# Cutaneous Tolerance Induction - A Possible Strategy for the Treatment of Autoimmune Disease

by

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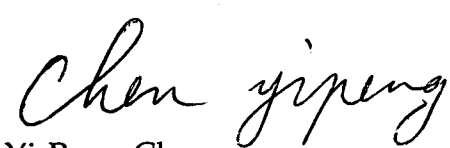

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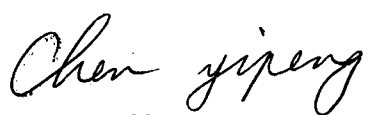

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# Abstract

Exposure of murine skin to carcinogens such as 7,12-dimethylbenz[a]anthracene (DMBA) or ultraviolet light B (UVB) irradiation depletes the epidermal Langerhans cells (LC) and alters the local environment such that antigen applied through the treated skin causes the development of antigen specific immunosuppression. This ability to induce suppression was used as a strategy to downregulate an established immune response in mice sensitised to a contact sensitiser or mice with autoimmune disease.

Mice immune to picryl chloride or 2,4,6-trinitrochlorobenzene (TNCB) were treated with either DMBA or UVB irradiation which was then followed by TNCB through the treated skin. The DMBA followed by TNCB treatment downregulated both contact hypersensitivity (CHS) and antibody production in an antigen specific manner, whereas UVB followed by TNCB treatment could only downregulate the CHS response. When spleen cells were transferred from TNCB tolerant mice (i.e. naive mice treated with DMBA followed by TNCB) to TNCB-immune mice, both an established CHS and an established antibody response were downregulated in an antigen specific manner. Although there was significant downregulation, the reduction was not complete as it did not reach the background level.

The ability to downregulate an established immune response provided an opportunity to treat autoimmune disease. A suitable model was experimental autoimmune gastritis (AIG) which develops following thymectomy 3 days after birth (3dnTx). Prior to evaluating these mice it was necessary to determine their immune function and to confirm that they could also develop antigen specific suppression. The 3dnTx mice had a reduced number of $CD4^+$ and $CD8^+$ T cells, a reduced lymphocyte proliferative response to PHA but a normal contact sensitivity response to TNCB. After treatment of the skin with DMBA these mice failed to develop contact sensitivity to TNCB. Adoptive transfer of splenocytes from these mice to naive mice transferred antigen-specific suppression, irrespective of whether the 3dnTx mice had developed autoimmune gastritis. Despite thymectomy the capacity of BALB/c mice to generate antigen-specific peripheral tolerance to TNCB was retained. These results suggest that precursor T cells which mediate suppression to antigens such as TNCB are present in 3dnTx mice and that these cells are likely to have developed in the thymus and exported to the periphery before 3 days after birth.

When 3dnTx mice with established autoimmune gastritis were treated with DMBA, followed by the proton pump autoantigen or its epitope a reduced disease severity was observed but DMBA alone caused similar effects. By replacing DMBA with TNCB, which could also deplete LC from the epidermis, and applying autoantigens through the TNCB treated skin failed to induce antigen specific suppression to AIG and consequently established

disease was not downregulated. At the preventative phase the results were more encouraging. When 3dnTx mice were treated on day 4 following birth with a combination of TNCB and the autoantigen peptide, peptide 19, none of these mice developed disease.

The key findings in this thesis were that the application of a contact sensitiser through skin depleted of normal LC can downregulate an established CHS response. When this approach was applied to an autoimmune disease, the application of autoantigen through skin depleted of LC did not reduce an established autoimmunity but application of autoantigen through neonatal skin was completely sufficient in preventing autoimmune gastritis development. Modified LC may therefore provide a basis for a potential vaccine against autoimmune disease.

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# Abbreviations

| | |
|---|---|
| 3dnTx | Three days neonatal thymectomy |
| AIG | Autoimmune gastritis |
| APC | Antigen presenting cells |
| BG | Birbeck Granules |
| BSA | Bovine serum albumin |
| CD | Cluster of differentiation antigen |
| CHS | Contact hypersensitivity |
| DD | Dermal dendrocytes |
| DETC | Dendritic epidermal T cell |
| DMBA | 7,12-dimethylbenz[a]anthracene |
| DNFB | 2,4-dinitrofluorobenzene |
| DTH | Delayed hypersensitivity |
| EAE | Experimental autoimmune encephalomyelitis |
| FCS | Foetal calf serum |
| FITC | Fluorescein 5 isothiocyanate |
| HIg | Human immunoglobulin G |
| HLA-DR | Human major histocompatibility complex class II antigen |
| IDC | Indeterminate dendritic cells |
| IFN | Interferon |
| Ia | Murine major histocompatibility complex class II antigen |
| IL | Interleukin |
| LC | Langerhans cells |

| | |
|---|---|
| MBP | Myelin basic protein |
| MHC | Major histocompatibility complex |
| NOD | Non-obese diabetic |
| PBS | Phosphate buffered saline |
| PKC | Protein kinase C |
| PLP | Proteolipid protein |
| TGF | Transforming growth factor |
| TNBS | 2,4,6-trinitrobenzenesulfonic |
| TNCB | 2,4,6-trinitrochlorobenzenesulfonic acid |
| TNF | Tumour necrosis factor |
| TPA | 12-*O*-tetradecanoylphorbol-13-acetate |
| SIS | Skin immune system |
| SAPC | Splenic antigen presenting cells |
| SLE | Systemic lupus erythematosus |
| SRBC | Sheep red blood cells |
| UV | Ultraviolet light |
| UVB | Ultraviolet light B |

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# References

# Foreword

Langerhans cells reside in the skin and belong to a population of professional antigen-presenting cells known as dendritic cells. These cells are crucial to the immune system as the initiation and direction of an immune response relies on the interaction of antigen-presenting cells with naive T cells. Under most circumstances a response is generated that results in immunity and elimination of the foreign antigen. However, when skin is treated with chemical or physical agents the Langerhans cells in the skin are depleted and the morphology of residual cells is altered in such a way that when antigen is applied through the treated skin an immunosuppressive response results. This ability of such a modified skin environment to induce suppressive immunity offers the opportunity to target disease conditions where the immune response can cause tissue damage eg. autoimmune disease.

The initial experiments in this thesis evaluated the ability of a modified skin environment to downregulate an established disease. For this aspect of the thesis the ability to downregulate an established contact sensitivity response was evaluated. Mice were made immune to TNCB and these TNCB-immune mice were then treated with DMBA or UVB irradiation which was then followed by TNCB. The results from these experiments would determine if established immunity could be manipulated.

Following these experiments, an autoimmune model of murine autoimmune gastritis was evaluated. As this model required neonatal mice to be thymectomised it was necessary to establish baseline immunological data and to determine if these thymectomised could also generate a population of regulatory cells, similar to the population of regulatory cells in normal mice, that could prevent induction of immune response.

The final aspect of this thesis was to utilise the neonatal thymectomy model of autoimmune gastritis and to treat these mice by applying autoantigen through skin, which contained a population of modified Langerhans cells. These experiments were designed to determine if immunisation of autoantigen through a modified Langerhans cell environment could provide a potential strategy to treat autoimmune disease at either the prevention or treatment levels.

# Chapter One
# Literature Review

## Introduction

The skin acts as a primary barrier against potentially harmful insults suffered by animals, but the overall structure and function of the skin is far more complicated than that of a mere physical barrier. In addition to its physical aspect, the skin also produces various substances and chemical components as well as containing different cell populations that react against stimulation by environmental agents. Of these environmental agents, chemical carcinogens and ultraviolet light are of particular interest as these two agents are able to induce a level of immune tolerance which may provide a potential strategy to regulate deleterious immune responses, including autoimmune disease. The following overview will examine the concept of the skin as an immune system and evaluate tolerance and autoimmune diseases with potential approaches for the treatment of autoimmune disease.

## 1.1 Skin immune system

The concept that the skin is an immune system is now well accepted. This is because the skin contains immune and non-immune cells which form an efficient network in response to a variety of environmental encounters. The term skin immune system (SIS) was first coined by Bos in 1986 and is now in widespread use, providing an accurate description of the

role of this large and major organ. The SIS is a dynamic structure and can be subdivided into cellular and humoral components. These include resident, recruited, and recirculating cell populations (Bos, 1997), the most important of these are the Langerhans cells. Other cells of SIS are keratinocytes, macrophages, and T cells, including αβ T cells as well as γδ T cells which are particularly unique to murine strains. Interaction of these immune cells is also vital for the effective function of SIS. Those cells which contribute to immunological events will be discussed here.

### 1.1.1 Langerhans cells

#### 1.1.1.1 Background

The most remarkable of the skin immune cells are the Langerhans cells (LC) which are bone marrow-derived dendritic cells (Tamaki *et al.*, 1980) featuring unique intracellular organelles, Birbeck Granules (BG) (Birbeck *et al.*, 1961). They also express the distinctive cell markers of Cluster of Differentiation (CD) 1a (Murphy *et al.*, 1981; Moulon *et al.*, 1991) and CD1c (Schmitt *et al.*, 1986) in humans. CD1a and Cd1c are not identified in murine skin, however, LC also express various other important cell markers including ATPase, CD45, the CD11 family, the ICAM family and the B7 molecules. Over 40 different cell markers have been found on normal or treated forms of LC (Teunissen *et al.*, 1997) as listed in Table 1.1. With the exception of the Birbeck Granules there is no phenotypic marker that uniquely identifies LC. The expression of cell markers depends on where and, in what conditions, LC reside.

| Marker | | Presence |
|---|---|---|
| **LC-specific** | | |
| "Lag" antigen | | +b |
| | | |
| **MHC molecules** | | |
| Class I | HLA-ABC | ++ |
| b2-microglobulin | | ++ |
| Class II | HLA-DR | ++ |
| Class II | HLA-DP | ++ |
| Class II | HLA-DQ | ++ |
| CD 74 | invariant chain | + |
| RFD1 | DQ-like | + |
| | | |
| **T cell makers** | | |
| CD1a | | ++ |
| CD1b | | - |
| CD1c | | + |
| CD2 | | - |
| CD3 | | - |
| CD4 | | + |
| CD5 | | - |
| CD7 | | - |
| CD8 | | - |
| abT cell receptor | | - |
| rd T cell receptor | | -(+)c |
| | | |
| **B cell markers** | | |
| CD10 | | - |
| CD19 | | - |
| CD20 | | - |
| CD22 | | - |
| CD24 | | - |
| CD40 | | +d |
| | | |
| **Monocyte/macrophage markers** | | |
| CD15 | LeuM1 | -(+)e |
| | LeuM2 | - |
| CD14 | LEUM3 | -/+ |
| CD33 | | + |
| CD68 | | -(+)F |
| OKM5 | | - |

| Marker | | Presence |
|---|---|---|
| **Receptors & Adhesion Molecules** | | |
| CD25 | IL-2R | - |
| CD64 | FcγRI | - |
| CD32 | FcγRII | + |
| CD16 | FcγRIII | - |
| | FcεRI | + |
| CD23 | FcεRI I | - |
| eBP | | + |
| CD35 | CR1 | - |
| CD21 | CD2 | - |
| CD11B | CR3; C3biR | + |
| CD11c | gp150/95 | + |
| CD18 | b chain of CD 11a,b,c | + |
| CD11a | LFA-1 | - |
| CD58 | LFA-3 | +(-)g |
| CD54 | ICAM-1 | +(-)g |
| CD102 | ICAM-2 | - |
| CD50 | ICAM-3 | ++ |
| CD80 | B7-1 | -(+) |
| CD86 | B7-2 | - |
| CD49a | VLA-1 | +h |
| CD49b | VLA-2 | +h |
| CD49c | VLA-3 | +h |
| CD49d | VLA-4 | ++h |
| CD49e | VLA-5 | +h |
| CD49f | VLA-6 | ++h |
| CD29 | b chain of VLA antigens | + |
| E-cadherin | | + |
| CD15 | sialy-Lewisx | + |
| HECA-452 | | + |
| | | |
| **other Markers** | | |
| S100 | | + |
| CD45 | pan-leukocyte | + |
| CD45RA | | - |
| CD45RB | | - |
| CD45RO | | +d |
| CD69 | | + |
| CD83 | | + |

**Table 1.1** LC makers. (From Marcel et al., 1997)

The most significant cell marker expressed by LC is the high level of major histocompatibility complex (MHC) class II. In normal epidermis, MHC II is only expressed by LC (Katz *et al.*, 1985). It is through MHC II that LC provide a powerful stimulatory function to activate naive T cells (Green *et al.*, 1980; Enk and Katz, 1995). Thus LC are now identified as major antigen-presenting cells (APCs) (Kapsenberg *et al.*, 1995). As such their *in vivo* role includes the characteristic skin reaction of a contact hypersensitivity (CHS), and type IV delayed hypersensitivity (DTH) (Teunissen *et al.*, 1997). It also been suggested that LC are essential in the immunosurveillance against skin tumours (Halliday and Muller, 1984). In addition, LC have been implicated in the induction of tolerance (Halliday and Muller, 1986; Halliday and Muller, 1987b; Cruz and Bergstresser, 1990; Simon *et al.*, 1991). Understanding the role of LC is necessary to potentially manipulate the skin immune system.

#### 1.1.1.2 Role in CHS and DTH reactions

Definitive evidence of LC as highly efficient antigen-presenting cells comes from studies of CHS reactions, particularly at the sensitisation stage. Contact hypersensitivity is a delayed type of hypersensitivity response specifically induced by a small antigen or hapten. When a hapten such as 2,4,6-trinitrochlorobenzenesulfonic acid (TNCB) is applied to the skin, it binds to a carrier protein in the epidermis . This protein-hapten complex is processed by LC which migrate to the draining lymph node where the processed antigen in association with MHC-II is presented to naive T cells,

which are then activated. In addition, the MHC II of LC can also be directly bound by a hapten (Silberberg *et al.*, 1976; Shelley and Juhlin, 1977).

A successful CHS response depends on LC density. The application of a hapten through normal dorsal or ventral skin, which has a high LC density, induces a strong CHS response. However, when a hapten is applied, through a depleted LC density an effective CHS response is impaired. This is evident by studies of mice exposed to ultraviolet light B (UVB) or chemical carcinogens, both of which have the ability to deplete epidermal LC. The initial experiments were performed in 1976 by Silberberg and colleagues who showed that UVB irradiation depleted epidermal LC. When antigen was applied through this LC-depleted skin there was a failure to induce CHS response (Lynch *et al.*, 1981; Gurish *et al.*, 1983). This was further supported by Toews *et al.* (1980) who demonstrated that LC numbers in the epidermis were important in the induction of immune suppression using UVB radiation. Halliday and colleagues (1986) showed that chemical carcinogens could deplete LC and that this correlated with the failure to induce a CHS response when antigen was applied through this depleted skin. More recently it has been shown that contact antigens can deplete LC, and when another antigen was applied through this skin, CHS failed to generate (Woods *et al.*, 1996). This later study also demonstrated that the induction of immune suppression is associated with the extent of LC depletion in the skin, higher doses of TNCB reduced LC numbers significantly. In addition,

mice tail skin which normally has a low LC density fails to generate a CHS response (Streilein *et al.*, 1983).

As previously mentioned, LC migrate to the draining lymph node. It is within the lymph node following exposure to a hapten, such as fluorescein 5 isothiocyanate (FITC), where these antigen-bearing LC present antigens to specific $CD4^+$ T cells and trigger a primary immune response (Hill *et al.*, 1993). The trigger for this migration occurs after exposure to antigen, although there is a low background level of migration occurring continuously (Dandie *et al.*, 1992). The mechanisms involved in this migration are unclear, although some aspects have been partly revealed. When considering the mechanisms involved in migration it is necessary to conceptualize the structure of the skin and the location of the LC within this structure.

The LC are located in the epidermis which is separated by a basement membrane. This structure consists of a variety of extracellular matrices, e.g. type IV collagen, laminin, heparan sulfate proteoglycan, and fibronectin (Laurie *et al.*, 1982). The travelling LC bearing the antigen must pass through the basement membrane to reach lymphatic vessels. A study by Le Varlet *et al.* (1991) suggested that the expression of integrins of the β 1 subfamily on LC may be important in this process. Production of cytokines by keratinocytes and LC upon hapten application also increases the mobility of LC (Enk and Katz, 1992). In fact two cytokines, IL-1β and TNF-α, have been

demonstrated to stimulate LC migration (Enk *et al.*, 1993; Cumberbatch and Kimber, 1992). In addition, *in vivo* hapten application such as TNCB or FITC enhanced LC migration, however, sodium lauryl sulfate , which is an inhibitor of the extracellular matrix reduced the LC migration. Thus, haptens play an important role in LC mobility (Kobayashi *et al.*, 1994). Another study showed that application of a hapten to the skin would stimulate the LC to produce the matrix metalloproteinases which are important factors for degrading gelatin and collagen (Kobayashi, 1997). The activation of protein kinase C (PKC) also has been identified as critical in the migration of LC to the draining lymph as the inhibition of PKC activity can stop the LC migration (Halliday and Lucas, 1993). Thus, it is through this complex series of events that LC migrate and present antigen after encountering a contact sensitiser.

The response of CHS to LC is antigen specific. When hapten-bearing LC migrate to a draining lymph node, they activate naive T cells. After this event the specific T cells expand and memory T cells are generated. The activated T cells produce interferon-gamma (IFN-$\gamma$) and interferon-alpha (IFN-$\alpha$), and travel back to the skin to induce a CHS immune response (Luger *et al.*, 1997).

#### 1.1.1.3 Role in skin immunosurveillance

The concept of immunosurveillance was first proposed by Macfarlane Burnet (Burnet, 1970). He postulated that immune cells have the ability to

survey the body for abnormal cells (eg. damaged or cancer cells) and to eliminate them. The relevance of this concept has been supported by the increased risk of skin cancers in patients using immunosuppressive drugs (Pritzker *et al.*, 1970). It has been reported by Marshall (1973)who showed that transplant patients have an increased incidence of skin tumors (from 8% to 17%) over a 4 years period. Further study by Sheil and co-workers found that in 10 years time, transplant patients had a 57% higher risk of skin tumours than normal individuals (Sheil *et al.*, 1979). Those skin tumours found in patients treated with immunosuppressive drugs include squamous cell carcinoma, basal cell carcinoma, Kaposi's sarcoma, malignant melanoma, and Bowen's disease (Penn, 1980; Penn, 1994).

Skin tumors also increased in patients with psoriasis, which is likely to be a consequence of their requirement for ultraviolet light treatment. This is because the ultraviolet light treatment can disturb the normal skin function. Patients with AIDS disease also have an increased risk of skin tumors as a result of reduced T cell function. With the AIDS viruses destroying $CD4^+$ T cells there is reduced immunosurveillance and the risk of aberrant cells escaping detection is increased, hence the increased incidence of Kaposi's sarcoma (Dalgleish *et al.*, 1984; Santucci *et al.*, 1988; Chachoua *et al.*, 1989).

At the experimental level, skin tumors develop following carcinogens treatment. This is due to the carcinogens altering the skin immune function, especially the LC in the skin, thus allowing abnormal tumour cells to occur (Qu *et al.*, 1997).

#### 1.1.1.4 Role in tolerance induction

It has been well documented that the induction of immunological tolerance can be achieved by application of a hapten through LC-depleted skin. Methods used to deplete LC in the skin have included chemical carcinogens (Muller *et al.*, 1985), UVB irradiation (Toews *et al.*, 1980), and tape stripping (Streilein *et al.*, 1982). The tolerance that develops in this situation is antigen specific and appears to be related to the presence of suppressor cells (Muller *et al.*, 1992). On the other hand, upregulation of LC numbers increases the ability of LC to activate T-cells (Meunier *et al.*, 1994). The reduction of LC density is not the only explanation for the induction of tolerance, as the function of the residual cells must be activated to induce immunosuppression. This has been demonstrated by Ragg *et al.* (1995) in a sheep model where afferent LC (ie. veiled cells) were collected as they migrated away from carcinogen treated skin. Following application of DMBA the LC displayed a reduced ability to process and present soluble antigen in an *in vitro* proliferation assay.

Tolerance induced by LC depletion was also found after UVB irradiation. Epidermal cells from UVB irradiated skin depleted of LC from C57BL/6 mice incubated with a hapten suppressed the induction of CHS response (Dai and Streilein, 1995). Study of human epidermal LC found that LC antigen-presenting function to T cells was impaired by UVB irradiation in the mixed epidermal cell-lymphocyte reaction experiment (Rattis *et al.*, 1995).

Consequently, when skin is depleted of LC by exposure to chemical carcinogens or UVB irradiation, one explanation for the failure to induce an immune response is that the dendritic cells are functionally impaired.

### 1.1.2 Other skin dendritic cells

The epidermis of the skin contains another population of dendritic cells termed indeterminate dendritic cells (IDC) which express CD1 and MHC II molecules, but lack Birbeck Granules in humans (Rowden *et al.*, 1979). Since IDC can only be distinguished from LC by the absence of BG they may be the precursor of LC (Breathnach, 1975). Indeed, a study by Hsiao (1989) of IDC in rats using immunoelectron microscopic showed that IDC belong to the lineage of LC. Recently, it has been suggested that IDC are true LC (Teunissen *et al.*, 1997).

The dermis also contains different populations of dendritic cells. These include LC that migrate into and out of the epidermis, and skin dermal dendritic cells, termed dermal dendrocytes (DD). The LC population in the

dermis is the precursor of epidermal LC and migrating LC, which carry antigens from the epidermis to the lymphoid organs. The dermal dendrocytes are the residential dendritic cells of the dermis. Although they have a dendritic shape the function of DD is largely different to that of the LC of epidermis. The phenotype of DD may closely resemble macrophages as many DDs express CD45, HLA-DR, Factor IIIa, CD11b and CD14 (Cerio *et al.*, 1989; Weber Matthiesen and Sterry, 1990; Rowden, 1997). Unlike LC, with a main function of antigen presentation, the main functions of DD are involved in inflammation, wound healing, and immunity. The immune functions relating to DD include the production of IL-2, IL-8, and TNF-α which has been shown in psoriasis in humans (Nickoloff *et al.*, 1991; Nestle *et al.*, 1994). However, DD have also been hypothesised to be involved in antigen presentation (Lappin *et al.*, 1996).

### 1.1.3 Keratinocytes

Keratinocytes are the major cell type and structure of the epidermis. They form the physical barrier in animals. However there is increasing evidence that keratinocytes are involved in skin immunity as they are able to express MHC II molecules, accessory molecules, produce various cytokines and play a role in the induction of tolerance (Chu and Morris, 1997).

### 1.1.3.1 MHC II expression

Keratinocytes have the ability to express MHC II in a variety of clinical conditions including graft vs. host disease (Lampert *et al.*, 1981), psoriasis (Morhenn *et al.*, 1982) and contact dermatitis (MacKie and Turbitt, 1983). All of these conditions are characterised by T cell infiltration, thus implicating keratinocytes in potential T cell activation. It has been determined that the T cell cytokine INF-γ is capable of inducing MHC II expression of keratinocyte *in vitro* (Basham *et al.*, 1985; Nickoloff *et al.*, 1985), and *in vivo* (Aiba *et al.*, 1984). Consequently a model can be proposed whereby an inflammatory response is generated following exposure to antigens when the T cell in the infiltrate would modulate IFN-γ, thereby up-regulating MHC II in keratinocytes which in turn can contribute to the maintenance of the immune response. It is not known whether keratinocytes can induce a primary immune response rather than maintain an established response. In fact there is evidence that MHC II bearing keratinocyte can anergise naive T cells (Gaspari *et al.*, 1988), possibly due to a lack of adequate costimulatory signaling, although in some instances accessory molecules have been expressed. In mice, application of the contact sensitiser TNCB induces Ia expression of keratinocyte within 3 days (Germain, 1981).

### 1.1.3.2 Accessory moleculars expression

Keratinocytes can express both adhesion and costimulatory molecules. They express intercellular adhesion molecule-1 (ICAM-1/CD54) which is vital for the initiation and evolution of localized inflammatory processes in

the skin and serve as a specific ligand for lymphocyte function-associated antigen-1 (LFA-1/CD18), a cell-surface protein expressed on all leukocytes (Wright Caughman *et al.*, 1992). Another important accessory molecule expressed by keratinocyte is LFA-3 (CD58) which is the ligand/receptor pair of LFA-3 (CD58)/LFA-2 (CD2) and is important for T cell activation (Chu and Morris, 1997).

With regard to costimulatory molecules, keratinocytes express BB1, a member of the B7 family, B7-1, B7-2. BB1 serves as the ligand for CD28 (Chu *et al.*, 1987). B7-1 and B7-2 are critical for T cell activation as the blockage of these two molecules by antibodies results in T cell anergy. However, the function of BB1 expressed on keratinocytes is not clear. BB1/CD28 blockage by monoclonal antibody did not have significant effect on keratinocyte induced T cell activation (Nickoloff and Turka, 1994).

#### 1.1.3.3 Cytokine production by keratinocytes

Within the skin immune system the most important function of keratinocyte is to produce a vast array of cytokines with inflammatory and immunological activities. The effective nature of the cytokine profile can be seen in Table 1.2. It should be noted that resting keratinocytes produce a number of cytokines but following activation this range is dramatically expanded. It should also be noted that the activation of these cytokines encompasses a broad range of immunological and pathological activities.

| | |
|---|---|
| Resting keratinocytes | IL-1α, IL-6, IL-11, IL-15<br>TNFα<br>GM-CSF, G-CSF<br>TGFβ |
| Activated keratinocytes<br>(UV, endotoxin, phorbol ester, cytokines) | IL-α/β, IL-6, IL-7, IL-10, IL-11, IL-12, IL-13, IL-15, IL-1RA,<br>TNF α<br>IL-8, Gro-α,β,γ, ENA–78, IP-10, MIP-2, MCP-1, RANTES<br>IL-3, G-CSF, M-CSF, GM-CSF, SCF<br>IFNα, IFNβ<br>TGFα, TGFβ, PDGF, bFGF |

Note: IL, interleukin; TNF, Tumor necrosis factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony stimulating factor; transforming growth factor; Gro, growth regulated oncogene; ENA-78, epthelial neutrophil-activating protein-78; IP-10, γ-interferon-inducible protein-10; MIP- 2, macrophage inflammatory protein-2; MCP-1, microbial cationic protein-1; RANTES, regulated upon activation, normal T expressed, and presumably secreted; M-CSF, macrophage colony-stimulating factor; SCF, stem cell factor; IFN, interferon; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor.

**Table. 1.2** Cytokine production by keratinocytes
(From Thomas et al., 1997)

Amongst these cytokines, IL-1 is critical in the activation of T cells. Keratinocytes secrete a low level of IL-1 in normal conditions but are upregulated when activated. TNFα is another important cytokine secreted by keratinocytes which increases following activation. This cytokine is involved in the inflammatory response and increases IL-1α production. Other important cytokine produced by keratinocytes involved in T cell activation include IL-12 and IL-15. IL-12 plays an important role in the induction phase of a CHS response as the topical application of allergens to skin in humans results in the induction of IL-12 within the epidermis (Muller *et al.*, 1995). It also regulates TGF-β production (Marth *et al.*, 1997). IL-15 has similar biological effects to IL-12, as IL-15 can enhance the proliferation and activation of T cells (Luger *et al.*, 1997).

Other important cytokines produced by keratinocytes include IL-6, IL-7 and IL-10. IL-6 is an important mediator of inflammation as the serum levels of IL-6 are elevated in patients with inflammatory or autoimmune diseases (Akira *et al.*, 1993). Keratinocyte derived IL-7 is a growth factor for the epidemal dendritic T cells as the cultured dendritic epidermal T cell (DETC) line, 7-17, and freshly isolated DETC exhibited dose- and time-dependent proliferative responses to the IL-7 receptor and these responses were blocked completely by anti-IL-7 antibodies (Matsue *et al.*, 1993). With keratinocytes irradiated by UVB the IL-7 production is inhibited (Takashima, 1995) also implicating IL-7 in UVB-induced immune suppression. IL-10 is important

in the regulation of immunosuppression and will be discussed in the next section.

### 1.1.3.4 Induction of immune tolerance

Antigen presentation by professional APC results in a positive immune response. However, antigen presented by non-APC such as keratinocytes which lack costimulator factors can result in a state of immune suppression (Bal *et al.*, 1990). Immunosuppression induced by these keratinocytes is antigen specific. It has been shown *in vitro* that hapten-modified $Ia^+$ keratinocytes specifically downregulate a contact allergen induced immune response to a Th1 cell clone (Gaspari *et al.*, 1988); *in vivo* hapten-modified $Ia^+$ keratinocytes also showed specific suppressive ability for a CHS response (Gaspari and Katz, 1991).

The ability of keratinocytes to induce immune suppression is related to suppressive cytokine production. IL-10 for example, a powerful immunosuppressive cytokine, is secreted by keratinocytes (Taga *et al.*, 1993). Enk and colleagues (1994) documented that IL-10 is directly involved in the induction of hapten-specific tolerance. IL-10 is also directly involved in the downregulation of contact sensitivity (Ferguson *et al.*, 1994; Niizeki and Streilein, 1997). Studies with IL-10 knockout mice demonstrated that without IL-10 these mice mounted an exaggerated contact sensitivity response to hapten application, and also an increased magnitude and duration of a contact sensitivity response when compared with wild type

mice (Berg *et al.*, 1995). Keratinocyte derived IL-10 also mediate systemic immunosuppressive effects from animals exposed to UVB irradiation. Ullrich and associates showed that UVB exposed PAM 212 keratinocytes which were neutralized with anti-IL-10 prior to injection into mice abrogated the suppressive effects of mediators on induction of delayed type hypersensitivity to subcutaneouly injected protein antigens which occurred with the PAM 212 keratinocytes (Ullrich, 1995a). This was also demonstrated when the mice were injected with antibodies to IL-10 and exposed to UVB irradiation (Ullrich, 1995b). Consequently the suppressive effect appear to be mediated by IL-10.

In skin, the production of keratinocyte IL-10 is increased after haptenization of the cells and after UVB irradiation. IL-10 is the product of Th2 cells and is able to suppress proliferation of Th1 cells. In the situation of induction of immunosuppression after UVB irradiation, it has been postulated that keratinocyte derived IL-10 converts LC as inducer APCs of contact sensitivity to tolerogenic APCs (Enk and Katz, 1995).

### 1.1.4 T Cells

T lymphocytes express αβ or γδ antigen receptors in humans and rodents. In the circulation the majority of T lymphocytes express αβ antigen receptors. However, in the cutaneous epidermis T lymphocytes populations are different between humans and rodents. In humans, the epidermis

contains few αβ T cells with a minor γδ T cell population. The function of these T cells is most likely to boost the immune response as most of the T cells are $CD45RO^+$ memory population (Bos *et al.*, 1993). Further studies from Davis *et al.* showed that these T cells are in an activated state as they express HLA-DR or CD25 molecules (Davis *et al.*, 1988).

On the other hand, the murine epidermis contains a unique T lymphocyte population referred to as dendritic epidermal T cells (DETCs). The αβ T cells of the epidermis participate in normal immune activation, while DETC are involved in a number of immune activities.

DETC are dendritic cells but belong to the T cell population because they express Thy-1, and CD3 antigens. DETC are derived from bone marrow (Breathnach and Katz, 1984). Because of their location, it has been proposed that DETC are part of a first line of defense (Asarnow *et al.*, 1988). Nixon Fulton *et al.* (Nixon Fulton *et al.*, 1986) demonstrated that the proliferation ability of DETC increased when incubated with concanavalin A and IL-2. DETC can also be activated *in vivo* by contact allergens (Kaminski *et al.*, 1993a). DETC have the capacity to kill tumour cells when stimulated by IL-2 (Okamoto *et al.*, 1988; Kaminski *et al.*, 1993b). Recently, Schuhmachers *et al.* (1995) suggested that the tumor killing ability of DETC may be mediated by 2B4 antigen.

DETC may serve as a regulatory population of cells as they are able to induce hapten-specific tolerance *in vivo* when DETC incubated with hapten are injected into mice (Welsh and Kripke, 1990). The capacity to cause tolerance may be due to an ability to inhibit activated T cells to proliferate (Welsh *et al.*, 1992) or to act as cytotoxic cells (Love Schimenti and Kripke, 1994). Studies by Cruz and co-workers demonstrated that DETC had an immunosuppressive function after low dose UVB irradiation (Cruz *et al.*, 1989). Intravenous injection of DETC derivatised with hapten can initiate down-regulation of the CHS response (Sullivan *et al.*, 1986).

### 1.1.5 Macrophages

Skin macrophages are related to LC and both are bone marrow-derived (Austyn, 1987). They can be found in various compartments of the skin but primarily in the superfical or papillary dermis (Weber Matthiesen and Sterry, 1990). The expression by macrophages of CD11b, CD14, and CD68 distinguishes macrophages from LC. Although CD11b is also expressed on LC the level of CD11b is significantly lower than that of macrophages.

The functions of dermal macrophages include anti-microbial defense in inflammation, contribution to repair in wound healing, antigen presentation, and immune suppression. Macrophages are phagocytes; they engulf microbial organisms and destroy them through oxidative and nonoxidative mechanisms. Macrophages participate in wound healing by producing growth factors for fibroblasts. Macrophages are also involved in

immune responses where they may act as the primary APC during induction of an immune response (Moller, 1978). Skin macrophages can downregulate or suppress an immune response. It has been shown by Cooper and associates that in human skin irradiated with ultraviolet light the numbers of $CD36^+$ $CD11b^+$ $CD1^-$ macrophage increase dramatically (Cooper *et al.*, 1986; Meunier *et al.*, 1995). Application of hapten through this macrophage enriched skin can lead to immune tolerance to the antigen (Hammerberg *et al.*, 1994). UV-induced tolerance could be reversed by anti-CD11b monoclonal antibody treatment, which further support the concept that macrophages are involved in UV-induced immune suppression (Hammerberg *et al.*, 1996).

### 1.1.6 Other skin immune cells

The skin contains other cells with immunological functions. Mast cells mediate immediate hypersensitivity and have a role in delayed-type hypersensitivity immune responses. They release histamine and serotonin following crosslinking of their IgE receptors. Vascular endothelial cells can act as APCs to activate T lymphocytes via MHC II antigen on their surface. They also secrete immunological cytokines such as IL-1, TNF and IFN$\alpha/\beta$ to increase the expression of adhesion molecules on epidermal cells (Pasyk *et al.*, 1997).

Merkel cells are also involved in the regulation of the skin immune function. They secrete vasoactive intestinal polypeptides (Hartschuh *et al.*, 1983) and serotonin (Garcia Caballero *et al.*, 1989) which are involved in inflammation. They also secrete calcitonin gene-related peptide (Alvarez *et al.*, 1988) which has been shown to regulate LC (Hosoi *et al.*, 1993) and macrophage function (Nong *et al.*, 1989).

### 1.1.7 Summary

The skin contains a complete and complex immune system which is not only a barrier to microbial invaders but provides for a range of immune responses including responses involved in anti-tumour immunity, autoimmunity, and transplantation reactions.

## 1.2 Tolerance

Tolerance is a specific absence of an immune response to an antigen and is essential as animals tolerate their own antigens. This is critical for the animal's survival as failure of tolerance results in autoimmune disease. As the immune system must also eliminate potentially harmful foreign antigens, mechanisms must exist whereby these antigens are eliminated and self antigens remain unaltered. Tolerance therefore is a consequence of how the immune system discriminates between harmless 'self' antigen and harmful 'non-self' antigens.

Induction and maintainance of tolerance is one of central themes in immunology. The main targets for tolerance are T and B cells with several mechanisms suggested to induce tolerance in T and B cells which include clonal deletion , clonal anergy, and suppressor cells.

### 1.2.1 Clonal deletion

Clonal deletion is the elimination of self-reactive lymphocytes (T and B cells). Although the mechanism of clonal deletion involves many cells, discussion here is centred on T and B cells.

#### 1.2.1.1 T cell clonal deletion

The primary place for T cell deletion is the thymus. In the thymus clonal deletion occurs in two waves, one of positive selection, the second negative selection. T cells that recognise self-MHC molecules are selected for positive selection whereas the T cells that fail to bind with self-MHC are clonally deleted. The second wave of clonal deletion occurs when T cells bind to dendritic cells or macrophages via self antigen. As these cells are potentially self reactive lymphocytes they are deleted.

Kappler and associates used a monoclonal antibody against the product of Vβ17a (a variable region gene segment of the β chain of the T cell receptor) to follow the fate of T cells expressing this Vβ17a (Kappler *et al.*, 1987). They found that in mice which expressed the E molecule of the MHC class II, the

Vβ17a bearing T cells were eliminated. In the thymus the first stage of immature T cells are $CD4^+8^+$ phenotypes and at this stage the thymus contains a proportion of Vβ17a bearing T cells. In a more mature stage the Vβ17a bearing T cells were depleted. This depletion of Vβ17a T cells is complete as no Vβ17a mRNA can be detected in the mature T cells.

Cells involved in deleting T cells are probably antigen-presenting cells. Experiments using T cell receptor transgenic mice demonstrate that splenic antigen-presenting cells (SAPC) could induce deletion of $CD4^+8^+$ T cells when these SAPC are incubated with thymocyte suspensions from TCR transgenic mice (Swat *et al.*, 1991). Thymic dendritic cells express MHC molecules. T cells that recognise the self-peptide/MHC complex with dendritic cells are deleted. Thus, tolerance could be maintained in T cells by clonal deletion.

However, the deletion mechanism in the thymus does not eliminate all the harmful autoimmune reactive T cells. Many self peptides and MHC complexes are not generated or can not be seen by T cells in the thymus. Thus, T cell clones specific for such peptide/MHC complexes would not be deleted. These T cells can be activated if spleen cells from the animals are stimulated with synthetic peptides of this type (Schild *et al.*, 1990).

#### 1.2.1.2 B cell clonal deletion

Clonal deletion also occurs with B cells. Studies using transgenic mice have shown that transgenic mice of $MHC^d$ background which expressed an IgM B cell receptor specific for MHC class I molecules $K^k$ and $D^k$, contained 25-50% splenic B cells expressing this receptor (Nemazee and Burki, 1989). When crossed with $MHC^k$ mice all B cells expressing the IgM receptor disappeared. No transgenic receptor-bearing cells were detected in the spleen but a very low to undetectable amount was detected in the bone marrow. Thus B cell clonal deletion occurs in bone marrow.

### 1.2.2 Clonal anergy

Another mechanism that silences auto-reactive T and B lymphocytes is clonal anergy. As not all the self-reactive lymphocytes were eliminated from the primary lymphoid organs (ie. thymus, bone marrow), the escaped self-reactive lymphocytes must be suppressed in the periphery. This functional inactivation is termed clonal anergy.

#### 1.2.2.1 T cell anergy

T cell anergy has been demonstrated in *in vivo* and *in vitro* models. In *in vivo* study Jacobsson and colleagues (Jacobsson *et al.*, 1976) found an intravenous injection of Mtv-$7^+$ spleen cells into MHC-compatible Mtv-$7^-$ mice prevented a subsequent mixed lymphocyte response to these spleen cells. This study was further developed by Rammensee *et al.* (1989). They used an antibody to $V\beta6^+$, a T cell antigen that can interact with and be

stimulated by the Mtv-7 antigen, to follow the track of Vβ6$^+$ T cells after intravenously injection. They reported that CD4$^+$ Vβ6$^+$ T cells survived, however, these T cells could not proliferate when stimulated with spleen cells expressing Mtv-7. These T cells also failed to make IL-2. Thus T cell anergy was induced *in vivo*. In the *in vitro* model, Lamb and co-workers (Lamb and Feldmann, 1984) found that a normal CD4$^+$ T cell clone, when stimulated with high concentration of a peptide fragment from the influenza virus haemagglutination, for which it was specific, failed to respond to restimulation by that antigen in the presence of antigen-presenting cells. The cells were alive but not functional. Those anergised T cells could be restimulated with the introdution of IL-2. Importantly, this reversal was achieved in all the anergic cells (Beverly *et al.*, 1992).

One of the mechanisms of anergy induction in T cells is the two signal model. Since a successful immune response require the peptide/MHC complex and a second signal such as CD28/B7 ligation, the absence of this second signal can lead to the T cell anergy. For example, cultures of T-cell clones in the presence of submitogenic doses of anti-CD3 without competent APC resulted in anergy, which could be prevented if anti-CD28 antibody was incorporated (Harding *et al.*, 1992; Lenschow *et al.*, 1996). In *in vivo* study, blockade of the B7-CD28 pathway has been demonstrated to result in long lasting tolerance to human xenoantigen in mice (Lenschow *et al.*, 1992).

#### 1.2.2.2 B cell anergy

The induction of B cell anergy has been found in the two signal model. In B cell activation, two elements are required, the first is that the Ig receptor of the B cell must interact with the antigen. The second signal involves the peptide/MHC complex and CD40/CD40 ligand. If B cells receive the first signal but not the second signal, the B cells are anergic. The reversal of B cell anergy can be achieved by lipopolysaccharides or strong T cell help.

### 1.2.3 Suppressor T cells and self-tolerance

Existence of suppressor T cells has been sought as a population of regulatory cells with an important role in fail-safe mechanisms to prevent an overeactive immune response and to contain self-reactive cells. Early evidence for the existance of suppressor T cells came from Gershon's study who showed that spleen cells can suppress the activation of normal T cells in an antigen specific manner (Gershon and kondo, 1971). In their study, immunological tolerance of sheep red blood cells could be transferred from one mouse to another. This was attributed to a subset of $CD8^+$ T cells and was termed suppressor cells. One of the major limitations to the acceptance of suppressor T cells has been the inability to isolate and characterise these cells. This is due to the absence of reliable phenotypic markers of suppressor cells. Nonetheless, many experimental models have demonstrated the phenomena of suppressor T cells and indicated possible markers of these cells.

In humans, a subset of $CD4^+$ T cells expressing glycoprotein 2H4, a member of the CD45 group of leukocyte common antigens, has been shown to induce suppressor cell activity in an *in vitro* study (Baadsgaard *et al.*, 1987). These cells are $CD4^+CD45RA^+$ suppressor-inducer cells which have been shown to increase their numbers after UV irradiation, depending on the dose (Baadsgaard *et al.*, 1987). These $CD4^+CD45RA^+$ suppressor cells have also been shown to have a reduced number in patients with SLE disease indicating $CD4^+CD45RA^+$ cells indeed play a role in the induction of immunosuppression (Morimoto *et al.*, 1987).

In mice, suppressor cells have been proposed as the IJ-restricted T cells. In this proposed system, suppressor cells are induced by various suppressive factors (Figure 1.1) . In this scheme, $I\text{-}J^+$ antigen specific $CD4^+$ or $CD8^+$ cells contain a dominant cross-reactive idiotype. These cells, termed Ts1, produce the T suppressor cells factor TsF1. With the help of TsF1 and antigen stimulation, Ts2 cells are generated which are $CD8^+$ $I\text{-}J^+$ cells and produce TsF2. The interaction of primed antigen and TsF2 generate Ts3 cells which secrete nonspecific factors that are final mediators of suppression (Gershon *et al.*, 1981).

Elimination of functional T cells can also be achieved by veto cells. The T cell that recognises an antigen on the surface of the veto cell is rendered nonfunctional as a result of that interaction; the cell that becomes unresponsive determines the specificity of this type of unresponsiveness,

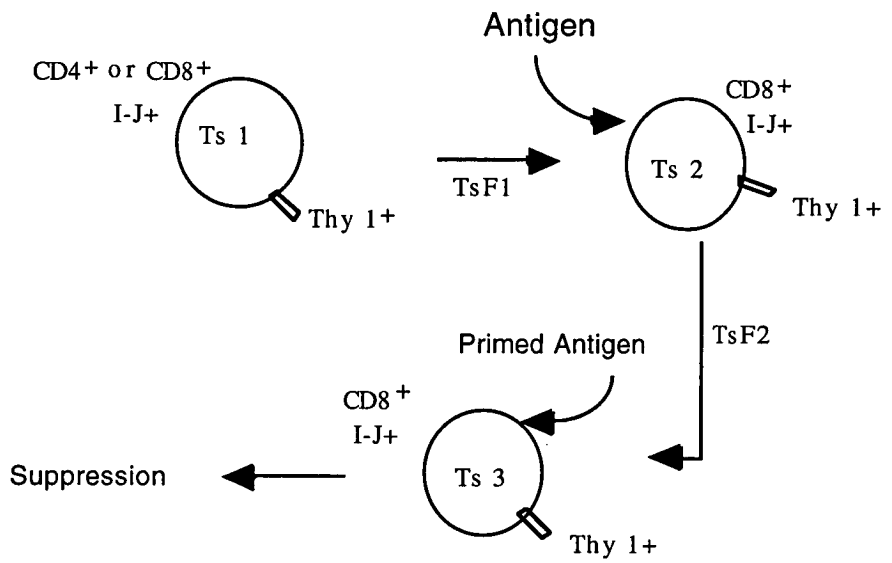



**Figure 1.1** Interaction of proposed T suppressor cell subsets (Graph modified from Weiner et al., 1988)

and self is defined by antigens expressed on the veto cell surface (Fink *et al.*, 1988).

Another demonstration of the existence of suppressor cells is that organ specific autoimmune disease can be induced in thymectomised mice (more detail in section 1.3.2). For example, autoimmune thyroiditis was found to occur without immunisation in thymectomised and X-ray irradiated rats (Volpe, 1994). However, reconstruction of these rats with lymph node cells will prevent the thyroiditis.

## 1.3 Autoimmunity

An intact immune system must tolerate self-antigens. When this fails autoimmunity results. In general, autoimmune disease can be defined as organ-specific eg. thyroiditis or non-organ specific eg. systemic lupus erythematosus. Although it is convenient to use such a classification, many autoimmune diseases appear to be both eg. multiple sclerosis. Another classification of autoimmune disease is either MHC class II associated or MHC class I associated autoimmune diseases (Sinha *et al.*, 1990).

It remains unclear how autoimmune diseases are initiated. However, studies from animals have revealed different mechanisms leading to the induction process. For example, certain strains of mice spontaneously develop autoimmune diseases, eg. non-obese diabetic mice (Gill and Haskins, 1993); antigen or chemical injection may induce autoimmune

diseases, eg. collagen-induced arthritis (Wilder, 1989) ; exposure to foreign antigens which are similar to self determinants eg. mycobacterial extract induced arthritis (Roitt *et al.*, 1992) ; and 3 day neonatal thymectomy also result in autoimmune diseases, eg. autoimmune gastritis (Fukuma *et al.*, 1988).

The study of animal models with autoimmune disease provide the opportunity to understand the autoimmune diseases. Here some important animal models will be discussed.

### 1.3.1 Animal models of autoimmune diseases

An important challenge for the study of autoimmune disease is to develop animal models that realistically reflect similar autoimmune diseases in humans. Several autoimmune diseases have been induced in animals similar to that of human autoimmune disease. Examples include: (1) Neonatal thymectomy causing autoimmune gastritis in BALB/c mice which is analogous to pernicious anaemia in humans (Toh *et al.*, 1993). (2) Non-obese diabetic (NOD) mice or BB rats spontaneously develop diabetes with similar symptoms to insulin-dependent diabetes mellitus (Murase *et al.*, 1990; Georgiou and Mandel, 1995). (3) Experimental autoimmune encephalomyelitis (EAE) can be induced following immunisation with myelin basic protein which produces a disease similar to multiple sclerosis (Swierkosz and Swanborg, 1977). (4) Collagen type II or mycobacterial extracts induce arthritis which is akin to rheumatoid arthritis (Stuart *et al.*, 1984). (5)

New Zealand Black mice spontaneously develop systemic lupus erythematosus similar to SLE of humans (Howie and Helyer, 1968). (6) Nephritis in rats occurs following exposure to $HgCl_2$ in the drinking water and is analogous to glomerulonephritis in humans (Karp *et al.*, 1991). Such models provide useful starting points to the mechanisms of autoimmune disease.

### 1.3.2 Experimental autoimmune gastritis

Autoimmune gastritis (AIG) can be induced in BALB/c mice by thymectomising these mice in the neonatal period (Nishizuka, 1982). The best time to induce autoimmune gastritis by thymectomy in mice is 2-4 days after birth. The disease can not be induced at day 0 or day 7 post birth (Taguchi *et al.*, 1990). Thymectomising mice 3 days after birth is the most common method to induce autoimmune gastritis and is termed 3dnTx mice. Transferring spleen cells from autoimmune gastritis diseased mice to nude syngenic mice induces the disease (Taguchi and Nishizuka, 1987). Further studies have shown that $CD4^+$ T cells of diseased mice are the key cellular element to induce autoimmune gastritis, as transfer of adult $CD4^+$ but not $CD8^+$ T cells to 3dnTx mice prevents 3dnTx mice from developing autoimmune gastritis (Fukuma *et al.*, 1988). The pathological changes include lymphocytic infliltration of the gastric mucosa, destruction of parietal and chief cells and the production of autoantibodies to parietal cells (Kojima *et al.*, 1980). These changes are also found in human pernicious anaemia.

Autoantibodies are directed towards the parietal cells, which are polarised epithelial cells, with the basal membranes oriented towards the lamina propria and the apical membranes facing the lumen of the gastric gland (Toh *et al.*, 1997). These parietal cells are specialised cells that secrete hydrochloric acid into the stomach. This acid secretion is performed by a membrane-bound enzyme termed gastric $H^+/K^+$-ATPase (also called proton pump) (Gleeson and Toh, 1991; Morley *et al.*, 1992). The $H^+/K^+$-ATPase consists of two major gastric membrane antigens, a 95 kDa protein, also defined as α-subunit and a 60-90 kDa glycoprotein, also defined as β-subunit (Toh *et al.*, 1990; Gleeson and Toh, 1991; Toh *et al.*, 1992). Transgenic mice that express the β-subunit of the $H^+/K^+$-ATPase in thymus fail to develop anti-parietal cell autoantibodies when mice are thymectomised 3 days after birth, thus providing evidence that $H^+/K^+$-ATPase is the autoantigen (Alderuccio *et al.*, 1993). Furthermore, immunisation of mice with $H^+/K^+$-ATPase dissolved in adjuvant results in the short term induction of autoimmune gastritis further supporting that $H^+/K^+$-ATPase is indeed the autoantigen of autoimmune gastritis (Scarff *et al.*, 1997). Prevention of AIG has been achieved by adoptive transfer of spleen cells from normal mice (Taguchi and Nishizuka, 1987) or intra-thymic injection of parietal cells (Nishio *et al.*, 1995).

### 1.3.3 Experimental diabetes

Type 1 diabetes mellitus is an autoimmune disease caused by autoantibodies against islet cells of pancreas and has been proposed to be mediated by Th1-like $CD4^+$ T cell immunity (Bradley *et al.*, 1992). Two stains of murine, the Bio-Breeding (BB) rat and nonobese diabetic (NOD) mouse, can develop this disease spontaneously. BB rats develop the type 1 diabetes with insulitis and autoantibodies against 64-kD β-cell antigen of the islet. Preventing BB rats from developing the disease can be achieved by treatment with anti-T-cell antibodies (Rossini *et al.*, 1985).

NOD mice begin to develop insulitis at the age of 5 to 8 weeks. At 6 to 7 months, 70% of females and 40% of males become diabetic (Rossini *et al.*, 1985). The disease can also be induced by transfering T cells from diabetic mice to newborn NOD mice (Bendelac *et al.*, 1989). Prevention and treatment of diabetes in NOD mice has been approached from two directions: non islet antigen-specific therapies and islet antigen-specific therapies. In the non-islet antigen-specific therapies, BCG vaccination to NOD mice (Harada *et al.*, 1990) or injection of complete Freund's adjuvant in NOD mice (Sadelain *et al.*, 1990) is efficient to prevent the onset of diabetes in NOD mice. In the islet antigen-specific therapies, complete prevention of diabetes is achieved by injecting syngeneic islets into the thymus of NOD mice at birth (Charlton *et al.*, 1994). Oral feeding of porcine insulin to nonobese mice can prevent the disease (Zhang *et al.*, 1991) as can intranasal administration of insulin peptide B: 9-23 (Daniel and Wegmann,

1996a; Daniel and Wegmann, 1996b) or glutamic acid decarboxylase (Schloot *et al.*, 1996).

### 1.3.4 Experimental allergic encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory disease of the central nervous system induced in laboratory animals by the injection of brain or spinal cord tissue, containing myelin basic protein (MBP) and proteolipid protein (PLP), with adjuvant. Paralysis begins 2-3 weeks after the hind legs are challenged with brain tissue. Injection of either MBP or PLP in normal mice is sufficient to induce EAE. Induction of EAE can also be achieved by transfering MBP-specific $CD4^+$ T cells into normal recipients (McDonald and Swanborg, 1988). MBP is a complex protein and contains many epitopes. Some of these epitopes are pathogenic, depending on the animals. For instance, human MBP peptide residues 114-122 are pathogenic to guinea pigs but not to rabbits (Samson and Smilek, 1995). Prevention and treatment of EAE has been reported in many ways. Oral feeding of PLP 139-151 peptide (Karpus *et al.*, 1996) or MBP (Brod *et al.*, 1991) prevent the EAE disease. Nasal administration of MBP also prevents the relapsing of EAE (Bai *et al.*, 1997). Suppression of EAE after clinical signs has also been reported by Racke and co-workers who showed that intraveneous injection of MBP twice daily, three times over 5 days, can suppress passively induced EAE (Racke *et al.*, 1994). Brocke and colleague used altered peptide of MBP injected intraperitoneally to EAE diseased animals and were able to suppress the disease (Brocke *et al.*, 1996).

### 1.3.5 Experimental arthritis

Induction of experimental arthritis can be achieved by immunising mice with collagen or adjuvant. In collagen-induced arthritis, immunisation of mice with native type II collagen induces an inflammatory polyarthritis within 20 days (Goldschmidt *et al.*, 1992). The disease can be induced in mice with a cocktail of monoclonal anticollagen antibodies. Further, the disease can be transferred from mice with collagen-induced to syngenic mice via their serum (Stuart *et al.*, 1984). In adjuvant arthritis, an injection of *Mycobacterium tuberculosis* in oil (complete Freund's adjuvant) causes transient inflammation of the joints. The main pathologic finding in the joints is granuloma formation. The lesion can be transferred to syngeneic animals by T cells from rats with the disease. Prevention of collagen-induced arthritis can be achieved by oral feeding of collagen II (Trentham *et al.*, 1993) or i.v. injection of its protein (Myers *et al.*, 1989) or peptide (Samson and Smilek, 1995).

### 1.3.6 Other experimental autoimmune diseases

Experimental nephritis can be induced by repeated injections of Brown Norway rats with mercuric chloride (Hinglais *et al.*, 1979). Repeated immunisation of mice with rat red blood cells results in the production of erythrocyte autoantibodies (Young and Hooper, 1993). Mice immunised with retinal antigen interphotoreceptor retinoid-binding protein induces experiment autoimmune uveoretinitis (Rizzo *et al.*, 1994). Experimental autoimmune neuritis can be induced in rats by treatment of bovine spinal

root myelin or neuritogenic P2-specific T cells (Gehrmann *et al.*, 1992). In addition, injection of viable syngenetic testicular germ cells can induce experimental autoimmune orchitis (Tokunaga *et al.*, 1993). Mice infected with cardiotropic virus coxsackievirus B3 lead to autoimmune myocarditis (Neumann *et al.*, 1994).

### 1.3.7 Summary

Experimental induced autoimmune diseases provide an ideal tool to look at the inside story of similar diseases in humans. The treatment method used in treating autoimmune diseased animals may be useful for applying to human treatments. However, different autoimmune diseases require different approaches for the treatment of autoimmune diseases. Some of the disease can be prevented before onset. The following section will discuss a possible new method, skin-induced tolerance, for applying to the treatment of autoimmune disease.

## 1.4. Skin and cutaneous tolerance

It has long been understood that immune tolerance can be induced via the skin. Application of antigen through chemical or physical light treated skin results in immunosuppression. Whether this skin-induced immunosuppression is suitable for the treatment of autoimmune disease requires answers. However, many facts which will be discussed below, may provide clues for the use of cutaneous tolerance induction as a possible treatment strategy for autoimmune disease.

### 1.4.1. Chemical substances and cutaneous tolerance

Chemical substances such as carcinogens and allergens can alter the skin environment. Application of carcinogens such as 7,12-dimethylbenz[a]anthracene (DMBA) can reduce the LC number in the skin as can contact allergens such as TNCB, FITC and 2,4-dinitrofluorobenzene (DNFB) (Woods *et al.*, 1996). Application of antigen through DMBA or TNCB-treated and LC-depleted skin results in immunological tolerance (Halliday and Muller, 1986; Halliday and Muller, 1987a; Halliday and Muller, 1987b; Woods *et al.*, 1996). More importantly the tolerance is transferable as spleen cells transferred from mice treated with DMBA/antigen or TNCB/antigen to syngeneic mice, induced tolerance in the recipient mice. The cutaneous tolerance generated is antigen specific as responses to different antigens remain unaltered. The induction of DMBA-induced cutaneous tolerance which acts on both cellular and humoral immunity; this has been demonstrated by Halliday and co-workers showing contact sensitivity and antibody production are both reduced (Halliday and Muller, 1986; Halliday and Muller, 1987b). Further, the specific tolerance induced by DMBA/antigen treatment is long lasting (Halliday and Muller, 1987b).

Possible mechanisms underlying the development of specific immune tolerance induced in DMBA/antigen treated mice have been studied. Halliday *et al.* (Halliday *et al.*, 1988) demonstrated that activation of suppressor cells may be the key element in DMBA/antigen induced tolerance. Cells that mediate the induction of suppressor cells in DMBA-

treated mice have been indicated as the DMBA-resistant, Ia-positive, Thy-1$^-$, IJ-restricted epidermal cell (Halliday *et al.*, 1990). When antigen is applied through normal skin containing LC a positive immune response resulted, whereas antigen applied through LC-depleted skin containing the DMBA-resistant, Ia-positive, Thy-1$^-$, IJ-restricted epidermal cell immunosuppression resulted. Thus LC depletion is an important indication of whether immune response results in activation or suppression.

DMBA treatment of the skin not only depletes LC but also affects their function. Study in sheep found that dendritic cells migrating after DMBA treatment had a reduced antigen-presenting function (Ragg *et al.*, 1995). Study in mice found that cluster formation between CD4$^+$ T cells and dendritic cells were reduced in DMBA-treated mice compared with normal controls (Chilcott, 1994). Analysis of the ability of DMBA-treated dendritic cells to induce apoptosis in T cells showed no changes between DMBA-DC incubated with normal T cells and Normal-DC incubated with T cells (Grzegorczyk, 1994). However, an *in vivo* study showed DMBA-DC could induce apoptosis to T cells indicating tolerance induction by DMBA treatment may in part be through apoptosis (Reed, 1996).

Further study on DMBA-treated mice revealed that DMBA did not alter IL-12 production of LC when compared with normal control (Malley, 1996). However, a decreased IL-1β production of draining dendritic cells after DMBA treatment has been observed in sheep (Ragg *et al.*, 1997).

Other chemicals can also induce an immunosuppressive effect when applied to the skin. The LC depletion remains the important indication of whether suppression results. The tumour promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) can deplete LC from the epidermis which leads to an impaired immune response whereas urethane, a tumour initiator does not impair LC numbers and function, and the immune response remains intact (Halliday *et al.*, 1987).

### 1.4.2 Ultraviolet light and cutaneous tolerance

Ultraviolet (UV) radiation is part of sunlight and is divided into three regions: UVA (320-400 nm), UVB (290-320 nm), and UVC (200-290 nm). UVB in particular has been directly implicated in the cause of human skin cancer. One of the reasons why skin tumours are induced by UVB irradiation is the association with an impaired skin immune function. In classical experiments, Kripke demonstrated that UVB-induced tumours grafted to syngenic normal mice were rejected. However, when these tumours were grafted to UVB irradiated syngeneic mice the tumour survived (Fisher and Kripke, 1976; Kripke, 1977; Kripke and Fisher, 1977). This indicated that UVB not only initiates skin tumours but has the ability to suppress immune functions.

Further assessment of UVB-induced immune suppression revealed that UVB irradiation could suppress contact hypersensitivity (CHS) and delayed-type hypersensitivity (DTH) responses and in some cases a humoral immune response. In clinical terms CHS is a common skin disease, presenting as allergic contact dermatitis. Contact hypersensitivity is a prototype of DTH which is a T-cell mediated reaction. However CHS and DTH are not exactly the same. CHS can be mediated by MHC class I-restricted $CD8^+$ cells and class II-restricted $CD4^+$ cells whereas DTH only can be mediated by MHC class II-restricted $CD4^+$ cells (Gocinski and Tigelaar, 1990). When mice are treated on the skin with UVB irradiation and then hapten is applied through this UVB-treated skin, CHS is not induced. These animals become specifically unresponsive to the chemical contact, and are unable to mount effective hypersensitivity reactions if presented subsequently with an immunogenic regimen (Streilein *et al.*, 1980; Toews *et al.*, 1980; Kripke and Morison, 1985). In addition, the murine CHS response can be suppressed by spleen cells or draining lymph node cells from UVB and hapten-treated mice. This suppression is due to the hapten-specific suppressor cells as transfer of UVB and other hapten-treated spleen cells could not suppress the normal CHS response (Elmets *et al.*, 1983).

Similar to CHS, the DTH response can also be impaired using the UVB regimen. When mice were irradiated and sensitised with large antigens such as sheep red blood cells (SRBC) or bovine serum albumin (BSA), the DTH responses were suppressed on subsequent challenge (Ullrich *et al.*, 1986a).

This was due to antigen-specific suppressor cells as spleen cells of immune suppressed mice transferred to recipients suppress the DTH response on subsequent antigen application (Ullrich *et al.*, 1986a).

Most of the evidence indicates that UVB irradiation selectively suppresses the cell-medicated part of the immune response (Brissert and Granstein, 1997). However, there are some reports suggesting that humoral immunity can also be suppressed. Spellman and co-workers showed that in naive mice, application of human immunoglobulin G (HIg) through UVB irradiated skin failed to reduce anti-HIg antibodies whereas in HIg immunised mice, a normal humoral immune response was evident (Spellman *et al.*, 1984). In addition, it has been shown that when mice were treated with UVB irradiation followed by hapten application, plaque-forming cells to the hapten were significantly reduced (Ullrich *et al.*, 1986b). Recently, Brown and colleagues showed that the reduction of antibody production by UVB irradiated is selective. They found that *Borrelia burgdorferi* immunization to UVB irradiated mice reduced IgG2a and IgG2b antibody production but not IgG1 antibody production (Brown *et al.*, 1995).

The relationship of the dose of UVB and the volume of hapten applied to the UVB irradiated skin is critical in the induction of tolerance. A study using C3H/HeN mice showed that UVB-induced local immune suppression was related to the dose of UVB, the divided exposure of UVB, the hapten concentration, the volume of the hapten, and the time course of UVB

irradiation in the suppression of CHS (Miyauchi and Horio, 1995). The strain of mice used in the experiment also effects the degree of UV-induced tolerance (Streilein and Bergstresser, 1988).

Mechanisms involved in UV-induced immune suppression are complex. For example, in mice, UV radiation is able to convert LC from potent antigen-presenting cells to suppressive antigen-presenting cells (Simon *et al.*, 1991), Thy-1$^+$ epidermal cells have been documented as involved in the induction of UV-induced immunosuppression (Sullivan *et al.*, 1986), Epidermal I-J$^+$, Ia$^+$ and Thy-1$^-$ cells have been suggested as the important cells in the UV-induced suppressor T cell generation (Granstein, 1985), CD4$^+$ T cells have also been demonstrated as the suppressor cells induced after UVB irradiation followed by hapten application. These CD4$^+$ T cells belong to the Th2 subset (Elmets *et al.*, 1983; Simon *et al.*, 1990). In humans, Granstein *et al.* showed that after UV irradiation the CD4$^+$CD45RA$^+$ suppressor-inducer T cells were responsible for the induction of immunologic tolerance and this only occurred when human skin was irradiated with UVB or UVC (Cooper *et al.*, 1985; Baadsgaard *et al.*, 1987).

The chemical mediators of UVB-induced tolerance include cytokines, cis-urocanic and histamine, and other agents. The most studied cytokine in UVB-induced cutaneous tolerance is IL-10. It has been shown that *in vivo* hapten-specific tolerance induced by UVB is mediated by IL-10 (Enk *et al.*, 1994) and this is due to the inhibition of LC antigen-presenting function

(Enk *et al.*, 1993). TNFα has also been shown to play a role in the UVB irradiation-induced immune suppression (Rivas and Ullrich, 1994). Injection of TNF-α intradermally mimicked the effects of UVB irradiation in regulating immune responses against contact sensitisers in mice. TNF-α is reported to facilitate LC migration and thus neutralizing the production of TNF-α results in immune suppression (Moodycliffe *et al.*, 1994). IL-4 is another cytokine participating in the UVB-induced suppression. When transferring suppressor cells of UVB irradiated mice in combination with antibodies to IL-4, all immunosuppressive activity was abrogated (Rivas and Ullrich, 1994). It has been suggested that because the cytokines produced after UVB irradiation were Th2 cytokines the suppressor cells may be Th2-like cells (Ullrich, 1995b). As IL-12 is critical in the induction of immune response, UV irradiation can reduce the production of IL-12 indicating UV-induced suppression may be due to insufficient IL-12 production (Schwarz *et al.*, 1996). IL-1α and IL-1β are also critical in the activation of the immune response; reduction of these cytokine also leads to immune suppression (Enk *et al.*, 1993; Grabbe *et al.*, 1994).

Urocanic acid is another molecule that takes part in UVB-induced immunosuppression. It is a product of histidine and is abundant in the upper layer of the epidermis. Histidine is the precusor of both trans-urocanic acid and histamine. Irradiation of the skin with UVB causes isomerisation of trans-urocanic acid to cis-urocanic acid. The cis-urocanic acid is

immunosuppressive (De Fabo and Noonan, 1983; Noonan and De Fabo, 1992). This can be demonstrated by local injection of cis-urocanic acid to the epidermis which decreases the number of LC (Kurimoto and Streilein, 1992) when mice are treated with cis-urocanic acid their splenic antigen-presenting cells function are affected (Noonan *et al.*, 1988). Injection of cis-urocanic acid has also been shown to extend the acceptance of heart grafted animal (Noonan and De Fabo, 1992). Histamine, another product of histidine in the epidermis also behaves like cis-urocanic acid to inhibit CHS responses (Hart *et al.*, 1997). Investigation of how cis-urocanic acid or histamine induce immune suppression revealed that both molecules can induce $PGE_2$ production suggesting the $PGE_2$ plays a role in the cis-urocanic acid induced immunosuppression (Jaksic *et al.*, 1995). Studies by Kurimoto and Streilein found that cis-urocanic acid can increase TNF-α secretion from the epidermis, therefore, preventing LC migration and resulting in immunosuppression (Kurimoto and Streilein, 1992).

Other agents are also involved in the mediation of UV-induced immunsuppression. UVB has the ability to directly mutate DNA to form pyrimidine dimers. This leads to the concept that DNA damage may be the result of UVB-induced tolerance. Study by Kripke and colleagues domonstrated that pyrimidine dimers in DNA were involved in the initiation of systemic immunosuppression in UV-irradiated mice as application of liposomes which repair DNA damage, together with UVB irradiation prevents the induction of immunosuppression (Kripke *et al.*,

1992). Aloe barbadensis gel extract also prevents UVB irradiation-induced immunosuppression but this prevention is not due to the repair of DNA damage (Strickland *et al.*, 1994). Serum from UVB irradiated mice is also reported to have the ability to induce immunosuppression (Harriott Smith and Halliday, 1988). Supernatants from irradiated keratinocyte cell line also contain immunosuppressive ability and this has since been identified as IL-10 (Ullrich, 1995a)

## 1.5 Methods of tolerance induction

Other methods to induce experimental tolerance can be achieved by oral, respiratory, eye chamber, and intrathymic or intravenious injection of antigens or antibodies. Such methods have been reported to treat established or autoimmune responses (Kosiewicz *et al.*, 1994; Liblau *et al.*, 1997; Weiner, 1997a). However, one thing in common with cutaneous tolerance is all the methods used to induce specific tolerance require specific antigen and involve participation of antigen-presenting cells. For example, oral feeding of antigen can induce antigen specific immunosuppression. This tolerance induction involves antigen and APC as demonstrated by Liu *et al.* (1993) who showed that intestinal dendritic cells can process and present antigen from intestinally fed antigen leading to tolerance. Respiratory tolerance is another form of experimental tolerance which utilises inhalation or nasal challenge of antigen to generate specific tolerance. It has been demonstrated that antigen-presenting alveolar macrophages are responsible for the induction of tolerance in animals (Thepen *et al.*, 1991). Nasal challenge of

antigen can also lead to tolerance via Th2 cytokine production and this may be related to the nasal mucosa dendritic cell (Tian *et al.*, 1996).

Induction of tolerance via the anterior chamber of the eye has been shown to suppress the immune response if soluble antigen is applied through the eye chamber before (Williamson and Streilein, 1989) and after antigen application (Kosiewicz *et al.*, 1994). This method of induced tolerance also appears to involve APC. To support this view Steptoe and colleague, showed that MHC II-positive dendritic cells migrated from the eye chamber after antigen treatment (Steptoe and Thomson, 1996). Suppressor cells have been isolated after this treatment (Wilbanks and Streilein, 1990) suggesting a strong link between APC and suppressor cells in eye tolerance.

Induction of tolerance can be achieved via intrathymic injection (Staples *et al.*, 1966; Ellison and Waksman, 1970). Oluwole and co-workers demonstrated that the mechanism of this tolerance was associated with thymic DC (Oluwole *et al.*, 1995). Intraveneous injection of antigen can also induce tolerance and this form of immunosuppression has been suggested to involve blood dendritic cells (Liblau *et al.*, 1996; Liblau *et al.*, 1997).

## 1.6 Summary

An immune response driven by antigen towards a full immune response or a suppressed immunity is dependant on the quantity and quality of antigen application as well as where the antigen is applied. Utilizing skin

as the tolerance-driven organ, requires deletion of LC in the epidermis. This has been demonstrated in chemically and UVB manipulated skin. Other methods of induced tolerance also require certain conditions in the treated organ.

Cutaneous tolerance has been used mainly for allegic dermatitis, unlike other forms of tolerance induction which have been used to treat established immunity as well as autoimmune diseases. It is of interest that cutaneous tolerance may be an effective method for the treatment of unwanted immune response. So far cutaneous tolerance has been shown to prevent induction of contact sensitivity however the use of cutaneous tolerance to suppress established immunity has yet to be fully examined. This thesis will examine the ability of cutaneous tolerance to downregulate established immunity as a possible treatment for autoimmune disease.

# Chapter Two
# Materials and Methods

## 2.1 Animals

BALB/c mice were obtained from the Central Animal House, University of Tasmania. All mice were used with the approval of the Animal Ethics Committee of the University of Tasmania (permit number 94049). BALB/c mice were housed in the Animal House, Division of Pathology with free access to water and food. The nu/nu BALB/c mice were purchased from Animal Resources Centre, Western Australia and housed in special designed cages (Model AS 980, Able Scientific, West Australia) in the Division of Pathology with free access to sterilised water and food.

## 2.2 Reagents

### 2.2.1 Solutions

#### 2.2.1.1 Phosphate Buffered Saline (PBS)

16.00g NaCl
7.30g $Na_2PO_4 \cdot 12H_2O$
0.40g $KH_2PO_4$
0.40g KCl
Dissolved in 2 litres of Milli-Q water and autoclaved.

#### 2.2.1.2 Glucose-Phosphate Buffer Saline (G-PBS)

I) 1.0 M Phosphate Saline
a). 1.0 M $KH_2PO_4$: 136.1 g/liter
b). 1.0 M Na2HPO4.2H2O: 156.01g/liter.
Combine 910 ml of 1.0 M $Na_2HPO_4 \cdot 2H_2O$ and 90 ml of 1.0 M $KH_2PO_4$.
II) Saline and Glucose
a). 0.14 M NaCl: 8.2g/litre--1000 ml

b). D-Form Glucose--10g
Combine (a) and (b).
All solutions were prepared in Milli-Q water.
10 ml of solution (I) was added to 1 litre of solution (II) and sterilised by filtration through 0.22 µm millipore filters.

### 2.2.1.3 10% FCS in G-PBS

10ml of fetal calf serum (FCS Commonwealth Serum laboratories, Australia) was added to 90ml G-PBS.

### 2.2.1.4 0.28 M Cacodylate Buffer pH 6.9

39g cacodylic acid sodium ($C_2H_6AsO_2Na$) (Sigma, U.S.A.) was dissolve in 900 ml distilled water. pH was adjusted to 6.9 with 2 M HCl (73g/liter). Volume adjusted to 1000 ml with Milli-Q water and sterilised through 0.22 µm millipore filters.

### 2.2.1.5 EDTA/PBS/0.001% trypsin

7.2g EDTA $Na_4$ salt
0.001g trypsin (BDH, Product 39041, Australia)
Volume made to 1 litre

### 2.2.1.6 0.25% trypsin

0.25g of trypsin was added to 100ml G-PBS.

### 2.2.1.7 RPMI medium

104g of RPMI powder (ICN Biomedicals, INC., U.S.A. Cat. Number 10-601-22) was added to 10 litres of Milli-Q water and sterilised through 0.22 µm millipore filters.

### 2.2.1.8 10%FCS/RPMI

100ml Fetal Calf Serum (FCS Commonwealth Serum Laboratories, Australia) was added to 1 litre RPMI medium.

100ml 100X Vitamins (Flow Laboratories) and 100mg L-Glutamine (Sigma, U.S.A.) was added to 1 litre RPMI medium.

### 2.2.1.9 PBS + 2%BSA + 0.2% Azide

1 litre PBS
20g crude bovine serum albumin (BSA) (Commonwealth Serum Laboratories)
2g $NaN_3$ (BDH, Australia)
20g of BSA and 2g of $NaN_3$ was floated on the top of PBS (1 litre) and allowed to gently settle into solution until all the BSA dissolved and sterilised through 0.22 μm Millipore filters. This solution could not be shaken or stirred as this would result in the formation of large sticky lumps of BSA.

### 2.2.1.10 FACS Fixative

500ml PBS
10g glucose (Ajax Chemicals)
1.09ml 15% $NaN_3$
13ml 40% formaldehyde
Add above together

### 2.2.1.11 Glycerin Jelly

10g gelatin
60ml Milli-Q water
70ml glycerin
0.25g phenol
Dissolve the gelatin in distilled water using gentle heat. Add the glycerin and phenol, mix well. For use, melt in water bath at 56-60$^0$ C, avoid shaking to speed this process otherwise mountant will be full of air bubbles.

### 2.2.1.12 Trypan Blue

0.25g Trypan blue powder (Chroma-Gesellschaft, Schmid & Co. German) was added to 100ml of Milli-Q water and sterilised through 0.22 μm millipore filters.

### 2.2.2 Contact sensitisers

(1) Picryl Chloride or 2,4,6-trinitrochlorobenzene (TNCB): Tokyo-Kasei, Japan, Cat. Number C0307.
(2) Picryl Sulfonic Acid Sodium Salt or 2,4,6-trinitrochlorobenzenesulfonic acid (TNBS): Tokyo-Kasei, Japan, Cat. Number FC005.
(3) 2,4-dinitrofluorobenzene (DNFB): Sigma Chemical Company, U.S.A., Cat. Number F-7250.

### 2.2.3 Antigens

(1) Gastric $H^+/K^+$ ATPase (Proton Pump): The proton pump was provided by my collaborator Prof. Toh of Monash University Australia. Mouse and pig gastric $H^+/K^+$ ATPase was purified from gastric membranes by tomato-lectin affinity chromatography (Callaghan *et al.*, 1992).

(2) Peptide 19: The peptide 19 of proton pump was purchased from Dr Cenk Suphioglu of the Melbourne University, Australia. Sequence: LLNVPKNMQVSIVCKILADHVTFNN

### 2.2.3 Carcinogens

(1) 7,12, dimethylbenz(a)anthracene (DMBA): Sigma Chemical Company, U.S.A., Cat. Number D-3254.

### 2.2.4 Vehicle

(1) Acetone: BDH, Australia.
(2) Olive oil: Bertolli, Italy.

### 2.2.5 Antibodies

(1) TIB 120: Cultured hybridoma supernatant, anti-mouse I-A, I-E (American Type Culture Collection) (Davignon *et al.*, 1981; Bhattacharya *et al.*, 1981).
(2) Goat anti - rat Ig - peroxidase conjugate: Southern Biotechnology Associates Inc., Birmingham, USA., Cat E295-W365D.
(3) Anti-mouse immunoglobulin $F(ab)_2$ fraction affinity isolated fluorescein conjugated: Silenus Laboratories Victoria, Australia, Cat 5023DDAF0591.
(4) Sheep anti-mouse immunoglobulin stabilised serum: Silenus Laboratories Victoria, Australia, Cat. Number 5034DS0591.

### 2.2.6 Others

(1) Veet Hair Remover: Reckitt and Colman Pharmaceuticals, Australia
(2) Diaminobenzidine tetrahydrochloride (DAB): Sigma Chemical Company, U.S.A., Cat. Number Ki390.

## 2.3 General methods (methods used in two or more chapters)

### 2.3.1 Sensitisation of mice

Immune mice were prepared by immunising adult mice with two applications of the hapten 2,4,6-trinitrochlorobenzene (TNCB) (dissolved in a 4:1 solution of acetone: olive oil) at days 0 and 7 by applying 100µl of 0.5% or 2%TNCB to the shaved dorsal trunk or abdominal skin.

### 2.3.2 DMBA treatment

Adult mice were treated with 100µl of 1% DMBA dissolved in acetone to the shaved dorsal skin.

### 2.3.3 Assessment of contact sensitivity

Contact sensitivity was elicited by applying 20 µl of sensitising agent (0.5% TNCB in 4:1 acetone-olive oil ) to the dorsal surface of the right ear, 5 days following the final treatment (eg. TNCB applied through DMBA-treated skin). The contact sensitivity to the TNCB appeared as an increase in ear thickness. The thickness of the challenged and unchallenged ears was measured 24 or 48 hr later with an engineer's spring-loaded micrometer. The contact sensitivity response was determined by measuring the increase

in ear thickness and expressed as a percentage increase as calculated by the formula:

$$\frac{\text{thickness of challenged ear - thickness of unchallenged ear}}{\text{thickness of unchallenged ear}} \times 100.$$

One exception of the measurement was the UVB study of chapter 4. In chapter 4, the ear thickness before and after challenge was measured. The contact sensitivity response was expressed as a percentage increase in ear thickness as calculated by the formula:

$$\frac{\text{ear thickness after ear challenge - ear thickness before ear challenge}}{\text{ear thickness before ear challenge}} \times 100.$$

**2.3.4 Measurement of anti-TNP antibodies**

Two hours following ear measurement the mice were bled by cardiac puncture while under Halothane (Fluothane, ICI Pharmaceuticals, Melbourne, Australia) anaesthesia. The blood was allowed to clot for 1 hour at 37°C and left overnight at 4°C before the serum was separated and stored at -20°C. Specific anti-trinitrophenyl (anti-TNP) antibodies formed in response to sensitisation with TNCB were measured using an indirect haemagglutination technique based on procedures described by Mishell (1980). Trinitrophenylated derivatives of sheep red blood cells (SRBC) were

prepared by reacting SRBC with 2,4,6-trinitrobenzenesulfonic (TNBS). Fresh SRBC were washed in PBS and 300 µl was added to 2.1 ml TNBS-cacodylate buffer (pH 6.9) and incubated at room temperature for 30 minutes. The TNBS-cacodylate buffer was prepared by dissolving 0.075g TNBS in 4 ml cacodylate buffer and centrifuging at 3000rpm for 15 minutes and the upper 2.1 ml of TNBS-cacodylate buffer was collected. The SRBC and TNBS-cacodylate buffer mixture was mixed for 30 minutes and washed 4 times in cold PBS buffer containing 2% w/v glucose. Finally, TNBS conjugated SRBC (TNP-SRBC) were resuspended at 2% in Phosphate Buffered Saline solution. At all times the tubes were wrapped in aluminum foil to prevent photodecomposition of TNBS. For indirect haemagglutination, 96 well round bottom plates were used and 25µl serum samples were added in doubling dilution to the wells of a 96 well round bottom plate. A total of 25 µl of 2% TNP-SRBC was then added to each well and the plate incubated at 37°C for 45 minutes. Plates were washed once at 400g for 7 minutes. Unbound serum was removed by washing the well with 50µl PBS, the plates were centrifuged at 400g for 7 mins and the supernatant carefully removed. In each well 50 µl sheep anti-mouse IgG diluted 1:500 with PBS was added to the washed pellet and mixed by repeated pipetting. Plates were incubated overnight at room temperature.

### 2.3.5 Spleen cell preparation and adoptive transfer

Mice from the spleen cells transfer experiments were sacrificed by inhalation of Halothane anaesthesia and their spleens were removed. A

single cell suspension was prepared by gently teasing the spleen through 80 mesh stainless-steel sieve into RPMI medium (HyClone U.S.A.). The cell suspension was collected into 10 ml centrifugation tubes and centrifuged at 400g for 10 minutes, and the supernantant discarded. The cells were then washed twice in RPMI medium by centrifugation. The cell viability was estimated by their exclusion of trypan blue. The number of viable lymphocytes was counted using a haemocytometer. The concentration of the spleen cells was adjusted to 1 X $10^8$/ml. Approximately 5 X $10^7$ viable cells were injected into tail vein of syngeneic host mice.

### 2.3.6 Epidermal LC identification

#### 2.3.6.1 Preparation of epidermal sheets

The dorsal area of the mice was shaved with animal clippers, and mice were then sacrificed by $CO_2$ in air asphyxiation. The remaining hair was removed with a chemical depilitant containing thioglycollate (Veet Hair Remover). This was rubbed into the coat of the animal and left for 10 minutes. The dorsal area was then washed in cold water and the skin rubbed dry. The keratin layer was removed by applying good quality cellotape to the shaved skin and then stripping it off until the skin glistened. A fresh strip of cellotape was applied to the shaved skin and the area dissected from the animal.

The skin/tape combination was then cut into approximately 1cm$^2$ segments, placed with the dermis side down into wells containing 20mM EDTA/PBS/0.001% trypsin and incubated at 37°C for 2 hours with slight agitation (Baker and Habowsky, 1983). The dermis was separated from the epidermis with fine forceps using a dissecting microscope. The epidermis was left attached to the sticky tape. The epidermal sheets were then fixed in acetone for 10 -15 minutes at room temperature.

### 2.3.6.2 Identification of Langerhans Cells

The sheets were washed three times in PBS with the shaking epidermis facing downward and then incubated with 200µl of undiluted TIB 120 hybridoma supernatant, to identify $Ia^+$ cells at 37°C with slight agitation for 2 hours and then incubated at 4°C overnight. The sheets were washed in PBS three times and incubated in goat anti - rat Ig - peroxidase conjugate (1/100 ) for two hours at 37°C. After washing three times with PBS staining was then visualised with diaminobenzidine tetrahydrochloride, by incubation at room temperature for 5 - 10 minutes. The sheets were then washed three times with MilliQ water and mounted in glycerin jelly on clean glass slides. The slides were stored at 4°C when not being used.

### 2.3.6.3 Dendritic cell count

Cells were counted by means of an image analyser using the NIH Image 1.6 program on a Macintosh Power PC computer. Cell counts were calculated from epidermal sheets stained with the TIB 120 monoclonal antibodies at 40 times magnification. Six fields were counted and the number of cells per $mm^2$ was then calculated. The mean count for each age group was then calculated.

### 2.3.7 Detection of autoantibody production of autoimmune gastritis

Normal BALB/c mice were sacrificed and their stomachs dissected. Approximately 1mm strips of the fundus of the stomachs were frozen by being placed on aluminum foil to float on a 2-Methyl butane (BDH Laboratory Supplies. Poole, England) and liquid nitrogen (Union Carbide, Australia) solution. Frozen sections of approximately 6µm thickness were then mounted on aquadhere slides. Serum was removed from the tail blood samples after spinning in a microcentrifuge at 12,000 rpm for 30 seconds. The tail blood serum was then double diluted in PBS commencing with a 1/8 dilution to a 1/ 2024 dilution. Serum dilutions (10µl per section) were then incubated on the stomach section slides for thirty minutes at room temperature. The slides were then gently washed twice with PBS and slightly agitated with PBS on the slide for five minutes after each wash.

10µl of anti-mouse immunoglobulin conjugated with fluorescein isothiocyanate (1 : 80) was added to each slide and incubated for 30 minutes at room temperature. The slides were washed as previously with PBS. Slides were then viewed using a fluorescence microscope and the degree of fluorescence was assessed and scored when compared with positive and negative controls, strongly positive (score of three) and negative (score of 0). The titre was then determined as the last dilution at which a positive reaction could be scored.

### 2.3.8 Histological assessment

After the end point of each experiment, the mice were sacrificed and their stomachs dissected. The fundus of each stomach had a strip of approximately 1mm removed, which was fixed in formalin solution. Sections of this part of the stomach were mounted on clean glass slides and stained by means of H&E staining and sectioned to determine the histological presence of disease. The sections were viewed at 10X and 20X magnification and were allocated a score for the degree of atrophy and inflammatory cellular infiltration present in each section. A score of 3 was given to those stomachs with the greatest degree of gastric atrophy (loss of parietal cells) and inflammatory cellular infiltration and a score of 0 to those stomachs with normal gastric mucosa and minimal cellular infiltration. The stomach of each mouse was scored according to the extent of the presence or absence of these parameters.

### 2.3.9 Neonatal thymectomy

Normal BALB/c (H-$2^d$) mice were thymectomised on day 3 (day of birth designated as day 0) (3dnTx) under cold anaesthesia. The sternum was opened at the midline and after exposing the thymus, each lobe was aspirated with a Pasteur pipette attached to a vacuum. These mice were checked for the remaining thymus using the fine forceps. If any residual thymus was detected it was removed by forceps. Mice were then warmed with a 60 watt lamp and returned to their mother.

### 2.3.10 Statistical analysis

An unpaired Student's t-test was performed to analyse experimental data. Macintosh computer Statview (Version 1.1 Abacus Concepts, Inc.) program was used for this analysis.

## 2.4 Specific methods (methods used only in one chapter indicated)

### 2.4.1 Methods used in chapter 3

#### 2.4.1.1 Downregulation of established contact response

TNCB immune mice were treated with 100µl 1% DMBA on shaved dorsal skin. Five days later, 100µl of 0.5% TNCB was applied to the DMBA-treated dorsal trunk of each mouse. For testing the specificity of immune suppression, the same procedure was carried out using 0.5% DNFB. A further 5 days later the mice were ear challenged and contact sensitivity was determined.

### 2.4.1.2 Spleen cell transfer

1% DMBA or acetone was applied onto the dorsal trunk of naive adult mice and a further 5 days later, 100µl of 0.5% TNCB was applied to the same site. Five days later, spleens of these mice were removed and adoptively transferred via the tail vein to TNCB immune mice. For examining the specificity of immunosuppression, 0.5% DNFB was used instead of 0.5% TNCB.

### 2.4.1.3 Epidermal cell preparation

The dorsal skin of the mice was shaved using an electric clipper and the remaining fur removed by chemical depilitant (Veet; Ricketts and Colman Ltd, U.K.). The keratin layer was removed by repeated attachment and removal of cellophane until the skin was shiny. Treated skin was excised and floated dermal side down in 0.25% trypsin dissolved in G-PBS for 18 hours at 4° C. After 18 hours, fetal calf serum (FCS; Commonwealth Serum Laboratories, Melbourne, Australia) was added (final concentration of 10%) to neutralise the trypsin. Epidermal cells were obtained by using a glass rod to vigorously rub the skin surface. The epidermal cells removed by this method were washed once in G-PBS containing 10% FCS and once in G-PBS by centrifugation at 400g for 7 minutes. The viability was assessed by trypan blue exclusion.

### 2.4.1.4 Conjugation of epidermal cells with TNCB

ECs were incubated at 37° C in a fully humidified atmosphere with 5% $CO_2$ in air. One hour later the cells were washed twice in G-PBS. These epidermal cells were mixed with to an equal volume 20mM 2,4,6-trinitrobenzenesulphonic acid for 15 minutes at 37° C. Cells were then washed twice in G-PBS with 10% FCS. The final concentrations of ECs was adjusted to $1 \times 10^5$/ml or $1 \times 10^7$/ml.

Cells prepared by both these methods were subsequently injected into immunised mice. No less than $1 \times 10^4$ cells was subcutaneously injected into each mouse or $1 \times 10^6$ cells was intravenously injected into each mouse.

### 2.4.1.5 Transfer of epidermal cells treated with DMBA followed by TNCB

ECs from mice treated with either 100µl 1% DMBA followed by TNCB or either 100µl acetone followed by TNCB were prepared (section 2.4.1.3). The final concentrations of ECs was adjusted to $1 \times 10^5$/ml or $1 \times 10^7$/ml.

Cells prepared by both these methods were subsequently injected into immunised mice. No less than $1 \times 10^4$ cells was subcutaneously injected into each mouse or $1 \times 10^6$ cells was intravenously injected into each mouse.

### 2.4.2 Methods used in chapter 4

#### 2.4.2.1 UVB irradiation protocols

For treatment with UVB, the dorsal trunk was shaved and the mice were anaesthetised by intraperitoneal injection of 600μg pentobarbital sodium (Nembutal, Boehringer Ingelheim, Australia) diluted 1: 10 in 0.9% sodium chloride. Irradiation was carried out using a bank of six FS40 sun lamps (UVS Ultraviolet, Scoresby, Victoria, Australia) with high efficiency silverlux reflective film (3M Australia Pty Ltd) behind the lamps. Lamps were prewarmed for at least fifteen minutes to stabilise output. The UV light emitted by these lamps was within the range of 280 nm to 400 nm with a major emission peak at 313 nm. UVB (280 nm to 320 nm) output was 22.4 watts/$m^2$ representing approximately 64% of total UV light emission. UV dose was measured using a IL-1700 research radiometer (International Light, Newburyport M.A., U.S.A.). Mice were irradiated at a distance of 15 cm from the light source and were exposed to either 1, 3, 7 or 13 kJ/$m^2$ of UVB irradiation. In the study of LC density, the TNCB immune mice were exposed to 3, 7, 10 or 13 kJ/$m^2$. In multi exposure experiments, mice were exposed to three doses of 7 kJ/$m^2$. In all irradiation protocols the ears of the mice were protected from the UVB source by shielding with aluminum foil.

### 2.4.3 Methods used in chapter 5

#### 2.4.3.1 Lymphocyte proliferation assay

Lymph node (axillary, brachial and inguinal) cell suspensions were prepared by pressing the lymph nodes through nylon mesh. The cells were washed twice in RPMI culture medium (HyClone, U.S.A.), resuspended at $1x10^6$ cell/ml in complete RPMI medium containing 10% fetal calf serum, Gentamicin (150µg/ml), L-glutamine (2mM) (CSL, Melbourne, Australia), 2-mercaptoethanol (SERVA, New York) and placed into 96-well round-bottom microtitre plates (Sarstedt-Group, Adelaide, Australia) in a final volume of 200µl which contained doubling dilutions of PHA from 50µg/ml to 1µg/ml. The cultures were incubated for 3 days at $37^0$C, 5% $CO_2$ and 1µCi/well of methyl [$^3$H] thymidine (specific activity 2 Ci/mMol; Amersham Life Sciences, Australia) was added for the last 6 hours. Cultures were set up in triplicate and harvested on an automatic cell harvester (Skatron) and cellular incorporation of [$^3$H] thymidine counted using a liquid scintillation counter (LKB Wallac) and expressed as counts per minute (c.p.m).

#### 2.4.3.2 Flow cytometry

Lymphocytes ($1 X 10^6$) from lymph nodes of 3dnTx mice, at 6-8 weeks of age, were incubated on ice for 30 minutes with either biotinylated anti-mouse CD4 (Clone RM4-5, PharMingen), biotinylated anti-mouse CD8 (Clone 53-6.7, PharMingen) for T cell identification or phycoerythrin conjugated anti-mouse B220 (Clone RA3-6B2 , PharMingen) for B cell

identification. After washing, the B220 labeled cells were immediately prepared for flow cytometry whereas streptavidin-phycoerythrin (Code No. R0438, DAKO A/S, Denmark) was added to the biotinylated CD4 and CD8 preparations and incubated on ice for a further 30 minutes. Cells were analysed on a Coulter EPICS Elite ESP flow cytometer equipped with a Coherent Innova 90 argon ion laser and analysed with the ELITE workstation software.

#### 2.4.3.3 Induction of immunosuppression in 3dnTx mice and nu/nu mice

Groups of age matched 3dnTx mice (TNCB immune or non-immune) or nu/nu adult mice were treated on the dorsal skin with 100µl 1% DMBA. Five days later 100µl 2% TNCB was applied through DMBA-treated skin. The control groups were treated with acetone instead of DMBA. For antigen specificity test, DNFB was used instead of TNCB in the DMBA-treated group. Groups of 3dnTx mice were separated into autoimmune gastritis positive and negative groups.

#### 2.4.3.4 Adoptive transfer of spleen cells from thymectomised and nu/nu mice

Thymectomised or nu/nu mice were shaved and treated with 1% DMBA in acetone. Five days later, 2% TNCB was applied through the DMBA-treated skin. A further five days later, spleens from the DMBA-TNCB-treated mice were removed and a single cell suspension prepared in RPMI medium containing 10% FCS. These cells were washed 3 times with

RPMI and 5 x $10^7$ viable cells, as determined by trypan blue exclusion, were injected into naive mice. Control mice received spleen cells from acetone-TNCB-treated mice. For the antigen specific control 2,4-dinitrofluorobenzene (DNFB), instead of TNCB, was applied through the treated skin and the spleen cells were transferred as above. Suppressor spleen cells were detected by their ability, following adoptive transfer, to suppress the host response to TNCB sensitisation.

### 2.4.4 Methods used in chapter 6

#### 2.4.4.1 Treatment of autoimmune gastritis (AIG)

Mice thymectomised 3 days with AIG (12-15 weeks old) were treated with 1% DMBA followed by $H^+K^+$-ATPase (50µg per mouse) or peptide 19 (50µg per mouse) dissolved in PBS applied through the DMBA-treated area. Control groups were treated with DMBA alone or Acetone followed by $H^+K^+$-ATPase (50µg per mouse). Mice were bleed at day 35, and 50. For one experiment the DMBA was replaced by 2% TNCB followed by peptide 19 application.

#### 2.4.4.2 Prevention of autoimmune gastritis (AIG)

Four day old 3dnTx mice were treated with peptide 19 (10µg per mouse) dissolved in 2% TNCB. Control groups were 2% TNCB alone. The solvent alone (acetone) and peptide 19 (10µg per mouse) dissolved in acetone groups were also assessed.

# Chapter Three
# Downregulation of Established Immune Response by Chemical Carcinogen

## 3.1 Introduction

Skin antigen-presenting cells, the Langerhans cells (LC), play an important role in the activation of naive T cells (Silberberg *et al.*, 1976). Loss of LC from the skin impairs the skin immune system's ability to mount an immune response against contact sensitisers and to detect unwanted cells such as tumour cells (Muller *et al.*, 1997). Further, the application of hapten or antigen through LC-depleted skin results in the induction of antigen specific tolerance (Spellman *et al.*, 1984; Halliday and Muller, 1986). For example, the chemical carcinogen 7, 12-dimethylbenz(a)anthracene (DMBA), which has been shown to deplete LC in the epidermis, establishes an environment in which the application of a contact sensitiser through the treated skin results in the induction of immunosuppression to the contact sensitiser (Halliday and Muller, 1986). High concentrations of 2,4,6-trinitrochlorobenzene (TNCB) also deplete LC from the skin. As with DMBA-mediated LC depletion, when haptens, such as DNFB, are applied through TNCB-treated skin, tolerance rather than immunity to DNFB is induced (Woods *et al.*, 1996). The tolerance induced by applying antigen through LC-depleted skin is antigen specific.

It has been proposed that this antigen specific suppression was due to antigen specific suppressor cells, as the transfer of spleen cells from hapten

tolerant mice to naive mice suppressed the development of an immune response when the naive mice were subsequently challenged with the same hapten (Halliday and Muller, 1987b). It would appear that the spleen contained antigen specific cells that were important 'effector' cells, that is, they were able to prevent the induction of an immune response at the effector stage. Further analysis at the inducer stage of tolerance induction revealed that the residual antigen-presenting cells were unable to induce immunity as naive mice immunised with epidermal cells harvested from DMBA-treated skin and pulsed with hapten failed to generate an immune response to the same hapten (Halliday *et al.*, 1988). These cells induced antigen specific suppression as the immune response to other antigens was unaffected. Consequently, antigen specific tolerance can be divided into the induction phase, which is due to antigen presentation by aberrant epidermal cells and at the effect phase, due to antigen specific cells present in the spleen.

The prevention of contact sensitivity development by pretreatment of murine skin with an LC-depleting agent such as DMBA has been clearly established. However the work so far has concentrated on prevention, rather than reversal of an established contact sensitivity response. The ability to downregulate an established, and deleterious immune response has obvious important clinical implications. Consequently this chapter evaluates the potential for manipulating the skin immune system to downregulate an established contact sensitivity response.

## 3.2 Results

### 3.2.1 Langerhans cell density after DMBA treatment

In normal mice, depletion of LC from the epidermis following exposure to DMBA is central to the development of antigen specific tolerance. These LC-depletion studies were determined using naive mice and as this chapter is evaluating the ability to downregulate an established contact sensitivity response, immune mice rather than naive mice were examined to ascertain that LC depletion was also observed. All the mice in these experiments had been immunised with the contact sensitiser and immunisation was performed through ventral skin. The evaluation of DMBA-mediated LC depletion was performed by treating these immune mice with DMBA through dorsal skin, because this was the site that was to be treated for the downregulation experiments.

Application of 1% DMBA to TNCB-immune mice depleted LC from the skin when compared with the acetone treated control (Figure 3.1). The epidermis of DMBA-treated TNCB-immune mice contained approximately 100 LC per $mm^2$ whereas the acetone treated (control) group contained approximately 1300 LC per $mm^2$. Consequently, DMBA not only depleted epidermal LC in normal mice but had the ability to deplete epidermal LC in immune mice.

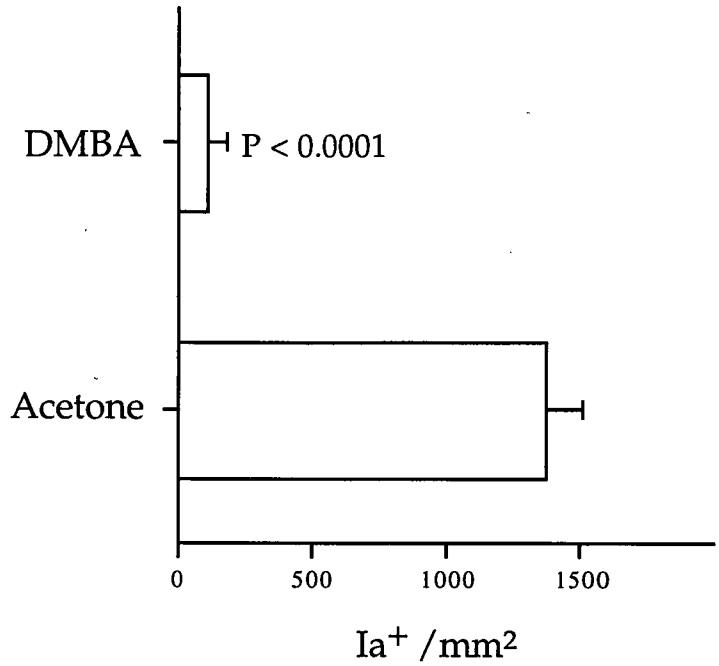



**Figure 3.1** Number of LC residing in the epidermis five days after DMBA (test) or acetone treatment (control).

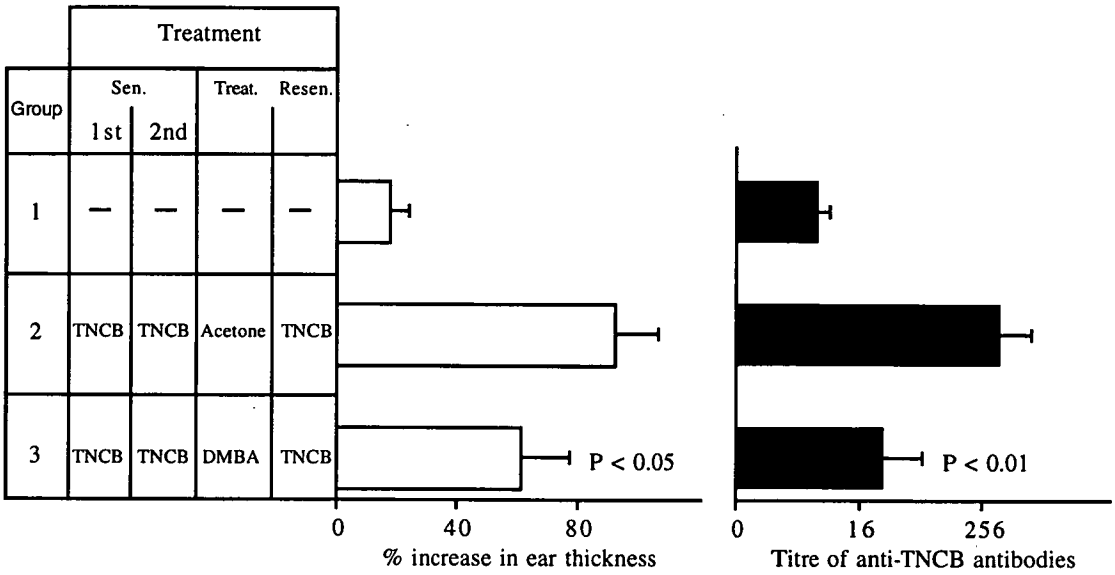
Results represent the mean ± standard deviation from at least 6 mice. P value was determined by Student's unpaired t-test.

### 3.2.2. DMBA/TNCB treatment reduced contact sensitivity and antibody production in TNCB-immune mice

When the antigen TNCB was applied through the DMBA-treated skin of TNCB-immune mice, there was a significant reduction in the level of contact sensitivity and antibody production to TNCB (Figure 3.2). Although this reduction was significant, it was not a complete reduction as both contact sensitivity and antibody levels to TNCB were higher than the control group (group 1), which had not been exposed to TNCB until they were ear challenged. It can be concluded that the application of TNCB through DMBA-treated skin causes a significant, but not complete reduction, of both cell mediated and humoral immunities.

### 3.2.3 Reduction of contact sensitivity and antibody production in TNCB-immune mice by DMBA/TNCB treatment was antigen specific

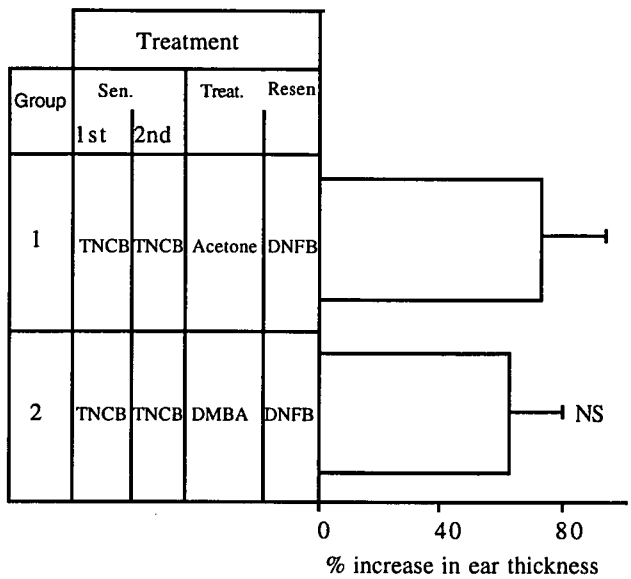
To determine if the reduction of contact sensitivity and antibody production was antigen specific the non-cross reactive antigen DNFB was applied through the DMBA-treated skin of TNCB-immune mice to render the mice tolerant to DNFB. When these mice were assessed for contact sensitivity to TNCB there was no significant reduction compared to the control TNCB-immune mice that were treated with acetone followed by DNFB (Figure 3.3). Consequently, applying DNFB through DMBA-treated skin did not reduce established contact sensitivity to TNCB.




Sen. = Sensitisation
Treat. = Treatment
Resen. = Resensitisation

**Figure 3.2** Reduction of established contact sensitivity and antibody response following DMBA/TNCB treatment.

TNCB-immune mice were sensitised by immunising through the skin with two separate applications (1st, 2nd) of 0.5% TNCB. These mice were then treated with acetone (group 2) or 1% DMBA (group 3) and five days later they were resensitised through the treated skin with 100µl of 0.5% TNCB. A further 5 days later contact sensitivity was assessed by ear challenge and increase in ear thickness was determined (left panel) and anti-TNCB antibody levels were assessed by indirect haemagglutination (right panel). Naive mice (group 1) were untreated throughout but were ear challenged with TNCB. Results represent the mean ± standard deviation from at least 6 mice. P values were determined by comparing group 2 and 3 using Student's unpaired t-test.




Sen. = Sensitisation
Treat. = Treatment
Resen. = Resensitisation
NS = No significant difference

**Figure 3.3** Downregulation of established immune response by DMBA/TNCB is antigen specific.

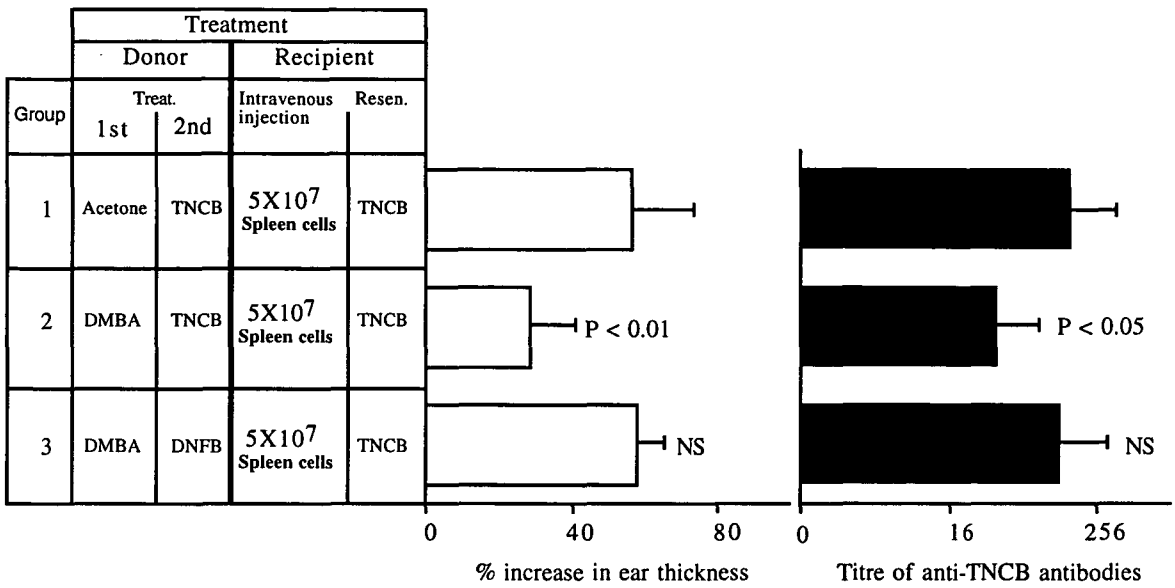
TNCB-immune mice were sensitised by immunising through the skin with two separate applications (1st, 2nd) of 0.5% TNCB. These mice were then treated either with acetone (group 1) or 1% DMBA (group 2) and five days later they were resensitised through the treated skin with 100µl of 0.5% DNFB. A further 5 days later, contact sensitivity to TNCB was assessed by ear challenge and increase in ear thickness was determined. Results represent the mean ± standard deviation from at least 6 mice. P value was determined with Student's unpaired t-test.

### 3.2.4 Reduction of contact sensitivity and antibody production in TNCB-immune mice following adoptive transfer of spleen cells from TNCB-tolerant mice

Suppression can be transferred by spleen cells. To determine whether spleen cells from TNCB-tolerant mice (i.e. naive mice) treated with DMBA and TNCB could also downregulate cell mediated and humoral immune responses of TNCB-immune mice, spleen cells were transferred from TNCB-tolerant mice to TNCB-immune mice and contact sensitivity and antibody levels to TNCB were evaluated. As shown in Figure 3.4, the transfer of spleen cells from TNCB-tolerant mice (group 2) to TNCB-immune mice caused a significant reduction in both contact sensitivity and antibody responses to TNCB. This reduction was antigen specific as spleen cells transferred from DNFB-tolerant mice (group 2) to TNCB-immune mice did not reduce either contact sensitivity or antibody levels to TNCB. Consequently the transfer of spleen cells from TNCB-tolerant mice to TNCB-immune mice caused an antigen specific reduction in both cell mediated and humoral immunity.

### 3.2.5 Effect of contact sensitivity and antibody production in TNCB-immune mice following adoptive transfer of epidermal cells after DMBA followed by TNCB treatment

Downregulation of an established contact sensitivity response can be achieved by the transfer of spleen cells from tolerant mice. The next phase of this study was to determine if downregulation could be achieved at the




Treat. = Treatment
Resen. = Resensitisation
NS = No significant difference

**Figure 3.4** Downregulation of established immune response by spleen cells generated from DMBA/TNCB treated mice.

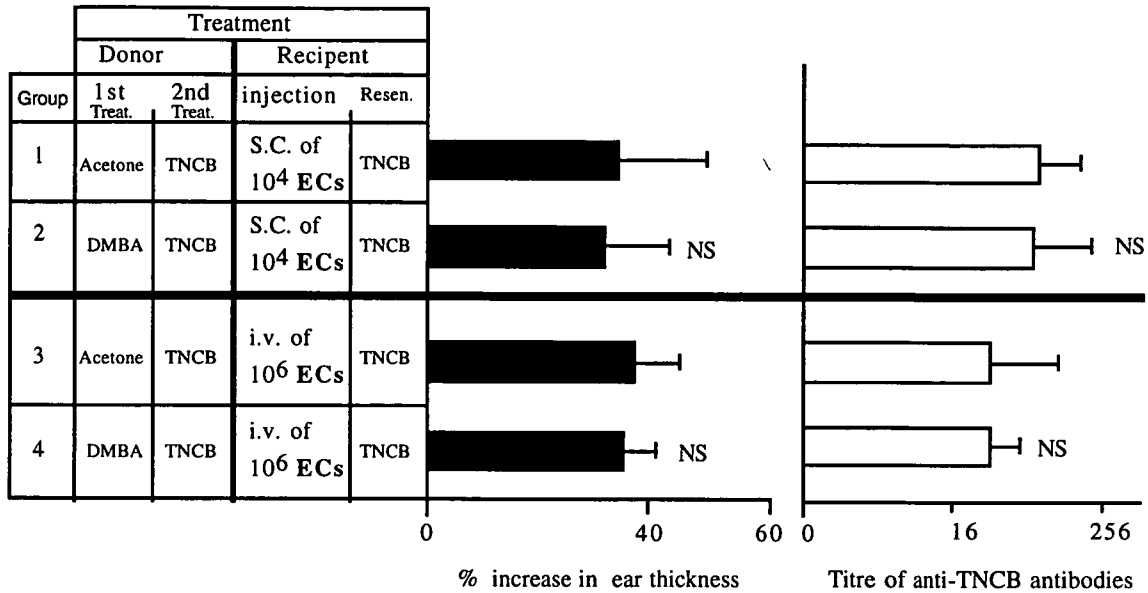
Recipent TNCB-immune mice were sensitised by immunising through the skin with two separate applications (1st, 2nd) of 0.5% TNCB. These mice received spleen cells from donor mice treated either with acetone followed 5 days later by 0.5% TNCB (group 1 - control), 1% DMBA followed 5 days later by 0.5% TNCB (group 2 - TNCB tolerant), or 1% DMBA followed 5 days later by 0.5% DNFB (group 3 - DNFB tolerant). A further 5 days following spleen cell transfer contact sensitivity was assessed by ear challenge and increase in ear thickness was determined (left panel) and antibody levels were assessed by indirect haemagglutination (right panel). Results represent the mean ± standard deviation from at least 6 mice. P values were determined by comparing group 1 with group 2 and group 1 with group 3 using Student's unpaired t-test.

inducer phase. To investigate this possibility epidermal cells from DMBA-TNCB-treated mice were transferred to TNCB-immune mice.

Figure 3.5 shows that epidermal cells from DMBA/TNCB-treated donors did not cause a downregulation of either contact sensitivity or antibody production following either a subcutaneous (sc) injection of $1 \times 10^4$ epidermal cells or an intravenous (iv) injection of $1 \times 10^6$ epidermal cells. This indicates that after DMBA/TNCB treatment epidermal cells could not transfer a suppressive signal strong enough to reduce the response in TNCB-immune mice.

### 3.2.6 Effect upon contact sensitivity and antibody production in TNCB-immune mice following adoptive transfer of TNP coupled epidermal cells from DMBA-treated mice

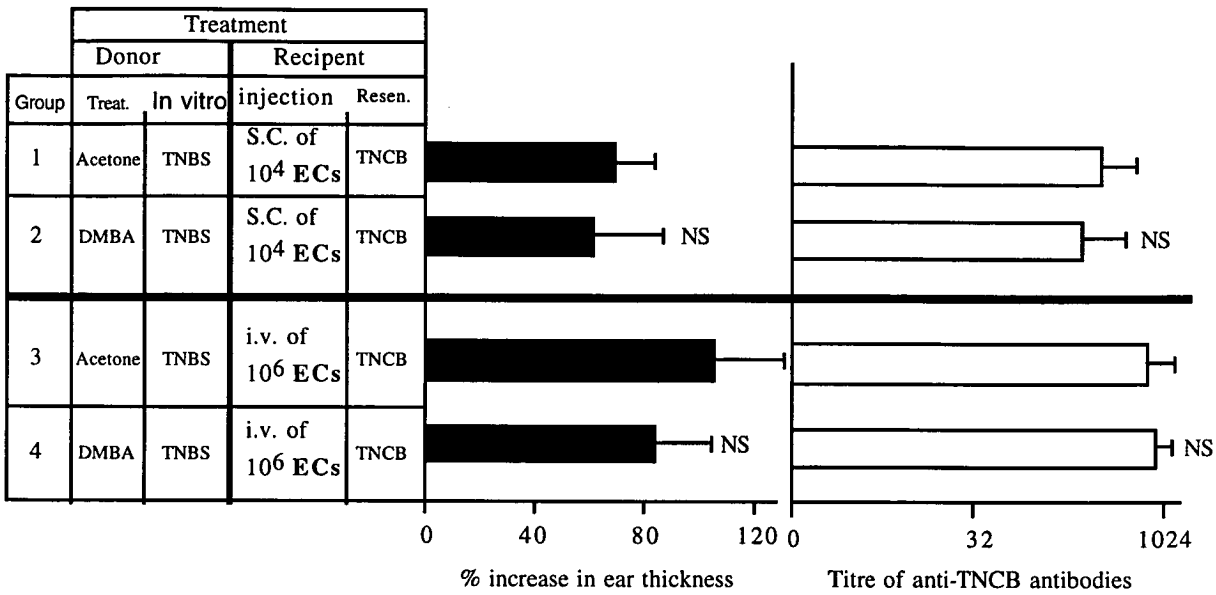
Epidermal cells obtained from treated with DMBA and incubated *in vitro* with hapten results in the suppression of CHS in naive mice. The next aspect of this study was to determine if an established immune response could be suppressed by DMBA-treated and TNP-coupled epidermal cells. Figure 3.6 shows that neither subcutaneous injection nor intravenous injection of DMBA-treated and TNP-coupled epidermal cells could downregulate the subsequent TNCB challenge induced immune responses. Neither contact sensitivity nor anti-TNCB antibody production was downregulated.




Treat. = Treatment
Resen. = Resensitisation
NS = No significant difference

**Figure 3.5** Failure to downregulate an established immune response by transfer of epidermal cells from DMBA followed by TNCB treated mice.

Recipent TNCB-immune mice were sensitised by immunising through the skin with two separate applications (1st, 2nd) of 0.5% TNCB. These mice received epidermal cells by s.c or i.v. routes from donor mice treated either with acetone followed 5 days later by 0.5% TNCB (group 1and 3 - control), or 1% DMBA followed 5 days later by 0.5% TNCB (group 2 and 4- test). A further 5 days following epidermal cell transfer contact sensitivity was assessed by ear challenge and increase in ear thickness was determined (left panel) and antibody levels were assessed by indirect haemagglutination (right panel). Results represent the mean ± standard deviation from at least 6 mice. P values were determined by comparing group 1 with group 2 and group 3 with group 4 using Student's unpaired t-test.




Treat. = Treatment
Resen. = Resensitisation
NS = No significant difference

**Figure 3.6** Epidermal cells from DMBA treated mice coupled with TNBS could not suppress an established immune response.

Recipent TNCB-immune mice were sensitised by immunising through the skin with two separate applications (1st, 2nd) of 0.5% TNCB. These mice received epidermal cells by s.c or i.v. routes from donor mice treated with either acetone (group 1and 3 - control), or 1% DMBA (group 2 and 4- test) and coupled with TNBS. A further 5 days following epidermal cell transfer contact sensitivity was assessed by ear challenge and increase in ear thickness was determined (left panel) and antibody levels were assessed by indirect haemagglutination (right panel). Results represent the mean ± standard deviation from at least 6 mice. P values were determined by comparing group 1 with group 2 and group 3 with group 4 using Student's unpaired t-test.

## 3.3 Discussion

One of the first steps, leading to the development of antigen specific immunosuppression is the depletion of LC from the epidermis. Halliday and Muller have clearly shown that DMBA has the ability to deplete LC from the skin and application of a contact sensitiser through this skin result in antigen specific suppression (Halliday and Muller, 1986). Adapting this procedure to downregulate an established immune response required confirmation that DMBA caused LC depletion in mice that have previously been immunised through the skin with a contact sensitiser. LC depletion is not a unique phenomenon to carcinogen as the application of this contact sensitisers TNCB or DNFB on its own can cause significant LC depletion (Woods *et al.*, 1996) and there is evidence showing that when an animal is treated with contact sensitiser the LC migrate from the skin to the draining lymph (Woods *et al.*, 1996). This is the same result with DMBA treatment of the skin which induced LC migration (Dandie *et al.*, 1992; Dandie *et al.*, 1994). The consequence of the pretreatment of TNCB is unknown. However, the results in this thesis demonstrate that LC depletion from the epidermis of TNCB-immune mice occurred following exposure to DMBA, but it is important to note that the DMBA application was applied to a different site than the original site of TNCB immunisation. This was undertaken as a deliberate measure to mimic as closely as possible the situation in naive mice that had not been exposed to antigen.

In both naive and immune mice, the same treatment resulted in a downregulation of established immunity suggesting that similar mechanisms that caused antigen specific suppression in naive mice also triggered immune suppression in immune mice. However in immune mice, unlike naive mice, where the immune response was completely abolished to hapten, the downregulation did not reach the background level. It is possible that although similar suppressive factors or signals were generated in both naive and immune mice, these signals did not have the strength ability to completely reverse or to silence previously activated T lymphocytes, possibly due to the fact that secondary T cell responses are more intense than primary response. Consequently suppression of naive T cell activation is more effective than suppression of previously activated T cells.

The ability to transfer suppression from tolerant mice to naive mice via spleen cells (Halliday and Muller, 1987b) indicates that the spleen contains a population which can prevent the induction of an immune response in an antigen specific manner. This population could also downregulate an established immune response and therefore provide further support for the concept that similar mechanisms of suppression operate at the cellular level in both naive and immune mice. However, in immune mice, these suppressive mechanism may not be as effective and as a result, an established immune response is only partially downregulated.

Coupling of hapten to DMBA-treated cells *in vivo* or *in vitro* did not suppress CHS or antibody production when these cells were transferred to immune mice. In contrast, the immune response of naive mice became tolerant when these mice received DMBA-treated hapten-coupled epidermal cells (Halliday *et al.,* 1988). It is likely that inactivation of memory T cells requires suppressor effector cells rather than suppressor inducer cells. The suppressive signals generated by DMBA-treated epidermal cells may not be sufficiently strong enough to suppress memory T cells. Alternatively the number of DMBA-treated epidermal cells required to suppress an established immune response may be greater than the number required to prevent the induction of immune response in naive mice. This might be a consequence of the greater number of antigen specific cells present in immune mice following clonal expansion in response to antigen stimulation.

It is concluded from this study that manipulation of the skin immune system by DMBA was able to reduce an established immune response either by *in vivo* treatment or by spleen cells transfer. This suppression has parallel outcomes for both cellular and humoral immune responses with a downregulation of both. However, modified epidermal cells did not have the ability to suppress an established immune response using current methods. These basic observation have clinical relevance for the treatment of autoimmune disease as the downregulation of established immunity may be sufficient to benefit over-reactive immunity.

# Chapter Four
# Downregulation of Established Immune Response by Physical Carcinogen

## 4.1 Introduction

The previous chapter has shown that an established immune response can be downregulated by modifying epidermal LC by DMBA treatment. However, as DMBA is a toxic carcinogen it does not lend itself to the ultimate goal of human trials. Alternatively, a workable agent that has a similar ability to suppress the immune response through the skin treatment is ultraviolet light (UVB) irradiation. It has been well established that cutaneous exposure to UVB irradiation alters the immune response and leads to the suppression of CHS to haptens applied through the irradiated epidermis in mice (Toews *et al.*, 1980) and in humans (Yoshikawa *et al.*, 1990). Since LC play a role in the induction of immunity and as LC are depleted from the skin by UVB irradiation, it has been suggested that the loss of LC contributes to the induction of tolerance (Stingl *et al.*, 1981; Aberer *et al.*, 1981). Various other factors are also involved in UVB-induced immune suppression. For example, isomerization of urocanic acid from trans to cis following UVB irradiation has been linked with the induction of immune tolerance (Noonan and De Fabo, 1992). An increase of Interleukin-10 after UVB irradiation also been correlated with the induction of immunosuppression (Rivas and Ullrich, 1994). Macrophages have also been shown to be involved in UVB-induced immune suppression as there is an increase in their number in the draining lymph nodes and at the site of

UVB irradiation (Cooper *et al.*, 1986; Duraiswamy *et al.*, 1994). Consequently, UVB irradiation can induce immune suppression via a number of potential mechanisms, however, LC depletion is most likely to play a prominent role. This immune suppression is also antigen specific as spleen cells adoptively transferred from UVB and hapten-treated mice to naive syngeneic mice transfer antigen specific tolerance (Elmets *et al.*, 1983; Simon *et al.*, 1992). It has been proposed that hapten-specific suppressor cells may play a key role in the induction of tolerance (Greene *et al.*, 1979; Ullrich, 1995b). The ability to induce antigen specific suppression by UVB and hapten treatment thus provides an alternative strategy to manipulate the immune system in an antigen-specific manner, such that inappropriate or deleterious immune responses including those associated with autoimmunity, could be modulated.

Indeed UVB radiation has been used in the treatment of various immune disorders in humans. A combination therapy of psoralen and UVA has been well established for the treatment of psoriasis, atopic dermatitis, vitiligo, and T cell lymphoma (Ullrich *et al.*, 1989). UVB combined with coal tar also has been shown to be effective in treating psoriasis (Stern *et al.*, 1980). Narrow-Band UVB wavelengths situated around 313 nm have been used to effectively treat atopic dermatitis (Green *et al.*, 1988). Other beneficial effects of UVB therapy include reduction of prostaglandins which mediate inflammation (Katayama and Hori, 1984), increase of vitamin D synthesis (Staberg *et al.*, 1988), and immunosuppression (Teunissen *et al.*, 1993).

## 4.2 Results

### 4.2.1 Effect of different UVB doses on LC depletion

The study with DMBA showed that LC were depleted 5 days after DMBA treatment in TNCB-immune mice. To ascertain that UVB irradiation causes LC depletion in TNCB-immune mice rather in addition to naive mice and to determine its optimal dose the LC numbers of the dorsal skin were evaluated 5 days after different UVB doses. The results, showed that 5 days after UVB irradiation LC numbers were significantly reduced when the skin was exposed to doses of 7 $kJ/m^2$ or higher (Figure 4.1).

### 4.2.2 Time course of LC depletion following UVB-irradiation

Unlike DMBA, maximum LC depletion may not occur 5 days after irradiation. Previous studies have shown a time dependent reduction in LC number following UVB irradiation, and the kinetics of this reduction was driven by the initial UVB dose (De Rie and Bos, 1997). Consequently it was important to determine the appropriate time after UVB-irradiation for maximum LC-depletion in TNCB-immune mice and therefore development of immunosuppression. As 7 and 13 $kJ/m^2$ caused significant LC depletion at 5 days post irradiation, these doses were utilized. The dose of 7 $kJ/m^2$ was the minimum dose that caused LC depletion whereas 13 $kJ/m^2$ was the maximum dose that did not cause excessive erythema and discomfort for the mice (Doses of 20 $kJ/m^2$ or higher were abandoned due to ethical constraints). As shown in Figure 4.2, both 7 and 13 $kJ/m^2$ UVB doses could significantly deplete LC numbers at 3 and 5 days after irradiation. The

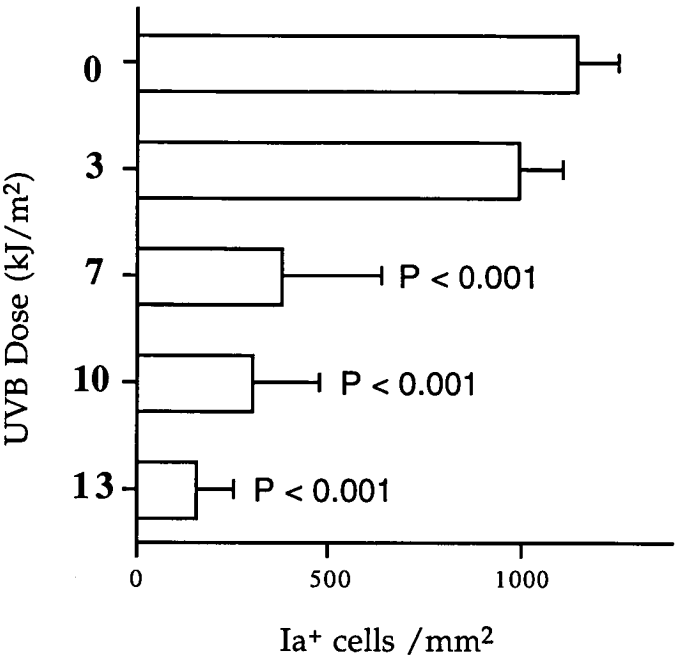



**Figure 4.1** Number of LC in the epidermis of TNCB-immune mice five days after UVB irradiation.

Mice were sensitised by immunising through the skin with two separate application of TNCB, 5 days apart. The TNCB-immune mice were then treated with UVB irradiation and a further 5 days later the number of $Ia^+$ cells was determined. Results represent the mean ± standard deviation from at least 6 mice. P values were determined by comparing the control group (0 $kJ/m^2$) and test groups using Student's unpaired t-test.

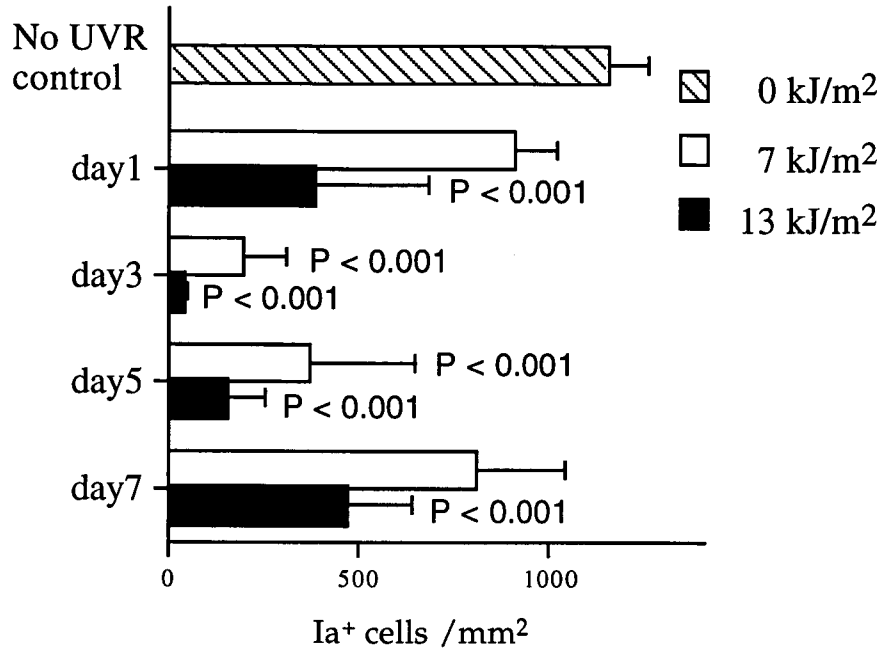



**Figure 4.2** Number of LC in the epidermis of TNCB-immune mice at 1,3,5 or7 days following UVB irradiation.

Mice were sensitised by immunising through the skin with two separate application of TNCB, 5 days apart. The TNCB-immune mice were then treated with UVB irradiation at 7 (□) or 13 kJ/m$^2$ (■) and a further 1,3,5 or 7 days later the number of Ia$^+$ cells was determined. Results represent the mean ± standard deviation from at least 6 mice. P values were determined by comparing control group (0 kJ/m$^2$) and test groups using Student's unpaired t-test.

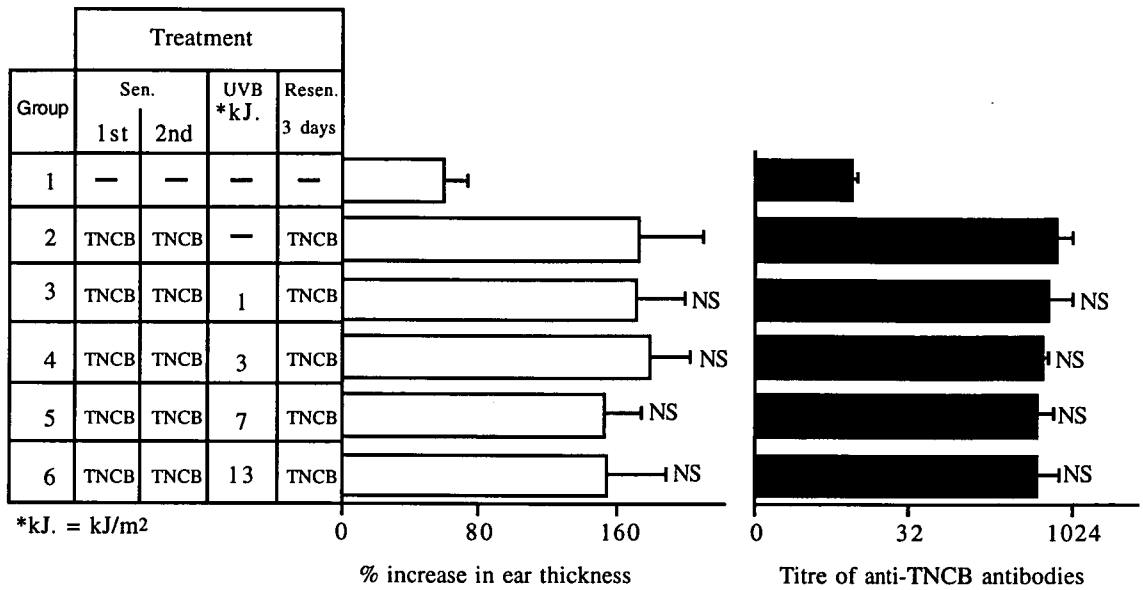
13 kJ/m$^2$ UVB reduced LC numbers from day 1 to day 7 after irradiation whereas 7 kJ/m$^2$ UVB reduced LC numbers from day 3 to day 5 after irradiation. In current protocol, the most effective time of LC depletion for both doses was 3 days after irradiation.

### 4.2.3 Application of TNCB three days after UVB irradiation did not reduce contact sensitivity or antibody production in TNCB immune mice

As LC depletion was the most significant 3 days after UVB irradiation it was predicted that hapten application at this time would lead to antigen specific downregulation of an established immune response. As shown in Figure 4.3, when TNCB was applied through the skin of TNCB-immune mice 3 days after UVB irradiation there was no significant reduction in contact sensitivity. A slight, but not statistically significant reduction was seen following UVB doses 7 kJ/m$^2$ or higher. At all doses, antibody levels to TNCB were not affected (Figure 4.3). Hence, the established TNCB immunity, neither contact sensitivity nor antibody production was significantly downregulated if TNCB was applied to skin 3 day after UVB irradiation.

### 4.2.4 Application of TNCB five days after UVB irradiation reduced contact sensitivity but not antibody production in TNCB immune mice

Although 3 days after UVB irradiation caused maximum LC depletion, this time course did not cause significant immune suppression. Miyauchi and Horio (1995) reported similar findings in naive mice as



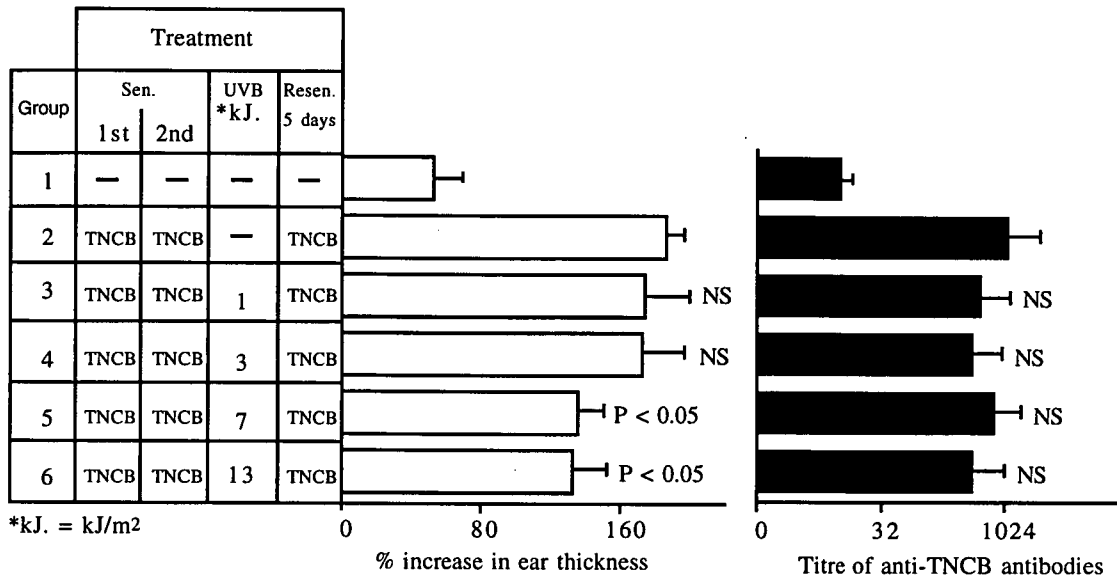

Sen. = Sensitised with 2% TNCB
Resen. = Resensitised with 2% TNCB
NS = No significant difference

**Figure 4.3** Unaffected contact hypersensitivity and antibody responses following UVB/TNCB treatment.

Mice were sensitised by immunising through the skin with two separate applications (1st, 2nd) of 2% TNCB, 5 days apart. Control mice (group 1) were treated with acetone alone. TNCB-immune mice were then treated with varying doses of UVB irradiation and three days later they were resensitised through the treated skin with 100µl of 2% TNCB. A further 5 days later contact sensitivity to TNCB was assessed by ear challenge and increase in ear thickness was determined (left panel) and antibody levels were assessed by indirect haemagglutination to TNCB (right panel). Naive mice (group 1) were untreated but were ear challenged with TNCB alone. Remaining groups (2-6) received 0,1,3,7 or 13 kJ/m$^2$ UVB irradiation respectively. Results represent the mean ± standard deviation from at least 6 mice. P values were determined by comparing group 2 with groups 3,4,5 and 6 using Student's unpaired t-test.

maximal LC depletion did not generate suppression in UVB irradiated mice. In effect, a maximum suppression occurred 2 days after maximum LC depletion (Miyauchi and Horio, 1995). As application of TNCB 3 days following UVB irradiation did not downregulate an established immune response the next procedure was to increase this period to 5 days after UVB irradiation. As shown in Figure 4.4, when TNCB was applied through the UVB irradiated skin of TNCB-immune mice there was a dose dependent reduction in contact sensitivity to TNCB. When given as a single dose, at least 7 $kJ/m^2$ UVB irradiation was required to cause a significant reduction in contact sensitivity. However, at all doses, antibody levels to TNCB were not affected. Although the reduction in contact sensitivity was significant, it was not a complete reduction, as in all UVB doses tested the contact sensitivity was much higher than the control mice (group 1) that had not been exposed to TNCB until they were ear challenged. Therefore a single UVB dose of 7 $kJ/m^2$ or more, significantly downregulated cell mediated immunity but not humoral immunity in TNCB-immune mice.

As 7 $kJ/m^2$ was the minimum dose that caused significant immune suppression, this dose was used throughout the remainder of this thesis.




Sen. = Sensitised with 2% TNCB
Resen. = Resensitised with 2% TNCB
NS = No significant difference

**Figure 4.4** Reduction of established contact sensitivity but not antibody response following UVB/TNCB treatment.

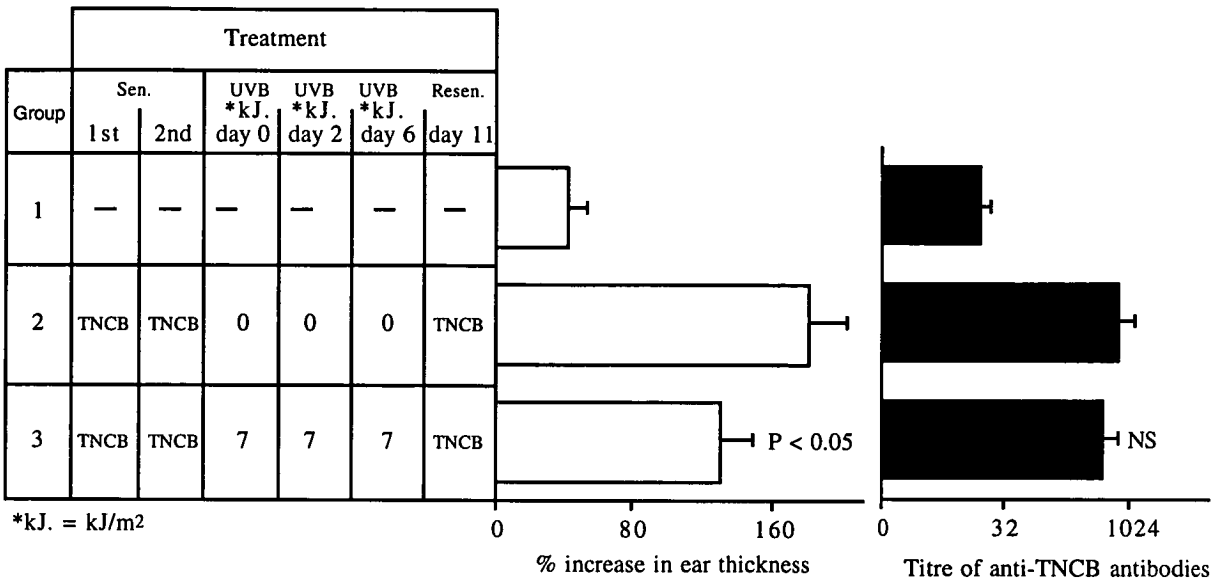
Mice were sensitised by immunising through the skin with two separate applications (1st, 2nd) of 2% TNCB, 5 days apart. Control mice (group 1) were treated with acetone alone. TNCB-immune mice were then treated with varying doses of UVB irradiation and three days later they were resensitised through the treated skin with 100µl of 2% TNCB.A further 5 days later contact sensitivity to TNCB was assessed by ear challenge and increase in ear thickness was determined (left panel) and antibody levels were assessed by indirect haemagglutination to TNCB (right panel). Naive mice (group 1) were untreated but were ear challenged with TNCB alone. Remaining groups (2-6) received 0,1,3,7 or 13 kJ/m$^2$ UVB irradiation respectively. Results represent the mean ± standard deviation from at least 6 mice. P values were determined by comparing group 2 with groups 3,4,5 and 6 using Student's unpaired t-test.

### 4.2.5 Three consecutive doses of UVB irradiation followed by TNCB reduced an established contact sensitivity response but not antibody production

Although an established contact sensitivity response could be downregulated by application of antigen 5 days following UVB irradiation, this reduction did not reach the background level. The next aspect of this study was to increase the downregulation of an established immune response. Consecutive doses of UVB irradiation have a greater immunosuppressive effect on the induction of immunosuppression. TNCB application following 3 consecutive doses of 7 $kJ/m^2$ UVB irradiation to immune mice caused a significant reduction of contact sensitivity but antibody levels to TNCB remained unaffected (Figure 4.5). The reduction in contact sensitivity, although significant, still did not reach the background level.

### 4.2.6 Three consecutive cycles of UVB irradiation followed by TNCB reduced an established contact sensitivity response but not antibody production

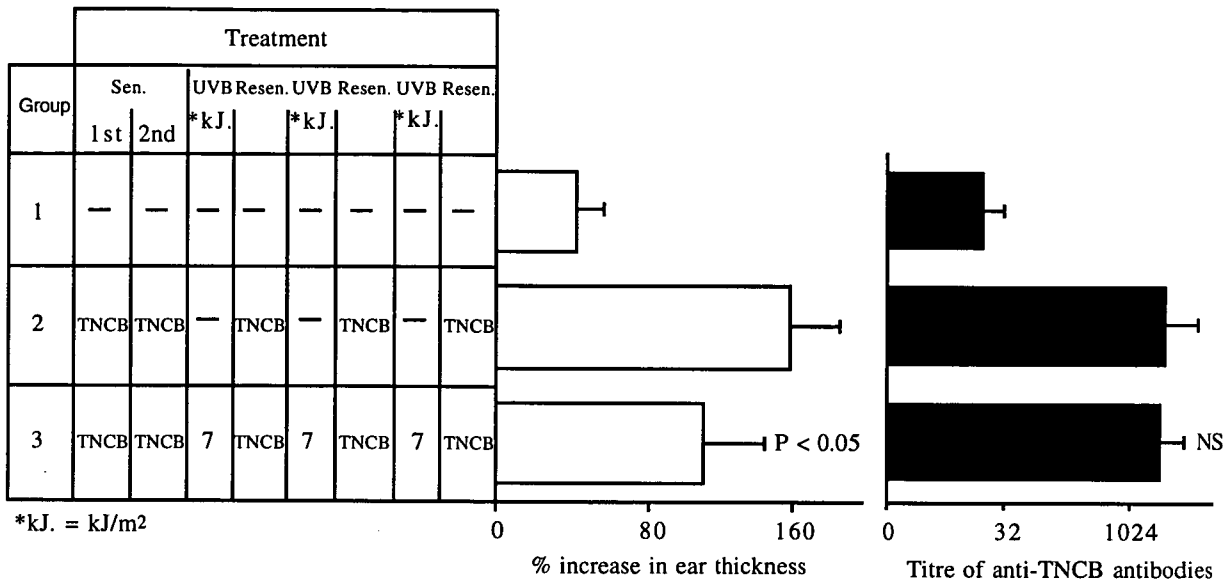
In a further attempt to completely reduce contact sensitivity an alternative strategy was employed. Since the generation of suppressor cells came from the treatment of application of hapten through the LC-depleted skin, TNCB-immune mice were treated with 3 consecutive cycles of 7 $kJ/m^2$ UVB irradiation followed by TNCB. As shown in Figure 4.6 contact sensitivity was significantly reduced but antibody levels to TNCB remained




Sen. = Sensitised with 2% TNCB
Resen. = Resensitised with 2% TNCB
NS = No significant difference

**Figure 4.5** Reduction of established contact sensitivity but not antibody response following 3 consecutive doses of UVB.

Mice were sensitised by immunising through the skin with two separate applications (1st, 2nd) of 2% TNCB, 5 days apart. Control mice (group 1) were treated with acetone alone. The TNCB-immune mice were either untreated (group 2) or treated with 3 consecutive doses of 7 kJ/m$^2$ UVB irradiation 2 days apart (group 3). Five days following the final UVB irradiation they were resensitised through the treated skin with 100µl of 2% TNCB. A further 5 days later contact sensitivity to TNCB was assessed by ear challenge and increase in ear thickness was determined (left panel) and antibody levels to TNCB were assessed by indirect haemagglutination (right panel). Naive mice (group 1) were untreated but ear challenged with TNCB. Results represent the mean ± standard deviation from at least 6 mice. P values were determined by comparing group 2 and group 3 using Student's unpaired t-test.




Sen. = Sensitised with 2% TNCB
Resen. = Resensitised with 2% TNCB
NS = No significant difference

**Figure 4.6** Reduction of established contact sensitivity but not antibody response following 3 consecutive UVB -TNCB treatments.

Mice were sensitised by immunising through the skin with two separate applications (1st, 2nd) of 2% TNCB, 5 days apart. Control mice (group 1) were treated with acetone alone. The TNCB-immune mice were then either treated with 2%TNCB alone (group 2) or with 3 consecutive cycles of 7 kJ/m$^2$ UVB followed 5 days later with TNCB and the cycle repeated after another 5 days (group 3). Five days following the final TNCB treatment contact sensitivity to TNCB was assessed by ear challenge and increase in ear thickness was determined (left panel) and antibody levels to TNCB were assessed by indirect haemagglutination (right panel). Naive mice (group 1) were untreated but ear challenged with TNCB alone. Results represent the mean ± standard deviation from at least 6 mice. P values were determined by comparing group 2 and group 3 using Student's unpaired t-test.

unaffected. Again, although the reduction in contact sensitivity was statistically significant, it did not reach background level.

## 4.3 Discussion

The ability to prevent the development of an immune response provides an opportunity to manipulate the immune system and avoid the induction of inappropriate immune responses including autoimmune diseases. When contact sensitisers were painted on UVB pre-treated skin, a state of immunosuppression was induced instead of a normal CHS response. This was due to the loss of skin dendritic cells, the LC. In the present study, the LC were depleted at day 3 or day 5 after UVB irradiation, when UVB doses of 7 $kJ/m^2$ or greater were given. In the situation of pre-treatment with UVB, the degree of LC depletion often correlated with the degree of immunosuppression. Interestingly, in mice with an established immune response, when hapten was applied to the skin 3 days after a dose of UVB irradiation which resulted in the most severe loss of LC, suppression of CHS response was not achieved. However, suppression of an established immune response was obtained when the hapten was applied through skin that, 5 days before, had been irradiated with a high dose of UVB. This suggested that the stronger immune suppressive signals were generated after 5 days of UVB irradiation.

The comparison between multiple doses in naive and immune mice showed a different result. It has been demonstrated that multiple doses of

UVB irradiation could lead to more effective tolerance induction compared to a single dose of UVB irradiation (Miyauchi and Horio, 1995). In the present study, multiple doses did not create a greater immune suppression, as the suppression was no greater than a single high UVB dose. There are few reports describing experiments designed to downregulate an established CHS response. One group that did evaluate contact sensitivity was Kosiewcz *et al.* (1994). They failed to detect a reduction in contact sensitivity when DNFB-immune mice were treated with 4 daily UVB doses of 0.4 $kJ/m^2$ followed 1 hour after the final UVB dose with DNFB. This regime was able to induce suppression in naive mice but not in immune mice, thus contrasting with the present study. The apparent discrepancy may reflect the UV irradiation protocols and timing of antigen application. This thesis study found that a single dose of 7 $kJ/m^2$ was required to cause a significant downregulation of established immunity. This is substantially higher than the 4 consecutive doses of 0.4 $kJ/m^2$ undertaken by Kosiewcz and colleagues. Other differences may relate to the strains of mice (BALB/c compared to C3H) or the timing of antigen application. This thesis study found that application of antigen 3 days post-UVB exposure was not able to induce significant downregulation. At least 5 days post-UVB exposure was required to cause significant downregulation. As Kosiewcz *et al.* applied antigen immediately after the final UVB irradiation, the microenvironment in the skin may not have changed to the extent required for a suppressor signals to be generated.

The results presented here did not show complete downregulation of the immune response, even after repeated treatments with UVB irradiation. This may relate to the ability of UVB followed by hapten to stimulate memory T cells. The activation requirements of naive T cells include the appropriate antigen/TCR interaction as well as a range of costimulatory signals including the B7/CD28 system and soluble mediators such as the cytokines IL-1 (Simon *et al.*, 1991) and IL-12 (Kang *et al.*, 1996). Memory T cells can be restimulated via TCR ligation and a reduced requirement for costimulation (Katz, 1988; Van de Velde *et al.*, 1993; Croft *et al.*, 1994). It may therefore be possible that following UVB-treatment, some functional antigen-presenting cells remain in the skin, which induces the activation of the memory T cells. An alternative explanation may relate to the nature of the suppressor signal which prevents the activation of naive T cells more effectively than memory cells.

UVB irradiation did not affect antibody production in most of the study involving in the use of naive mice (Kim *et al.*, 1990). However, some reports may suggest that UVB irradiation may affect antibody production. Ullrich and colleagues (Ullrich *et al.*, 1986b) showed in an *in vivo* study that suppressive cells generated by hapten applied through 40 $kJ/m^2$ UVB irradiated skin and transferred to naive mice could reduce the number of direct plaque-forming cells of the recipient against hapten. This was antigen specific as the plaque-forming cells against other antigens were not reduced. Another study by Spellman et al. documented that application of

immunoglobulin through the UVB irradiated skin did result in the suppression of antibody production (Spellman *et al.*, 1984). The present study, used high doses of UVB radiation, both signal and multiple applications, were unable to suppress the antibody production in immune mice. It appears that tolerance induced by UVB had little effect on suppression of antibody production in immune mice.

In comparison, both contact sensitivity and antibody production were downregulated in immune mice following DMBA treatment. However, contact sensitivity but not antibody mediated immunity was reduced when immune mice were treated with UVB followed by hapten. This difference in suppression by DMBA and UVB is consistent with the observed suppression in naive animals and reflects the ability of DMBA/TNCB treatment to prevent both Th1 and Th2 responses, whereas UVB irradiation is more likely to prevent Th1 responses, probably by inducing the production of Th2 cytokines, including IL-4 and IL-10 (Rivas and Ullrich, 1992). Such a consistent response observed with both naive and immune animals indicates that a similar underlying mechanism of suppression was involved.

These responses propose that suppressor cells were generated as the transfer of spleen cells from TNCB-tolerant mice to TNCB-immune mice also resulted in antigen specific suppression and reduced the immune response. The transfer of suppression to naive mice is due to antigen specific

suppressor cells which are induced after the application of antigen to LC-depleted skin (Halliday *et al.*, 1988). This cellular suppressor signal has the ability to prevent the induction of an immune response and as shown in this study is also able to significantly reduce an established response.

The ability to reduce an established immune response in an antigen specific manner has important implications for the potential treatment of allergic and autoimmune diseases where the causative antigens have been identified. Although immunisation through carcinogen modified skin only partially suppressed an established immune response, this reduction may be sufficient to ameliorate the symptoms of an autoimmune disease. Future studies in this area could be directed at a greater reduction or ablation of an ongoing immune response.

# Chapter Five
# Peripheral Tolerance Induction in Thymectomised Mice by Immunisation Through Chemical Carcinogen Altered Skin

## 5.1 Introduction

Following the demonstration that downregulation of an established immune response could be achieved by applying hapten through altered skin, the next step of this study was to evaluate whether an autoimmune disease could be modulated by a similar approach. The first consideration before such an undertaking was to determine an appropriate model. As the induction of antigen specific immunosuppression requires at least two components, modification of the skin and an antigen that could be applied through this modified environment, it was important to select a model of autoimmunity where the autoantigen had been well characterised. Furthermore, for effective monitoring of disease progression it was crucial that a measure of autoimmunity, such as levels of autoantibodies could be routinely performed. One such model is autoimmune gastritis (AIG). The identification of the autoantigens came from tests examining the specificity of parietal cell autoantibodies present in the circulation of animals with AIG. The autoantibodies are parietal cell specific and can be routinely monitored by immunofluorescence using serum samples on stomach sections (Fukuma *et al.*, 1988).

The target of the autoantibodies in the parietal cells is the $H^+K^+$-ATPase (also known as the proton pump), the enzyme responsible for acidification of gastric juice. More specifically the autoantigen is the 95 kDa α-subunit and more specifically the 60-90 kDa glycoprotein β-subunit of the $H^+K^+$-ATPase (Jones *et al.*, 1991; Gleeson and Toh, 1991; Toh *et al.*, 1992) and peptide mapping of this antigen has identified the active epitope (Toh *et al.*, 1990).

Experimentally, this disease can be induced by two main procedures. Firstly, immunisation of BALB/c mice with rat gastric parietal cells in Freund's complete adjuvant leads to the induction of gastritis and circulating antibodies to the $H^+K^+$-ATPase (Kontani *et al.*, 1992). Gastritis could be induced by immunising dogs with gastric mucosal extracts (Hennes *et al.*, 1962). Immunisation of rhesus monkeys with gastric mucosal extracts in Freund's complete adjuvant also resulted in gastritis (Andrada *et al.*, 1969). More recently, normal BALB/c mice immunised with purified murine gastric $H^+K^+$-ATPase emulsified in complete Freund's adjuvant developed autoimmune gastritis (Scarff *et al.*, 1997). However this approach, requires continued immunisation with the gastric $H^+K^+$-ATPase to maintain the disease.

The second approach is to use mice thymectomised 3 days after birth (3dnTx). These mice generate autoimmune gastritis in approximately 60 percent of animals (Kojima and Prehn, 1981; Murakami *et al.*, 1993). The mice have similar pathological lesions to those in pernicious anaemia in

humans (Kojima and Prehn, 1981; Gleeson *et al.*, 1996) and the disease is maintained throughout the life of the mouse without any further treatment (Kojima and Prehn, 1981). As with the immunisation model, the autoantigen is the gastric $H^+K^+$-ATPase and the disease can be monitored by screening for autoantibodies in the blood. As the 3dnTx immunisation model results in life-long disease and reflects an altered immunoregulation mechanism it was selected as the appropriate model to use in this investigation.

It has been hypothesized that progenitor autoreactive T cells are present in the periphery (Bonomo *et al.*, 1995a; Bonomo *et al.*, 1995b), but appear to be in a state of anergy (Jones *et al.*, 1990; Kruisbeek *et al.*, 1992). Analysis of this hypothesis has lead to the proposal that following thymectomy, particularly at day 3 after birth, populations of immunoregulatory T cells lost. Analysis of the lymphocyte populations in these mice indicates that an absence of the regulatory T cells that control self-reactive cells was responsible for this autoimmune disease (Bonomo *et al.*, 1995b). Evidence that these regulatory T cells exist in adult mice was provided by adoptive transfer of normal spleen cells (Taguchi and Nishizuka, 1987; Taguchi *et al.*, 1990), particularly the $CD4^+$ $CD25^+$ T cells (Asano *et al.*, 1996) from healthy adult mice to 3dnTx mice. This prevents the development of AIG implicating a role for these T cells in the maintenance of peripheral tolerance to the gastric antigens.

These background observations provide a number of interesting questions relating to immunoregulation and the fate of T cell subpopulations. Of particular interest to the studies in this thesis was the question of whether active suppression could be induced following application of antigen through LC-depleted skin in the 3dnTx mice. The ability to generate suppression via this approach would confirm the validity of this model and provide evidence that peripheral tolerance could still be induced thus providing a strategy for the treatment of autoimmune gastritis.

Prior to undertaking the specific experiments to evaluate the potential of applying antigen through a modified skin environment to produce systemic antigen specific suppression it was important that the immune status of the 3dnTx mice was evaluated. This chapter summarises the immunological status of 3dnTx mice assessed by enumeration of $CD4^+$ and $CD8^+$ T cells; the capacity of spleen cells from these mice to respond to the mitogen PHA; evaluation of contact hypersensitivity responses to TNCB, and determination of the ability of these mice to generate antigen specific suppressor cells to TNCB induced contact hypersensitivity and antibody production. As an extension of this study nude mice that respond to TNCB immunisation were also examined in a similar manner to determine the absolute requirement of the thymus for the generation of suppression.

## 5.2 Results

### 5.2.1 Development of autoimmune gastritis

Thymectomised mice developed autoimmune gastritis as assessed by autoantibody titres ≥ 32. AIG negative mice were defined by an autoantibody < 8. Titres of 8 and 16 were classified as indeterminate and mice were not used. Mice with titres ≥ 32 comprised 50-60 percent of the 3dnTx mice; 10 percent of 3dnTx mice were in the undetermined group and the rest were AIG negative mice.

### 5.2.2 Proliferative responses of 3dnTx mice splenic lymphocytes pre and post DMBA treatment

The splenic lymphocytes from the 3dnTx mice showed a reduced ability to respond to PHA (Figure 5.1). After cutaneous application of 1% DMBA there was a further reduction in the PHA-induced proliferation from 3dnTx mice as well as a reduction in proliferation of cells from normal control mice (Figure 5.2). Thus thymectomy and/or topical application of DMBA reduced the ability of lymphocytes to proliferate in response to PHA.

### 5.2.3 LC density in 3dnTx mice following DMBA-treatment

As reduced LC density is a critical component for the induction of antigen specific tolerance, LC density in 3dnTx mice following DMBA application was examined. Results shown in Figure 5.3 demonstrate that application of DMBA caused a significant reduction in LC density compared to acetone treated controls.

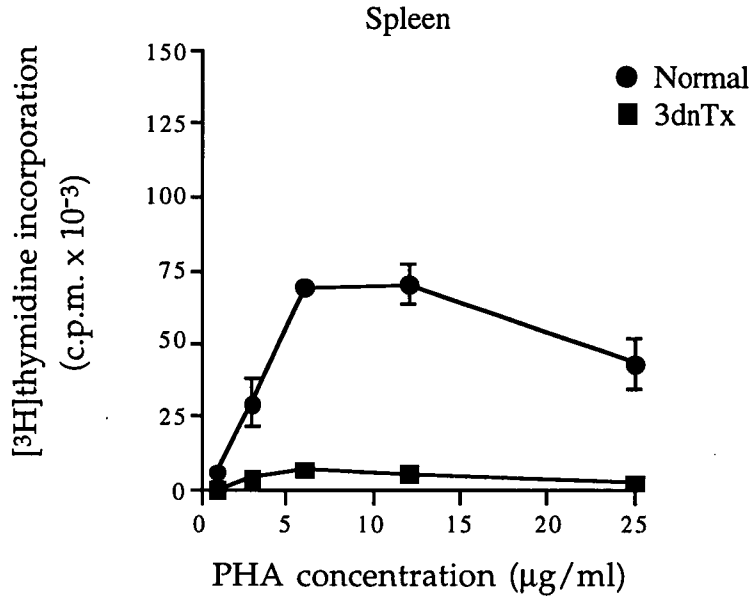



**Figure 5.1** PHA induced proliferation of lymphocytes from 3dnTx mice.

Spleen cells from normal or 3dnTx mice at $10^5$/well were incubated with various concentrations of PHA for 3 days at 37°C, 5%$CO^2$ and proliferation was determined by [$^3$H] thymidine incorporation. Results represent the mean ± standard deviation of triplicate cultures from a representative experiment.

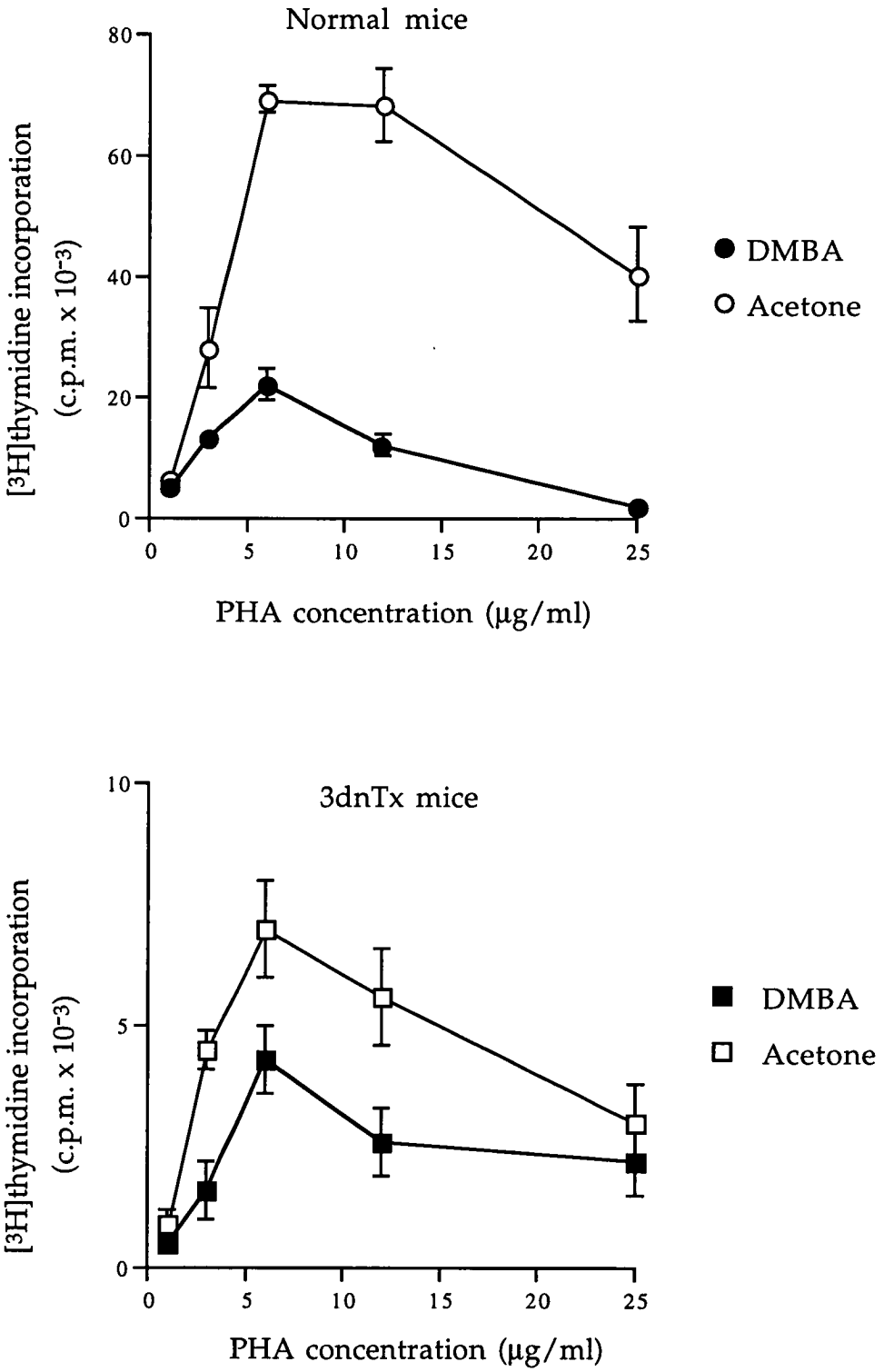



**Figure 5.2** PHA induced proliferation of lymphocytes from DMBA or acetone treated normal or 3dnTx mice.

Spleen cells from DMBA or acetone treated normal or 3dnTx mice at $10^5$/well were incubated with various concentrations of PHA for 3 days at 37°C, 5%$CO_2$ and proliferation was determined by [$^3$H] thymidine incorporation. Results represent the mean ± standard deviation of triplicate cultures from a representative experiment.

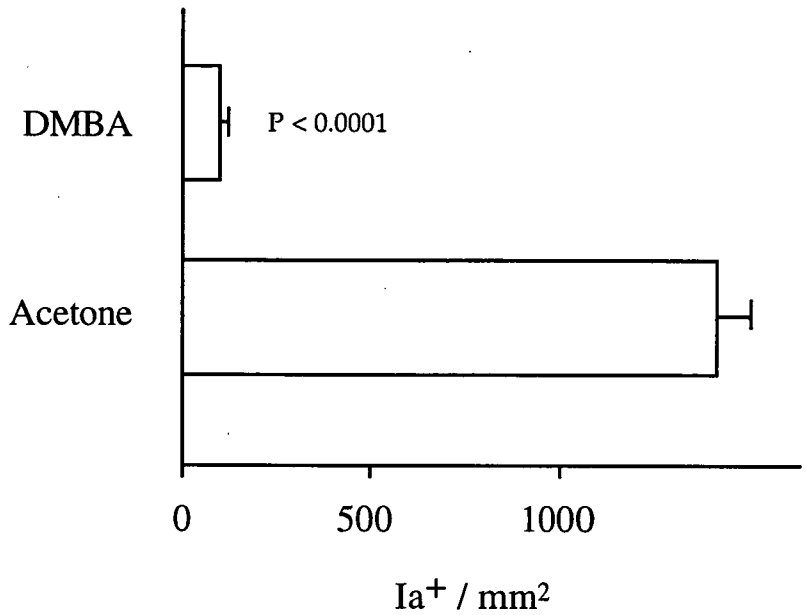



**Figure 5.3** Number of LC residing in the epidermis five days after DMBA (test) or acetone treatment (control).

Results represent the mean ± standard deviation from at least 6 3dnTx mice. P value was determined by Student's unpaired t-test.

### 5.2.4 Lymphocyte numbers in 3dnTx mice

There was overall reduction in the total number of lymphocytes from the spleen of 3 dnTx mice (Table 5.1). When the relative number of T and B cells was evaluated the percentage of $B220^+$ B cells was found to be unchanged in the 3dnTx mice compared to normal mice whereas the percentages of $CD4^+$ and $CD8^+$ T cells were decreased (Figure 5.4). This was observed with all thymectomised mice, irrespective of whether they developed autoimmune gastritis. As the relative proportion of $CD4^+$ and $CD8^+$ remained unchanged in the 3dnTx mice this indicates that thymectomy did not alter the balance of these cells. Following cutaneous exposure to DMBA the ratio of T and B cells was unaffected but the total number of spleen cells was further reduced (Table. 5.1).

### 5.2.5 Induction of immunosuppression in 3dnTx mice

As changes in lymphocyte population were observed with 3dnTx mice it was important to evaluate immune tolerance induction. When TNCB was applied through the DMBA-treated skin of thymectomised mice there was neither a contact sensitivity nor antibody response (Figure 5.5). This occurred with all the DMBA-treated thymectomised mice, irrespective of whether they developed AIG.

| Mice | Treatment | Average numbers of spleen cell per mouse |
|---|---|---|
| Normal mice | Acetone | $10.00 \times 10^7 \pm 0.95 \times 10^7$ |
| | DMBA | $5.10 \times 10^7 \pm 0.41 \times 10^7$ |
| 3dnTx mice (AIG positive) | Acetone | $3.75 \times 10^7 \pm 0.45 \times 10^7$ |
| | DMBA | $3.15 \times 10^7 \pm 0.13 \times 10^7$ |
| 3dnTx mice (AIG negative) | Acetone | $4.05 \times 10^7 \pm 0.32 \times 10^7$ |
| | DMBA | $3.25 \times 10^7 \pm 0.16 \times 10^7$ |

**Table 5.1** Number of spleen cells from normal and 3dnTx mice.

Mice treated with DMBA showed a reduced number of total spleen cells. Normal mice contained more spleen cells than 3dnTx mice. Result represent the mean± standard deviation from 6 mice per group.

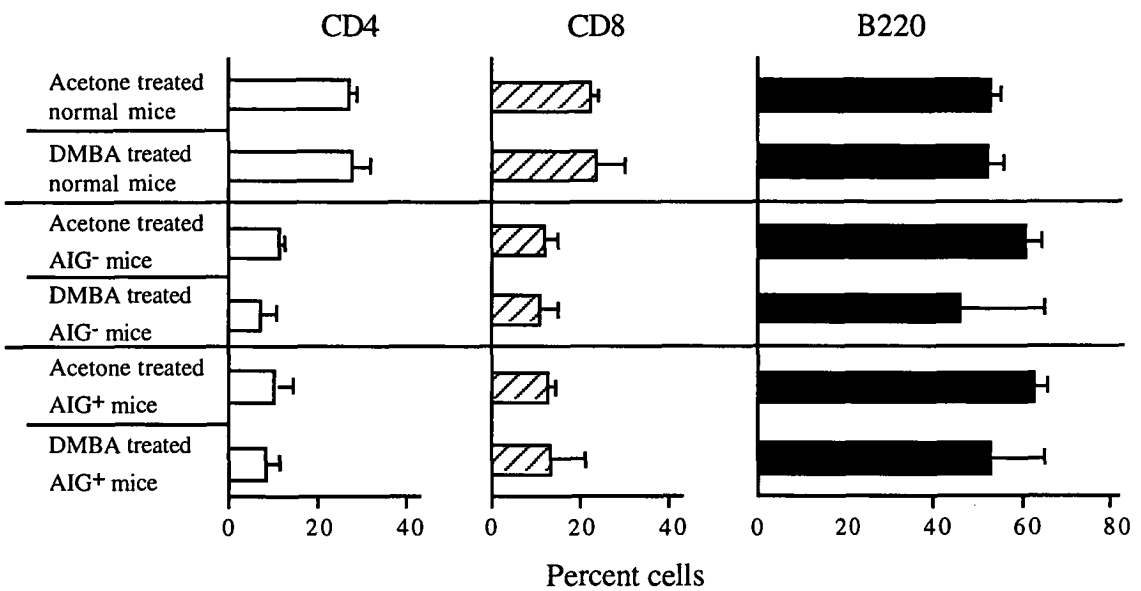
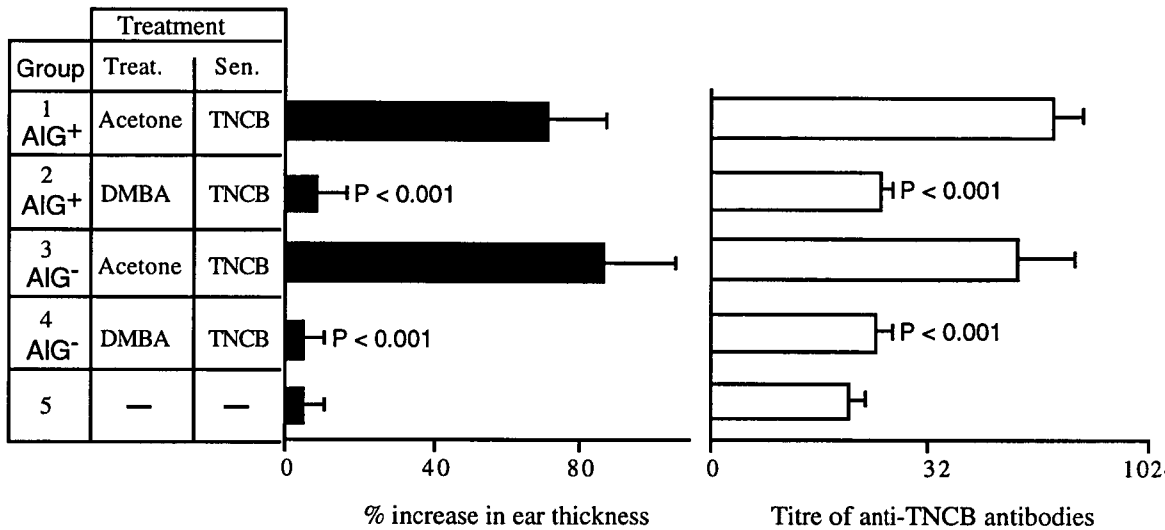



**Figure 5.4** Phenotypic analysis of lymphocytes from the spleen of normal and 3dnTx mice.

Normal mice (upper panel), AIG- 3dnTx mice (middle panel), or AIG+ 3dnTx mice (lower panel ) were treated with either the acetone vehicle or DMBA and the spleen removed 5 days later. Results represent the mean ± standard deviation for each phenotypic marker from each group.




Treat. = Treatment
Sen. = Sensitisation

**Figure 5.5** Induction of immunosuppression following DMBA/TNCB treatment of 3dnTx mice.

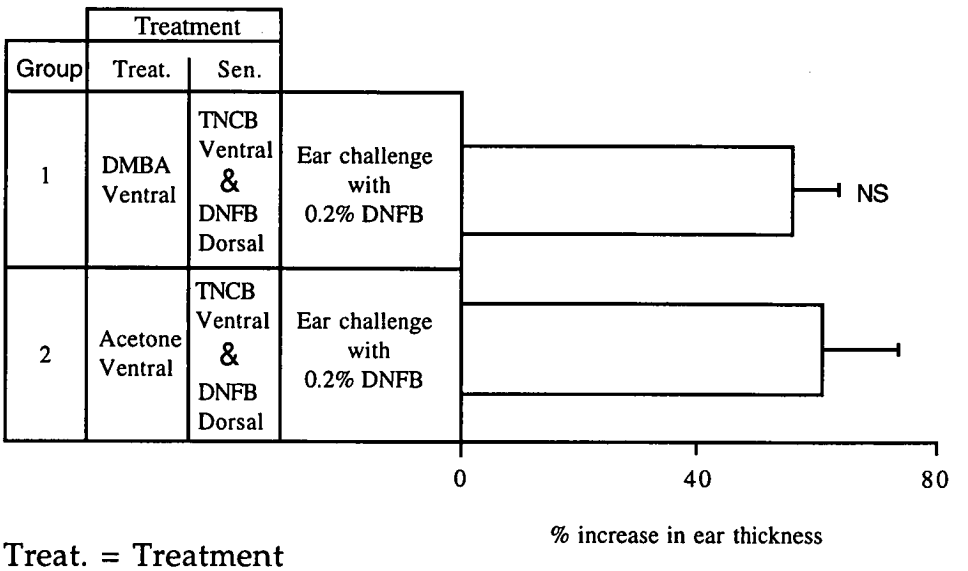
Thymectomised mice were subdivided into mice with disease (AIG+; groups 1 and 2) and mice without disease (AIG-; groups 3 and 4) and were treated with either 1% DMBA or acetone followed 5 days later with 2% TNCB. A further 5 days later contact sensitivity was assessed by ear challenge and increase in ear thickness was determined (left panel) and antibody levels were assessed by indirect haemagglutination (right panel). Control mice (group 5) received no treatment prior to ear challenge. Results represent mean ± standard deviation from at least 6 mice. P values were determined by comparing group 1 with group 2 and group 3 with group 4 using Student's unpaired t-test;

### 5.2.6 Induction of immunosuppression in 3dnTx mice by DMBA/TNCB treatment was antigen specific

To determine if the reduction of cell mediated response was antigen specific the non-cross reactive antigen DNFB was applied through the DMBA-treated skin of 3dnTx mice to render the mice tolerant to DNFB. When these mice were assessed for contact hypersensitivity to TNCB there was no significant reduction in cell mediated immunity compared to the control mice which were treated with acetone followed by DNFB (Figure 5.6). Consequently, applying DNFB through DMBA-treated skin did not induce tolerance to TNCB.

### 5.2.7 Induction of immunosuppression in 3dnTx mice by DMBA treatment had a systemic effect

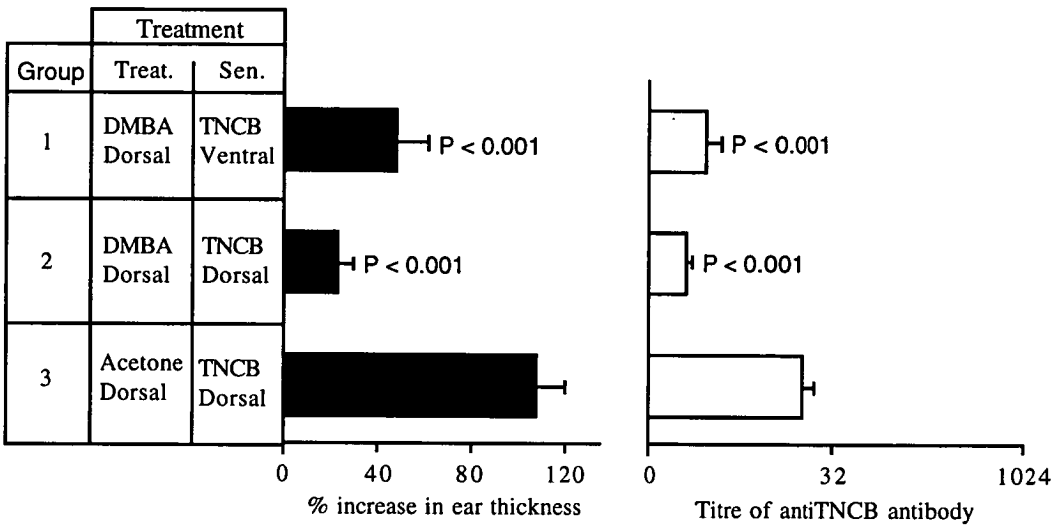
Normal mice treated with DMBA develop antigen specific suppression when antigen is applied through the LC-depleted skin (Halliday and Muller, 1986), but not when antigen is applied through normal untreated skin. Results shown in Figure 5.7 demonstrate that when antigen is applied through the abdominal skin of 3dnTx mice treated with DMBA on the dorsal skin there is a significant reduction in both contact sensitivity and in antibody production. Thus in 3dnTx mice DMBA-treatment resulted in a systemic effect on immunosuppression.




Treat. = Treatment
Res. = Resensitisation
NS = No significant difference

**Figure 5.6** Induction of immunosuppression in 3dnTx mice is antigen specific.

Groups of mice were treated with acetone or DMBA on shaved abdominal skin. Five days later, groups of mice were sensitised by applying TNCB to the treated (abdominal) skin and DNFB to the untreated (dorsal skin). A further 5 days later contact sensitivity to DNFB was assessed by ear challenge and increase in ear thickness was determined (CHS). Results represent the mean ± standard deviation from at least 6 mice. P value was determined by comparing group 1 with group 2 using Student's unpaired t-test.




Treat. = Treatment
Sen. = Sensitisation

**Figure 5.7** Induction of immunosuppression in 3dnTx mice is local and systemic.

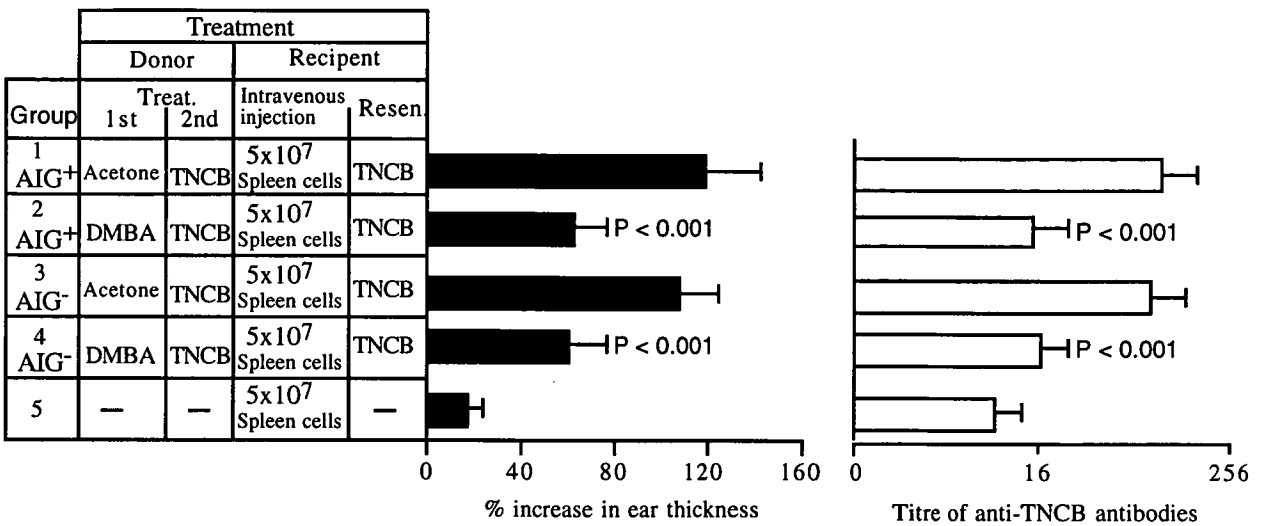
Groups of mice were treated with acetone or DMBA on dorsal skin. Five days later, the mice were sensitised by applying TNCB to the treated (dorsal) skin or to the untreated (ventral) skin. A further 5 days later contact sensitivity to TNCB was assessed by ear challenge and increase in ear thickness was determined (CHS) and antibody levels were assessed by indirect haemagglutination to TNCB (Ab production). Results represent the mean ± standard deviation from at least 6 mice. P values were determined by comparing group 1 with group 2 and group 1 with group 3 using Student's unpaired t-test.

### 5.2.8 Generation of transferable suppression in 3dnTx mice

The ability to induce immunosuppression in 3dnTx mice posed the question of the ability of these mice to produce antigen specific suppressor cells. Results illustrated in Figure 5.8 demonstrate that transferable suppression was induced by DMBA/TNCB treatment of 3dnTx mice. Adoptive transfer of the spleen cells from these mice transferred suppression as the ability of the recipient mice to respond to TNCB was significantly reduced when compared to the control mice which received normal spleen cells. This transfer of suppression was observed in all 3dnTx mice irrespective of whether they developed AIG, and both contact sensitivity and antibody production was suppressed.

### 5.2.9 Generation of antigen specific suppressor cells in 3dnTx mice

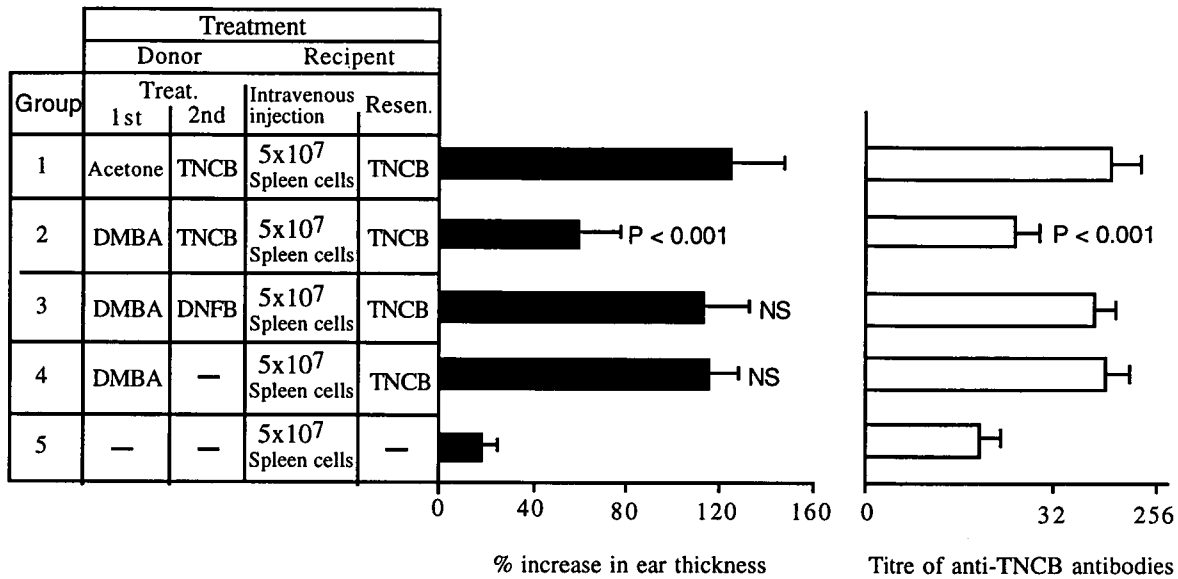
To determine whether spleen cells from TNCB-tolerant 3dnTx mice (i.e. 3dnTx mice treated with DMBA and TNCB) could also suppress cell mediated and humoral immune responses in an antigen specific manner, spleen cells were transferred from other hapten-tolerant 3dnTx mice to 3dnTx mice. Spleen cells of DMBA alone treated 3dnTx mice were also transferred to 3dnTx mice to determine whether DMBA alone could generate suppressor cells. As shown in Figure 5.9, recipient mice that received spleen cells from 3dnTx mice treated with DMBA followed by the antigen DNFB, did not have any reduction in either contact sensitivity or antibody production to TNCB, whereas recipient normal mice that received spleen cells from 3dnTx mice treated with DMBA followed by TNCB had a




Treat. = Treatment
Resen. = Resensitisation

**Figure 5.8** Adoptive transfer of immunosuppression.

Spleen cells were transferred to naive mice from TNCB tolerant 3dnTx mice which had been subdivided into mice with disease (AIG+: groups 1 and 2) and mice without disease (AIG-: groups 3 and 4) and were treated cutaneously with either 1% DMBA or acetone followed 5 days later with 2% TNCB at the same site. Five days after transfer all mice (except group 5) were sensitized with 2% TNCB and a further 5 days later contact sensitivity was assessed by ear challenge and increase in ear thickness was determined (left panel) and antibody levels were assessed by indirect haemagglutination (right panel). Control mice (group 5) received spleen cells from 3dnTx mice that received no treatment. Results represent mean ± standard deviation from at least 6 mice. P values were determined by comparing group 1 with group 2 and group 3 with group 4 using Student's unpaired t-test.




Treat. = Treatment
Resen. = resensitisation
NS = No significant difference

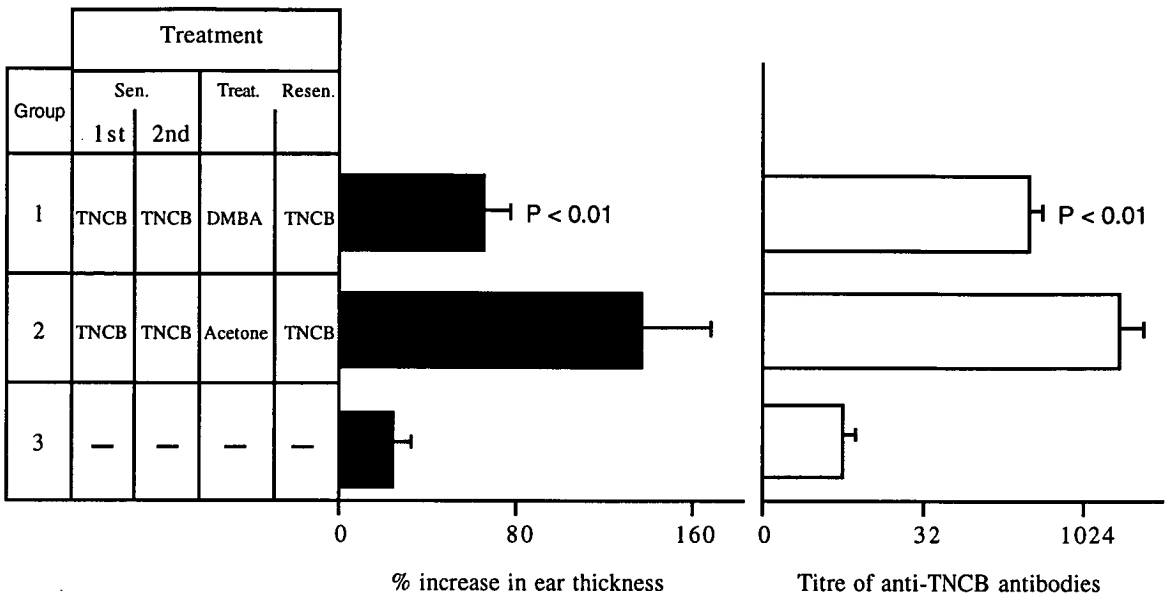
**Figure 5.9** Adoptive transfer of antigen specific immunosuppression.

Spleen cells were transferred from TNCB or DNFB tolerant 3dnTx mice which had been treated cutaneously with either acetone or 1% DMBA followed 5 days later with 2% TNCB (group 1 and group 2), 1% DMBA followed 5 days later by 2% DNFB (group 3), 1% DMBA only (group 4). Five days after transfer all mice sensitized challenged with 2% TNCB and a further 5 days later contact sensitivity was assessed by ear challenge and increase in ear thickness was determined (left panel) and antibody levels were assessed by indirect haemagglutination (right panel). Control mice (group 5) received no treatment prior to ear challenge. Results represent mean ± standard deviation from at least 6 mice. P values were determined by comparing group 1 with group 2 and group 3 with group 4 using Student's unpaired t-test.

significantly reduced contact sensitivity and antibody response when challenged with TNCB thus indicating antigen specificity (Figure 5.9). To determine whether a single application of DMBA alone to the dorsal skin of 3dnTx mice also had the ability to generate transferable suppressor cells, the spleen cells of these DMBA-treated mice were transferred to normal mice and no suppression was observed, thus DMBA treatment alone does not cause antigen-specific suppression.

### 5.2.10 DMBA/TNCB treatment reduced contact sensitivity and antibody production in TNCB-immune 3dnTx mice

To determine whether DMBA/TNCB treatment of 3dnTx mice could also suppress cell mediated and humoral immune responses of mice with an established immune response to TNCB, 3dnTx mice pre-immunised with TNCB were treated with DMBA followed by TNCB application. The control was acetone followed by TNCB. When the antigen TNCB was applied through the DMBA-treated skin of TNCB-immune 3dnTx mice there was a significant reduction in the level of contact sensitivity and antibody production to TNCB (Figure 5.10). Although this reduction was significant, it was not a complete reduction as both contact sensitivity and antibody levels to TNCB were higher than the control group (group 3), that had not been exposed to TNCB until they were ear challenged. Consequently, the application of TNCB through DMBA-treated skin causes a significant but not complete reduction of both cell mediated and humoral immunity in immune 3dnTx mice.




Sen. = Sensitisation
Treat. = Treatment
Resen. = Resensitised with 2% TNCB

**Figure 5.10** DMBA/TNCB treatment in 3dnTx mice reduces established contact sensitivity and antibody responses.

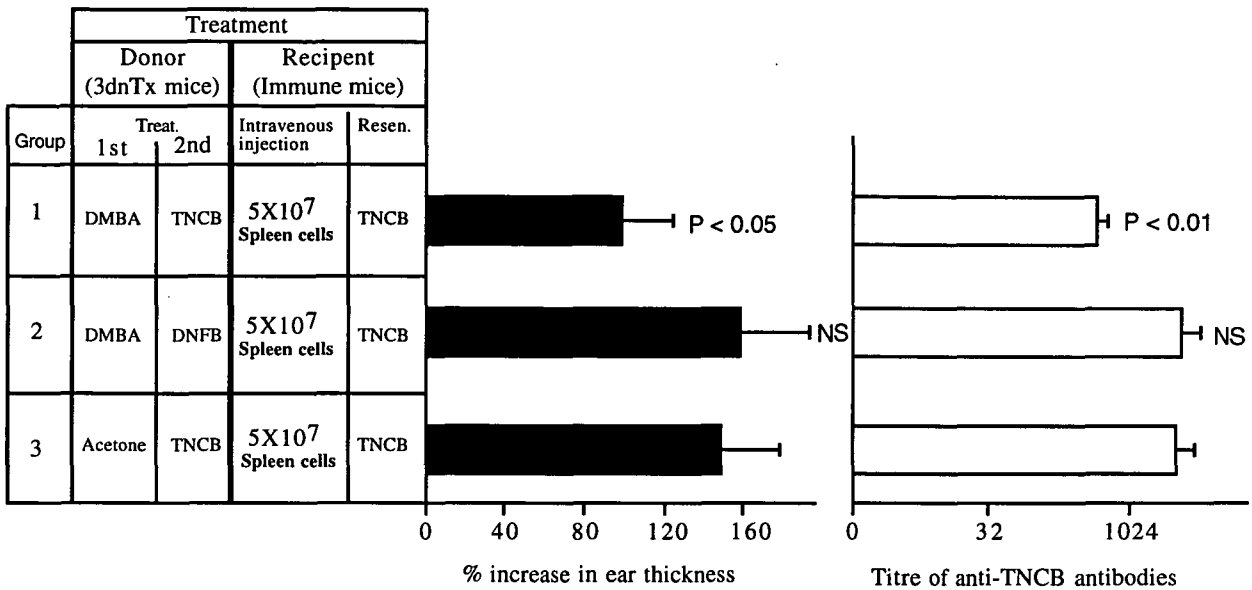
TNCB-immune 3dnTx mice were sensitised by immunising through the skin with two separate applications (1st, 2nd) of 2% TNCB. These mice were then treated with acetone (group 2) or 1% DMBA (group 1) and five days later they were resensitised through the treated skin with 100µl of 2% TNCB. A further 5 days later contact sensitivity was assessed by ear challenge and increase in ear thickness was determined (left panel) and anti-TNCB antibody levels were assessed by indirect haemagglutination (right panel). Naive mice (group 1) were untreated throughout but were ear challenged with TNCB. Results represent the mean ± standard deviation from at least 6 mice. P values were determined by comparing group 2 and 3 using Student's unpaired t-test.

### 5.2.11 Reduction of contact sensitivity and antibody production in TNCB-immune mice following adoptive transfer of spleen cells from TNCB-tolerant 3dnTx mice

It was next determined if spleen cells from TNCB-tolerant mice could also downregulate cell mediated and humoral immune responses of TNCB-immune mice. This was performed by transferring spleen cells from TNCB-tolerant 3dnTx mice to TNCB-immune mice and contact sensitivity and antibody levels to TNCB were evaluated. As shown in Figure 5.11, the transfer of spleen cells from TNCB-tolerant 3dnTx mice (group 1) to TNCB-immune mice caused a significant reduction in both contact sensitivity and antibody responses to TNCB. This reduction was antigen specific as spleen cells transferred from DNFB-tolerant 3dnTx mice (group 2) to TNCB-immune mice did not reduce either contact sensitivity or antibody levels to TNCB. Consequently the transfer of spleen cells from TNCB-tolerant 3dnTx mice to TNCB-immune mice caused antigen specific reduction in both cell mediated and humoral immunity.

### 5.2.12 LC density in nu/nu mice following DMBA-treatment

As the induction of immunosuppression was observed in 3dnTx mice, the next logical step was to determine if the induction of immunosuppression could also be observed in athymic mice. For these experiment nu/nu BALB/c mice were used. Before examining this question, LC depletion following DMBA treatment was evaluated as LC depletion is an important indication of skin containing the ability to generate




Treat. = Treatment
Resen. = Resensitisation
NS = No significant difference

**Figure 5.11** Downregulation of established immune response by suppressor cells generated from DMBA/TNCB treated 3dnTx mice.

Recipent TNCB-immune mice were sensitised by immunising through the skin with two separate applications (1st, 2nd) of 2% TNCB. These mice received spleen cells from donor mice treated either with acetone followed 5 days later by 2% TNCB (group 3 - control), 1% DMBA followed 5 days later by 2% TNCB (group 1 - TNCB tolerant), or 1% DMBA followed 5 days later by 0.5% DNFB (group 2 - DNFB tolerant). A further 5 days following spleen cell transfer contact sensitivity was assessed by ear challenge and increase in ear thickness was determined (left panel) and antibody levels were assessed by indirect haemagglutination (right panel). Results represent the mean ± standard deviation from at least 6 mice. P values were determined by comparing group 3 with group 1 and group 3 with group 2 using Student's unpaired t-test.

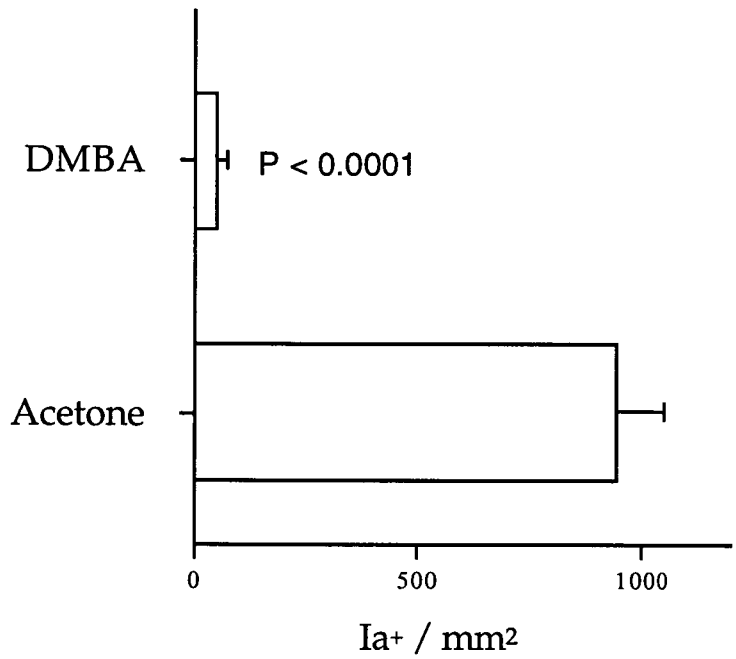



**Figure 5.12** Number of LC residing in the epidermis of nu/nu mice five days after DMBA (test) or acetone treatment (control).

Results represent the mean ± standard deviation from at least 6 mice. P value was determined by Student's unpaired t-test.

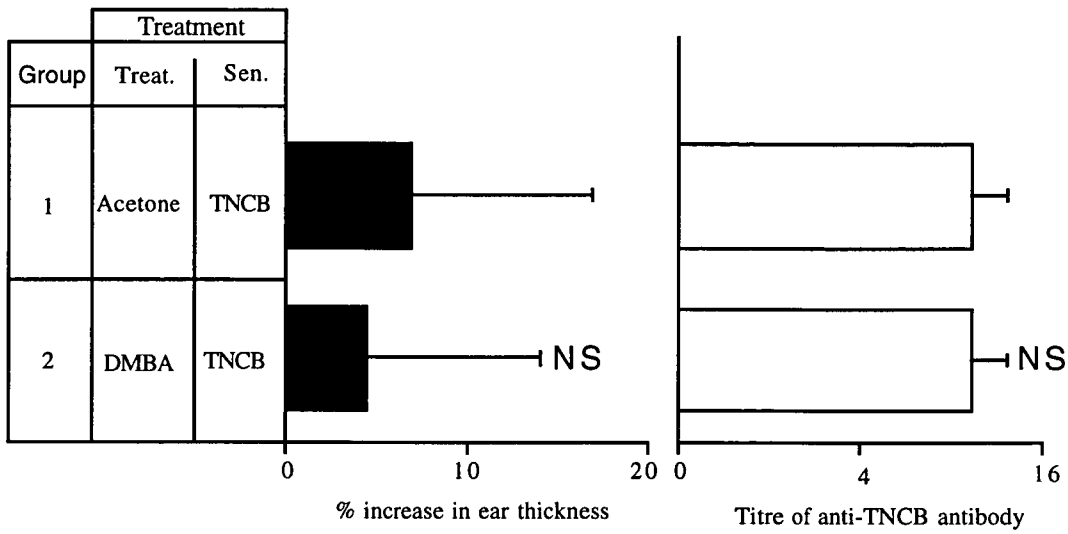
immunosuppression. As with normal and 3dnTx mice, LC numbers were significant depleted in DMBA-treated nu/nu mice (Figure 5.12).

### 5.2.13 Contact hypersensitivity and antibody production following DMBA/TNCB treatment in nu/nu mice

When TNCB was applied through the DMBA-treated or vehicle treated skin of nu/nu mice there was neither a contact sensitivity nor antibody response (Figure 5.13). While this demonstrates that T cells have a critical role in the contact sensitivity response it does not provide any additional information on the generation of suppression.

### 5.2.14 Contact hypersensitivity and antibody production of normal mice after transferring spleen cells from nu/nu mice following DMBA/TNCB

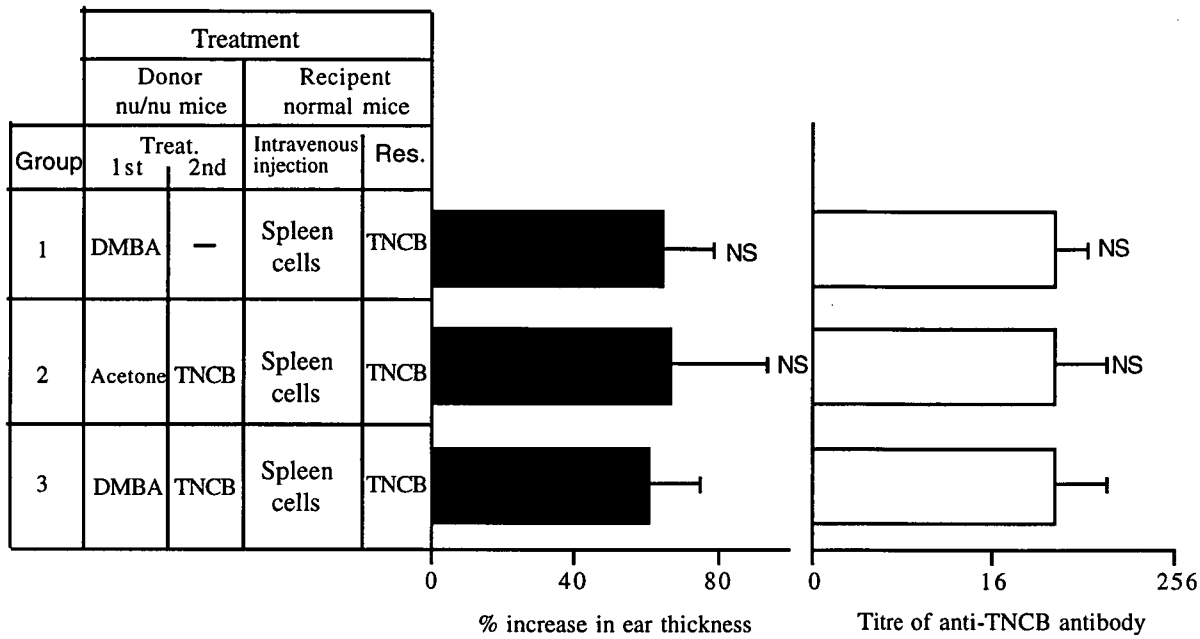
A key element for the induction of tolerance in this study is the generation of suppressor cells. Spleen cells from nu/nu mice treated with DMBA or acetone followed by TNCB or DMBA alone were adoptively transferred to the normal naive mice. Results show that none of the three groups transferred tolerance indicating that thymic derived cells are important for the generation of antigen specific suppression (Figure 5.14).




Treat. = Treatment
Res. = Resensitisation
NS = No significant difference

**Figure 5.13** Effect of DMBA/TNCB treatment on contact sensitivity in nu/nu mice.

The nu/nu mice were treated with either 1% DMBA or acetone followed 5 days later with 2% TNCB. A further 5 days later contact sensitivity was assessed by ear challenge and increase in ear thickness was determined (left panel) and antibody levels were assessed by indirect haemagglutination (right panel). Results represent mean ± standard deviation from at least 6 mice. P values were determined using Student's unpaired t-test;




Treat. = Treatment
Res. = Resensitisation
NS = No significant difference

**Figure 5.14** Adoptive transfer of immunosuppression following transfer of spleen cells to naive mice from nu/nu mice.

Nu/nu mice were treated with either 1% DMBA or acetone followed 5 days later with 2% TNCB. Five days after transfer all mice (except group 1) were challenged with 2% TNCB and a further 5 days later contact sensitivity was assessed by ear challenge and increase in ear thickness was determined (left panel) and antibody levels were assessed by indirect haemagglutination (right panel). Results represent mean ± standard deviation from at least 6 mice. P values were determined by comparing group 1 with group 2 and group 1 with group 3 using Student's unpaired t-test.

## 5.3 Discussion

When mice are treated with agents that deplete epidermal Langerhans cells an immunosuppressive environment is established such that any new antigen applied through the skin causes an antigen-specific immunosuppression (Halliday and Muller, 1986) that is transferable via spleen cells (Halliday and Muller, 1987b). In this study, we evaluated the capacity of 3dnTx mice to generate this form of antigen-specific suppression. As T cells develop into $CD4^+$ and $CD8^+$ T cells in the thymus, removal of the thymus in the neonatal period should not be expected to shift the balance of the T cell subpopulations which have already been exported to the periphery. However, it is not known whether neonatal thymectomy would remove the precursor T cells which mediate antigen-specific suppression. Further, as thymectomy 3 days after birth results in autoimmunity in more than half of these mice (Kojima and Prehn, 1981) and as autoimmunity can occur as a consequence of perturbations in maintenance of tolerance mechanisms the ability of 3dnTx mice to develop peripheral suppressor mechanisms by applying antigen through LC-depleted skin was evaluated. The 3dnTx mice had a reduced ability to respond to PHA, an observation which is consistent with that of Ward and colleagues (Ward *et al.*, 1986). This is most likely a consequence of the reduced number of T cells while B cells remained unaltered, hence there was a relative increase in the proportion of B cells which would not respond effectively to PHA. The reduction of T cells was not due to a selective loss of a specific lymphocyte

population as both $CD4^+$ and $CD8^+$ T cells were present in the spleen at a normal ratio.

In contrast to the reduced ability to respond to PHA stimulation, the 3dnTx mice displayed a relatively normal contact sensitivity response. This was an interesting observation and suggests that there exists a relatively large pool of precursor T cells that can respond to TNCB, which is normally coupled to proteins within the skin in order to be recognised. Further, PHA-responsiveness relies on cell proliferation whereas the ability to generate a CHS relies not only on cell proliferation but inflammation and T cell infiltration, which are still functional in the 3dnTx mice. When TNCB was applied through DMBA-treated skin of 3dnTx mice there was a failure to induce humoral and cellular immunity in both the $AIG^+$ and $AIG^-$ groups of 3dnTx mice. This failure to induce immune responses was antigen specific as another hapten, DNFB, applied through DMBA untreated skin did not induce immunosuppression. However, a suprising result was TNCB applied through DMBA untreated skin also caused immune suppression in 3dnTx mice. This was opposite to the results with normal mice treated with the same procedure and of 3dnTx mice treated with another hapten. As normal mice have more effective T cells available for activation than 3dnTx mice, normal mice can response to contact sensitisers more vigorously. While it has been shown that DMBA is a immunosuppressive agent (Thurmond *et al.*, 1987; Thurmond *et al.*, 1988; Ha *et al.*, 1993) the induction of CHS in normal mice was not affected by DMBA treatment. In the 3dnTx

mice, DMBA suppressed most T cell activity thus systemic immune suppression appeared. As DNFB induced immunity through DMBA untreated skin, this may be explained by the 3dnTx mice receiving two strong haptens (TNCB and DNFB) together, and although T cells have been suppressed by DMBA, these two haptens could still revive the immune response. It is also possible that these two haptens stimulate different subset of T cell populations. Hence, DNFB may activate a T cell population that is not actively suppressed by DMBA.

The present study found that antigen specific suppression was generated in both groups of thymectomised mice since adoptive transfer of spleen cells from these mice to naive mice also transferred antigen specific suppression. Confirmation that DMBA alone did not cause antigen specific suppression was obtained when transfer of spleen cells from mice treated with only DMBA did not transfer suppression. Thus 3dnTx mice can develop peripheral tolerance to the hapten TNCB and this developed regardless of whether the mice developed AIG.

Reflection on the above results leads to the suggestion that induction of peripheral tolerance may be possible in athymic mice, as immunosuppression was generated in 3dnTx mice. Study on nu/nu mice demonstrated that suppressor cells could not be detected through DMBA modified skin. The most likely reason that no suppressor cells generated in nu/nu mice is that they lack of T cells from the beginning of their life. As

thymus is the place for T cell maturation and T cells are responsible for the generation of immunosuppression, that no suppressor cells generated in nu/nu mice is not surprising.

While LC depletion was evident in the skin of nu/nu mice treated with DMBA, application of contact sensitiser through such skin did not induce comparable immune suppression. The incapability of nu/nu mice to generate suppressor cells may be due to the abnormal function of LC, as Grabbe and colleagues have demonstrated that LC of athymic nude mice have a deficient antigen-presenting function (Grabbe *et al.*, 1993). In the same study they also showed that transplantation of thymus can restore LC function suggesting the thymus is important for the complete function of LC.

The demonstration in this chapter that the downregulation of TNCB established immune response can be achieved by the application of TNCB through DMBA modified skin. This is an important observation and provides support for the notion that the treatment of AIG disease could be achieved through a similar procedure.

Recent investigations have provided important insights into the intriguing question as to why thymectomy should predispose to autoimmune gastritis. One proposition is that an alteration of homeostatic mechanisms will allow autoreactive T cells to be activated, rather than be suppressed, and that

autoimmmunity can be prevented following the restoration of homeostasis (Bonomo *et al.*, 1995b). This is consistent with the early observations that the transfer of normal spleen cells to 3dnTx mice can prevent the induction of autoimmunity and that the population of regulatory spleen cells appeared to map to a T cell ($Thy1.2^+$, $Ig^-$) population (Taguchi and Nishizuka, 1987; Taguchi *et al.*, 1990). Further refinement by Sakaguchi and coworkers has identified a subpopulation of $CD4^+$ T cells that regulate AIG induction (Sakaguchi *et al.*, 1996). This subpopulation of $CD4^+$ T cells is $CD25^+$ and adoptive transfer of this specific population from normal adult mice to 3dnTx mice prevents the development of AIG (Asano *et al.*, 1996) suggesting that this cell population controls the function and expansion of pathogenic $CD4^+$ T cells. It has been further proposed by Asano *et al.* (1996) that pathogenic autoimmune $CD4^+$ T cells are produced before day 3 and that the immunoregulatory $CD4^+$ $CD25^+$ T cells are produced after day 3 following birth. This explains why thymectomy at day 3 allows the pathogenic, but not immunoregulatory T cells to develop, hence predisposing to autoimmunity. The actual role and function of these cells awaits further clarification.

In contrast, the regulatory T cells involved in suppression of a contact sensitivity response following application of antigen through LC-depleted skin remain poorly defined. Our studies here suggest that within 3 days of birth precursor cells, that have the potential to develop into immunoregulatory cells against foreign antigens, such as TNCB, have

developed. From this information it is likely that these cells differ from the immunoregulatory $CD4^+$ $CD25^+$ T cells that control development of autoimmune gastritis. Nonetheless, preliminary results have also implicated a population of activated $CD4^+$ T cells as suppressing the induction of contact sensitivity (Rist, personal communication, manuscript in preparation). This being the case, it suggests that suppression is analogous to the activated $CD4^+$ $CD25^+$ regulators in autoimmune gastritis, with a different timing of their thymic development. While it is unlikely that there is a direct link between developing autoimmune gastritis and developing antigen specific suppression by immunising through DMBA-modified skin, the results from the present study indicate that peripheral tolerance can still be generated by the presentation of antigen by altered dendritic cells and as such all tolerance generating mechanisms were not abrogated in the 3dnTx mice that developed autoimmune gastritis. Moreover, despite the decline in lymphocyte numbers and the diminished PHA response, antigen-specific suppression was still retained.

In summary, a reduction in LC numbers in the skin of BALB/c mice allows for the development of antigen specific suppression, despite removal of the thymus 3 days after birth. Therefore in these mice, although lymphocyte numbers are reduced and the lymphocytes show a diminished proliferative response to PHA, precursor cells that account for this type of active immunosuppression are retained. The retained immunosuppressive ability of the peripheral T cells is trasferrable and is antigen specific. This provides

an opportunity to develop a unique strategy for the treatment of autoimmune disease.

# Chapter Six
# Treatment of Autoimmune Gastritis Through LC-depleted Skin

## 6.1 Introduction

Previous chapters have demonstrated that the application of a contact sensitiser through DMBA modified skin of either normal or thymectomised mice leads to the induction of antigen specific immunosuppression. Using this procedure both an established CHS and an antibody response were significantly downregulated. From these observations, and coupled to the fact that this suppression is long lived and systemic, it may be possible to regulate other immune responses by inducing tolerance through the skin. This is a particularly attractive proposal as it provides an opportunity to treat immune diseases, including autoimmunity. As antigen specific suppression was also observed with 3dnTx mice that were prone to autoimmune gastritis, the application of a defined antigen through LC-depleted skin in these mice could be exploited in order to downregulate autoimmune gastritis.

The 3dnTx autoimmune gastritis model has been well characterised and was discussed in the previous chapter. The autoantigen in this model is the immunogenic $H^+/K^+$ ATPase (proton pump). Immunisation with $H^+/K^+$ ATPase in Freund's complete adjuvant causes the development of AIG (Scarff *et al.*, 1997). Induction of antigen specific immunosuppression is an immune response that also requires an immunogenic antigen to be applied

through LC-depleted skin. The $H^+/K^+$ ATPase antigen was an appropriate antigen for these studies. The $H^+/K^+$ ATPase consists of α and β subunits (Toh *et al.*, 1990). Studies by Alderuccio *et al.* (1993) demonstrated that thymectomy-induced gastritis was prevented in transgenic mice when the β subunit of the gastric $H^+/K^+$ ATPase was expressed in the thymus. This suggested that the β subunit is a major autoantigen recognised by the pathogenic T cells. BALB/c mice which develop autoimmune gastritis following immunisation with murine gastric $H^+/K^+$ ATPase react with $H^+/K^+$ ATPase in lymphocyte proliferation assays *in vitro*. Using a series of 21-mer overlapping peptides, the immunogenic peptide reactive with these splenic T cells was identified and hereinafter will be referred to as "peptide 19" which was located at the C-terminus of the β subunit of the $H^+/K^+$ ATPase (B.H. Toh - personal communication). During the progress of this thesis peptide 19 became available and its soluble nature combined with an ability to stimulate T cells when appropriately presented, made it ideally suitable for use with skin applications. Although the 3dnTx mice had a reduced level of immunity they retained their capacity to generate a CHS response indicating that the skin contained functional LC and that there was a sufficient pool of naive T cells to be stimulated. More importantly, and by virtue of their ability to respond to contact sensitisers, these mice could also generate antigen specific suppression when treated with DMBA followed by the contact sensitiser. This antigen specific suppression could be adoptively transferred by spleen cells to normal mice, thus providing evidence that

3dnTx mice could generate antigen specific suppression. The selection of the 3dnTx model of autoimmune gastritis was because this disease induced by thymectomy persists for a lifetime, can be routinely screened during experimental procedures without the need for a further challenge of antigen and it represents an alteration in immunoregulation which is likely to mimic the disease situation. As the immunisation model needs to be regularly immunised this model would not be as effective in analysing the down regulation experiments.

In this chapter approaches to either down regulate autoimmune gastritis or to prevent development of disease were evaluated. In one series of experiments $H^+/K^+$ ATPase or peptide 19 was applied through DMBA-treated skin to generate antigen specific suppression as an approach to treat established disease. In an alternative series of experiments, peptide 19 was applied through neonatal skin as an approach to prevent development of disease.

## 6.2 Results

### 6.2.1 Stomach histology in mice with AIG

Histologically, the development of AIG in 3dnTx BALB/c mice was classified into three stages (Figure 6.1). Histology of normal stomach is shown in Figure 6.1A. In stage 1 (Figure 6.1B), mononuclear cells initially infiltrated along the subglandular region of the lamina propria and the parietal cells remain intact. In stage 2 (Figure 6.1C), these inflammatory cells

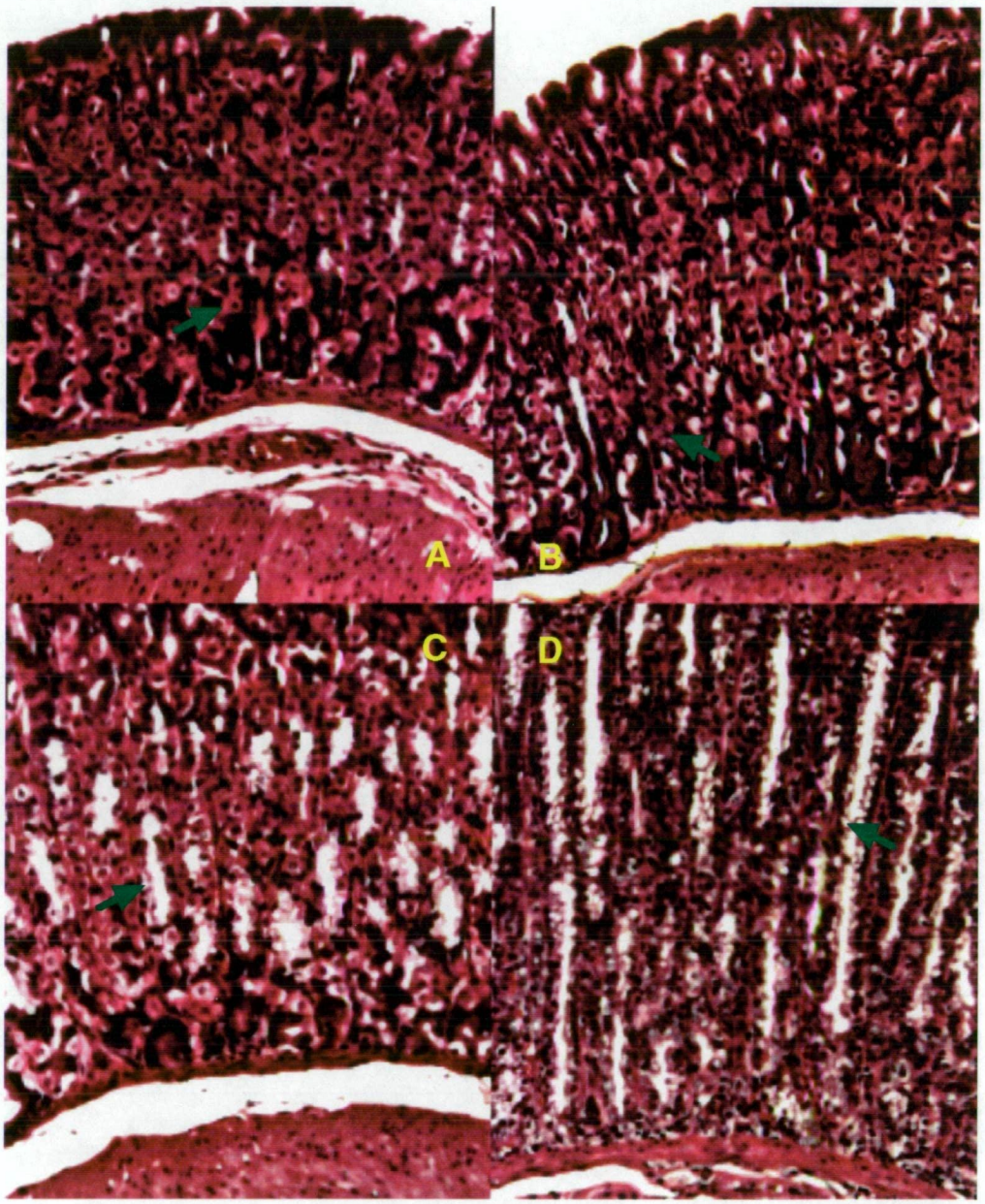



**Figure 6.1** Histology of AIG in 3dnTx BALB/c mice. The normal (A), first (B) , second (C) and third (D) stage of AIG spontaneously developing in 3dnTx BALB/c mice. Arrow in panel A represents normal parietal cell. Arrow in panel B represents lymphocytes start to infiltrate. Arrow in panel C represents initial destruction of parietal and chief cells. Arrow in panel D represents parietal cells replaced by mucous neck cells. (Original magnification X100).

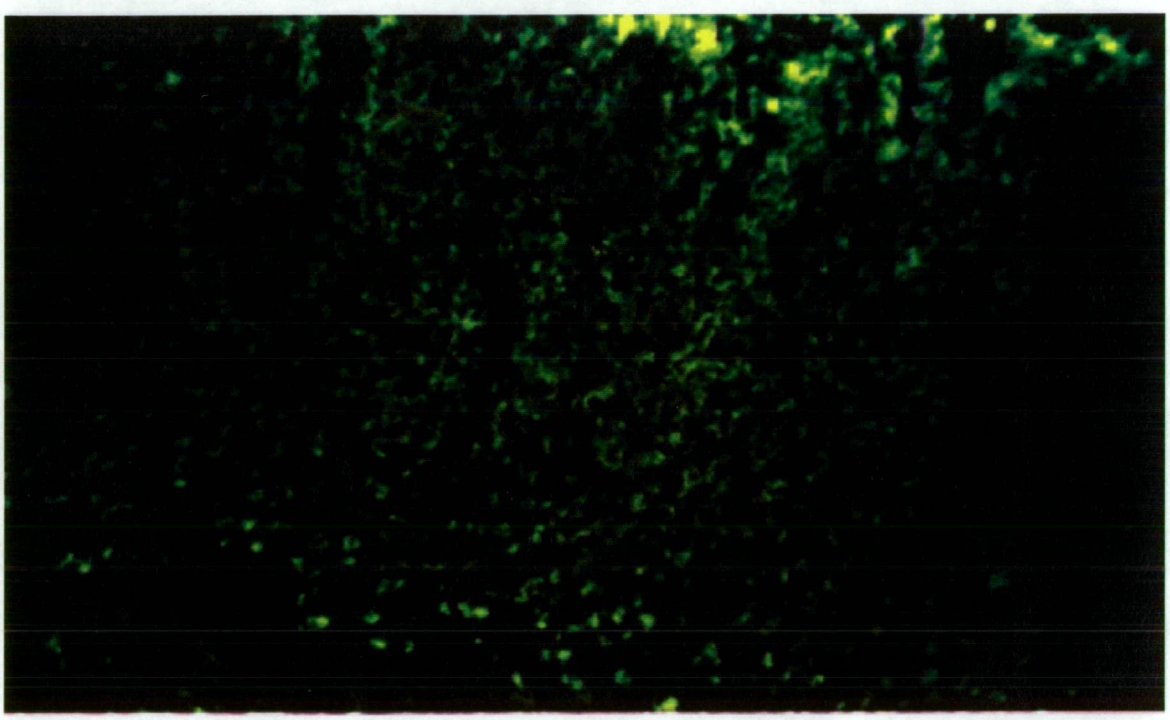


**Figure 6.2** Indirect immunofluorescence staining of the gastric mucosa of a normal mouse by 1:256 dilution of autoantibody from a 3dnTx mouse in stage 3 of AIG. (Original magnification X100).

expanded upwardly along the gland, coupled with the destruction of both parietal and chief cells. In stage 3, the gland became atrophic and was replaced by proliferating mucous neck cells (Figure 6.1D). In all mice showing histological features of AIG, autoantibodies against parietal cells were detected by immunofluorescence (Figure 6.2). The titres of autoantibodies generally correlated with the pathological changes observed with the different stages of AIG. The immunofluorescence titres were approximately $\leq 32$ in stage 1, between 64 and 128 in stage 2, and $\geq 256$ in the stage 3. However there was an occasional exception.

### 6.2.2 Treatment of 3dnTx mice with established autoimmune gastritis

Thymectomised mice with autoimmune gastritis and autoantibody titres $\geq 32$ which were treated with DMBA followed 5 days later by $H^+/K^+$ ATPase or peptide 19 showed significantly decreased autoantibody titres compared to mice treated with acetone alone. This was observed at both 35 and 50 days post treatment (Figure 6.3). However, a significantly reduced autoantibody titre was also observed with the group treated with DMBA alone. Consequently application of DMBA to the skin of 3dnTx mice with AIG caused a reduction in autoantibody titres.

A summary of the histological changes following DMBA treatment is shown in Table 6.1. Of the 5 mice treated with DMBA followed by $H^+/K^+$ ATPase (group 2), 4 mice had evidence for either stage 2 or stage 3 pathological changes. Of the 5 mice treated with DMBA followed by peptide

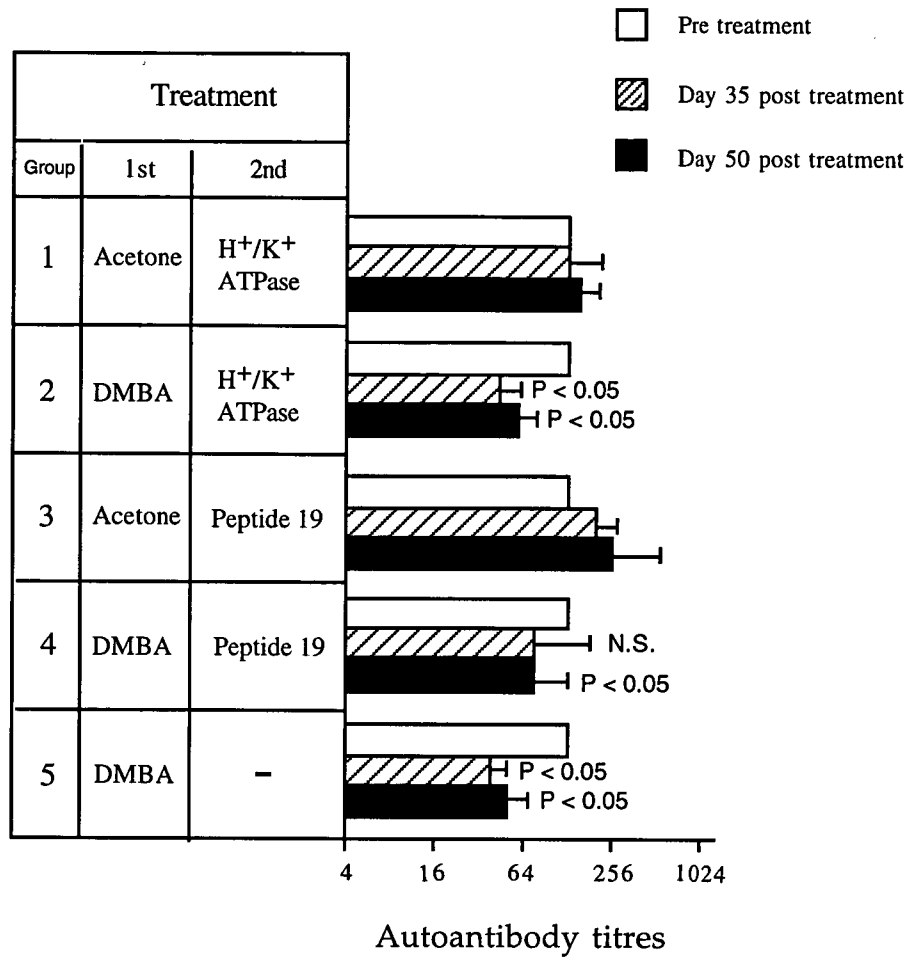



**Figure 6.3** Autoantibody titres of AIG positive mice after treatment with DMBA followed by H+/K+ ATPase or peptide 19.

The shaved dorsal trunk skin of 3dnTx mice with AIG was treated with either acetone followed by H+/K+ ATPase (group 1), DMBA followed by H+/K+ ATPase (group 2), acetone followed by peptide 19 (group 3), DMBA followed by peptide 19 (group 4), or DMBA alone (group 5). The open columns represent the autoantibody titres of the 3dnTx mice prior to treatment. The hatched columns represent autoantibody titres after 35 days since first treatment and the solid columns represent autoantibody titres tested after 50 days since first treatment. Results represent mean ± standard deviation from 5 mice per group. Comparisons were made between the different treatments at day 35 and day 50, group 1 was compared to group 2, group 3 was compared to group 4, and group 5 was compared to group 1 or group 3, using Student's two-tailed, unpaired t-test. DMBA followed by H+/K+ ATPase or peptide 19 or DMBA alone appeared to reduce autoantibody titres.

| Treatment | Pathology | Autoantibody titre | Number |
|---|---|---|---|
| (Group 1; 5 mice) | Normal | | 0 |
| Acetone followed by $H^+/K^+$ ATPase | Stage 1 | 128 | 1 |
| | Stage 2 | 128 | 1 |
| | Stage 3 | 128 128 256 | 3 |
| (Group 2; 5 mice) | Normal | | 0 |
| DMBA followed by $H^+/K^+$ ATPase | Stage 1 | 32 | 1 |
| | Stage 2 | 64 64 | 2 |
| | Stage 3 | 64 64 | 2 |
| (Group 3; 5 mice) | Normal | | 0 |
| Acetone followed by peptide 19 | Stage 1 | 64 | 1 |
| | Stage 2 | 256 256 | 2 |
| | Stage 3 | 256 1024 | 2 |
| (Group 4; 5 mice) | Normal | | 0 |
| DMBA followed by peptide 19 | Stage 1 | 32 | 1 |
| | Stage 2 | 32 64 | 2 |
| | Stage 3 | 64 128 | 2 |
| (Group 5; 5 mice) | Normal | | 0 |
| DMBA alone | Stage 1 | 32 | 1 |
| | Stage 2 | 32 64 64 | 3 |
| | Stage 3 | 64 | 1 |

**Table 6.1** Comparison of autoantibody titres and pathological changes of 3dnTx mice with autoimmune gastritis 50 days post treatment.

19 (group 4), 4 mice had evidence for either stage 2 or stage 3 pathological changes. This was the same result for the DMBA alone treatment (group 5) and acetone followed by $H^+/K^+$ ATPase (group 1) or peptide 19 (group 3) treated group. Consequently, the evidence indicates that DMBA treatment may suppress autoantibody production but it does not affect lymphocyte infiltration towards the base of the glands and the destruction of both parietal and chief cells.

### 6.2.3 Effect of spleen cell transfer from normal mice treated with DMBA and $H^+/K^+$ ATPase to 3dnTx mice with autoimmune gastritis

Although DMBA followed by $H^+/K^+$ ATPase appeared to cause downregulation of autoantibody production it might still be possible that antigen specific suppressor cells were generated. To assess this possibility normal mice were treated with DMBA followed by $H^+/K^+$ ATPase and the spleen cells were adoptively transferred to 3dnTx mice with established AIG. As there was no evidence for a reduction in autoantibody levels (Figure 6.4), it was unlikely that antigen specific suppressor cells were produced that could downregulate established autoimmune gastritis when the intact $H^+/K^+$ ATPase was applied through DMBA-treated skin. Confirmation that adoptive spleen cell transfer did not suppress disease development can be seen from the histology (Table 6.2). All of the 5 mice that received spleen cells from donor mice treated with DMBA followed by $H^+/K^+$ ATPase had evidence for stage 2 and stage 3 pathological changes. This is the same result for DMBA alone and acetone followed by $H^+/K^+$ ATPase treatment.

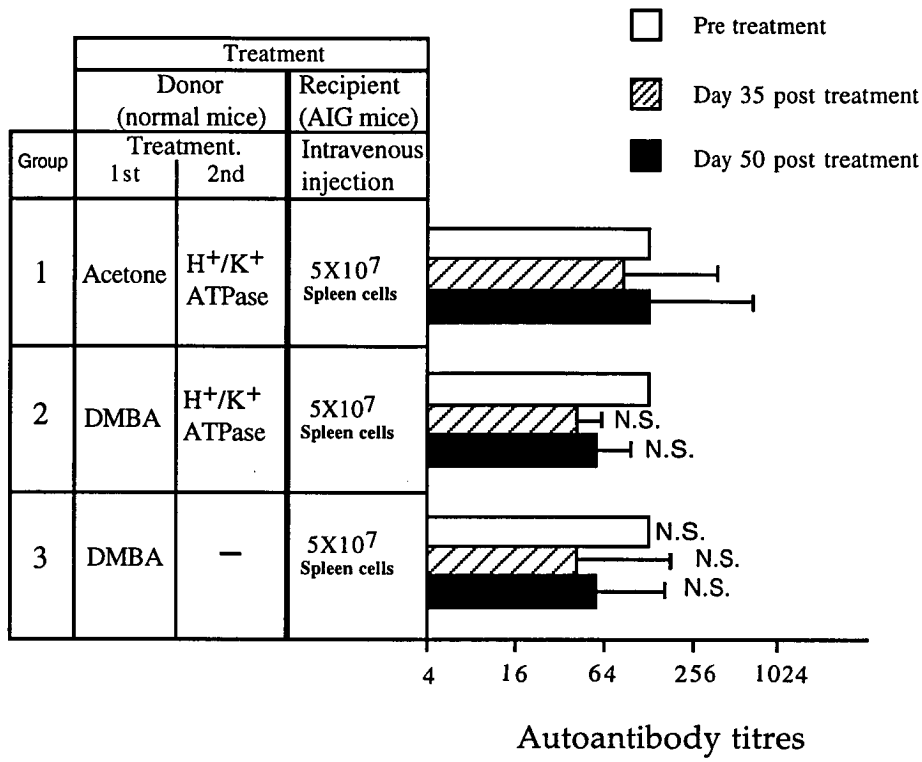



**Figure 6.4** Autoantibody titres of AIG mice after receiving spleen cells from normal mice treated with DMBA followed by $H^+/K^+$ ATPase.

Shaved dorsal trunk skin of 3dnTx mice with AIG was treated with either acetone followed by $H^+/K^+$ ATPase (group 1) DMBA followed by $H^+/K^+$ ATPase (group 2), or DMBA alone (group 3). The spleen cells from these were adoptively transferred to 3dnTx mice with AIG. The open columns represent the autoantibody titres of the 3dnTx mice that were selected prior to treatment. The hatched columns represent autoantibody titres after 35 days since spleen cells transfer and the solid columns represent autoantibody titres tested after 50 days since spleen cells transfer. Results represent mean ± standard deviation from 5 mice per group. Comparisons were made between the different treatments at day 35 and day 50, group 1 was compared to group 2 and to group 3 using Student's two-tailed, unpaired t-test. DMBA followed by $H^+/K^+$ ATPase or DMBA alone did not appeare to reduce autoantibody titres.

| Treatment | Pathology | Autoantibody titre | | | | Number |
|---|---|---|---|---|---|---|
| (Group 1; 5 mice) | Normal | | | | | 0 |
| AIG mice received spleen cells from Acetone followed by $H^+/K^+$ ATPase treated mice | Stage 1 | | | | | 0 |
| | Stage 2 | 16 | | | | 1 |
| | Stage 3 | 64 | 64 | 512 | 1024 | 4 |
| (Group 2; 5 mice) | Normal | | | | | 0 |
| AIG mice received spleen cells from DMBA followed by $H^+/K^+$ ATPase treated mice | Stage 1 | | | | | 0 |
| | Stage 2 | 32 | 32 | | | 2 |
| | Stage 3 | 64 | 64 | 128 | | 3 |
| (Group 3; 5 mice) | Normal | | | | | 0 |
| AIG mice received spleen cells from DMBA alone treated mice | Stage 1 | | | | | 0 |
| | Stage 2 | 16 | 32 | 64 | | 3 |
| | Stage 3 | 32 | 256 | | | 2 |

**Table 6.2** Comparison of autoantibody titres and pathological changes of 3dnTx mice with autoimmune gastritis 50 days after receiving spleen cells.

Consequently, the evidence indicates that DMBA treatment does not induce suppression that can be transferred by spleen cells.

### 6.2.4 Alternative strategies to deplete LC and establish an immunosuppressive environment

As DMBA treatment caused non-specific suppression an alternative strategy was required to deplete LC without causing generalised suppression. The contact sensitiser TNCB was selected because at a high dose it is able to deplete epidermal LC in normal mice; when a second antigen is applied through this skin antigen specific suppression results (Woods *et al.*, 1996).

#### 6.2.4.1 LC density in 3dnTx mice following TNCB-treatment

The ability of TNCB to deplete LC from 3dnTx mice was evaluated to confirm that LC depletion also occurred in 3dnTx mice and that the mice did not suffer any stress or discomfort. Results shown in Figure 6.5 demonstrate that topical application of 2% TNCB caused a significant reduction in LC density compared to acetone treated controls. The mice did not exhibit any undue distress or discomfort.

#### 6.2.4.2 Effect of 2% TNCB followed by peptide 19 in 3dnTx mice with autoimmune gastritis

Thymectomised mice with autoimmune gastritis and autoantibody titres $\geq 32$ which were treated with 2% TNCB followed by peptide 19 did not demonstrate any evidence of antigen specific suppression as there was no

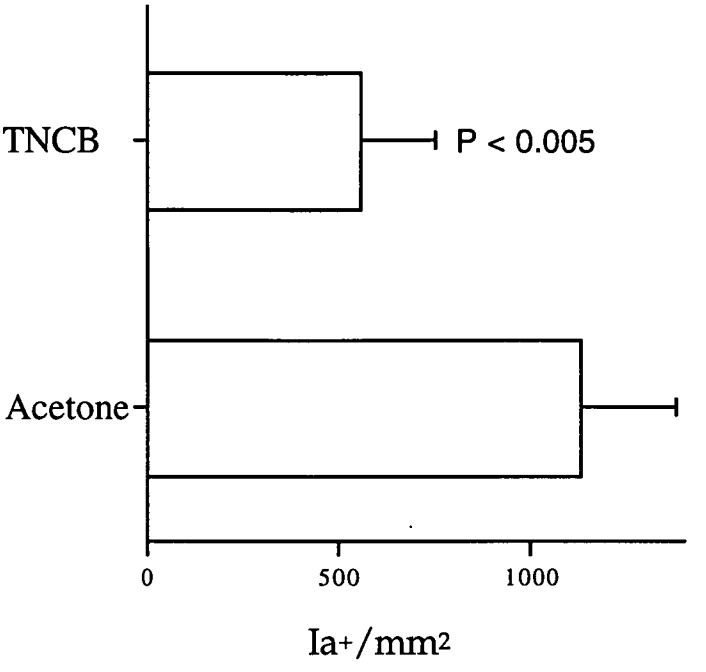



**Figure 6.5** Number of LC in the epidermis five days after TNCB (test) or acetone treatment (control).

Results represent the mean ± standard deviation from at least 6 mice. P value was determined by Student's unpaired t-test.

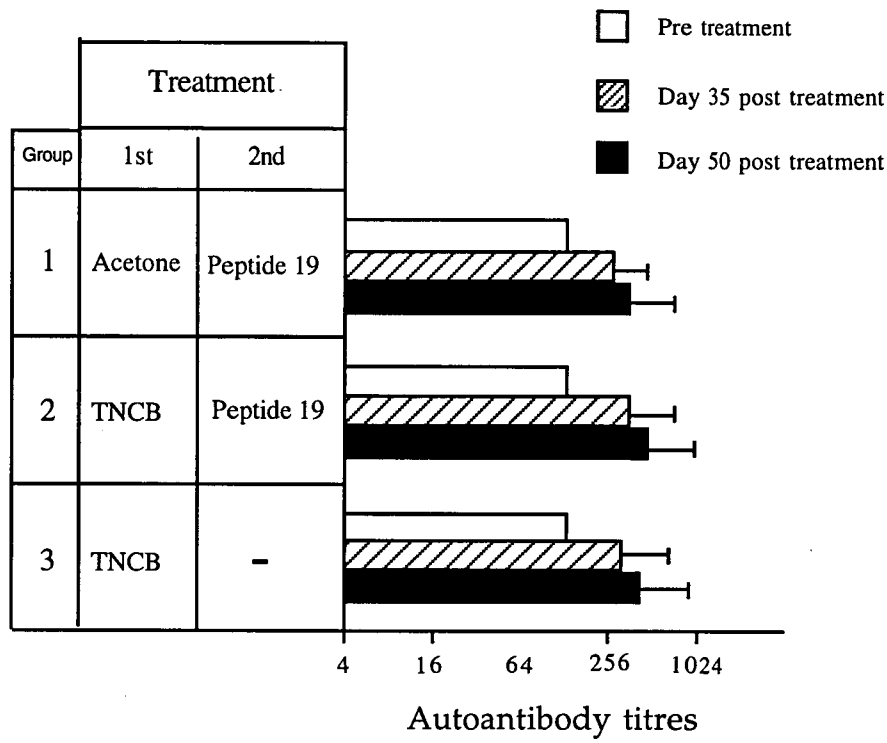



**Figure 6.6** Autoantibody titres of AIG positive mice after treatment with TNCB followed by peptide 19.

Shaved dorsal trunk skin of 3dnTx mice with AIG was treated with either acetone followed by peptide 19 (group 1), TNCB followed by peptide 19 (group 2), or TNCB alone (group 3). The open columns represent the autoantibody titres of the 3dnTx mice that were selected prior to treatment. The hatched columns represent autoantibody titres after 35 days since first treatment and the solid columns represent autoantibody titres tested after 50 days since first treatment. Results represent mean ± standard deviation from 6 mice per group. Comparisons were made between the different treatments at day 35 and day 50, group 1 was compared to group 2 and to group 3 using Student's two-tailed, unpaired t-test. TNCB followed by peptide 19 or TNCB alone did not reduce autoantibody titres.

| Treatment | Pathology | Autoantibody titre | Number |
|---|---|---|---|
| (Group 1; 6 mice) | Normal | | 0 |
| Acetone followed by peptide 19 | Stage 1 | | 0 |
| | Stage 2 | 128 128 | 2 |
| | Stage 3 | 128 256 256 1024 | 4 |
| (Group 2; 6 mice) | Normal | | 0 |
| TNCB followed by peptide 19 | Stage 1 | | 0 |
| | Stage 2 | 128 256 | 2 |
| | Stage 3 | 256 256 512 512 | 2 |
| (Group 3; 6 mice) | Normal | | 0 |
| TNCB alone | Stage 1 | | 0 |
| | Stage 2 | 128 128 256 | 3 |
| | Stage 3 | 256 512 512 | 3 |

**Table 6.3** Comparison of autoantibody titres and pathological changes of 3dnTx mice with autoimmune gastritis 50 days post treatment.

difference in the autoantibody titres of these mice compared to both 2% TNCB treatment alone and acetone treatment groups (Figure 6.6).

A summary of the histological changes following TNCB treatment is shown in Table 6.3. Of the 5 mice treated with TNCB followed by peptide 19, all 6 mice had evidence for either stage 2 or stage 3 pathological changes which was similar to both the acetone followed by peptide 19 treated and TNCB alone treated groups. Consequently, the evidence indicates that TNCB followed by peptide 19 treatment does not cause antigen specific suppression of autoantibody production and has no effect on the pathological changes.

### 6.2.5 Prevention of the development of autoimmune gastritis disease by treating 3dnTx mice at 4 days of age with peptide 19

In an associated study undertaken in the Division of Pathology (Mary Power-Connon, Advanced Studies Project) it was revealed that the epidermis of 4 day old mice contained dendritic cells that appeared immature, had yet to form a network, had shortened dendrites and when a contact sensitiser was applied through this skin suppression, rather than immunity, resulted. As there was a striking resemblance between the epidermal Langerhans cells of the 4 day old mice and the epidermal Langerhans cells of adult mice following exposure to DMBA, it was proposed that these neonatal and immature LC would induce suppression in a similar manner to the LC from DMBA-treated skin. Consequently, experiments were initiated with the application of peptide 19 through the

epidermis of 3dnTx mice at day 4. As shown in Figure 6.7a, 58% mice thymectomised at day 3 and treated at day 4 with 10μg of peptide 19 dissolved in acetone developed autoantibody titres ≥ 32. This compared to 46% mice treated with acetone alone. It thus appeared that application of peptide 19 through neonatal skin did not prevent the development of autoimmune gastritis. As peptide 19 was a small self derived peptide it may not have provided an appropriate signal (eg. danger signal) to the epidermis and was therefore ignored by the immune system. In order to increase the signals to the immune system the next stage was to apply peptide 19 dissolved in the contact sensitiser, TNCB. With this strategy TNCB would serve as an adjuvant and cause some minor skin irritation and inflammation. This approach provided encouraging evidence that autoimmune gastritis could be prevented. As shown in Figure 6.7b, 13 mice were thymectomised at day 3 and treated at day 4 with peptide 19 dissolved in TNCB and not one of these mice (0%) developed autoantibody titres ≥ 32, compared to the control group, treated with TNCB alone where 50% mice developed autoantibody titres ≥ 32.

The histology is summarised in Table 6.4. There are two important points to note. Firstly, there was a consistent correlation of antibody titre with pathological stage. It should also be noted that thymectomy does not always result in AIG, consequently there are some mice in the control groups which failed to develop disease. These mice were not cured but simply failed to develop disease. Secondly, and more importantly, none of mice treated with

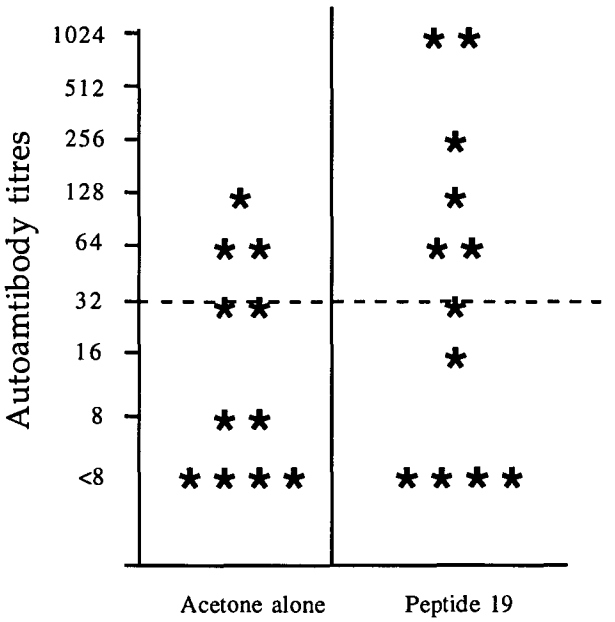



**Figure 6.7a** Autoantibody titres of 3dnTx mice after treatment with peptide 19.

Dorsal trunk skin of 3dnTx mice was treated at day 4 following birth with either acetone or peptide 19. In the acetone alone group, 45% of 3dnTx mice developed autoantibody titres ≥ 32. In the peptide 19 alone group, 58% of 3dnTx mice developed autoantibody titres ≥ 32.

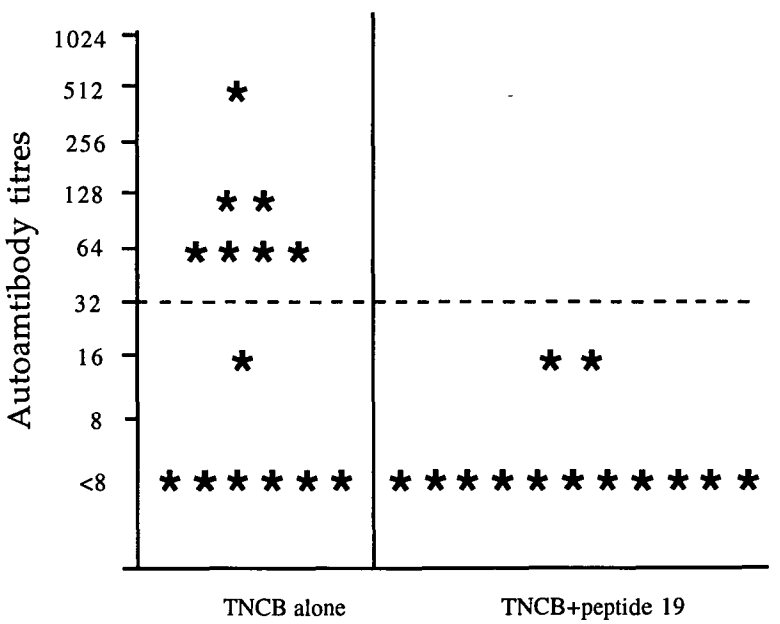



**Figure 6.7b** Autoantibody titres of 3dnTx mice after treatment with peptide 19 dissolved in TNCB.

Dorsal trunk skin of 3dnTx mice was treated with either TNCB or peptide 19 dissolved in TNCB at day 4. In the TNCB alone treated group, 50% of 3dnTx mice developed autoantibody titres ≥ 32. In the TNCB + peptide 19 treated group, 0% of 3dnTx mice developed autoantibody titres ≥ 32.

| Treatment | Pathology | Autoantibody titre | | | | | | Number |
|---|---|---|---|---|---|---|---|---|
| (Group 1; 11 mice) | Normal | 0 | 8 | | | | | 2 |
| Acetone alone | Stage 1 | 0 | 0 | 0 | 8 | | | 4 |
| | Stage 2 | 32 | 32 | 64 | 64 | 128 | | 5 |
| | Stage 3 | | | | | | | |
| (Group 2; 12 mice) | Normal | 0 | 0 | | | | | 2 |
| Peptide 19 | Stage 1 | 0 | 0 | 16 | | | | 3 |
| | Stage 2 | 32 | 64 | 128 | | | | 3 |
| | Stage 3 | 64 | 256 | 1024 | 1024 | | | 4 |
| (Group 3; 14 mice) | Normal | 0 | 0 | | | | | 2 |
| TNCB alone | Stage 1 | 0 | 0 | 0 | 16 | 64 | 64 | 6 |
| | Stage 2 | 0 | 64 | 64 | | | | 3 |
| | Stage 3 | 128 | 128 | 512 | | | | 3 |
| (Group 4; 13 mice) | Normal | 0 | 0 | 0 | 0 | 0 | 0 | 6 |
| Peptide 19 dissolved in TNCB | Stage 1 | 0 | 0 | 0 | 0 | | | 4 |
| | Stage 2 | 0 | 16 | 16 | | | | 3 |
| | Stage 3 | | | | | | | |

**Table 6.4** Comparison of the autoantibody titres and the stomach pathology of 2dnTx mice treated at day 4 with acetone (group1) or peptide 19 (group2), TNCB (group3),or peptide 19 dissolved in TNCB (group4).

peptide 19 dissolved in TNCB developed stage 3 pathological changes and only 23% showed signs of stage 2 changes, which also had very low (<32) autoantibody titres. This compares to the acetone alone treatment group where 46% of the mice developed stage 2 or stage 3 changes, the peptide 19 treatment group were 58% developed stage 2 or stage 3, and 43% TNCB alone treatment group developed stage 2 or stage 3 changes and all of these mice developed relatively high titre (≥ 32).

Consequently, application of peptide 19 dissolved in TNCB through day 4 neonatal skin of mice thymectomised on day 3 not only significantly inhibited the development of autoantibodies, but also prevented the infiltration of lymphocytes and prevented pathological changes.

## 6.3 Discussion

BALB/c mice thymectomised 3 days after birth are prone to developing autoimmune gastritis, presumably due to an alteration in the balance of immunoregulatory T cells (Sakaguchi *et al.*, 1996). In this chapter, 3dnTx mice with the symptoms of autoimmune gastritis were treated with DMBA to deplete the epidermal LC, followed by the gastric $H^+/K^+$ ATPase autoantigen or the immunogenic epitope, referred to as peptide 19 to induce antigen specific suppression. The justification for this strategy was based on earlier observations that application of antigen through LC-depleted skin could downregulate an established CHS response (Chapter 3) and that 3dnTx mice retain their capacity to generate antigen specific suppression (Chapter

5). As an established immune response could be downregulated and as 3dnTx mice could generate antigen specific suppression, it was reasonable to propose that antigen specific suppression could be generated with this treatment. This suppression could therefore be directed against the autoreactive cells responsible for causing the tissue damage which leads to autoimmune gastritis.

The strategy of applying autoantigen through skin previously exposed to DMBA did result in a downregulation of autoantibody levels, however, this appeared to be primarily due to a direct effect of DMBA which caused non-specific immune suppression. This non-specific suppression following exposure to DMBA had not been observed with the contact sensitivity experiments outlined in Chapter 3 nor had it been observed by Halliday *et al.* (1986) who performed the early work on DMBA-induced antigen specific suppression of contact sensitivity. In these experiments, when DMBA was applied through the dorsal skin and a contact sensitiser applied through the ventral skin, there was no evidence for a reduction in contact sensitivity, hence no evidence for non-specific suppression (Halliday and Muller, 1986). Nonetheless, there are other reports suggesting that DMBA can be immunosuppressive. Thurmond and colleagues showed that mice injected intravenously with a low dose of DMBA and then immunised with sheep erythrocytes failed to produce antibody forming cells in a plaque-forming assay (Thurmond *et al.*, 1987). They also showed that splenic lymphocytes had a reduced ability to proliferate *in vitro* when they were cultured in the

presence of DMBA (Ward *et al.*, 1984; Thurmond *et al.*, 1988). This reduced proliferative ability may have been due to a suppressed production of IL-2 as Con A stimulated splenic lymphocytes have a reduced ability to produce IL-2 when the cells have been exposed to DMBA (Thurmond *et al.*, 1988). Thus it appears that in certain circumstances DMBA can cause non-specific immunosuppressive effects.

A contact sensitivity response is an efficient cell mediated immune response as a low dose of contact sensitiser can induce an optimal immune response (Sullivan *et al.*, 1990) and consequently the non-specific suppression caused by DMBA might be insufficient to overcome the strength of this response. In contrast, intact proteins are generally weak immunogens when applied through the skin and it has been reported that protein antigens often require the assistance of some form of adjuvant to induce an optimal immune response (Ada, 1993). The ability of TNCB to cause non-specific suppression may be observed with less effective immune responses.

Peptides may also require the addition of cytokines or repeated applications to produce an effective immune response (Berzofsky and Berkower, 1993), therefore, induction of antigen specific suppression in this system may require modification such as the inclusion of adjuvant or repeated applications. As antibody responses appear to be sensitive to DMBA, evidenced by reduced plaque-forming cells (Thurmond *et al.*, 1987), and as $H^+/K^+$ ATPase and its peptide are self antigens and therefore unlikely to

elicit an efficient immune response, it is highly plausible that the suppression induced by DMBA at the level of antibody mediated immunity might be sufficient on its own to reduce the level of autoantibodies. The stomach pathology supports this conclusion as there was no evidence for a reduced lymphocyte infiltration. The encouraging aspect from this reduction in autoantibody titres was that autoantibody production could at least be downregulated. With this in mind, the next strategy to induce auto-antigen specific suppression required procedures that did not involve exposure to DMBA.

The requirement for depleting epidermal LC without causing non-specific immune suppression in the 3dnTx mice, led to the concept of applying TNCB through the skin which not only results in LC depletion, but an immunosuppressive environment is established such that a second antigen applied through this skin results in antigen specific suppression (Woods *et al.*, 1996). The approach of applying autoantigen through LC-depleted skin following TNCB application did overcome the problem of non-specific suppression, however there was still no evidence for specific suppression at the autoantigen level. It is possible that the amount of peptide used in this study was not optimal to induce tolerance. Evidence for this comes from a related study which suggested that a large antigen dose is required to induce immune tolerance when introduced intravenously (Critchfield *et al.*, 1994). Another possible explanation may be that peptide 19 is not sufficiently immunogenic and therefore would be unable to produce a negative signal

when applied through LC-depleted skin. Downregulation of autoimmunity would not result as the strength of the suppressive signal could not compete effectively with the established autoimmune response. To increase the immunogenicity, and therefore produce a more effective immunosuppressive response, it may be appropriate to modify peptide 19 or use another peptide derived from the parent protein $H^+K^+$ ATPase. This strategy has been successful in studies involving mice with experimental autoimmune encephalomyelitis (EAE). In this autoimmune disease it has been shown that myelin basic protein, like $H^+/K^+$ ATPase in autoimmune gastritis, is the autoantigen target of EAE. Experimental autoimmune encephalomyelitis can be prevented by administering myelin basic protein through oral (Miller *et al.*, 1993), nasal (Bai *et al.*, 1997), or intravenous routes (Gaur *et al.*, 1992). However, to suppress established EAE (eg. after clinical symptoms have developed) the normal myelin basic protein requires modification. Brocke and colleagues discovered that by altering peptides derived from myelin basic protein the symptoms of established EAE could be downregulated, whereas the normal peptide derived from myelin basic protein could not treat established EAE (Brocke *et al.*, 1996). Consequently, it would appear that to treat established autoimmune disease a modification of the immunising autoantigen is required whereas prevention of disease requires the native conformation of the autoantigen. This does not imply that autoimmune gastritis can not be treated by applying autoantigen via modified dendritic cells, but it does suggest that

further manipulation of this system would be required to achieve optimal results.

The ability to prevent development of autoimmunity has been demonstrated in other disease such as experimental autoimmune encephalomyelitis (Hauser *et al.*, 1984; Bai *et al.*, 1997), type 1 diabetes of non-obese mice (Harada *et al.*, 1990; Muir *et al.*, 1995; Daniel and Wegmann, 1996b), experimental arthritis (Thompson *et al.*, 1993), and experimental uveitis (Sasamoto *et al.*, 1992; Gregerson *et al.*, 1993). Consequently it was reassuring that the induction of autoimmune gastritis could be prevented by immunising neonatal mice with peptide 19 dissolved in TNCB. The requirement for simultaneous administration of TNCB was a different approach from the earlier experiments where the aim in these experiments was to treat established disease. For the previous experiments, the application of TNCB or DMBA to the skin of adult mice with autoimmune gastritis was designed to cause LC depletion and the cells that were present in the treated area 5 days later induced antigen specific suppression. Results from collaborative work in the Department of Pathology revealed a similarity between the LC in the epidermis of adult mice treated with DMBA and the LC in the epidermis of neonatal mice. The similarity was that both populations appeared to be immature. Furthermore, application of TNCB through neonatal skin failed to induce immunity (Mary Power Connon and Kathleen Doherty, personal communication). Consequently, application of antigen through neonatal skin, and therefore immature LC, is more likely to

result in tolerance than immunity. The results from this chapter indicated that application of peptide 19 through neonatal skin did not prevent the development of autoimmune gastritis, however, when peptide 19 and TNCB were combined there was a remarkable reduction in both autoantibody production and pathological changes. The requirement for TNCB is unlikely to be due to the need to cause LC depletion (as in adult suppression experiments) but to serve as an adjuvant for the self peptide, peptide 19, thus making this peptide more visible to the immune system. In addition, inflammatory changes associated with the effect of TNCB may also contribute to the increased immune response. Consequently future strategies at either the prevention or treatment stages may benefit from the simultaneous administration of peptides ( either unmodified for prevention strategies, or modified for the treatment strategies) and some form of adjuvant that will allow the peptide to be recognised by the residual antigen-presenting cells.

The preventative immunisation strategy of peptide 19 dissolved in TNCB not only prevented autoantibody titres from developing, but also significantly reduced, and in most cases prevented, the lymphocyte infiltration towards the base of the glands and the destruction of both parietal and chief cells. As there was no lymphocyte infiltration, there was no damage to the stomach mucosa and therefore no opportunity for autoantigen to be released and therefore there was no evidence for autoantibodies. Consequently, the preventative phase of this approach

successfully targeted the disease process and therefore provides a potential strategy for immunological manipulation of autoimmune disease.

# Chapter Seven
# General Discussion

Autoimmunity is one of the major medical problems in today's society with approximately 5% of the population suffering from some form of autoimmune disease (Peakman and Vergani, 1994). Most of these conditions are chronic (eg. diabetes, rheumatoid arthritis, and multiple sclerosis), and therefore in both medical and social terms, autoimmune disease is a high-cost disorder. To reduce the medical and social cost related to autoimmune disease, efficient and cost-effective treatment strategies are required that target the disease process and reduce the associated symptoms. Current methods of treating autoimmune diseases are successful in reducing the symptoms but are more palliative, rather than curative, as most treatments are aimed at non-specific reduction of the immune response. A more desirable way to treat autoimmune disease would be to target the treatment against the cause of the disease without producing detrimental side effects. It was the aim of this thesis to design and evaluate a strategy for antigen specific treatment of autoimmune disease.

One way of achieving antigen specific immunosuppression is by immunising through skin that has been modified. In normal situations the application of a contact sensitiser through skin induces an immune response against the contact sensitiser (Stingl *et al.*, 1980; Katz *et al.*, 1985; Katz, 1988; Streilein and Grammer, 1989). This is primarily due to the Langerhans cells which transport the antigen to the local lymph nodes

where it is presented to antigen specific T cells (Silberberg and Thorbecke, 1980). Application of a complete carcinogen such as DMBA or exposure to a physical carcinogen such as UVB irradiation can significantly reduce the density and alter the morphology of the epidermal LC population (Greene *et al.*, 1979; Bergstresser *et al.*, 1980; Kripke, 1984; Dandie *et al.*, 1992; Muller *et al.*, 1993). When a contact sensitiser is applied through this modified skin environment, contact sensitivity is not induced but antigen specific suppression results. The ability to prevent the development of an immune response through a modified skin environment provides an ideal opportunity to manipulate the immune system to induce antigen specific immunosuppression and avoid the induction of an inappropriate immune response, such as autoimmunity.

Central to the induction of antigen specific immunosuppression is the depletion, and/or modification, of LC from the skin as well as exposure to the antigen. To utilise the skin to induce immunosuppression against autoimmune disease, the skin needs to be depleted of LC and autoantigen applied through this modified skin. For this approach to be successful, in an animal model of autoimmune disease, the autoantigen must be characterised. It would also be necessary to be able to effectively monitor these animals for evidence of disease by evaluating autoantibody titres and pathological changes. Autoimmune gastritis was selected because the animal model closely resembles the human disease and therefore a direct comparison could be made (Toh *et al.*, 1997) and the autoantigen has also

been identified as the $H^+/K^+$ ATPase (proton pump). More specifically it is the β-chain of the 'proton pump' that is the major target autoantigen (Alderuccio *et al.*, 1997) and peptide mapping analysis has identified an epitope (peptide 19) which reacts with the autoreactive T cells (B-H Toh, personal communication). Furthermore AIG is the underlying cause of pernicious anaemia, a disease that affects 0.1% of the general western population. However, above the age of 60 it affects 1% of the population, and is a representative organ specific autoimmune disease (McGee *et al.*, 1992).

As autoimmune disease is the result of an established immune response, and as the previous work on skin-induced tolerance involved naive mice, prior to the induction of contact sensitivity, the initial experiments in this thesis were designed to determine if an established contact sensitivity response could be downregulated. In chapters 3 and 4, an established immune response to TNCB was shown to be effectively downregulated by treating skin with DMBA or UVB irradiation followed by TNCB. This downregulation was observed for both contact sensitivity and antibody production following DMBA treatment, whereas only contact sensitivity was downregulated following UVB irradiation. The observation that downregulation was not a complete suppression of immunity, may have been due to a number of factors. These include the possibilities that memory cells, or previously activated cells, are more difficult to regulate than non-activated T cells or that an established contact sensitivity response, which is a potent response, would be difficult to suppress due to its intensity (Gray,

1992; Ahmed and Gray, 1996). Nonetheless, downregulation was observed and consequently a similar DMBA treatment protocol could prove to be effective in downregulating an autoimmune response. The next stage of the study was to evaluate this possibility.

Thymectomy of BALB/c mice at day 3 following birth is a well established and reliable procedure for inducing AIG and therefore provides an effective animal model for studying an autoimmune disease (Toh *et al.*, 1993). The removal of the thymus at such an early age means that the full repertoire of T cells would not have fully developed and the question of the ability of these mice to generate regulatory T cells to either prevent, or downregulate, an already established immune response was evaluated in chapter 5. It has been suggested that a population of $CD4^+$ T cells regulates the development of autoreactive T cells that contribute to the prevention of AIG (Fukuma *et al.*, 1988; Fowell *et al.*, 1991; Mason, 1993; Nishio *et al.*, 1994). The proposal is that a failure of these regulatory $CD4^+$ T cells to develop allows autoreactive T cells to be activated, and/or expand, resulting in damage to the parietal cells eventually causing AIG. The $CD4^+$ T cell population has been proposed to belong to a $CD5^{high}$ or $CD45RB/C^{low}$ T cell subset because elimination of either of these populations in normal mice resulted in the development of AIG and their reconstitution prevented autoimmune development (Powrie and Mason, 1990; Mason and Fowell, 1992; Fowell *et al.*, 1995).

Further analysis revealed that a $CD25^+$ subpopulation of $CD4^+$ cells was more effective in regulating AIG development as adoptive transfer of these cells from normal mice to 3dnTx mice prevented the induction of AIG (Sakaguchi *et al.*, 1995; Asano *et al.*, 1996). Consequently it would appear that these regulatory $CD25^+$ T cells, or at least their precursors, developed at, or after 3 days following birth, whereas the autoreactive T cells were exported to the periphery before day 3, thus explaining why thymectomy at this time predisposes to autoimmunity. This is consistent with the observation that the $CD4^+$ $CD25^+$ cell are not detected in peripheral lymphoid organs of normal mice within 3 days following birth (Sakaguchi *et al.*, 1995).

It is unlikely that these same regulatory T cells also regulate contact sensitivity responses as 3dnTx mice, which still produced an effective contact sensitivity response, could also produce regulatory T cells that suppressed this contact sensitivity response. Thus the precursor cells that can regulate a contact sensitivity response were produced within 3 days following birth whereas the $CD4^+$ $CD25^+$ regulatory cells, which regulate the T cells that contribute to autoimmune gastritis, were exported at, or after 3 days following birth. It is attractive to consider that the $CD25^+$ T cells, which are specific for AIG prevention, and exported from the thymus after 3 days following birth, contribute to 'central' tolerance. In contrast the precursor cells to the regulatory T cells that are induced following inappropriate antigen presentation when a antigen is applied through DMBA-treated skin, have been exported from the thymus within 3 days following birth. It is this

population of cells that contributes to 'peripheral' tolerance. The nature of these cells has yet to be determined, however, they exhibited similar characteristics to the regulatory T cells from normal mice as they were present in the spleen and adoptive transfer prevented the induction of a contact sensitivity response. In preliminary work it appears that these cells belong to a population of $CD4^+$ T cells that express CD11b (Rist personal communication). As 3dnTx mice could still generate cells capable of preventing a contact sensitivity response, the application of autoantigen through DMBA-treated skin may produce a similar population of T cells that regulate autoreactive T cells via peripheral tolerance mechanisms.

The experiments undertaken in chapter 6 evaluated this possibility and it was shown that established autoimmune gastritis was not downregulated in an antigen specific manner. This was different to contact sensitivity induced immunity where both contact sensitivity and antibody production were specifically downregulated by applying contact sensitiser via DMBA modified skin. This may be a consequence of the different mechanisms involved in the induction of immunity via immunisation and autoimmunity induced via thymectomy. Thymectomy does not expose the mice to new antigen but is likely to alter immunoregulatory T cell balance (Heath *et al.*, 1996) whereas contact sensitisation involves the exposure to a foreign antigen and therefore is a reactive immune response. The concept proposed is that when an exogenous antigen is involved, downregulation can be achieved but when T cell immunoregulation is affected, an

established immune response is difficult to downregulate. This proposal gains support from studies of other autoimmune diseases such as EAE, which require an immunising antigen and can be downregulated once the disease has been established (Sun *et al.*, 1996). Autoimmune diseases that occur spontaneously in genetically susceptible mice such as diabetes in NOD mice, have yet to be downregulated, when the disease has been established.

An additional explanation may also relate to the nature of the antigen. In autoimmune gastritis, the autoantigen is continuously present whereas with the contact sensitivity experiment the immunising antigen was only present for a short term. Hence, the downregulation of an established immune response may be made more difficult due to the continued presence of antigen. It is possible that the downregulation method may be useful for the treatment for autoimmune disease that develops following exposure to autoantigen.

Although suppression of established AIG was not achieved, this same autoimmune disease was successfully prevented by treating neonatal mice with antigen dissolved in a contact sensitiser. Two important elements lead to this prevention. Firstly, adjuvant assistance in the form of TNCB was required. Peptide 19, being a self peptide was not highly immunogenic on its own, therefore an adjuvant, such as contact sensitiser, which is a powerful antigen, could assist the skin to respond to the antigen. This approach has also been successful in delivering antigen through the skin when antigen

was combined with cholera toxin (Glenn *et al.*, 1998). In this situation bovine serum albumin alone did not produce an effective immune response, however when cholera toxin was used as an adjuvant the immune response was boosted and immunity developed. The second element leading to successful immunisation against AIG was the nature of neonatal skin which was comprised of an immunosuppressive environment. As previously discussed it is most likely that the immature LC in neonatal skin are responsible for producing the immunosuppressive signal. Neonatal mice can accept allografts from adult mice (Jamshidi *et al.*, 1991) and a combined transplant of (neonatal and adult) skin can prolong the survival of adult skin allografts and induce long-term tolerance in recipients given short-term immunosuppression (Markees *et al.*, 1989; Markees *et al.*, 1992; De Fazio *et al.*, 1992). Thus neonatal skin can provide a tolerising environment which, in the experiments performed in this thesis, appeared to generate a population of regulatory cells. These cells protected against AIG development when peptide 19 (antigen) and TNCB were simultaneously applied through the skin of mice thymectomised on day 3 and immunised on day 4. These cells belong to a different population to the $CD25^+$ T cells that regulate against autoimmune gastritis as these cells were not present in the 3dnTx mice (Asano *et al.*, 1996). It would therefore appear that two separate populations of regulatory cells can prevent the development of AIG. The precursor to one population is the $CD25^+$ population that did not arise until at least 3 days after birth and contributes to 'central' thymic derived tolerance. The precursor to the other population

develops within 3 days following birth and can be induced following exposure to antigen via modified LC. It is likely to belong to a similar population of regulatory cells that suppress a contact sensitivity response and contribute to peripheral tolerance. While this population of cells can be induced by immunisation, further challenges will be to adapt these cells, and their counterparts, to downregulate an established disease.

Other immunological approaches to specifically target autoimmune disease have induced tolerance via the oral, nasal, or intravenous routes. These procedures have all induced a form of peripheral tolerance via various mechanisms. For example, the induction of Th2 (IL-4/IL-10) regulatory cells was achieved by oral feeding of a low dose of antigen (Weiner *et al.*, 1994); deletion or anergy of both Th1 and Th2 cells has been achieved by oral feeding of a high dose of antigen (Weiner, 1997b); induction of regulatory $CD4^+$ (Fuller *et al.*, 1993) or $CD8^+$ (Mukasa *et al.*, 1994) T cells was achieved by intravenous injection of autoantigen or its associated antigen. Dendritic cells cultured *in vitro* and incubated with IL-10 have a greatly reduced capacity to stimulate $CD4^+$ T cells (Steinbrink *et al.*, 1997). It was proposed that these IL-10 pulsed dendritic cells induced alloantigen-specific anergy in $CD4^+$ T cells and also induced peptide-specific anergy in the influenza haemagglutinin-specific T cell clone HA1.7 (Steinbrink *et al.*, 1997).

In this thesis, application of antigen through skin depleted of LC by prior carcinogen treatment was used to downregulate an established contact

sensitivity response whereas immunisation of an autoantigen peptide through neonatal skin could be used to prevent the spontaneous development of autoimmune gastritis. The combined interpretation of these result provides evidence that modified Langerhans cells, (or modified dendritic cells), produce signals that can alter the outcome of an immune response such that potentially damaging responses can be avoided. Although the risk of cancer development limits the use of carcinogen the information gained from this thesis provides potential as it is not the carcinogen that is necessary but the depletion of LC which can occur by other treatments (eg. by TNCB) (Woods *et al.*, 1996) that results in the generation of antigen specific suppression. The results outlined in the previous paragraph indicate that immunisation through oral, nasal, or intravenous routes protect against damaging immunity and it is most likely a consequence of the interaction of the dendritic cell populations found in these areas. Consequently the results are more compelling that modified dendritic cells can be used to alter the direction of an immune response, and the appropriate use of such knowledge could provide therapeutic strategies.

In summary, the insight gained from this thesis provides encouraging evidence that detrimental immune responses, as well as established immunity, can be manipulated in an antigen specific manner. This implies that immunotherapy protocols targeted at specific antigens could provide potential therapeutic strategies. The future challenges will be to determine the most appropriate antigens, (or preferably peptides), the best

immunisation routes and the nature of modified dendritic cells that establish the immunosuppressive response.

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