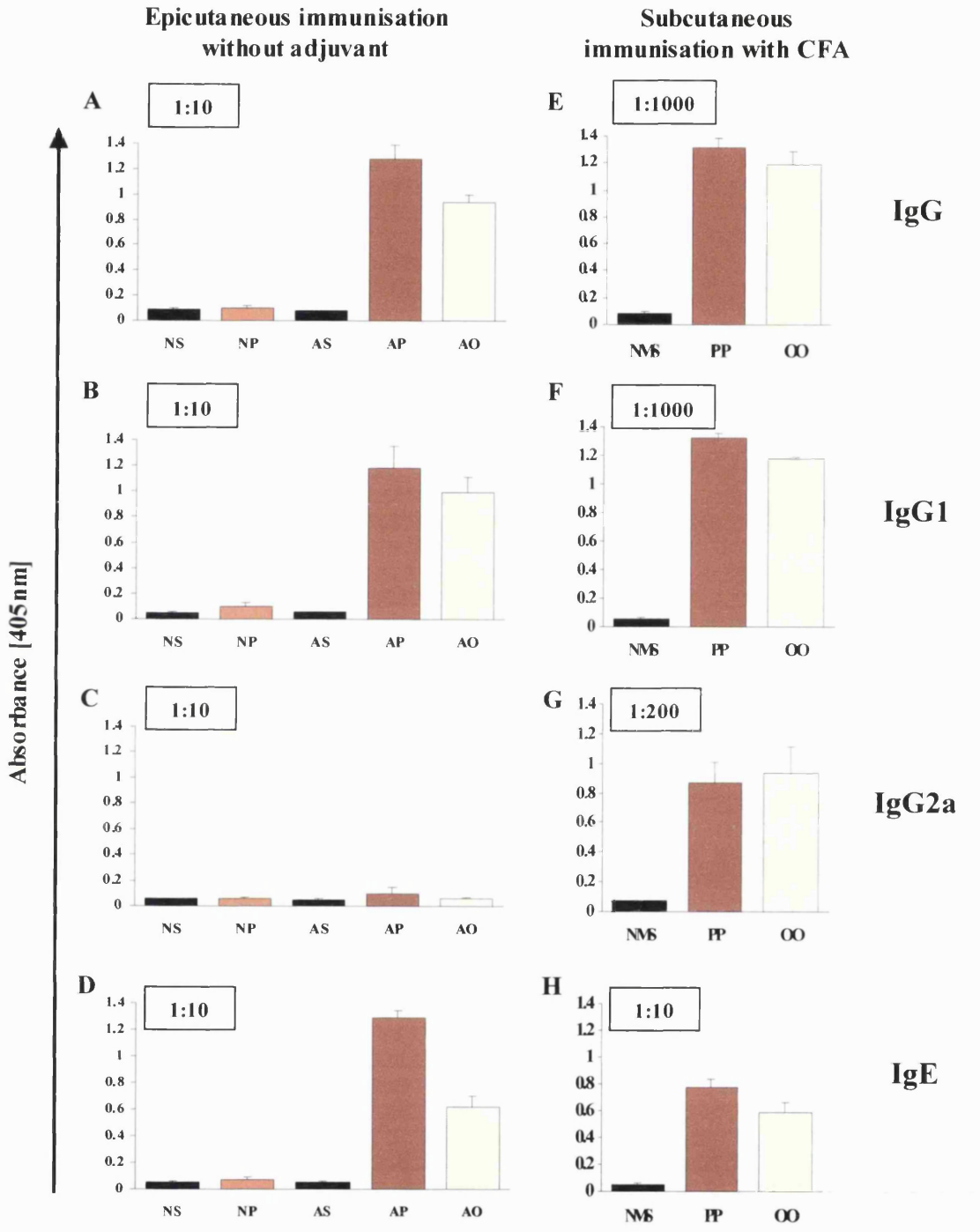


Fig. 5.4 Antigen-specific antibody responses following epicutaneous immunisation or subcutaneous immunisation with CFA

Peanut- and OVA-specific antibody responses following epicutaneous immunisation (A-D) or subcutaneous tail-base immunisation with CFA and recall immunisation (E-H). Antibodies were measured 20 days after epicutaneous immunisation or 4 weeks after immunisation with CFA. Sera were diluted 1:10 times following epicutaneous immunisation and between 10 and 1000 times following subcutaneous immunisation with CFA (dilution factor shown on individual graphs). Results are expressed as mean antibody levels + 1 SEM ($n \geq 6$). Similar results were obtained from at least 8 individual experiments. [NS: Normal skin painted with saline; NP: Normal skin painted with peanut; AS: Abraded skin painted with saline; AP: Abraded skin painted with peanut; AO: Abraded skin painted with OVA; NMS: Naïve mouse serum; PP: Peanut immunisation with CFA and recall immunisation with peanut; OO: OVA immunisation with CFA and recall immunisation with OVA]

The immune responses following epicutaneous immunisation with peanut protein and OVA were explored further by examining antigen-specific T cell responses in cultures of cells from the draining skin lymph nodes (CLN), the spleen and from the draining lymph nodes of the gut (MLN).

Six days after epicutaneous immunisation with either peanut protein (Fig. 5.5A) or OVA (Fig. 5.5B), a strong antigen-specific proliferative response was obtained with T cells from the CLNs, which was not seen in control animals. Similar levels of T cell proliferation were observed in cultures of spleen cells, indicating systemic sensitisation. No proliferation to peanut protein or OVA was observed in cultures of MLN cells, consistent with sensitisation through the skin and not via gastrointestinal exposure through grooming (Fig. 5.5). Similar peanut- and OVA-specific proliferative T cells responses were found in CLN and spleen at 10 and 20 days after the initial epicutaneous immunisation (data not shown). A much weaker but definite peanut-specific T cell proliferation was observed in cultures of both CLN and spleen cells from animals epicutaneously exposed to peanut protein but without prior skin abrasion (Fig. 5.5, NP).

Cytokine patterns produced by CLN, spleen and MLN cells collected at 6, 10 and 20 days after epicutaneous immunisation with peanut protein or OVA were analysed following ***in vitro*** culture with peanut protein or OVA. Levels of IFN-γ produced by CLN cells 6 days after the epicutaneous immunisation were around 3500pg/ml but the levels decreased significantly after 10 days and even further to around 600pg/ml 20 days after the initial immunisation (Fig. 5.6A). In contrast, IL-4 levels were elevated above background to 25pg/ml 6 days after the epicutaneous immunisation and increased substantially to around 155pg/ml when analysed 10 and 20 days after the initial epicutaneous immunisation (Fig. 5.6B). A similar cytokine pattern was produced by spleen cells although the levels of cytokines were lower (especially IFN-γ). No detectable cytokines were produced by MLN cells or by CLN cells from control animals epicutaneously exposed to saline. Cytokine secretion was not detectable when cells were stimulated with a control antigen or when left un-stimulated (data not shown).

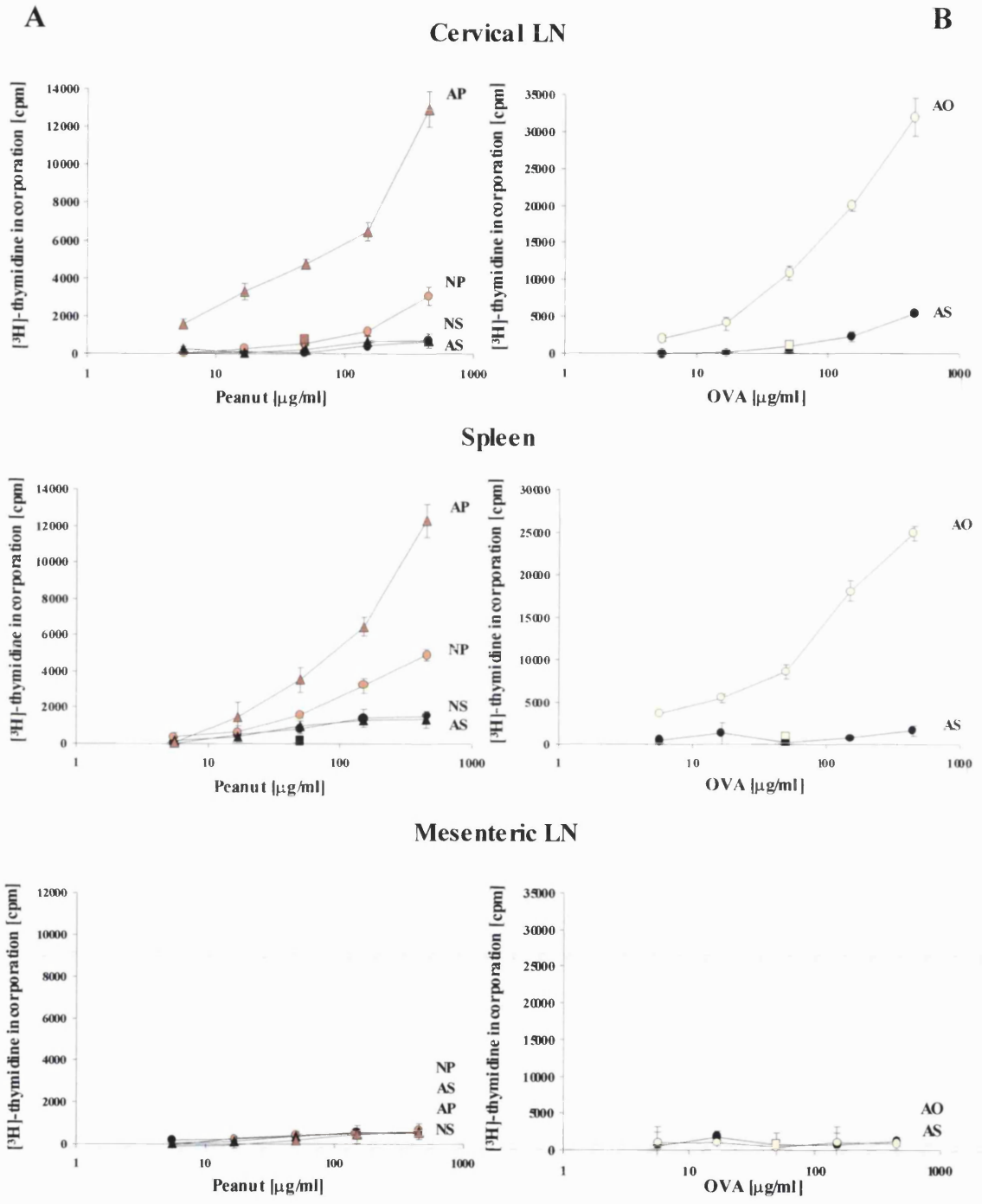


Fig. 5.5 Antigen-specific T cell proliferation in response to epicutaneous immunisation
Peanut- (A) or OVA- (B) specific proliferation by CLN, spleen and MLN cells collected 6 days after the initial epicutaneous immunisation. Cells were pooled from 6 mice per group and each data point represents the mean cpm of triplicate cultures for 90 hr ± 1 SEM. Background proliferation when no antigen was present has been subtracted. Squares (where visible) represent proliferation to a control antigen. All groups proliferated with similar vigour to mitogenic stimuli with ConA. Similar proliferation patterns were seen both 10 and 20 days after the epicutaneous immunisation. The experiment was repeated at least 8 times with similar results. [AP (▲): Abraded skin painted with peanut, AS (▲): Abraded skin painted with saline, NP (●): Normal skin painted with peanut; NS (●): Normal skin painted with saline; AO ( ): Abraded skin painted with OVA; AS (●): Abraded skin painted with saline]

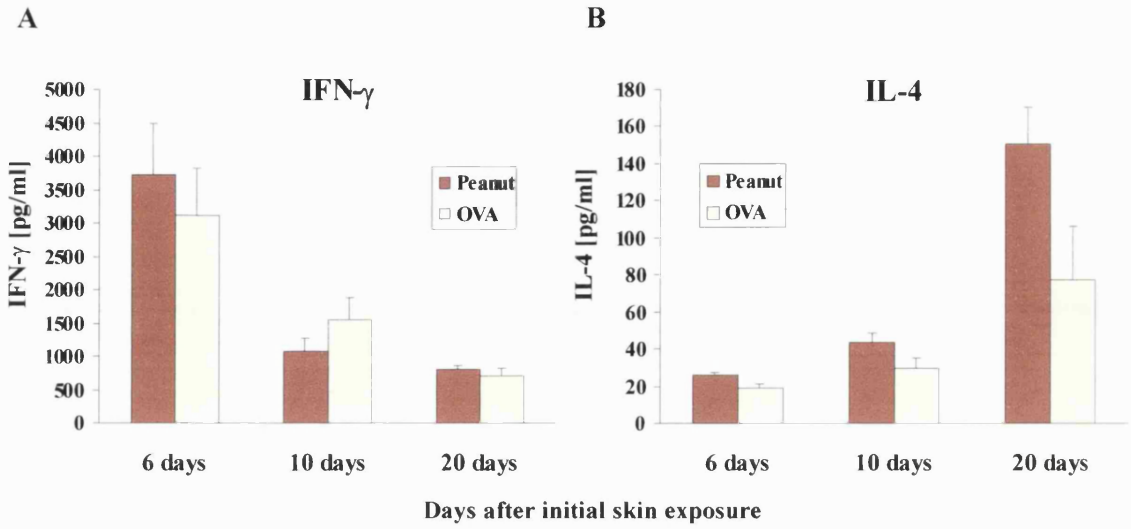


Fig. 5.6 Cytokine production by CLN cells following epicutaneous immunisation
Cytokine production by draining CLN cells collected 6, 10 and 20 days after the initial epicutaneous immunisation with peanut protein or OVA. Secretion of IFN-γ (A) and IL-4 (B) was analysed after 96 hr *in vitro* stimulation with peanut protein or OVA. Cells were pooled from 6 mice per group and analysed in triplicate. Results are expressed as mean cytokine levels + 1 SEM of 2-4 individual experiments.

5.3.3 Differential impact of the route of immunisation

The impact of the route of immunisation was investigated by directly comparing responses to epicutaneous- and subcutaneous immunisation with peanut protein. Animals were either epicutaneously exposed to 100μg peanut protein on abraded skin for 3 days or injected subcutaneously with 100μg peanut protein in PBS at the base of each ear for 3 consecutive days. Twenty days after the initial epi- or subcutaneous immunisation, animals were injected with 100μg peanut protein in PBS in the footpad. At the same time a group of naïve control animals were given a similar footpad injection. DTH responses were measured as the footpad thickness before and 24 hr after this immunisation. Substantial DTH responses were demonstrated after epicutaneous immunisation, which were significantly higher than observed after subcutaneous immunisation (Fig. 5.7A). The subcutaneous immunisation did however give rise to small DTH responses, that were significantly higher than in the animals subjected only to the footpad immunisation, which did not respond at all (Fig. 5.7A). The epicutaneous route of immunisation also gave rise to significant antigen-specific T cell proliferation in

cultures of CLN cells and spleen cells. Very little or no specific T cell proliferation was detectable when animals were immunised via the subcutaneous route in the absence of adjuvant (Fig. 5.7B).

Analysing the cytokine responses by CLN cells 20 days after the initial epi- or subcutaneous immunisation with peanut protein revealed strikingly different patterns following the two routes of immunisation (Fig. 5.7C). The subcutaneous route of immunisation induced production of large amounts of IFN-γ and some IL-10 but only low levels of IL-4 by draining CLN cells. In contrast, the epicutaneous route yielded high levels of IL-4 but low amounts of IFN-γ and IL-10 (Fig. 5.7C).

Antigen administered subcutaneously gave rise to some specific antibody production. The nature of the antibody response was different to that seen after epicutaneous immunisation with a predominance of antibodies requiring Th1 help. Subcutaneous immunisation induced significantly less antigen-specific IgG1 and IgE was barely detectable. In contrast, significantly more specific IgG2a was induced after sub- than epi-cutaneous immunisation (Fig. 5.7D), suggesting that the epicutaneous route gave rise to a Th2-skewed response, while the immune response after subcutaneous immunisation was Th1-predominant. This observation was consistent with the cytokine data.

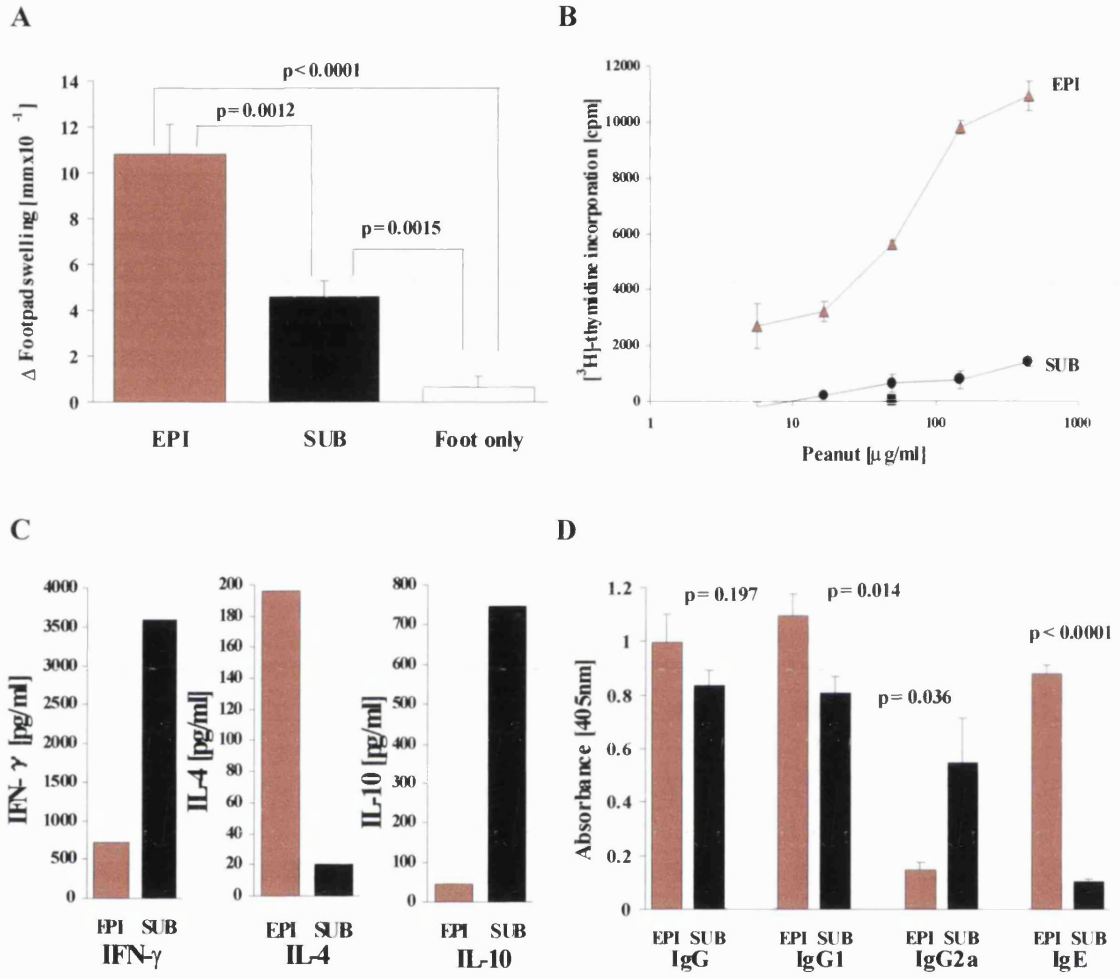


Fig. 5.7 Direct impact of the route of immunisation

Animals (n=6) were immunised with peanut protein epicutaneously or subcutaneously for 3 consecutive days and the peanut-specific responses compared 20 days after the initial epi/subcutaneous immunisation. (A) DTH responses following injection with peanut protein in the footpad. (B) Peanut-specific CLN T cell proliferation. CLN cells were pooled from 6 mice per group and each data point represents the mean cpm of triplicate cultures for 90 hr ± 1 SEM. Background proliferation when no antigen was present has been subtracted. Squares represent proliferation to a control antigen. The two groups responded equally to ConA. Similar proliferation patterns were seen by spleen cells. (C) Cytokine secretion from cultured CLN cells. Cytokines were measured following 96 hr stimulation with peanut *in vitro*. Cells were pooled from 6 animals per group and analysed in triplicate. (D) Peanut-specific IgG, IgG1, IgG2a and IgE in serum. Sera samples were diluted 1:10 prior to analysis. All results are expressed as mean + 1 SEM. [EPI: epicutaneous immunisation; SUB: subcutaneous immunisation].

5.3.4 Epicutaneous immunisation also induces potent Th2-biased immunity in C57BL/6 and CBA/C9 mice

To exclude the possibility that the effectiveness of the epicutaneous immunisation and the apparent Th2 dominance of the resulting immune response was linked to the phenotype of the BALB/c mice, immune responses following similar epicutaneous immunisation to peanut protein on two other mouse strains; C57BL/6 and CBA/C9 were investigated. The epicutaneous immunisation induced strong *in vivo* DTH responses in both C57BL/6 and CBA/C9 mice, which were significantly larger than in controls (Fig. 5.8A,B). Similarly, epicutaneous immunisation of both C57BL/6 and CBA/C9 mice induced significant specific proliferation by T cells from CLN and spleen, but not by T cells from the MLN (spleen and MLN data not shown) (Fig. 5.8C,D). As observed in the BALB/c mice, the resulting immune responses were predominantly Th2-like with significant amounts of IL-4, IgG1 and IgE but little IFN-γ and IgG2a (Fig. 5.8E,F – cytokine data not shown). This was true for both C57BL/6 and CBA/C9 mice with only minor strain variance in the amounts of cytokines secreted and specific antibodies produced.

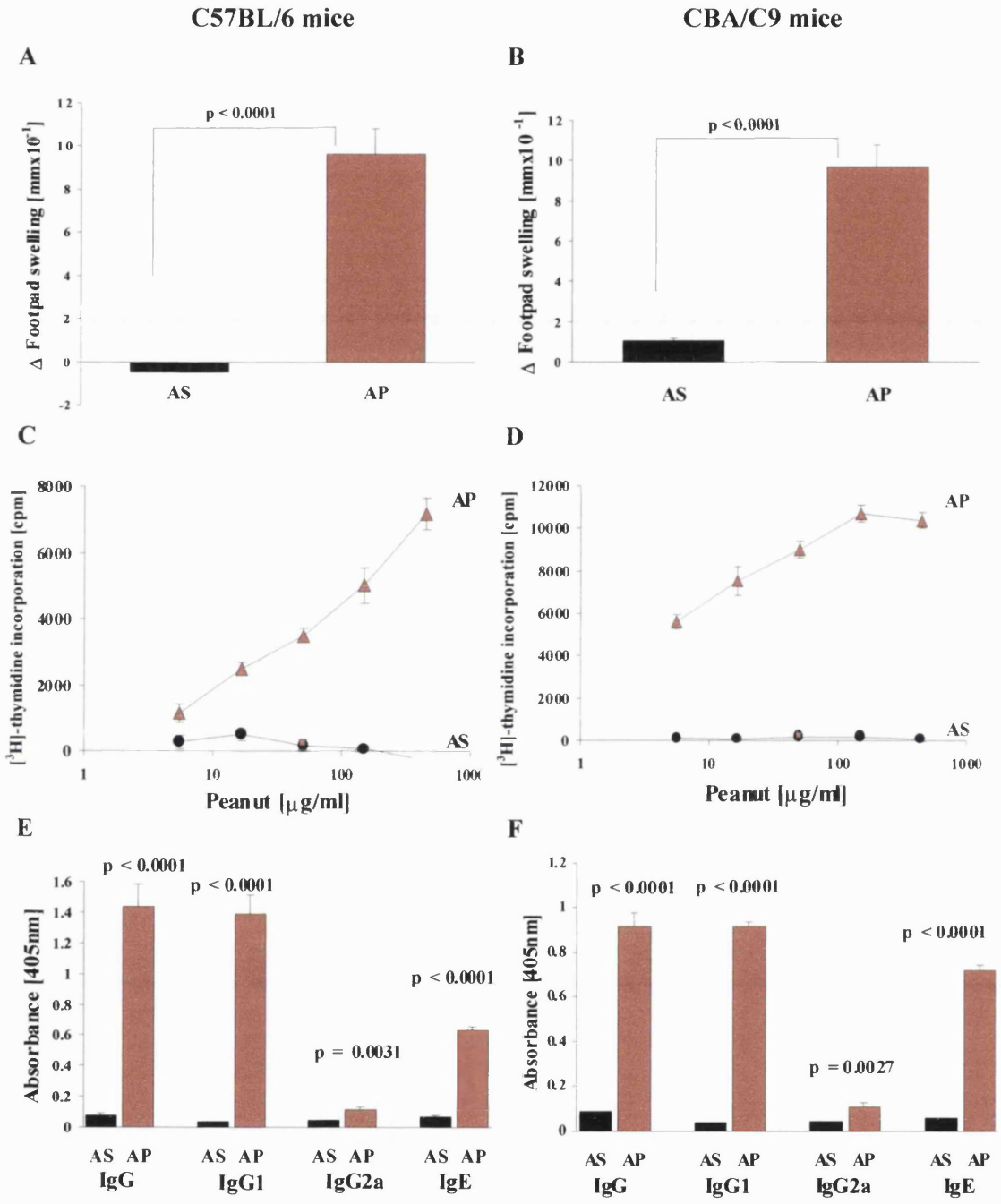


Fig. 5.8 Epicutaneous immunisation of C57BL/6 and CBA/C9 mice
C57BL/6 (A, C, E) and CBA/C9 (B, D, F) mice were epicutaneously immunised with peanut and the peanut-specific responses assessed 20 days later. (A) and (B) Mean DTH responses following a single peanut injection in the footpad. (C) and (D) Peanut-specific T cell proliferation in CLN cultures from C57BL/6 and CBA/C9 mice respectively. CLN cells were pooled from 6 mice per group and each data point represent the mean cpm of triplicate cultures for 90 hr ± 1 SEM. Background proliferation without antigen has been subtracted. Squares represent proliferation to a control antigen. CLN cells from all groups responded with similar cpm when cultured with ConA. Similar proliferation patterns were seen in spleen cell cultures, but no proliferation was observed in MLN cell cultures. (E) and (F) Levels of peanut-specific antibodies in serum. Sera samples were diluted 1:10 prior to analysis. All results are expressed as the mean + 1 SEM (n = 6). [AS (●): Skin abraded and painted with saline; AP(▲): Skin abraded and painted with peanut]

5.3.5 Skin abrasion alters Langerhans cell morphology and activation status

The results presented in this Chapter demonstrated that the effectiveness of immunisation through the skin was dependent on epidermal exposure and that immune responses induced by epicutaneous exposure to antigens were dramatically augmented by prior abrasion of the skin. This suggested that the epidermal cells were activated by the skin abrasion in a way to augment the antigen-specific immune responses. Langerhans cells (LC) are the professional antigen-presenting cells in the epidermis, and the effects of abrasion (removing the stratum corneum) on LCs were therefore investigated. Epidermal sheets from animals with intact skin, with abraded skin and after epidermal exposure to protein antigens were examined by confocal microscopy, and LC morphology and markers of maturation were investigated *in situ*. LCs express MHCII *in situ* and can be visualised in the normal epidermis as a uniform network of large MHCII$^+$ cells with dendritic morphology (Fig. 5.9).

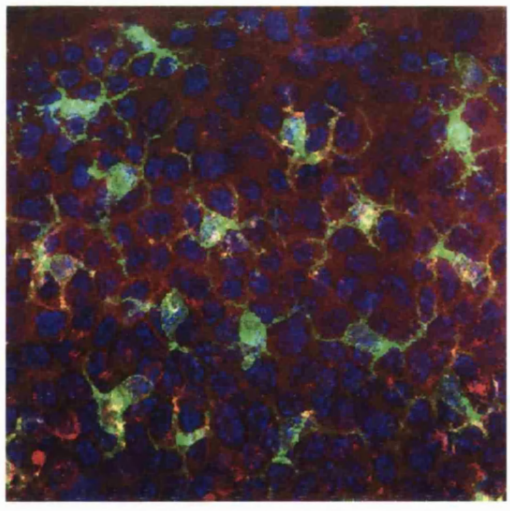


Fig. 5.9 Epidermis with Langerhans cells
Confocal fluorescence microscopy of normal mouse epidermis from ear skin. Fresh epidermal sheets were stained for MHCII (green), F-actin with phalloidin (red) and cell nuclei with TO-PRO (blue). Langerhans cells are seen as large MHCII$^+$ cells with dendritic morphology. Original magnification x100

In the normal epidermis, long MHCII$^+$ dendrites were seen extending out from the spindly cell body creating a contiguous network of brightly MHCII$^+$ stained LCs on top of the dimly MHCII$^+$ keratinocytes. The keratinocytes were visualised as cells forming a 'cobblestone' pattern underneath the LCs (Fig. 5.10 and 5.11A).

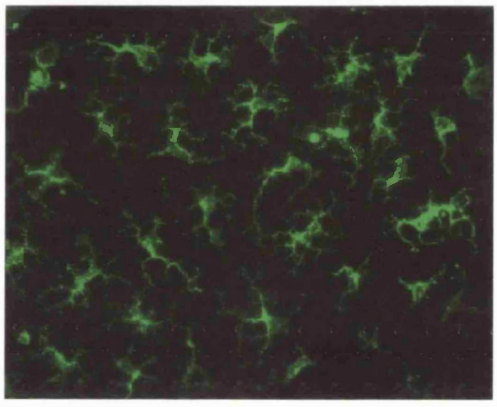


Fig. 5.10 Epidermis with MHCII$^+$ Langerhans cells and keratinocytes
Epidermal sheets from normal naïve mouse ear skin stained for MHCII (green/FITC) and analysed by confocal fluorescence microscopy. Original magnification x40

Resting Langerhans cells measured on average around 40μm in diameter, including the thick dendrites. As early as 2 hr after abrasion of the skin, the morphology of the LCs was seen to change (Fig. 5.11B). The thick dendrites were gradually pulled in and the cell body widened, making the cell size measurements smaller. Twelve hr after skin abrasion, the cells were rounded with few visible dendrites (Fig. 5.11D). By 24 hr after abrasion the morphology of the LCs had completely changed. They were now seen as smaller (on average around 30μm), totally rounded or oval cells with a ruffled appearance (Fig. 5.11E). The surface phenotype of the LCs had also changed by 24 hr and they were staining more brightly for MHCII (Fig. 5.11E). By 48 hr after the abrasion procedure, the morphology of the LCs in the epidermis and the appearance of the epidermis as a whole had returned to normal (Fig. 5.11F).

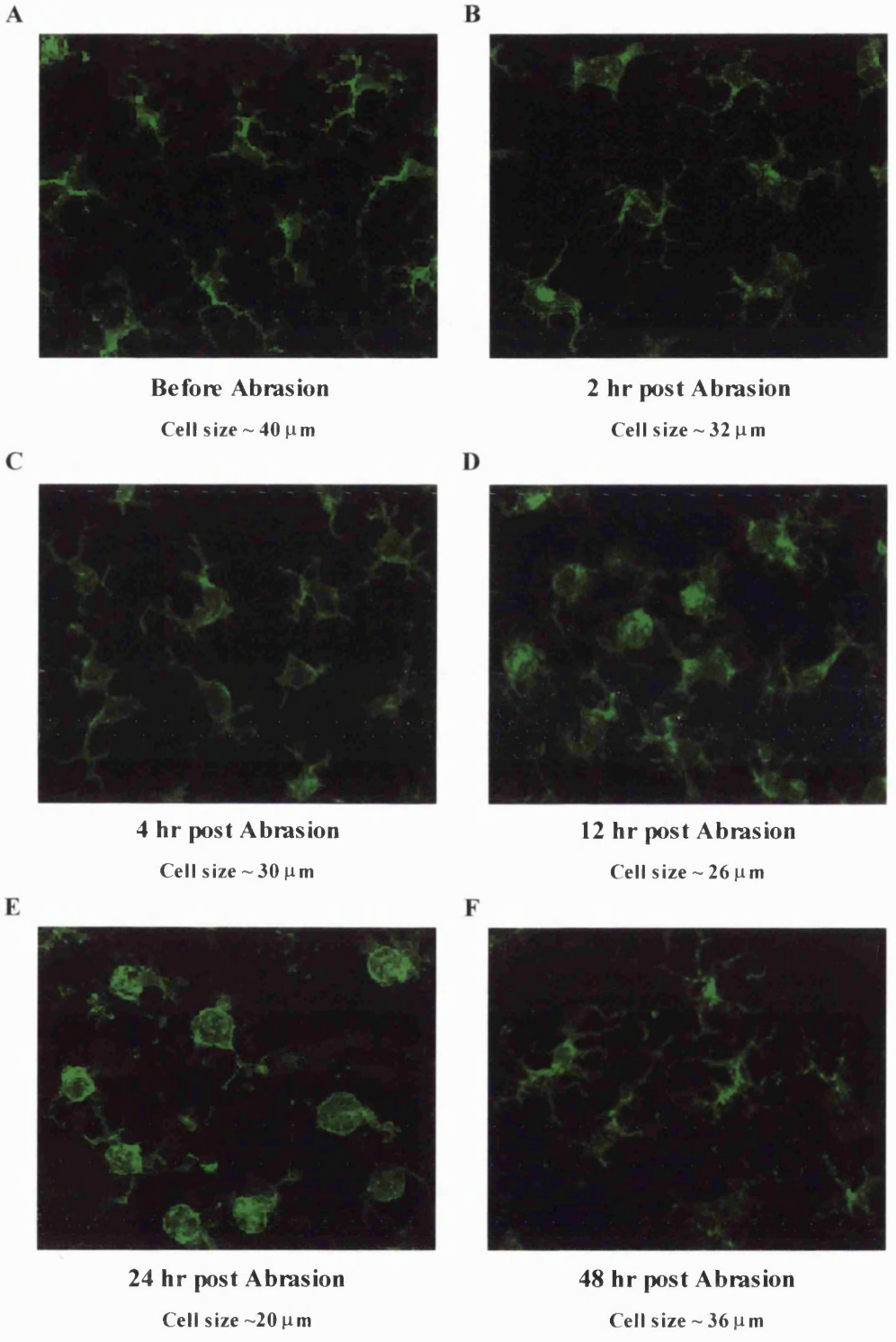


Fig. 5.11 Morphologic alteration of Langerhans cells in response to skin abrasion
Confocal fluorescence microscopy of MHCII$^+$ cells in the epidermis before and after mechanical disruption of the corneum barrier by skin abrasion. Fresh epidermal sheets from the ear were stained for MHCII (green/FITC) before abrasion of the skin (A), 2 hr (B), 4 hr (C), 12 hr (D), 24 hr (E) and 48 hr (F) after the abrasion technique. Original magnification x100 for all pictures

The Langerhans cells additionally expressed MHCII in a differential way before and 24 hr after the abrasion procedure. MHCII expression of resting LCs cells in normal skin was visualised mostly within intracellular vesicles creating a dotted MHCII$^+$ pattern (Fig. 5.12A,B). In contrast, 24 hr after the abrasion technique, the LCs expressed large amounts of MHCII on the cell surface and less intracellular MHCII was visible (Fig. 5.12C,D).

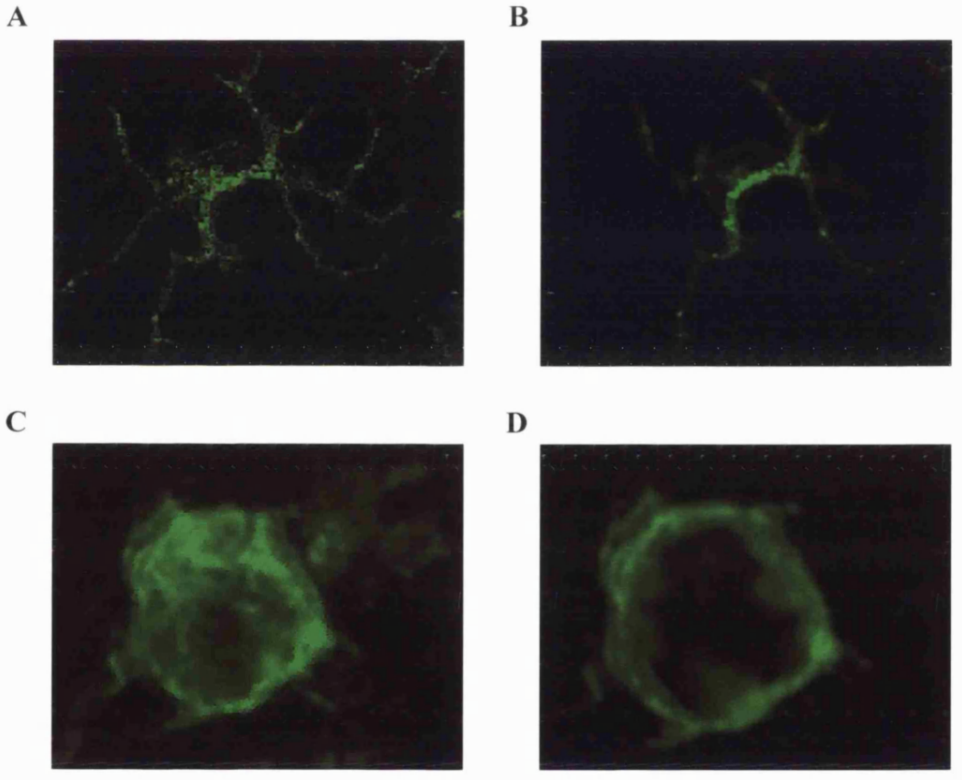


Fig. 5.12 MHCII$^+$ expression by Langerhans cells in normal epidermis and in epidermis after skin abrasion
Confocal fluorescence microscopy analysis of MHCII expression by Langerhans cells in epidermal sheets from normal ear skin (A, B) and 24 hr after abrasion of the skin (C, D). (A) and (C) Average of the entire confocal stack showing the whole cell. (B) and (D) Middle cross-section of the confocal stack demonstrating the centre of each cell. MHCII expression represented by green/FITC staining. Original magnification x300 (A, B) and x600 (C, D)

The changes in LC morphology, the transport of MHCII from intracellular compartments to the cell surface and the significantly upregulated expression of MHCII as a whole seemed to mimic the well-documented maturation process of DCs [Banchereau and Steinman, 1998]. To investigate this further, the expression of important co-stimulatory and accessory molecules on LCs in normal intact skin and 24 hr after disruption of the skin barrier by abrasion was examined. Freshly prepared epidermal sheets were double stained for MHCII and either CD86, CD40, CD54 or CD11c and analysed by confocal microscopy. In naïve skin with an intact barrier the expression of CD86, CD40, CD54 and CD11c on LCs was very low or not detectable. In contrast, 24 hr after abrasion the expression of all of these molecules was significantly enhanced and could easily be visualised (Fig. 5.13). The expression of both CD86, CD40, CD54 and CD11c mostly co-localised with the expression of MHCII on the cell surface, although some co-stimulatory molecules appeared to be expressed separately from MHCII (Fig. 5.14). The epidermal keratinocytes did not express either CD86, CD40, CD54 or CD11c, either before or after the abrasion when monitored by fluorescence staining of epidermal sheets, but they did express some MHCII.

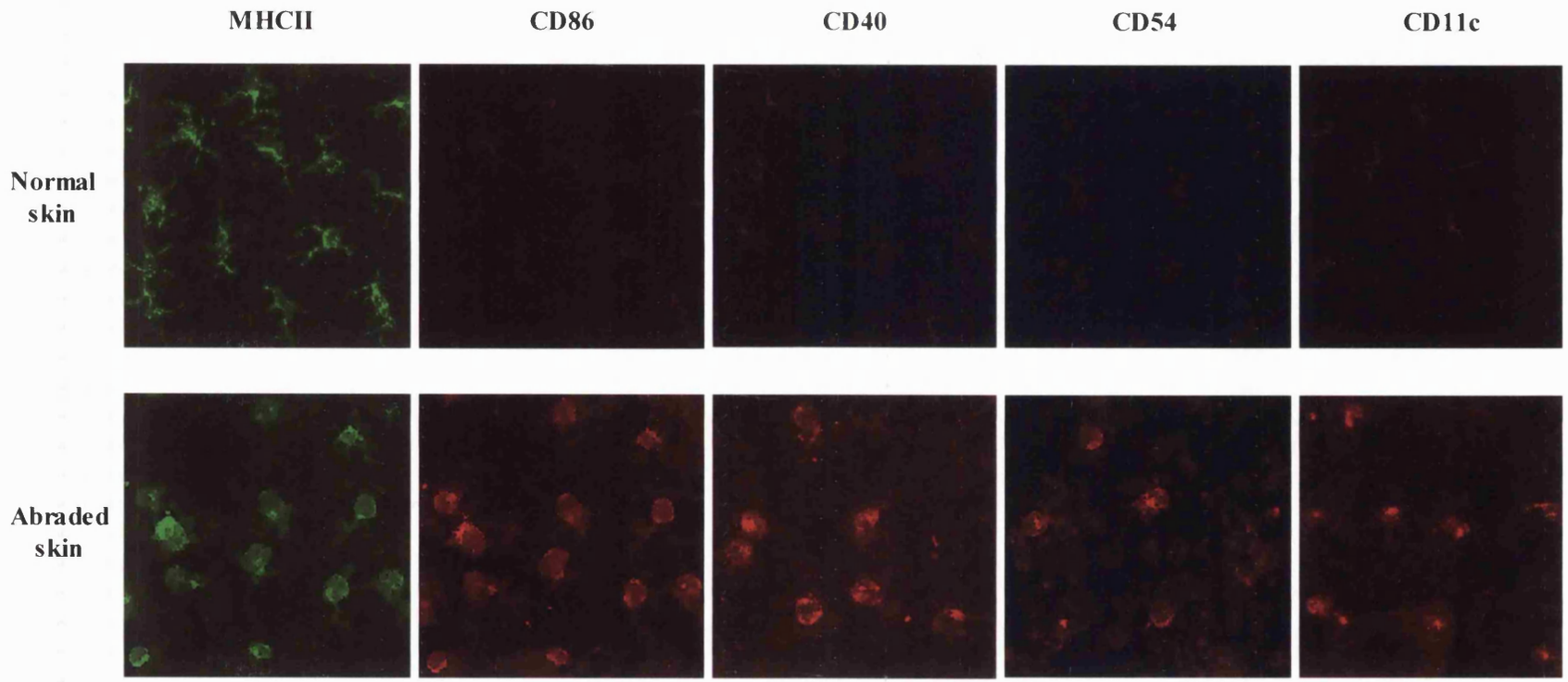


Fig. 5.13 Alteration of Langerhans cell morphology and surface phenotype in response to skin abrasion
Confocal fluorescence microscopy analysis of epidermal sheets from the ear before and after skin abrasion by tape-stripping. Expression of MHCII, CD86, CD40, CD54 and CD11c by Langerhans cells *in situ* in the epidermis are demonstrated in normal intact skin and 24 hr after skin abrasion. $MHCII^+$ cells in green (FITC) and $CD86^+$, $CD40^+$, $CD54^+$ or $CD11c^+$ cells in red (TRITC). Original magnification x100

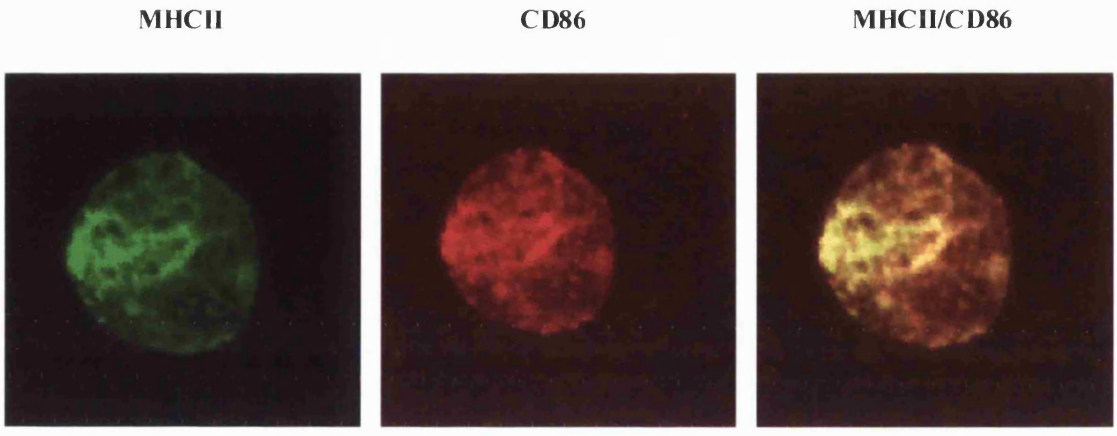


Fig. 5.14 Co-expression of MHCII and co-stimulatory molecules after skin abrasion
Confocal fluorescence microscopy of Langerhans cells *in situ* in the epidermis 24 hr after corneum barrier-disruption by abrasion of the ear skin. Epidermal sheets were double stained for MHCII$^+$ cells and CD86$^+$, CD40$^+$, CD54$^+$ or CD11c$^+$ cells. An example of MHCII/CD86 analysis is shown where MHCII expression is represented in green (FITC) and CD86 expression in red (TRITC). Co-localisation of MHCII/CD86 appears yellow. Similar images were seen when analysing expression of MHCII/CD40, MHCII/CD54 and MHCII/CD11c. Original magnification x500

5.3.6 Maturation and exposure to antigen is required for Langerhans cell migration

In this study, it was observed that skin abrasion by tape-stripping induced maturation of LCs in the epidermis and allowed for strong systemic immune responses to be initiated through epicutaneous exposure to proteins. This gave rise to the hypothesis that the abrasion technique enhanced LC migration to the draining LN. Confocal examination of epidermal sheets however showed no decline in epidermal LC numbers after skin abrasion alone (Fig. 5.11 and 5.13) or after abrasion and epicutaneous exposure to saline (Fig. 5.15). The LCs were rounded, ruffled in appearance and expressed large amounts of MHCII but they did not appear to migrate, and 14 hr after epicutaneous exposure to saline the appearance of the epidermis returned to normal with a contiguous network of immature LCs. In contrast, a rapid migration from the epidermis was observed when abraded skin was epicutaneously exposed to peanut antigens. Two hr after epicutaneous application of peanut antigen the majority of LCs had migrated away from the epidermal layer and many visual fields were nearly devoid of LCs. Epidermal LC numbers were significantly lower than in saline-exposed

controls at this time point ($p < 0.0001$) (Fig. 5.15). The epidermal keratinocytes stained brightly for MHCII and the LCs were seen in between the keratinocytes, suggesting migration through the keratinocyte tight junctions (Fig. 5.15 and Fig. 5.16). The same pattern was seen 4 hr after antigen application. Fourteen hr after the epicutaneous antigen exposure there were still significantly fewer LCs visible in the epidermis, although some repopulation had occurred. By 24 hr, more LC were appearing in the epidermis with features of normal immature LCs. Complete repopulation of the epidermis with a normal network of immature LCs displaying extended dendrites did not occur until 48 hr (Fig. 5.15).

Langerhans cells leaving the epidermis following abrasion and epicutaneous exposure to peanut protein were difficult to photograph as many were in the same plane as the keratinocytes. They could be visualised in deeper layers of the epidermis 'squeezed' in between the MHCII positive keratinocytes, while the epidermal LCs following abrasion and saline exposure remained in a layer on top of the keratinocytes (Fig. 5.16). The migrating LCs in the epidermis of animals with abraded and peanut painted skin could additionally be seen loaded with peanut antigens. (Fig. 5.17). Peanut antigens visualised within the epidermis 2 hr and 4 hr after epicutaneous peanut exposure were nearly all in associated with LCs and co-localised with MHCII expression. Some LCs could still be seen in conjunction with peanut antigen 14 hr after the epicutaneous exposure, but no peanut antigens could be demonstrated on or in the LCs at 24 hr or 48 hr after cutaneous exposure. No peanut positive staining could be detected in saline painted control animals (Fig. 5.17).

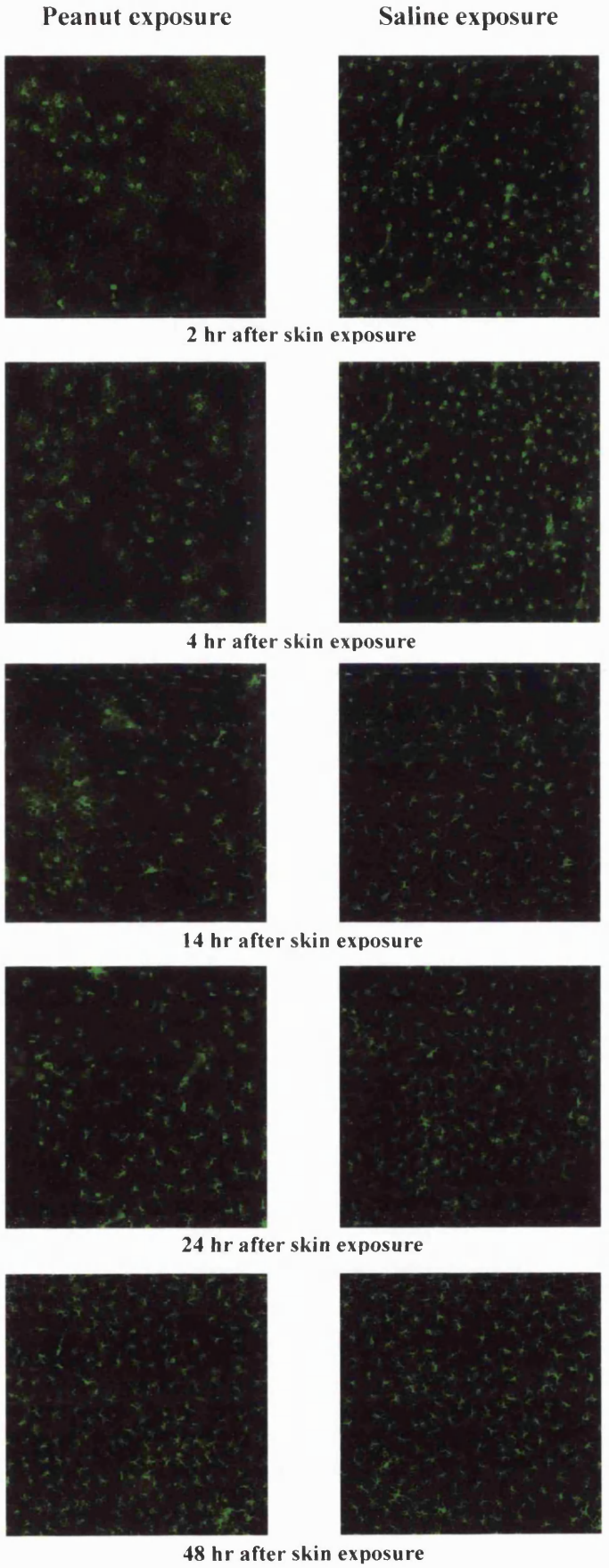


Fig. 5.15 Immunohistological analysis of Langerhans cell migration from the epidermis

Skin on the ear lobes were abraded and 24 hr later exposed to either 100μg peanut protein (left column) or saline (right column). At time points between 2 and 48 hr after epicutaneous exposure to peanut protein or saline ear skin was collected and epidermal sheets prepared. LCs were stained for MHCII expression and examined by confocal microscopy. $MHCII^+$ cells can be seen as green ($FITC^+$) cells. Confocal analysis of the epidermal sheets showed rapid migration of $MHCII^+$ LCs from the epidermis following skin abrasion and epicutaneous exposure to peanut protein but only very low or no migration following skin abrasion and epicutaneous exposure to saline. Quantification of LCs in the epidermis 2 hr after peanut protein or saline exposure demonstrated mean LC counts ± 1 SEM in defined visual grids of the epidermis of 40 ± 5 when the skin was exposed to peanut protein and 268 ± 29 when exposed to saline ($p < 0.0001$), which was similar to the LC density of 293 ± 32 in normal naïve skin. Note the 'cobblestone' pattern of the $MHCII^+$ keratinocytes. Original magnification x20

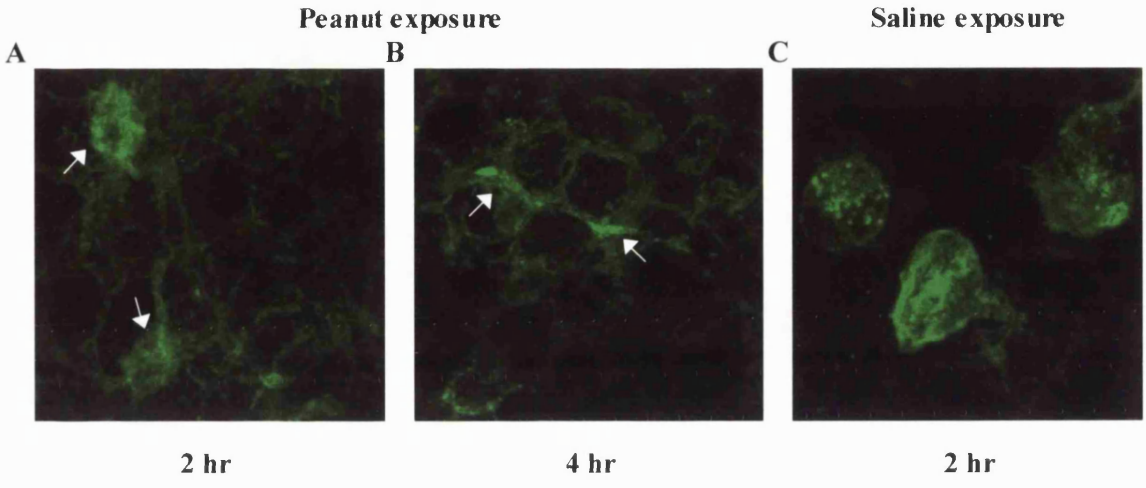


Fig. 5.16 Langerhans cells migrating through the keratinocyte layer of the epidermis
Confocal fluorescent microscopy of MHCII$^+$ LCs and keratinocytes in the epidermis following abrasion of the skin and epicutaneous exposure to either peanut protein or saline. MHCII$^+$ LCs in bright green and MHCII$^+$ keratinocytes in dull green. (A) and (B) LCs in the epidermis of animals 2 hr and 4 hr after peanut protein exposure respectively. The LCs (arrow) are visualised within the 'cobblestone' pattern of the keratinocytes or in between the keratinocytes. (C) Mature, round, MHCII$^+$ LCs in the epidermis of animals 2 hr after exposure to saline. These LCs were visualised in a distinct layer on top of the keratinocytes. A similar picture was seen 4 hr after saline exposure. Original magnification x300

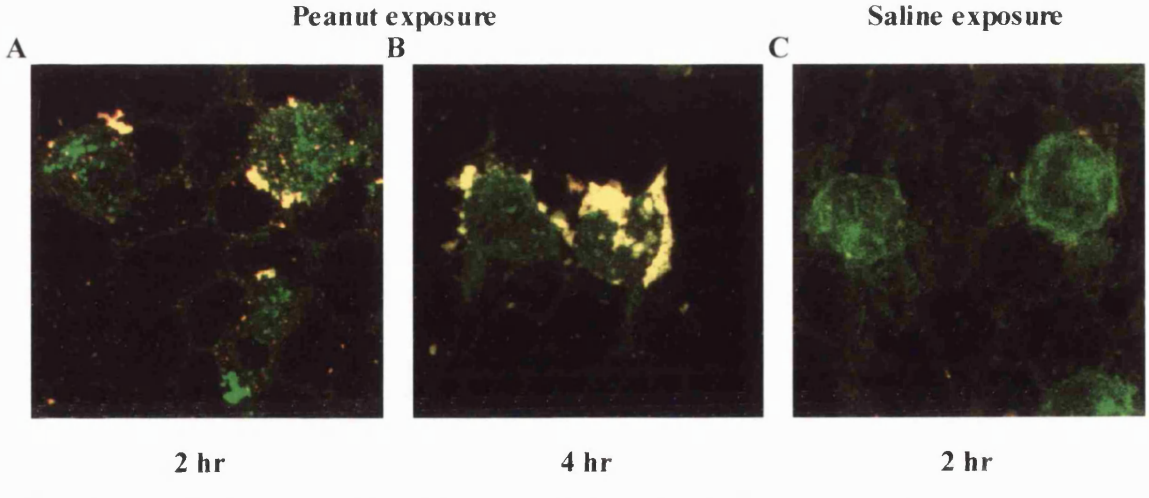


Fig. 5.17 Langerhans cells loaded with peanut antigens following epicutaneous exposure
Confocal fluorescent microscopy of epidermal sheets following abrasion of the skin and epicutaneous exposure to peanut protein or saline. Epidermal sheets were stained for MHCII$^+$ (green/FITC) and peanut antigens (red/TRITC). Co-localisation of MHCII and peanut antigens shows yellow. (A) and (B) LCs carrying peanut antigen in the epidermis of animals 2 hr and 4 hr after peanut exposure respectively. (C) Mature, round, MHCII$^+$ LCs in the epidermis of animals 2 hr after exposure to saline. Saline-exposed skin was stained for peanut antigens similar to (A) and (B). A similar picture was seen 4 hr after saline exposure. Original magnification x300

To further analyse LC migration following skin abrasion and epicutaneous antigen exposure the skin draining CLN were examined. Detection of epidermal LC migration to the draining LN has commonly been done by skin painting with FITC diluted in the enhancer vehicle acetone/dibutylphtalate [Macatonia et al., 1987; Thomas et al., 1980]. Here, peanut extract was conjugated to FITC and painted onto intact or abraded skin without any other enhancers or organic solvents. LCs that had migrated to the draining CLN were identified by flow cytometry 18 hr later as $CD11c^+$ cells that had taken up FITC-conjugated protein. Analysis of the total number of $CD11c^+$ cells in CLNs showed higher numbers in CLN from animals epicutaneously exposed to peanut protein than to saline on abraded skin ($p = 0.032$), suggesting significant migration of $CD11c^+$ cells from the epidermis only following abrasion *and* antigen exposure (Fig. 5.18). This was consistent with the histology. Total numbers of $CD11c^+$ cells were also higher in CLN from animals epicutaneously exposed to peanut protein on abraded skin compared to intact non-abraded skin ($p = 0.046$). Major migration of LCs following skin abrasion *and* antigen exposure was confirmed by analysis of CD11c and FITC double-positive cells in CLN. Significantly higher numbers of double-positive cells were found in CLN from animals epicutaneously exposed to peanut-FITC on abraded skin compared to normal intact skin ($p = 0.0008$), suggesting that more peanut-FITC bearing LCs reached the draining LN when the skin was abraded prior to peanut-FITC exposure (Fig. 5.18). CLN cells from control animals epicutaneously exposed to saline contained no $CD11c^+$ $FITC^+$ cells. The animals with abraded and peanut-FITC painted skin exhibited a general hyperplasia of the draining LN with significantly higher total cell numbers than any other group ($p < 0.05$), indicating not only enhanced LC migration but that some T cell proliferation and/or accumulation was already initiated (Fig. 5.18). Repeating the topical peanut-FITC application for 3 consecutive days resulted in accumulation of CD11c peanut-FITC double-positive cells in the CLN, demonstrated as slightly higher overall percentages of $CD11c^+$ peanut-$FITC^+$ cells (results not shown).

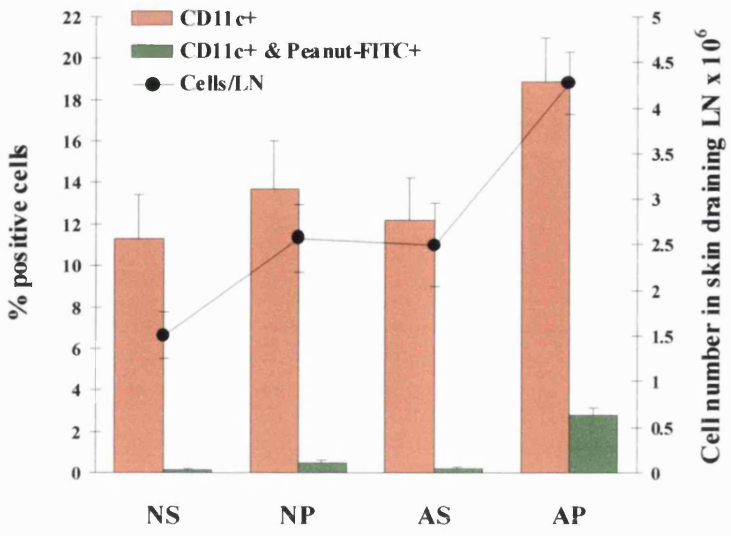


Fig. 5.18 Langerhans cell migration from epidermis to draining skin lymph nodes
Analysis of draining cervical lymph node cells 18 hr after a single topical application of saline or peanut-FITC conjugate on normal ear skin (NS and NP respectively) or on abraded ear skin (AS and AP respectively). Results are expressed as mean ± 1 SEM of four individual experiments

5.4 Discussion

The incidence and severity of allergic and atopic disorders has steadily increased in the industrialized world over the past three decades [Holgate, 1999; von Mutius, 1998]. Most of these diseases are associated with an increased and sustained allergen-specific IgE production, which mediates immediate Type I allergies upon re-exposure to the specific allergen. The mechanism of primary sensitisation to protein allergens is clearly important in pathogenesis of the disease. In young children, the skin is often strongly exposed to food antigens. In addition, many cosmetics, lotions and bath additives contain vegetable oils including peanut oil with small amounts of protein allergens. Children with eczema or atopic dermatitis often have various ointments containing vegetable oils applied frequently to the affected areas, to control disease. The use of peanut-containing creams has been shown in a cohort study to be more widespread in children who become allergic to peanut than in control infants [Lack et al., 2003].

The current study investigated the systemic effects of epicutaneous exposure to high molecular-weight food proteins. Peanut proteins or OVA applied to corneum barrier-disrupted skin elicited strong systemic immune responses. Both the cellular and humoral arms

of the immune system were affected with elevated antigen-specific T cell proliferation, DTH responses and induction of antigen-specific immunoglobulins. The immune response initiated by epicutaneous exposure was strongly Th2-biased with increased IL-4 and reduced IFN-γ production by T cells from draining LNs. In addition, levels of antigen-specific IgG1 and IgE, was increased, whereas IgG2a was barely detectable, consistent with a Th2 response. It is intriguing that the mice challenged through barrier-disrupted skin gave rise to a strong DTH response without dominant induction of Th1 cytokines. It has been reported, however, that IL-4 can have proinflammatory activities [Tepper et al., 1990] and also that DTH responses may be initiated by IgE antibodies [Ptak et al., 1991]. The epicutaneous allergen exposure selectively induced IgE to a larger extent than any other immunoglobulins. The levels of IgE produced in response to epicutaneous sensitisation were significantly higher than those induced after subcutaneous injection with adjuvant and recall immunisation. Other studies have reported that prolonged application of OVA to shaved murine skin can result in elevated levels of serum IgE [Spergel et al., 1998; Wang et al., 1996]. In these studies however, OVA was applied to shaved skin by an occlusive patch over a period of 7 weeks, which may cause some abrasion, induce immunological changes in the skin and aid penetration of proteins as a result of local dilation of the blood vessels. Other transcutaneous immunisation protocols have used lipid solvents or strong adjuvants such as cholera toxin or enterotoxin of *Escherichia coli* [Beignon et al., 2001; Glenn et al., 1998]. In contrast, the results presented here clearly show that a systemic Th2 immune response *can* be triggered by application of aqueous antigen to the skin without occlusion, addition of solvents or adjuvants. Impaired function of the epidermal barrier, even in non-lesional skin, is characteristic of atopic dermatitis [Kondo et al., 1998; Wollenberg et al., 2000]. The high level of IgE produced by epicutaneous immunisation on barrier-disrupted skin, suggests that the skin is a potential potent source of primary allergic sensitisation to food allergens such as peanut. Skin exposure may as such explain some allergic sensitisation and may be particularly relevant in subjects already suffering from other skin associated atopic diseases. In addition, it is possible that excessive washing with rough materials or using syndets and other de-fattening agents and/or frequent use of adhesive tape found for example on disposable nappies may cause abrasion of the skin of babies and young children and promote allergic sensitisation to food and other environmental antigens.

The method of skin abrasion used here was shown to remove the stratum corneum exposing the epidermis and LCs without disrupting the living epidermal layer. Application of antigen to

the abraded skin gave direct access to the epidermis, but not the dermis or dermal DCs. Such epicutaneous exposure to antigens gave rise to strong Th2-dominant immune responses. In contrast, subcutaneous immunisation delivered antigen directly into the dermis, gave rise to predominately Th1 responses and was a less potent route of immunisation. This suggests that the epidermal micromilieu and antigen exposure to LCs favours the induction of Th2 immunity. The mechanisms underlying this bias are unknown at present, but some observations indicate that LCs are naturally prone to make T cells enter a Th2 path. It has been shown that T cells grown with cultured LCs secrete IL-4 and stimulate IgE production by B cells, consistent with a Th2 bias by this route of antigen presentation [Hauser et al., 1989]. Immunological sites at other body surfaces have been reported to induce predominant Th2 responses when challenged with protein antigen without adjuvant. Exposure of the lung to aerosolized OVA without adjuvant results in IgE production and airway hyperresponsiveness in the mouse [Renz et al., 1992]. Furthermore, it has been shown that resting DCs in the respiratory tract preferentially stimulate Th2 responses and require additional cytokine signals for induction of Th1 immunity [Stumbles et al., 1998]. Intranasal exposure to OVA has also been shown to induce Th2 type responses with IgE production [McMenamin et al., 1994; Nelde et al., 2001]. Other DCs residing at body surfaces may have a specialised role by inducing tolerance rather than immunity. Gut lamina propria DCs usually reside in an immature state lacking a full range of costimulatory molecules such as CD80/CD86, CD40 and CD54. These cells may be tolerogenic if presenting antigen in absence of inflammation [Finkelman et al., 1996; Viney et al., 1998]. Indeed it has been shown that expanding but not maturing gut DCs *in vivo* with Flt3L enhances the induction of oral tolerance [Viney et al., 1998] while expanding DCs *in vivo* and simultaneously maturing them with IL-1α to express high levels of MHCII and costimulatory molecules abrogates the induction of oral tolerance and active immunity is achieved [Williamson et al., 1999b]. It is thus tempting to speculate that LCs and other DCs found at body surfaces have an inherent or default mechanism to promote tolerance and/or anti-inflammatory Th2 responses to dampen down Th1 responses that may be elicited by harmless symbiotic microbes found on our skin and in our gut. To elicit a strong Th1 type immunity at body surfaces, injury allowing antigen access to deeper layers and/or additional signals might be required.

In addition to the natural phenotype of LCs in the epidermis, the local response to cutaneous barrier-disruption may play a role in the Th2 dominance of the immune response after

epicutaneous antigen exposure. The response to barrier-disruption may result in cytokine production or other molecular signals in the skin that promote Th2 responses. This could explain the Th2 response to the hapten picryl chloride observed after acute barrier-disruption, compared to the normal Th1 contact hypersensitivity response [Kondo et al., 1998]. It is thus conceivable that the cascade of molecular events that occur after perturbation of the epidermal barrier play a role in diminishing Th1-type responses without influencing Th2-type immunity. Among the cytokines produced after acute disruption of the stratum corneum are an array of proinflammatory cytokines as well as GM-CSF and IL-10 [Nickoloff et al., 1994; Nickoloff and Naidu, 1994; Wood et al., 1992]. Normal intact skin is devoid of IL-10, but it is reported to be expressed in the epidermis soon after barrier-disruption [Nickoloff et al., 1994; Nickoloff and Naidu, 1994] and may play a role in suppressing Th1 cytokine production. IL-4 is also reported to be expressed in the skin after barrier-disruption and exposure to antigen [Kondo et al., 1998; Matsui and Nishikawa, 2002]. The resident LCs in the skin may be primarily affected by this production of immunoregulatory cytokines. IL-4 has been shown to make LCs more efficient in taking up and processing naïve protein [Elbe-Burger et al., 2002] and GM-CSF either alone or in combination with IL-4 augments the rate of pinocytosis by precursor DCs [Lutz et al., 1996].

Removal of the stratum corneum in the experiments described here was found to alter the immunological phenotype and function of the epidermal LCs *in situ*. The data suggested that efficient epicutaneous immunisation and T cell priming was dependent on this altered immunological function of the LCs. In intact naïve epidermis, LCs were found in a resting state as large cells with a typical dendritic morphology forming a network throughout the epidermis. MHCII staining gave a punctuate appearance, and analysis of individual cross-sections by confocal microscopy showed that most MHCII was expressed inside the cell. Other studies using similar techniques have found this punctuate MHCII pattern in LCs to co-localise with intracellular lysosomal markers [Pierre et al., 1997]. In addition, the LCs in intact epidermis expressed only very low or no costimulatory molecules such as CD86, CD40, CD54 and CD11c. Following disruption of the epidermal barrier, the LCs appeared to undergo a classical maturation process *in situ*. The morphology changed and the cells appeared smaller, rounder and with a ruffled cell body. MHCII staining progressively became brighter, less concentrated in the cell body and extended throughout each cell to the cell membrane. In addition, the expression of CD86, CD40, CD54 and CD11c was substantially increased,

consistent with maturation and antigen presentation. Barrier-disruption and the subsequently altered immune function did not however induce LCs migration to the draining LN. In contrast, barrier-disruption *and* antigen exposure caused rapid migration from the epidermis to the draining LN. This LC migration was not likely induced by LPS contamination of the peanut protein preparation as no LPS could be detected by limulus assay. LCs were found by confocal microscopy in conjunction with peanut protein in the epidermis, squeezed in between the keratinocytes and appeared in deeper layers of the epidermis. Eighteen hours after antigen exposure LCs were observed with peanut proteins-FITC in the draining LN. These results strongly suggest that maturation as defined by increased expression of MHCII, CD86, CD40, CD54 and CD11c is not sufficient to induce migration but that additional signals are required. Similar observations were made in two recent *in vitro* studies, where it was reported that maturation of human monocyte derived DCs was not sufficient to induce migration and that a second signal, which could be delivered by PGE_2, was needed [Luft et al., 2002; Scandella et al., 2002]. Additional studies have indicated that LC maturation and migration can be independently regulated events, as it was shown that LCs accumulating in the draining LN of chronically inflamed skin were of a typical immature phenotype [Geissmann et al., 2002]. It is conceivable that IL-10 produced in the epidermis following barrier-disruption [Nickoloff et al., 1994; Nickoloff and Naidu, 1994] may be involved in limiting migration of the mature LCs from the skin and may thereby minimise inflammation. This possible effect of IL-10 was overcome by additional antigenic stimuli, and IL-10 was subsequently downregulated in the draining LN of animals exposed to peanut following barrier-disruption. Enhanced LC migration from the skin has been shown in IL-$10^{-/-}$ mice [Wang et al., 1999].

Understanding the mechanisms that lead to tolerance versus immunity have been a central issue for many years. Recent reports have suggested that continuous activation of resident APCs can lead to a breakdown of tolerance to self and possibly to environmental antigens. Continuous activation of LCs in the skin by overexpressing CD40-ligand in the epidermis was shown to result in chronic skin inflammation and in systemic autoimmunity [Mehling et al., 2001]. Similarly, it was shown in the rat insulin promotor-glycoprotein double transgenic mouse model that T cell interactions with peptide only induced autoimmunity when APCs were continuously activated with anti-CD40 and not when peptide was presented in a resting or naïve environment [Garza et al., 2000]. In skin diseases such as AD there are increased numbers of IgE-bearing epidermal LCs both in lesional and non-lesional skin [Leung, 2000;

Wollenberg et al., 1996]. Continual activation of these cells through scratching and abrasion of the pruritic skin excoriations may break the homeostatic balance and tolerance induced in the skin, and sensitisation to innocuous environmental and food antigens could occur as a result. Thus, immunodysregulation may be initiated by continuous activation of resident DCs, and the key to whether peripheral and systemic tolerance or immunity are induced lie in the local microenvironment wherein an antigen is encountered.

As discussed in Chapter 3, every food protein could potentially be an allergen. In the current Chapter, epicutaneous sensitisation to both peanut protein and OVA resulted in Th2-type responses; however, peanut protein repeatedly gave rise to significantly higher levels of IgE than similar exposure to OVA. The crude peanut extract is a mixture of many proteins and thus the concentration of any given protein is much lower than that of OVA when applied to the skin. This suggests that in addition to route of sensitisation and the local microenvironment at the time of exposure, the nature of the antigen can play an important role in determining the immunological outcome of an allergen encounter.

In this Chapter it has been shown that protein antigens can easily enter through abraded skin and cause primary systemic Th2-biased immune responses. The results suggest that the LCs in the epidermis play a pivotal role in promoting the Th2 responses. The evidence from this and other studies thus demonstrates that the skin is an active immunological site and that cutaneous allergen exposure should be considered when therapeutic regimens for atopic diseases are developed. Regulation or restoration of proper cutaneous barrier function may be a potential target for immunotherapeutic intervention to correct the cytokine imbalance seen in atopic dermatitis and eczema and may as such circumvent some allergic and classical DTH responses. The findings presented here, as well as changes in skin care and cleanliness, may provide an alternative explanation for the increase in allergic responses to environmental allergens in the developed world.

Chapter 6

Epicutaneous immunisation prevents oral tolerance and enhances allergic sensitisation

6.1 Introduction

Food allergy is a group of disorders characterised by adverse hypersensisitivity reactions to food antigens. The key feature of disease is a Th2-predominant allergen-specific immune response, with production of IgE antibodies specific for the food allergen. The aetiology of food allergy remains unclear, but a failure to develop oral tolerance, or a breakdown in its maintenance, may be responsible. Alternatively, patients may be sensitised via routes other than the gastrointestinal tract and the GALT immune system.

Clinically, food allergy is closely associated with AD. Both diseases show systemic allergic responses and marked elevated serum IgE, which can passively sensitise mast cells, basophils and DCs throughout the body. It has been suggested that food allergy and AD may represent distinct organ manifestations of a common systemic allergic response [Schleimer and Togias, 2000]. It may be that a common sensitisation pathway exits for both diseases. Skin manifestations are the most frequent complication associated with food allergy [Werfel, 2001], but little is known of whether sensitisation through the skin can affect mucosal immunity in the GALT.

In Chapter 5 it was demonstrated that primary systemic sensitisation to protein allergens, which are relevant to atopic diseases, can occur through the skin. Sensitisation to protein allergens through the skin resulted in strong Th2-biased immunity with large amounts of allergen-specific IgE. In the current Chapter, the effects of epicutaneous sensitisation on the induction and maintenance of oral tolerance are analysed. Additionally, the effects of skin exposure to protein allergens on already orally-tolerised animals are assessed. The main purpose of the this study is to determine whether exposure to allergens through the skin can promote food allergy.

6.2 Immunisation protocol

The influence of epicutaneous protein exposure on oral tolerance induction was investigated using the epicutaneous immunisation protocol (section 5.2.1 and Fig. 5.1) together with the oral tolerance protocol (section 3.2 1 and Fig. 3.1). As outlined in Fig. 6.1, ear skin was abraded by tape-stripping and 24 hr later, 100μg peanut protein or OVA was applied to both sides of the earlobe. Control mice were painted with saline. Six or 20 days after the initial

topical exposure, animals were gavage fed a tolerogenic dose (100mg for peanut protein, 20mg for OVA) of the same antigen. Naïve mice were fed PBS as controls. One week after the gavage, all animals were immunised by tail base injection of 100μg of the appropriate protein antigen (peanut or OVA) emulsified 1:1 in CFA. Three weeks later, mice were recall immunised with 100μg peanut protein or OVA in the footpad and the DTH responses measured. Seven days after recall immunisation, mice were culled and PLN, spleens and serum were collected and assayed for immune responses to the specific protein antigen. Abbreviations used for the specific immunisation procedures are listed in Table 6.1.

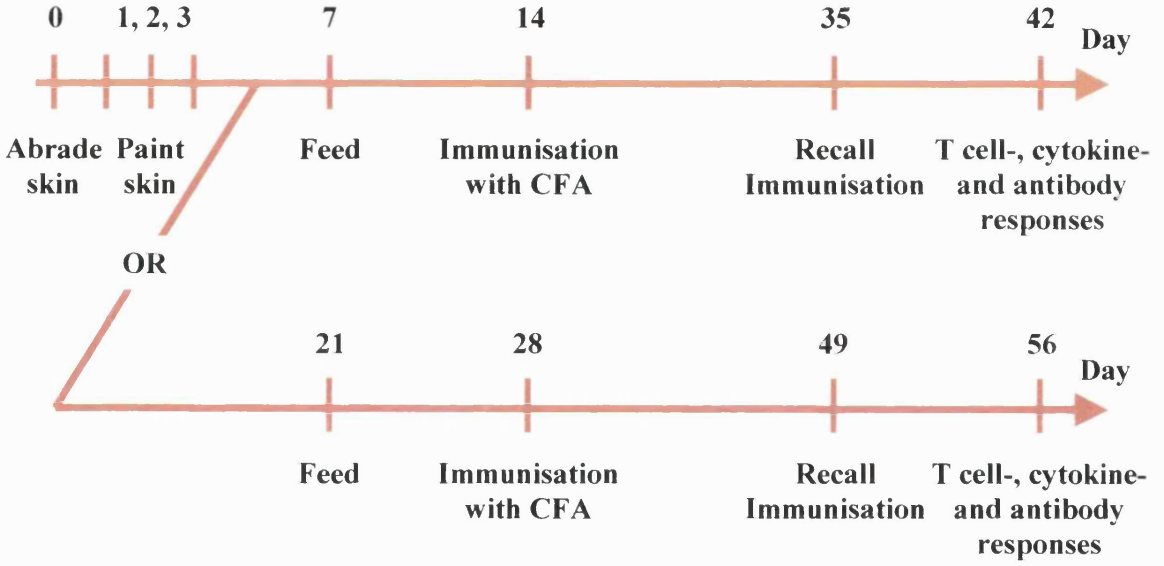


Fig. 6.1 Diagram of epicutaneous immunisation prior to oral tolerance induction
The schematic representation shows the experimental protocol used to study the influence of epicutaneous sensitisation on the subsequent induction of oral tolerance

Table 6.1 Abbreviations used for experimental protocols of epicutaneous immunisation prior to oral tolerance induction

Procedure / Abbreviation	Skin abrasion	Topical application	Feed	Immunisation with CFA	Recall immunisation
SPP	-	-	Saline	Peanut	Peanut
APPPP	Abrasion	Peanut	Peanut	Peanut	Peanut
ASPPP	Abrasion	Saline	Peanut	Peanut	Peanut
SOO	-	-	Saline	OVA	OVA
AOOOO	Abrasion	OVA	OVA	OVA	OVA
ASOOO	Abrasion	Saline	OVA	OVA	OVA

Conversely, to investigate whether an existing state of orally-induced tolerance could be disrupted by sensitisation through the skin, the following experimental protocol was adopted. Animals were fed a tolerogenic dose of peanut protein (100mg) or sham-fed PBS by gavage. One week after the intra-gastric feeding, ear skin on all animals was abraded and 24 hr later half of the peanut-fed animals were painted with 100µg of peanut protein and half were painted with saline. PBS-fed animals were painted with 100µg peanut protein on both sides of both ears in an identical manner. Nineteen days after the initial epicutaneous exposure to peanut protein animals were given a footpad injection with 100µg peanut protein. Twenty four hours later DTH responses were measured, where after the animals were culled and CLN, spleens and serum were collected and analysed. The experimental protocol is shown in Fig. 6.2 and abbreviations used for the particular immunisations are listed in Table 6.2.

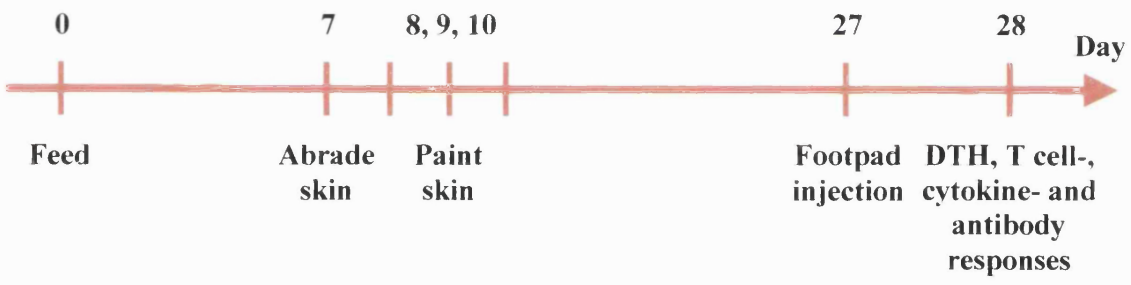


Fig. 6.2 Diagram of tolerance induction prior to epicutaneous immunisation
The figure shows the experimental protocol used to investigate whether antigen-specific oral tolerance can be interrupted by epicutaneous exposure to the same protein antigen

Table 6.2 Abbreviations used for experimental protocols of oral tolerance induction prior to epicutaneous immunisation

Procedure / Abbreviation	Feed	Skin abrasion	Topical application
SAP	Saline	Abrasion	Peanut
PAS	Peanut	Abrasion	Saline
PAP	Peanut	Abrasion	Peanut

6.3 Results

6.3.1 Epicutaneous antigen exposure prevents induction of oral tolerance

A tolerogenic dose of peanut protein or OVA fed 6 days after epicutaneous exposure was still able to suppress antigen-specific T cell responses upon systemic antigen challenge. *In vivo* DTH responses were significantly reduced ($p < 0.0001$) in mice fed antigen compared to mice fed saline prior to immunisation with peanut or OVA in CFA and recall immunisation (SPP) (Fig. 6.3A,B). There were no differences in DTH responses between animals epicutaneously exposed to peanut (APPPP) or OVA (AOOOO) and control animals exposed to saline prior to similar tolerogenic feeds (ASPPP and ASOOO) (Fig. 6.3A,B). In contrast, when a tolerogenic dose of peanut protein was fed 20 days after the initial epicutaneous exposure, animals were no longer hyporesponsive upon antigen challenge. DTH responses following antigen challenge in the footpad were as high as in saline-fed hypersensitive controls and significantly higher ($p < 0.0001$) than in tolerant control animals epicutaneously exposed to saline prior to a tolerogenic feed of peanut protein (Fig. 6.3C).

Antigen-specific T cell proliferation was measured *in vitro* in cells from PLN draining the peanut/CFA immunisation site and from the spleen 6 or 8 weeks after the initial epicutaneous sensitisation (5 weeks after oral challenge). PLN T cells from animals fed a tolerogenic dose of peanut protein or OVA 6 days after epicutaneous sensitisation were hyporesponsive to the fed antigen and proliferated significantly less than PLN T cells from saline-fed controls ($p < 0.001$) (Fig. 6.4A,B). PLN cells from animals epicutaneously sensitised to antigen did however show slightly higher antigen-specific T cell proliferation than the tolerant animals epicutaneously exposed to saline (Fig. 6.4A,B). Animals fed a tolerogenic dose of peanut protein 20 days after epicutaneous exposure no longer became hyporesponsive to the fed antigen. Suppression of T cell proliferation was prevented by the epicutaneous exposure and the feeding further enhanced sensitisation. PLN T cell proliferation was significantly enhanced ($p < 0.0001$) compared to proliferation of PLN T cells from peanut-tolerant animals epicutaneously exposed to saline prior to the tolerogenic feed (Fig. 6.4C). Specific-T cell proliferation was even demonstrated to be higher ($p < 0.05$) than in saline-fed hypersensitive controls (Fig. 6.4C). Similar proliferation patterns as shown in Fig. 6.4 were demonstrated in spleen cell cultures (data not shown).

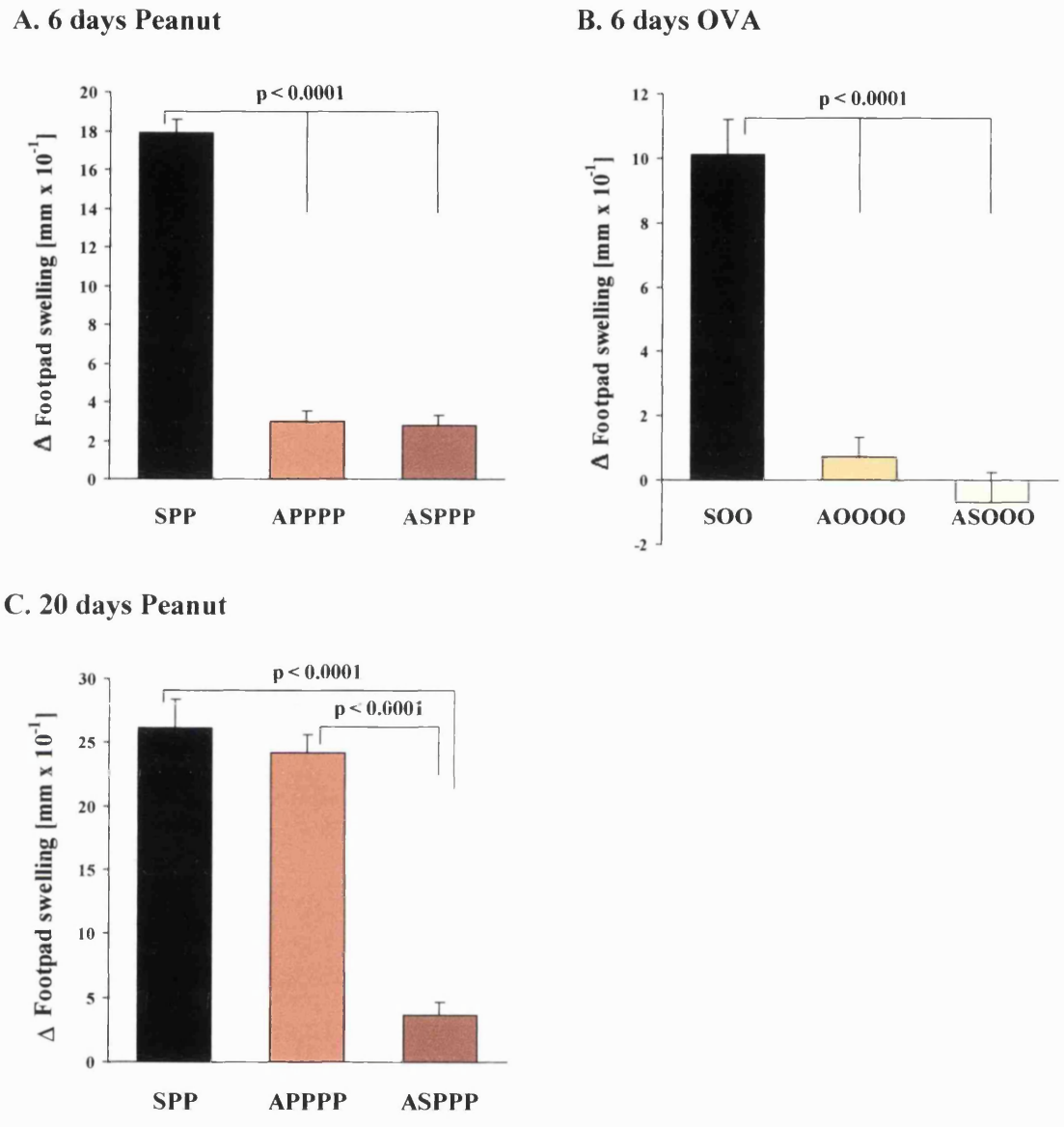
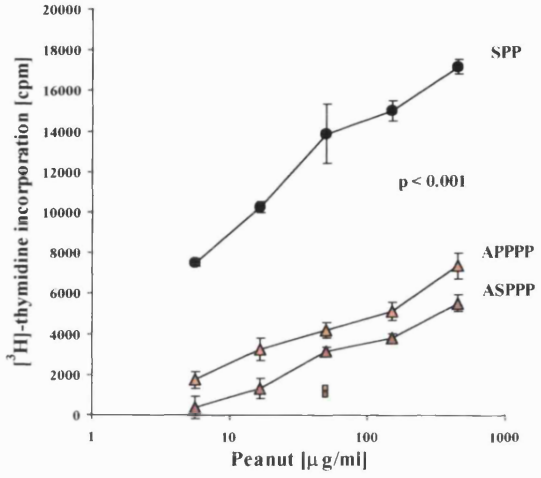


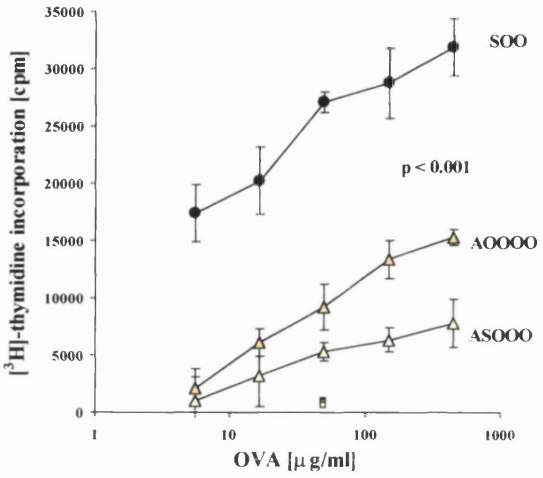
Fig. 6.3 Specific DTH responses after epicutaneous sensitisation followed by feeding of antigen

Animals were epicutaneously sensitised to peanut protein (APPPP), to OVA (AOOOO), to saline (ASPPP or ASOOO) or received no skin procedure (SPP or SOO) 6 (A and B) or 20 (C) days prior to a tolerogenic feed of peanut protein, OVA or saline as control. All animals were then immunised with peanut protein or OVA according to Fig. 6.1. DTH responses were measured in peanut exposed animals (A, C) and in OVA exposed animals (B) as the relative footpad swelling 24 hr after injection of peanut protein or OVA in the hind footpad. Results are expressed as the mean DTH responses + 1 SEM (n=6-8). The peanut experiments were repeated twice with similar results. [SPP/SOO: No skin procedure, fed saline, immunised with peanut/OVA in CFA, recall immunised with peanut/OVA; APPPP/AOOOO: Skin abraded, painted with peanut/OVA, fed peanut/OVA, immunised with peanut/OVA in CFA, recall immunised with peanut/OVA; ASPPP/ASOOO: Skin abraded, painted with saline, fed peanut/OVA, immunised with peanut/OVA in CFA, recall immunised with peanut/OVA]

A. 6 days Peanut



B. 6 days OVA



C. 20 days Peanut

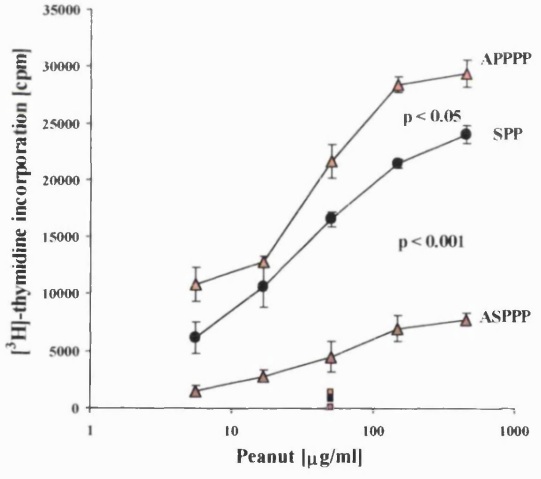


Fig. 6.4 Specific T cell proliferation after epicutaneous sensitisation followed by feeding of antigen

Animals were epicutaneously sensitised to peanut protein (APPPP), to OVA (AOOOO), to saline (ASPPP or ASOOO) or received no skin procedure (SPP or SOO) 6 (A and B) or 20 (C) days prior to a tolerogenic feed of peanut protein, OVA or saline as control. All Animals were then immunised as outlined in Fig. 6.1. Specific T cell proliferation was measured in cultures of PLN following 90 hr stimulation with peanut protein (A and C) or OVA (B) *in vitro*. PLN cells were pooled from 6-8 mice per group and results are expressed as the mean cpm of triplicate cultures ± 1 SEM. Background proliferation when no antigen was present has been subtracted. Squares represent proliferation to a control antigen. All groups responded with similar proliferation [cpm] when stimulated with ConA. The peanut experiments were repeated twice with similar results. [SPP/SOO (●): No skin procedure, fed saline, immunised with peanut/OVA in CFA, recall immunised with peanut/OVA; APPPP/AOOOO: Skin abraded, painted with peanut (▲)/OVA (▲), fed peanut/OVA, immunised with peanut/OVA in CFA, recall immunised with peanut/OVA; ASPPP/ASOOO: Skin abraded, painted with saline, fed peanut (▲)/OVA ( ), immunised with peanut/OVA in CFA, recall immunised with peanut/OVA]

A difference in peanut-specific T cell sensitivity between animals fed a tolerogenic dose of peanut protein 6 days after- or 20 days after epicutaneous peanut protein exposure was observed by measuring T cell proliferation with [^{3}H]-thymidine (Fig. 6.4). To confirm this, PLN cells were stained with CFSE and the number of cell divisions determined by analysing the CFSE intensity on the FACS. Fig. 6.5 shows proliferation of PLN T cells from animals fed a tolerogenic dose of peanut protein 6 days (Fig. 6.5A-C) or 20 days (Fig. 6.5D-F) after epicutaneous exposure to peanut protein. Feeding a tolerogenic dose of peanut protein 6 days after epicutaneous sensitisation rendered specific T cells hyporesponsive upon peanut challenge. The number of dividing cells after 90 hr *in vitro* stimulation with peanut protein were similar to the peanut-tolerant control animals (epicutaneously exposed to saline prior to a tolerogenic peanut protein feed) and as low as unstimulated cells. Proliferation of PLN cells from both the epicutaneously sensitised animals and the tolerant animals were significantly lower than proliferation of cells from the saline-fed animals (Fig. 6.5B). In contrast, feeding a tolerogenic dose of peanut protein 20 days after epicutaneous sensitisation failed to induce T cell hyporesponsiveness (Fig. 6.5E). The proliferative responses induced in the animals epicutaneously exposed to peanut were even higher than in the saline-fed hypersensitive animals (Fig. 6.5E). All groups proliferated vigorously and approximately equally to stimulation with ConA (Fig. 6.5C and F) and only minimal spontaneous proliferation was seen when cells were not stimulated (Fig. 6.5A and D). Results from the CFSE staining were thus consistent with those from incorporation of [^{3}H]-thymidine. The experiment was repeated twice with similar results.

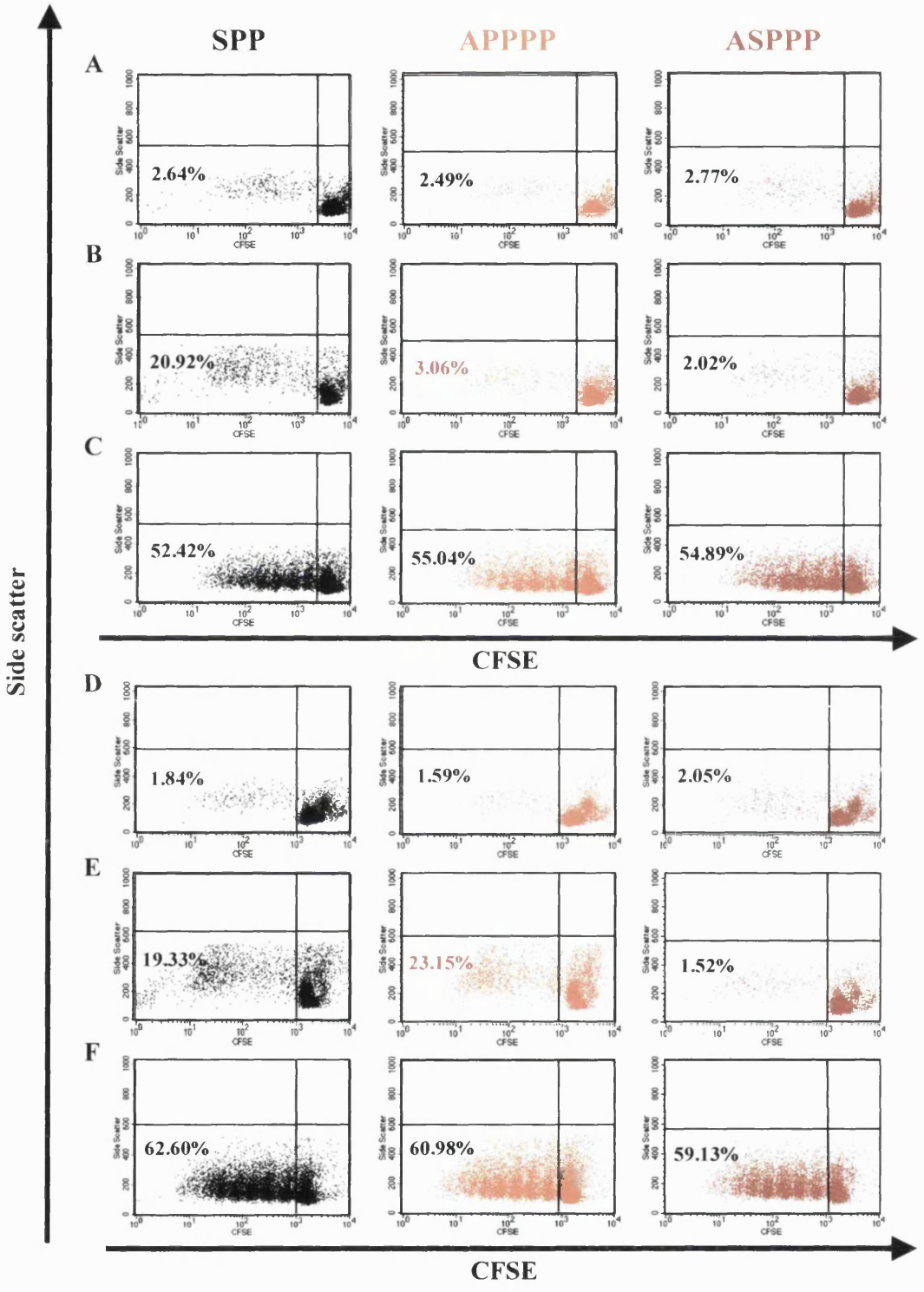


Fig. 6.5 T cell divisions in PLN cultures after epicutaneous sensitisation followed by feeding of antigen 6 or 20 days later

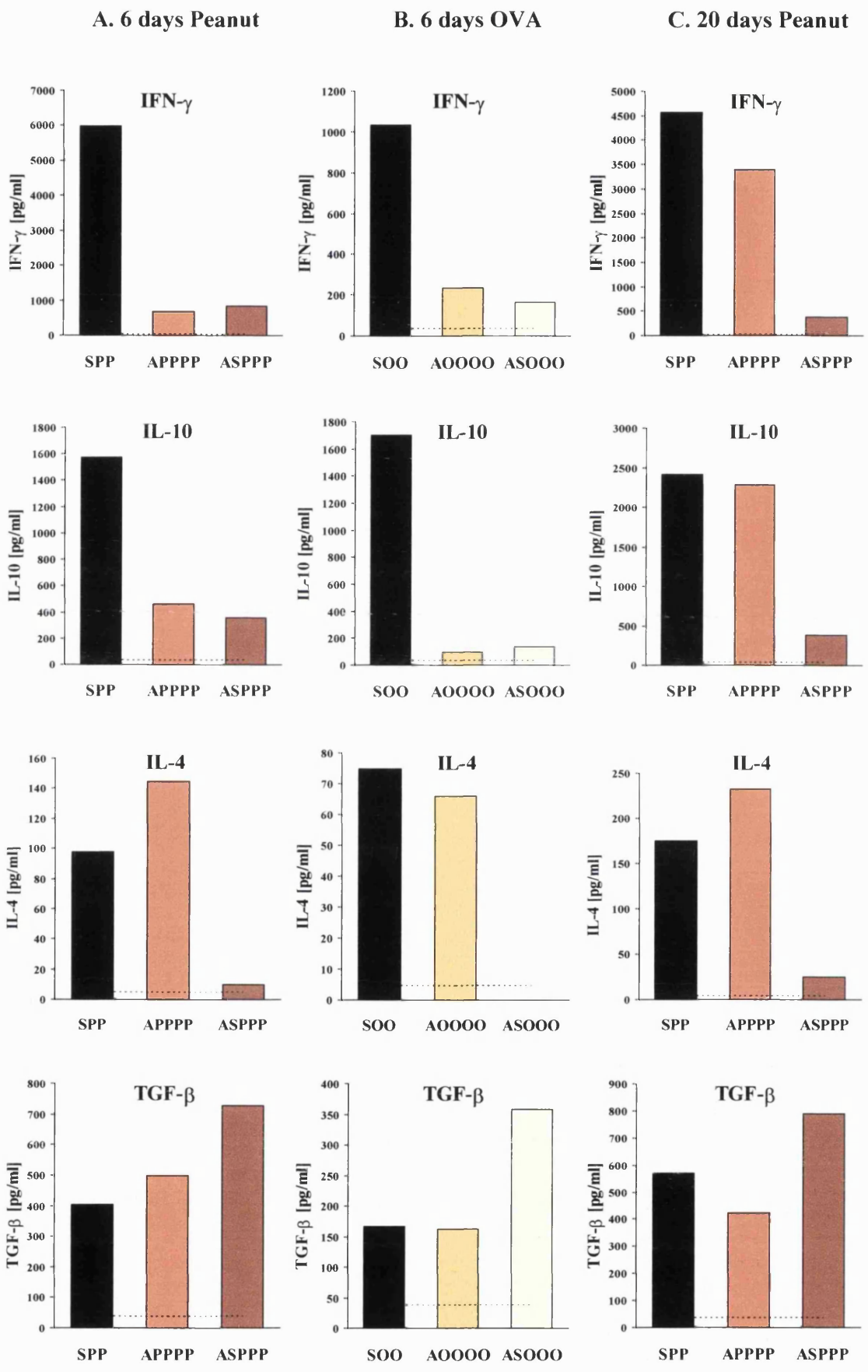
Animals were fed a tolerogenic dose of peanut protein 6 days (A-C) or 20 days (D-F) after epicutaneous exposure to peanut protein. PLN cells were stained with CFSE and T cell proliferation measured as decline in CFSE intensity. Cells were pooled from 8 mice per group and cultured without stimulation (A and D), with 200μg peanut protein/ml (B and E) or with 1μg ConA/ml (C and F) for 90 hr and then analysed by flow cytometry. [SPP (■): No skin procedure, fed saline, immunised with peanut in CFA, recall immunised with peanut; APPPP (■): Skin abraded, painted with peanut, fed peanut, immunised with peanut in CFA, recall immunised with peanut; ASPPP (■): Skin abraded, painted with saline, fed peanut, immunised with peanut in CFA, recall immunised with peanut]

Analysis of the cytokine production from PLN and spleen cells showed that animals fed a tolerogenic dose of peanut protein (APPPP) or OVA (AOOOO) 6 days after epicutaneous sensitisation suppressed IFN-γ and IL-10 responses to the specific antigens. These groups produced similar levels of IFN-γ and IL-10 to the tolerant animal groups which were epicutaneously exposed to saline prior to feeding of peanut or OVA, and the levels of IFN-γ and IL-10 were significantly lower than that secreted from the saline-fed control groups (SPP) (Fig. 6.6A,B). In contrast, IL-4 production was markedly upregulated in animals epicutaneously sensitised prior to the tolerogenic feed. IL-4 levels were significantly higher than in tolerant control animals and even higher than in saline-fed hypersensitive controls when exposed to peanut (Fig. 6.6A). TGF-β levels were lower than in tolerant control animals (ASPPP and ASOOO) and more similar to the saline-fed hypersensitive controls (Fig. 6.6A,B). Cytokine production by PLN and spleen cells from animals fed a tolerogenic dose of peanut protein 20 days after epicutaneous sensitisation showed a different response-pattern which was similar to the peanut-sensitive saline-fed animals. Levels of secreted IFN-γ, IL-10 and IL-4 were all significantly elevated, while TGF-β secretion was reduced compared to tolerant controls animals (Fig. 6.6C). Levels of IL-4 were in addition even higher than in the saline-fed hypersensitive animals. The specifically enhanced level of IL-4 was comparable to the results observed following a tolerogenic feed 6 days after epicutaneous sensitisation, where in contrast IFN-γ and IL-10 were reduced (Fig. 6.6A).

No cytokines were detected in any groups in response to a control antigen (OVA or peanut) or when cells were not stimulated. Similar patterns of cytokine production were observed from spleen cells (results not shown).

Fig. 6.6 Cytokine production by PLN cells after epicutaneous sensitisation followed by feeding of antigen

Animals were epicutaneously sensitised to peanut protein (APPPP), to OVA (AOOOO), to saline (ASPPP and ASOOO) or received no skin procedure (SPP or SOO) 6 (A and B) or 20 (C) days prior to a tolerogenic feed of peanut protein, OVA or saline as control. All animals were then immunised as shown in Fig. 6.1. Cytokine secretion by $2x10^6$ PLN cells from animals exposed to peanut protein (A and C) or OVA (B) were analysed following 96 hr re-activation *in vitro* with peanut protein. PLN cells were pooled from 6-8 mice per group and analysed in triplicate. Results are expressed as the mean cytokine level. Dotted lines demonstrate the limit of detection of each cytokine assay. The peanut experiments were repeated twice with similar results. [SPP/SOO: No skin procedure, fed saline, immunised with peanut/OVA in CFA, recall immunised with peanut/OVA; APPPP/AOOOO: Skin abraded, painted with peanut/OVA, fed peanut/OVA, immunised with peanut/OVA in CFA, recall immunised with peanut/OVA; ASPPP/ASOOO: Skin abraded, painted with saline, fed peanut/OVA, immunised with peanut/OVA in CFA, recall immunised with peanut/OVA]



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The apparent specific loss of Th2-type tolerance following epicutaneous sensitisation was also evident when antigen-specific antibody responses were analysed. While levels of peanut- and OVA-specific IgG and IgG2a were still significantly suppressed by feeding peanut protein or OVA 6 days after epicutaneous exposure, IgG1 and especially IgE were significantly higher than in tolerant control animals (Fig. 6.7A,B). Animals epicutaneously sensitised to peanut protein not only failed to downregulate IgE responses following a tolerogenic peanut feed, but produced levels of peanut-specific IgE significantly higher than even the saline-fed (SPP) hypersensitive controls ($p < 0.0001$) (Fig. 6.7A). Animals epicutaneously sensitised to OVA prior to a tolerogenic feed also had significantly higher OVA-specific IgE levels than tolerant controls ($p < 0.0001$) and higher IgE levels than the saline-fed group, although this was not statistically significant ($p = 0.0515$) (Fig. 6.7B).

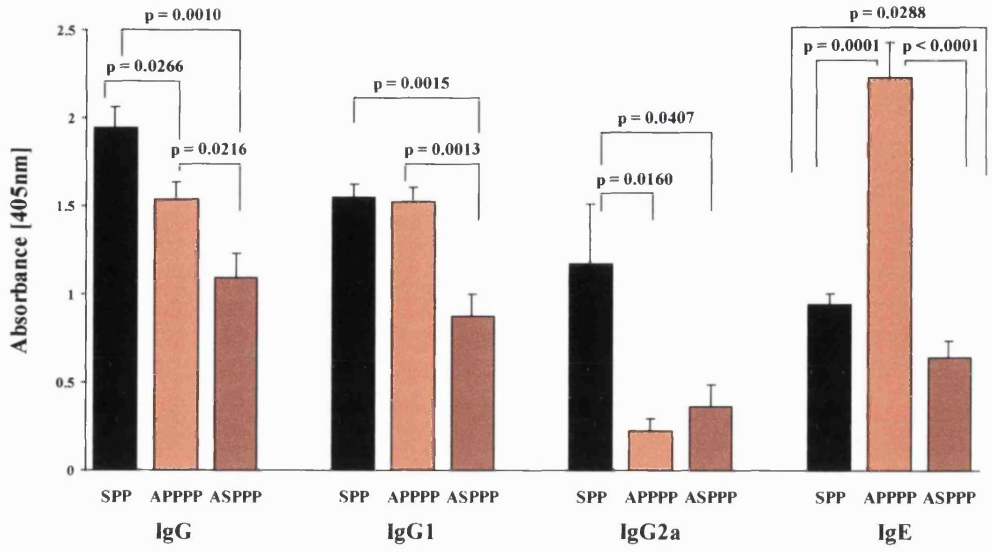
Analysis of peanut-specific immunoglobulins in serum from animals fed a tolerogenic dose of peanut protein 20 days after epicutaneous sensitisation showed that the epicutaneous peanut protein exposure completely prevented suppression of the antibody response. Levels of peanut-specific IgG, IgG1, IgG2a and IgE were all significantly higher than in tolerant control animals that were epicutaneously exposed to saline prior to a similar tolerogenic peanut protein feed (Fig. 6.7C). Levels of peanut-specific IgE were additionally significantly higher than in the saline-fed hypersensitive animals ($p = 0.0015$), suggesting that the peanut feed instead of tolerising the epicutaneously exposed animals had sensitised them further. The entire *in vivo* peanut exposure experiment was repeated twice with similar immunoglobulin patterns between groups.

In summary, feeding a tolerogenic dose of protein antigen 6 days after the initial epicutaneous sensitisation resulted in suppressed DTH-, T cell proliferative-, IFN-γ-, IL-10-, IgG- and IgG2a- responses to the fed antigen, similar to normal induction of oral tolerance (Chapter 3). However, inhibition of IL-4, IgG1 and IgE was prevented when the animals were pre-sensitised by epicutaneous antigen exposure. In contrast, epicutaneous exposure to peanut protein 20 days prior to feeding of a tolerogenic dose of peanut protein completely prevented the induction of oral tolerance Results showed significantly increased DTH-, T cell proliferative-, IFN-γ-, IL-10-, IL-4-, IgG-, IgG1-, IgG2a- and IgE-responses compared to tolerant animals. TGF-β appeared to be down-regulated. Epicutaneous exposure to peanut particularly affected IL-4 and IgE with all animals showing further peanut sensitisation upon oral challenge and levels of IL-4 and IgE elevated significantly above the saline-fed animals.

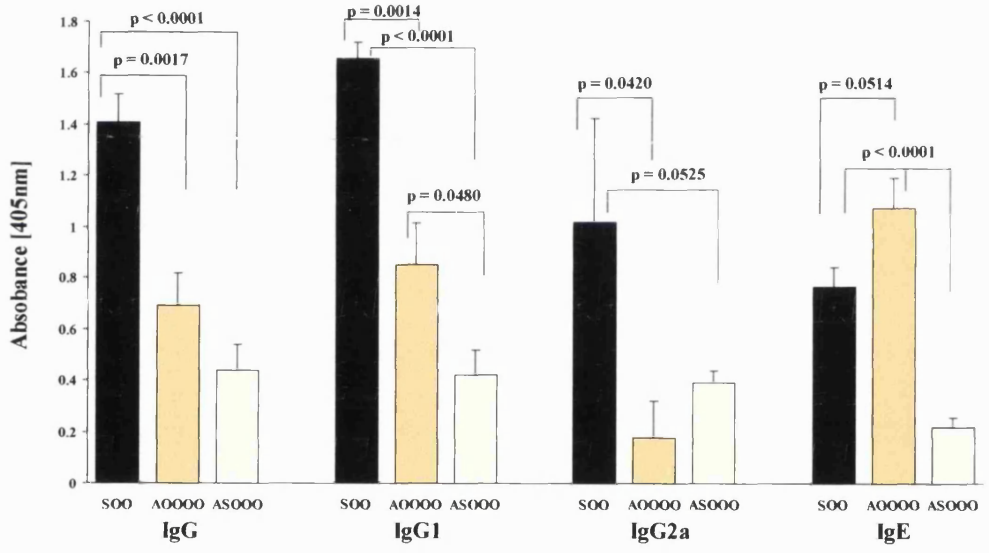
Fig. 6.7 Specific antibody responses after epicutaneous sensitisation followed by feeding of antigen

Animals were epicutaneously sensitised to peanut protein (APPPP), to OVA (AOOOO), to saline (ASPPP and ASOOO) or received no skin procedure (SPP or SOO) 6 (A and B) or 20 (C) days prior to a tolerogenic feed of peanut protein, OVA or saline as control. All animals were then immunised as outlined in Fig. 6.1. Peanut- (A and C) or OVA- (B) specific immunoglobulins were measured in serum 6 or 8 weeks after the epicutaneous sensitisation (5 weeks after gavage feeding). Serum samples were diluted 1:1000 for IgG and IgG1, 1:200 for IgG2a and 1:10 for IgE prior to analysis. Results are expressed as the mean antibody levels + 1 SEM (n=6-8). [SPP/SOO (■): No skin procedure, fed saline, immunised with peanut/OVA in CFA, recall immunised with peanut/OVA; APPPP/AOOOO: Skin abraded, painted with peanut (■)/OVA ( ), fed peanut/OVA, immunised with peanut/OVA in CFA, recall immunised with peanut/OVA; ASPPP/ASOOO: Skin abraded, painted with saline, fed peanut (■)/OVA ( ), immunised with peanut/OVA in CFA, recall immunised with peanut/OVA]

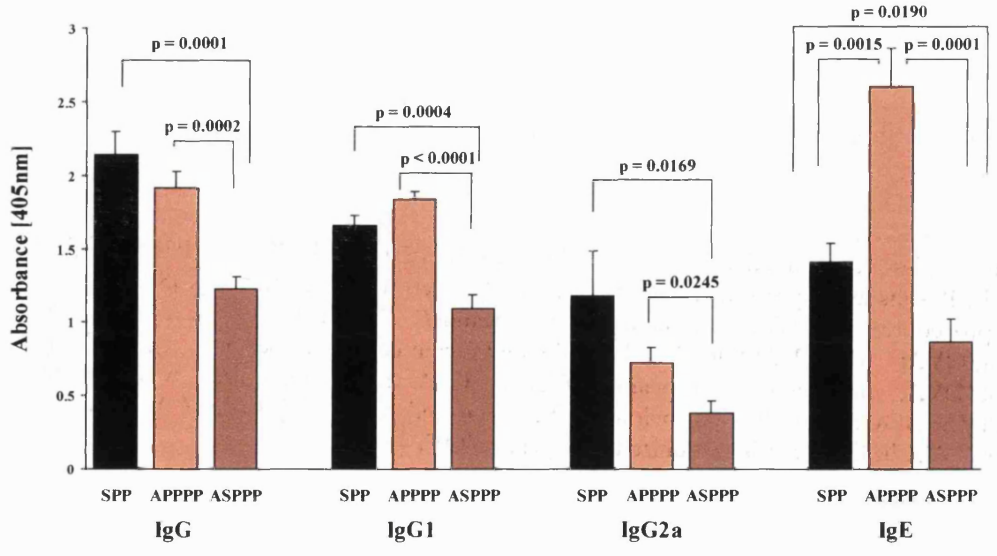
A. 6 days Peanut



B. 6 days OVA



C. 20 days Peanut



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6.3.2 Tolerance to peanut may be disrupted by epicutaneous exposure

To investigate whether an existing state of peanut-specific tolerance, or orally-induced systemic hyporesponsiveness, could be modulated by epicutaneous exposure to peanut protein, animals were fed a tolerogenic dose of peanut protein 7 days prior to epicutaneous sensitisation.

Three weeks later, a peanut protein footpad challenge demonstrated that T_{DTH} cells were no longer hyporesponsive in animals epicutaneously exposed to peanut protein, but had become activated (Fig.6.8A). These animals showed DTH responses well above that of animals only given oral antigen and even significantly higher than tolerant control animals subsequently immunised with peanut protein in CFA (as in Chapter 3) ($p < 0.0001$). However, the tolerogenic feed conferred some protection from sensitisation as the DTH responses were lower than in animals fed saline prior to epicutaneous peanut protein exposure ($p = 0.0061$) (Fig. 6.8A).

In vitro peanut-specific T cell proliferation was measured in CLN draining the skin sensitisation site and in the spleen. CLN T cell proliferation in tolerant animals subsequently exposed to peanut protein on the skin was significantly higher ($p < 0.01$) than in tolerant animals epicutaneously exposed to saline (Fig. 6.8B). The observed proliferation was however significantly lower ($p < 0.01$) than by CLN cells from peanut-sensitive animals fed saline prior to epicutaneous peanut protein exposure which proliferated strongly to stimulation with peanut protein (Fig. 6.8B), suggesting that the initial feed inhibited the response to epicutaneous peanut protein exposure. Similar proliferation patterns were observed with splenic T cells (data not shown). The measurements of *in vivo* and *in vitro* T cell responses thus gave similar response patterns between experimental groups.

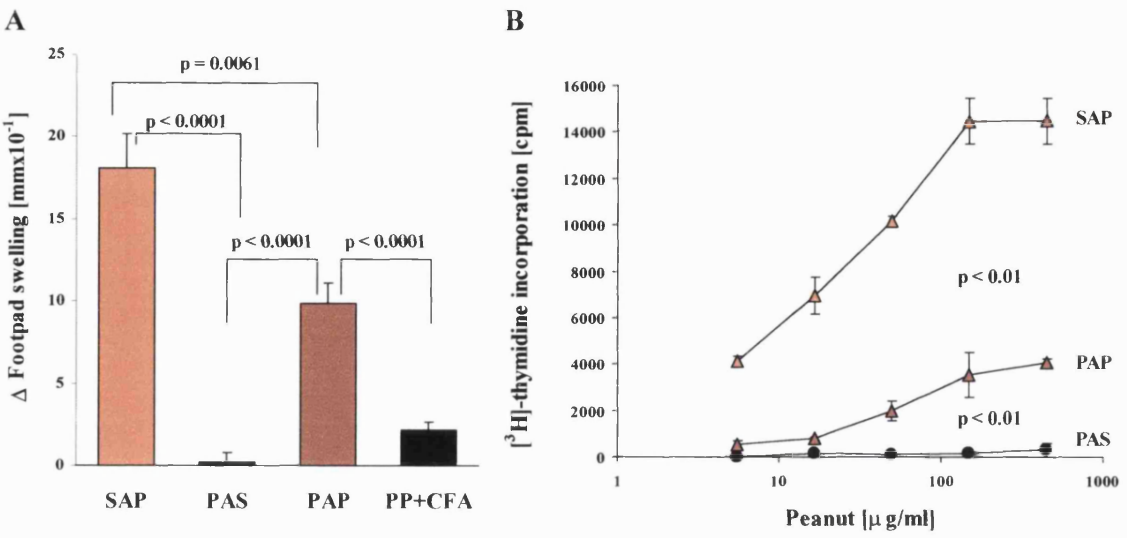

Fig. 6.8 Influence of epicutaneous sensitisation on *in vivo* and *in vitro* T cell responses in previously tolerant or naïve animals

Animals were fed 100mg peanut protein or saline by gavage 1 week prior to epicutaneous exposure to peanut protein or saline. (A) DTH responses to peanut protein three weeks after epicutaneous sensitisation or subcutaneous immunisation with peanut protein in CFA. T_{DTH} activity was measured as net footpad increments 24 hr after subcutaneous injection of 100μg peanut protein in the hind footpad. Results are the expressed as the mean footpad swelling + 1 SEM (n = 6) (B) Peanut-specific T cell proliferation in CLN three weeks after epicutaneous sensitisation. CLN cells were pooled from 6 mice per group and results are expressed as the mean cpm of triplicate cultures for 90 hr ± 1 SEM. Background proliferation when no antigen was present has been subtracted. Squares represent proliferation to a control antigen. All groups proliferated strongly and equally when stimulated with ConA. [SAP (▲): Fed saline, skin abraded, painted with peanut; PAS (●): Fed 100mg peanut, skin abraded, painted with saline; PAP (▲): Fed 100mg peanut, skin abraded, painted with peanut, PP+CFA: Fed 100mg peanut, immunised with peanut in CFA]

Cytokine secretion from CLN and spleen cells was measured after *in vitro* culture with peanut protein for 96 hr –144 hr. T cells from draining CLN of animals fed saline before epicutaneous exposure to peanut protein produced high amounts of IL-4 and IL-10 which increased over the 6 day culture period. Animals fed a tolerogenic dose of peanut protein prior to epicutaneous exposure to peanut protein also produced high levels of IL-4 and moderate levels of IL-10, both of which were well above animals epicutaneously exposed to saline, although lower than saline-fed animals. There was no difference in secretion of IFN-γ and TGF-β between groups (Fig. 6.9). A similar pattern of cytokine production was observed from splenic T cells, although the levels of cytokines were lower (results not shown).

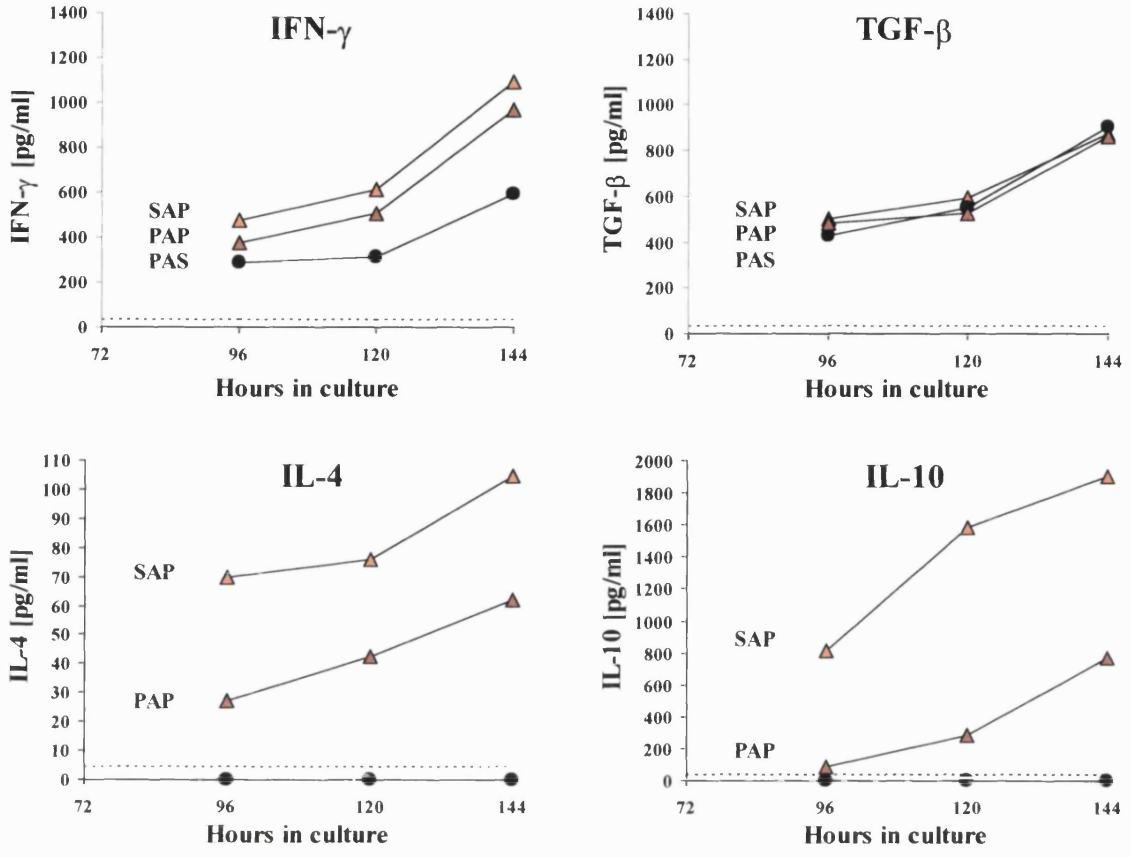

Fig. 6.9 Effect of epicutaneous sensitisation on cytokine production in previously tolerant or naïve animals

Animals were fed 100mg peanut protein or saline by gavage 1 week prior to epicutaneous exposure to peanut protein or saline. Levels of secreted IFN-γ, TGF-β, IL-4 and IL-10 after *in vitro* stimulation with peanut protein were analysed from CLN cells three weeks after the epicutaneous sensitisation. CLN cells were pooled from 6 mice per group and analysed in triplicate. Results are expressed as the mean cytokine levels. Dotted lines represent the limit of detection of each cytokine assay. No cytokines were produced by any group in response to a control antigen or when cells were not stimulated. [SAP (▲): Fed saline, skin abraded, painted with peanut; PAS (●): Fed 100mg peanut, skin abraded, painted with saline; PAP (▲): Fed 100mg peanut, skin abraded, painted with peanut]

Peanut-specific antibodies were measured in serum three weeks after the epicutaneous sensitisation. Analysis of specific IgG responses showed that feeding of 100mg peanut protein inhibited the responses to subsequent epicutaneous sensitisation with peanut. IgG and IgG1 levels were lower than in animals fed saline prior to epicutaneous sensitisation, but higher than in animals fed peanut protein and epicutaneously exposed to saline. IgE responses were however no longer suppressed and levels of IgE were similar to saline-fed peanut-sensitive animals (Fig. 6.10). Very low levels of specific IgG2a were produced in any experimental group indicating the Th2-biased nature of the epicutaneous sensitisation route (Fig. 6.10).

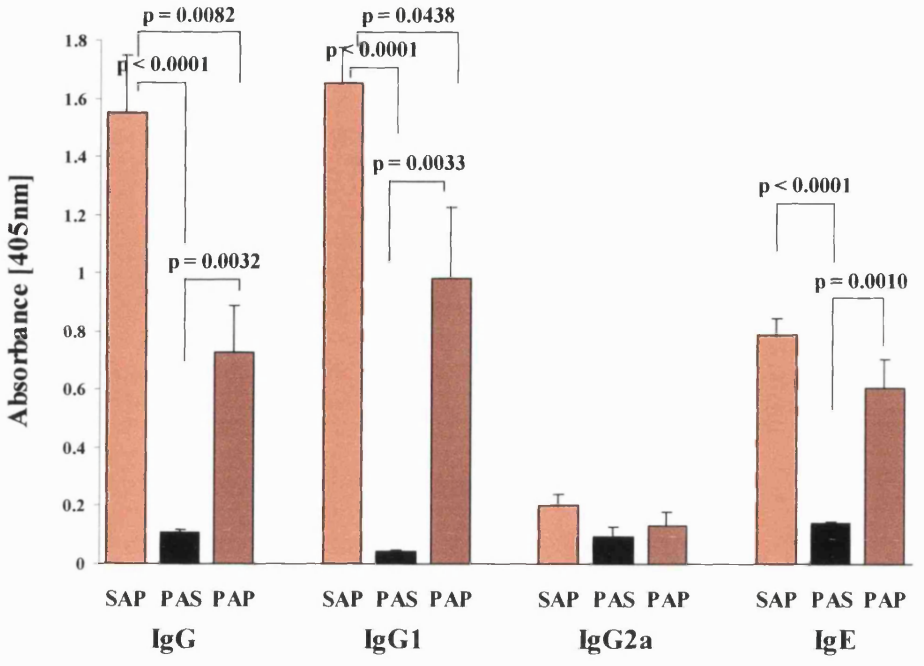

Fig. 6.10 Influence of epicutaneous sensitisation on levels of specific antibodies in previously tolerant or naïve animals

Animals were fed 100mg peanut protein or saline by gavage 1 week prior to epicutaneous exposure to peanut protein or saline. Peanut-specific immunoglobulins were measured in serum three weeks after epicutaneous sensitisation. Serum samples from individual animals were diluted 1:10 prior to analysis of specific-IgG, IgG1, IgG2a and IgE. Results are expressed as the mean antibody concentrations + 1 SEM (n=6). [SAP: Fed saline, skin abraded, painted with peanut; PAS: Fed 100mg peanut, skin abraded, painted with saline; PAP: Fed 100mg peanut, skin abraded, painted with peanut]

In summary, these experiments demonstrated that an existing state of orally-induced systemic tolerance confers protection from epicutaneous sensitisation. T cell and IgG responses were lower in animals fed a tolerogenic dose of peanut protein than in animals fed saline prior to epicutaneous sensitisation with peanut protein. However, the results may also suggest that an existing state of tolerance can be broken by epicutaneous allergen exposure. DTH responses in fed animals were significantly higher following epicutaneous immunisation than subcutaneous immunisation with CFA. Additionally, the feeding did not inhibit the development of specific-IgE responses. Results showed equally high levels of IgE in animals fed a tolerogenic dose of peanut protein prior to epicutaneous sensitisation as in naïve animals fed saline prior to a similar epicutaneous sensitisation.

6.4 Discussion

Peanuts contain potent allergens which cause severe allergic reactions in sensitised children and adults. The clinical features of peanut allergy are usually expressed as an acute IgE-mediated reaction after ingestion or inhalation of peanut protein [Bock et al., 2001; Helm and Burks, 2000; Sicherer et al., 1999]. However, the primary route of sensitisation is not clear, and may not always be through the gastrointestinal tract. In this study it was demonstrated that animals epicutaneously exposed to peanut protein produced high levels of peanut-specific IL-4 and IgE upon oral challenge. This is consistent with the induction of a potent Th2 response and may suggest that peanut allergy can be induced by exposure to peanut protein through the skin. Similar results were obtained with OVA, suggesting that the strongly sensitising effect of epicutaneous antigen exposure is a more general phenomenon and not restricted to the highly allergenic proteins in peanut.

Primary epicutaneous exposure to peanut protein or OVA interfered with normal tolerance induction. A time-dependent threshold for the induction of oral tolerance was observed. Feeding 6 days after the epicutaneous exposure still induced T- and B cell hyporesponsiveness in most parameters assessed. However, TGF-β secretion was reduced, perhaps as a sign of failing tolerance induction. Importantly, IL-4 was increased and specific IgE production was significantly higher in epicutaneously sensitised animals following an oral feed (Fig. 6.6A,B and 6.7A,B). Twenty days after epicutaneous exposure, the systemic Th2 response was persistent and could not be suppressed by induction of oral tolerance (Fig. 6.3 – 6.7). This supports the notion that it is more difficult to suppress an ongoing immune response than to induce tolerance *de novo* [Strobel and Mowat, 1998]. Epicutaneous sensitisation not only prevented the normal induction of tolerance, but affected mucosal responses in a way that led to further enhanced Th2 responses and IgE production upon gastrointestinal exposure. These results reinforces my (Chapter 5 and 7) and others [Spergel et al., 1998; Wang et al., 1996] previous findings that epicutaneous exposure to allergens specifically drives Th2 responses. Following gastrointestinal challenge, the epicutaneously sensitised mice additionally developed symptoms of systemic anaphylaxis. The symptoms appeared 10-15min. after challenge and included puffiness around the eyes, decreased activity, pilar recti and cyanosis around the eyes, tail and feet. Some animals were inactive even after prodding, but no animals were lost during oral challenge and they usually recovered within three hours of challenge. The saline sensitised control group showed no symptoms after challenge. The symptoms were however not

formally evaluated using a scoring system as has been described in other models of hypersensitivity [Li et al., 2000]. These results demonstrate that local exposure to antigen on the skin can affect mucosal responses in the GALT and mediate systemic disease manifestations.

An existing state of orally-induced systemic hyporesponsiveness was demonstrated to confer some protection from sensitisation through the skin. Immune responses following epicutaneous exposure to peanut protein on peanut-tolerant animals were not as high as in naïve animals exposed to peanut protein in an identical way. However, IgE was a notable exception, with high production of peanut-specific IgE also following cutaneous exposure on tolerant animals (Fig. 6.10). This suggests that existing oral tolerance can be modified by subsequent epicutaneous exposure to peanut protein and may partly explain why some patients develop food allergies, such as peanut allergy, after years of tolerance [Crespo and Rodriguez, 2003].

Protein antigen exposure at mucosal surfaces, such as the gut, nose and lung normally leads to immunological hyporesponsiveness [Bilsborough and Viney, 2002; Garside and Mowat, 2001; Strobel and Mowat, 1998; Weiner, 1997]. In contrast, antigen exposure through the skin results in strong Th2 immunity, and may play an important role in giving rise to various allergic diseases. As demonstrated here, epicutaneous exposure to allergens can cause food allergy-like systemic responses with high levels of IgE upon oral challenge. Similar results were recently published in a study showing that prolonged epicutaneous exposure to OVA may result in elevated levels of plasma histamine as well as histological changes in the intestine and lungs following oral challenge with OVA [Hsieh et al., 2003]. Epicutaneous exposure to allergens can also result in asthma-like airway hyperresponsiveness following challenge in the lung [Spergel et al., 1998]. It may be, that diseases such as asthma, allergic rhinitis and food allergy reflect different facets of a single disease, where the diseases manifestation is determined by the route of challenge. Epicutaneous sensitisation could potentially represent a common sensitisation pathway for these allergic diseases.

AD usually predates the development of both asthma and food allergy [Sampson, 1999; Sicherer and Sampson, 1999]. In inflammatory skin diseases such as AD, psoriasis and eczema the barrier properties of the stratum corneum are diminished. Impaired barrier function of the

epidermis also in skin areas without lesions is characteristic of atopic dermatitis [Kondo et al., 1998; Wollenberg et al., 2000]. In the experiments presented in this study, the stratum corneum was disrupted by abrasion, prior to peanut administration, to mimic the desquamated skin of AD and other inflammatory skin conditions. It is proposed here, that allergen exposure through barrier-disrupted skin could serve as a natural sensitisation pathway for food allergy and other allergic hypersensitivity diseases. Its potential role in the pathogenesis of various allergic diseases should be considered and further investigations initiated. The mechanisms underlying the Th2-predominant responses induced in the cutaneous microenvironment also require further investigation.

Most studies examining sensitisation and allergy to foods use intraperitoneal sensitisation protocols and strong adjuvants such as cholera toxin and aluminium hydroxide are commonly used [Li et al., 1999; Roy et al., 1999]. A murine model of peanut allergy where animals are sensitised through the oral route exists but relies on concomitant and repeated feeding of cholera toxin during peanut sensitisation [Li et al., 2000]. Indeed, allergic sensitisation via the gastrointestinal tract might be facilitated by injury in an as yet undefined way. Several studies, including those described here, have demonstrated that the route of antigen delivery profoundly affects the nature of an immune response. Models using intraperitoneal food sensitisation and challenge are thus not satisfactory for studying human food allergy, which is provoked by mucosal allergen exposure. The novel model presented here using epicutaneous sensitisation and oral challenge could be used as a more physiologically relevant model for studying peanut-induced sensitisation and clinical anaphylaxis. It may additionally be useful for studying interactions between AD and food allergy as well as the effects of epicutaneous sensitisation on other allergic hypersensitivity diseases. This model appears to be IgE-mediated, and resembles physiologic and immunologic phenomena seen in human subjects. How closely it mimics human pathophysiology remains to be seen.

Chapter 7

Epicutaneous immunisation modifies subsequent and established antigen-specific immune responses

7.1 Introduction

In the previous Chapters it was shown that epicutaneous immunisation through barrier-disrupted skin induces strong antigen-specific responses which are more potent than those induced by subcutaneous immunisation. It was also shown that epicutaneous immunisation can modify immune responses following oral antigen challenge and specifically drive Th2-type responses.

In this Chapter the potential of epicutaneous antigen immunisation to modify immune responses to subsequently encountered antigen and established antigen-specific immune responses is further investigated. The ability of epicutaneous immunisation to boost immune responses following injection of antigen at a distant immunological site is examined. The potential of epicutaneous immunisation to interfere with the development of Th1-type immune responses is analysed after immunisation with antigen in CFA. Finally, the ability of epicutaneous immunisations to modify the nature of established Th1-type immune responses is explored and the potential application of epicutaneous immunisation to vaccination and therapy is discussed.

7.2 Immunisation protocols

7.2.1 Epicutaneous immunisation followed by footpad immunisation without adjuvant

To investigate the potential of epicutaneous immunisation to prime animals for strong immune responses upon secondary challenge at a distant site, mice were immunised in the footpad with 100μg peanut protein in sterile PBS 20 days after epicutaneous immunisation with peanut proteins. Control animals were epicutaneously immunised with saline or left naïve prior to a similar footpad immunisation and 24 hr later DTH responses were measured in all animals. Three weeks after the footpad immunisation, animals were culled and popliteal lymph nodes (PopLN), spleens and serum were collected and analysed for T cell responses and antibody production. The immunisation protocol is outlined in Fig. 7.1 and abbreviations used are listed in Table 7.1.

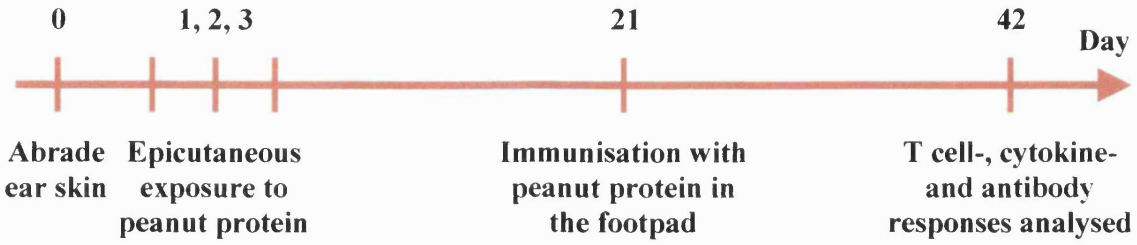

Fig. 7.1 Experimental protocol for epicutaneous immunisation followed by subcutaneous footpad immunisation without adjuvant
Schematic representation of the experimental protocol used for studying the effect of epicutaneous immunisation on immune responses in a distal site following footpad immunisation

Table 7.1 Abbreviations used for protocol of epicutaneous immunisation prior to footpad immunisation

Procedure / Abbreviation	Skin abrasion	Topical application	Footpad immunisation
ASP	Abrasion	Saline	Peanut
APP	Abrasion	Peanut	Peanut
P	-	-	Peanut

7.2.2 Epicutaneous immunisation prior to subcutaneous immunisation with CFA

To investigate the ability of epicutaneous immunisation to modulate the outcome of a subsequent potent subcutaneous immunisation, mice were epicutaneously immunised with peanut protein and three weeks later tail-base immunised with 100μg of peanut protein emulsified 1:1 in CFA. Three weeks later, the mice were challenged in the footpad with 100μg peanut protein in PBS and DTH responses were measured. Animals were sacrificed 1 week after the footpad immunisation and para-aortic lymph nodes (PLN), spleen and serum were collected and analysed for T cell responses and antibody production. Fig. 7.2 shows a schematic diagram of the experimental protocol and the abbreviations used are listed in Table 7.2.

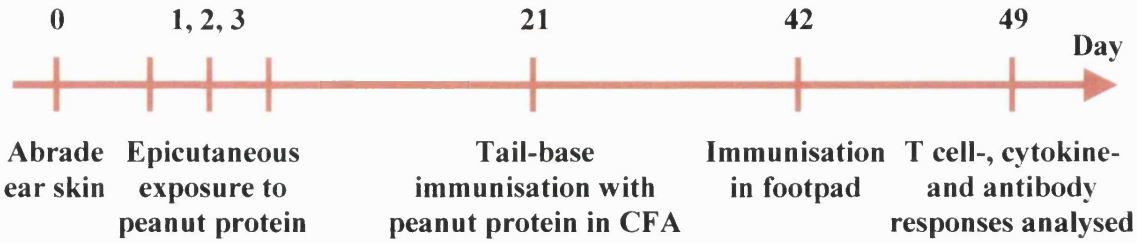

Fig. 7.2 Diagram of epicutaneous immunisation prior to immunisation with antigen in CFA
Schematic representation of the experimental protocol used to study the effect of epicutaneous immunisation on subsequent Th1-type immunisation with antigen in CFA

Table 7.2 Abbreviations used for protocol of epicutaneous immunisation prior to immunisation with antigen in CFA

Procedure / Abbreviation	Skin abrasion	Topical application	Immunisation with CFA	Footpad immunisation
ASPP	Abrasion	Saline	Peanut	Peanut
APPP	Abrasion	Peanut	Peanut	Peanut
PP	-	-	Peanut	Peanut

7.2.3 Subcutaneous immunisation with CFA prior to epicutaneous immunisation

To additionally investigate the potential of epicutaneous immunisation to modify established antigen-specific immune responses, animals were tail-base immunised with 100µg of peanut protein in CFA first and three weeks later immunised epicutaneously with peanut protein. Three weeks after the epicutaneous immunisation mice were immunised in the footpad with 100µg of peanut protein in PBS and the DTH responses measured. One week later, the animals were culled and CLN, spleen and serum were collected and analysed for T cell responses and antibody production. The experimental protocol is shown in Fig. 7.3 and abbreviations listed in Table 7.3.

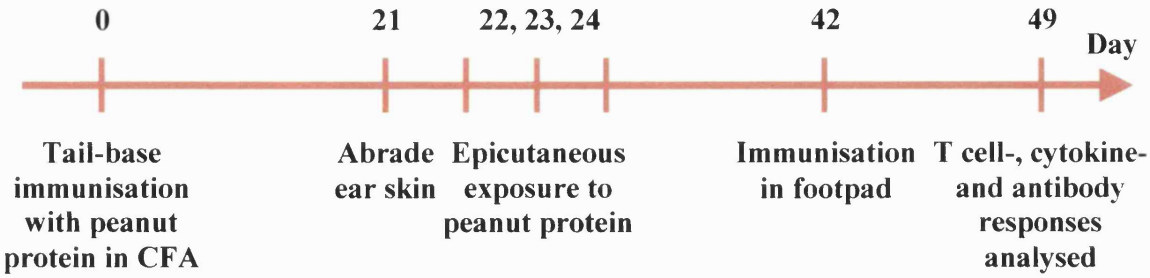

Fig. 7.3 Diagram of immunisation with antigen in CFA prior to epicutaneous immunisation
Schematic representation of the experimental protocol used to study whether epicutaneous immunisation effects the nature of an established Th1-biased specific immune response

Table 7.3 Abbreviations used for protocol of subcutaneous immunisation with antigen in CFA prior to epicutaneous immunisation

Procedure / Abbreviation	Immunisation with CFA	Skin abrasion	Topical application	Footpad immunisation
PASP	Peanut	Abrasion	Saline	Peanut
PAPP	Peanut	Abrasion	Peanut	Peanut

7.3 Results

7.3.1 Epicutaneous immunisation enhances responses to a subsequent footpad immunisation

DTH responses to peanut protein were measured 24 hr after footpad injection and results showed that DTH responses were enhanced by the epicutaneous immunisation (Fig. 7.4A). Only the animals epicutaneously exposed to peanut protein prior to the subcutaneous footpad immunisation produced a strong DTH response to peanut. DTH responses in these animals were significantly higher than in animals epicutaneously exposed to saline or in animals receiving no skin procedure ($p < 0.0001$) (Fig. 7.4A). Three weeks after the footpad immunisation, peanut-specific proliferation and cytokine production of cells from the PopLN draining the footpad immunisation site and from the spleen were measured. In cultures of PopLN cells from animals epicutaneously immunised with peanut, a relatively weak but

distinct peanut-specific T cell proliferation was observed. This proliferation was significantly higher ($p < 0.001$) than that detected in animals epicutaneously immunised with saline or in animals given no skin procedure prior to footpad immunisation with peanut protein (Fig. 7.4B). Similar results were observed with splenic T cells (data not shown).

Analysis of cytokines secreted from PopLN cells showed that cells from animals epicutaneously exposed to saline and animals receiving no cutaneous treatment prior to the footpad immunisation produced around 140pg/ml IFN-γ after 96 hr *in vitro* stimulation with peanut protein (Fig. 7.5A). PopLN cells from animals epicutaneously immunised with peanut protein prior to footpad immunisation secreted roughly 50% less IFN-γ (Fig. 7.5A). In contrast, these cells produced high levels of IL-4 (100pg/ml) upon stimulation with peanut protein, while the animals epicutaneously exposed to saline or receiving no skin treatment produced levels of IL-4 below- or around the 5pg/ml detection point for the assay (Fig. 7.5B). None of the groups produced detectable amounts of IL-10. Similar cytokine patterns were produced from splenic cells, although no IFN-γ was detectable in animals epicutaneously immunised with peanut protein and no IL-4 was detectable in animals epicutaneously exposed to saline and in animals receiving no skin procedure prior to the footpad injection (data not shown).

Levels of peanut-specific antibodies produced by the experimental animals were also investigated. Animals that were not epicutaneously immunised with peanut protein produced some IgG antibodies in response to the footpad immunisation, but the levels of IgG and IgG1 were significantly lower than in the epicutaneously immunised animals and no IgE was observed. There was no significant difference in levels of IgG2a between the groups, but the animals epicutaneously immunised with peanut protein produced significantly larger amounts of IgE (Fig. 7.6).

Taken together, these data show that epicutaneous immunisation can prime animals to respond with a Th2-bias to a second antigen challenge distant from the original immune induction site.

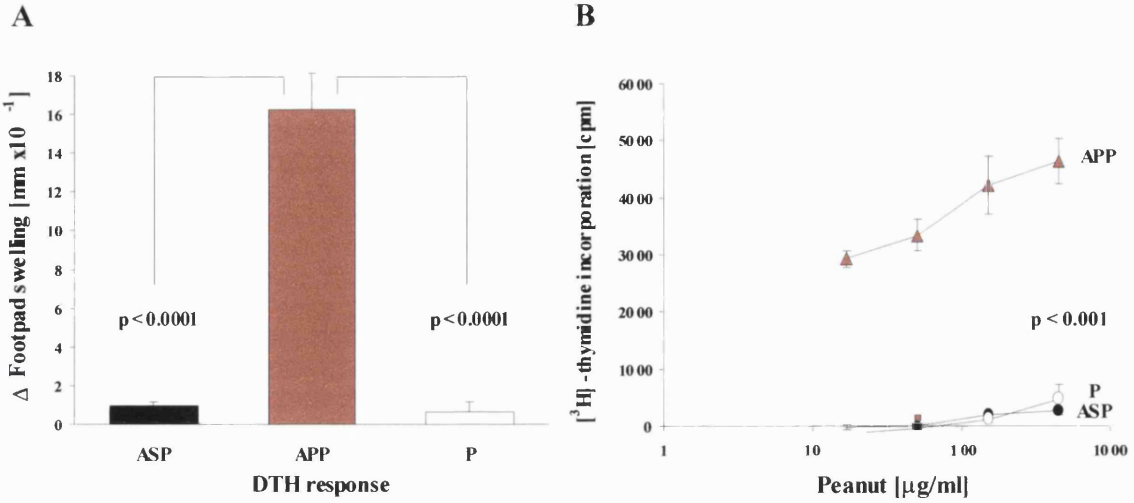

Fig. 7.4 Specific T cell responses after epicutaneous immunisation followed by footpad immunisation without adjuvant

Animals were epicutaneously immunised with peanut protein (APP), with saline (ASP) or left untreated (P) prior to similar footpad immunisations with peanut protein in PBS. (A) DTH responses 24 hr after injection of peanut protein in the footpad. Results are expressed as the mean DTH response + 1 SEM (n = 6). (B) Peanut-specific PopLN T cell proliferation 3 weeks after footpad immunisation. PopLN were pooled from 6 mice per group and results are expressed as the mean of triplicate cultures for 90 hr ± 1 SEM. Background proliferation without antigen has been subtracted. Squares represent proliferation to a control antigen. All groups responded with similar [cpm] when stimulated with ConA. [ASP (●): Skin on ears abraded, painted with saline, immunised with peanut in the footpad; APP (▲): Skin on ears abraded, painted with peanut, immunised with peanut in the footpad; P (○): No skin procedure, immunised with peanut in the footpad]

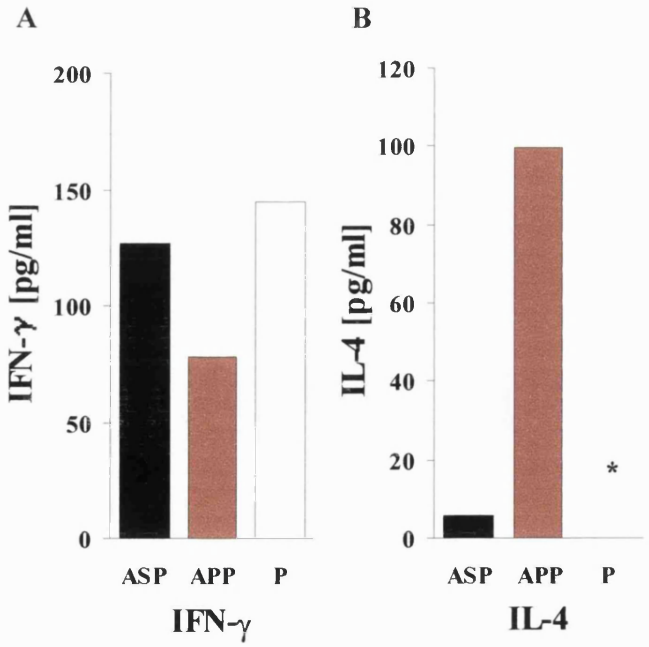

Fig. 7.5 Cytokine responses after epicutaneous immunisation followed by footpad immunisation without adjuvant

Animals were epicutaneously immunised with peanut, saline or left untreated prior to a single peanut protein injection in the footpad. Three weeks later, the secretion of IFN-γ (A) and IL-4 (B) from PopLN cells after 96 hr reactivation with peanut protein *in vitro* were measured. PopLN cells were pooled from 6 mice per group and analysed in triplicate. No cytokines were detected when cells were not stimulated or stimulated with a control antigen (OVA). [ASP: Skin on ears abraded, painted with saline, immunised with peanut in the footpad; APP: Skin on ears abraded, painted with peanut, immunised with peanut in the footpad; P: No skin procedure, immunised with peanut in the footpad; *: Below detection limit (5pg/ml) of the assay]

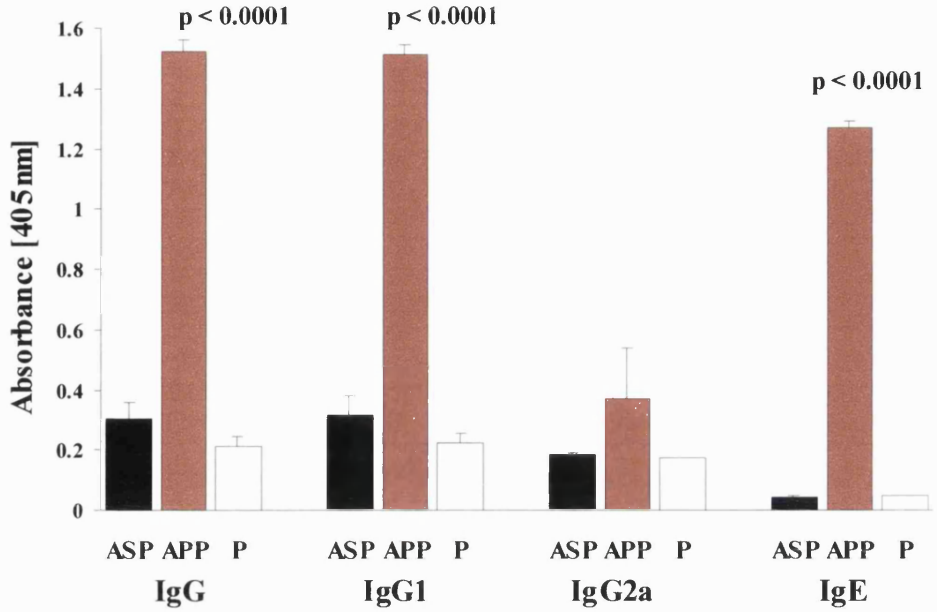

Fig. 7.6 Peanut-specific antibody production after epicutaneous immunisation followed by footpad immunisation without adjuvant
Animals were epicutaneously immunised with peanut protein (APP), saline (ASP) or untreated (P) prior to subcutaneous immunisation with peanut protein in the footpad. Relative peanut-specific antibody levels in serum were analysed three weeks after the footpad injection. All serum samples were diluted 1:10 prior to analysis of IgG, IgG1, IgG2a and IgE. Results are expressed as the mean antibody level + 1 SEM (n=6). [ASP: Skin on ears abraded, painted with saline, immunised with peanut in the footpad; APP: Skin on ears abraded, painted with peanut, immunised with peanut in the footpad; P: No skin procedure, immunised with peanut in the footpad]

7.3.2 Epicutaneous immunisation enhances and modifies responses to subsequent immunisation with CFA

As demonstrated in Chapter 5 and 6 as well as in the above section, epicutaneous immunisation gives rise to a Th2-predominant specific immune response. To examine the persistence of this Th2-type immunity and the potential of epicutaneous antigen immunisation to modify immune responses to subsequently encountered antigen, mice were epicutaneously immunised to peanut protein and subsequently given a Th1-type subcutaneous immunisation with peanut protein in CFA (immunisation protocol shown in Fig. 7.2).

Epicutaneous immunisation significantly enhanced the vigour of the response to a subsequent peanut/CFA immunisation. DTH responses to peanut protein were larger in the epicutaneously immunised animals compared to control animals epicutaneously immunised

with saline (p = 0.030) or animals receiving no skin procedure (p = 0.003) prior to peanut/CFA immunisation (Fig. 7.7A).

In vitro T cell proliferation to peanut protein in cultures of PLN cells draining the peanut/CFA immunisation site 1 week after recall immunisation showed similar response patterns to the *in vivo* DTH response. The epicutaneous peanut protein immunisation 8 weeks prior to measuring the proliferative response enhanced PLN T cell proliferation ($p < 0.01$) compared to PLN cells from animals epicutaneously exposed to saline and from animals without skin procedures prior to a similar peanut/CFA and recall immunisation protocol (Fig. 7.7B). Similar results were obtained when measuring peanut-specific proliferative responses in splenic T cells (results not shown).

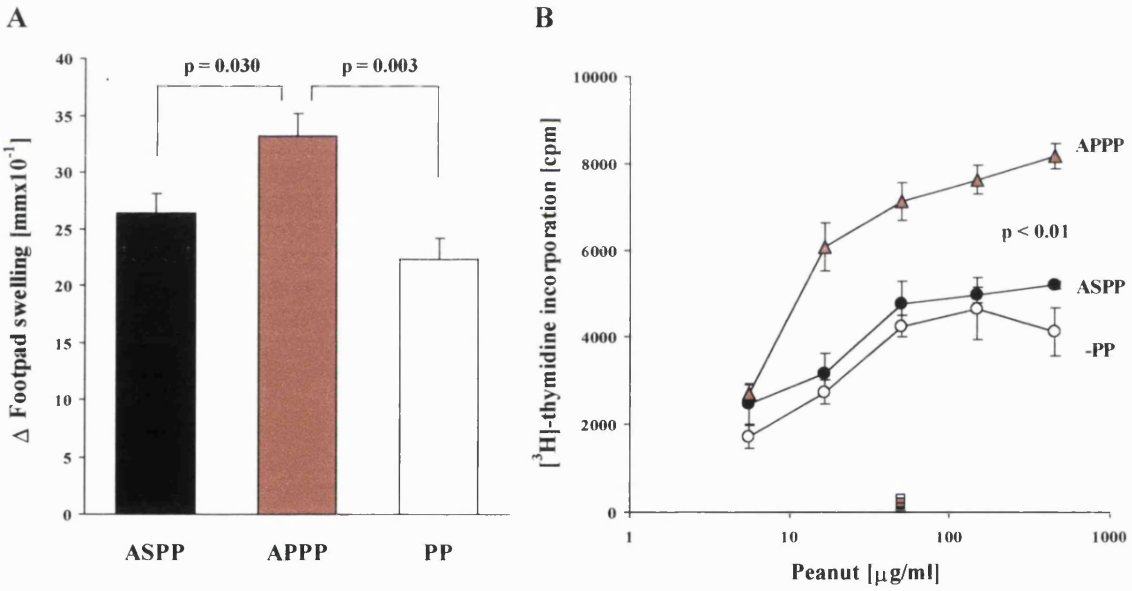

Fig. 7.7 *In vivo* and *in vitro* T cell proliferation after epicutaneous immunisation followed by immunisation with CFA

Animals were epicutaneously immunised with peanut protein (APPP), with saline (ASPP) or untreated (PP) 3 weeks prior to tail-base immunisations with peanut protein in CFA and recall immunisation with peanut. (A) DTH responses 24 hr after footpad injection of peanut protein in PBS. Results are expressed as mean footpad swelling + 1 SEM (n=6 for ASPP and PP, n=7 for APPP). (B) Peanut-specific T cell proliferation in cultures of PLN draining the peanut/CFA immunisation site. PLN were pooled from 6-7 mice per group and results are expressed as the mean of triplicate cultures for 90 hr ± 1 SEM. Background proliferation when no antigen was present has been subtracted. Squares represent proliferation to a control antigen (OVA). All groups responded with similar [cpm] when stimulated with ConA. [ASPP (●): Skin abraded, painted with saline, immunised with peanut in CFA, recall immunised with peanut; APPP (▲): Skin abraded, painted with peanut, immunised with peanut in CFA, recall immunised with peanut; PP ϕ): No skin procedure, immunised with peanut in CFA, recall immunised with peanut]

Moreover, the cytokines produced by PLN cells draining the peanut/CFA immunisation site of animals epicutaneously exposed to peanut protein showed an inverse pattern to the cytokines produced by PLN cells from control animals. As shown in Fig. 7.8A-C, epicutaneous immunisation to peanut protein prior to peanut/CFA immunisation significantly decreased levels of IFN-γ and IL-10 but enhanced levels of IL-4. These data are consistent with a Th2-predominant response and suggests that the epicutaneous immunisation biased responses to antigen in CFA from Th1- to Th2-type immunity.

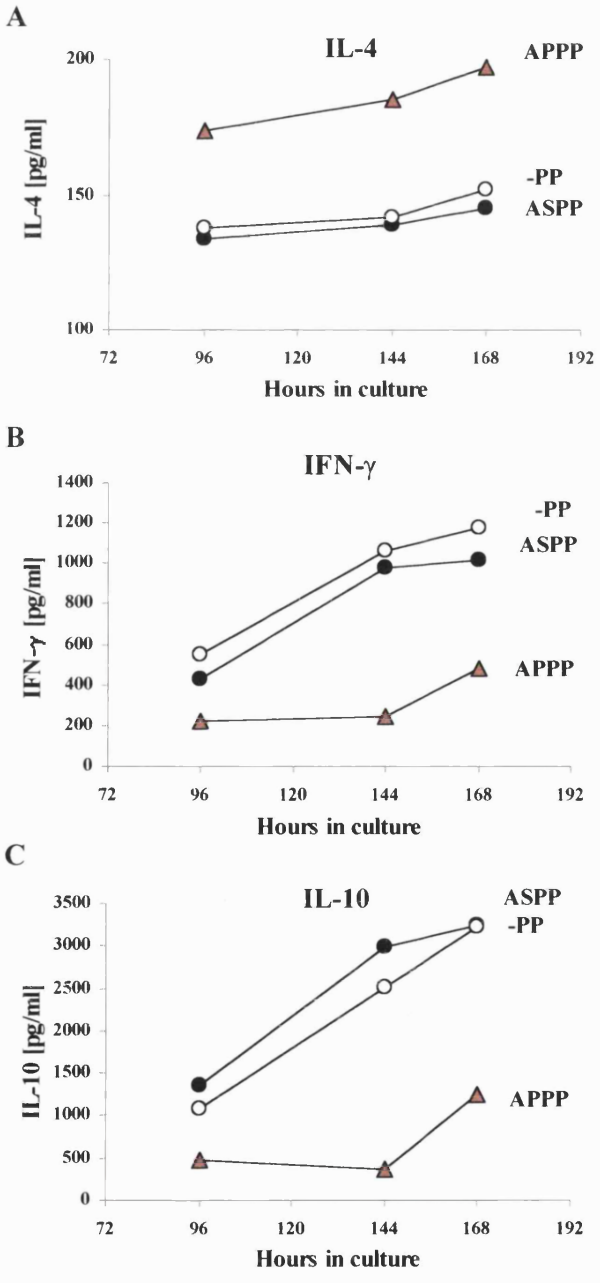

Fig. 7.8 Cytokine response after epicutaneous peanut immunisation followed by immunisation with CFA

Animals were epicutaneously immunised with peanut protein, saline or left untreated prior to tail-base immunisation with peanut protein in CFA and recall immunisation. IFN-γ (A), IL-4 (B) and IL-10 (C) secretion from PLN cells draining the CFA immunisation site were analysed four weeks after the CFA immunisation (7 weeks after epicutaneous immunisation with peanut). PLN cells were pooled from 6-7 mice per group and analysed in triplicate after *in vitro* stimulation with peanut protein for 96 – 168 hr. No cytokines were produced in response to OVA as a control antigen or when cells were not stimulated. Similar results were obtained with spleen cells. [ASPP (●): Skin abraded, painted with saline, immunised with peanut in CFA, recall immunised with peanut; APPP (▲): Skin abraded, painted with peanut, immunised with peanut in CFA, recall immunised with peanut; PP (○): No skin procedure, immunised with peanut in CFA, recall immunised with peanut]

Analysis of peanut-specific antibody responses showed that epicutaneous immunisation with peanut protein significantly enhanced levels of specific IgE and IgG1 following peanut/CFA immunisation compared to controls. In contrast, levels of specific IgG2a were reduced compared to control animals epicutaneously immunised with saline or untreated prior to peanut/CFA immunisation (Fig. 7.9). This Th2-domiant antibody pattern in animals epicutaneously immunised with peanut protein was consistent with the cytokine analysis. There was no difference in overall production of peanut-specific IgG between the experimental groups (Fig. 7.9).

These results demonstrate that the immune responses induced by peanut/CFA immunisation were both significantly enhanced and modified by prior epicutaneous immunisation. Together, these experiments show that a persistent and dominant antigen-specific Th2-type response was induced by the epicutaneous exposure to peanut antigens on barrier-disrupted skin.

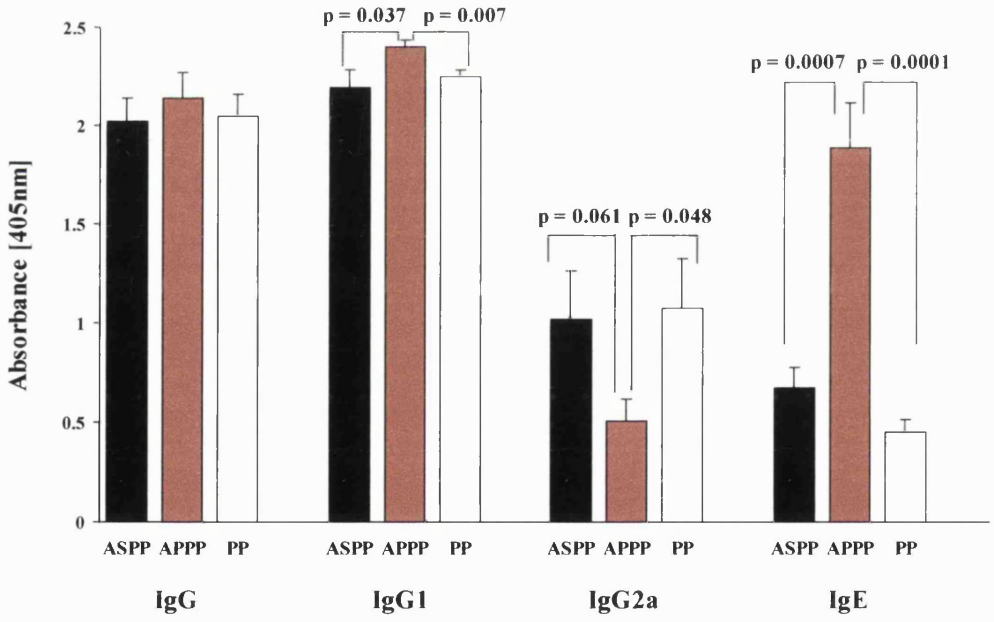

Fig. 7.9 Peanut-specific antibody production after epicutaneous immunisation followed by immunisation with CFA

Animals were epicutaneously immunised with peanut protein (APPP), saline (ASPP) or left untreated (PP) prior to tail-base immunisation with peanut protein in CFA and recall immunisation. Peanut-specific antibody levels in serum were analysed 4 weeks after CFA immunisation (7 weeks after epicutaneous immunisation). Serum samples were diluted 1:1000 for IgG and IgG1, 1:200 for IgG2a and 1:10 for IgE prior to analysis. Results are expressed as mean antibody levels + 1 SEM (n=6-7). [ASPP: Skin abraded, painted with saline, immunised with peanut in CFA, recall immunised with peanut; APPP: Skin abraded, painted with peanut, immunised with peanut in CFA, recall immunised with peanut; PP: No skin procedure, immunised with peanut in CFA, recall immunised with peanut]

7.3.3 Epicutaneous immunisation converts an established antigen-specific Th1- to a Th2-type response

To investigate whether the Th2-biased epicutaneous immunisation could modify an established Th1-type immune response to the same antigen, animals were tail-base immunised with peanut protein in CFA and three weeks later epicutaneously immunised with peanut protein or with saline as controls. Three weeks after epicutaneous peanut exposure a recall immunisation was given in the footpad and the DTH responses measured.

Animals epicutaneously immunised with peanut protein after the peanut/CFA immunisation responded vigorously to the recall immunisation with footpad swellings of 37 ± 4 [mmx10^{-1}]. These high DTH responses were enhanced, although non-significantly ($p = 0.063$), compared to animals immunised with peanut protein in CFA and then epicutaneously exposed to saline (Fig. 7.10). *In vitro* peanut-specific T cell proliferation in cultures of CLN cells from mice receiving the epicutaneous peanut immunisation in addition to the subcutaneous peanut/CFA immunisation was significantly greater ($p < 0.001$) than in cultures of CLN cells from control mice (Fig. 7.11A). This results was however difficult to interpret as the control mice tail-base immunised with peanut/CFA followed by epicutaneous exposure to saline did not show proliferation of CLN cells. However, when analysing spleen cell responses there was also a significantly higher T cell proliferation in splenic cultures from mice epicutaneously immunised to peanut following the peanut/CFA immunisation compared with cultures of spleen cells from control mice ($p < 0.01$) (Fig. 7.11.B).

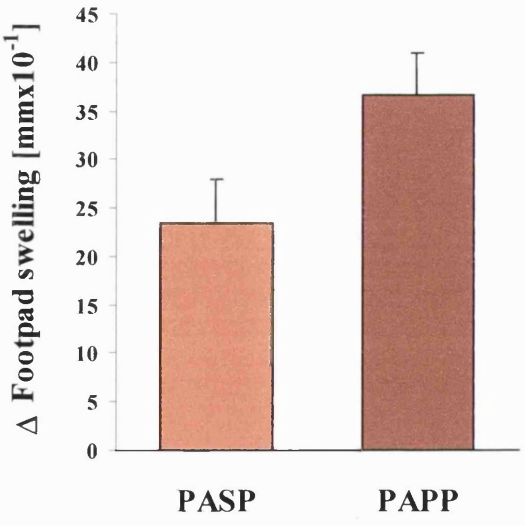

Fig. 7.10 DTH responses to peanut after immunisation with CFA followed by epicutaneous immunisation
Animals were tail-base immunised with peanut protein in CFA 3 weeks prior to epicutaneous immunisation with peanut protein (PAPP) or saline (PASP). Three weeks later animals were recall immunised with peanut in the footpad and DTH responses measured 24 hr after. Results are expressed as the mean footpad swellings + 1 SEM (n=4 for PASP, n=5 for PAPP). [PASP: Immunised with peanut in CFA, skin abraded, painted with saline, recall immunised with peanut; PAPP: Immunised with peanut in CFA, skin abraded, painted with peanut, recall immunised with peanut]

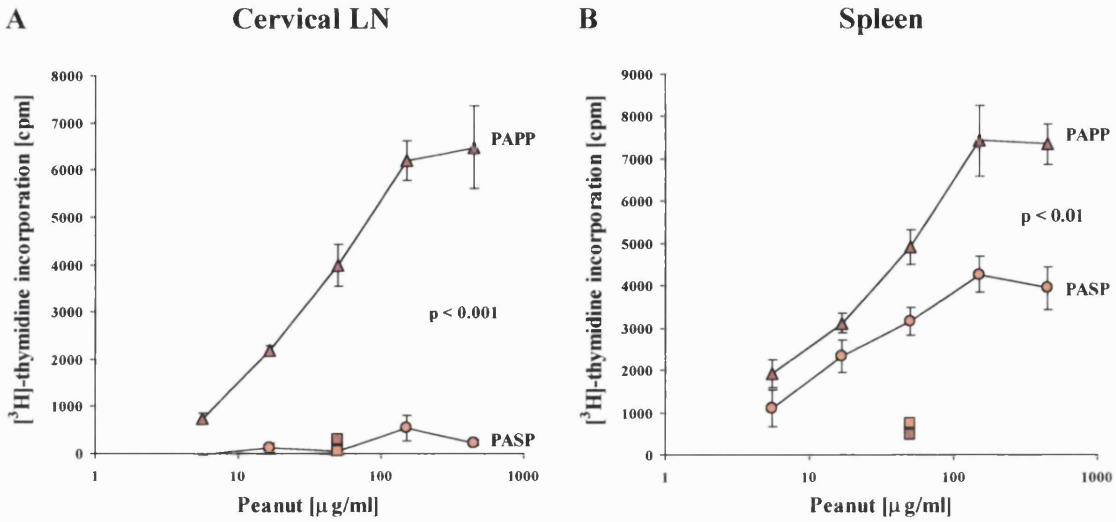

Fig. 7.11 Specific T cell proliferation after immunisation with CFA followed by epicutaneous immunisation

Animals were tail-base immunised with peanut protein in CFA 3 weeks prior to epicutaneous immunisation with peanut protein (PAPP) or saline (PASP). Peanut-specific T cell proliferation in cultures of CLN draining the epicutaneous immunisation site (A) and in spleen cell cultures (B) were measured 4 weeks after the epicutaneous immunisation. Cells were pooled from 4 (PASP group) or 5 (PAPP group) mice per group and results are expressed as the mean of triplicate cultures for 90 hr ± 1 SEM. Background proliferation when no antigen was present has been subtracted. Squares represent proliferation to a control antigen (OVA). The two groups responded with a similar proliferation [cpm] when stimulated with ConA. [PASP (●): Immunised with peanut in CFA, skin abraded, painted with saline, recall immunised with peanut; PAPP (▲): Immunised with peanut in CFA, skin abraded, painted with peanut, recall immunised with peanut]

Epicutaneous immunisation not only enhanced the potency of the ongoing immune response, but analysis of cytokine responses showed that it also changed the nature of the established peanut-specific immune response (Fig. 7.12). In CLN cultures it was predominantly cells from animals epicutaneously immunised to peanut protein following the peanut/CFA immunisation that produced cytokines in response to *in vitro* peanut protein stimulation. These CLN cells produced much higher levels of Il-4 and Il-10, but similar amounts of IFN-γ and TGF-β, compared to CLN cells from control animals epicutaneously exposed to saline (Fig. 7.12A). Splenic cells from the control animals with an established immune response to peanut protein produced large amounts of IFN-γ and IL-10, but little IL-4. In contrast, animals epicutaneously immunised to peanut following the peanut/CFA immunisation showed significantly reduced levels of IFN-γ but enhanced levels of IL-4. There were no differences between the experimental groups in the level of secreted IL-10 or TGF-β (Fig. 7.12B).

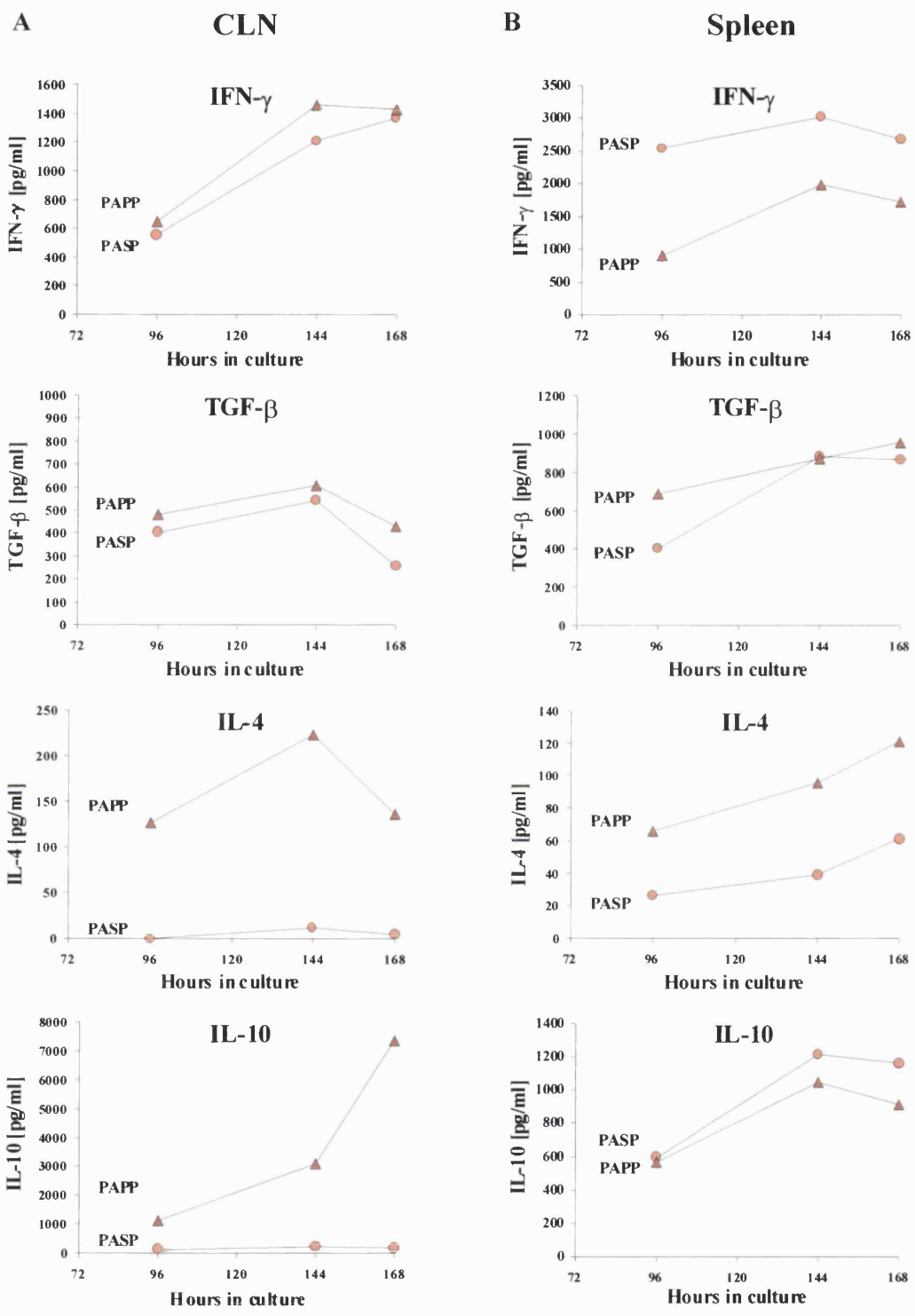

Fig. 7.12 Cytokine production after immunisation with peanut protein in CFA followed by epicutaneous immunisation

Animals were tail-base immunised with peanut protein in CFA 3 weeks prior to epicutaneous immunisation with peanut protein (PAPP) or saline (PASP) and recall immunisation with peanut. Cytokine levels in the supernatant of cultured CLN cells (A) and splenocytes (B) were analysed following stimulation with peanut protein *in vitro* for 96 hr, 144 hr and 168 hr. Cell were pooled from 4 (PASP) or 5 (PAPP) animals and analysed in triplicate. No cytokines were detected when cells were stimulated with a control antigen (OVA) or left unstimulated. [PASP (●): Immunised with peanut in CFA, skin abraded, painted with saline, recall immunised with peanut; PAPP (▲): Immunised with peanut in CFA, skin abraded, painted with peanut, recall immunised with peanut]

Modulating effects of the epicutaneous immunisation on the established CFA-induced immune responses were also evident when levels of peanut-specific antibodies were analysed. Peanut-specific IgG, IgG1, IgG2a and IgE were analysed in serum from all animals and relative levels of the antibodies are compared in Fig 7.13. There were no differences in levels of specific IgG or IgG1 between the two experimental groups. However, the epicutaneous exposure to peanut protein significantly reduced the level of IgG2a antibodies while the level of IgE was significantly increased compared to control animals immunised with peanut protein in CFA prior to epicutaneous exposure to saline (Fig. 7.13).

Taken together, these results suggest that epicutaneous immunisation modified established antigen-specific immune responses by specifically decreasing Th1-type responses and enhancing Th2-type responses.

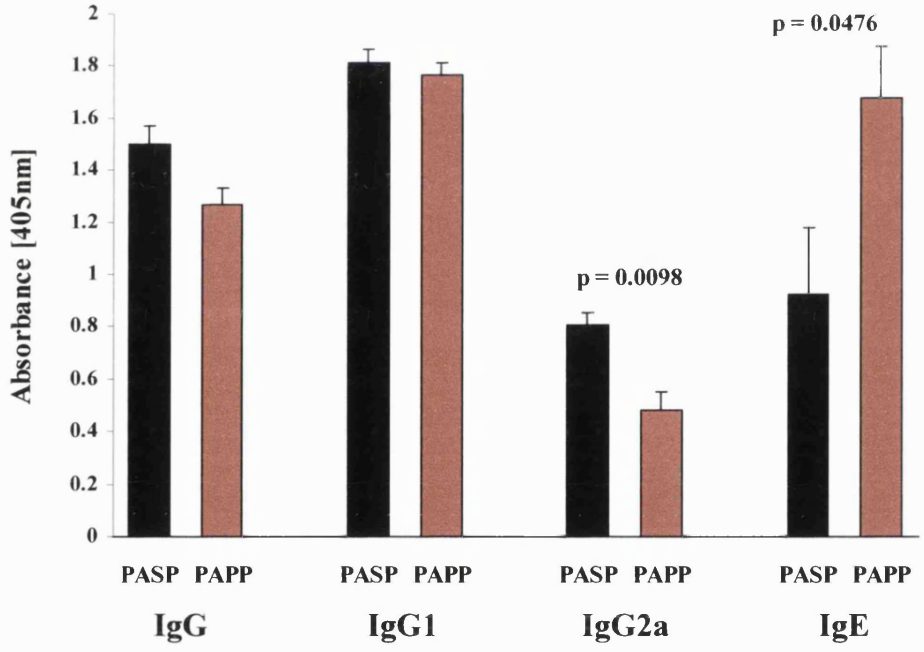

Fig. 7.13 Specific antibody response after immunisation with peanut protein in CFA followed by epicutaneous immunisation
Animals were tail-base immunised with peanut protein in CFA 3 weeks prior to epicutaneous immunisation with peanut protein (PAPP) or saline (PASP) and recall immunisation with peanut protein. Relative peanut-specific antibody levels in serum were analysed 4 weeks after the epicutaneous immunisation. Serum samples were diluted 1:1000 for IgG and IgG1, 1:200 for IgG2a and 1:10 for IgE prior to analysis. Results are expressed as mean antibody levels + 1 SEM (n=4 or 5). [PASP: Immunised with peanut in CFA, skin abraded, painted with saline, recall immunised with peanut; PAPP: Immunised with peanut in CFA, skin abraded, painted with peanut, recall immunised with peanut]

7.4 Discussion

The results presented here show that immune responses induced by epicutaneous immunisation are potent and persistent and can both enhance and modify the nature of subsequent immune responses to the same antigen, including strong adjuvant-driven responses. Epicutaneous immunisation primed animals to induce strong specific immune responses upon a secondary antigen challenge by footpad immunisation and caused increased IL-4 and decreased IFN-γ production in the PopLN, the most distant site from the initial epicutaneous immunisation. Importantly, the Th2 responses elicited by epicutaneous immunisation were found to be dominant over the development of subsequent Th1 responses obtained by subcutaneous immunisation with CFA. Strong T cell responses induced by epicutaneous immunisation could be demonstrated in PLNs draining the CFA immunisation site 8 weeks after the initial epicutaneous immunisation. This potent response was not merely acting in synergy with the adjuvant-driven response, since the epicutaneous immunisation predisposed animals to shift immune responses to the subsequent CFA immunisation from Th1 to Th2.

Antigen delivery onto other body surface organs like the gut, the nose and the lung generally leads to immunological hyporesponsiveness, which has hampered the development of mucosal vaccines. Experimentally, oral tolerance can be overcome and active immunity against orally administered antigens achieved by combining soluble proteins with strong mucosal adjuvants such as cholera toxin or heat-labile enterotoxin from *Escherichia coli* [Bilsborough and Viney, 2002]. However, many of the toxin-based experimental mucosal adjuvants are not appropriate for humans, and alternatives are still being sought. The effectiveness of epicutaneous immunisation and the way LCs and the immune system of the skin deals with antigens and promote active immunity makes the skin a potential alternative and attractive non-invasive route for vaccine delivery. There has recently been some interest in exploiting the immune system of the skin for vaccine administration and strategies for 'transcutaneous immunisation' have been developed. These new strategies, as well as the more conventional- and the mucosal immunisation protocols, rely on strong non-specific adjuvants for good immunity to high-molecular weight proteins. Transcutaneous immunisation strategies have utilised coadministration with cholera toxin and heat-labile enterotoxin of *Escherichia coli* on the skin [Beignon et al., 2001; Beignon et al., 2002; Glenn et al., 1998; Hammond et al., 2001]. The use of such toxins is required for the induction of immunity in these protocols, but non-

specific immune activation as well as antibody and cell-mediated immune responses to irrelevant antigens in the adjuvant are induced in addition to responses to the desired protein antigens. The inclusion of such toxins are as mentioned generally not acceptable for human vaccines. In contrast, the simple abrasion technique described here (Chapter 5-7) activates LCs into potent T cell stimulators and enables the generation of effective immune responses without the use of adjuvants and toxins. Most of the currently available vaccines are delivered via parenteral routes. As a result, immunisation requires trained medical staff, is expensive, may be painful, may lead to injection site reactions and in certain instances to infection by blood-borne pathogens (e.g. human immunodeficiency virus or hepatitis viruses) because of the use of contaminated needles [Kane et al., 1999]. A strategy of utilising the *in situ* modulation of LCs as a natural adjuvant for vaccine protocols would not only avoid the use of needles or non-specific adjuvants, but be a simple, non-invasive and economical route to vaccine delivery. As such this immunisation strategy might be advantageous for mass vaccination programmes in Third World countries.

From a clinical point of view the strong Th2-bias of the immune responses following epicutaneous antigen delivery may not be appropriate for all inoculation programmes. However, inclusion of agents that promote Th1 responses may allow for modulation of the dominant Th2 response. This has yet to be tested in the model presented here. It was recently shown that transcutaneous immunisation with the cholera toxin B subunit (the non-toxic receptor-binding moiety of cholera toxin) selectively induced Th1 responses and suppressed IgE antibodies [Anjuere et al., 2003]. The Cholera toxin B subunit, however, only promoted modest antibody responses to a coadministered antigen and required repeated coapplications. The fact that cholera toxin B suppresses systemic Th1 responses when given by a mucosal route, but potentiates these responses when applied onto the skin, underscores the role of the epithelial microenvironment in the regulation of immune responses. Understanding the mechanisms underlying the induction and modulation of epicutaneous immune responses is therefore of pivotal importance and will greatly enhance the prospects of applying this immunisation method for the control of a wide range of diseases. Further studies are awaited with great interest.

The Th2 dominance of the responses to epicutaneous immunisation may have therapeutic applications in Th1-type diseases such as collagen induced arthritis, diabetes, thyroiditis and

EAE. Autoimmune diseases, such as these, result from dysregulated immune responses to self-antigens, which in many cases are driven by Th1 autoreactive T cells. Autoimmune deviation and disease remains one of the more obscure areas of immunology, and the pathogenesis of autoimmunity is poorly understood. The paradoxical application of self-antigens to prevent or delay the onset of disease has been validated in many animal models of autoimmune disease [Harrison and Hafler, 2000; Weiner, 1997]. Orally- or mucosally induced tolerance has been the predominant mode of antigen-specific therapy, but has proven less successful in treating established disease and awaits demonstration of reproducible clinical efficacy in human autoimmune disease [Harrison and Hafler, 2000; Weiner, 1997]. Recently, also epicutaneous administration of autoantigenic MBP was shown to protect transgenic mice from induced EAE [Bynoe et al., 2003]. In this study MBP or PLP peptides were applied twice on an occlusive patch on shaved skin for two weeks and EAE was subsequently induced by subcutaneous immunisation with MBP or PLP peptides in CFA. The disease resistance was shown to be mediated by $CD4^+$ suppressor T cells but was not dependent on $CD4^+CD25^+$ T cells [Bynoe et al., 2003]. The therapeutic potential of this epicutaneous autoantigen administration was not determined. However, if treatment of humans is to become a therapeutic reality, the ability to modulate an ongoing immune response and re-establish immunological balance or tolerance is crucial.

In this Chapter it was demonstrated that epicutaneous immunisation was able to alter an established Th1-, CFA-driven immune response. Antigen-specific immunity was significantly modified by epicutaneous immunisation, which decreased Th1-type responses such as IFN-γ and IgG2a and increased Th2-type responses such as IL-4 and IgE. The results were not as striking as those seen when the epicutaneous immunisations were given prior to CFA immunisation and the development of Th1-type immunity. This may support the findings from oral tolerance experiments that it is more difficult to alter an ongoing immune response than the induction of one, or it may simply reflect the small number of animals in these experiments (n=4 and 5). However, the potency of the epicutaneous immunisations to induce Th2 responses and the efficacy in modifying even established Th1-type responses, may render this route of application of self-antigen useful for therapy of Th1-type autoimmune diseases. This possibility of using skin abrasion and LC activation as a natural vaccine adjuvant is currently being explored in our laboratories using murine models of Th1-type autoimmune disease.

Chapter 8

General discussion

8.1 Introduction

This Thesis has examined the immunological outcome of exposure to common food proteins at the intestinal mucosae and skin surfaces. Although it has merely been able to touch on the potential plasticity of responses that are available at body surfaces several key findings have emerged. Gastrointestinal- or cutaneous exposure to OVA and peanut proteins can induce either systemic hypo- or hyperresponsiveness upon re-challenge depending on the nature of the primary exposure. The route of the primary exposure is crucial in determining the nature of the systemic immune responses. This is clearly demonstrated in the skin where epidermal exposure to antigens gives Th2-type responses and exposure to antigens at the deeper layers of the skin induces Th1-type responses. The GALT has the potential to induce suppressive T_{reg}-type cells.

Epicutaneous exposure to antigens can affect the outcome of a secondary intestinal exposure and vice versa. Local immunoregulation and control of information being relayed systemically thus appear to be the key for determining whether antigens encountered at the body surfaces induce tolerance or immunity. The pleiotropy of choice for the epithelial-associated lymphoid tissues is highlighted by the dramatic changes in immunological outcome when the GALT is exposed to different doses of antigen or antigenic mixtures, and when the barrier-properties of the SALT are diminished. Understanding the immune mechanisms operating at the body surfaces, where the majority of pathogens and environmental antigens enter the host, as well as understanding the relay of information between local and systemic responses provides the starting point for elucidating differential immune reactivity.

8.2 Tolerance

The mammalian immune system must specifically recognise and eliminate foreign invaders but refrain from damaging the host. How immune systems distinguish between 'self' and 'non-self' – or how tolerance to self or foreign antigens is regulated – is a fundamental issue that has troubled scientists for decades and remains largely unresolved.

There is no direct evidence to suggest that mechanisms of oral tolerance to foreign proteins is related to self tolerance. However, these phenomena are often linked as many experiments have been aimed specifically at establishing tolerance to self by feeding of autoantigens

(section 1.2.7). To understand the mechanisms of orally-induced immunoregulation and tolerance it may indeed prove useful to also study immunological mechanisms underlying self-tolerance.

Current evidence suggests that self-reactive lymphocytes can be inactivated at all stages of their development by a variety of mechanisms. In the thymus, immature T cells are deleted if they express too high affinity for the self antigens expressed there. However, this process alone cannot account for tolerance, which also exists toward antigens that are not expressed in the primary lymphoid organs, antigens that are only expressed at certain developmental stages of an organism and the effectively infinite variety of innocuous environmental antigens present in the diet and air. To control responses to antigens not present in the thymus and responses of self-reactive lymphocytes that escape central tolerance, several mechanisms of peripheral tolerance exist. It has been shown recently that the thymus may play an additional role in peripheral tolerance as $CD4^+CD25^+$ regulatory T cells are selected there as a unique lineage of lymphocytes that can control autoreactive thymic escapees [Bensinger et al., 2001; Itoh et al., 1999]. Although this appears to be an efficient mechanism for the maintenance of tolerance, these $CD4^+CD25^+$ T_{reg} cells share the potential paradox of central tolerance, in that they cannot control lymphocytes specific for non-thymic antigens. As such, evidence for several subsets of peripherally generated regulatory cells, with distinct regulatory mechanisms, is emerging.

All healthy adults have peripheral T cell clones specific for food, other environmental antigens and even self antigens. These T cell clones are controlled in the periphery. At mucosal surfaces, immune responses have been suggested to be controlled by the generation of so-called Tr1 and Th3 cells, which regulate responses through the secretion of IL-10 and TGF-β respectively [Groux and Powrie, 1999; Weiner, 2001; Weiner, 1997]. In this Thesis, the feeding of 100mg of crude peanut protein extract resulted in systemic hyporesponsiveness and the emergence of a population $CD4^+CD25^+$ T cells with immunoregulatory properties, something that was not observed feeding smaller doses of peanut protein. This population of antigen-specific cells appears to be similar to the thymic population of $CD4^+CD25^+$ T_{reg} in that they are anergic, proliferate poorly, suppress the proliferation of other lymphocytes and express CTLA-4 (Chapter 4). These orally-induced regulatory T cells may be produced *de novo* from peripheral $CD4^+$ T cells and contribute to the pool of $CD4^+CD25^+$ T_{reg} produced in the

thymus. Alternatively, they may represent expansion of an existing pool of thymus-derived $CD4^+CD25^+$ T_{reg} cells that show cross-reactivity to peanut antigen. Peripheral induction of a population of $CD4^+CD25^+$ T cells has been reported once previously in mice continuously exposed to OVA in the drinking water [Thorstenson and Khoruts, 2001].

The mechanisms involved in the induction of all of these regulatory cells are unclear, as is the relationship between them. It may be that the Tr1, the Th3 and the $CD4^+CD25^+$ T_{reg} cells generated in this study are very similar if not representatives of the same cells. The Th3 cells generated by gastrointestinal exposure to antigen notably secrete TGF-β, as do the $CD4^+CD25^+$ T_{reg} cells induced following feeding of 100mg peanut protein in the current study (Chapter 3 & 4). It has been suggested that the Tr1 cells represent the naturally occurring regulatory $CD45RB^{lo}CD4^+$ T cell population [Groux and Powrie, 1999]. The T_{reg} cells capable of inhibiting the development of IBD in this population have additionally been found to predominantly exist within the $CD4^+CD45RB^{lo}CD25^+$ population [Read et al., 2000]. However, a major discrepancy between the Tr1, Th3 and the $CD4^+CD25^+$ T_{reg} generated in this study is the dose of antigen involved in their induction. While it is generally believed that only small and/or repetitive antigenic stimuli can induce regulatory Tr1 and Th3 cells, it is shown in this Thesis that a single large oral dose of crude peanut protein extract can produce cells with immunoregulatory properties. As the mechanisms involved in the generation of any of these T_{reg}-type cells remain elusive, one can only speculate that the induction of T_{reg} cells in this study may be related to the processing of antigenic mixtures in the GALT. No other study on the induction of T_{reg} cells at mucosal surfaces has used antigenic mixtures but has utilised single, highly purified proteins or peptides. Moreover, most other studies on oral tolerance and the generation of T_{reg}-type cells have used transgenic mouse models with a high frequency of antigen-specific T cell precursors. The immune responses induced in these mice and the antigen dose-response relationship are probably not comparable to conventional mice with normal immune systems.

The focus of studies on oral tolerance has traditionally, as well as in this Thesis, been on T cells. The handling of antigen at the body surface and the nature of the cell that presents the antigen is, however, likely to play a pivotal role in the development of T_{reg} cells and of tolerance and immunity in general. An investigation of such differential APC responses in tolerant versus sensitised animals was beyond the scope of this Thesis. DCs with their unique

capacity to prime naïve T cells have taken centre stage in studies of immune responses and are also believed to be able to orchestrate tolerogenic immune responses [Jonuleit et al., 2001b; Rissoan et al., 1999; Shortman and Heath, 2001; Steinman et al., 2003; Weiner, 2001]. It is likely that the many different DC subsets at the body surfaces, and their extreme plasticity in responding to changes in the microenvironment, represent the decision makers of T cell differentiation and thus control the T cell regulators. It has been suggested that the local mucosal milieu with abundant secretion of IL-10 and especially TGF-β as well as antigen-presentation by immature and/or specialised subsets of DCs is especially favourable for the induction of T_{reg} cells and tolerogenic immune responses [Steinman et al., 2003; Weiner, 2001]. Skin, in a similar way to gut tissue, is rich in TGF-β and harbours various DC subsets. It is thus plausible that a similar kind of regulatory and tolerogenic mechanism can be induced also through skin exposure to antigens. This has, however, yet to be tested. The dramatic differences in immunological outcome when the GALT is exposed to different doses of orally administered antigen (Chapter 3), may be a consequence of different amounts of antigen reaching different immune sites of the GALT. Antigen may thus, dependent on the dose, be taken up and presented by different APCs, in unique microenvironments and under varying degrees of stress or 'danger' signals. This could potentially be looked at by *in situ* immune staining of the tissue and the use of novel optical imaging techniques such as multiphoton laser microscopy combined with electron microscopy. The tissue's unique microenvironment may be the ultimate regulating factor deciding what type of response is appropriate (compare below section 8.5).

8.3 Allergic sensitisation

The first question the immune system must consider when faced with foreign antigen is whether to respond. Having decided to respond it must decide what kind of immune response to make. There has been a significant increase in the prevalence of allergic and atopic diseases in the Western world over the past 2 to 3 decades [Holgate, 1999; von Mutius, 1998], and their prevalence in developing countries is considerably lower [ISAAC Steering Committee, 1998]. Why the immune system in developed countries at an increasing rate is over-responding to environmental antigens by making IgE and pathogenic Th2 responses is not known. Why certain antigens seem to make the immune system prone to respond with the production of specific IgE is also still elusive, although the potential allergenicity of proteins has been extensively studied. The potent allergens in peanut do not always cause hypersensitisation and

hypersensitisation and generation of IgE, as shown by the normal induction of oral tolerance. The GALT immune system is efficient in inducing tolerance, but it is equally efficient in inducing sensitisation (Chapter 3). It thus appears that allergenicity is a consequence of a complex series of interactions involving not only the nature of the allergen but also the dose, the sensitising route, the induction- or coapplication of molecules with an adjuvant effect, and the genetic constitution of the recipient. Some allergens may cause damage to the host, by, for example, having protease activity, which could alter the microenvironment and the nature of the cells that process and present the antigen. However, exposure to allergens such as those in peanut is common throughout the world, yet mostly in the Western world is a rise in aberrant reactions against them observed. Antigens with allergenic properties are not novel in the Western world, either - however, the pathogenic immune responses to them are. As the allergens of common foods and plant pollens have not changed – have we, or the way we are exposed to them?

It has been the general belief that most allergic IgE responses are initiated at mucosal surfaces and thus in response to allergens that enter the body either by ingestion or inhalation. In this Thesis it has been demonstrated that in addition to sensitisation through the GALT (Chapter 3), potent Th2-type responses can be induced through the skin. In fact, the skin appears to be a particularly powerful route of induction of Th2-type sensitisation with very high IgE responses and may as such be a potential route of primary allergic sensitisation to protein antigens (Chapter 5). Primary sensitisation through the skin was observed to interfere with the normal induction of oral tolerance. Instead of inducing tolerance, gastrointestinal exposure to antigen further sensitised the animals and elevated specific IgE responses as well as promoting a clinical appearance of anaphylaxis (Chapter 6). Many different immunisation routes were utilised in the present study - oral, subcutaneous with and without adjuvant and epicutaneous immunisation. The epicutaneous route was the most potent inducer of IgE and Th2-type responses. The mechanism underlying this phenomenon is not well understood. IgE-bearing LCs from AD skin lesions, but not LCs that lack surface IgE, are capable of presenting house dust mite allergen to T cells [Beck and Leung, 2000]. This suggests that cell-bound IgE on LCs can facilitate capture and internalisation of allergens before their processing and presentation to T cells. It is known that epidermal LCs from patients with AD have increased expression of FcεRI throughout the skin [Wollenberg et al., 1996], but resting LCs in normal skin also express FcεRI [Larregina and Falo, 2001]. IgE-bearing LCs that have captured

allergen may thus activate memory Th2 cells in already atopic skin, but are likely to also migrate to the lymph nodes and stimulate uncommitted T cells to initiate or further expand a pool of Th2 cells. Subsequently, these Th2 cells are distributed systemically through the circulatory system. In addition, this study showed that activated LCs express CD86, a costimulatory molecule thought to skew T cell differentiation toward the Th2 phenotype [Freeman et al., 1995; Kuchroo et al., 1995]. Expression of CD80 was not determined in the present study, but other studies of LCs in atopic skin have shown that they predominantly express CD86 [Ohki et al., 1997].

Allergy and hypersensitivity responses to innocuous antigens are ultimately aberrant responses that can be dangerous to the host. Th2 and IgE responses are, however, necessary for protection against parasitic worm infections [Yazdanbakhsh et al., 2001; Yazdanbakhsh et al., 2002]. Since the parasitic burden is low in Western societies, IgE may have become temporarily redundant. Although the common food proteins used in this study are not parasitic worms, the epidermal immune system targets them with an IgE-class response. The effector response may thus not be tailored to the targeted pathogen as has been the general belief, but instead tailored to the tissue in which the response occurs. The epidermis may be particularly efficient in inducing Th2 and IgE responses when distressed, as in many cases this is the entry or destination point for parasitic worms, and the immune system is as such conferring protection against the invasion. A Th2-biased, anti-inflammatory response at the body surface may additionally be advantageous to dampen down responses that may be elicited by the commensal flora of the skin or gut.

The dramatic increase of allergic disorders in the industrialised world has popularly been explained by the *hygiene hypothesis*, which argues that insufficient stimulation of Th1 responses allows the expansion of Th2 responses [Strachan, 1989]. However, not only Th2 diseases are rising, and a positive correlation is found between diagnosis of allergic Th2- and autoimmune Th1 diseases [Sheikh et al., 2003]. Additionally, Th2 and IgE responses *per se* do not promote allergy or atopy. This is demonstrated by the strong Th2 and IgE responses observed in heavily helminth infected subjects, but the low prevalence of allergic diseases in these populations [Yazdanbakhsh et al., 2001; Yazdanbakhsh et al., 2002]. The observations from helminth infected populations prompted Yazdanbakhsh et al. to suggest that the difference in prevalence of allergy may be explained by the different exposure to pathogens. High exposure

would educate the immune system and establish a strong regulatory network, whereas low exposure to pathogens in the Western world would lead to a weak regulatory network with low capacity for controlling allergic disease [Yazdanbakhsh et al., 2002]. This Thesis offers an alternative to these two hypotheses, and suggests that it is not necessarily the lack of infections or exposure to pathogens that is promoting an increased frequency of allergy in the Western world, but the way that we are exposed to the allergens. It is possible that excessive washing, the frequent use of syndets and other de-fattening agents and the use of adhesive tape found for example on disposable nappies may cause abrasion of the skin in children. In adults, washing, shaving, waxing and the frequent use of various cosmetics may cause a similar surface abrasion of the skin. Exposure of such distressed, but not injured, skin to otherwise innocuous proteins causes induction of Th2 and IgE responses, that with no 'worm to chase' may promote allergic sensitisation to food and other environmental antigens.

8.4 Power to the tissues

It is generally thought that immunity is controlled by the lymphocytes of the adaptive immune system or by the DCs, macrophages etc of the innate immune system. As alluded to in the previous two sections, however, it appears more likely that the ultimate power in deciding if and how to respond lies with the tissues. This interpretation is consistent with the latest adaptation of Polly Matzinger's 'danger model' [Matzinger, 2002]. The basic response, or non-response, from the tissues may shape the DCs to shape the lymphocyte response. Different tissues appear to have different means of determining the effector class of an immune response. This was clearly demonstrated by the starkly different immune responses observed when antigen was applied on the skin or just under the skin. While epidermal exposure to peanut protein gave rise to a Th2 response, subcutaneous injection, which delivered peanut protein directly into the dermis, gave rise to a Th1 response (Chapter 5). This is probably due to the local microenvironment and to the specialised DCs residing in the different tissue compartments. The possible difference in function of epidermal and dermal DCs could be studied in more detail by separating these cells *in vitro* and investigating what T effector cell response they promote. In addition, it would be of interest to investigate to what extent this spatial separation of epidermal and dermal DCs determines what APCs process antigen applied epicutaneously. By multiphoton laser microscopy it may be possible to examine how deeply the epicutaneously applied antigen penetrates, whether it can breach the very dense

collagen meshwork of the basement membrane and under what conditions antigen may be picked up by different LC or DC subsets.

All body surface organs, and the immune networks within, appear to dampen down Th1 responses without affecting Th2 responses when exposed to antigens only at the surface. This may be a mechanism to prevent inflammation at the body surface and to avoid eliciting damaging immune responses towards harmless symbiotic microbes. Inappropriate Th1 immune responses generated at body surfaces may cause injury allowing microbes access to deeper layers. On the other hand, strong Th1-type immunity may be initiated - and needed - if foreign antigen has invaded further into the body as a result of injury or damage to the surface integrity.

Understanding the vast and intricate properties of various tissues and the specialised adaptive and innate immune cells within is clearly no easy task. More attention needs to be aimed at trying to understand the normal mechanisms and types of immune responses in different tissues, as well as to understand responses in stressed and/or injured tissues. Such knowledge may enable us to manipulate immune responses 'gone wrong' in the tissues. Additionally, different tissues could be utilised as natural therapeutic tools in gaining a particular class of immune response against unwanted antigens.

8.5 Therapeutic applications

The ease with which potent immunity is induced by application of antigens to the skin of mice may, if proven possible in humans, revolutionise the way vaccines can be administered. Topical delivery of vaccines would be advantageous for several reasons. It elicits both systemic and mucosal immunity, which is important as the majority of pathogens enter the host via mucosal surfaces [Partidos et al., 2003]. Epicutaneous immunisation would eliminate the use of needles, which is often associated with pain and may therefore increase compliance to vaccination programmes. Additionally, the common practise of reusing needles and syringes in developing countries would be eliminated, and with it the risk of transmission of blood-born infections [Kane et al., 1999]. Topical administration of vaccines to the skin would be simple, non-traumatic and thus allow for (repetitive) self-administration.

All of the above could also be applied to orally delivered vaccine strategies. However, the desirability of developing oral vaccines that are protective against pathogens has been confounded by the GALT's inherent property to induce systemic unresponsiveness. It is possible to actively immunise against orally administered antigens by combining soluble proteins with strong mucosal adjuvant or toxins. However, the commonly used experimental mucosal adjuvants are not appropriate for human vaccines because of their toxicity. This is a dilemma for all mucosal vaccination – efficacy versus toxicity. Appropriate alternative adjuvants are actively being sought by several scientific groups. When utilising the skin as shown in this study, a natural adjuvant effect was achieved simply by disrupting the stratum corneum of the epidermis. This transformed the resting LCs into vigorous T cell stimulators and ultimately resulted in potent systemic immunity. This study has also shown the potential therapeutic benefit of combining epicutaneously-delivered antigens with orally-delivered antigens. Chapter 6 demonstrated a synergistic effect of skin and oral exposure to antigens. The systemic response induced by epicutaneous immunisation was enhanced by subsequent oral exposure to the same antigen. As such, epicutaneous administration may act as an adjuvant for the mucosal delivery of the same antigen. This may be a particularly advantageous way to induce potent antigen-specific immune responses and its potential for vaccine administration and therapy should be further studied.

The antigen-specific Th2-bias induced by epicutaneous immunisation (Chapter 5-7) may have direct clinical applications in Th1-dominated diseases. This was suggested by the results of Chapter 7, where epicutaneous immunisation was shown to prevent the development of subsequently induced Th1 responses. More importantly it was shown that epicutaneous immunisation could direct an established antigen-specific Th1 response to a Th2 response, which suggests that this immunisation strategy may have therapeutic potential. This possibility is currently being investigated in our labs using murine models of Th1-type autoimmune diseases such as diabetes, EAE, collagen induced arthritis and Hashimoto's thyroditis. The idea that epicutaneous delivery of autoantigens may interfere with the development of inflammatory autoimmune diseases where Th1 cells play a central role was confirmed in a recent publication showing prevention of EAE induction following epicutaneous immunisation with MBP and PLP peptides [Bynoe et al., 2003]. Results from the first experiments investigating whether this approach also has therapeutic benefits on autoimmune diseases are eagerly awaited.

Autoimmune therapy has predominantly been investigated through oral- or nasal application of autoantigens. A range of experimental animal models have shown the efficacy of such mucosally administered autoantigens in preventing autoimmunity (compare section 1.2.7.1). The oral or nasal application has, however, proven less successful in treating autoimmunity, which may partially explain the disappointing results of initial human disease trials. Although the ultimate goal is to re-establish tolerance in the autoimmune patient, utilising 'oral tolerance' as a vaccine strategy may not prove powerful enough, and a different strategy inducing active immunity may be necessary. With this aim, several groups are investigating the potential of using mucosal adjuvants. Additionally, a further understanding of the optimal way to induce and manipulate T_{reg}-type cells both *in vivo* and *in vitro* may have therapeutic potential for re-induction of immune tolerance in a range of diseases including autoimmune- and allergic diseases. It was suggested recently that epicutaneous immunisation with autoantigenic peptides may induce T cells with a suppressive function, which can transfer protection against EAE [Bynoe et al., 2003]. This is an interesting possibility that requires further investigation. Efforts must however focus on imprinting antigen-specificity on induced T_{reg} cells to not hamper protective host immunity in general.

Epicutaneous immunisation with the induction of systemic and mucosal Th2-type responses and high levels of antigen-specific IgE may have another direct application as a potential protection against parasites. Around 1 billion people worldwide are heavily infected with helminths and suffer from the resultant nutritional, growth and cognital deficiencies [Yazdanbakhsh et al., 2002]. No preventive vaccination is available against infection. Whether epicutaneous immunisation with helminth antigens would induce active immunity and whether such responses would confer protection against infection is completely unknown. The idea however deserves to be explored further.

Development of an effective preventive measure or vaccine for atopic disorders is equally as desirable. Allergic and atopic diseases are a major health hazard in the industrialised world and currently no effective long term treatment exist. For food allergic subjects, strict avoidance of the allergenic food is the only means of preventing potential life-threatening reaction. Avoidance is extremely difficult and unintended ingestion happens frequently, sometimes with fatal results [Bock et al., 2001]. Several different forms of immunomodulatory therapies are currently under investigation, but although experimentally encouraging results have been

achieved, no clinically reproducible therapy has yet emerged (compare section 1.3.3). The Th2-predominance of responses induced by the epicutaneous immunisation described in this Thesis makes direct use of this vaccine strategy against atopic diseases unlikely. However, inclusion of Th1-promoting agents may yet render the epicutaneous immunisation useful. Co-application of live or dead bacteria, CpG oligodeoxynucleotides or recombinant cytokines with the relevant allergen may modulate the allergen-specific Th2 response and prevent allergy. Such Th1-promoting agents have previously proved promising in preventing atopic diseases [Burks et al., 2001; Wohlleben and Erb, 2001]. Additionally, transcutaneous immunisation with the cholera toxin B subunit has recently been shown to suppress IgE antibody responses and selectively induce Th1 responses [Anjuere et al., 2003]. Although this only induced weak systemic immunity, it demonstrates that the dominant Th2 responses of the skin can be effectively modulated and it thus highlights the potential use of epicutaneous vaccine delivery also for atopic (and perhaps infectious) diseases. Epicutaneous immunisation against atopic disorders could prove especially advantageous as the resultant mucosal and skin immunity may protect against the manifestations of atopy at the body surfaces.

While the potential of using epicutaneous immunisation as a novel vaccine strategy looks promising, the dangers of therapeutic manipulation of the Th1-Th2 balance must be kept in mind. In directly utilising the Th2-bias of skin-induced responses in the treatment of autoimmunity, the possible induction of pathogenic IgE responses must be monitored carefully. The epicutaneous immunisation presented in this study did not, however, rely on adjuvants for Th2 potency, and thus appears to have the advantage of initiating antigen-specific Th2 responses. The possibilities of pathogenic responses are probably greater in treatments involving adjuvants that non-specifically direct responses toward Th1 or Th2. Although, many of the experimentally successful allergy-therapies have been associated with immune deviation from Th2 to Th1 responses, and do not appear to have resulted in autoimmune or otherwise aberrant Th1-mediated diseases [Burks et al., 2001]. However, there is naturally a serious risk of oversimplifying the concepts, if disease is solely explained by polarisation of Th1- versus Th2 effector cells. Polarisation of Th cells is rarely complete, and an efficient mutually negative regulation by Th1 and Th2 cytokines implied by the concept of a Th1-Th2 'see-saw' is not always evident *in vivo* (Chapter 3). As discussed elsewhere, the direct correlation between the rise of Th1-mediated autoimmune- and Th2-mediated atopic disorders suggests a general immunodysregulation or deficiency of regulatory-cell activity.

Ideally, the direct induction of regulatory cells would have the capacity to control both Th1 and Th2 mediated disease, although with the potential hazardous draw-back of antigen-nonspecific effector responses and generally suppressed immunity. However, re-establishing a balanced Th1/Th2 response may relieve symptoms of disease and ultimately re-establish healthy immune regulation and tolerance.

8.6 Concluding remarks

During the course of writing this Thesis, results have been looked at over and over again, many and varied papers have been read, thoughts have been twisted and turned. Yet what stands clearest at the end, are the challenges that still lie ahead in fully understanding the immune system. This is highlighted by the amazing pleiotropy of immune responses that are available to a mammal at any given time and how small differences in deliverance of antigens appear to govern dramatic changes in immune responses. Many immunoregulatory decisions may well lie outside what we normally think of as the immune system, and to fully understand the regulation of immune responses a lateral and broader view of immunity is probably necessary. Many big questions have already been answered and the framework of immunology set by landmark discoveries relating to, for example, hematopoiesis, organisation of the MHC, rearrangement of TCR genes, thymic selection, expression of immunoglobulin genes and discovery of many cytokines and chemokines. However, much remains to be learnt about the intricate ways that these immune segments function and communicate together. Ultimately, understanding and managing immune responses as those associated with allergic and autoimmune diseases probably means coming to terms with complexity both at the inter- and intra-cellular levels. Indeed, one may have to realise that the simplistic schemas the human mind can encompass are insufficient to deal with the full extent of biological complexity.

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Appendix

Chapter 2 Reagents, materials and methods

Throughout this Thesis optimal conditions were determined for all experimental assays:

DTH (p. 85): In the first experiments DTH responses were measured at both 24 hr, 48 hr and 72 hr following antigen challenge in the footpad. There was no difference in the footpad swelling at 24 hr and 48 hr but swelling had minimised by 72 hr. DTH responses were then routinely measured and compared between experimental groups 24 hr after antigen challenge.

T cell proliferation measured by [^{3}H]-thymidine incorporation (p. 86): In initial experiments cells were harvested for assay of [^{3}H]-thymidine incorporation following 3, 4, 5 and 6 days incubation with antigen. Fairly low counts of antigen-specific T cell proliferation were obtained following only 3 days incubation. After 4 and 5 days incubation, similarly high [cpm] counts were obtained and the difference in proliferation was similar between experimental groups. Following 6 days incubation the high-responder cells were out-growing the wells at the highest antigen-concentration and cells stimulated with ConA were dying. Four days of incubation was chosen as the optimal time for comparison of antigen-specific T cell proliferation between experimental groups.

Antibodies (p. 92): In initial experiments of all immunisation protocols a full titration of the experimental sera was undertaken. From these titration curves the optimal dilution for comparing antibody levels in sera between experimental groups were determined.

Cytokines (p. 94): A kinetic analysis of cytokine production was carried out at the beginning of each new experiment. The cytokine kinetics was determined by collecting cell culture supernatants at 24 hr intervals between 24 hr and 168 hr of incubation with antigen and assaying these for cytokine levels. Generally the 96 hr time point was chosen for comparison of cytokine levels between different experimental groups.

Only limited *in vivo* kinetic studies were carried out on immune responses developing following the different immunisation protocols. Analysis of responses following epicutaneous exposure to antigen (Chapter 5) was carried out at 3 different time points; day 6, 10 and 20 following primary antigen exposure. The effect of epicutaneous antigen exposure on the induction of oral tolerance (Chapter 6) was determined at two different time points. The effect of feeding various doses of peanut protein or OVA on the induction of systemic hypo- or hypersensitivity (Chapter 3) was however only determined at day 35 following the feed (1

week after footpad challenge). As such the enhanced response to peanut protein shown following feeding of 0.02mg or 0.2mg peanut protein and the reduced response shown following feeding of 100mg peanut protein could simply reflect a faster or slower response compared with the control groups. With the presented data it is not possible to conclude whether the enhanced or reduced responses demonstrate true hyper- or hyporesponsiveness to peanut protein or solely kinetic differences between the two groups. The novel model of oral tolerance and sensitisation to peanut protein was however based on previously described OVA models, where the induction of oral tolerance does not appear to reflect only a short delay in the response [Strobel and Ferguson, 1987].

Chapter 3 A novel model of oral tolerance and sensitisation to peanut protein

This Chapter shows the development and partial characterisation of a novel murine model of oral tolerance and sensitisation to peanut protein. In the dose-response studies in this Chapter animals fed various doses of peanut protein are compared to animals fed the diluent saline but otherwise treated the same. A limitation in the interpretation of the data from these studies arises from the use of saline-fed controls as these animals have been exposed to peanut protein one less time than the peanut-fed animals. To control for the number of times animals have been exposed to antigen an additional and more appropriate control may be the use of animals fed a similar dose of antigen in an immunogenic form for example with an adjuvant such as CT.

Repetitive antigen stimulation inducing large numbers of cell divisions during clonal expansion would be expected to lead to shortening of telomeres and predisposition to replicative senescence - or clonal exhaustion. The reduced responses to peanut in animals fed 100mg peanut protein could be argued to be due to such clonal exhaustion. Although this possibility cannot be excluded by the presented data (as the controls were exposed to peanut protein one less time) it is unlikely that this immunising regime would induce the 40-50 cell divisions that exhaustion of specific clones normally require. Clonal exhaustion is mostly described in $CD8^+$ T cells under conditions with very high antigenic load as in persistent virus infections [Ou et al., 2001; Welsh and McNally, 1999]. In addition, the experiments described in this Chapter argue against the *number* of antigen exposures alone being responsible for the reduced responses following feeding of 100mg peanut protein as animals exposed to peanut protein an

equal amount of times but fed a lesser dose did not show hyporesponsiveness. However, whether the combination of one extra antigen exposure (compared to control animals) combined with the high dose of peanut protein (100mg) required for oral tolerance induction does induce clonal exhaustion should be controlled for by comparing with responses in animals fed 100mg peanut protein in an immunogenic form but otherwise treated identically.

In this Chapter T cell proliferation following feeding and immunisation with peanut protein is presented both as incorporation of [^{3}H]-thymidine and as dilution of CFSE staining on dividing cells. A very high percentage of cells from peanut-immunised animals are shown by CFSE staining to proliferate when stimulated with peanut protein and are suggested to be antigen-specific. These results were reproducible in 3 independent experiments (Table 1A). One representative example is shown in the Thesis (p. 107 and Exp. 1 in Table 1A). The reasons for this very high level of apparently antigen-specific proliferation compared to other studies are unknown at present. It may be that peanut protein in itself possess some adjuvanticity or has mitogenic effects, although cells from animals fed 100mg peanut protein do not proliferate when stimulated with peanut (Table 1A) and neither do cells from peanut-naïve OVA-sensitive animals (shown throughout the Thesis). Another explanation could be selective death of non-peanut-specific cells during the incubation period and thus concentration of peanut-specific cells in the gating of live cells on the FACS machine.

Table 1A: T cell proliferation following a feed of 0.2mg peanut protein, 100mg peanut protein or saline in 3 independent experiments

	Fed: / *In vitro* Stimulation:	Saline	100mg Peanut	0.2mg Peanut
Exp. 1	None	1%	1%	1%
	Peanut	16%	2%	22%
	ConA	64%	65%	64%
Exp. 2	None	1%	1%	1%
	Peanut	17%	3%	24%
	ConA	65%	65%	66%
Exp. 3	None	1%	2%	2%
	Peanut	13%	3%	18%
	ConA	59%	60%	58%

Chapter 4 Mechanisms involved in oral tolerance

In this Chapter, possible mechanisms involved in the maintenance of oral tolerance to peanut protein is explored and in particular the possible induction of a population of regulatory $CD4^+CD25^+CTLA\text{-}4^+$ T cells with suppressive properties is discussed. Co-culture experiments show that inducing T cells from orally tolerised animals into the culture of cells from peanut-sensitive animals suppresses the proliferation, IFN-γ, IL-10 and IL-4 production of these cells possibly through a mechanism involving CTLA-4 and TGF-β. However, in order to fully conclude that it is the putative regulatory cells from the orally tolerised animals conferring this suppression, additional experiments of co-culturing peanut-sensitive cells with completely naïve cells should be carried out. The question of antigen-specificity in the effector function of these T_{reg} cells could additionally be addressed in co-culture experiments by culturing cells from animals orally tolerised to peanut protein with cells from OVA-sensitive animals.

The only difference between the peanut-tolerant animals and the controls is that the peanut-tolerant animals have been fed 100mg peanut protein. It is however difficult to fully identify whether it is this single feed that induces the apparent induction of $CD4^+CD25^+$ T_{reg} cells. As discussed under Chapter 3 in this Appendix the interpretation of results would have benefited from the use of orally primed animals as controls. It would be of additional interest to look at the possible development of these T_{reg} cells immediately following feeding and identify any differences in the specific T cell development between animals fed tolerogenic or immunogenic forms of antigen. It may be that antigen administered in different forms reaches different parts of the GALT and thus is presented in different microenvironments. This could possibly be examined *in situ* with the use of optical imaging techniques such as multiphoton laser microscopy and electron microscopy. It is also possible that the final development of T_{reg} cells requires some form of inflammatory environment, which in the experiments presented in this Thesis may be conferred by the subsequent challenge with peanut in CFA.

Finally, the regulatory function of the apparent T_{reg} population induced by feeding of 100mg peanut protein must be substantiated *in vivo* by adoptive transfers to syngeneic recipients. It is important to acknowledge that regulatory function *in vitro* may not correlate with suppressive activity *in vivo*. The suppressive and regulatory function of $CD4^+CD25^+$ T cells *in vivo* has commonly been shown in various lymphopenic mouse models. It has recently been suggested, that T cell regulation in these models may partially be due to normal homeostatic mechanisms,

expansion of the transferred T cell clones and competition for shared resources [Barthlott et al., 2003]. It would as such be of particular interest to examine the *in vivo* regulatory capacity of the orally induced T_{reg} cells in this study by transfer into fully immunocompetent animals.

Chapter 5 Epicutaneous exposure to protein antigen induces systemic Th2-biased immunity

This Chapter shows that exposure to protein allergens such as peanut protein and OVA on barrier-disrupted skin induces potent systemic sensitisation with a Th2-bias. The early response following abrasion of the skin and allergen exposure can be described as a mixed Th1/Th2 response as IFN-γ is produced as well as IL-4. Over time, however, the immune response appear to polerise more towards a Th2 response, as shown by diminished IFN-γ production, enhanced IL-4 production and high levels of antigen-specific IgG1 and IgE with no or very low levels of IgG2a. That these animals mount a DTH response is therefore curious, as DTH responses are normally associated with dominant induction of Th1 cytokines. The skin as an immune organ appear to be particularly well suited for the induction of DTH responses and it may be that low levels of Th1 cytokines released locally in the skin is enough to induce DTH. Alternatively (as discussed on p. 184), the DTH response may be supported by proinflammatory activities of IL-4 or even initiated by IgE, IgG1 and/or vasoactive serotonin as has been reported previously [Ptak et al., 1991].

This Chapter also shows evidence that Langerhans cells pick up peanut antigens in the epidermis and migrate to the draining LN. Migration was shown to occur only following abrasion of the skin *and* exposure to peanut protein and not by the abrasion technique alone. This is intriguing as it suggests that endocytosis of antigen or exposure to antigen confers some second signal which allow the emigration of Langerhans cells. Whether this extensive migration from the epidermis following abrasion and exposure to peanut protein is peculiar to peanut was however not formally assessed in this study. It could be that peanut has mitogenic effects which are responsible for the induction of this large migration and these migration studies should thus be repeated with other proteins in order to be fully conclusive. It must however be assumed that a similar migration occurs following epicutaneous exposure to OVA on abraded skin as strong systemic OVA-specific responses were shown following such exposure to OVA in this study.

The work on Langerhans cell morphology and activation status shown in this Chapter was conducted with immunofluorescence micoscopy. This work shares the limitations of most immunofluorescence microscopy in that results are largely qualitative. The confocal microscopy images presented show few cells, however these are representative examples of a whole body of work analysing many cells, many visual fields in the microscope and many whole epidermal sheets. Conclusions are thus not based only on the presented images but on the bulk of work behind them.

Isotype control staining was carried out for all immunofluorescence staining and no background staining was detected as stated in Chapter 2, p. 90-91. The isotype controls are not shown in for example Fig. 5.13, p. 177 as it can be noted that there is no staining with the same antibodies in the normal intact skin.

Chapter 6 Epicutaneous immunisation prevents oral tolerance and enhances allergic sensitisation

This Chapter shows that epicutaneous exposure to allergens can prevent the normal induction of oral tolerance and specifically enhance Th2 responses such as IL-4 and antigen-specific IgE upon gastrointestinal challenge. Several more in depth experiments could be carried out to substantiate these initial observations. It would be of interest to examine whether allergen administration via one route could change local responses elicited via another exposure route. For example, it could be explored whether prior epicutanoeus allergen exposure would induce mucosal pathology following challenge in the gut. A thorough investigation of mucosal histology, histamine release, local production of cytokines and gut permeability would aid the understanding of the data presented here.

Chapter 7 Epicutaneous immunisation modifies subsequent and established antigen-specific immune responses

This Chapter shows that epicutaneous exposure to peanut protein on abraded skin can interfere with the development of Th1-type immunity and also modify established Th1 responses. After epicutaneous peanut protein exposure, Th1 immune responses to a subsequent immunisation with peanut in CFA were reduced while Th2 responses were enhanced. These observations and possible implications of the results are intriguing, but the experimental set-up confers some limitations in the interpretation of the data. As the control animals in this study are epicutaneously exposed to saline it is impossible to determine

whether the changes in the immune responses induced by epicutaneous peanut exposure are simply a consequence of one additional exposure to peanut protein. However, the results are not consistent with the epicutaneous exposure solely conferring an 'add-on' effect as IFN-γ and IgG2a responses to the CFA immunisation are specifically downregulated following epicutaneous immunisation. The limitations in data interpretation as a consequence of inadequate control for the *number* of antigen exposures is also discussed under Chapter 3 in this Appendix.

From the data presented in this Chapter it cannot be determined whether the deviation towards Th2 responses induced by epicutaneous immunisation prior or post immunisation with CFA are specific to this *route* of exposure. It was shown in Chapter 5 that subcutaneous injection with antigen exposure in the dermis promoted Th1-type responses. To address the question of whether the changes in CFA-induced immune responses following epicutaneous immunisation is specific for this route of exposure it would be suitable to compare with subcutaneous antigen exposure. This would additionally control for the total number of antigen exposures.

New references in Appendix

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