GRAFT-VERSUS-HOST REACTION AND THE MUCOSAL IMMUNE RESPONSE

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For my Mother and Father

DECLARATION

I declare that this thesis has been composed by myself, and that the work contained within it, except on occasions which are clearly stated, was performed by myself.

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SUMMARY

A GvHR in nonirradiated (C57Bl/6JxDBA/2) BDF_1 mice manifests systemically in an acute immunostimulatory phase followed by immunosuppression, often progressing to a chronic immunostimulatory form. An intestinal GvHR can occur due to the presence of activated T cells in the mucosa. The aim of this thesis was to study the effects of GvHR-induced T cell activation on mucosal structure and humoral immunity.

The GvHR produced an intestinal inflammatory response which resulted in an evolution of changes to the mucosal architecture, correlating with the progression of the systemic reaction. The lesion progressed from crypt hyperplasia, and increased intraepithelial cells (IEL) to crypt hyperplasia followed by crypt atrophy, villus atrophy and decreased IEL counts. The animals remained healthy and the mucosa recovered from this destructive lesion.

Total immunoglobulin concentrations were analysed in the gut washings of mice with GvHR, using a newly developed technique. An increase in IgA (day 12) and IgM (days 11-14) levels in gut washings accompanied by increased plasma cell counts in the lamina propria was observed. No changes in IgG plasma cell counts were detected. The effects on mucosal Ig levels were confined to the gut, and no dissemination to distant mucosal sites was observed. A pattern of increased serum IgA, IgM and IgG emerged, reflecting the systemic GvHR. Increased total cell numbers were observed in PP, MLN and SP, which although transient

in the mucosal-associated lymph nodes, remained high in the SP, with a dramatic increase on day 32. Similarly on day 32, Ig levels in the spleen and gut washings were dramatically increased, as was the spleen index, which possibly reflected the onset of the chronic GvHR. After 18mths, mice with chronic GvHR developed an autoimmune-type reaction, which did not affect mucosal Ig levels. The effect of a GvHR on mucosal Ig production was not exaggerated in young immunologically immature mice or aged mice with altered immune function.

I suggest that the effects of the GvHR on both the mucosal structure and Ig levels are the result of cytokines released from activated T cell subsets.

Oral tolerance studies revealed that in normal mice 50mgs betalactoglobulin (BLG) was required to suppress the systemic humoral and CMI responses. Preliminary studies using a transgenic mouse colony, which carries a cloned sheep BLG gene and expresses sheep BLG in milk during lactation, suggested that possession and expression of the gene are important in determining the antibody response to BLG, presented in the milk during the first weeks of life, in the transgenic offspring.

Finally, the mucosal immune response to the dietary proteins, ovalbumin (OVA) and BLG were investigated, using the GvHR as a model of mucosal cell activation. Initial studies revealed that feeding OVA during the initial phase of GvHR produced IgA antibodies to OVA in gut washings, comparable to the IgA anti-OVA antibody response observed when OVA was fed with the IgA immunopotentiating agent,

cholera toxin, and which was not observed in mice fed OVA alone. However further studies revealed that an IgA antibody response to OVA or BLG was dependent on the number of feeds the animals received. Interestingly, systemic hyporesponsiveness and mucosal immunity occurred simultaneously in normal animals fed dietary antigen.

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ABBREVIATIONS

APC	--- Antigen presenting cell
BLG	--- Betalactoglobulin
BSA	--- Bovine serum albumin
BMT	--- Bone marrow transplant
CCPR	--- Crypt cell production rate
CD4/CD8	--- T helper/suppressor cell markers (human)
CD3	--- T cell marker (human)
CFA	--- Complete Freund's adjuvant
CMI	--- Cell mediated immunity
CMPI	--- Cow's milk protein intolerance
Con A	--- Concanavalin A
CT	--- Cholera toxin
CTL	--- Cytotoxic T lymphocyte
CyA	--- Cyclosporin A
DC	--- Dendritic cell
DNA	--- Deoxyribonucleic acid
DTH	--- Delayed-type hypersensitivity
ELISA	--- Enzyme-linked immunosorbent assay
ELISPOT	--- Enzyme-linked immunospot assay
FAE	--- Follicle-associated epithelium
FcR	--- Fc receptor
FCS	--- Foetal calf serum
F_1	--- First generation
GALT	--- Gut-associated lymphoid tissue
GvHR/D	--- Graft-versus-host reaction/disease
H-2	--- Histocompatability locus (mouse)
H-2K/D	--- Subregions of murine H-2 (class 1)
H&E	--- Haemotoxylin and eosin

HGG	---	Human gamma-globulin
HLA-D/DR	---	Histocompatability locus (human)
HSA	---	Horse serum albumin
HRP	---	Horseradish peroxidase
Ia	---	Immune associated antigen
I-A, I-E	---	Subregions of murine H-2 (class II)
IBF	---	Immunoglobulin binding factor
IEL	---	Intraepithelial lymphocyte(s)
IFN	---	Interferon
IgA	---	Immunoglobulin A
IgE	---	Immunoglobulin E
IgG	---	Immunoglobulin G
IgM	---	Immunoglobulin M
Ig(s)	---	Immunoglobulin(s)
Ig-SC	---	Immunoglobulin-secreting cell
Ig-SFC	---	Immunoglobulin-spot forming cells
IL	---	Interleukin
IPP	---	Intra-Peyer's patch
KLH	---	Keyhole limpet haemocyanin
LP	---	Lamina propria
LPS	---	Lipopolysaccharide
Lyt	---	T lymphocyte associated antigen (mouse)
L3T4	---	T helper cell marker (mouse)
M cell	---	Microfold cell
MHC	---	Major histocompatability complex
MLN	---	Mesenteric lymph node
MLS	---	Non-MHC locus (mouse)
MRC OX8	---	T suppressor cell marker (rat)
mRNA	---	Messenger ribonucleic acid

NK	--- Natural killer
OVA	--- Ovalbumin
PAV	--- Patch-associated villi
PBS	--- Phosphate buffered saline
PEG	--- Polyethylene glycol
PFC	--- Plaque-forming cells
PP	--- Peyer's patch
PPD	--- Purified protein derivative
PMN	--- Polymorphonuclear leucocyte
PWM	--- Pokeweed mitogen
r	--- Recombinant
RBC	--- Red blood cell(s)
SI	--- Spleen index
SP	--- Spleen
SRBC	--- Sheep red blood cells
TBS	--- Tris-buffered saline
Tcs	--- T contrasuppressor cell(s)
$T_{H1}/_{H2}$	--- T helper cell subsets 1/2
TNF	--- Tumour necrosis factor
W3/25	--- T helper cell marker (rat)

LENGTH

cm	--- centimetre
mm	--- millimetre
um	--- micrometre
nm	--- nanometre

VOLUME

l	--- litre
mls	--- millilitres
ul	--- microlitres

WEIGHT

g	--- gram
mg	--- milligram
ug	--- microgram
ng	--- nanogram

TIME

mths	--- months
wks	--- weeks
hr	--- hour
min	--- minute

CONCENTRATIONS

N	--- Normal
M	--- Molar
mM	--- millimolar

MISCELLANEOUS

i.d.	--- intra-dermal
i.g.	--- intra-gastric
i.p.	--- intra-peritoneal

i.v.	--- intra-venous
O.D.	--- optical density
pH	--- reciprocal $\log_{10}$ hydrogen ion concentration
%	--- percent
RT	--- room temperature
U	--- international units

STATISTIC SYMBOLS

s.d.	--- Standard deviation
SEM	--- Standard error of the mean
P	--- Probability
<	--- Less than
>	--- Greater than
=	--- Equal to
NS	--- Not significant

INTRODUCTION TO EXPERIMENTS

The mucosal surface of the gastrointestinal tract shows a fascinating dichotomy of functional roles. Physiologically, this is the site of absorption of dietary nutrients; it is also an immunological barrier against environmental antigens.

The mucosal immune responses and the regulation of immunity are totally different from the rest of the body.

The predominant immunoglobulin secreted from the mucosae is IgA, and regulation of the mucosal IgA response is T cell dependent.

Presentation of certain antigens at the mucosal surface is believed to produce oral tolerance, recognised as a suppression of the systemic humoral and cell-mediated immune responses. This systemic hyporesponsiveness is also under T cell regulation, and T cell reactions at the gut level can alter oral tolerance induction.

The phenomenon of oral tolerance is thought to protect the body from harmful systemic immune reactions, and is believed to be the normal immunological response to innocent dietary proteins.

The relationship between mucosal immunity and oral tolerance is as yet uncertain, but there is evidence to suggest a potential reciprocal balance, in that "switching on" of a secretory IgA response effectively abrogates oral tolerance.

The purpose of my work was to explore the effects of T cell activation within the gut mucosa.

Specifically I aimed to measure:-

i) The production and isotype distribution of mucosal immunoglobulins and antibodies.

ii) Having observed striking changes in immunoglobulin production the work was extended to study where, in the mucosal B cell maturation pathway, T cells were exerting their effect. Several areas of possible influence exist such as isotype-switching and proliferation in the Peyer's patches, migration, and at terminal differentiation in the lamina propria.

iii) Finally the influences of antigen presentation and mucosal T cell activation on oral tolerance induction were to be examined.

The murine Graft-versus-host reaction (GvHR) model was used in these studies. The injection of parental, immunocompetent cells into nonirradiated F_1 hybrid animals produces a GvHR, during which class II-restricted, activated T cells present in the mucosa. There is an extensive amount of literature describing T cell-mediated hypersensitivity reactions and cytotoxic T cell and NK cell activity during the GvHR. However, apart from the findings of Gold, Kosek, Wanek and Baur (1976), in which an immunodeficiency of IgA- and IgM-containing plasma cells in the gut was observed in neonatal mice, very little is known about mucosal immunoglobulin production during a GvHR.

To answer the questions on intestinal immunoglobulin production it was first necessary to develop a method for measuring secretory immunity. A gut lavage technique was

used. To allow analysis of T cell influences on B cell development an ELISPOT assay was developed.
In the studies of antigen and mucosal influences on oral tolerance I selected ovalbumin, which has been extensively studied, and betalactoglobulin, an important dietary protein implicated in milk allergy. In addition, betalactoglobulin was selected as I had the unique source of a transgenic mouse strain, which expresses sheep betalactoglobulin in milk during lactation.

In this thesis I shall first review the literature on the induction and immunoregulation of the mucosal immune response (chapter 1), and the murine GvHR model (chapter 2).
In chapter 4 are described the different phases that occur during the GvHR, with specific attention to the effects on the histological architecture of the gut.
Chapter 5 explains the development of the gut lavage method for the collection of gut luminal contents.
Having established this technique, a definitive, descriptive study of the effect of the GvHR on the concentrations of mucosal immunoglobulins was performed, and is documented in chapter 6.

Significant changes were observed and attempts to address the possible regulatory mechanisms were undertaken.
Experiments investigating the possible dissemination of immunity to other sites of immune expression are detailed in chapter 7. The influences of the GvHR at the extremes of life, in neonates with potential immunodeficiency and

possible accentuated gut damage, and in aged animals, 18 months after the initial GvHR when an autoimmune-type disease occurs, are analysed in chapter 8. The last chapter, dealing with studies on regulation (chapter 9), describes the experiments undertaken to determine at what stage in mucosal B cell differentiation the GvHR exerts its effect.

The final two chapters concentrate on the mucosal and systemic immune responses to clinically relevant dietary proteins both in normal mice, and mice with a GvHR.

CHAPTER ONE

INDUCTION AND IMMUNOREGULATION OF THE MUCOSAL IMMUNE RESPONSE

INTRODUCTION

The mucosal surface of the gastrointestinal tract is involved in the absorption of dietary nutrients, but perhaps more important, is its function as a barrier against the constant pervasion of potentially antigenic material. A wide range of antigens may be encountered within the gut; these include ingested proteins, commensal microorganisms and visiting pathogens such as bacteria, viruses, protozoa and helminths.

While maintaining a tolerance to dietary proteins and commensal microorganisms, and allowing absorption of essential nutrients, the gut must be able to mount a rapid, isolated and efficient immune response to the invading pathogen. This ability lies in the close association between the mucosa and the gut-associated lymphoid tissue (GALT) and the range of mucosal immune responses that can occur within the GALT.

The intestinal mucosa is composed of an inner epithelial layer in direct contact with the gut lumen, an outer layer of connective tissue (the lamina propria), and finally the muscularis mucosae which separates the mucosa from the submucosa (reviewed in Trier and Madara, 1981).

The GALT, the largest immune compartment in the body, is composed of lymphoreticular cells within the epithelial layer, the lamina propria, the organised lymphoid aggregates (Peyer's patches) situated along the length of the intestine, and the draining mesenteric lymph node (MLN).

This chapter will concentrate on the induction and immunoregulation of the mucosal immune response within the GALT, oral tolerance induction to enterically presented antigens, and the relationship between mucosal immunity and oral tolerance.

INDUCTION OF A MUCOSAL IMMUNE RESPONSE

The induction of the mucosal immune response requires the initial uptake of antigen across the gut wall, and subsequent presentation to mucosal effector cells.
Peyer's patches (PP) are generally believed to be the main sites of engagement of antigens with the lymphoid system of the gut, and of induction of the immune response. However recent evidence suggests that the villus enterocyte may be an important site of antigen presentation and immunoregulation. Both theories will be reviewed.

ANTIGEN PRESENTATION WITHIN THE PEYER'S PATCH

PP, present in all mammals, are organised lymphoid follicles distributed along the length of the small intestine, with the greater number positioned distally. Lymphoid aggregates, similar to PP but without an organised structure, are also found the length of the intestine including the large bowel.
In man, approximately 240 PP are present at puberty, and this number declines with age (Cornes, 1965). In mice and rats 8-10 PP can usually be determined in each animal.

The structure of the Peyer's patch

The structure of the PP is comparable to that of other lymph nodes, however, the PP is not encased in a capsule and does not have an afferent lymph system.

Each patch is composed of a dome region, lymphoid follicles (B cell zone) and a parafollicular region (T cell zone). The dome region consists largely of T and B lymphocytes and macrophage (Carlson and Owen, 1987). Enzymatic treatment of PP releases equal numbers of T and B cells. 85-92% of B cells are surface (s) $sIgM^+$, the remainder being $sIgA^+$ (Frangakis *et al*, 1982; Kiyono *et al*, 1982a). The mature T cells are predominantly T helper cells bearing the $Lyt\text{-}1^+$ (40-50%) and the $L3T4^+$ (57-63%) surface markers, however 15-20% of PP T cells bear the T suppressor/cytotoxic, $Lyt\text{-}2^+$ surface marker (reviewed Mestecky and McGhee, 1987).

The germinal centres of the follicles are the sites of antigen induced T-dependent B cell differentiation.

Follicle-associated epithelium

The important feature of the PP is the specialized epithelium overlying the dome region. The columnar follicle-associated epithelium (FAE) is derived from the same crypt stem cells as the adjacent patch-associated villi (PAV), however it possesses important characteristics which enable it to transport antigen. For example, mature goblet cell numbers are reduced in FAE as compared to the adjacent epithelium (Bockman and Cooper, 1973) and there is

greater contact between antigen and epithelial cells due to the relative absence of mucus. The most important feature is the presence of specialized antigen transport cells, M cells, which are interspersed among the columnar epithelial cells of the FAE (Owen and Jones, 1974).

M cells have microfolds or irregular shaped microvilli on their surface (Owen and Jones, 1974; Carlson and Owen, 1987), and lymphocytes, accessory cells and PMN within the central, basal indentation (Owen, 1977). There is substantial evidence to suggest that M cells are the cells through which luminal antigens are sampled and introduced to the PP effector cell environment. Horseradish peroxidase (HRP) (Owen, 1977) and the lectins, *Ricinus communis* agglutinin II and wheat germ agglutinin (Neutra *et al*, 1982) have all been shown to adhere to M cells and be transported into the PP. Similarly M cell transport of viruses such as reovirus II (Wolf *et al*, 1983), and bacteria such as *Vibrio cholerae* (Owen *et al*, 1986) has been demonstrated.

It is unknown however if M cells are capable of antigen presentation.

Antigen presentation in the Peyer's patch

Before their recognition by T lymphocytes, soluble antigens require processing by antigen-presenting cells (ie. macrophages or dendritic cells), and re-expression on the cell surface in association with the MHC gene products (Shimonkevitz *et al*, 1983).

Macrophages are present in the PP, however their low expression of all types of Fc receptors (FcR), and Ia antigens (Vetvicka *et al*, 1987), and their inability to effectively present antigen (Barr *et al*, 1985) suggests they are not directly involved in presentation of antigen. They do however appear to have a phagocytic role and phagocytosis of ferritin (Lause and Bockman, 1981), latex particles (Vetvicka *et al*, 1987), and *Giardia* trophozites (Owen, Allen and Stevens, 1981) have been demonstrated.

A non-adherent accessory cell population, dendritic cells (DC), has been extracted from PP (Mayrhofer, Pugh and Barclay, 1983; Barr *et al*, 1985). DC are found in the skin (Langerhan's cells) and there they play an important role in antigen presentation. In the PP, cells with a dendritic morphology occur in very low numbers (Barr *et al*, 1985). Nevertheless, a non-adherent population extracted from PP are capable of presenting ovalbumin (OVA), human gamma-globulin (HGG) and purified protein derivative (PPD) to sensitized lymph node cells and to antigen-specific T cell clones (Barr *et al*, 1985). PP DC are Ia^+ indicating their state of activation (Mayrhofer *et al*, 1983). In culture, the interaction between both PP and splenic B cells with a PP DC-T cell mixture produces a significant polyclonal secretion of IgA, which may indicate the importance of DC in the induction of a mucosal immune response.

The PP is widely recognised as the preferential source of IgA^+ B cells, and the site of induction and differentiation of B cells after enteric antigen stimulation (Craig and Cebra, 1971). There are two prominent theories as to why the GALT is so rich in IgA^+ B cells.

One theory suggests that the persistent presentation of antigen, occurring at the PP surface, drives a continuous cell division of PP B cells which in turn leads to a vectorial deletion of heavy-chain C-region-encoding genes. A B cell population is thus arrived at with the ultimate heavy-chain C-region (*Calpha*) characteristic of IgA B cells. The theory of antigen dependent B cell differentiation is at odds however with the current understanding of IgA B cell differentiation. In particular, are the aspects concerned with the molecular biology of immunoglobulin (Ig) genes. Although the gene encoding *alpha* heavy-chain C-region is the ultimate in the murine system, in humans one *alpha* heavy-chain C-region is in the middle of the gene sequence and one at the end, hence in the human model, continuous gene deletion would not lead inevitably to IgA expression. Similarly, the theory predicts that during some stage in the development of an IgA response, B cells would express IgG and then switch to IgA production. However cells bearing sIgG and sIgA have never been observed (reviewed in Strober and Jacobs, 1985).

The second theory, and the one more readily accepted, suggests that the differentiation of B cells into IgA

producing plasma cells is completely under T cell regulation.

T CELL REGULATION OF IGA B CELL DIFFERENTIATION.

There is now quite substantial evidence supporting T cell regulation of IgA B cell differentiation and two important stages have been defined.
The initial stage involves isotype-switching of $sIgM^+$ PP B cells to $sIgA^+$ B cells and the second stage is the terminal differentiation of $sIgA^+$ PP B cells into IgA-producing plasma cells (Fig 1:1).

Isotype-switching during IgA B cell differentiation

The definition of an IgA-specific isotype-switch T cell came largely from Kawanishi and co-workers studying Concanavalin A (Con A) stimulated cloned T cells, and their effect on lipopolysaccharide (LPS)-driven B cells.
The co-culture of purified PP $sIgM^+$ B cells with cloned PP T cells has been shown to produce an increase in PP B cells expressing sIgA, with a simultaneous decrease in $sIgM^+$ B cells. This isotype-switch did not occur in the presence of cloned SP T cells, or when PP $sIgG^+$ B cells were used (Kawanishi, Saltzman and Strober, 1983a). The co-culture of cloned PP T cells with SP $sIgM^+$ B cells also produced an isotype-switch to sIgA expression, although to a lesser extent. In addition the co-culture of $sIgM^+$ B cells and cloned T cells, both derived from the SP, resulted in an

increase in sIgG expression concomitant with a decrease in $sIgM^+$ B cells (Kawanishi, Saltzman and Strober, 1983a; Kawanishi and Strober, 1983).

These results suggest that isotype-switching is T cell dependent and that the origin of the T cell, not the B cell, determines the expression of an isotype. Thus T cells resident in the PP direct $sIgM^+$ B cells to express sIgA, whereas splenic T cells direct $sIgM^+$ B cells to express sIgG. The switch T cells do not act by causing proliferation of cells which have reached the level of IgA differentiation by random processes, but rather by acting on B cells in such a way as to affect the course of DNA rearrangement.

Importantly, the isotype-switch T cell does not induce terminal differentiation of $sIgA^+$ B cells, and thus no increase in IgA synthesis occurs (Kawanishi, Saltzman and Strober, 1983a).

Post-switch IgA B cell differentiation.

After PP B cells have undergone isotype-switching from $sIgM^+$ to become committed IgA^+ B cells, they finally undergo terminal differentiation to become IgA-producing plasma cells.

Initial studies by Elson, Heck and Strober (1979) had shown that Con A stimulated PP T cells were able to induce IgA synthesis while suppressing both IgG and IgM, indicating that despite the absence of plasma cells within the PP, the regulatory-effector cells were present.

Further studies by Kawanishi and co-workers have shown that after isotype-switching of PP B cells from $sIgA^-$ to $sIgA^+$, in the presence of cloned PP T cells, the addition of MLN or SP T cells or $Lyt\text{-}2^+$-depleted T cell subsets or soluble T cell products, induces IgA synthesis (Kawanishi, Saltzman and Strober, 1983b; Kawanishi and Strober, 1983).

From these observations it would appear that the terminal differentiation of IgA-committed PP B cells is either activated in the PP or during the migration of the B cells from the PP to the draining MLN and SP. The post-switch T cell is a T helper cell but its mode of regulation is as yet unclear.

T cell clones from human GALT (appendix) mediate both an isotype-switch and terminal differentiation of IgA B cells, whereas T cell clones from peripheral blood direct terminal differentiation only (Benson and Strober, 1988); both mechanisms were mediated by soluble factors.

Fc*alpha*R and IgA B cell terminal differentiation

Quite separate studies performed by Kiyono and co-workers, again using cloned PP T cells, have demonstrated the existence of a PP T helper cell bearing the Fc*alpha*R which selectively promotes terminal differentiation of $sIgA^+$ B cells (Kiyono *et al*, 1982b; 1984).

T cells bearing the Fc*alpha*R have been described in both mice and humans (Strober *et al*, 1978; Lum *et al*, 1979).

In addition, the Fc*alpha*R can be released as an IgA binding factor (IBF*alpha*) which has been shown to both suppress

(Yodoi *et al*, 1983), and enhance (Endoh *et al*, 1981; Suga *et al*, 1985) IgA synthesis in pokeweed mitogen (PWM)-driven SP B cell cultures. More detailed studies of free IBF*alpha* have demonstrated that higher concentrations of this factor suppress, while lower concentrations enhance, IgA responses (Kiyono *et al*, 1985).

These findings suggest that the terminal differentiation of $sIgA^+$ B cells is regulated by T helper cells in an isotype-specific manner, rather than by non-specific regulation as indicated by Kawanishi and Strober (1983).

The IgA response to T cell-dependent antigens may further be regulated, either upwards or downwards, by the presence of IBF*alpha*.

Lymphokine mediated terminal differentiation

The initial work by Kawanishi and Strober (1983), demonstrated that IgA synthesis can be promoted by the addition of B cell differentiation factor (BCDF). BCDF is now recognised as IL-6, a lymphokine involved in the terminal differentiation of B cells into immunoglobulin-secreting cells (Kishimoto and Hirano, 1988). Recent studies of lymphokine production and IgA synthesis have produced reasonable evidence that IL-5 is a "mucosal lymphokine" (Harriman and Strober, 1987), in that it preferentially enhances IgA synthesis from $sIgA^+$ B cells but not $sIgA^-$ B cells, and is therefore important in IgA B cell terminal differentiation (Harriman *et al*, 1988; Matsumoto *et al*, 1989). IL-4 appears to enhance the action

of IL-5 by two-three fold (Murray *et al*, 1987).

Recently, Mosmann *et al* (1986) described the existence of two subsets of T helper cell clones that differ in their pattern of lymphokine secretion. One type, T_{H1}, secretes IL-2, IL-3 and IFN-*gamma*, whereas the second type, T_{H2}, secretes IL-4 and IL-5. It is possible that the population of T helper cells within the mucosa are predominantly of the T_{H2} phenotype, which on activation release IL-4 and IL-5 causing a predominantly IgA response.

The influences of T cell activation and lymphokine secretion on immunoglobulin production within the mucosa will be discussed in greater detail in the discussion chapter of this thesis.

ANTIGEN PRESENTATION BY VILLUS ENTEROCYTES

The intestinal epithelium represents the barrier between the gut lumen and the lymphoid system of the gut. The position of the enterocyte, its absorptive function, MHC-class II (Ia) expression, and close association with intraepithelial (IEL) and lamina propria (LP) lymphocytes indicates a pertinent role of the gut enterocyte in antigen presentation and immunoregulation of a mucosal immune response.

Intraepithelial lymphocytes

Approximately 15-20% of cells in the epithelium are lymphocytes. The IEL are a heterogeneous population, and

although sharing surface antigens with T lymphocytes, no T lymphocyte marker encompasses the whole IEL population in any species (reviewed Mayrhofer, 1984). There are no B cells, plasma cells or macrophages found within the epithelium.

The IEL population are located in the basal one-third of the epithelium at the basement membrane of the enterocyte, and can be found crossing this membrane in either direction (Toner and Ferguson, 1971).

The majority of IEL carry the T suppressor/cytotoxic ($CD8^+$) surface marker, with less than 15% possessing the T cell helper/inducer ($CD4^+$) surface marker (Dobbins, 1986; Mowat, 1987a). In the mouse, a population of IEL bear the CD3-associated *gamma-delta* T cell receptor, as opposed to the *alpha-beta* T cell receptor. The function of this phenotypically diverse population is as yet unclear. Interestingly, approximately half of the $CD8^+$ IEL do not express Thy 1 on their cell surface which is unprecedented for murine T cells (Bonneville *et al*, 1988; Goodman and Lefrancois, 1988).

In vitro studies have shown that rodent IEL have cytotoxic T lymphocyte (CTL) activity (Davies and Parrott, 1981) and natural killer (NK) cell activity (reviewed in chapter 2). They can secrete minimal amounts of IL-2, and intermediate amounts of IFN-*gamma* and IL-3 on *in vitro* stimulation with Con A (Dillon, Dalton and MacDonald, 1986). Other *in vitro* studies have demonstrated a poor proliferating capacity of murine IEL in response to phorbol esters and calcium ionophores, both powerful stimulators of cell proliferation

(Mowat, McInnes and Parrott, 1989). The authors suggested that IEL are IL-2 unresponsive and their poor proliferative response is because the cells are fully activated *in situ*. Certainly, murine IEL have been shown to express markers associated with T cell activation, such as CT1 (Klein, 1986; Lefrancois, 1987) and human IEL have an activated morphology *in situ* (Marsh, 1975a). It is possible that a significant proportion of IEL are recently derived from a rapidly dividing precursor pool (Marsh, 1975b; Ropke and Everett, 1976) most probably from the LP. In this respect, Monk *et al*, (1988) have shown that *in situ* activation of LP T cells in human foetal intestine culture, using the T cell activator anti-CD3 antibody, produced an increase in IEL numbers/100 epithelial cells 1-3 days after culture. Only LP T cells were found to be activated, as determined by the presence of the CD25 surface marker, and the authors suggested that the IEL were activated T cells which had migrated from the LP.

The *in vivo* cell effector function of IEL has not yet been elucidated. However, raised IEL counts are found in experimental cell-mediated immune (CMI) responses such as in graft-versus-host reactions (GvHR) (see chapter 2) and allograft rejection (MacDonald and Ferguson, 1976) and in clinical disorders associated with intestinal inflammation such as coeliac disease (Ferguson, 1974) and cow's milk protein intolerance (CMPI) (Phillips *et al*, 1979).

Class II expression on intestinal epithelium

Expression of MHC class II molecules on intestinal epithelium has been described in guinea pigs (Wilman *et al*, 1978), mice (Parr and MacKensie, 1979), rats (Mason, Dallman and Barclay, 1981) and humans (Scott *et al*, 1981). This constitutive expression is confined to the small intestine, and is not normally displayed in the large bowel. In rat intestinal epithelium, as with other species, the class II (Ia) molecules are expressed on the mature enterocyte in the distal two-thirds of the villi, with no constitutive expression on the crypt stem cells (Mayrhofer *et al*, 1983). The molecule is restricted to the basolateral membrane (Bland and Warren, 1986).

An increased expression of Ia on villus enterocytes occurs during intestinal inflammation both in experimental animals and clinical disease (see chapter 2).

The association of IEL with the Ia molecule has raised the interesting possibility that epithelial cells may perform class II-dependent genetically restricted transport and presentation of luminal antigens to IEL.

Antigen presentation by villus enterocytes

Presentation of OVA by villus enterocytes, to primed lymph node T cells has been shown to induce proliferation of the T cells after 18hrs in co-culture. This accessory function of the enterocytes was Ia-restricted as determined by blocking with monoclonal anti-sera (Bland and Warren,

1986). Similarly class II-restricted presentation of tetanus toxoid to primed peripheral blood T cells by human enterocytes has been demonstrated (Mayer and Shlien, 1987). In both reports the authors demonstrated that T cells of the suppressor/cytotoxic phenotype (MRC $OX8^+/CD8^+$) were activated when antigen was presented in such a manner.

Using a class II negative cell line, Cerf-Bensussan *et al*, (1984) showed that class II expression could be induced in this cell line when cultured with Con A supernatants of spleen cells or IEL. *In vivo*, the expression of Ia antigens was associated with an increased IEL count. As with class II induction in other cell types, the Ia-inducing factor is most likely to be IFN-*gamma*.

The preferential induction/activation of $CD8^+$ T cells either in the LP or epithelium (IEL) may explain the suppressive nature of the response to enterically presented antigens. However a recent publication has described the class II-restricted presentation of Keyhole limpet haemocyanin (KLH), by mouse enterocytes, to a specific class II-restricted ($L3T4^+$) T cell hybridoma. This presentation resulted in a production of IL-2 (Kaiserlain, Vidal and Revillard, 1989).

There still remain many unanswered questions as to the ability of the villus enterocyte to present antigen and influence the outcome of the immune response. The hypothesis proposed by Bland (1985; 1988) is that the enterocyte selectively absorbs luminal antigens in endocytic vesicles, whereby antigenic fragments (epitopes)

would become complexed to locally synthesized Ia molecules. The complex would then be re-expressed on the lateral epithelial cell membrane, and be presented to an adjacent IEL. The IEL would then migrate into the LP where it would exert a regulatory signal directing terminal differentiation of immunoglobulin-secreting cells. The regulatory T cells for IgA terminal differentiation are autoreactive and may be activated by antigen in association with MHC class II (Ia) molecules to release lymphokines such as IL-4, IL-5 or IL-6 *in situ*.

Aicher *et al* (1989), have recently demonstrated IEL in Balb/c mice which express mRNA for IL-5 in relatively high amounts compared to SP T cells. This IEL population also possessed IgA F*calpha*R. Binding of IgA to this receptor down-regulated the production of IL-5, which in turn down-regulated the synthesis of IgA in an antigen-specific manner.

Intra-PP (IPP) immunisation of rats with KLH results in a population of antigen-specific T helper cells for IgA (Dunkley and Husband, 1987). Interestingly, KLH-specific IgA helper T cells were subsequently detected within the IEL and LP populations. Unlike other forms of oral antigen presentation, no SP suppressor cells were formed which again suggests that enterocytic presentation of antigen is involved in systemic suppression, while PP antigen presentation is involved in the induction of a mucosal response.

ORAL TOLERANCE

Intra-gastric presentation of antigen can result in a down-regulation of both the systemic humoral and cell mediated immune responses; a phenomenon recognised as oral tolerance. Oral tolerance can be induced to a variety of soluble proteins such as OVA (Hanson *et al*, 1977), gamma-globulins (Chiller, Habicht and Weigle, 1970), to particulate antigens, such as sheep red blood cells (SRBC) (Mattingly and Waksman, 1978), and to contact-sensitizing agents (Asherson *et al*, 1977).

The degree of suppression depends on the dose of antigen given, the frequency of administration, and the timing of feeding relative to challenge with the antigen (Bienenstock and Befus, 1980). In addition, the genetic background of the animal is critical (Stokes, Swarbrick and Soothill, 1983; Mowat, Lamont and Bruce, 1987), as is the age of the animal at the time of feeding. Neonatal mice fed OVA in the first days of life are primed rather than tolerized (Strobel and Ferguson, 1984), similarly aged mice are more difficult to tolerize (Szewczuk and Wade, 1983).

Many different mechanisms have been implicated in the induction and maintenance of oral tolerance. Serum factors (which on transfer to naive recipients induce oral tolerance) such as circulating immune complexes (André *et al*, 1975), anti-idiotypic antibodies (Kagnoff, 1978), tolerogenic forms of antigen (Strobel *et al*, 1983) which have been intestinally processed (Bruce and Ferguson, 1986), have been reported.

Cellular control mechanisms have been extensively studied, and although direct clonal anergy of T helper cells has been demonstrated (Vives *et al*, 1980; Titus and Chiller, 1981), T cell mediated suppression seems to be the predominant mechanism (Mattingly and Waksman, 1978; Miller and Hanson, 1979; Mowat, 1985). Adoptive transfer of cells from antigen fed animals into naive syngeneic recipients demonstrates, *in vivo*, that T suppressor cells act on both arms of the systemic immune response, and the cells act on the afferent phase of the CMI response, as they are effective only when transferred before or shortly after the time of sensitization (Miller and Hanson, 1979).

The suppressor cells appear to be first stimulated within the GALT, and are found within PP 3 days after feeding antigen. By 7 days post-feed they have migrated to the SP (Richman *et al*, 1981). However, oral tolerance to SRBC can be induced in rats after surgical removal of PP (Enders, Gottwald and Brendel, 1986). Although this procedure does not eliminate the role of unorganised lymphoid aggregates in oral tolerance induction, it does suggest, once again, a possible role of enterocytic antigen presentation in oral tolerance induction.

A number of mechanisms therefore are implicated in the induction and maintenance of systemic hyporesponsiveness, and within each experimental situation different mechanisms may be in play.

In this final section the relationship between the systemic and mucosal responses to orally presented antigens will be discussed.

SYSTEMIC TOLERANCE AND MUCOSAL IMMUNITY

There are conflicting reports as to the relationship between mucosal immunity and oral tolerance. Prior feeding of a tolerizing dose of OVA has been shown to reduce subsequent absorption of that antigen, presumably a function of local immunity, as well as induce systemic hyporesponsiveness (Swarbrick, Stokes and Soothill, 1979). In a more specific study, Challacombe and Tomasi (1980), found that feeding OVA, or the particulate antigen *Strep. mutans*, produced both a suppression of the systemic response to either antigen and significant salivary antibodies. The demonstration of antigen-specific T helper cell activity for IgA anti-OVA and antigen-specific T suppressor cell activity for IgG anti-OVA in PP after feeding (Richman *et al*, 1981) explained the co-existence of both responses.

It has been suggested by Green and Martin (1983), that the maintenance of a mucosal immune response and oral tolerance is under the regulation of the contrasuppressor T cell (Tcs) network.

There are three cell types involved in the contrasuppressor circuit, Tcs inducer ($Lyt\text{-}1^{-}2^{+}, I\text{-}J^{+}$), Tcs transducer ($Lyt\text{-}1^{+}2^{+}$, $I\text{-}J^{+}$) and Tcs effector ($Lyt\text{-}1^{+}2^{-}$, $I\text{-}J^{+}$) cells. The Tcs effector cell can be positively selected on the basis of its adherence to plastic dishes coated with the *Vicia villosa* lectin (Gershon *et al*, 1981).

Murine PP are enriched in T cell subsets with helper activity and with contrasuppressor activity (Green and

Martin, 1983). The Tcs effector cell is believed to render T helper cells resistant to suppressor signals (Green and Martin, 1983), and when adoptively transferred to mice the previously induced oral tolerance to SRBC can be abrogated (Suzuki *et al*, 1986; Kitamura *et al*, 1987). An IgA response is potentiated as the Tcs cells bear Fc*alpha*R with which they bind to isotype-specific T helper cells (Suzuki *et al*, 1986).

These findings suggest that the contrasuppressor T cell network preferentially protects the mucosal IgA response from suppressor signals, but not the systemic IgG and IgM responses. In this way orally encountered antigens can induce protective immunity and systemic tolerance.

In complete contrast, Elson and co-workers have provided substantial evidence that mucosal immunity and oral tolerance do not occur simultaneously, but exist in a reciprocal relationship.

Using a method for sampling intestinal contents and directly measuring secretory antibody responses (Elson, Ealding and Lefkowitz, 1984) these workers found that while KLH induced systemic hyporesponsiveness when given orally, there was no detectable IgA response in the gut contents (Elson and Ealding, 1984ab). However when KLH was fed concomitantly with Cholera Toxin (CT), a recognised immunopotentiator of an IgA response, the oral tolerance to KLH was abrogated and an IgA response, both to CT and KLH, was stimulated (Elson and Ealding, 1984b). Further *in vitro* studies of both SP anti-CT and PP anti-CT antibody

production demonstrated that the induction of both the mucosal IgA response and the systemic IgG response originated in the GALT and then disseminated to other tissues.

CT is a potent immunogen of both the systemic and mucosal immune responses (Elson and Ealding, 1984ab; Lycke and Holmgren, 1986), however the way in which CT affects the immunoregulatory network of the immune response is as yet unclear. *In vitro* studies have shown that CT induces IL-1 production from antigen presenting cells, but not expression of Ia antigens, increased B cell proliferation and production of IgG and IgA from activated B cells, and either inhibition or stimulation of T cells (Lycke *et al*, 1989).

This combination of effects of CT on the important cells within the mucosal immunoregulatory network may favour an up-regulation of T helper cells and down-regulation of systemic T cell-mediated suppression; abrogation of systemic hyporesponsiveness and the induction of a secretory response would thus be the end result and unrelated antigen presented to the GALT at this time, would produce the same response. CT however is an atypical antigen and one must be careful in drawing analogies between its effects on the mucosal response and that of other proteins.

A breakdown in oral tolerance is an important factor in the immunology of food hypersensitivities such as coeliac disease and CMPI, and although an increase in secretory

antibody does occur, the important clinical manifestation is an antigen-specific delayed-type hypersensitivity (DTH) response in the gut. In this respect animal studies have shown that feeding of OVA, after the abrogation of oral tolerance, can lead to a DTH response in the gut manifesting in crypt hyperplasia and IEL infiltration. This reaction only occurs when T suppressor cells are depleted or APC are activated (Mowat, 1987b), or after challenging mice whose first encounter with OVA was during the neonatal period (Strobel, 1983).

The intestinal GvHR is an animal model of a DTH response in the gut (reviewed in chapter 2), and although not specific to a dietary protein, the manifestations are similar to those found in clinical enteropathy.

The effects of the local DTH response and T cell activation on the mucosal immune response is the subject of this thesis.

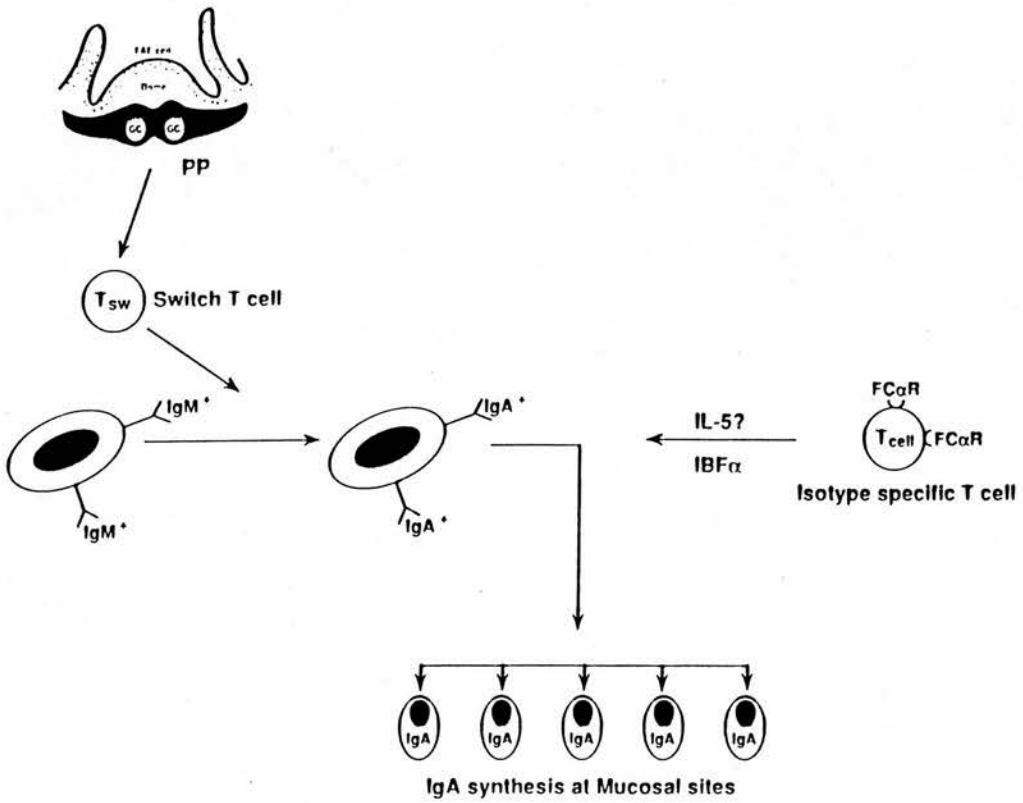


FIGURE 1:1 T cell regulation of IgA B cell differentiation in GALT.
(adapted from Mestecky and McGhee, 1987)

CHAPTER TWO

THE GRAFT-VERSUS-HOST REACTION MODEL

In this chapter I shall review the literature on the Graft-versus-host reaction (GvHR). The review will be predominantly concerned with the reaction in animals. However, the GvHR is clinically important and where appropriate the consequences of the reaction in humans will be described. The first section will detail the general consequences of a systemic GvHR, and the cellular and genetic basis of the reaction. In the second section I shall review the intestinal GvHR.

INTRODUCTION

The demonstration and description of the GvHR came in the 1950s and 1960s from studies of transplantation immunology and immunological tolerance. While studying tolerance induction in mice, Billingham and Brent (1957) found that in certain strain combinations the injection of spleen cells from one strain into newborn mice of another produced alarming mortality rates. In some animals the attack was acute, whereas in others, the disease manifested in a chronic form, and mice became undersized runts with retarded growth and development before eventual death.

With the discovery of the immunologically competent lymphocyte and its recirculation properties (Simonsen, 1960; Gowans, 1962), it was soon recognised that this "runting disease" was the end result of introducing graft lymphocytes which had the ability to react against the foreign antigens of the host: hence the Graft-versus-host reaction (Simonsen, 1962).

The mature T cell is responsible for the induction of a GvHR (Cantor, 1972) and stimulation of the donor T cells by the differing host MHC antigens, and/or non-MHC antigens results in a complex series of events affecting both lymphoid and parenchymal organs.

While the GvHR in animals provides an excellent model for investigating MHC-restricted cellular reactions, Graft-versus-host disease (GvHD) is a serious clinical problem in bone marrow transplant (BMT) patients, and in 5-10% of cases GvHD is associated with fatal complications (Tsoi, 1982).

GRAFT-VERSUS-HOST DISEASE IN MAN

There are two forms of GvHD in bone marrow transplant patients; acute and chronic. Acute GvHD occurs in 30-50% of BMT patients within 3 months of transplantation (Renkonen, 1987) and is a primary or contributory cause of death in 15-40% of patients (Gale, 1985). The target organs are the skin, liver, gastrointestinal tract and lymphoid tissue. Chronic GvHD can affect 15-50% of patients who have survived 180 days post-transplantation, and is often a transition from the acute form (Graze and Gale, 1979). However in 20-30% of cases chronic GvHD may develop without a previous acute reaction (Gale, 1985). Effects are seen in skin (Siimes, Johansson and Rapola, 1977), mouth, oesophagus, (McDonald *et al*, 1981) and liver (Sullivan *et al*, 1981).

A spectrum of immunological disorders have been described;

these include B cell hyperplasia, hypergammaglobulinaemia, immune complex deposition and autoantibody production (Shulman *et al*, 1980), and are suggestive of an autoimmune-type disease. In contrast to the autoimmune-type reactivity, a decreased ability to produce an immune response to exogenous antigens occurs, resulting in recurrent and often fatal bacterial infections (Atkinson *et al*, 1979).

EXPERIMENTAL GRAFT-VERSUS-HOST REACTIONS IN ANIMALS

The outcome of an experimental GvHR in animals depends on the strain combinations used and the immune status of the host.

The reaction can be divided into a proliferative phase and an effector phase (Klein, 1976), and the evolution of either phase depends on the animal model under investigation.

In irradiated hosts there is an initial proliferative phase during which the donor cells are induced to proliferate after stimulation by differing host cell membrane antigens. This is followed by an effector phase characterised by the generation of donor derived effector lymphocytes that can attack and destroy host tissue.

During the proliferative phase in the semi-allogeneic GvHR in F_1 hybrid hosts, alloactivation of donor cells occurs, followed by a non-specific activation of host cells. The consequences of this activation depend on the genetic differences between the donor cells and the recipients.

In both cases the proliferative phase leads to an enlargement of spleen and lymph nodes and the progression of the reaction can be monitored by the splenomegaly assay (Simonsen, 1962).

The severity of the effector phase is usually monitored by changes in body weight, mortality rate or cumulative mortality (Bril and Benner, 1985).

The animals most commonly used in experimental GvHR are mice and rats.

The two models most widely studied are:-

i) reconstitution of irradiated hosts with semi-allogeneic or allogeneic donor cells (referred to in the text as GvHR in irradiated hosts), and,

ii) a semi-allogeneic GvHR produced by injection of parent cells into F_1 hybrid hosts, (referred to as a semi-allogeneic GvHR in F_1 hybrids).

Both models produce a distinctive pathology. The systemic effects in each model will be discussed separately below. Due to the nature of the intestinal GvHR, both models will be discussed in conjunction in that section.

General consequences of a GvHR in irradiated hosts.

The reconstitution of irradiated hosts by a graft containing high numbers of T cells results in death within 30 days (reviewed Bril and Benner, 1985). This acute reaction affects the skin, liver, gastrointestinal tract and lymphoid system. The skin is the initial target and

lesions range in severity from a mild rash and epidermal necrolysis, to a loss of epidermis. Liver damage can result in hepatocellular necrosis or bile duct injury. The intestinal lesions will be discussed in full below.

A delayed form of GvHR can occur when bone marrow grafts, considered T cell depleted, are contaminated with either relatively low numbers of mature T cells (Lowenberg *et al*, 1977), or immature T cells and haemopoietic stem cells which mature under the influence of the host thymus (Goedbloed and Vos, 1965; Wolters *et al*, 1979). The clinical features of delayed GvHR are not well documented, but it is reported to have an almost 100% mortality rate (Bril and Benner, 1985).

General consequences of a semi-allogeneic GvHR in F1 hybrids.

The semi-allogeneic GvHR induced in the parent to F_1 hybrid model can produce an acute and a chronic form.
The acute form of the reaction is characterised by pancytopenia, aplastic anaemia and hypogammaglobulinaemia. This phase of suppressive pathology is often preceded by a brief phase of stimulation depending on the strain combinations used (Rolink, Pals and Gleichmann, 1983; Rolink and Gleichmann, 1983; Pals, Radaszkiewicz and Gleichmann, 1984a; Pals, Gleichmann and Gleichmann, 1984). Specific lesions are detectable in the gut and these will be described in full below.

The chronic phase of this GvHR produces a stimulatory pathology with B lymphocyte hyperplasia leading to hypergammaglobulinaemia and the formation of autoantibodies. Characteristics of systemic lupus erythematosus (SLE) such as antibodies against nuclear antigens, double-stranded DNA, erythrocytes and thymocytes have been described (Gleichmann, Van Elven, and Van der Veen, 1982). Symptoms often include severe immune complex glomerulonephritis and deposition of immunoglobulins along the basement membrane of the skin, suggestive of collagen vascular disease. Injury to the liver is also observed. (Gleichmann *et al*, 1984). In certain parent to F_1 combinations the acute form may precede the chronic form.

GENETIC BACKGROUND TO THE GRAFT-VERSUS-HOST REACTION

Major histocompatability complex antigens and GvHR.

The strongest proliferative response, as measured by the splenomegaly assay, occurs when the donor and host cells differ at the class II, H-2I region of the MHC (Klein and Park, 1973). The main loci of stimulation is the I-A subregion (Klein, 1976). Disparities at the H-2K/D region produce a weak proliferative response, whereas mutations in the H-2K or H-2D region have a strong proliferation-inducing capacity (Klein and Egorov, 1973).

With regard to the effector phase, the most severe reactions, as measured by 100% cumulative mortality, occur across an entire H-2 disparity or H-2K plus H-2I (I-A)

disparity. A less severe mortality rate occurs in H-2K, I or D singular disparities (Klein, 1976).

In man the strongest GvHR occurs across an HLA-D/DR incompatability which is analogous to the class II disparity in animals.

Class III products do not participate in the GvHR.

Non-Major histocompatability complex antigens and GvHR.

A lethal GvHR can occur across an H-2 identical, non-H-2 disparity in irradiated recipients (Korngold and Sprent, 1983). This type of GvHR is obviously clinically important in BMT patients receiving HLA-matched bone marrow cells.

The outcome of non-H-2 incompatable GvHR depends on where the incompatability lies, and on there being at least three minor H antigen differences (Korngold and Sprent, 1987).

Non-MHC antigen products of the MLS locus in mice, MLSa, MLSd and MLSe, are strongly stimulatory in the mixed lymphocyte culture assay, whereas MLSc is intermediate and MLSb not at all (Festenstein, 1973; Coutinho *et al*, 1977).

In the vast majority of H-2 compatable, non-H-2 incompatible strain combinations, the GvHR is directed by class I-restricted T cells and class II-restricted GvHR is less common.

CELLULAR REQUIREMENTS

T cell markers

T cells can be divided into two distinct subsets according to differences in phenotypic expression and function, and in using monoclonal antibodies directed against surface markers, individual T cell subsets can be isolated for investigation.

T helper cells are now recognised as $CD4^+$ and T suppressor/cytotoxic cells as $CD8^+$. However in past research other antibody markers were used, and T helper cells were recognised as Lyt-1^+2^- and T suppressor/cytotoxic cells as Lyt-1^-2^+ (Shiku, Kisielow, Bean, 1975; Cantor and Boyse, 1975). More recently the Lyt-1 surface marker was found to be present on all mouse T cells (Ledbetter *et al*, 1980), and the monoclonal antibody to the L3T4 molecule (Dialynas *et al*, 1983) has facilitated the separation of T cells into two non-overlapping populations; $L3T4^+$ and Lyt-2^+. Except for isolated T cell clones (Fink, Rammensee and Bevan, 1984) expression of both molecules by peripheral T cells is rare.

Lyt-1^+2^-/$L3T4^+$/$CD4^+$ T cells are helper cells for B cell and DTH reactions. They are class II (I-A, I-E) MHC-restricted, both in recognising antigens and alloantigens. Lyt-1^-2^+/$CD8^+$ T cells are predominantly cytotoxic cells and are class I (H-2K/D) MHC-restricted.

The induction of GvHR is caused by mature T cells (Cantor, 1972), and in the following section the role of individual T cell subsets in GvHR will be discussed. The nomenclature

of the T cell subsets will be that used by the authors whose work is cited.

T cell subsets and GvHR in irradiated hosts.

The original studies analysing the role of Lyt-1^+ and Lyt-2^+ T cells in GvHR in irradiated mice produced conflicting results. Vallera, Soderling and Kersey (1982) found Lyt 1^+ T cells to be responsible for a lethal GvHR across a complete H-2 complex disparity, while Jadus and Peck (1983), described cytotoxic T cells as the predominant subset. Using the L3T4$^+$ and Lyt-2^+ T cell markers, Korngold and Sprent (1985), found that a GvHR in irradiated mice directed against the complete H-2 complex was induced by L3T4$^+$ T cells, confirming the original findings of Vallera *et al*, (1982).

In the class I (H-2K or D) mismatched GvHR, Piguet (1985), detected only Lyt-2^+ T cells in the recipient. Conversely, in the class II (I-A or I-E) mismatched GvHR, Jadus and Peck (1983), found that Lyt-2^+ T cells were not involved. However both Lyt-1^+ and Lyt-2^+ T cells have been isolated from recipients with a class II disparate GvHR (Piguet, 1985). Using the L3T4$^+$ T cell marker, Sprent *et al*, (1986) showed that L3T4$^+$ T cells are able to mediate a class II mismatched GvHR, with no detectable effect by Lyt 2^+ T cells. Similarly, in rats, T helper cells with the W3/25$^+$ surface marker are very potent inducers of GvHR (Mason, 1981).

Lyt-2^+ T cells are also fully capable of mediating GvHR

independently but only across an allelic or bm1 mutant H-2 K/D difference (Korngold and Sprent, 1985).

This MHC-restricted function of both $L3T4^+$ and $Lyt\text{-}2^+$ T cells has also been demonstrated in *in vitro* assays (Sprent and Schaefer, 1985).

T cell subsets and GvHR in F1 hybrid hosts.

The parent into F_1 hybrid GvHR mouse model provides more evidence regarding the role of each T cell subset in the eventual outcome of the GvHR.

The persistent B cell hyperplasia and autoantibody production as described in the chronic form of this GvHR model is produced by $Lyt\text{-}1^+$ T cells activated by a class II (I-A) disparity (Van Rappard Van der Veen, Rolink and Gleichmann, 1982; Rolink *et al*, 1983).

The induction of the suppressive acute GvHR requires both $Lyt\text{-}1^+$ and $Lyt\text{-}2^+$ T cells with a difference at both class I and class II MHC regions in the F_1 recipient (Rolink *et al*, 1983).

Interestingly, in the (C57B1/10 x DBA/2) BDF_1 mouse strain, introduction of C57B1/10 parental cells into the BDF_1 recipients produces a suppressive-type GvHR which is preceded by a brief initial stimulatory phase of alloactivation. This occurs in the first 2 weeks. The alloactivation of $Lyt\text{-}1^+$ donor cells produces a transient proliferation with B cell activation. This phase is followed by alloactivation of donor $Lyt\text{-}2^+$ T cells and the suppressive pathology (Rolink *et al*, 1983; Pals *et al*,

1984ab).

The induction of a GvHR with DBA/2 parental cells, produces a stimulatory reaction. Only donor T helper cells are recovered from the recipients and no or only a few F_1 specific donor T suppressor/cytotoxic cells (Gleichmann *et al*, 1984). The lack of Lyt-2^+ T cells in the DBA/2 induced GvHR has been attributed to an inadequate number of functioning Lyt-2^+ T cells in the donor inoculum (Via, Sharrow and Shearer, 1987).

This information suggests that the L3T4^+ T cells are involved in the proliferative and stimulatory reactions occurring during the GvHR, while Lyt-2^+ T cells are involved in the effector mechanisms, such as suppression of the host immune system and damage of the host tissue.

MECHANISMS OCCURRING DURING THE GRAFT-VERSUS-HOST REACTION

There are a range of anti-host reactions that occur during the GvHR depending on the animal model under investigation. Cytotoxic T cell activity, helper T cell activity, suppressor T cell activity, DTH responses, effects on immunoglobulin and antibody production, NK cell activity and enhanced class II (Ia) expression have all been described, and are detailed below. Those activities important in the intestinal GvHR will be discussed in the second section of this chapter, while the effects on systemic T and B cells will be discussed below.

Anti-host delayed-type hypersensitivity responses in the irradiated host.

During the GvHR in irradiated mice a DTH response, directed against host histocompatability antigens, may occur.
The degree of anti-host DTH reactivity can be determined in an *in vivo* assay by transferring lymphoid cells from the irradiated recipient with GvHR into a nonirradiated naive host, syngeneic to the original GvHR donor. This animal is then challenged in the right hind footpad with spleen cells syngeneic with the original irradiated recipients. The anti-host DTH response to the challenge cells is measured as the difference in thickness of the footpad 24hrs later.

The early proliferation of mature donor T cells is required for the anti-host DTH response to occur (Wolters and Benner, 1978). The response is detectable, in irradiated recipients, 1 day after the transplantation of spleen cells, and peaks on day 4 (Wolters and Benner, 1978). It is class II MHC-restricted, the I region antigens providing the proliferative stimulus (Wolters and Benner, 1981). For a maximum anti-host DTH response, H-2I alloantigens must be presented with syngeneic H-2K/D molecules (Benner *et al*, 1985). In H-2 compatible and non H-2 incompatible strain combinations no consistent anti-host DTH responses occur unless the recipient has a disparity at a stimulatory MLS-locus such as MLSa, MLSd or MLSe (Bril *et al*, 1984).
A long-lived recirculating population of T DTH effector precursor cells (Bril *et al*, 1984), which are Lyt-$1^{+}2^{-}$, and designated T2 cells (Cantor and Asofsky, 1972), are

believed to be responsible for the anti-host DTH response. A second population of short-lived, sessile spleen cells of the Lyt-1^+2^+ phenotype and designated T1 cells are believed to "amplify" the T2 response, although they are unable to mediate an anti-host DTH response alone (Wolters and Benner, 1981). T2 cells are activated by an H-2I region disparity and T1 cells require H-2K/D antigens to elicit their amplifier effect (reviewed Benner *et al*, 1985).

T cell synergism may not be dependent on direct cell contact, but may be mediated by soluble factors released from the activated cells.

Suppressor T cells, able to suppress both the induction of the anti-host DTH response as well as already activated anti-host T DTH effector cells, can be selectively induced by intra-venous preimmunisation of spleen or bone marrow cell donors with recipient type irradiated allogeneic lymphoid cells (Bril and Benner, 1985). The suppressor cells are long-lasting, are of the Lyt-2^+ phenotype, and require proliferation in order to display their effect. Although H-2 identity between donor and host cells is required to activate the T suppressor cells, they are able to mediate both alloantigen specific and alloantigen non-specific suppression (Benner *et al*, 1985).

Anti-host cytotoxic responses in irradiated hosts.

The sensitization of donor cells to host histocompatability antigens during a GvHR in irradiated mice or rats has been shown to activate cytotoxic T cells (CTL) (Cerottini,

Nordin and Brunner, 1971; Singh, Sabbadini and Sehon, 1972; Mowat *et al*, 1988). Anti-host CTL specifically lyse host target cells, and this activity may contribute to the host tissue damage that occurs during the GvHR.

The specific anti-host cytotoxic activity can be assessed, *in vitro*, by measuring the release of ^{51}Cr from labelled tumour target cells cultured with cells from the GvHR recipient (Borland, Mowat and Parrott, 1983).

Lyt-2^+ CTL are class I MHC-restricted and in a class I directed GvHR or non H-2 incompatibility they are potent mediators of the disease.

Lyt-2^+ T cells and specific anti-host CTL are detectable in the spleens of irradiated mice 4-6 days post-transplantation of donor cells (Cerottini *et al*, 1971; Singh *et al*, 1972; Mowat *et al*, 1988). Donor-derived CTL-precursors are also detectable in the thymus, and although initially few in number their numbers start to increase between 10-20 days after bone marrow transplantation (Ceredig and MacDonald, 1982).

The ability of Lyt-2^+ T cells to produce a lethal GvHR in the absence of L3T4$^+$ T cells suggests that in certain strain combinations Lyt-2^+ T cells are able to function without exogenous help from L3T4$^+$ T helper cells or recombinant (r)IL-2 (Sprent *et al*, 1986).

A GvHR elicited across a class I MHC disparity appears in a more chronic form (Piguet, 1985), and in mice transplanted with donor-marrow and Lyt-2^+ T cells, mortality normally occurs 6-8 weeks post-transplantation (Sprent and Korngold, 1987). If however, animals are

simultaneously injected with rIL-2 or $L3T4^+$ T cells of the donor type, death occurs more quickly. Transfer of $L3T4^+$ T cells with host type marrow produces a very acute mortality due to the CTL activity against the host cells (Sprent and Korngold, 1987).

Anti-host cytotoxic responses in F1 hydrid hosts

Kubota, Ishikawa and Saito (1983) described the presence of CTL in nonirradiated GvHR in F_1 mice, however, Borland *et al* (1983) were unable to detect CTL in their F_1 animal model.

An immunostimulatory GvHR is characterised by a complete absence of $Lyt\text{-}2^+$ T cells, with donor cells almost entirely of the T helper phenotype. Conversely in the suppressive GvHR, $Lyt\text{-}2^+$ T cells are the predominant phenotype (Rolink and Gleichmann, 1983). The absence of anti-host CTL activity allows the continuous presentation of antigen to donor $L3T4^+$ T cells thus resulting in non-specific help for F_1 B cells, and the immunostimulatory features.

The anti-host CTL activity in certain mouse F_1 hybrid combinations is believed to be under suppression. This has been shown *in vitro* (Shearer and Polisson, 1980; Hurtenbach and Shearer, 1983; Langlade Demoyen and Larsson, 1986) and appears to be effective at an early stage of the CTL differentiation pathway (Langlade Demoyen and Larsson, 1986).

Non-specific cytotoxic activity, NK cell activity, has also been described in both irradiated (Mowat *et al*, 1988) and

nonirradiated F_1 hybrid mice with GvHR (Borland *et al*, 1983; Pattengale *et al*, 1983). Due to the importance of NK cell activity in intestinal GvHR, this topic will be discussed later in the chapter.

Effects on B cells during the GvHR in F1 hybrid hosts

An important immunological feature of both acute and chronic stimulatory GvHD, in BMT patients and certain semi-allogeneic F_1 hybrid murine models, is the phase of B cell hyperplasia which may result in hypergammaglobulinaemia and autoantibody production.

In the brief phase of alloactivation occurring in class I plus class II MHC-restricted GvHR in mice, there is an initial phase of lymphoproliferation (Gleichmann *et al*, 1984), and increased numbers of plasma cells in the medullary areas of peripheral lymph nodes, and spleen have been reported (Lindholm, Rydberg and Stannegard, 1973; Rolink *et al*, 1982). By day 14, the plasma cells are still present, but in lower numbers (Lindholm *et al*, 1973) reflecting, possibly, the alloactivation of T suppressor cells and the onset of a suppressive GvHR (Rolink and Gleichmann, 1983).

An increase in host immunoglobulins has been reported in the serum of nonirradiated F_1 mice (Lindholm *et al*, 1973) and in the liver of irradiated rats transplanted with allogeneic bone marrow cells. In the latter case a decrease in immunoglobulin-producing cells was observed later in the GvHR (Renkonen, Wangel and Hayry, 1986).

In acute GvHD in BMT patients there is a sharp, transient increase in IgE levels in serum (Ringden, Persson and Johansson, 1983). Similarly, increased IgG-, IgM- and IgA-plaque-forming cells (PFC) have been reported in BMT patients, within the first 4 weeks post-transplantation, followed by a significant decrease, during weeks 6-12 post-transplantation, of IgM- and IgA-PFC. Long-term survivors had significantly reduced IgM (Ringden *et al*, 1987).

In the chronic form of GvHR in mice there is an increase in IgM- and IgG-secreting cells in the spleen, and although the IgM-secreting cell numbers return to normal levels by day 5, IgG-secreting cell numbers remain elevated (van Rappard van der Veen *et al*, 1984).

During the acute GvHR the T-B cell collaborations are between host B cells, and alloreactive donor T helper cells (Gleichmann *et al*, 1984). The initial increase in immunoglobulin production is attributed to polyclonal activation of stimulated B cells, which under the influence of alloreactive T helper cells, and their soluble mediators, proliferate, mature and release antibodies (Gleichmann *et al*, 1984; van Rappard van der Veen *et al*, 1984; Ringden *et al*, 1987).

The presence of foreign antigen at the onset of GvHR, can provide an activation signal to B cells which are induced to produce a specific IgG antibody response on receiving help from alloreactive T helper cells. This is known as the allogeneic effect (Osborne and Katz, 1972; Katz and Osborne, 1972), and it only occurs when the antigen

presented to the host, prior to the induction of GvHR, is a hapten coupled to either linear molecules or cell membranes. The production of these antibodies does not require a challenge immunisation of antigen.

A common feature of the antigens which can produce the allogeneic effect, is their repeating identical epitopes presented in a linear fashion on molecules with a rigid backbone (Osborne and Katz, 1973). This structural feature is believed to allow multipoint high-avidity binding to, and cross-linking of, the Ig-receptors on unprimed F_1 B cells, thus activating them, and making them sensitive to soluble mediators released by activated T cells.

The allogeneic effect is important in considering the underlying mechanisms of autoantibody production in chronic GvHR (Gleichmann *et al*, 1984). "Self-antigens" are believed to activate autoreactive B cells while the long-lived alloreactive donor T helper cells provide the second help signal, possibly in the form of soluble mediators. (Gleichmann *et al*, 1984; van Rappard van der Veen *et al*, 1984).

The importance of T helper cells in the induction of autoimmunity was shown using Cyclosporin A (CyA). CyA suppresses the T helper cell population in lethally irradiated rats reconstituted with syngeneic bone marrow. The induction of CyA-induced autoimmunity in these animals commences only on the cessation of CyA, when T helper cells have regenerated (Bos *et al*, 1988).

The "self antigens" which induce autoantibodies share similar molecular structures to those antigens capable of

inducing the allogeneic effect (Gleichmann *et al*, 1982). Thus, autoantibodies to double-stranded DNA and RBC are detectable, but autoantibodies to organ-specific globular proteins such as mouse thyroglobulin and insulin are not (Gleichmann *et al*, 1982; van Rappard van der Veen *et al*, 1984).

The binding of "self-antigen" to F_1 B cells may induce an isotypic-switch from IgM to IgG, causing decreased levels of IgM and hypergammaglobulinaemia. Autoantibodies to RBC results in haemolytic anaemia. Both phenomena are characteristic of the chronic disease.

Immunosuppression during GvHR in F1 hybrid hosts

As discussed, certain models of GvHR produce a suppressive disease, resulting in profound immunosuppression of both T- and B-cell functions.

The degree of suppression and the kinetics depend on the murine strain combinations used and the donor cell dosage. Maximal effects are seen between day 7 and day 14 after GvHR induction (Lapp, Weschler and Kongshavn, 1974; Shand, 1975) and can persist for an indefinite period. A second phase of immunosuppression was shown in mice 21 days after GvHR induction although no effector cell was detected (Shand, 1975).

The initial early suppression was previously attributed to an increase in macrophage numbers in the spleen (Sjoberg, 1972), however other workers have shown the presence of a parental-type T suppressor cell in host spleen that is

capable of the immunosuppression of normal spleen cell function (Shand, 1976; Pickel and Hoffman, 1977). Serum from mice with GvHR has been shown to suppress normal lymphoid function (McMaster and Levy, 1975), and the production of soluble suppressor factors has been demonstrated *in vitro* (Elie and Lapp, 1976). A depressed T helper cell function has also been observed (Moser *et al*, 1987).

Thymic dysplasia and atrophy have been reported after the entry of mature lymphocytes during the GvHR (Seemayer, Lapp and Bolande, 1977). The damage to the thymus is believed to result in defects in T cell function thus preventing the maturation of T cells into IL-2 secreting cells (Lapp *et al*, 1985).

During the GvHR the humoral response to systemically presented antigens is determined by, when in the progression of the GvHR, the antigen is administered. A potentiated antibody response to the thymus-independent antigen SIII occurred when the antigen was given immediately after the induction of the GvHR. In contrast, when the antigen was given to the mice at a later stage of the GvHR, a suppressed antibody response was observed (Byfield, Christie and Howard, 1973).

An interesting finding by Treiber and Lapp (1976), was the concomitant stimulation of a CMI response to SRBC and suppression of a humoral response during a GvHR. The authors suggested that two distinct T helper cells were involved, and the recent discovery of two subsets of T helper cell clones, T_H1 and T_H2 cells, giving help for CMI

and antibody responses respectively (Mosmann *et al*, 1986) enhances their original hypothesis. It is feasible that during the GvHR, different T cell subsets are stimulated, and this possibility will be explored in the discussion of my findings.

The role of suppressor mechanisms in the progression of GvHR in both animals and humans is important, and in some animal models of GvHR, suppressor cells were found to be more important than CTL in mortality of the host (Rolink *et al*, 1982).

The consequences of the GvHR are diverse. The close relationship between the lymphoid system and the parenchymal organs, both targets during the GvHR, raises interesting questions relating to the mechanisms underlying the damage found in the skin, liver and gastrointestinal tract. Is there a direct attack on the host tissues by anti-host cytotoxic cells, or is the damage a bystander effect of the many and varied alloantigen-stimulated DTH responses?

This following section describes the intestinal GvHR, and studies more closely the mechanisms of host tissue, cell damage.

GRAFT-VERSUS-HOST REACTION AND THE SMALL INTESTINE

The gastrointestinal tract is a major target organ in both human and animal GvHD. The clinical features include malabsorption, bloody diarrhoea and a protein-losing enteropathy (Weisdorf *et al*, 1983). In the rodent GvHD, this results in general wasting and the classic "runting" syndrome as described by Billingham and Brent (1957).

Distribution of lesions

In man, the whole length of the gastrointestinal tract is affected, with more severe lesions found in the distal ileum and colon (Slavin and Woodruff, 1974). In animal models, histological changes have also been observed in the ileum and the colon (Reilly and Kirsner, 1965), but the majority of information on GvHR and the intestine has been gained from studies of the jejunum.

PATHOLOGICAL CONSEQUENCES OF THE GRAFT-VERSUS-HOST REACTION

The earliest studies of intestinal GvHR in irradiated mice described destructive changes in the mucosal architecture, characterised by crypt lengthening, increased crypt cell mitosis, increase in cell numbers near the tips of villi, villus atrophy and cellular debris in the intestinal lumen (Reilly and Kirsner, 1965; Cornelius, 1970). In some mice, crypts at various stages of necrosis were also described (Cornelius, 1970). These studies were performed

in irradiated animals and the conclusions made did not allow for the damage occurring in the gut due to irradiation alone.

The simultaneous occurrence of increased crypt cell mitotic activity and villus atrophy during the GvHR was suggested, by these early workers, to be the direct result of a cytotoxic attack on the enterocytes. The increase in crypt cell mitosis was thus an attempt to redress the balance and produce normal functioning villi.

More detailed studies of the dynamic changes in intestinal morphology during the GvHR were obtained using the nonirradiated F_1 hybrid murine model.

Elson, Reilly and Rosenberg (1977), showed that 14 days after the induction of GvHR in adult nonirradiated (C57BL/6JxDBA/2) BDF_1 mice the crypts were elongated but there was no change in the villus length.

In nonirradiated neonatal F_1 hybrid mice, age 6-8 days at the onset of GvHR, crypt hyperplasia and an increased crypt cell production rate (CCPR) were observed 5-7 days after the induction of the GvHR (MacDonald and Ferguson, 1977; Mowat and Ferguson, 1982; Felstein and Mowat, 1988).

In very immature neonates, age 2-6 days at the onset of GvHR, a more interesting evolution of morphological changes has been described. After an initial distinct phase of crypt hyperplasia and increased CCPR, the lesion evolved into a severe, destructive lesion characterised by crypt hyperplasia, increased CCPR and villus atrophy (MacDonald and Ferguson, 1977; Felstein and Mowat, 1988).

This evolution of morphological changes has also been

observed in irradiated recipients. 1-3 days after the induction of GvHR in heavily irradiated (CBAxBALB/c) F_1 mice, there is an increase in CCPR and crypt hyperplasia. After 3 days, the CCPR falls to zero and the crypt and villus lengths significantly shorten (Mowat *et al*, 1988).

The enteropathy occurring during the GvHR has been suggested by Mowat and co-workers to present in two distinct phases of morphological change.

It has been suggested that the initial increase in CCPR and crypt hyperplasia is a proliferative form of enteropathy. This then evolves to a destructive form of enteropathy characterised by villus atrophy with or without crypt hyperplasia.

The proliferative form predominates in mature immunocompetent hosts, and can occur as an initial early phase of enteropathy in severe models of GvHR. The progression to the second, destructive form appears to be the outcome in neonatal animals and immunocompromised hosts (Mowat *et al*, 1988; Felstein and Mowat, 1988; Mowat and Felstein, 1989).

IMMUNOLOGICAL CONSEQUENCES

Alterations to the lymphoid cell populations in the mucosae appear to fall into a similar pattern. An early infiltration of mononuclear cells into the LP, with a later destruction and ulceration of PP and depletion of mucosal lymphoid cells, was described in early studies of intestinal GvHR (Reilly and Kirsner, 1965; Cornelius,

1970).

Influences on T cells, of both donor and host origin have now been extensively studied, however the effects on B cells, and non-lymphoid cell populations are less well documented.

GvHR and mucosal T cells

Both donor and host T cells may infiltrate the mucosae, depending on the animal model used. T cell infiltrates of the small intestine of lethally irradiated mice, are predominantly of donor origin, whereas a semi-allogeneic GvHR in F_1 hybrid mice produces a cell infiltrate generally of host origin.

T cells have been shown to infiltrate the crypt area, both LP and epithelium, an area in which T cells are not normally found (Guy-Grand and Vassalli, 1987).

Alloantigenic activation of T cells has been reported in PP, with subsequent migration to the gut (Guy-Grand, Griscelli and Vassalli, 1978), suggesting that the intestinal GvHR is a locally mediated response.

GvHR and Intraepithelial lymphocytes

In the initial proliferative form of enteropathy there is a substantial increase in the IEL count per 100 epithelial cells, making it a useful marker of the progression of the GvHR. The increase in IEL count coincides with the increased CCPR and crypt hyperplasia, and with the

progression of the systemic GvHR, as measured by splenomegaly (Mowat and Ferguson, 1982).

In the destructive form of enteropathy in irradiated hosts, a striking decrease in IEL numbers coincides with the decrease in CCPR, and crypt and villus length (Mowat *et al*, 1988).

The origin of IEL is as yet unclear. Reports of donor IEL and host IEL infiltration into the epithelium have been documented (Guy-Grand and Vassalli, 1986). Their role remains to be elucidated.

GvHR and mucosal B cells

A profound decrease in the numbers of IgA- and IgM-containing plasma cells in the LP of nonirradiated neonatal mice (Gold *et al*, 1976), and a secretory IgA deficiency in BMT patients with chronic GvHD (Beschorner *et al*, 1981) have been described. There is very little data on GvHR and mucosal B cells; the findings of this thesis will hopefully elucidate the effects of the GvHR on mucosal B cell responses.

GvHR and Mucosal Mast cells

An increase in mucosal mast cells (MMC) has been reported in both the mouse (Mowat and Ferguson, 1982) and rat (Ferguson *et al*, 1987) during GvHR. The increase in cell counts in the rat is paralleled by an increase in the MMC specific protease - rat mast cell protease II in jejunal

tissue and in serum (Ferguson *et al*, 1987; Cummins *et al*, 1988).

The induction of Class II expression on intestinal epithelium during a GvHR.

During a GvHR in rats, the expression of class II (Ia) molecules on villus enterocytes, is intensified and *de novo* expression occurs on the crypt stem cells (Mason, Dallman and Barclay, 1981; Barclay and Mason, 1982).
In human acute GvHD, HLA-DR (class II) expression has been described on rectal crypts in association with the characteristic histological appearance of GvHD, even in the absence of intestinal symptoms (Sviland *et al*, 1988). Interestingly, in other disorders of the gastrointestinal immune system, such as coeliac disease and dermatitis herpetiformis, where villus atrophy and crypt hyperplasia are classic features, both residual immature enterocytes and crypt cells express class II (Scott *et al*, 1981).
Using an *in vitro* organ culture of foetal small intestine, MacDonald, Weinel and Spencer (1988), showed that activation of T cells *in situ* using PWM, produced a cell-mediated immune reaction with enhanced expression of HLA-DR (class II) on villus and crypt epithelium. This effect was neutralized with an antibody raised against recombinant human IFN-*gamma*.
The Ia molecule is normally associated with the presentation of antigen to activated T cells. During GvHR in rats there is an increase in macrophages and dendritic

cells - antigen presenting cells - expressing class II molecules (Barclay and Mason, 1982; Mayrhofer *et al*, 1983). A possible role of enterocytic presentation of antigen either directly to T cells, or into the LP has been hypothesized (Bland, 1988; reviewed in chapter 1), but not proven.

During GvHR, the enhanced expression of Ia could feasibly be induced by the release of IFN-*gamma* during the DTH reaction, but the role it plays in the pathology of gut enteropathy remains to be elucidated.

THE CELLULAR AND GENETIC BASIS OF THE INTESTINAL GRAFT-VERSUS-HOST REACTION.

The intestinal GvHR is T cell dependent, and as with the systemic GvHR, T cell activation depends on the MHC disparity between donor and host.

Initial studies using the Lyt T cell markers suggested that Lyt-1^+2^- T cells alone induced the increase in IEL numbers, and a degree of crypt hyperplasia in nonirradiated F_1 hybrid mice, but the full crypt changes appeared to require both Lyt-1^+2^- and Lyt-1^-2^+ T cells. A disparity at the class II I-A locus alone was sufficient for these changes. Class I disparities did not produce the intestinal, proliferative changes (Mowat, Borland and Parrott, 1986).

The intestinal lesions produced in irradiated mice have been shown to occur solely across a class II disparity (Piguet, 1985).

More detailed information has evolved from the use of the T

cell markers, Lyt-2^+, and L3T4^+. While both phenotypes are able to migrate to the gut, Lyt-2^+ T cells are five times more efficient. Thus, even in a GvHR across a class II difference alone, both cell types are detectable in the gut (Guy-Grand and Vassalli, 1987). However, despite the lesser ability of L3T4^+ cells to migrate to the gut, they are more efficient in creating gut damage and stimulating host cells (Guy-Grand and Vassalli, 1986).

Despite these apparently clear-cut findings, other reports have shown that Lyt-2^+ cells are capable of causing intestinal injury, however this depends on the concentration of Lyt-2^+ cells in the donor inoculum (Guy-Grand and Vassalli, 1987).

The L3T4^+ and Lyt-2^+ T cells are able to function independently (Sprent *et al*, 1986). It would appear that both class I and class II MHC alloantigens can stimulate intestinal GvHR, but that the L3T4^+ class II-restricted T cells may be more potent inducers of intestinal GvHR, and that the Lyt-2^+ class I-restricted T cells may be involved at a different stage of the intestinal damage.

MECHANISMS OF GUT INJURY

The mechanisms underlying the intestinal GvHR are still under investigation. There are two possible mechanisms. It is feasible that the destruction of the mucosa is due to a local, specific anti-host cytotoxic response against the enterocytes. An alternative explanation is that a local DTH response is occurring and either through direct cell

contact or by the action of cytokines released during the reaction, profound alterations are made to the homeostasis of the villus/crypt unit.

The predominant involvement of class II MHC-restricted T cells in the induction of intestinal GvHR, suggests that the underlying mechanism is a class II MHC-restricted local DTH reaction.

The class II (I-A) restricted proliferative form of intestinal GvHR in both adult nonirradiated mice (Borland *et al*, 1983) and 7 day old neonatal mice (Felstein and Mowat, 1988) is not associated with CTL activity. However, the destructive form, observed in irradiated mice (Mowat *et al*, 1988) and in 1-2 day old neonatal mice (Felstein and Mowat, 1988) is paralleled by a marked CTL activity. Yet, CTL activity is not necessary for the destructive phase because, in 5-6 day old mice, which can develop villus atrophy, no CTL activity was detected (Felstein and Mowat, 1988).

There is in fact detailed evidence to suggest that the intestinal damage occurring during GvHR is a "bystander phenomenon" requiring no direct cellular attack on the mucosa.

The implantation of parental-type foetal small intestine under the kidney capsule of adult F_1 hybrid mice produces a graft which develops normal, parental intestine architecture and contains host bone marrow derived cells (Ferguson and Parrott, 1972). Induction of a GvHR in the F_1 hosts by injection of parental cells induces an increase

in IEL numbers, CCPR and crypt length in the implanted graft, similar to the host jejunum. (Elson *et al*, 1977; Mowat and Ferguson, 1981). Similarly, implanted foetal intestine in mice, irradiated prior to GvHR induction, demonstrates the destructive pathology as seen in the host jejunum (Mowat *et al*, 1988). Thus, both forms of intestinal pathology can occur when the gut offers no allogeneic stimulus for donor cells.

The evidence to date favours a local DTH response, over the cytotoxic attack, as the underlying mechanism of intestinal GvHR. And there is further evidence that locally produced cytokines play a key role.

Lymphokines and intestinal damage

The described forms of enteropathy are also observed in T cell mediated allograft rejection of foetal small intestine implants, and the damage has been attributed to lymphokines - "enteropathic" lymphokines released during a DTH reaction (Ferguson and Parrott, 1973; MacDonald and Ferguson, 1976; 1977).

Mucosal lymphocytes isolated from mice with acute GvHR are able to produce IFN-*gamma*, IL-2 and IL-3 (Guy-Grand and Vassalli, 1986). The production of IFN-*gamma* is probably responsible for the enhanced expression of class II (Ia) molecules on intestinal epithelium and increased NK activity (see below).

IFN-*gamma* is a potent stimulator of lymphocyte migration

into the skin during DTH (Issekutz, Stoltz and Meide, 1988), and interestingly, treatment of mice during GvHR with monoclonal antibodies to IFN-*gamma* prevents the increase in IEL counts (Mowat, 1989).
Injection of anti-TNF-*alpha* antibodies during GvHR almost entirely prevents intestinal lesions (Piguet *et al*, 1987), as does the injection of anti-IFN-*gamma* antibodies (Mowat, 1989). TNF-*alpha* cannot be detected in the blood and is most probably produced locally (Piguet *et al*, 1987).

The definitive role of lymphokines in intestinal GvHR is as yet uncertain, however the characteristics of certain soluble mediators would suggest that their production and release at various times throughout the GvHR plays a major part in the patterns of intestinal injury.

Natural Killer cell activity

Enhanced peripheral non-specific natural killer (NK) cell activity has been demonstrated in early human GvHD (Dokhelar *et al*, 1981), and in nonirradiated mice with GvHR (Borland *et al*, 1983), and parallels the progression of the systemic GvHR.
The IEL population in mouse small intestine exhibit NK cell activity (Tagliabue *et al*, 1981; Tagliabue *et al*, 1982) which is under suppressive regulation (Mowat *et al*, 1983).
During the proliferative form of intestinal GvHR, IEL exhibit enhanced NK activity which coincides with the

increase in IEL numbers (Borland, Mowat and Parrott, 1983). Depletion of resting NK activity by treatment of host mice with anti-asialo (As G_{m1}) antibody results in a less severe systemic GvHR (Ghayur, Seemayer, and Lapp, 1988) with no crypt hyperplasia, and reduced IEL numbers (Mowat and Felstein, 1987).

Both IL-2 and IFN-*gamma* can enhance NK cell activity (Roder, Karre and Keissling, 1981), suggesting their activity is part of the local DTH reaction.

In the destructive form of intestinal GvHR in irradiated mice, the onset of villus atrophy is marked by a complete loss of NK cell activity (Mowat *et al*, 1988).

The close proximity between these effector cells and the epithelium is potentially important, and their role in the intestinal GvHR obviously warrants further investigation.

Bacterial microflora and intestinal damage

Antibiotic decontamination of the intestinal tract, and the use of laminar air flow isolation to provide protection from exogenous microorganisms have been reported to reduce the incidence and severity of acute GvHD in mice (van Bekkum *et al*, 1974), and in BMT patients (Buckner, Clift and Saunders, 1978). Nevertheless, it is not certain if survival is improved. More specifically, the removal of aerobic gut flora prevents much of the gut damage (van Bekkum *et al*, 1974) and return to conventional conditions induces tissue damage and mortality. Despite the suggested

beneficial effects of a germ-free environment, the intestinal damage does occur in antigen free, foetal, small intestine implants in mice with GvHR (Elson *et al*, 1977; Mowat and Ferguson, 1981).

Increased levels of circulating endotoxin have been described in animals with GvHR (Walker, 1978). It is possible that an increased absorption of antigens with adjuvant properties such as LPS will exacerbate intestinal GvHR.

The evidence discussed suggests that the intestinal GvHR evolves from a proliferative form to a destructive form: both of which produce distinct morphological and cellular changes (Table 2:1).

The progression from the proliferative form to the destructive form depends on the immune status of the host. This suggests that the host mucosal lymphocytes play a major role in determining the evolution of enteropathy.

A GvHR induced in athymic mice produces a severe, destructive enteropathy (Mowat, Felstein and Baca, 1987), as does a GvHR in irradiated and 1-5 day old neonatal mice. These animals all lack normal, mature T cell function and may thus be unable to suppress or regulate the ongoing DTH reaction.

A continuous release of unregulated non-specific soluble mediators during the DTH reaction may thus be responsible for the development of the intestinal lesion.

Interestingly, the two distinct forms of enteropathy have

been described in coeliac disease (Marsh, 1988). This cell-mediated hypersensitivity to gluten is characterised by the simultaneous occurrence of villus atrophy and crypt hyperplasia.

The local DTH reaction in the gut, irrespective of the initial stimulus, appears to induce an initial proliferative form of enteropathy. If this reaction proceeds uncontrolled by the host, perhaps due to a lack of suppressor mechanisms or some deficiency in immunoregulation, the intestinal lesions progress to a destructive pathology characteristic of gut enteropathy in clinical disease.

There is evidence, which I have detailed, suggesting that cytokines released locally, during the DTH response, have a major influence on the mucosal architecture of the gut and the infiltration of cells into the epithelium.
The close spatial relationship between the epithelium and cells of the lymphoid system, and the effects observed during the intestinal GvHR point to an important immunoregulatory balance between the two.

The intestinal GvHR offers an excellent model for the study of cell mediated immunity at the gut level, and may thus aid and extend our understanding of similar types of reactions occurring in clinical diseases.

PROLIFERATIVE PHASE OF INTESTINAL GVHR

Observed in nonirradiated adult F_1 mice, and 7 day old mice.

FEATURES

- Increased CCPR
- Crypt hyperplasia
- Increased IEL
- Increased MMC
- Enhanced class II (Ia) expression on villus and crypt epithelium.
- Increased NK cell activity
- No specific anti-host CTL activity
- Normal villus height.

DESTRUCTIVE PHASE OF INTESTINAL GVHR

Observed in immunocompromised hosts i.e. irradiated mice, 1-2 day old and some 5 day old mice.

FEATURES

- Reduction in CCPR
- Crypt hyperplasia
- Decreased IEL
- Villus atrophy
- Loss of NK cell activity
- Appearance of specific anti-host CTL activity

TABLE 2:1 Morphological and cellular changes occurring during the intestinal GvHR.

CHAPTER THREE

MATERIALS AND METHODS

ANIMALS.

C57Bl/6J (H-2^b) mice, purchased from Bantam and Kingman, (Hull, England), were mated with DBA/2 (H-2^d) mice which were bred continuously at the Animal Unit, Western General Hospital, Edinburgh. The BDF_1 (H-$2^{b\backslash d}$) F_1 progeny were used throughout this work.

The transgenic mice were a kind gift from J. Paul Simons, Edinburgh Research Station, AFRC Institute of Animal Physiology and Genetics Research, King's Buildings, West main Road, Edinburgh. The female (C57Bl/6JxCBA) F_1 progeny were mated with (C57Bl/6JxCBA) F_1 males. The progeny of this cross were donated for this study.

The ages of the animals used are indicated in the relevant chapters. All animals were kept on a 12/12 hour light/dark cycle.

DIET

Animals were maintained on a standard small rodent diet, CRM(x), (Labsure; Cambridge, England) which is known to be free from both ovalbumin and betalactoglobulin. Water was available *ad libitum*.

ANAESTHESIA

Ether (May & Baker Ltd; U.K.) anaesthesia was used for procedures such as intra-gastric intubation, intra-

peritoneal and intra-footpad injections, retro-orbital plexus bleeds, and complete exsanguination.

Initial experiments in the development of a method for the collection of gut contents (Chapter 5) were carried out under barbital anaesthesia using Sagatal 60mg/ml (May & Baker Ltd; U.K.) diluted 1:10 in sterile water. Mice were injected intra-peritoneally with 0.01mg/g body weight.

BODY WEIGHTS

Mice were weighed using an Oertling TP40, top pan balance, accurate to 0.00g.

ORGAN WEIGHTS

Organs were dissected free of surrounding tissue and weighed using the Oertling TP40, top pan balance, accurate to 0.00g.

ANTIGENS

Ovalbumin five times crystallized, and bovine betalactoglobulin, three times crystallized, were obtained from Sigma, (England, U.K.).

Cholera toxin was obtained from List Biological Laboratories Inc., (California, U.S.A.).

Antigens were dissolved in sterile water or 0.15M saline (Travenol; U.K.) before feeding or injection.

ADMINISTRATION OF ANTIGENS

Oral administration

Experimental animals were not fasted unless otherwise stated. Animals were fed antigen by gastric intubation using a 19 gauge stainless steel dosing needle with a spherical shaped blunted end.

Antigens were administered in either 0.2ml or 0.4ml volumes depending on the concentration of antigen being administered.

Parenteral immunisation

In studies of systemic immunity mice were immunised intra-dermally (i.d.) in the right hind footpad. 200ug of antigen, dissolved in 50ul saline, was suspended in an equal volume of Complete Freund's Adjuvant (CFA) (H37 Ra; Difco, England). Each animal received 100ug of antigen suspended in 50ul of adjuvant.

When raising antisera to specific antigens, mice were given 100ug of antigen in 100ul of CFA and injected intra-peritoneally (i.p.).

COLLECTION OF BLOOD

In survival experiments 150ul of blood were obtained from the retro-orbital plexus, under light ether anaesthesia, using non-heparinised haematocrit capillary tubes (Hawksley; England). Each capillary tube was sealed at one

end with Cristaseal (Hawksley; England). Samples were allowed to clot in the upright position and then spun down in a micro-haematocrit centrifuge (Hawksley; England) for 2min at 12,000g. Serum was collected and then stored at -20°C.

Larger quantities of blood were obtained by complete exsanguination from the axillary vessels. Mice were kept under continuous ether anaesthesia. Blood was kept in non-heparinised blood tubes, or autoanalyser cups (Sterilin; England), and centrifuged at 400g for 10min in a Super Minor MSE bench centrifuge. Serum was collected and stored at -20°C.

COLLECTION OF SALIVA

Mice were given an i.p. injection of 200ug pilocarpine (Sigma; U.K.) dissolved in 0.2ml sterile water. 10-15mins later the animals would begin to salivate. Up to 100ul of saliva could be collected in non-heparinised capillary tubes, by holding the tips at the mouth of the mouse, gently massaging the mouth area, and allowing the saliva to be collected by capillary action. Animals were maintained under light ether anaesthesia during this procedure. Saliva was stored at -20°C.

COLLECTION OF MILK

Lactating mice were given an i.p. injection of 600ul (3U) oxytocin (Syntocinon: Sandoz, England). 15-

20min later, milk was collected in capillary tubes by gentle massage of the mammary gland area. Milk was stored at -20°C. This procedure did not require anaesthesia, as the oxytocin had a light anaesthetic effect. The mice suffered no ill-effects from this procedure.

ASSESSMENT OF SYSTEMIC DELAYED-TYPE HYPERSENSITIVITY

Mice were tested for delayed-type hypersensitivity responses by measuring the specific increment in footpad thickness 24hrs after an i.d. challenge of 100ug of antigen in 50ul saline into the non-immunised rear footpad.
The response is expressed as the difference in thickness (in mm) between the pre- and post challenge footpad.
Initial experiments were performed using Pocotest A microcalipers (Carobronze Ltd; U.K.). In the final year of experimental work I used a set of Microstat calipers (Moore & Wright, Microsystems Ltd; Sheffield, U.K.) which had been specifically adjusted and refined to measure small rodent footpads. A comparative study between the two types of calipers showed that the latter gave a more sensitive and reproducible measurement of the footpad thickness.
Control mice were immunised with antigen in CFA and tested with 50ul saline.

SACRIFICE OF ANIMALS

Mice were killed by cervical dislocation.

PREPARATION OF CELL SUSPENSIONS

Spleen, mesenteric lymph nodes, and Peyer's patches were removed immediately after sacrifice and dissected free from surrounding tissue. They were placed in medium and cut into small segments. For the SP cell suspensions to be used in the induction of GvHR, RPMI 1640 medium (Flow Labs; Irvine, U.K.) was used. For SP, MLN and PP cell suspensions to be analysed in the ELISPOT assay, RPMI 1640 containing 10% foetal calf serum (FCS) (Gibco; U.K.) was used.

The segments of tissue were gently passed through a stainless steel fine gauge wire mesh using the plunger of a 5ml syringe (Sterilin; U.K.). The suspensions were transferred to sterile universals (Sterilin; U.K.). SP cell suspensions to be used in GvHR experiments were passed through a wide pore sterile gauze to remove debris. The cell suspensions were then washed three times at 400g in a MSE bench centrifuge.

After the final wash, the cells were counted in an improved Neubauer haemocytometer using white cell diluting fluid. Cell viability was determined by the trypan blue exclusion test. Viability was generally 80-85%. The final cell pellet was made up to the required concentration of viable cells using the appropriate medium.

INDUCTION OF A GRAFT-VERSUS-HOST REACTION

A semi-allogeneic GvHR was induced in nonirradiated female BDF_1 mice by an i.p. injection of 10^8 adult female C57BL/6J

spleen cells suspended in 0.2ml RPMI 1640. Control animals received either the equivalent concentration of syngeneic cells i.p. or medium alone.

In the experiments studying the effects of GvHR in neonatal mice (chapter 8), BDF_1 mice aged 7 days old received $7x10^5$ cells, mice aged 14 days received $5x10^7$ cells, and mice age 21 days received the adult dose, 10^8 cells. Control animals were given medium alone.

ASSESSMENT OF THE GRAFT-VERSUS-HOST REACTION

The progression of the GvHR was assessed using the Simonsen spleen weight assay (Simonsen, 1962). Mice were weighed prior to sacrifice, the spleen removed free of fat and weighed. The relative spleen weight was expressed as mg spleen/10g body weight.

The splenic index (SI) was calculated using the formula:-

$$SI = \frac{\text{mean relative spleen weight in GvHR mice}}{\text{mean relative spleen weight in control mice}}$$

THE SOLID-PHASE ENZYME-LINKED IMMUNOSPOT (ELISPOT) ASSAY FOR THE ENUMERATION OF IMMUNOGLOBULIN SECRETING CELLS

The ELISPOT assay, as described by Czerkinsky *et al* (1983), and Sedgwick and Holt (1986), was set up in this laboratory with the technical assistance of Mr N. Anderson. The assay was modified to suit our requirements and reagents, and was used for the detection of IgA-, IgM- and IgG-immunoglobulin secreting cells in PP, MLN and SP from mice.

Twenty-five well tissue culture plates (Replidishes, Sterilin; U.K.) were coated with 500ul of the isotype-specific antibody diluted in carbonate/bicarbonate buffer pH 9.6. (See solutions and buffers).

Coating antibodies

For the detection of IgA-secreting cells: Goat anti-mouse IgA affinity purified antibody (Sigma; U.K.) at a 1:500 dilution.
For the detection of IgM-secreting cells: Rabbit anti-mouse IgM affinity purified antibody (Litton Bionetics; U.S.A.) at a 1:250 dilution.
For the detection of IgG-secreting cells: Goat anti-mouse IgG affinity purified antibody (Sigma; U.K.) at a 1:1000 dilution.

The plates were incubated overnight at $4^{o}C$ in a "moist box", and then washed twice with PBS (pH 7.2) containing 0.05% Tween 20 (BDH Ltd; U.K.), with a final wash with PBS alone. The final SP, PP, MLN cell suspensions were made up in 500ul of RPMI 1640 containing 10% FCS (Gibco; U.K.), and 10^{6} cells were added to the first well. 3 subsequent doubling dilutions were made. The plates were incubated at $37^{o}C$ for 2hrs, then further washed with PBS/0.05% Tween 20. 500ul of the appropriate conjugate, detecting antibody diluted in PBS/0.05% Tween 20/1% BSA at a 1:1000 dilution for all three isotypes was added.

Conjugated antibodies

For the detection of IgA-secreting cells: Goat anti-mouse IgA alkaline phosphatase conjugated antibody (Sigma; U.K.).
For the detection of IgM-secreting cells: Goat anti-mouse IgM alkaline phosphatase conjugated antibody (Jackson Labs; U.S.A.).
For the detection of IgG-secreting cells: Goat anti-mouse IgG alkaline phosphatase conjugated antibody (Jackson Labs; U.S.A.).

The plates were incubated for 5hrs at room temperature (RT) in a "moist box". After a further three washes with PBS/0.05% Tween 20 the plates were placed on a level surface and 500ul of warm (45^{o}C) agarose-substrate mixture (containing 1mg/ml of 5-bromo-4-chloro-3-indolyl phosphate (5-BCIP: Sigma; U.K.) in 0.6% w/v agarose) added to each well.
After about 1min the agarose-substrate mixture hardens. Macroscopic blue spots became visible within 30mins at RT. The spots were counted after 1-2hrs. If the spots were small or not clearly visible, the plates were left overnight at RT in a "moist box" to allow further colour development.
The substrate-enzyme reaction was stopped by adding 200ul/well of 3M NaOH (BDH Ltd; U.K.). The plates could then be stored at 4^{o}C.
The spots were studied under a dissecting microscope (x32 magnification: Zeiss Stereomicroscope 4B) and the visible blue spots scored. By moving through the field of focus,

definite blue spots could be marked and blue stained debris eliminated.

The results are expressed as spot-forming cells (SPC) per 10^6 cells.

REMOVAL OF TISSUES

Immediately after sacrifice, pieces of intestine (approximately 5-10mm in length) were removed, free from PP. They were opened, placed on card, villus surface upwards and immersed in fixative.

FIXATIVES AND HISTOLOGY

For conventional histology, and for staining of plasma cells of IgA, IgM and IgG isotypes, intestine specimens were fixed in 4% buffered formalin (see solutions and buffers) for 24hrs and subsequently stored in 70% ethanol. The specimens were embedded in paraffin wax, and sections 3um thick were cut.

For conventional histology and counts of intraepithelial lymphocytes, tissues were stained with haemotoxylin and eosin (H&E).

INTRAEPITHELIAL LYMPHOCYTE COUNTS

Intraepithelial lymphocytes (IEL) were counted by the method described by Ferguson and Murray (1971). Sections

were examined under x1000 (oil immersion) magnification, and only sections with a single epithelial cell layer were considered. Lymphoid cell nuclei, above the basement membrane, were counted. A total of 600 epithelial cells were counted per specimen and counts were expressed as number of IEL/100 epithelial cells.

STAINING PROCEDURE FOR THE DETECTION OF IMMUNOGLOBULIN-CONTAINING CELLS IN TISSUE SECTIONS

IgA-, IgM- and IgG-containing plasma cells were detected separately using a peroxidase anti-peroxidase (PAP) technique.

Cut sections 3um thick were placed on lysine (Sigma; U.K.) coated slides, allowed to dry overnight at 37^{o}C, and dewaxed and rehydrated through histoclear and decreasing strengths of alcohol.

After a 5mins wash in tap water followed by a rinse in distilled water, the sections were treated with 1% periodic acid (BDH Ltd; U.K.) for 5mins, rinsed in running tap water for a further 5mins and treated with 0.2% potassium borohydride (BDH Ltd; U.K.) for 5mins. This effectively blocked endogenous peroxidase.

Sections were washed in running tap water for 5mins, rinsed in Tris buffer saline (TBS) (pH 7.6), and treated with normal donkey serum (SAPU; Scotland) in TBS (1:4 dilution) for 30mins.

The relevant primary antibody was then added at the appropriate dilution (in TBS) for 2hrs.

Primary antibodies

For the detection of IgA: Rabbit anti-mouse IgA affinity purified antibody (Litton Bionetics; U.S.A) at a 1:600 dilution.

For the detection of IgG: Rabbit anti-mouse IgG affinity purified antibody (Litton Bionetics; U.S.A.) at a 1:100 dilution.

For the detection of IgM: Rabbit anti-mouse IgM affinity purified antibody (Litton Bionetics; U.S.A.) at a 1:100 dilution.

The sections were rinsed in two changes of TBS, 5mins each, and the secondary antibody, a donkey anti-rabbit Ig (SAPU; Scotland) diluted 1:40 in TBS was then added for a further 2hrs.

After a further two rinses in TBS the rabbit peroxidase anti-peroxidase (PAP) complex, at a 1:80 dilution in TBS was added, for 1hr.

The sections were rinsed in TBS, washed in tap water for 5mins and treated with 3-amino-9-ethyl carbozole (Sigma; U.K.) for 20-30mins.

After a further wash in tap water for 5mins the sections were treated with haematoxylin to lightly stain the nuclei, the sections were washed and then "blued" with lithium carbonate (BDH Ltd; U.K.) solution.

After a final wash the sections were mounted in glycerine jelly.

All cells have dark blue nuclei. Positively stained cells have a red, grainy cytoplasm. The other cells have light

blue cytoplasms.

All sections were examined under a Leitz ortholux II or a Leitz dialux EB20 microscope.

Histological processing was carried out by Mr J Bode.

PLASMA CELL COUNTS

Positively stained plasma cells within the lamina propria were counted using a square-grid eyepiece, calibrated to give the number of cells per mm^2 of mucosa. The eyepiece was aligned with the lower edge lying on the upper surface of the muscularis mucosae close to the bottom of the crypts. The tissue was examined under x1000 magnification, 40 areas of each section were counted and the numbers expressed as cells/mm^2 mucosa. No correction was made for the area containing epithelium.

TISSUE PROCESSING FOR MICRODISSECTION

Pieces of intestine were removed immediately after sacrifice, opened, placed on card and immersed in Clarke's fixative (see solutions and buffers) for 24hrs, then stored in 70% ethanol. To allow for repeated examination, only one half of the tissue was stained by the modified feulgen reaction. Pieces of gut were immersed in 50% ethanol for 10mins, followed by 10mins in tap water, and 7mins hydrolysis in 0.1N HCL at 60^{o}C. The tissue was then rinsed three times in tap water, and stained with Schiff's reagent (Difco Ltd; U.K.) for 30mins at room temperature. The

specimens could then be stored in tap water for a maximum of 48hrs before microdissection.

The tissue was examined under the dissecting microscope (Zeiss Stereomicroscope 4B) and the muscularis mucosae removed using fine forceps. Thin layers of mucosa containing a few villi and the adjacent crypts were dissected from the tissue using a cataract knife (Weiss Ltd; U.K.). The pieces were placed on a microscope slide with a drop of 45% acetic acid, covered with a coverslip and examined under a Wild M20, Heerbrugg Microscope (Switzerland) with a previously calibrated eyepiece micrometer.

In each specimen the lengths of 10-15 complete villi (under x100 magnification) and crypts (under x400 magnification) were measured and the means calculated for group comparisons.

PRECIPITIN ASSAY FOR THE DETERMINATION OF THE CROSS-REACTIVITY BETWEEN BOVINE AND OVINE BETALACTOGLOBULIN.

A standard precipitin assay based on the Ouchterlony double diffusion method (Weir, 1973) was used to determine the cross-reactivity between bovine and ovine BLG (chapter 10). Antibodies to bovine BLG were raised in BDF_1 mice by an i.p. injection of 100ug BLG in 50ul CFA followed three weeks later by a booster dose of BLG in incomplete CFA. The presence of IgG antibodies to BLG was determined by ELISA.

The precipitin assay was performed in an agarose medium.

7mls of a 1% solution of pure agarose (Marine Colloids, Inc.; U.K.) in PBS pH 7.2, containing 0.1% sodium azide (BDH. Ltd; U.K.) were pipetted into a 5cm diameter plastic petri dish (Sterilin; U.K.), and allowed to solidify overnight. Three wells were cut into the agarose using a no. 2 cork borer, and sealed with a drop of molten agarose. A range of concentrations of bovine BLG and ovine BLG, (extracted from milk from the Dorset Horn sheep; a kind gift from J. Paul Simons, Edinburgh Research Station, AFRC Institute of Animal Physiology and Genetics Research. Edinburgh), were added to wells 1 and 2 in a series of plates and the detecting antisera against bovine BLG added to the final well (well 3) in each plate. A positive control was incorporated into the assay.

The plates were incubated in a "moist box" for 48hrs at RT and then washed over a period of two days with PBS, which was frequently changed.

The plates were allowed to dry, the precipitin lines were examined over a light source, and the lines of identity between the antisera to bovine BLG and the ovine BLG determined.

DETERMINATION OF TOTAL PROTEIN CONTENT IN GUT LAVAGE FLUID

Measurment of total protein concentrations in gut secretions from mice was performed using a diagnostic kit, the BCA protein assay (Pierce Chemical Company; U.K.).

The protein concentration is determined by the reaction of the protein, in the sample, with alkaline copper II to

produce copper I. The bicinchoninic acid (BCA) reagent reacts with the copper I to form an intense purple colour, which can be detected spectrophotometrically (I used a Pye Unicam PU 8610 spectrophotometer) at O.D. 562nm.

The concentrations of protein in the gut lavage fluid were extrapolated from the standard curve which was produced using a range of known concentrations of bovine serum albumin (BSA: Sigma; U.K.), plotted against their corresponding absorbance values.

The standard curve (Fig 3:1) for this assay is extremely accurate and reproducible.

THE GUT LAVAGE TECHNIQUE

(Elson, Ealding and Lefkowitz, 1984)

Materials

The lavage solution contained:-

25mM NaCl (BDH Ltd. U.K.)

40mM Na_2SO_4 (BDH Ltd. U.K.)

10mM KCl (BDH Ltd. U.K.)

20mM $NaHCO_3$ (BDH Ltd. U.K.)

48.5mM Polyethylene glycol (PEG) MW 3350 (Sigma; U.K.)

The solution was made up in 1 litre distilled water, with a final osmolarity of 530 osmM.

Protease Inhibitors

1. EDTA/Soybean trypsin inhibitor.

0.1mg/ml soybean trypsin inhibitor (Sigma; U.K.) in 50mM ethylenediaminetetra-acetic acid (disodium salt) (EDTA: BDH Ltd. U.K.).

2. 100mM phenylmethyl sulphonyl fluoride (PMSF: Sigma; U.K.) dissolved in 95% ethanol.

Method

Under anaesthesia, four doses of 0.5mls lavage solution were given intra-gastrically at 15mins intervals. 30mins after the last dose each animal was given 100ug of pilocarpine (Sigma; U.K.) in 100ul saline, i.p. Each animal was then placed on an animal cage lid, under which was positioned, a 100mmx15mm plastic petri dish (Sterilin; U.K.) containing 3mls of the EDTA/soybean trypsin inhibitor solution. A 500ml glass laboratory beaker was put over the animal to keep it in position (Fig 3:2).

The desired action of the pilocarpine begins within 15-20mins. The mice salivate, and defaecate, discharging faecal pellets and some intestinal fluid into the petri dish. Solid material not retained by the wire was removed.

The fluid was transferred to 15ml glass, graduated, conical centrifuge tubes and fibrous material fragmented using a glass rod. The volume obtained was recorded and the samples made up to 6mls with PBS (pH 7.2). Each sample was vortexed and centrifuged in a MSE bench centrifuge at 650g for

15mins.

3mls of supernatant were transferred into round bottom polycarbonate centrifuge tubes. 30ul of PMSF were added, and the samples were further clarified by ultracentrifugation at 27,000g in a MSE ultracentrifuge for 20mins at 4^{o}C.

2, 1ml aliquots of the supernatant were transferred into sterile bijoux bottles (Sterilin; U.K.) and a further 20ul PMSF plus 10ul sodium azide added to each.

After 15mins standing, 50ul FCS (Gibco; U.K.) were added to each aliquot and the samples were stored at -20^{o}C.

THE GUT WASHING TECHNIQUE

Mice were given 4x0.5ml doses of the lavage solution (as described above). After 30mins each animal was sacrificed, and the intestine from the duodenum up to and including the caecum carefully removed, so as not to damage or puncture the intestinal wall. The contents of the caecum were collected, because after this length of time they do contain a large volume of liquid (see chapter 5).

A 10ml syringe (Sterilin; U.K.), attached to a blunt ended feeding tube, containing 3mls EDTA/soybean trypsin inhibitor, plus 3mls of air was inserted into the proximal duodenum. The caecum was placed into a graduated, conical centrifuge tube, and the bottom snipped to allow the contents to empty into the tube. The contents of the syringe were gently flushed through the gut (Figure 3:3). The collected samples were immediately placed on ice and

processed using the method described for the lavage technique. The samples were finally stored at -70^{o}C.

ELISA METHODS

I developed the ELISA methods for the detection of total IgA in serum, gut washings, milk and saliva, total IgM in serum and gut washings, and total IgG in serum. This was achieved by a checkerboard analysis of the optimum concentrations of coating antibody, standard reference serum and conjugated antibody for each isotype.
The materials and the methodology for all the ELISAs are in the appendix to this chapter.
The initial studies of total IgA concentrations in the lavage fluid were performed using an ELISA set up prior to my arrival in the laboratory (Appendix A:1). In all subsequent investigations, using the gut washing technique, total IgA concentrations were determined using my own newly developed ELISA (Appendix A:2).

The ELISAs for the detection of IgG and IgM antibodies to OVA and BLG had been developed prior to my arrival in the laboratory. Using the same principles of development I set up an ELISA for the detection of IgA antibodies to OVA and BLG in gut washings.

STATISTICAL EVALUATIONS

The results for body and spleen weights, villus and crypt

lengths and IEL counts, are presented as mean±s.d., as indicated in the graphs and tables. A student's t-test was used to compare differences between groups.

Immunoglobulin concentrations, antibody responses and DTH responses, are presented as mean±SEM, and as a non-parametric distribution of this data was likely, a Wilcoxon rank sum test was used to compare differences between groups.

Where the analysis of variance within and between groups, as determined by the MINITAB computer programme, is given in the Tables, the Wilcoxon rank sum test significance values are given in the text.

In all tests $P>0.05$ was taken as not significant, a value of $P<0.05$ as significant, and $P<0.01$ as highly significant.

Calculations were performed on a Casio fx-180P calculator, or in cases of large group numbers, a MINITAB computer programme was used.

SOLUTIONS AND BUFFERS

Carbonate/bicarbonate buffer :

1:20 dilution of stock carbonate/bicarbonate buffer diluted in distilled water (Northeast Biomedicals; U.K.). The solution was used at pH 9.6.

4% Buffered formalin:

40mls Formaldehyde, 960mls distilled water, 4g $NaH_2PO_4.2H_2O$, and 6.5g Na_2HPO_4.

Clarke's fixative:

750mls ethanol, and 250ml Glacial acetic acid.

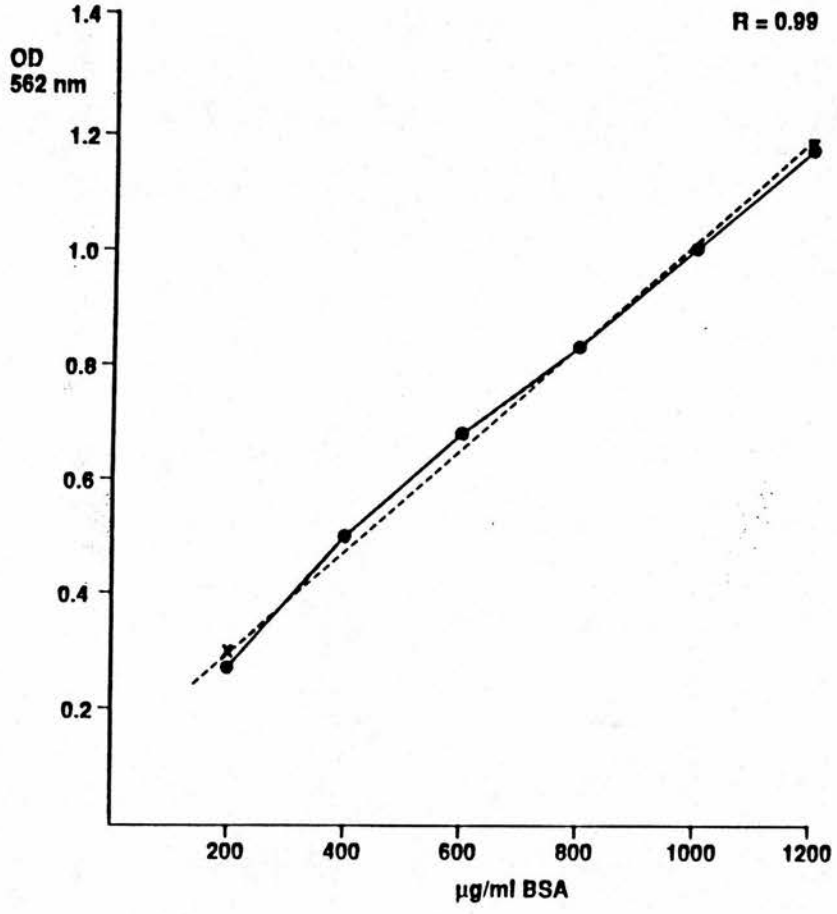


FIGURE 3:1 Protein assay standard Bovine serum albumin (BSA) curve.
Both the OD 562nm values (solid line) and the best fitting line (broken line), as determined by linear regression analysis, are shown.

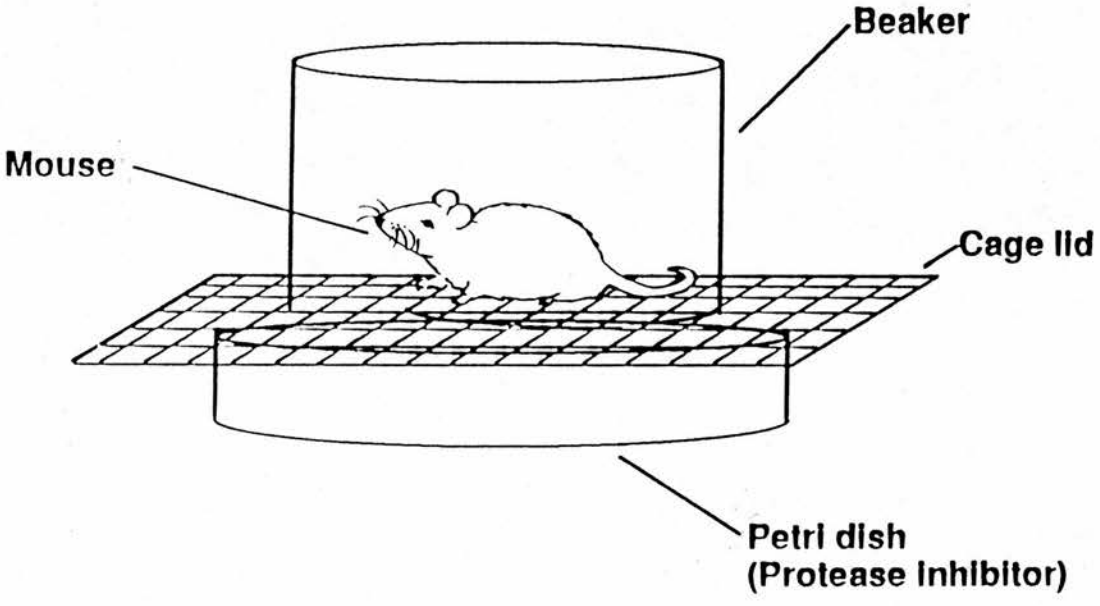


FIGURE 3:2 Diagrammatic representation of the apparatus used to collect lavage fluid using the gut lavage technique.

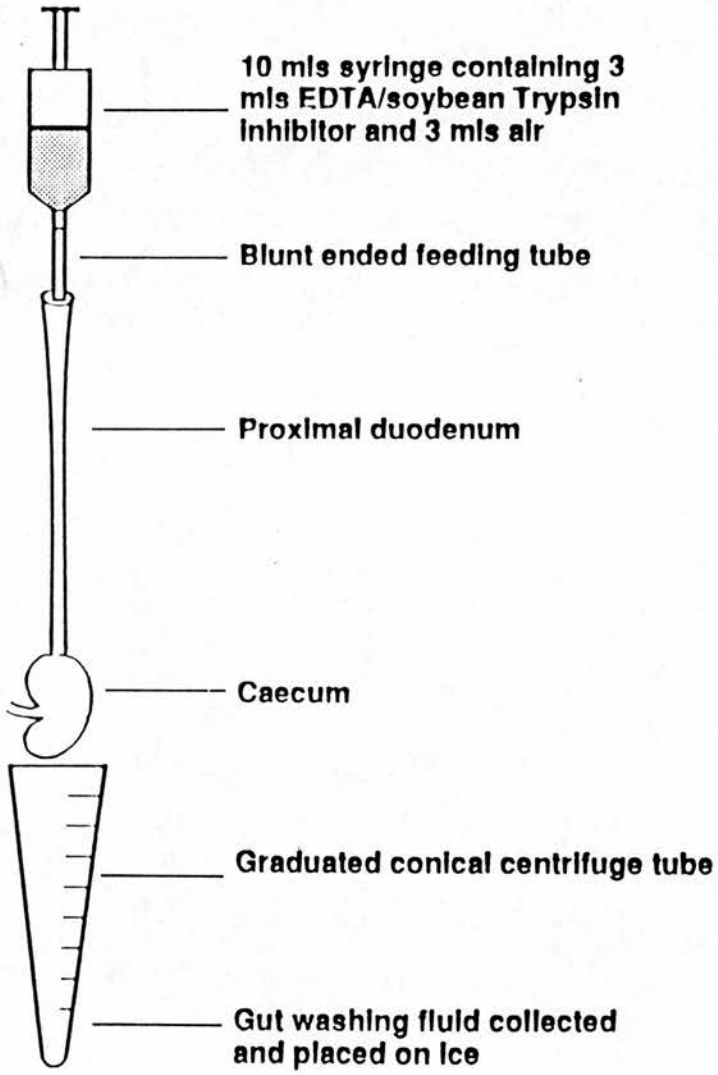


FIGURE 3:3 Diagrammatic representation of the method used to collect gut washings.

CHAPTER THREE

APPENDIX

ELISA DEVELOPMENT

To determine the optimum concentrations, of both coating and conjugated antibodies, to be used in each isotype-specific ELISA, a checkerboard analysis was performed. Briefly, a 96 well microtitre ELISA plate (as described below) was coated with one dilution of coating antibody (125ul/well;diluted in carbonate/bicarbonate buffer (for all solutions see below). The plate was incubated overnight at 4^{o}C in a "moist box", washed, and divided into 4 sections (each section 6 by 4 wells). Doubling dilutions of standard normal mouse reference serum (ICN Immunobiologicals), from neat to near extinction (diluted in serum diluent), were added to each section of the plate. The plate was incubated overnight at 4^{o}C, washed, and 4 different dilutions of the conjugate antibody (eg. 1:1000, 1:2000, 1:3000 or 1:4000) added;1 dilution/section. The plate was incubated at RT for 5hrs, washed and developed. Readings were taken continuously until the OD values for the doubling dilutions of reference serum gave a linear curve. This type of analysis was repeated, using various combinations of coating antibody and conjugated antibody concentrations, until the optimum dilutions, giving the most linear standard curve of reference serum, were determined. The ELISAs for the detection of total IgA, IgM and IgG were developed in this way.

The ELISA protocols described below are divided into two sections. The first section describes the materials and methods used for the detection of total immunoglobulins in

murine serum, saliva, milk and intestinal contents. The second section describes the materials and methods for the detection of specific antibodies in serum and gut washings. The ELISAs are grouped according to their method. The materials used for each ELISA will be detailed first, followed by the common method.

ELISA FOR THE DETECTION OF TOTAL IGA, IGM AND IGG

A.1 ELISA FOR THE DETECTION OF TOTAL IGA IN MOUSE LAVAGE FLUID

This ELISA was used in the initial experiments determining the concentration of total IgA in intestinal fluid, collected from mice using the lavage technique (as described in chapters 3 and 5). The general method for this ELISA is the same as the other ELISAs in this group. However, some of the antibodies used in this ELISA required different incubation times and temperatures, also there is an additional step, that of adding the detecting antibody prior to adding the conjugate. For this reason the incubation time for each antibody is given in the materials section.

MATERIALS.

Coating antibody

Rabbit anti-mouse IgA affinity purified antibody (Litton Bionetics; U.S.A) used at a 1:1000 dilution. Incubation: 2hrs at 37°C.

Standard curve

The standard curve was produced using the following dilutions of normal mouse reference serum (Miles; U.K.):- 100ug/ml, 10ug/ml, 1ug/ml, 100ng/ml, 31.6ng/ml, 10ng/ml. (Fig: A:1).

Sample dilutions

Lavage fluid: Neat

Detecting antibody

Rabbit anti-mouse Ig affinity purified antibody (Northeast Biomedicals; U.K.) used at a 1:500 dilution. Incubation: 5hrs at RT.

Conjugate antibody

Goat anti-rabbit globulin affinity purified antibody conjugated to alkaline phosphatase (Northeast Biomedicals; U.K.) used at a 1:1500 dilution. Incubation: overnight at 4°C.

A.2 **ELISA FOR THE DETECTION OF TOTAL IGA IN GUT WASHINGS, SERUM, MILK AND SALIVA.**

MATERIALS

Coating antibody

Goat anti-mouse IgA affinity purified antibody (Sigma; UK) used at a 1:5000 dilution.

Standard Curve

The standard curve was produced using the following dilutions of a normal mouse reference serum (ICN Immunobiologicals; U.K.):-

100ug/ml, 10ug/ml, 1ug/ml, 100ng/ml, 31.6ng/ml and 10ng/ml. (Fig: A:2).

Sample Dilutions

Serum:	1:400, 1:800, 1:1600, 1:3200.
Gut Washings:	Neat.
Milk:	1:10, 1:20.
Saliva:	1:10, 1:20.

Detecting Antibody

Goat anti-mouse IgA affinity purified alkaline phosphatase conjugated antibody (Sigma; UK), diluted 1:1000.

A.3 **ELISA FOR THE DETECTION OF TOTAL IGM IN SERUM AND GUT WASHINGS.**

MATERIALS

Coating Antibody

Rabbit anti-mouse IgM (Litton Bionetics; U.S.A) affinity purified antibody, diluted 1:2000.

Standard Curve

The standard curve was produced using the following dilutions of normal mouse reference serum (ICN Immunobiologicals; U.K.): 1000ng/ml, 500ng/ml, 250ng/ml, 125ng/ml, 62.5ng/ml, 31.25ng/ml and 15.62ng/ml. (Fig: A:3).

Sample Dilutions

Serum:	1:2000, 1:4000, 1:8000, 1:16000
Gut washings:	Neat

Detecting Antibody

Goat anti-mouse IgM alkaline phosphatase conjugated antibody (Jackson Labs Ltd. U.S.A) diluted 1:1000.

A.4 **ELISA FOR THE DETECTION OF TOTAL IGG IN SERUM.**

MATERIALS

Coating Antibody

Goat anti-mouse IgG affinity purified antibody (Sigma; U.K.) at a 1:2000 dilution.

Standard Curve

The following dilutions of normal mouse reference serum (ICN Immunobiologicals; U.K.) were used to produce the standard curve: 500ng/ml, 250ng/ml, 125ng/ml, 62.5ng/ml, 31.25ng/ml and 15.62ng/ml. (Fig: A:4).

Sample Dilutions

Serum: 1:80,000, 1:160,000, 1;320,000, 1:640,000.

Detecting Antibody

Goat anti-mouse IgG alkaline phosphatase conjugate antibody (Jackson Labs Ltd. U.S.A) used at a 1:5000 dilution.

ELISA METHOD

The ELISAs for the detection of all three isotypes were performed on EIA microtitre plates, number 129A (Dynatech Ltd. U.K.), and the working volume used throughout was 125ul.

The appropriate dilution of coating antibody was made in carbonate/bicarbonate buffer (see solutions), and added to each well. The plates were incubated overnight at 4°C in a "moist box". The plates were washed three times with an ELISA wash (see solutions), and blocked for 10-15mins with serum diluent (solutions).

The correct dilutions, of both the samples and the standards, were made up in serum diluent, and added to the plates. The plates were incubated in a "moist box" at either RT for 5hrs or overnight at 4°C.

After a further three washes, the conjugated antibody diluted in serum diluent, was added and the plates incubated in a "moist box" again at either RT for 5hrs or overnight at 4°C.

The plates were washed three times and the substrate solution, 1mg/ml p-nitrophenyl phosphate (Sigma; U.K.) in 10% Diethanolamine buffer (see solutions), added to the plates. The plates were kept uncovered at RT, in daylight, until the reaction developed.

Each plate was read, using a MR580 Microelisa autoreader (Dynatech Ltd. U.K.), at a wavelength of 405nm, when the highest concentration of standard reference serum reached an absorbance value of 1.00.

The IgG ELISA developed within 10-15mins, the IgA and IgM ELISAs took 40-45mins.

The standard concentrations were transformed into log values, and these were plotted against the corresponding absorbance values (Fig: A.1, A.2, A.3 and A.4).

The concentrations of total IgA, IgM and IgG immunoglobulins, in each sample, were extrapolated from the standard curves.

ELISA METHOD FOR THE DETECTION OF IGG, IGM, AND IGA ANTIBODIES TO OVA, AND BLG IN SERUM AND GUT WASHINGS.

A.5 ELISA FOR THE DETECTION OF IGG, IGM AND IGA ANTIBODIES TO OVA AND BLG IN SERUM.

MATERIALS

Coating antigen concentrations

Ovalbumin (Sigma; U.K.) - 10ug/ml

Betalactoglobulin (Sigma;U.K.) - 500ug/ml

Sample concentrations

Serum antibodies to Ovalbumin - IgG - 1:400

IgM - 1:50

IgA - 1:50

Serum antibodies to Betalactoglobulin - IgG - 1:200

IgM - 1:50

IgA - 1:50

Standard curves

The positive reference serum for each ELISA was obtained by immunising BDF_1 mice with 100ug of the appropriate antigen in 100ul of CFA. Serum to be used in the IgM detecting ELISA was collected from animals 10 days after immunisation, while serum to be used in both the IgA and IgG ELISAs was collected 21 days post-immunisation. Samples

were pooled, and aliquots stored at -20°C.

A range of doubling dilutions of the positive serum, starting at 1:400 for IgG, at 1:50 for IgM and 1:50 for IgA were added to the desired plate. Dilutions were made in serum diluent.

Detecting antibodies for IgG, IgM, and IgA antibodies to OVA and BLG.

For the detection of IgG antibodies, a 1:5000 dilution of Goat anti-mouse IgG antibody conjugated to alkaline phosphatase (Jackson Labs Ltd. U.S.A.) was used.

Antibodies of the IgM isotype were detected using a 1:1000 dilution of Goat anti-mouse IgM alkaline phosphatase conjugated antibody (Sigma; U.K.).

Antibodies of the IgA isotype were detected using a 1:1000 dilution of a Goat anti-mouse IgA alkaline phosphatase conjugated antibody (Sigma; U.K.).

ELISA METHOD

The appropriate antigen was dissolved in carbonate/bicarbonate buffer, and 125ul added to each well of a 129B EIA 96 well microtitre plate (Dynatech Ltd. U.K.). The plates were incubated overnight at 4°C in a "moist box".

The plates were washed 3 times with ELISA wash (see solutions), and blocked with serum diluent (see solutions); 15mins block for IgG, 30mins for IgM and 45-60mins for IgA.

Serum samples, in the appropriate dilutions, and the standard serum dilutions, again at 125ul, were added to the plates, and the plates incubated at RT for 2.5hrs, in a "moist box".

The plates were washed 3 times with ELISA wash, and the appropriate dilution of conjugated antibody, dissolved in serum diluent, added. Plates were incubated for either 3.5hrs at RT or overnight at 4^{o}C.

After a further washing, 125ul of substrate solution (1mg/ml p-nitrophenyl phosphate in 10% DEA buffer)(see solutions), was added to each well. The plates were allowed to develop, and were read when the first standard serum dilution gave an absorbance reading of 1.00.

A.6 **ELISA METHOD FOR THE DETECTION OF IGA ANTIBODIES TO OVA AND IGA AND IGM ANTIBODIES TO BLG IN GUT WASHINGS.**

MATERIALS

Coating antigen concentrations

Ovalbumin (Sigma; U.K.) - 10ug/ml

Betalactoglobulin (Sigma; U.K.) - 500ug/ml

Sample concentrations

Gut washings - Neat

Standard Curve

As described above. A 1:8 dilution of positive serum was used as the starting dilution.

Detecting antibody

Antibodies of the IgA isotype were detected using a 1:1000 dilution of a Goat anti-mouse IgA alkaline phosphatase conjugated antibody (Sigma; U.K.).

Antibodies of the IgM isotype were detected using a 1:1000 dilution of a Goat anti-mouse IgM alkaline phosphatase conjugated antibody (Sigma; U.K.).

ELISA METHOD

The appropriate antigen was diluted in

carbonate/bicarbonate buffer (see solutions), added to the plates and incubated overnight at 4°C. After washing 3 times with ELISA wash (see solutions) and blocking (60mins), with serum diluent (see solutions), the samples and standards were added, and the plates incubated at RT for 5hrs. The plates were washed, the detecting antibody added and incubated overnight at 4°C.

After a further wash, 125ul of substrate solution (1mg/ml p-nitrophenyl phosphate in 10% DEA buffer (see solutions)), was added to each well. The plates were allowed to develop and were read when the first standard serum dilution read 1.00.

SOLUTIONS

Carbonate/bicarbonate buffer:

1:20 dilution of stock carbonate/bicarbonate buffer (Northeast Biomedicals; U.K.) diluted in distilled water. Used at pH 9.6.

Elisa wash:

0.05% Tween 20 (BDH Ltd. U.K.) in physiological saline.

Serum diluent:

0.05% Tween 20, 0.02% sodium azide (BDH Ltd. U.K.) and 1% adult bovine serum (SAPU. Scotland), in physiological saline.

10% Diethanolamine buffer:

100mls DEA (Diethanolamine buffer: BDH Ltd. U.K.), 800mls distilled water, 0.1015g $MgCl_2.H_2O$ (BDH Ltd. U.K.), 0.2g Sodium azide (BDH Ltd. U.K.). The buffer was used at pH 8.6.

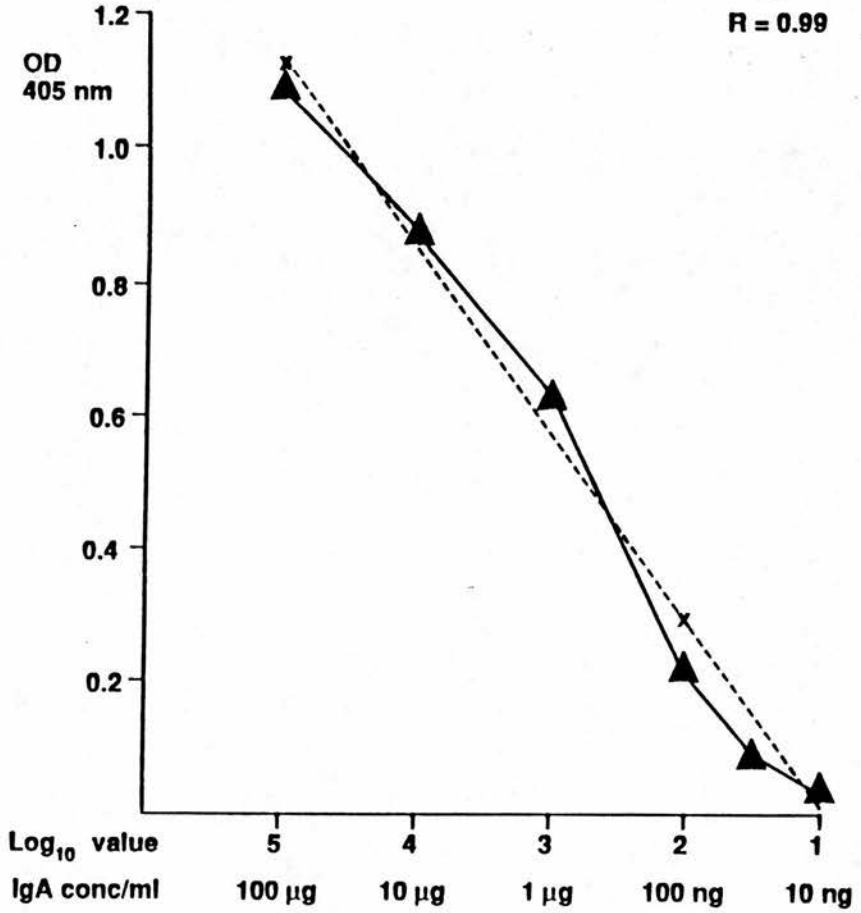


FIGURE A:1 Total IgA ELISA standard curve.
Both the OD 405nm values (solid line) and the best fitting line (broken line), as determined by linear regression analysis, are shown.

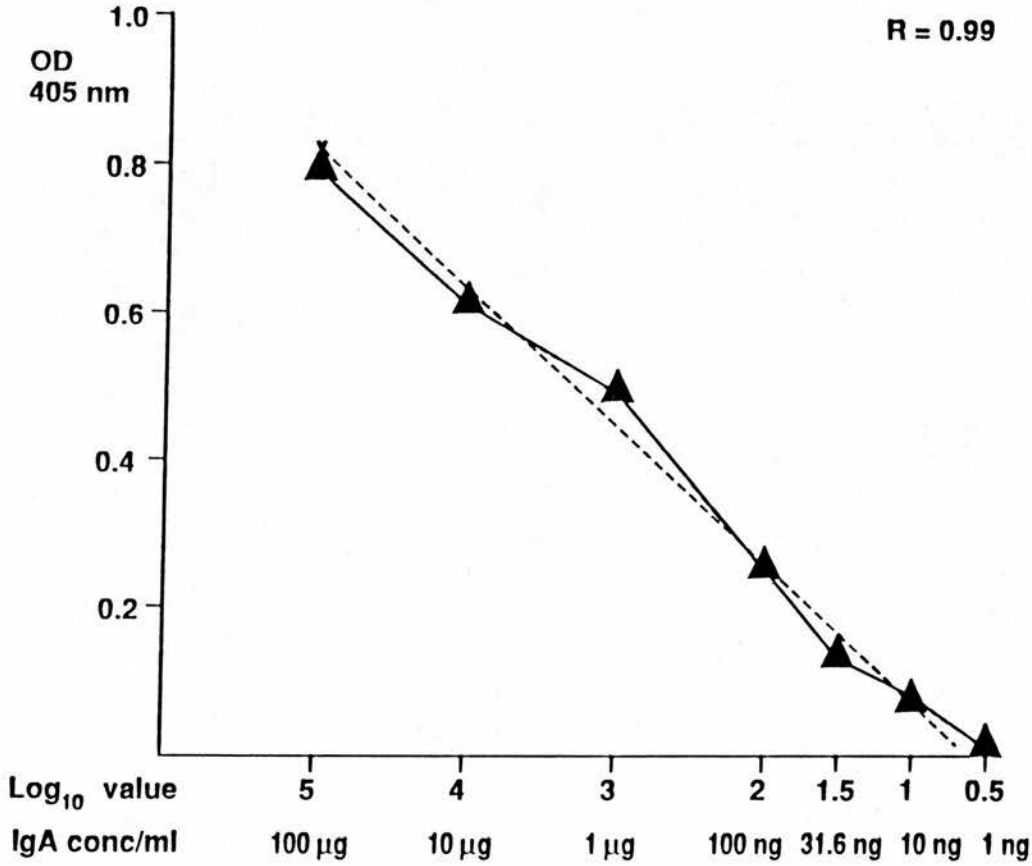


FIGURE A:2 Total IgA ELISA standard curve.
Both the OD 405nm values (solid line) and the best fitting line (broken line), as determined by linear regression analysis, are shown.

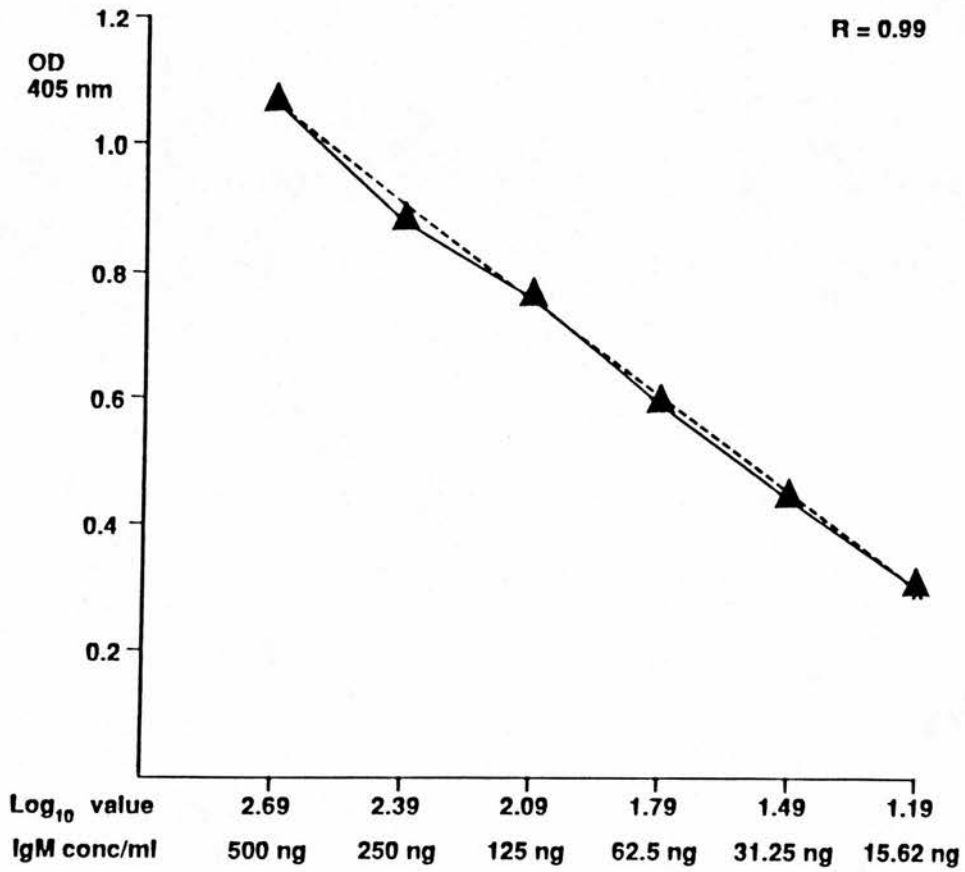


FIGURE A:3 Total IgM ELISA standard curve.
Both the OD 405nm values (solid line) and the best fitting line (unbroken line), as determined by linear regression analysis, are shown.

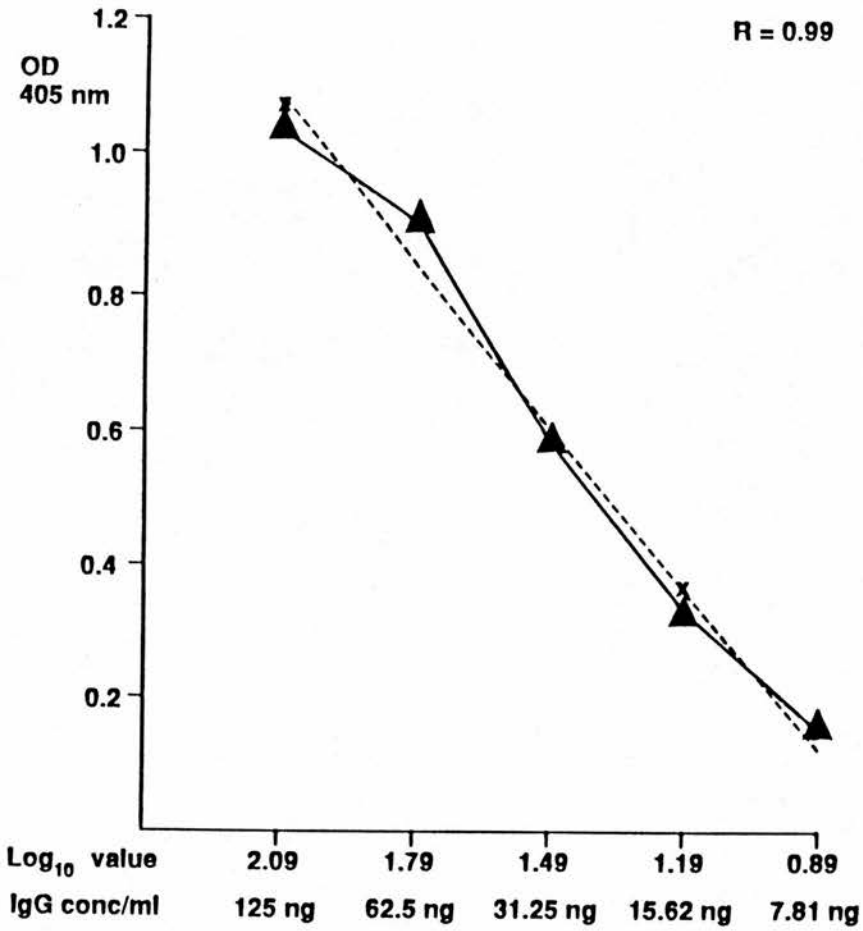


FIGURE A:4 Total IgG ELISA standard curve.
Both the OD 405nm values (solid line) and the best fitting line (unbroken line), as determined by linear regression analysis, are shown.

CHAPTER 4

INTESTINAL GVHR: A STUDY OF THE MUCOSAL CHANGES OCCURRING DURING A GVHR IN ADULT MICE

INTRODUCTION

Prior to studying the effects of a GvHR on the secretory immune response, it was first necessary to establish the changes in mucosal architecture that occur as a result of this reaction.

The intestinal GvHR in nonirradiated (C57Bl/6JxDBA/2) BDF_1 hybrid mice is known to produce alterations to the small intestine which are indicative of a local DTH response. An initial proliferative form of enteropathy, characterised by crypt hyperplasia, increased CCPR and infiltration of IEL, and a destructive form, characterised by villus atrophy, crypt hyperplasia and decreased IEL numbers, have been described (chapter 2).

A progression from the proliferative to the destructive form of intestinal GvHR has been described in immunocompromised mice such as neonates and irradiated hosts. However, in mature, adult nonirradiated F_1 hybrid mice, the evolution of the intestinal GvHR is not well defined.

The first set of experiments was designed to describe and define the morphology of the small intestine, during the initial phase of immunostimulation, in the adult, nonirradiated (C57B1/6J x DBA/2) BDF_1 murine GvHR model.

In the light of Mowat's definitions of the intestinal GvHR (chapter 2), briefly outlined above, the second set of experiments was designed to study, in the same animal model, the possible progression of the initial

proliferative form of intestinal GvHR, to a second destructive form of enteropathy.

The experiments were designed to monitor changes in the mucosal architecture up to 5 weeks after the induction of the GvHR. In conjunction, the protocol was modified in that a group of animals received a large donor cell inoculum, to try to produce severe, destructive lesions in the intestine.

The parameters used to study the changes in mucosal architecture were villus and crypt lengths, and IEL infiltration into epithelium.

A: **MUCOSAL CHANGES DURING THE INITIAL PHASE OF GVHR.**

Experimental protocol

Female BDF_1 mice, aged 6-8 weeks, were injected with 10^8 C57B1/6J female spleen cells. Age-matched controls received 10^8 F_1 spleen cells or medium alone. At various time intervals between day 4 and day 14 after the induction of GvHR, mice from control and experimental groups were sacrificed. Body and spleen weights were measured and the spleen index calculated. Sections of jejunum, taken from the mid-gut region were fixed and processed for microdissection.

Development of the GvHR

All animals remained healthy throughout the experiment.

Those animals with GvHR did show a slight decrease in body weight, on days 12 and 14, as compared to controls, however this did not reach statistical significance (Fig 4:1).

Splenic hypertrophy, as assessed by the spleen index, was detectable as early as day 4, reaching a peak of 3.20 on day 10, with a slow decline thereafter (Fig 4:1).

Mucosal architecture during GvHR

Microdissection analysis of jejunal samples revealed that the villus length was unchanged during this phase of GvHR (Table 4:1). The crypt length was significantly increased on day 10 of the GvHR ($P<0.05$) as compared with controls, and continued to rise until day 14 ($P<0.001$), the last day studied.

Intraepithelial lymphocyte counts during GvHR

IEL counts (Table 4:1) showed a significant increase on day 10 ($P<0.001$), as compared to controls, reaching a peak on day 12 ($P<0.001$). By day 14 the IEL counts were in decline, but still significantly greater ($P<0.001$) than controls.

Comments

The development of the GvHR was assessed by following the increase in spleen weight. The peak of splenomegaly

occurred on day 10, and from this time point the crypts remained longer than controls. IEL counts were also increased from day 10, however after reaching a peak on day 12 the counts began to decline. No change in villus length was observed.

These findings are in accordance with data from other researchers, and confirm the morphological changes occurring in the initial phase of GvHR in this murine model.

B: **EVOLUTION OF MUCOSAL CHANGES DURING GVHR**

Experimental protocol

The mucosa of the small intestine was studied at various intervals up to 32 days after the induction of GvHR. Architectural changes were monitored in mice receiving the standard donor cell dose and in mice receiving a large donor cell inoculum.

Group A and B animals were adult BDF_1 mice, aged 6-8 weeks at the onset of the experiment. The donor cells were C57Bl/6J spleen cells. Group A, received the standard donor cell dose, 10^8 spleen cells, while animals in group B received $5x10^8$ donor spleen cells. Age-matched controls received medium alone.

On days 4, 7, 11, 13, 19, 24, 27 and 32 after the induction of GvHR, 4 animals from each group were sacrificed, the body and spleen weights measured, and sections of jejunum fixed and processed for histological and microdissection

analysis.

The experiment was duplicated, and the results detailed below represent the findings.

Development of the GvHR

The animals remained well throughout the time of the studies, with no change in body weights (Fig 4:2).

Figure 4:3 shows the spleen index for both Groups A and B. The animals receiving 10^8 donor spleen cells (Group A) exhibited a peak in spleen hypertrophy, with a spleen index of 3.11 on day 11, followed by a decline reaching 1.7 on day 19 and 1.5 on day 27. On day 32, however, there was a substantial rise in spleen index to 4.70, much higher than the initial peak. The animals receiving $5x10^8$ spleen cells (Group B) revealed a much greater spleen hypertrophy which peaked at 4.90 on day 13, declined to 2.2 on day 19 and then rose to a second, higher peak, of 5.30, on day 32.

Mucosal architecture during the GvHR

Fig 4:4 depicts the various phases of alterations to both the villus and crypt lengths during the GvHR induced by the injection of 10^8 donor spleen cells.

a. Villus and crypt lengths remained as controls until day 11.

b. On day 11 there was a brief point of reduced villus length ($P<0.05$) coinciding with crypt hyperplasia

(P<0.001).

c. Villus length returned to normal by day 13, although the crypts remained hyperplastic (P<0.001).

d. By day 19, the crypts had returned to normal lengths but the villi were significantly reduced in length (P<0.001).

e. Day 24 revealed the most severe changes to the mucosal architecture, with very short villi (359um±31 vs 530um±32, P<0.001) and once again crypt hyperplasia (P<0.001).

f. The crypts entered an atrophic stage on day 27 (P<0.001). The villi, although longer than on day 24, were still shorter than controls.

g. While the crypts returned to normal length by day 32 the villi still remained shorter than controls.

In Fig 4:5 the changes in villus and crypt lengths of animals injected with $5x10^8$ (Group B) donor spleen cells are represented.

a. Villus and crypt lengths remained as controls until day 11.

b. On day 11 the villi were decreased in length (P<0.001) and the crypts hyperplastic (P<0.001).

c. Both villi and crypt lengths returned to normal by day 13.

d. On day 19, the villi became atrophic (P<0.001) and the crypts hyperplastic (P<0.001),

e. The villi reached their shortest length (353um±43 vs 530um±32, P<0.001) by day 24, although the crypt lengths had returned to control levels.

f. The crypts entered an atrophic phase on day 27 ($P<0.02$).
g. Both the villus and crypt lengths reached control length by day 32.

In both groups A and B the evolution of changes in mucosal architecture followed a similar pattern.

Intraepithelial lymphocyte counts during GvHR

IEL were counted on H&E stained sections of jejunum. Fig 4:6 shows that during GvHR, mice injected with 10^8 donor spleen cells (Group A) had an increased IEL count from day 7 ($P<0.02$) reaching a peak on day 11 ($P<0.001$). Thereafter there was a slow decline in IEL numbers, although the counts were always significantly higher than controls ($P<0.001$). On day 24, there was a striking decrease in IEL numbers ($P<0.001$), however, by day 27 the IEL count was higher than control values ($P<0.05$). This rise continued until the end of the study, day 32 ($P<0.001$).

BDF_1 mice receiving $5x10^8$ (Group B) donor spleen cells did not show such dramatic changes in IEL numbers (Fig 4:7). There was a significantly increased IEL count on day 7 ($P<0.005$), which after a slight decline, although still remaining significantly higher than controls ($P<0.005$), reached a peak on day 13 ($P<0.001$). The IEL count dropped, and on days 19 and 24 there was no statistically significant difference in IEL counts between mice with GvHR and controls. A significant decrease did occur on day 27

($P<0.02$), with a return to control levels by day 32.

As with the changes in mucosal architecture, the infiltration of IEL into the epithelium followed a very similar pattern in both groups.

Histological examination of mouse jejunum during GvHR

Photographs of H&E stained sections of control jejunum (Fig 4:8) and jejunum taken on days 11 (Fig 4:9) and day 24 (Fig 4:10; 4:11) of the intestinal GvHR (Group A) are submitted. The photographs show quite elegantly the changes in mucosal architecture that occur during the GvHR, and reinforce the findings using the microdissection technique.

During the initial phase, at day 11, (Fig 4:9), the villi are smaller in length, and of greater width as compared to the controls (Fig 4:8). The crypts are visibly longer, and an increase in IEL numbers is obvious.

Examination under x250 magnification of jejunum taken on day 24 (Fig 4:10) of the GvHR, when more destructive lesions were observed, reveals extreme damage to the villi, and elongated crypts. Interestingly, the presence of a large number of mitotic figures can be seen under x400 magnification (Fig 4:11), suggesting an increase in CCPR at this time.

Comments

The results detailed in this chapter not only confirm the

existence of two distinct forms of intestinal GvHR, but further indicate an evolutionary pattern of changes to the small intestine throughout the GvHR in adult mice.

A similar pattern in both the systemic progression (Fig 4:3), the mucosal alterations (Figs 4:4, 4:5) and the infiltration of IEL (Fig 4:6, 4:7) was observed. The pattern of changes did not appear to depend on the dose of donor cells given to induce the GvHR.

In Fig 4:12 I have brought together, diagrammatically, the evolution of effects both systemically and in the mucosa.

a. All parameters normal until day 7 when there was a rise in spleen index, and an increase in IEL count. No change observed in the gut.

b. By day 11 both the spleen index and the IEL count had reached a peak. At this point a transient decrease in villus length was observed and the crypts were hyperplastic.

c. By day 13 the spleen index was on a decline, as were the IEL counts. Although the villi had returned to normal length, the crypts remained hyperplastic.

d. On day 19 the spleen index and IEL counts remained as day 13. However subtle changes in the mucosae had produced normal crypts but villus shortening.

e. On day 24 the spleen index remained as before, but a dramatic decrease in IEL counts was observed accompanied by villus atrophy and crypt hyperplasia.

f. By day 27 the spleen index had increased and the IEL count had risen dramatically above normal. The villi remained atrophic and interestingly the crypts became atrophic as well.

g. A striking increase in spleen index and IEL counts occurred on day 32, even greater than had been observed on day 11. The crypt lengths had returned to normal and the villi were returning to normal, although they remained a little shorter than controls.

In summary, the findings show that during the intestinal GvHR, many changes occur in the mucosa. Several of the pathological alterations are however transient such as the shortening of villi on day 11, and the changes in crypt length between days 19 and 27.

Although two distinct forms, a proliferative and a destructive form, can be determined, the intestinal GvHR in adult mice appears to affect the gut in such a way, as to induce an evolution of changes to the mucosal architecture producing a severe, destructive type of enteropathy during week 4. However, by week 5 the mucosa had recovered.

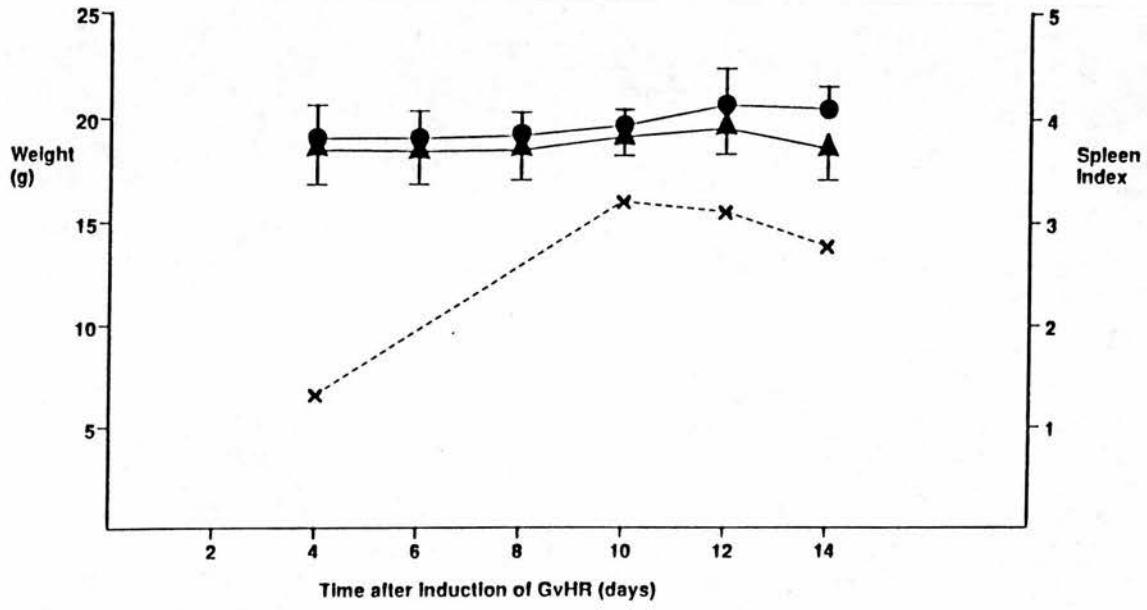


FIGURE 4:1 Progression of the GvHR in adult BDF1 mice. Measurements of body weights (means±s.d.) of control (closed circle) and GvHR groups (closed triangle), and spleen index (X).

Group n = 6-8	Villus length (μm)	Crypt length (μm)	IEL (no./100 epithelial cells)
Control	555 ± 19	116 ± 4	10 ± 2
Day 6 GvHR	550 ± 19	110 ± 10	11 ± 2
Day 8 GvHR	550 ± 10	110 ± 10	9 ± 1
Day 10 GvHR	540 ± 50	122 ± 4 *	16 ± 2 **
Day 12 GvHR	560 ± 20	126 ± 5 **	19 ± 1**
Day 14 GvHR	540 ± 50	132 ± 7 **	16 ± 2 **

* $p < 0.05$
** $p < 0.001$

TABLE 4:1 Changes in mucosal architecture during the initial phase of GvHR.
Villus and crypt lengths (mean±s.d.) and intraepithelial lymphocyte (IEL) counts (mean±s.d).

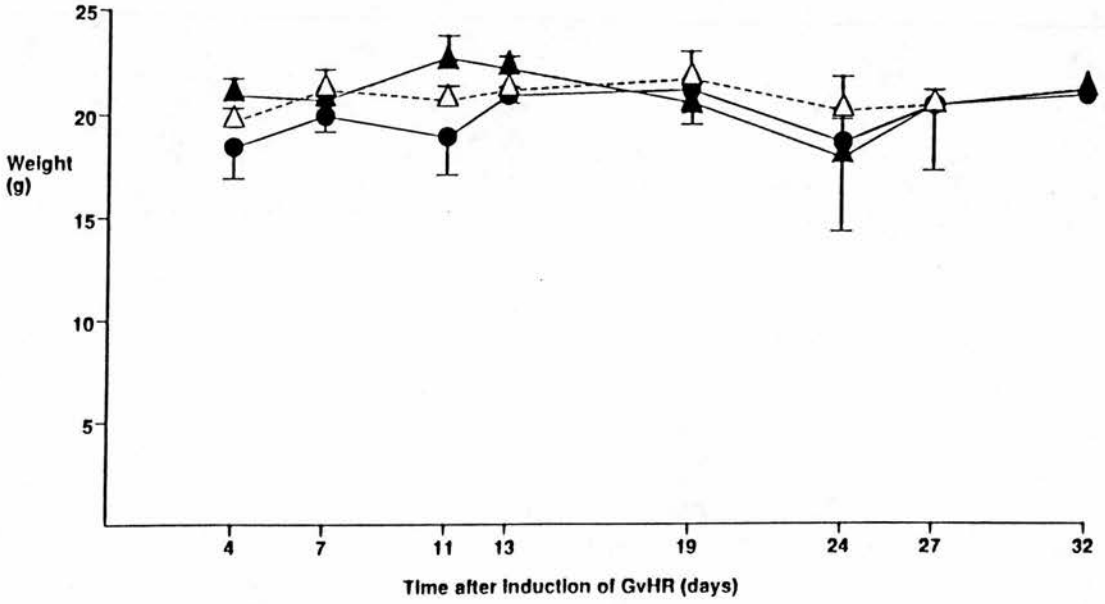


FIGURE 4:2 Measurement of body weights during GvHR in adult BDF1 mice.
Body weights of controls (closed circle) and GvHR mice having received 10^8 donor cells (Group A: closed triangle) or $5x10^8$ donor cells (Group B: open triangle).

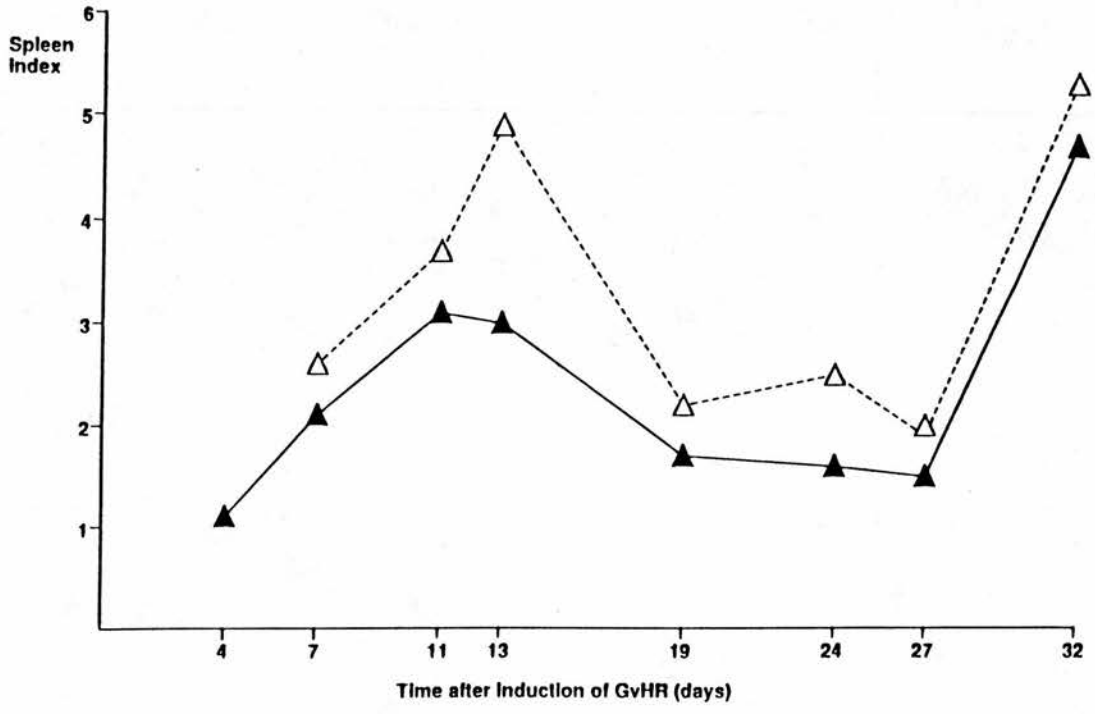


FIGURE 4:3 Progression of the GvHR in adult BDF1 mice. Spleen index of GvHR mice having received 10^8 donor cells (Group A: closed triangle) or $5x10^8$ donor cells (Group B: open triangle).

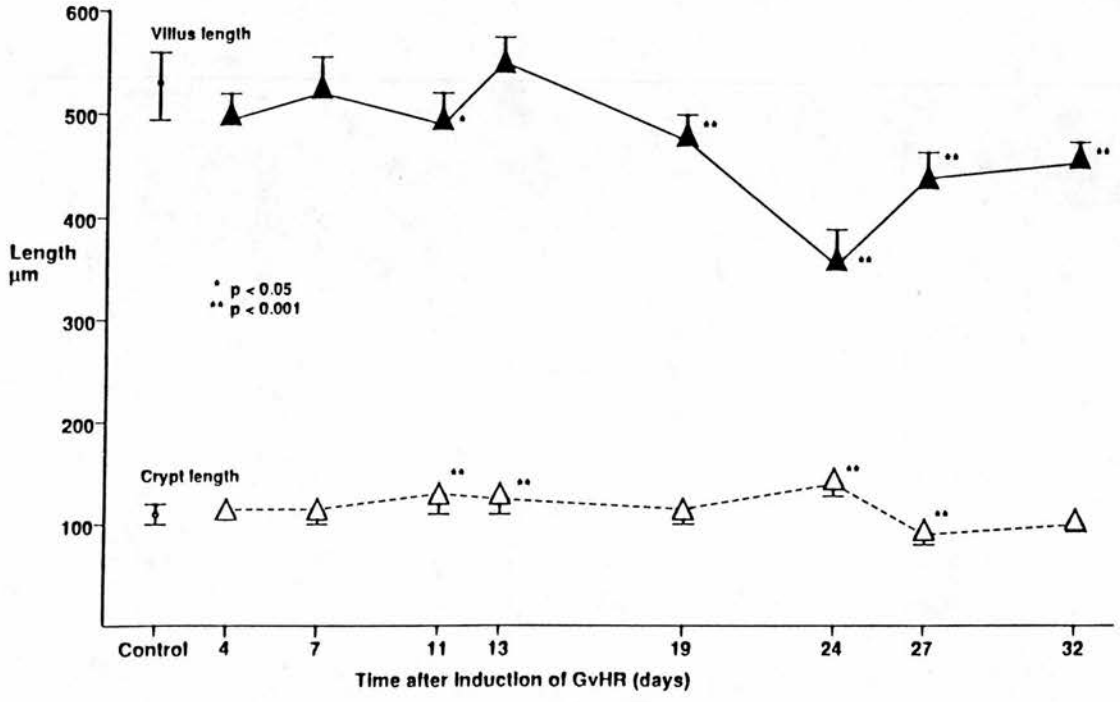


FIGURE 4:4 Changes in mucosal architecture during a GvHR. Villus and crypt lengths (mean±s.d.) of jejunum taken from mice injected with 10^8 donor cells (Group A). The group means±s.d. of villus and crypt lengths for age-matched controls are given on the left of the graph.

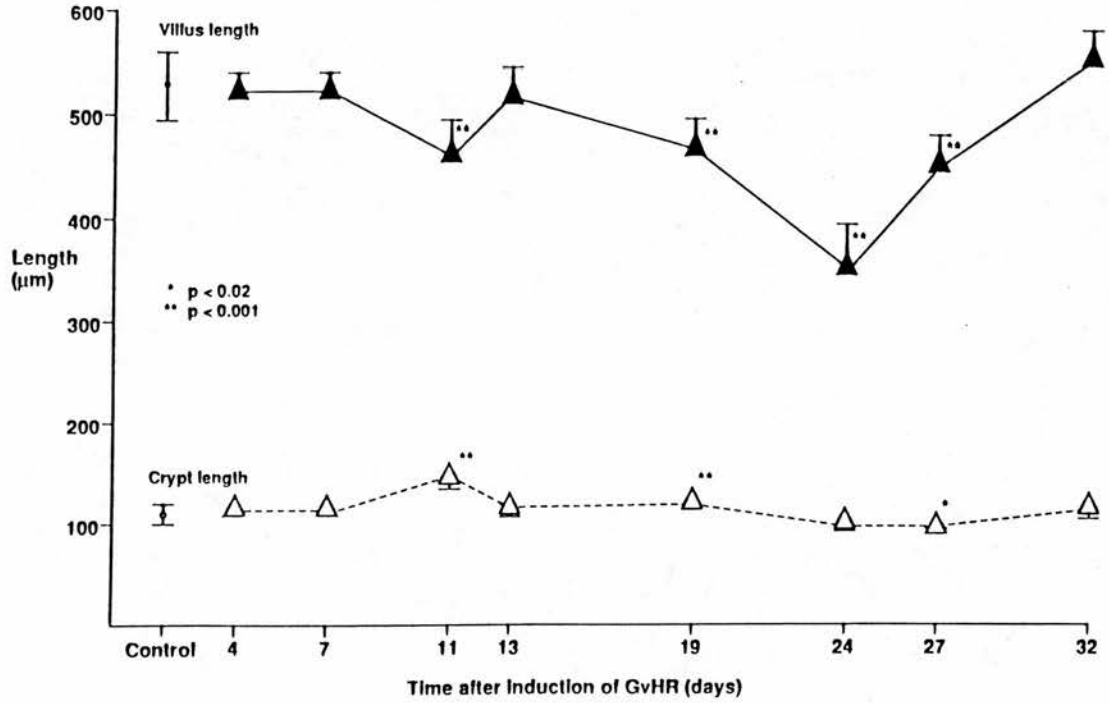


FIGURE 4:5 Changes in mucosal architecture during a GvHR. Villus and crypt lengths (mean±s.d.) of jejunum taken from mice injected with $5x10^8$ donor cells (Group B). The group means±s.d. of villus and crypt lengths for age-matched controls are given on the left of the graph.

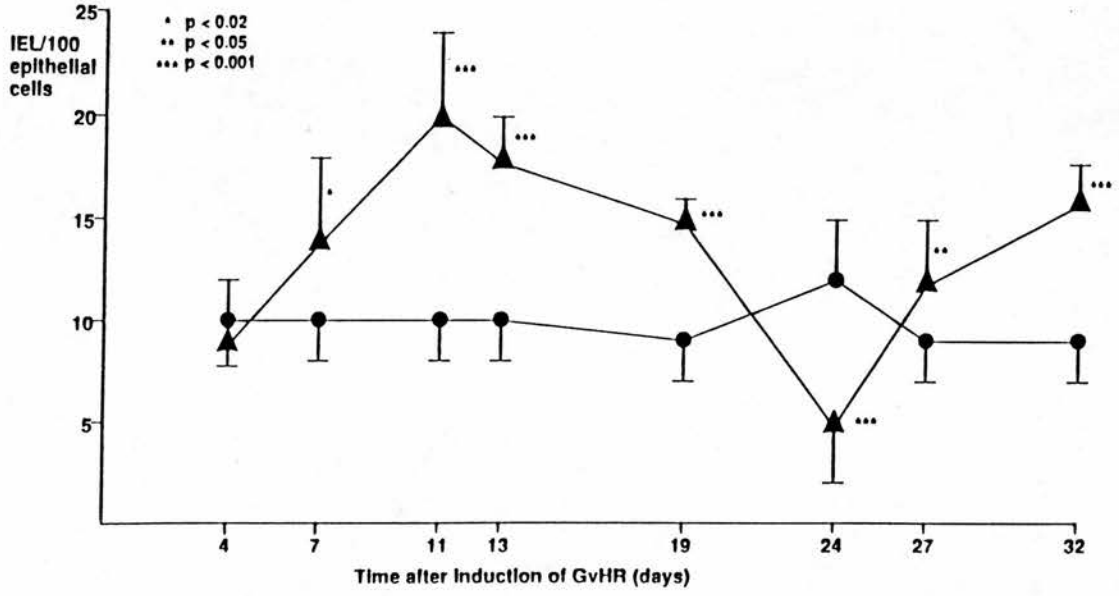


FIGURE 4:6 Cellular infiltration during a GvHR. Intraepithelial cell (IEL) counts (mean±s.d.) of mice injected with 10^8 donor cells (Group A: closed triangle), and age-matched controls (closed circle).

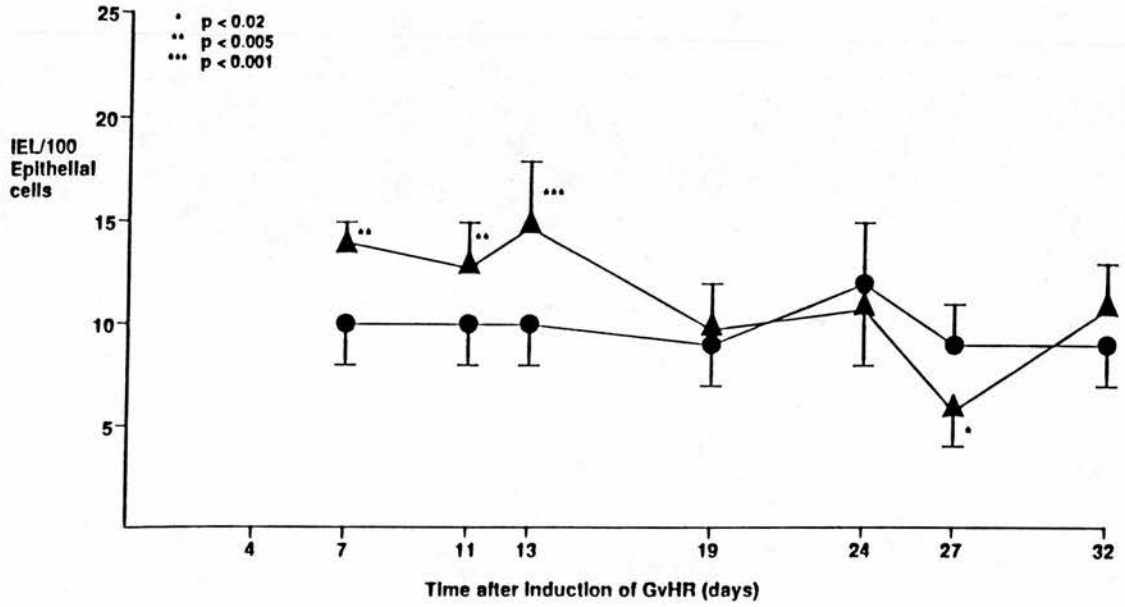


FIGURE 4:7 Cellular infiltration during a GvHR. Intraepithelial cell (IEL) counts (mean±s.d.) of mice injected with $5x10^8$ donor cells (Group B: closed triangle) and age-matched controls (closed circle).

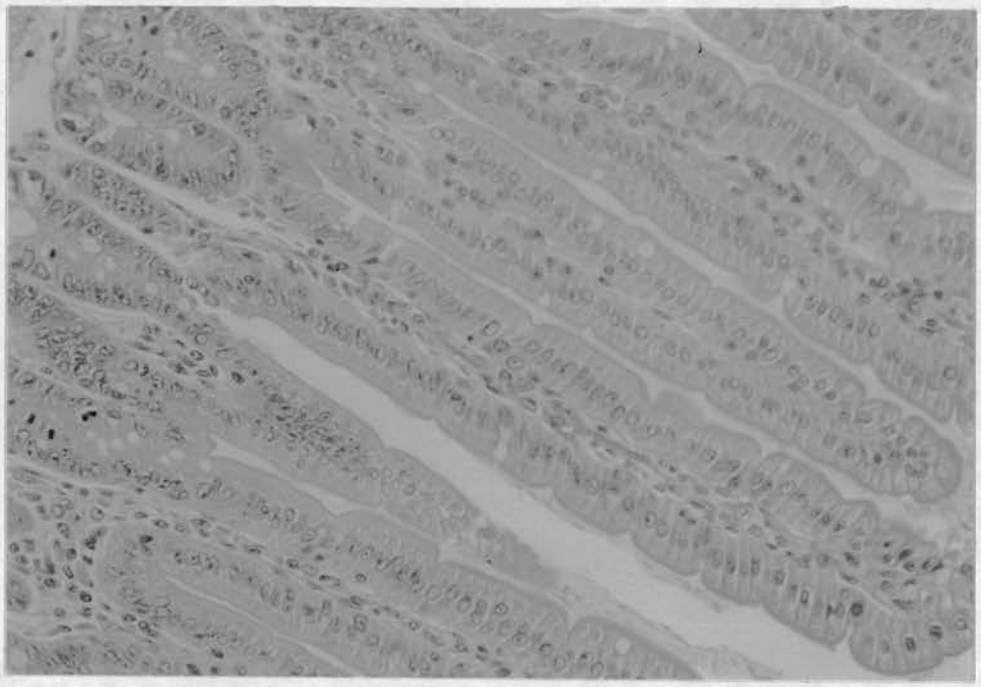


FIGURE 4:8 H&E stained section of jejunum taken from a control mouse. (x250 magnification).

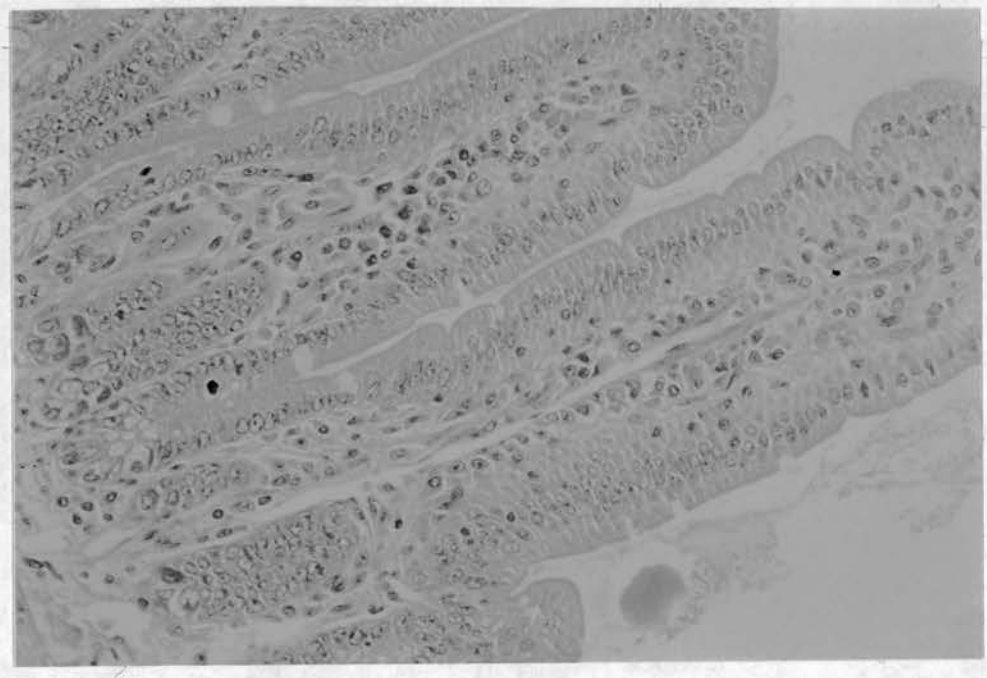


FIGURE 4:9 H&E stained section of jejunum taken on day 11 of the GvHR (Group A). (x250 magnification).

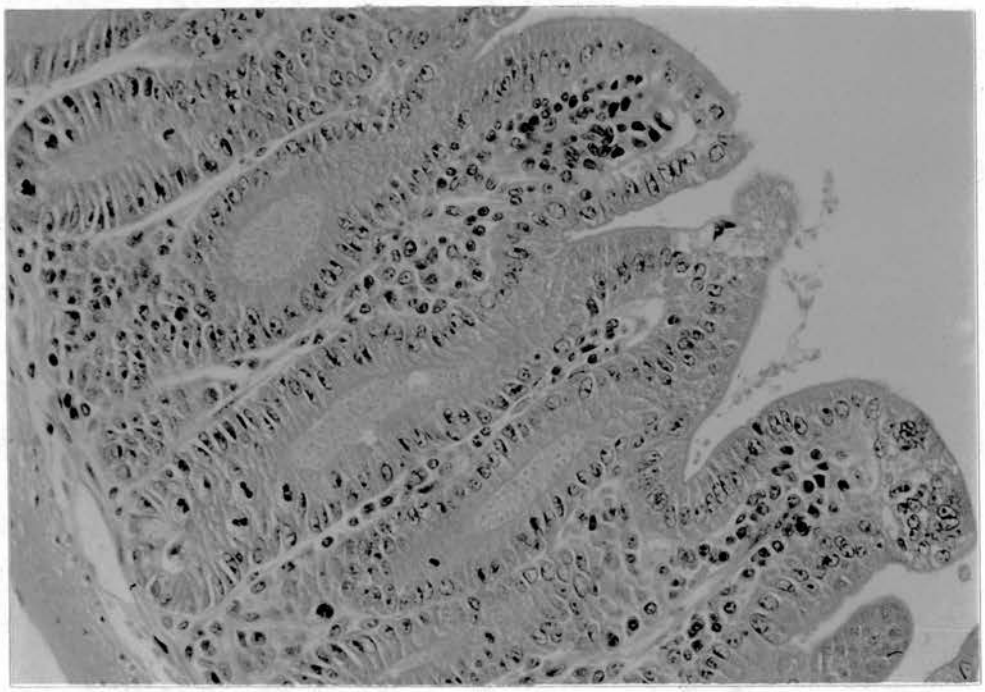


FIGURE 4:10 H&E stained section of jejunum taken on day 24 of the GvHR (Group A). (x250 magnification).

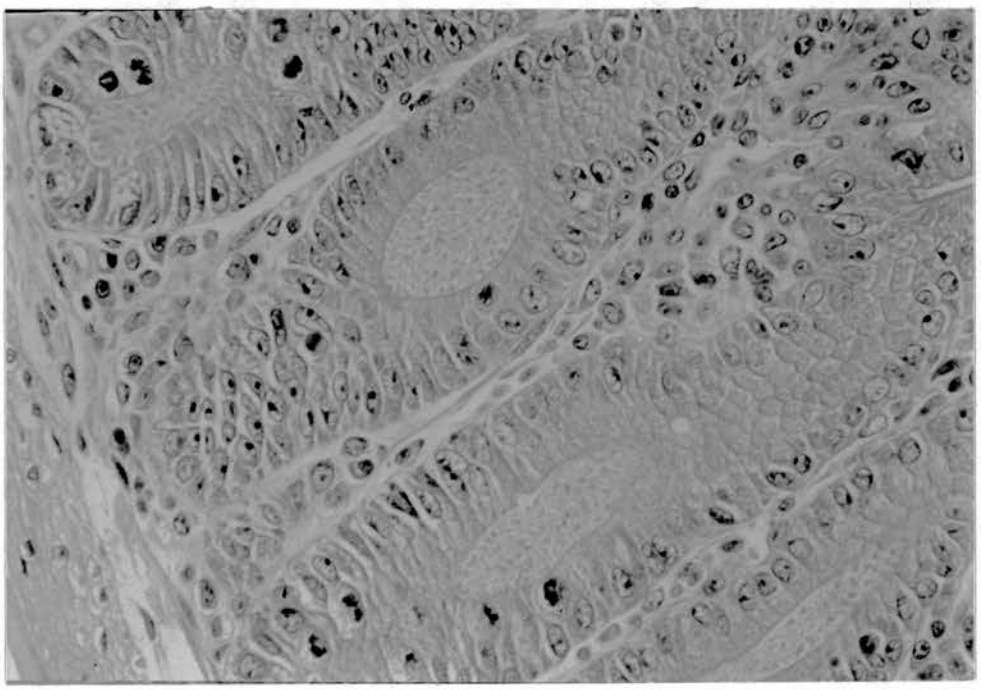


FIGURE 4:11 H&E stained section of jejunum taken on day 24 of the GvHR (Group A). (x400 magnification).

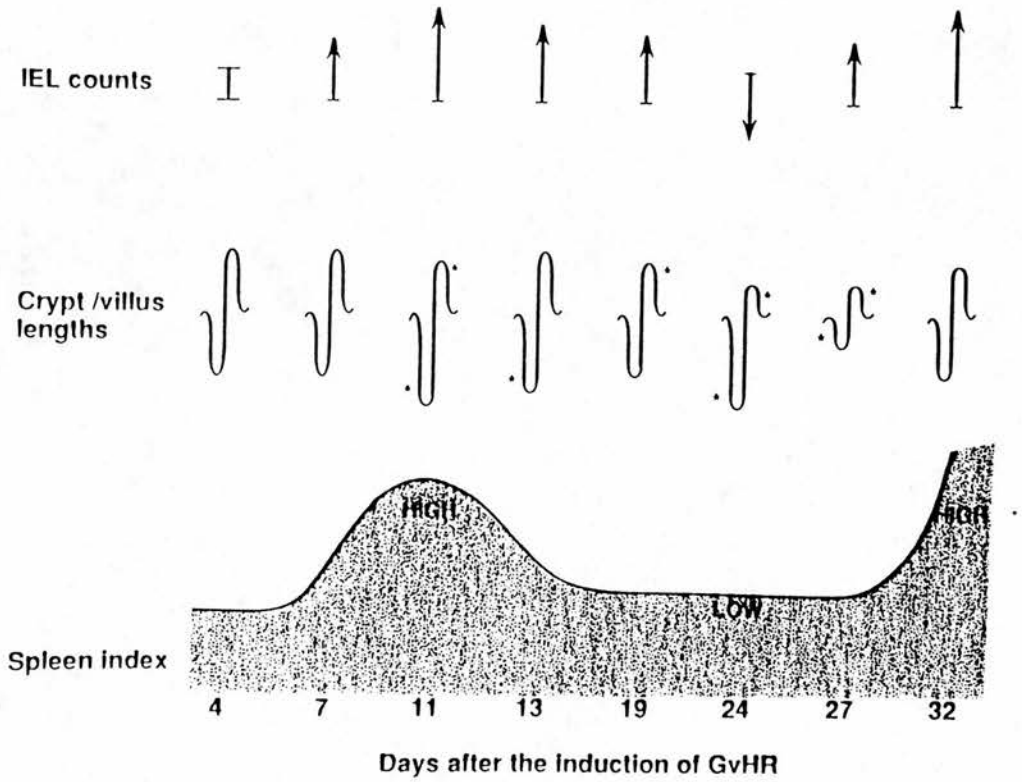


FIGURE 4:12 Diagrammatic representation of the evolution of effects on the mucosa and spleen during a GvHR. (see text).

CHAPTER 5

DEVELOPMENT OF A GUT WASHING TECHNIQUE FOR THE STUDY OF MURINE INTESTINAL HUMORAL IMMUNE RESPONSES

INTRODUCTION

The study of the intestinal secretory immune response in animals or humans, requires a specific technique for directly measuring the secretion of immunoglobulins and antibodies into the gut lumen.

Until the description by Elson, Ealding and Lefkowitz (1984), of a repeatable lavage technique for measuring IgA antibody in mouse intestinal secretions, most studies of mucosal humoral immunity were performed using isolated intestinal loops. Large animals such as rabbits (Hamilton *et al*, 1981; Keren *et al*, 1982) and rats (Husband and Gowans, 1978; Lightman, Sherman and Forstner, 1986) were used. More recently, simple gut wash collections (Elson and Ealding, 1984b; McKensie and Halsey, 1984) and gut perfusions (Ebersole, Smith and Taubman, 1985), have been described. Bile duct cannulations (Schmucker *et al*, 1988), have often been used to study the concentrations of dimeric IgA present in the bile, as a measure of the secretory antibody response. However, concentrations of IgA in the bile, are not representative of concentrations of IgA secreted at the gut level.

The lavage technique (Elson *et al* 1984), was described as an accurate and easily performed technique, allowing temporal studies of IgA antibody secretion in individual mice. The method consisted of feeding mice a lavage solution containing non-absorbable polyethylene glycol (PEG). Defaecation was induced by the cholinergic agent, pilocarpine. The intestinal fluid and faecal pellets were

collected, processed, the important proteins protected with a variety of protease inhibitors, and antibody content measured by ELISA.

The ability to measure accurately the immunoglobulin and antibody concentrations in intestinal contents of mice, was central to my work. This chapter describes my findings using the Elson method (1984), the problems I encountered, and the final method I developed to collect and analyse murine gut washings.
The gut washing technique was used in all of my studies of the intestinal, humoral immune responses.

THE LAVAGE TECHNIQUE. (Elson *et al* 1984)

Experimental Protocol

Initial studies were performed to assess the reproducibility of this technique.
On 4 separate occasions the same group of adult BDF_1 mice were given 4x0.5ml of the lavage solution. 30mins after the last dose, they were injected with pilocarpine and the intestinal contents collected. The complete method is described in chapter 3. The volume recovered was recorded, the concentrations of total IgA were determined by ELISA (chapter 3; Appendix:A.1), and the concentrations of total protein were determined using the Pierce BCA protein assay (chapter 3). The anaesthesia used, and the general methods of feeding are also described in chapter 3.

Results

The results for the volumes (mls), total IgA (ug/ml) concentrations and total protein (mg/ml) concentrations for each experiment are given in Table 5:1.

Volume of gut contents collected.

The collections from these animals contained both faecal pellets and lavage fluid. The volume of lavage fluid recovered varied significantly from day to day (P=0.001) and there was a wide range within each group.

Concentrations of total IgA in intestinal contents.

Table 5:1 also shows the striking variation of total IgA concentrations (ug/ml) between days (P=0.014), and the wide range within each group.

Total protein concentrations in intestinal contents.

The concentrations of total protein (mg/ml) in the intestinal contents were not significantly altered between experiments. Total protein concentrations were not determined in samples collected from experiment 4.

Comments

The volumes and total IgA concentrations, of intestinal

contents collected from these mice, were extremely variable both between animals and between days. Although the concentrations of protein appear to remain constant from day to day of the study, the significant variation in the IgA concentrations still remained when the results were expressed as ug total IgA/mg protein (Figures not shown).

There have been many reports describing the effects of pilocarpine on IgA concentrations (Wilson *et al*, 1982; Smith *et al*, 1982) and volume of fluid collected from mice (Elson *et al*, 1984), rats (Wilson *et al*, 1982) and hamsters (Smith *et al*, 1982).

The cholinergic activity of this agent increases the volume of fluid secreted, both in salivary glands and intestine, and subsequently produces a net decrease in IgA concentrations.

It is possible that the cumulative effect of the pilocarpine in these animals, altered the recoverable volume and thus the concentrations of total IgA.

The data shown in Table 5:1, is evidence that this technique is unreliable as a method for obtaining fluid for subsequent analysis of IgA concentrations in gut luminal contents.

A study of the movement of lavage fluid along the gut.

The volume of lavage fluid fed to the mice was 2mls, however, despite the large variation, the volume of lavage fluid recovered was on only one occasion 2mls. The average

amount collected was approximately 1ml.

In a further experiment, I fed adult BDF_1 mice the 4x0.5ml dose of lavage fluid containing 2 drops of Evans blue dye (Sigma; U.K.).
At times 0, 10, 20 and 30mins after the last lavage dose, 3 animals were sacrificed and the stomach, small intestine caecum and colon examined for the presence of Evans blue colouring.
A second group of mice were given the four 0.5mls of lavage, and after 30mins, they were injected with 100ug of pilocarpine. At times 0, 10, 20, 30, 40 and 80mins, after the administration of pilocarpine, the volume and colour of the fluid secreted were recorded. The animals (n=3) were then sacrificed, and the stomach, small intestine, caecum and colon examined for presence of blue colour.

The results are not presented here as the conclusions drawn from them simply reinforce the findings described above.
30mins after the last dose of lavage fluid was given, there was blue lavage fluid in the caecum and colon. The maximum volume of intestinal contents was recovered 30mins after the injection of pilocarpine (the maximum effect of pilocarpine is known to occur within 30mins (Elson *et al*, 1984)). The fluid collected in the petri-dish was blue in colour, but on sacrifice of the animals, a large amount of blue fluid still remained in the gut.

Comments

From the results obtained it appeared that the lavage technique could not be maximized in any way. The lavage fluid fed to the animals was not completely deposited, therefore the fluid collected for analysis was not representative of whole gut luminal contents. The variation, in volume recoverable and IgA concentrations, between animals and days was too great, and I therefore considered the technique to be unreliable.

THE GUT WASHING TECHNIQUE

I developed a gut washing technique which encompassed the positive aspects of the lavage technique, such as the lavage solution, the use of protease inhibitors and the actual processing of the samples. However, instead of inducing the animals to defaecate, they were sacrificed and the contents washed out.

Normal adult BDF_1 mice were fed the lavage solution, and after 30 mins the animals were sacrificed, their intestines from the duodenum up to and including the caecum removed and washed out. The complete method is described in chapter 3. Again the volumes of gut washings were recorded and the total IgA concentration determined by ELISA (chapter 3; Appendix:A.2).

Results

The results, shown in Table 5:2, are representative of the volumes of gut washings collected.
The concentrations of IgA (ng/ml) (Table 5:3) were obtained from two experiments, performed on normal mice. The technique was performed many times, and the results presented here are representative of other experiments.

Volumes of gut washings obtained

Of the total 5mls of fluid, 2mls lavage fluid plus 3mls of EDTA/soybean trypsin inhibitor passed through the gut, 90-100% was collected for analysis (Table 5:2).

Concentration of total IgA in gut washings.

As hoped, there was no significant difference between the two groups of animals (Table 5:3), although the range within each group (trial 1- 85ng/ml-274ng/ml and trial 2- 50ng/ml-251ng/ml) was still quite extensive.

Comments

In conclusion, the gut washing technique is reliable, reproducible and easy to perform.
The major disadvantage of this method, over the lavage technique, is that temporal studies of the secretory immune response in individual mice cannot be performed.

The concentrations of IgA in the gut washings, were lower than those obtained in the faecal fluid after whole gut lavage (ng/ml rather than ug/ml). This is probably because the gut lavage method produces a concentrate of gut luminal contents, and when the amount of IgA is expressed per ml of faecal fluid, a falsely high result is obtained.

A further observation using the gut washing technique was, that if less than 3mls of volume were collected from an animal, the concentration of total IgA was falsely high. Samples of less than 3mls volume were thus eliminated from future studies.

The range of results within a group of animals is rather wide, but the statistical methods used take account of this. Using large numbers of mice, and adequate controls for each experiment should eliminate any problems regarding statistical analysis.

An excellent advantage of using the gut washing technique is that sections of the jejunum can be fixed and stained for plasma cell counts. A record therefore of IgA concentrations in the intestinal lumen and IgA^{+}-plasma cell counts in the same animal can be obtained. This enables more involved studies of relative changes in the migration of plasma cells to the lamina propria, and production and/or secretion of immunoglobulin into the gut lumen.

Experiment	Volume (mls) (Range)	µg Total IgA/ml (Range)	mg Protein/ml (Range)
1 (n=12)	0.8 ± 0.37 (0.2 - 1.4)	18.90 ± 13.70 (0.46 - 168.69)	12.49 ± 8.02 (0.75 - 92.22)
2 (n=12)	0.63 ± 0.16 (0.4 - 1.0)	58.38 ± 16.70 (6.73 - 213.38)	7.29 ± 2.67 (0.31 - 22.93)
3 (n=12)	1.23 ± 0.54 (0.3 - 1.8)	1.38 ± 0.32 (0.12 - 4.26)	10.32 ± 1.94 (2.10 - 22.08)
4 (n=6)	1.45 ± 0.6 (0.3 - 2.1)	17.14 ± 3.1 (0.74 - 79.62)	ND
Analysis of variance	P = 0.001	P = 0.014	N.S.

FIGURE 5:1 The lavage technique.
Volumes (mean±SEM), total IgA (mean±SEM) and protein concentrations (mean±SEM) of lavage fluid collected on four separate occasions from a group of BDF1 mice.

Mouse	mls	% washing recovered (Total 5 mls given)
1	4.5	90%
2	4.5	90%
3	5.0	100%
4	4.9	98%
5	4.5	90%
6	5.0	100%
7	5.0	100%
8	4.7	94%
9	4.8	96%
10	5.0	100%

FIGURE 5:2 The gut washing technique.
Volume of gut washing and % washing recovered from individual BDF1 mice.

ng Total IgA/ml

Animal	Trial 1	Animal	Trial 2
1	121	1	223
2	169	2	125
3	274	3	251
4	186	4	177
5	186	5	223
6	250	6	63
7	250	7	177
8	256	8	50
9	170	9	158
10	85	10	177
Mean ± S.E.M	194.70 ± 19.73	Mean ± S.E.M.	162.40 ± 21.02
Range	(85-274)		(50-251)

FIGURE 5:3 The gut washing technique.
Concentrations of total IgA in gut washings collected from two experiments.

CHAPTER SIX

MUCOSAL IMMUNOGLOBULIN CONCENTRATIONS THROUGHOUT A GVHR IN ADULT MICE

INTRODUCTION

In comparison to the many investigations into the effects of a GvHR on systemic B cell responses and immunoglobulin production, the effect on secretory immunoglobulin production has not yet been elucidated.

In 1976 Gold *et al*, studied plasma cell numbers in the duodenum of neonatal mice with GvHR. Their findings showed, that 3 weeks after the induction of a semi-allogeneic GvHR in nonirradiated newborn (C57Bl/KAxBalb/c) F_1 mice, there was a virtual absence, in the LP, of IgA and IgM plasma cells, with no detectable IgG plasma cells.

Systemically it has been shown that the parent to F_1 hybrid murine model of GvHR produces a brief, initial phase of immunostimulation in which T helper cells are alloactivated. B cell hyperplasia and hypergammaglobulinaemia are common features. This is immediately followed by immunosuppression. During the initial immunostimulatory phase, activated T cells are known to be present in the mucosa (chapter 2).

The aim of this study was to investigate, in detail, what effect the presence of activated T cells, in the adult mouse gut, would have on mucosal B cell properties as reflected by plasma cell counts in the LP, and immunoglobulin secretion into the gut lumen.

Having established the gut washing technique, I was able to measure directly the concentrations of immunoglobulins in the gut luminal contents throughout the GvHR, as well as

determine the plasma cell counts in the small intestine LP.

General protocol

The same protocol was used throughout the following experiments.

A GvHR was induced in adult BDF_1 mice, aged 6-8 weeks, by i.p. injection of 10^8 C57Bl/6J donor spleen cells. Control animals received medium alone.

At various time points thereafter, gut washings were collected (as described in full in chapter 3) from both control and GvHR groups.

Sections of mid-gut were collected for staining of IgA-, IgM- and IgG-containing plasma cells (chapter 3).

Immunoglobulin concentrations were determined by ELISA (chapter 3; Appendix A:2, A:3).

8-10 animals/group were studied at each time point.

Due to the large numbers of animals required for such a time course experiment, it was impossible to study daily changes in immunoglobulin concentrations in gut washings in one experiment. A series of experiments were therefore performed to study selected time points.

The concentrations of total immunoglobulins in the gut washings from experiment 1-5, are detailed in Table 6:1 and similarly the corresponding plasma cell numbers are given in Table 6:2.

EXPERIMENT 1

In this first experiment, total IgA concentrations, and IgA-containing plasma cells numbers in the LP were analysed on days 7, 14 and 21.

These time points were chosen as being representative of the phases of GvHR. The onset of splenic hypertrophy occurs on day 7. By day 14 the GvHR is at the peak of severity, and within the period of T cell alloactivation. By day 21 the GvHR has entered a period of reduced activity.

Results

A significant increase ($P<0.01$) in total IgA (ng/ml) in gut washings was found on day 14 (Table 6:1-experiment 1), accompanied by an increase ($P<0.01$) in IgA plasma cell numbers (Table 6:2-experiment 1).

EXPERIMENT 2

After finding this striking rise in total IgA on day 14 of the GvHR, a second experiment was performed in order to duplicate this finding and study other time points.

Total IgA concentrations were analysed on days 4, 7, 10, 14, and 29 after the induction of a GvHR. Plasma cell counts were not performed on this occasion.

Results

Unfortunately, at no time point was there a significant increase in total IgA concentrations (Table 6:1- experiment 2).

EXPERIMENT 3

Despite there being no increase in total IgA concentrations on day 14 in the latter experiment, the initial results of experiment 1 revealed such a striking increase on day 14 that a 3rd experiment was set up to study more closely around this time.

In this experiment both total IgA and IgM concentrations in gut washings were studied. Simultaneously IgA, IgM and IgG plasma cell numbers were counted. Days 10, 11, 12, 13, and 14 after the induction of the GvHR were investigated.

Results

A significant increase ($P<0.05$) in total IgA concentrations (Table 6:1-experiment 3), concomitant with a significant rise ($P<0.05$) in IgA plasma cell numbers (Table 6:2-experiment 3) was observed on day 12 of the GvHR.
An increase in total IgM concentrations (Table 6:1-experiment 3) on day 11 ($P<0.01$), day 12 ($P<0.001$), day 13 ($P<0.01$) and day 14 ($P<0.05$) was observed. IgM plasma cell numbers (Table 6:2-experiment 3) were increased on days 12 ($P<0.05$) and 14 ($P<0.05$).

No change in IgG plasma cell counts were observed.

Because IgG plasma cells were found in such low numbers, and no changes in numbers were observed during the GvHR, total IgG concentrations in gut washings were not analysed.

This experiment was repeated and the same changes were observed.

EXPERIMENT 4

Results

The study of total IgA and IgM concentrations (Table 6:1-experiment 4) and IgA, IgM and IgG plasma cells numbers (Table 6:2-experiment 4) on days 15 and 18 revealed no significant difference between animals with GvHR and controls.

EXPERIMENT 5

A second phase of immunostimulation has been reported in this murine model of GvHR, the features of which are suggestive of an autoimmune-type disease. The onset of this phase is believed to occur many weeks after the onset of GvHR.

My previous studies of the progression of the GvHR (chapter 4) revealed a dramatic increase in spleen index around day 32 (Fig 4:3), suggesting that at this time there is an increase in systemic cell proliferation.

A study of immunoglobulin concentrations and plasma cell numbers was therefore performed on day 31.

Results

A striking increase in both total IgA concentrations ($P<0.05$) and total IgM concentrations ($P<0.01$) (Table 6:1-experiment 5) was observed at this time point.
No change was found in IgA plasma cell numbers (Table 6:2-experiment 5), and unfortunately the staining procedure for IgM plasma cells failed, and no results were obtained.

Comments

The results of this investigation show, that during the semi-allogeneic GvHR in nonirradiated adult BDF_1 mice, there were increased plasma cell counts in the LP and increased immunoglobulin concentrations in the gut lumen.
A transient increase in total IgA concentrations, accompanied by an increase in IgA plasma cell numbers in the LP, occurred on day 12 after the induction of a GvHR. By day 13 both the immunoglobulin levels and plasma cell numbers had returned to normal.
A prolonged increase in total IgM concentrations in gut luminal contents was observed from day 11 up to and including day 14. A peak of statistical significance was reached on day 12, suggesting that this day was the peak of the total IgM response. IgM plasma cell numbers were increased on days 12 and 14.

Both total IgA and IgM concentrations remained as controls until the second striking increase found on day 31 of the GvHR.

The increases in total immunoglobulin concentrations in the gut followed a similar pattern as that of the systemic GvHR, as monitored by the spleen index (Fig 4:3). It was therefore possible that the effects observed at the gut level were a direct result of serum leakage into the gut lumen, at a time when the gut is undergoing many morphological changes (chapter 4). Evidence against this is that an increase in LP plasma cell counts of the IgA and IgM isotypes but not IgG was observed. Secondly I performed a small experiment (data not shown) in which Evans' blue dye was injected intra-venously into mice on day 14 of the GvHR and into age-matched controls. No detectable leakage of Evans' blue dye, monitored spectrophotometrically, was found in the gut washings of either control mice or mice with GvHR.

The increase in IgA and IgM concentrations in the gut lumen on day 31, was not accompanied by an increase in plasma cell counts, and serum leakage may be relevant at this time. Other considerations, such as an increase in immunoglobulin production within each plasma cell, or increased immunoglobulin concentrations in bile secretions, must be taken into account.

In summary, during the initial phase of GvHR there was a transient increase in levels of IgA in the gut lumen, on day 12, and a prolonged increase in mucosal IgM. A similar

increase in plasma cell counts was also observed.
This dichotomy in mucosal immunoglobulin production suggests that the GvHR may be affecting mucosal B cells early in their maturation pathway.
The second rise in both IgA and IgM concentrations, occurring on day 31 of the GvHR, was discovered late in my work. It is possible that this increase reflects the onset of the chronic, immunostimulatory form of the reaction, however further study is required.

The data presented in this chapter should however be interpreted with great caution. A large degree of variability in total IgA concentrations, was observed in control animals, and the increase in IgA levels described during the GvHR was not observed on every occasion. In addition, some values for both IgA and IgM concentrations were near the limit of resolution of the ELISA. It is therefore possible that the gut washing technique is not sufficiently powerful to detect meaningful differences consistently.

Experiment		Total IgA (ng/ml) CON	Total IgA (ng/ml) GvHR	Total IgM (ng/ml) CON	Total IgM (ng/ml) GvHR
1 day	7	35.40 ± 8.17	20.66 ± 3.01		
	14	85.66 ± 27.48	419 ± 33**		
	21	43.25 ± 10.63	74.28 ± 22.95		
2 day	4	36.80 ± 2.72	44 ± 5.05		
	7	66.66 ± 14.06	140.50 ± 47.13		
	10	40.12 ± 12.25	34.62 ± 5.43		
	14	30.70 ± 2.05	41.14 ± 24.22		
	29	21.53 ± 1.48	33.62 ± 8.88		
3 day	10	201.78 ± 29.25	194.65 ± 60.04	14.95 ± 7.43	26.41 ± 10.83
	11	217.25 ± 19.39	228.25 ± 165.67	8.62 ± 1.48	43.06 ± 17.50**
	12	123.71 ± 10.53	272.16 ± 43.50*	4.48 ± 0.56	58.82 ± 25.90***
	13	154.81 ± 22.42	172 ± 27.99	8.92 ± 2.27	31.27 ± 10.91**
	14	107.11 ± 17.65	146.29 ± 22.84	8.29 ± 2.06	21.31 ± 7.47*
4 day	15	150.72 ± 24.28	157.39 ± 19.07	9.00 ± 1.68	10.04 ± 4.80
	18	150.72 ± 24.28	170.16 ± 21.54	9.00 ± 1.68	8.02 ± 3.73
5 day	31	415.84 ±136.57	777.85 ± 84.51*	17.06 ±11.24	262.66 ± 138.87**

* P <0.05
** P <0.01
*** P <0.001

TABLE 6:1 Total IgA and IgM concentrations in gut washings collected at various times throughout the GvHR. Figures represent the mean±SEM.

		IgA+		IgM+		IgG+	
Experiment		CON	GvHR	CON	GvHR	CON	GvHR
1 day	7	187 ± 38	126 ± 10				
	14	334 ± 52	574 ± 46**				
	21	226 ± 33	249 ± 4				
3 day	10	357 ± 37	368 ± 7	224 ± 12	220 ± 16	33 ± 7	31 ± 2
	11	327 ± 4	351 ± 5	210 ± 7	219 ± 18	31 ± 3	27 ± 5
	12	300 ± 25	421 ± 2*	239 ± 3	327 ± 13*	25 ± 2	32 ± 3
	13	309 ± 16	350 ± 16	269 ± 28	310 ± 10	19 ± 4	21 ± 3
	14	248 ± 11	247 ± 10	192 ± 4	257 ± 13*	19 ± 4	19 ± 4
4 day	15	300 ± 28	303 ± 36	213 ± 38	154 ± 9	21 ± 6	22 ± 4
	18	300 ± 32	314 ± 8	213 ± 38	146 ± 3	20 ± 1	20 ± 1
5 day	31	320 ± 17	366 ± 5	-	-	-	-

* $P < 0.05$
** $P < 0.01$

TABLE 6:2 IgA-, IgM-, and IgG- containing plasma cell counts in mid-gut sections taken at various times throughout the GvHR.
Figures represent the means±SEM.

CHAPTER SEVEN

IMMUNOGLOBULIN CONCENTRATIONS IN SALIVA, MILK AND SERUM DURING A GVHR IN ADULT MICE

INTRODUCTION

In chapter 6 the striking, enhancing effect of the GvHR on secretory immunoglobulin concentrations in the gut lumen was defined. On day 12 of the GvHR there was an increase in IgA plasma cell numbers in the intestinal LP, and an increase in IgA in the gut lumen.

The hypothesis of this investigation was that during the GvHR, the stimulation of IgA precursor B cells in PP may produce an increase in IgA precursor cells migrating to other sites of the common mucosal immune system; thus producing an increase in IgA in saliva and milk.
For completion, serum IgA, IgM and IgG concentrations were also studied throughout the GvHR; thus reflecting systemic B cell stimulation.

A: THE EFFECT OF A GVHR ON IGA PRODUCTION IN SALIVA AND MILK

There is much evidence to support the existence of a common mucosal immune system in animals. The theory is that after local sensitization of B cells in the GALT, the activated cells, of which a large number will be IgA precursor B cells, leave the gut and enter the MLN. Subsequently the cells enter the circulation through the thoracic duct and then home to the LP of the intestinal and respiratory tracts and salivary, lacrimal, mammary and cervical uterine glands. The IgA precursor B cells then differentiate into IgA plasma cells and produce antibody

(reviewed Mestecky and McGhee, 1987).

Total IgA concentrations in saliva during a GvHR.

Experimental protocol

A semi-allogeneic GvHR was induced in nonirradiated BDF_1 mice by i.p. injection of 10^8 C57Bl/6J donor spleen cells. Controls received medium alone.

On days 11, 12, 13, 14 and 18 after the induction of GvHR, 6-8 mice/group were given pilocarpine i.p. and the saliva collected (chapter 3). The technique used to collect saliva was quite easily performed although only small volumes could be obtained.

Total IgA concentrations were determined by ELISA (chapter 3-Appendix A:2).

Results

The results, presented in Table 7:1, show that at no time point after the induction of a GvHR, was there a significant difference in total IgA concentrations (ng/ml) as compared with controls.

Total IgA concentrations in milk during a GvHR.

Experimental protocol

The gestation period of the mouse is approximately 21 days from the first sign of mating; determined by the appearance

of a vaginal plug of semen, and commonly known as plugging. A semi-allogeneic GvHR was induced in nonirradiated, female BDF_1 mice (as described above) 10 days after plugging, thus the birth of the litter, and the release of milk coincided with the peak of effect of the GvHR. The control group, which consisted of females at the same stage of pregnancy, were given medium alone. 4-5 animals/group were used.

The offspring of the BDF_1 mothers, with an ensueing GvHR during pregnancy, suffered no ill effects and thrived as the offspring from the control mothers.

On days 1, 2, 3 and 4 post-parturition the mice were given an i.p. injection of oxytocin and the milk was collected in capillary tubes (chapter 3). This technique proved quite difficult and only small volumes of milk were collected from each animal.

The above time points coincided with days 11, 12, 13 and 14 of the GvHR, and are shown in Table 7:2 as such.

Total IgA concentrations were determined by ELISA (chapter 3-Appendix A:2).

Results

The results, given in Table 7:2, show that there was no significant difference in the total IgA concentrations (ng/ml) of milk from mice with GvHR as compared with controls.

Comments

The results show quite clearly that there was no effect on total IgA concentrations in saliva or milk during the GvHR. However, the levels of IgA in saliva did appear to be raised on days 13 and 14 of the GvHR, and although statistical significance was not reached, the possibility does exist that IgA concentrations are affected at these points during the reaction. Plasma cell counts of salivary and mammary glands were not studied, therefore it is not known if an increased number of IgA precursor cells migrated to these sites. The apparent unchanged concentrations of IgA in saliva and milk suggest that the GvHR does not induce increased cell migration and immunoglobulin production in distant mucosal sites, but rather the mucosal effect is confined to the gut.

B: SYSTEMIC IMMUNOGLOBULIN PRODUCTION DURING A GVHR

The immunostimulatory phase of the acute semi-allogeneic GvHR in nonirradiated mice, has been shown to produce B cell hyperplasia, increased plasma cell numbers in the medullary areas of lymph nodes and hypergammaglobulinaemia (chapter 2).

The aim of this study was to determine the concentrations of total IgA, IgG and IgM in the systemic circulation, throughout the time course of the GvHR.

Experimental protocol

A semi-allogeneic GvHR was induced in nonirradiated BDF_1 mice, aged 6-8 weeks, as described above. Control animals received medium alone.

On days 4, 7, 11, 12, 13, 20, 24, 27 and 32 after the induction of the GvHR, serum was collected from both control and GvHR groups, by total body exsanguination (chapter 3).

Total IgA, IgM and IgG concentrations were determined by ELISA (chapter 3-Appendix A:2, A:3 and A:4).

In order to allow the collection of serum on all the above days in one experiment, only 4 animals/group were used. The experiment was then repeated. The results of the two experiments were almost identical, therefore the values for each time point were grouped together to allow statistical evaluation.

Total IgA concentrations in serum

The results are shown in Fig 7:1. The concentrations of IgA (ug/ml) were significantly greater on days 4 ($P<0.001$), 7 ($P<0.001$) and 11 ($P<0.001$) of the GvHR.

The levels reached a peak on day 12 ($P<0.001$), had dropped by day 13, although they still remained significantly higher than controls ($P<0.001$).

By days 20, 24 and 27 the concentrations of IgA did not differ significantly from controls.

A striking increase (5 fold greater than the control) was observed on day 32 ($P<0.001$).

Total IgM concentrations in serum

The results are shown in Fig 7:2. Total IgM concentrations

(ug/ml) were significantly greater than controls on day 4 ($P<0.001$), 7 ($P<0.001$) and 11 ($P<0.001$).
By day 12 the levels were rising further ($P<0.001$), reaching a peak on day 13 ($P<0.001$).
By day 20 the levels had dropped but were still significantly greater than controls ($P<0.001$). However, by days 24 and 27, the concentrations were significantly lower than controls ($P<0.02$ and $P<0.05$ respectively).
A significant increase ($P<0.001$) was observed on day 32, but of a much lower magnitude than observed on day 13.

Total IgG concentrations in serum

The results are shown in Fig 7:3. There was no significant difference in total IgG concentrations (mg/ml) on days 4, 7, 11 and 12 of the GvHR.
On day 13 a significant increase ($P<0.001$) in the levels of IgG did occur, and this appeared to be the peak.
The concentrations remained higher than controls on day 20 ($P<0.001$) but appeared to be on the decline, and by days 24 and 27 no significant difference could be detected.
An increase, to a similar level as day 13, occurred on day 32.

Comments

The GvHR in adult mice produced significant changes in the systemic IgA, IgM and IgG concentrations, in a pattern very similar to the changes in splenic cell proliferation, as

monitored by the spleen index (Fig 4:3).

Concentrations of IgA and IgM were the first to become elevated, this occurred only 4 days after the induction of the GvHR. Levels of IgA reached a peak on day 12, and IgM on day 13. Concentrations of IgG did not increase until day 13. These alterations are in accord with the effects of immunostimulation described in the initial phase of the GvHR (chapter 2).

Following this brief initial immunostimulatory phase, a period of reduced activity has been shown to occur (chapter 2). The results presented here confirm this, in that the concentrations of systemic immunoglobulins subsequently dropped to that of the control group. Interestingly, the concentrations of IgM decreased to levels significantly lower than controls.

A second striking increase, particularly of IgA, was observed on day 32, again in conjunction with the dramatic increase in spleen index. It is possible that at this point the mice have developed the chronic form of GvHR.

In summary, while the GvHR does not appear to influence immunoglobulin production at the distant mucosal sites of the salivary and mammary glands, there are dramatic changes systemically, both during the initial phase of GvHR and at the onset of the chronic form.

Day after induction of GvHR		ng Total IgA/ml	Range (ng/ml)
Day 11	CON	179.08 ± 21.21	111.90 - 315.45
	GvHR	216.04 ± 36.20	99.75 - 397.15
Day 12	CON	246.56 ± 31.10	165.55 - 397.15
	GvHR	246.56 ± 14.37	194.50 - 310.25
Day 13	CON	151.85 ± 23.23	104.46 - 308.20
	GvHR	235.05 ± 41.24	119.94 - 488.62
Day 14	CON	337.10 ± 32.92	190.98 - 578.91
	GvHR	433.83 ± 86.59	83.17 -1294.36
Day 18	CON	193.49 ± 28.16	117.00 - 315.45
	GvHR	205.82 ± 34.88	115.53 - 322.80

TABLE 7:1 Total IgA concentrations in saliva during a GvHR. Figures represent mean±SEM.

Day after induction of GvHR		ng Total IgA/ml	Range (ng/ml)
Day 11	CON	403.20 ± 45.99	286.00 - 578.51
	GvHR	399.00 ± 37.78	273.10 - 475.53
Day 12	CON	293.81 ± 39.87	223.35 - 397.15
	GvHR	271.00 ± 21.41	208.40 - 301.25
Day 13	CON	263.74 ± 28.64	194.52 - 336.00
	GvHR	303.64 ± 64.39	213.30 - 488.62
Day 14	CON	231.03 ± 31.93	165.55 - 315.45
	GvHR	294.51 ± 41.23	213.25 - 397.15

TABLE 7:2 Total IgA concentrations in milk during a GvHR. Figures represent mean±SEM.

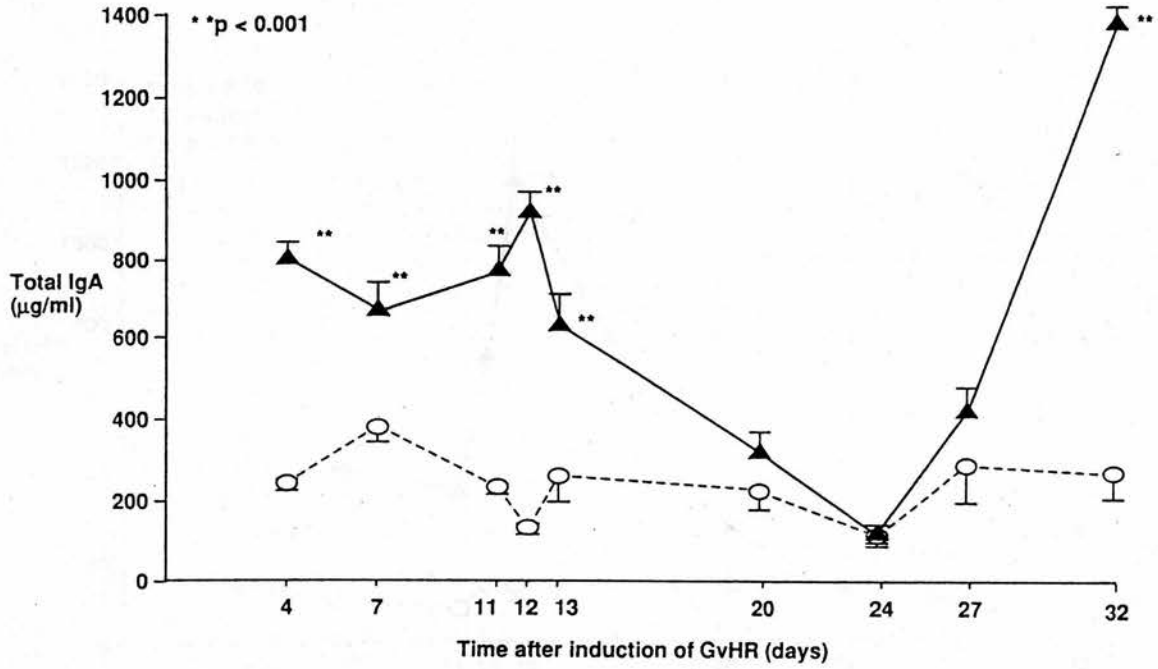


FIGURE 7:1 Serum IgA concentrations during a GvHR.
Total IgA concentrations (mean±SEM) in serum are given for both animals with GvHR (closed triangle) and age-matched controls (open circle).

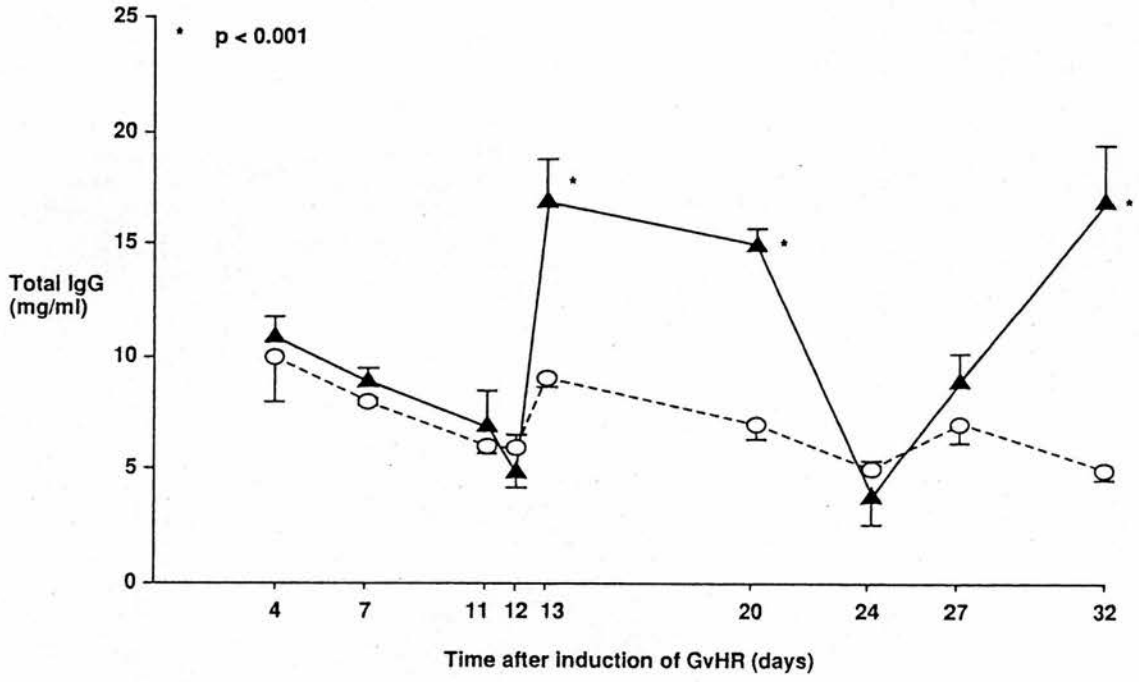


FIGURE 7:3 Serum IgG concentrations during a GvHR. Total IgG concentrations (mean±SEM) in serum are given for both animals with GvHR (closed triangle) and age-matched controls (open circle).

CHAPTER EIGHT

THE EFFECT OF A GVHR ON IMMUNOGLOBULIN PRODUCTION AT THE EXTREMES OF LIFE

INTRODUCTION

The stimulatory nature of the GvHR is well recognised in the adult murine model (chapter 2), and the work of this thesis has demonstrated the enhanced concentrations of mucosal immunoglobulins in the gut lumen (chapter 6). However, the effects on immunoglobulin production, particularly in the gut, are brief.

The aim of this study was to investigate a murine model in which the immunostimulatory influences of the GvHR would be exaggerated. The immune systems of both neonatal and aged mice are altered quite dramatically as compared to the young adult (see below) and thus both neonates and aged mice were studied.

A: MUCOSAL IMMUNOGLOBULIN PRODUCTION IN NEONATAL MICE.

In neonatal mice both humoral and cell mediated responses are immature. There are very few detectable immunoglobulin secreting cells (Ig-SC) in systemic and mucosal organs until weaning. At weaning, and in the following 3 weeks, the exposure of the animal to exogenous antigens, particularly in the gut (Ferguson and Parrott, 1972), stimulates an increase in numbers of Ig-SC in SP, bone marrow, MLN, PP and the small intestinal LP. T cell numbers are also known to increase, and the animal's immune responses mature (Van Der Heijden *et al*, 1988).

A GvHR induced in neonatal mice is known to produce a

destructive-type enteropathy (chapter 2) and, as discussed in chapter 6, a deficiency in immunoglobulin-containing cells in the duodenum of preweaned mice, 14 days after the induction of a GvHR, has been described (Gold *et al*, 1976). However, the increased stimulation of both B and T cells around the time of weaning, coupled with the immunostimulatory nature of the GvHR suggests that the effects of GvHR on mucosal immunoglobulin levels may be exacerbated in neonates.

Mucosal immunoglobulin production was investigated at and around the time of weaning.

Experimental protocol

A semi-allogeneic GvHR was induced in nonirradiated neonatal BDF_1 mice aged 7, 14 and 21 days. Mice were weaned at 21 days of age.
The concentration of donor spleen cell inoculum given to the recipients (chapter 3) was adjusted to take into account the age of the animals. Age-matched controls were given medium alone. 8-10 mice/group were used.
14 days after the induction of the GvHR, at ages 21, 28 and 35 days, body and spleen weights were recorded and gut washings collected from both control and GvHR groups. Collecting the gut washings on these days enabled the study of the effect of a GvHR on IgA and IgM concentrations before weaning (day 21) and 1 week (day 28) and 2 weeks (day 35) after weaning.

At this age (21-35 days), the mice were very small and unable to tolerate the 4x0.5mls of lavage fluid fed before the collection of the gut washings. In an initial pilot study, feeding this amount of lavage fluid resulted in stomach rupture and death. The mice were able to tolerate 2x0.5mls of lavage solution, after a 12hr fast, and on examination of the gut, both the small and large intestine contained fluid.

IgA and IgM immunoglobulin concentrations were determined by ELISA (chapter 3-Appendix A:2 and A:3).

Body weights and Spleen Index of neonatal mice with GvHR.

The body weights of both control and GvHR groups and the spleen index of the GvHR groups are given in Table 8:1.

Only those animals, age 14 days at the onset of GvHR had, on sacrifice, a significantly lower body weight ($P<0.01$) than age-matched controls. The spleen index indicates that a GvHR was in progress.

Total IgA concentrations in gut washings

In both control and GvHR groups there was a substantial rise in mucosal IgA concentrations (ng/ml) within the period of weaning (Table 8:2).

There was no significant difference in the total IgA concentrations in gut washings taken from GvHR mice, aged 7, 14 or 21 days at the induction of GvHR, as compared to the controls (Table 8:2).

Total IgM concentrations in gut washings.

As with IgA, the levels of IgM (ng/ml) in gut washings, from both control and GvHR groups, increased quite dramatically over the first 2 weeks after weaning (Table 8:2).

A significant increase ($P<0.01$) in IgM concentrations (Table 8:2) was observed on day 14 of the GvHR, in mice aged 7 days at the induction of the reaction. No significant changes were observed in the other age groups.

Comments

The results confirm previous reports (Van Der Heijden *et al*, 1988) that after weaning in normal mice the concentrations of IgA and IgM in the gut increase dramatically.

Despite the immunostimulatory nature of the GvHR there was no accentuation of IgA response in the gut lumen of neonatal mice. In mice aged 7 days at the onset of GvHR, an increase in total IgM was detected 14 days later. In contrast to the findings of Gold *et al*, (1976), no immunoglobulin deficiency was observed.

B: IMMUNOGLOBULIN PRODUCTION IN AGED MICE WITH CHRONIC GVHR.

In normal, aged animals the immune system undergoes a senescence that is characterised by a decreasing ability to

produce antibodies and to mount a CMI response. In contrast, an increase in autoantibody formation to polyclonal B cell stimulators, auto-anti-idiotypic antibodies, and a failure of tolerance induction have been described (Habicht, 1987). There is evidence to suggest that in aged mice the cells of the mucosal-associated lymph nodes do not have the decreased immune function that is observed in the systemic tissues (Szewczuk and Wade, 1983).

The semi-allogeneic GvHR in nonirradiated BDF_1 mice can progress to the chronic form; the characteristics of which are immunostimulatory in nature, and the most striking features are systemic polyclonal B cell stimulation, hypergammaglobulinaemia and autoantibody production. In the work from Gleichmann and co-workers, the features of the chronic GvHR were observed from 24 weeks after the induction of the GvHR (chapter 2).

The non-specific immunostimulatory effects of the GvHR are very similar to those occurring during the aging process, and as with the neonatal model, it was hypothesized that the effects on mucosal immunoglobulin production occurring during the GvHR would be exaggerated in aged mice. Systemic and mucosal immunoglobulin concentrations both in normal aged mice and in aged mice with chronic GvHR were studied.

The systemic immune response, both humoral and cell-mediated, to an enterically presented antigen, BLG, was also studied in these aged animals. This work is relevant to the investigations described in chapter 10 and will be

discussed there.

Experimental protocol

A semi-allogeneic GvHR was induced in adult BDF_1 mice aged 6-8 weeks, by i.p. injection of 10^8 C57Bl/6J spleen cells. Control animals received medium alone.
18 months later, body and spleen weights were recorded and gut washings and serum collected. Gut washings were collected from 10 animals/group, and serum from 16 animals/group.
Immunoglobulin concentrations were determined by ELISA (chapter 3 - Appendix A:2, A:3, A:4).

Body weights and relative spleen weights of aged mice

Throughout this experiment all the animals remained well.
A group of young, adult BDF_1 mice (aged 6-8 weeks) were sacrificed at the same time as the aged animals, and body and spleen weights recorded.
The body weights (Table 8:3) of the animals had, as expected, increased ($P<0.01$) with age. An interesting observation was the significantly lower ($P<0.01$) relative spleen weight (mgs spleen weight/10g body weight) of the aged animals compared to animals aged 6-8 weeks (Table 8:3).

Age and immunoglobulin concentrations in serum and gut washings

A comparison between the total IgA and IgM concentrations in gut washings and total IgA, IgM and IgG in serum from normal mice aged 6-8 weeks old, and normal mice aged 18 months is presented in Table 8:4. The values for normal mice aged 6-8 weeks are the means ± SEM of the results presented in chapters 6 and 7.

The concentrations of IgA and IgM in gut washings had quite obviously increased with age. The levels in aged mice were similiar in magnitude to those detected in early life, at the time of weaning (Table 8:2).

In serum (Table 8:4), both IgA and IgM concentrations were greatly increased in the 18 month old mice. Interestingly, the concentrations of IgG did not increase with age, and remained in the same range as the 6-8 week old mice.

Body weights and Spleen Index of aged mice with chronic GvHR.

Animals with chronic GvHR remained well throughout this experiment.

There was no significant difference in body weights between control and GvHR groups (Table 8:5). A spleen index of 1.52 (Table 8:5) indicated the late effects of the GvHR.

Mucosal immunoglobulin concentrations during chronic GvHR.

There was no significant difference (Table 8:6) in either IgA or IgM concentrations in gut washings of mice with chronic GvHR when compared to age-matched controls.

Serum immunoglobulin concentrations during chronic GvHR

The concentrations of serum IgA, IgM and IgG in mice with chronic GvHR are given in Table 8:7.
Mice with chronic GvHR had significantly higher ($P<0.005$) concentrations of IgA (ug/ml), and IgG (mg/ml) ($P<0.00001$). In contrast, serum IgM concentrations (ug/ml) were significantly lower ($P<0.05$) than controls.

Comments

The concentrations of both systemic and mucosal IgA and IgM increased with age. Interestingly, the concentrations of IgG remained within the same range as normal 6-8 week old mice. These results may reflect the dichotomy of effects of age on systemic and mucosal immunity.

The chronic form of the murine GvHR resulted in striking increases in the concentrations of IgA and IgG in serum, with a simultaneous decrease in IgM. There was no change in the immunoglobulin levels in the gut luminal contents.
Hypergammaglobulinaemia with a concomitant decrease in IgM has been observed in the chronic form of GvHR, in both experimental animals and BMT patients (chapter 2).

In summary, the GvHR in neonates did not exacerbate the production of IgA in the gut as had been hypothesized. 14 days after the induction of a GvHR in 7 day old mice an increase in IgM was observed, however, this increase was not observed in any other age group.

In chronic GvHR, no effect on the mucosal immunoglobulin response was observed. Systemically, both IgA and IgG levels were elevated, but IgM levels were much lower than controls, reflecting possibly, polyclonal stimulation and isotype-switching of activated B cells; both recognised features of the chronic form of GvHR.

Age at Onset of GvHR (days)	Age on testing (days)	Body Weights (g) Control	GvHR	Spleen Index
7	21	8.206 ± 0.65	7.828 ± 0.53	1.87
14	28	11.717 ± 0.27	10.261 ± 0.33*	1.79
21	35	18.546 ± 0.72	18.204 ± 0.57	1.80

* P<0.01

TABLE 8:1 Body weights and spleen index of neonatal BDF1 mice with GvHR.
Figures represent mean±s.d.

Age at Onset of GvHR (days)		Age on testing (days)	Total IgA ng/ml	Total IgM ng/ml
7	CON	21	166.42 ± 39.3	35.39 ± 3.03
	GvHR		289.70 ± 88.50	146.57 ± 20.69*
14	CON	28	406.62 ± 90.74	200.56 ± 39.46
	GvHR		393.27 ± 53.06	197.43 ± 50.51
21	CON	35	682.92 ± 116.93	523.20 ± 115.15
	GvHR		753.49 ± 118.92	573.82 ± 174.65

TABLE 8:2 Total IgA and IgM concentrations (mean±SEM) in gut washings collected from neonatal mice 14 days after the induction of a GvHR.

	Control Age : 6-8 wks	Control Age : 18 months
Body weights (g)	21.484 ± 0.38	35.812 ± 2.21*
Relative Spleen Weight (mgs)	35.41 ± 1.46	18.54 ± 2.28*

* $P<0.01$

TABLE 8:3 Body and spleen weights of aged BDF1 mice. Figures represent means±s.d.

	Control Age : 6-8 wks	Control Age : 18 months
Gut Washings		
Total IgA (ng/ml)	160.40 ± 21.38	1072.12 ± 327.19
Range	107.11 - 217.25	322.59 - 3027.12
Total IgM (ng/ml)	9.05 ± 1.68	30.38 ± 6.78
Range	4.48 - 14.95	8.48 - 67.76
Serum		
Total IgA (µg/ml)	243.55 ± 26.80	710.21 ± 71.66
Range	120 - 385	257 - 1531.49
Total IgM (µg/ml)	145.22 ± 12.88	486.41 ± 52.57
Range	86 - 215	191.05 - 1226
Total IgG (mg/ml)	7.12 ± 0.64	5.41 ± 0.21
Range	5.10 - 10.20	3.58 - 7.33

TABLE 8:4 The effect of age on serum and mucosal immunoglobulin concentrations.
Figures represent mean±SEM.

	Body weights (g)	Relative Spleen Weight (mgs)	Spleen Index
Control	35.812 ± 2.21	18.54 ± 2.28	-
GvHR	31.199 ± 1.40	28.11 ± 2.80	1.52

TABLE 8:5 Body weights and spleen index of aged BDF1 mice with chronic GvHR.
Figures represent mean±s.d.

	Total IgA (ng / ml)	Total IgM (ng / ml)
CON	1072.12 ± 327.19	30.38 ± 6.78
Range	322.59 - 3027.12	8.48 - 67.76
GvHR	1049.50 ± 267.50	35.31 ± 7.73
Range	452.98 - 2725.92	4.97 - 76.04

TABLE 8:6 Total IgA and IgM concentrations (mean±SEM) in gut washings collected from aged mice with chronic GvHR.

	Total IgA (µg/ml)	Total IgM (µg/ml)	Total IgG (mg/ml)
Control	710.21 ± 71.66	486.41 ± 52.57	5.41 ± 0.21
Range	257 - 1531	191 - 1226	3.58 - 7.33
GvHR	1177.37 ± 137.72**	318.37 ± 36.30*	8.59 ± 0.39***
Range	416 - 2635	139 - 600	5.55 - 11.34

* P<0.05
** P<0.005
*** P<0.00001

TABLE 8:7 Serum IgA, IgM and IgG concentrations (mean±SEM) in aged mice with chronic GvHR.

CHAPTER NINE

THE EFFECT OF A GVHR ON IMMUNOGLOBULIN-SECRETING CELLS IN PEYER'S PATCHES, MESENTERIC LYMPH NODES AND SPLEEN

INTRODUCTION

The findings presented in chapters 6 and 7, have highlighted the immunostimulatory effect of the GvHR on B cells and on immunoglobulin production.

Increased concentrations of IgA, IgM and IgG were detected in the circulation during the initial phase of GvHR. Quite separately, increased counts of IgA and IgM plasma cells, but not IgG, were observed in the LP. The transient increase in IgA concentrations in the gut lumen, and the prolonged increase in IgM suggests that the GvHR is affecting the immunoregulation of mucosal B cells early in their maturation pathway.

The source of mucosal immunoglobulin-secreting cells (Ig-SC) is believed to be the PP. During the migration of activated B cells from the PP to the LP, T cell dependent immunoregulation ensures isotype-switching from $sIgM^+$ B cells to $sIgA^+$ B cells, clonal proliferation and finally terminal differentiation into IgA-secreting plasma cells (reviewed in chapter 1).

On leaving the PP, B cells enter the MLN and from there enter the circulation via the thoracic duct. The cells may spend some time in the SP prior to homing to the gut and other distant mucosal sites.

The aim of this investigation was to assess the effect of a GvHR on the total cell counts and Ig-SC counts in the mucosal-associated lymph nodes and spleen to gain some preliminary information as to where in the maturation and immunoregulatory pathway of mucosal B cells the GvHR was

exerting an immunostimulatory effect.

General protocol

A semi-allogeneic GvHR was induced in adult BDF_1 mice, age 6-8 weeks, by i.p. injection of 10^8 donor spleen cells. Controls received medium alone.

On days 4, 7, 11, 13, 20, 24, 27 and 32 after the induction of a GvHR, PP, MLN and SP were removed (chapter 3). 4 mice/group were used at each time point. The lymph nodes were pooled, and a single cell suspension made (chapter 3). The total cell count was recorded and each suspension appropriately diluted and two aliquots containing 10^6 cells taken for use in the ELISPOT assay (chapter 3). The dilutions of coating and conjugated antibodies stated in chapter 3 were determined by using SP cells from normal nonimmunised BDF_1 mice. The concentrations of detecting antibody chosen, gave a count of IgA^+-, IgM^+- and IgG^+-SFC in the expected range (van der Heijden, Stok and Bianchi, 1987).

In this experiment, the ELISPOT assay was performed "blind" to the nature and origin of the samples. At the end of the experiment the data were collected and analysed.

The results are expressed in Fig 9:2, 9:3, and 9:4 (a, b, c) as numbers of Ig Spot-Forming Cells (SFC)/10^6 cells. This corresponds to the numbers of Ig-SC, as described in the text. The results given for those animals with GvHR, represent the mean value for each time point studied. The SEM is not given as the results are taken from pooled

samples. The results for the control samples, taken throughout the experiment, are grouped together and the mean±SEM displayed on the figures.

Macroscopic examination of lymph nodes

The enlargement of the spleen during a GvHR is well recognised. As well as splenomegaly, both PP and MLN were enlarged throughout the initial stages of the GvHR and consequently the MLN in mice with GvHR were easier to find and remove than in control mice.

A: **TOTAL CELL COUNTS OF PP, MLN AND SP THROUGHOUT THE GVHR**

The total cell counts of PP, MLN and SP are given in Fig 9:1 (a, b, and c).
The total cell counts in the lymph nodes dissected from normal, control mice were in the same order of magnitude as reported by van der Heijden *et al*, (1987).

Total cell count of PP

On day 7 of the GvHR there was a dramatic increase in the total cell count of PP (Fig 9:1a), which subsequently dropped to below control levels by day 11. On days 20 and 24 the total cell count was substantially lower than controls.

Total cell count of MLN

The total cell count of MLN (Fig 9:1b) was greater than controls on day 7 of the GvHR, and, although in decline the numbers remained high until day 13. A second small increase was observed on day 24, but by day 27 the count had returned to that of controls.

Total cell counts of SP

The total cell count of SP (Fig 9:1c) was greater than controls from day 7 of the GvHR and remained high until around day 24. On day 32 of the GvHR there was a dramatic increase in the total cell count, greater than that observed on day 7.

Comments

During the GvHR, the PP, MLN and SP were increased in size, and at certain time points throughout the reaction increased total cell counts were detected.

A substantial increase in total cell count was observed on day 7 of the GvHR in PP, MLN and SP. While the total cell count remained higher than controls in the SP throughout the GvHR, the numbers in the MLN and PP returned to normal by days 11-13. A striking increase in total cell count was observed on day 32, but only in the SP.

B: **IMMUNOGLOBULIN-SECRETING CELLS IN PP, MLN AND SP THROUGHOUT THE GVHR**

Total IgG-secreting cells in PP, MLN and SP throughout the GvHR.

The numbers of IgG^+-SC in the SP, MLN and PP of the control mice were of a similar order of magnitude as described by van der Heijden *et al* (1987), using a Plaque-forming-cell (PFC) assay.

Total IgG-secreting cells in PP

An increase in counts of $IgG^+SFC/10^6$ cells was observed on day 11 (Fig 9:2a) of the GvHR. The count returned to control levels by days 20-24 and remained so until the end of the study.

Total IgG-secreting cells in MLN

A substantial increase in IgG $SFC/10^6$ cells in the MLN (Fig 9:2b) was observed on day 11. The numbers dropped, but still remained higher than controls until the end of the study.

Total IgG-secreting cells in SP

The numbers of IgG $SFC/10^6$ cells in the SP (Fig 9:2c) were increased on day 7 of the GvHR. After a drop on day 11, the numbers remained higher than controls until a second

increase on day 32.

Total IgA-secreting cells in PP, MLN and SP throughout the GvHR.

The numbers of $IgA^+SFC/10^6$ cells in PP, MLN and SP taken from the control animals (Fig 9:3a,b,c) were extremely low compared to the findings of van der Heijden *et al*, (1987). Using their PFC-assay they found 1730 IgA^+-$SC/10^6$ PP cells, 1197 IgA^+-$SC/10^6$ MLN cells and 419 IgA^+-$SC/10^6$ SP cells. In this particular experiment only 2-3 IgA^+-$SC/10^6$ were detected; it would therefore appear that this ELISPOT assay had not worked.

Total IgM-secreting cells in PP, MLN and SP throughout the GvHR.

The numbers of IgM^+ cells in the PP, MLN and SP taken from control mice (Fig 9:4a,b,c) were also very low. van der Heijden *et al*, (1987) found 584 IgM^+-$SC/10^6$ PP cells, 142 IgM^+-$SC/10^6$ MLN cells and 2686 IgM^+-$SC/10^6$ SP cells.
In this experiment only 2-4 IgM^+-$SC/10^6$ cells were detected. Again this comparison of data would suggest that this ELISPOT assay did not work.
Despite the very low counts in the control group, the numbers of IgM^+-SC were higher during the GvHR.
In the PP and the MLN (Fig 9:4 a,b) an increase was observed on day 7 of the GvHR. In the SP (Fig 9:4c) the counts were higher than controls on days 4 and 7 and a

substantial increase was observed on day 32 of the GvHR.

General Comments

The investigations described in this study have shown that during the GvHR there is a substantial increase in the total cell count of PP, MLN and SP on day 7. While the total cell counts of the PP returned to control levels by day 11 and the MLN by day 13, the SP cell count remained high throughout the reaction. A dramatic increase was observed in the SP on day 32.

The results for the SP cell count parallel the increase in cell proliferation, as monitored by the SP index (Fig 4:3). The transient increase in PP and MLN cell numbers only occurs within the initial phase of the GvHR.

The initial pilot studies of the ELISPOT assays for the detection of IgA^+-SC and IgM^+-SC gave good results with clear, well defined spots and the same conditions were used in the "blind" experiment. Unfortunately, on this occasion the assay did not work and I did not have time to repeat the experiment, or re-analyse the assay. Further work optimising the assay is now underway in our laboratory.

Despite this, the numbers of IgM^+-SC during GvHR were raised in the PP, MLN and SP on day 7, followed by a dramatic increase, in the SP only, on day 32.

The ELISPOT assay for the detection of IgG^+-SC gave very good results, and again during the GvHR there were substantially increased numbers of IgG^+-SC in PP, MLN and SP. While the numbers in the PP and MLN were raised on day

11, and thereafter in decline, the numbers in the SP were raised on day 7 and remained higher than controls until the second increase on day 32.

In summary, during the initial immunostimulatory phase of the GvHR, the total numbers of cells in PP and MLN are transiently increased whereas in the SP the numbers remain high throughout the GvHR. The second increase in SP total cell count, on day 32, may reflect the onset of the chronic GvHR.

The effects appeared to be paralleled in the numbers of Ig-SC in the PP, MLN and SP in that a transient increase in IgG^+-SC numbers was observed in the PP and MLN but a prolonged increase was observed in the SP.

Unfortunately no conclusions can be drawn about the numbers of IgA^+- and IgM^+-SC as the assay did not work on this occasion. However, the increased numbers of IgM^+-SC detected in LN of mice with GvHR, and the lack of effect on IgA^+-SC, does warrant further study.

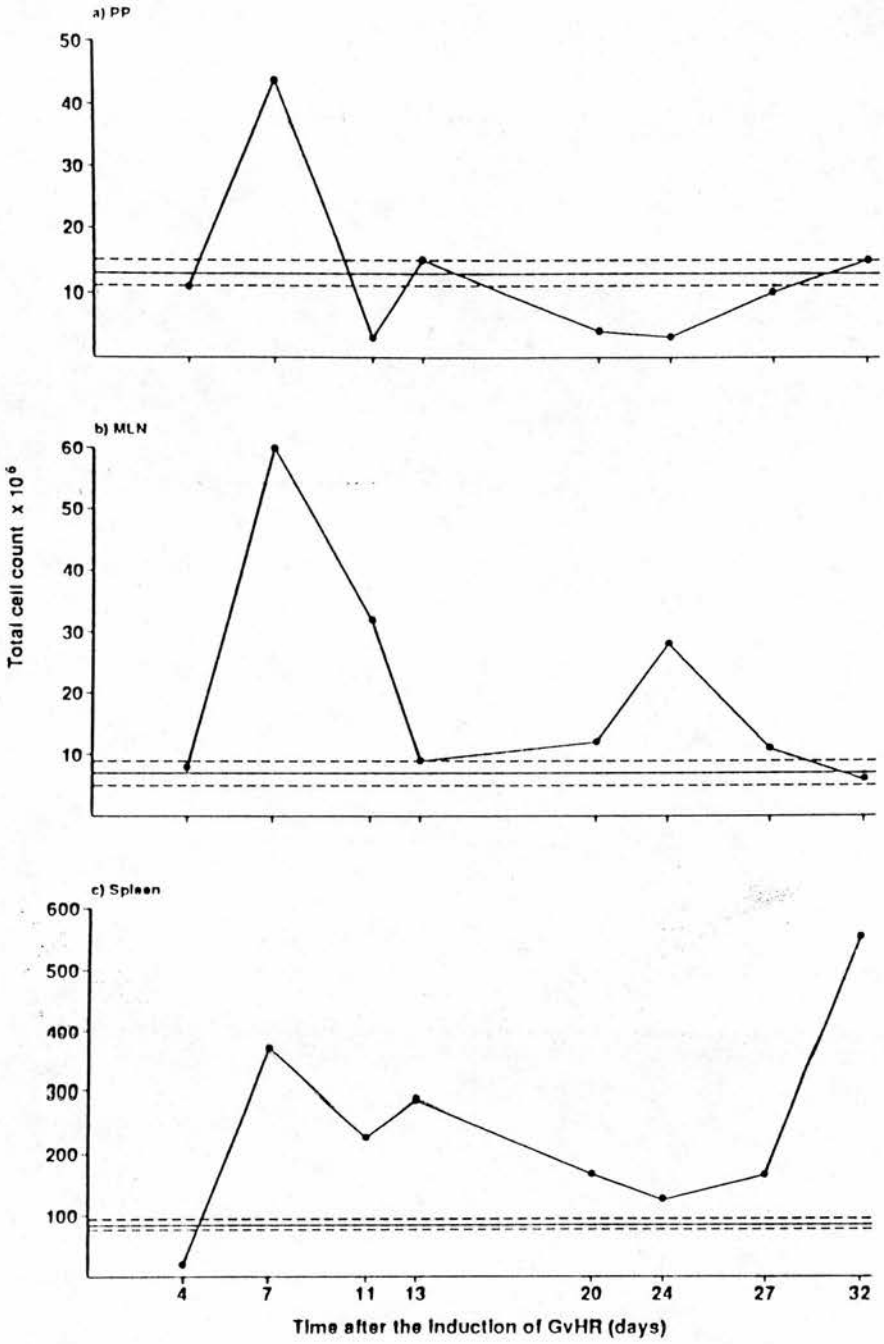


FIGURE 9:1 Total cell counts of PP (a), MLN (b), and SP (c), of adult BDF1 mice throughout the GvHR.
The group mean of the control animals is represented by the solid line and the SEM by the broken lines.

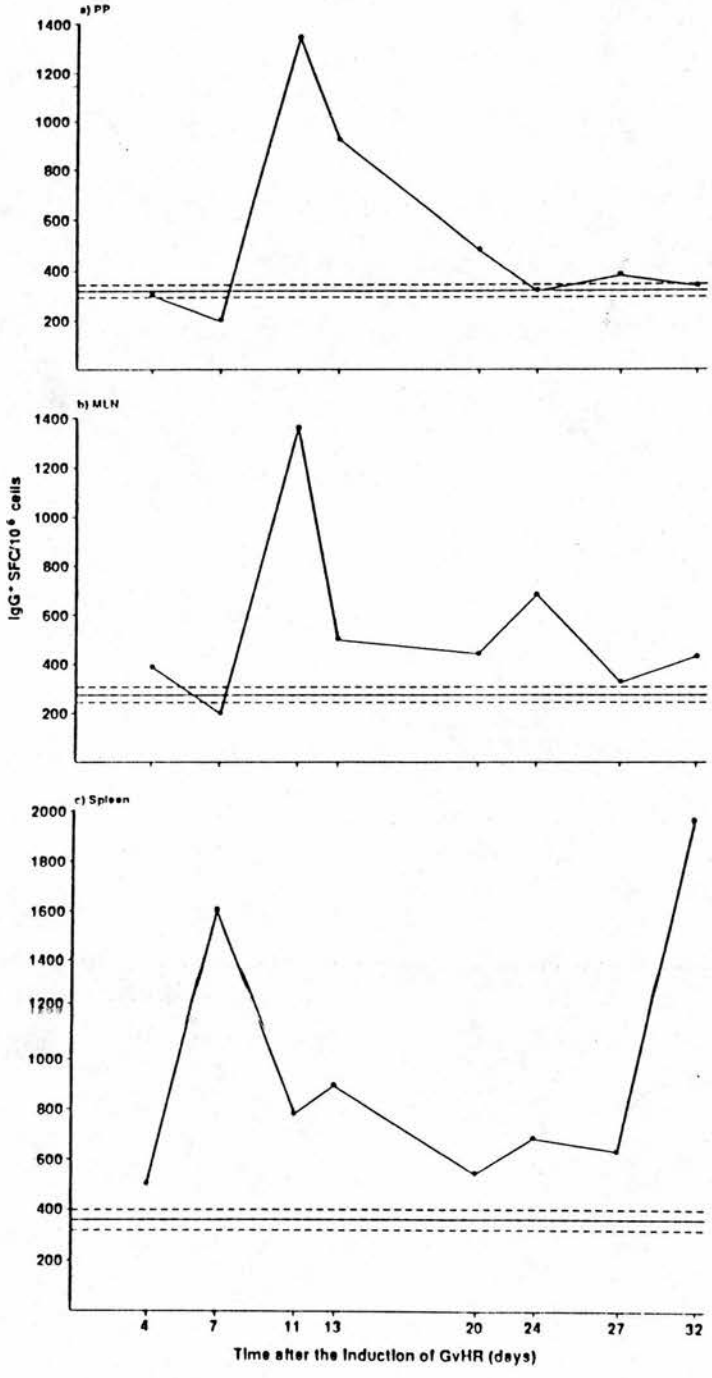


FIGURE 9:2 Total IgG-secreting cells in PP (a), MLN (b), and SP (c) of BDF1 mice throughout the GvHR.
The group mean of the control animals is represented by the solid line and the SEM by the broken lines.

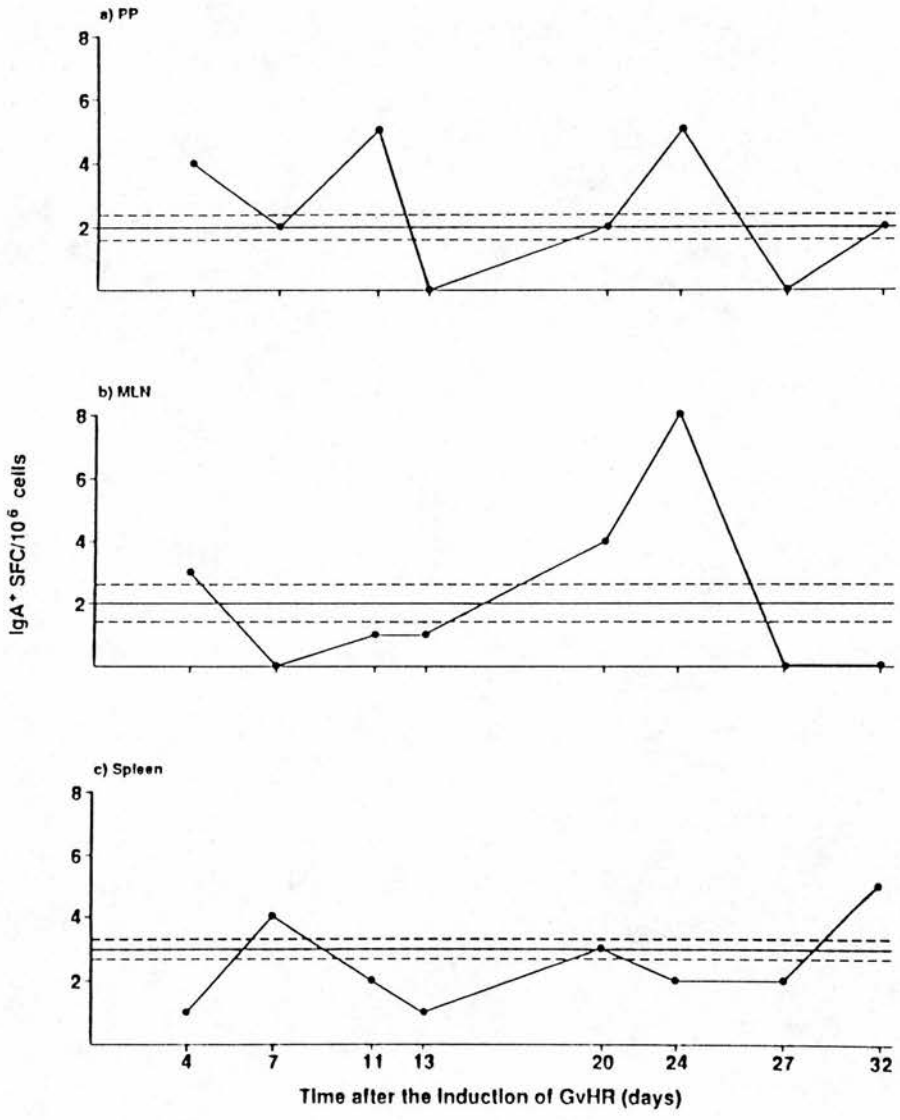


FIGURE 9:3 Total IgA-secreting cells in PP (a), MLN (b), and SP (c) of BDF1 mice throughout the GvHR.
The group mean of the control animals is represented by the solid line and the SEM by the broken lines.

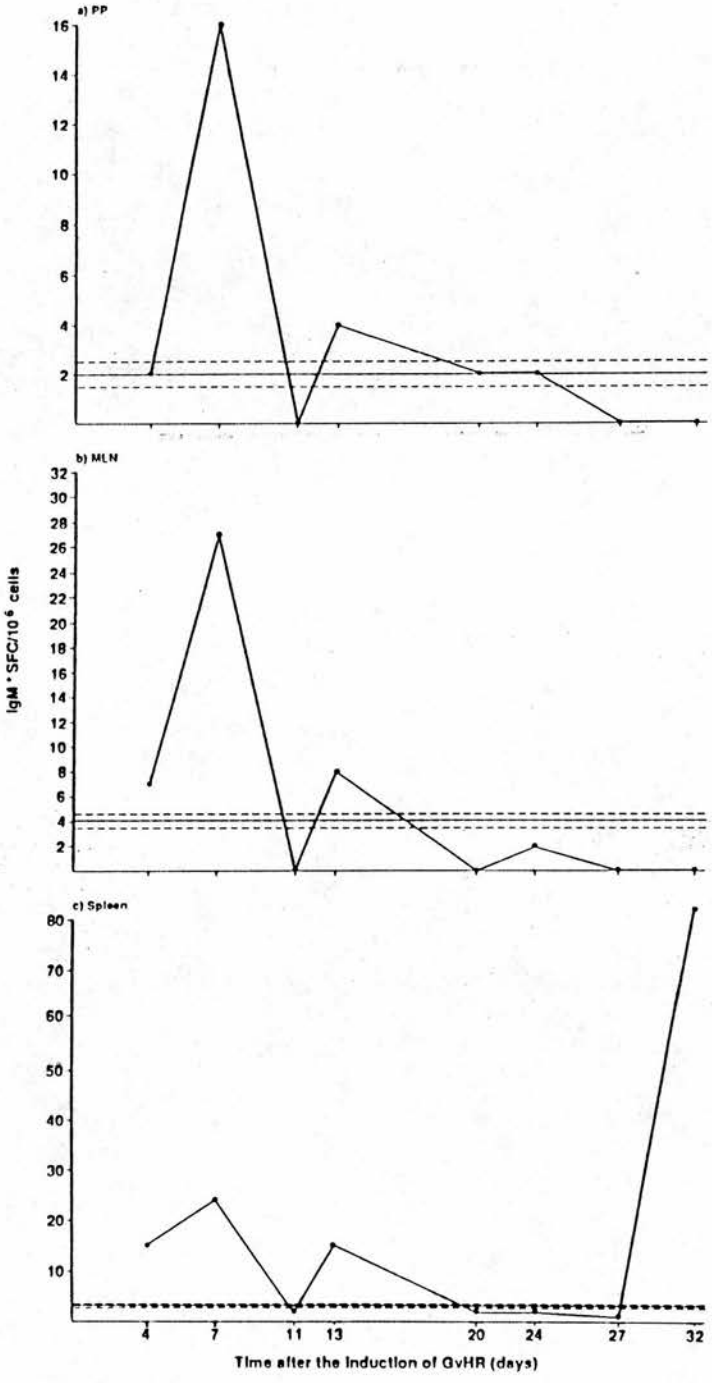


FIGURE 9:4 Total IgM-secreting cells in PP (a), MLN (b), and SP (c) of BDF1 mice throughout the GvHR.
The group mean of the control animals is represented by the solid line and the SEM by the broken lines.

CHAPTER TEN

ORAL TOLERANCE INDUCTION TO ENTERICALLY PRESENTED ANTIGENS IN BDF1 AND TRANSGENIC MICE

INTRODUCTION

The relationship between mucosal immunity and systemic hyporesponsiveness (oral tolerance) is as yet uncertain, and in chapter 11 the investigations into the murine systemic and mucosal immune responses to the clinically relevant dietary antigens, OVA and BLG, under normal conditions and conditions of T cell activation are described.

Prior to studying the secretory immune response to OVA and BLG, it was first necessary to investigate oral tolerance induction to these chosen antigens.

The phenomenon of oral tolerance is recognised as the suppression of systemic humoral and cell mediated immune responses to an enterically presented antigen, and is under T cell regulation (chapter 1).

A substantial amount of work on the mechanisms of oral tolerance to the dietary antigen OVA has been performed in this laboratory (Strobel, 1983; Bruce, 1985; Lamont, 1986), and therefore OVA was further used in these studies as a dietary antigen of known properties.

A second antigen, BLG, a protein found in cow's milk, and implicated in CMPI was also studied. This antigen was of particular interest as I had the unique resource of a colony of transgenic mice, transfected with a cloned sheep BLG gene, which expressed sheep BLG, in milk, during lactation.

The aims of the investigations described in this chapter were : -

a. to establish the technique of oral tolerance using the well studied antigen OVA.

b. to determine the dose of BLG required to induce oral tolerance in adult mice and to investigate a possible low dose priming effect, which has now been described with OVA (Lamont, Mowat and Parrott, 1989); a wide range of doses of BLG were studied. The antigen specificity of the reaction was also determined.

In addition, since aged animals were available from the experiments described in chapter 8, oral tolerance induction in aged mice was studied.

c. Finally, the systemic immune response to BLG in F_1 mice, having received BLG from the milk of transgenic mothers, from the first day of life, was assessed.

A: **ORAL TOLERANCE INDUCTION TO OVA**

Previous work from this laboratory had shown that systemic hyporesponsiveness to OVA could be induced when a dose of 25mgs OVA was fed to adult mice. Using the established protocol I performed an oral tolerance experiment, using OVA, to both learn the technique, and to determine the systemic hyporesponsiveness of the adult BDF_1 mice which were to be used in the studies described in chapter 11.

Experimental protocol of tolerance induction

The experimental design is described in Fig 10:1. This basic protocol was used in all of the studies of oral

tolerance. 7 days after feeding the desired amount of antigen (chapter 3), the animals were challenged systemically by i.d. injection of 100ug antigen in CFA (chapter 3). 3 weeks post-immunisation animals were bled via the retro-orbital plexus (chapter 3) and serum antibodies detected by ELISA (chapter 3, Appendix A:5). The DTH response was determined, by the 24hr footpad increment, after antigen challenge (chapter 3).

8 BDF_1 mice, aged 6-8 weeks, were used per group. Serum was collected from 6 mice/group, and the DTH response was assessed in all 8 mice.

Values for the % suppression of the DTH response were obtained as follows:-

$$\% \text{ Suppression} = \frac{\text{response in control group} - \text{response in tolerant group}}{\text{response in control group}} \times 100$$

Results

The results, presented in Table 10:1, show that feeding 25mgs of OVA prior to systemic challenge produces significantly lower ($P<0.01$) levels of IgG anti-OVA antibodies than in saline fed controls. Similarly the DTH response was significantly lower ($P<0.01$; 85% suppression) in mice fed OVA as compared with controls.

B: **ORAL TOLERANCE INDUCTION TO BLG IN ADULT BDF1 MICE**

Experimental protocol

The study was divided into 4 separate experiments, in which a range of doses (1ug-75mgs) of BLG were fed. The protocol used was as described in Fig 10:1. 8 BDF_1 mice (age 6-8 wks)/group were studied, and the antibody and DTH responses assessed as described above.

Results

Experiment 1

In this first experiment mice were fed 1mg, 10mgs, 25mgs, or 50mgs of BLG. The control group received saline alone.

While feeding 1mg BLG gave a significantly higher footpad increment ($P<0.05$) than controls, only in those mice fed 50 mgs BLG (Table 10:2), were both the IgG anti-BLG response ($P<0.05$) and the DTH response ($P<0.005$; 133% suppression) significantly lower than the saline fed controls.

Experiment 2

Mice were fed 10ug, 50ug, 100ug or 250ug BLG, and control mice received saline.

In those animals fed 10ug BLG, a significantly lower DTH response ($P<0.05$; 38% suppression) was observed. However no significant alteration in either the antibody or DTH

response was observed in the other groups.

Experiment 3

Mice were fed 500ug, 1mg, 25mgs or 50mgs of BLG. Controls received a saline feed.

In those animals fed 25mgs or 50mgs of BLG, the antibody response was significantly lower than controls ($P<0.01$). However, only those animals fed 50mgs of BLG had a significantly lower DTH response ($P<0.001$; 140% suppression) thus duplicating the findings of experiment 1.

Experiment 4

Mice were fed 1ug, 5ug, 10ug or 75mgs of BLG. The controls were fed saline.

Mice fed 75mgs of BLG had a significantly decreased antibody response ($P<0.01$) as compared with controls. No other concentrations of BLG induced oral tolerance.

Comments

The results described in Tables 10:2, :3, :4 and :5 show that the dose of BLG required to induce systemic hyporesponsiveness in both the antibody and DTH responses was 50mgs. A suppressed antibody response was also observed in mice fed 25mgs or 75mgs BLG. No low dose priming of the antibody and DTH responses was found. The disparate

effects to the DTH response after feeding 10ug or 1mg BLG were only significant at the $P<0.05$ level, and were not reproduced. The probable explanation is that these anomalies occurred by chance when such a large number of different experimental groups were being analysed. In addition, it should be noted that the DTH responses to BLG in some positive control groups were very low and this may complicate the interpretation of the findings on oral tolerance to BLG.

Antigen specificity of the induction of oral tolerance to BLG

In previous studies of oral tolerance induction to OVA, the antigen specificity of the response was determined by the inability to induce systemic hyporesponsiveness to an unrelated antigen, such as horse serum albumin (HSA) or bovine serum albumin (BSA), by prior feeding with OVA.
To determine the antigen specificity of the tolerance response to BLG, mice were challenged with OVA after prior feeding with BLG.

Experimental protocol

The experimental protocol described in Fig 10:1 was used in this experiment. The following 4 groups of 8 adult BDF_1 mice were studied. Group A, controls, were fed saline and challenged with BLG. Group B, were fed 50mgs BLG and challenged with BLG. Group C, the OVA control group, were fed saline and challenged with OVA, and Group D were fed 50mgs BLG and challenged with OVA. The antibody response, both IgG anti-BLG and IgG anti-OVA, was assessed in all groups, while the DTH response to BLG

was assessed in Groups A and B, and the DTH response to OVA assessed in Groups C and D.

Results

The results presented in Table 10:6 confirm that feeding 50mgs of BLG induces systemic tolerance for both IgG anti-BLG ($P<0.01$) and the DTH response ($P<0.001$; 100% suppression).
Feeding mice 50mgs BLG did not suppress either the humoral or DTH responses to OVA, indicating that oral tolerance induction to BLG is an antigen-specific phenomenon.

Oral tolerance induction in aged mice

Experimental protocol

The experimental protocol described in Fig 10:1 was used in this experiment. The following groups of BDF_1 mice (8 animals/group) were studied. Group A (age 6-8wks) were fed saline and immunised with BLG, Group B (age 6-8wks) were fed BLG (50mgs) and immunised with BLG, Group C (age 18mths) were fed saline and immunised with BLG and Group D (age 18mths) were fed BLG (50mgs) and immunised with BLG.

Results

The results are given in Table 10:7.
In mice age 6-8wks, prior feeding of BLG (50mgs),

significantly suppressed both the IgG anti-BLG response ($P<0.05$) and the DTH response ($P<0.01$; 112% suppression); thus repeating the findings described above.

In contrast, feeding a tolerizing dose of BLG to aged mice (age 18mths) did not induce oral tolerance for either the humoral or cell mediated immune response. In fact the DTH response of aged mice after immunisation, without prior feeding (Group C), was significantly greater ($P<0.05$) than that found in young adult mice (Group A).

C: **THE TRANSGENIC MOUSE MODEL**

A strain of transgenic mice, possessing the sheep BLG gene and expressing BLG in milk during lactation, was donated by J.P. Simons (Edinburgh Research Station, AFRC Institute of Animal Physiology and Genetics Research, King's Buildings Edinburgh).

The complete method of gene transfer used in producing the transgenic mouse strain is described in Simons, McClenaghan and Clark, (1987).

Briefly, a clone of the gene encoding sheep BLG was microinjected into pronucleus stage fertilized eggs obtained from superovulated (C57Bl/6xCBA)F_1 females after mating with F_1 males. The injected eggs were cultured overnight and cleaved embryos transferred into the oviducts of pseudopregnant F_1 females. Detection of the BLG gene in the offspring was by Southern blot analysis of sections of tail, at weaning.

Abundant BLG transcripts were detected only in the mammary

gland, although after long exposures extremely low levels of BLG could be detected in other sites (Simons *et al*, 1987).

A range of concentrations of sheep BLG from 3mg/ml to 23mg/ml could be found in the milk of lactating females (Simons *et al*, 1987), and production of the gene product was believed to be switched on, under hormonal control, as early as day 11 of gestation.

I was given a group of 8 females, known to possess the sheep BLG gene, but which had not yet expressed the protein, and 8 females, born of transgenic mothers, but not carrying the gene.

The transgenic mouse strain offered a possible animal model for the study of both the systemic and mucosal immune reactions of neonates to a novel, dietary antigen presented in the mothers milk, and administered to an essentially immature gut from the first day of life.

Previous work in this laboratory revealed that feeding a weight related dose of OVA to mice in the first weeks of life, or in the prenatal period, resulted in a primed antibody and DTH response on subsequent challenge (Strobel and Ferguson, 1984).

The investigations described in this section were a pilot study designed to determine the systemic response in adult mice having been fed a novel, dietary antigen, BLG, in the first few weeks of life.

CROSS-REACTIVITY BETWEEN OVINE AND BOVINE BLG

The BLG used in the studies of oral tolerance described above was the commercially available bovine BLG and the ELISA was designed to detect antibodies to bovine BLG. Prior to studying the systemic antibody and DTH responses in the transgenic mouse strain, it was first necessary to determine the cross-reactivity of bovine and ovine (sheep) BLG. Cross-reactivity between the two antigens would allow the challenge of mice with the bovine BLG, and detection of antibodies using the established ELISA.

The precipitin assay used to determine the cross-reactivity between the two antigens is described in chapter 3. 2 precipitin plates were set up as follows (Fig 10:2):-

Plate 1: A positive control, set up to assess the viability of the assay contained normal mouse serum (NMS) as the test antigen (in wells 1 and 2), and rabbit anti-mouse Ig (Dako, Immunoglobulins, Denmark) as the detecting antibody (well 3).

Plate 2: contained a 1:5 dilution of defatted Dorset Horn sheep milk (a kind gift from J.P. Simons), the test antigen in well 1, a 3mg/ml concentration of bovine BLG (Sigma) in well 2 and a neat solution of mouse serum containing IgG anti-bovine BLG antibodies, as determined by ELISA, in well 3.

Results

The results (Fig 10:2), depicted diagrammatically, show complete identity between bovine BLG and the BLG contained in sheep milk.

SYSTEMIC RESPONSE TO BLG IN THE OFFSPRING OF TRANSGENIC MICE

The following four groups were designed to assess the systemic response to BLG after prior feeding of the antigen (Group A a control, and Group B the test group).
In conjunction Groups C and D were designed to give some initial information on the effect of possessing the sheep BLG gene (although not having expressed the gene product) on the systemic response to BLG.

Group A: Offspring, both female and male, of a cross between nontransgenic females and CBA males (Animal Unit, Western General Hospital, Edinburgh). The offspring did not possess the sheep BLG gene, and were not exposed to sheep BLG during lactation; thus acting as controls.

Group B: Offspring of a cross between transgenic females and CBA males. All the offspring received sheep BLG during lactation. Some of the offspring may have received the sheep gene from the transgenic mothers, however this was not assessed.

Group C: Offspring of a cross between transgenic females

and CBA males. The offspring were then fostered onto normal lactating C57Bl/6J females (from Group D) within a few hours after birth. Some of the offspring would possess the sheep BLG gene, and the mice may have received sheep BLG, from the mother's milk, prior to being fostered.

Group D: Offspring of a cross between normal C57Bl/6J females and CBA males, fostered onto the transgenic mothers of group C. These animals would not possess the sheep gene, but would receive sheep BLG from milk, during lactation.

Experimental protocol

On day 21 of life the offspring from all 4 groups were weaned onto the standard small rodent diet (CRM(x)). 3 weeks post-weaning the mice were systemically challenged with 100ug bovine BLG in CFA injected i.d. into the right hind footpad. 3 weeks post-immunisation serum was collected and the DTH response assessed.

Results

Antibody response

The antibody response observed in Group A (Fig 10:3) was comparable to the response found in mice immunised with BLG but without a prior feed (see Tables 10:2, :3, :4 and :5). Although not statistically different from Group A, there was a wide range (OD values 0.129 - 1.086) in the antibody responses in Group B, suggesting that while some mice had a

suppressed antibody response to BLG after feeding, others did not.

The mice in Group C also had a wide range (OD values 0.419 - 1.134) of antibody response despite having only been in contact with BLG in the first few hours before being fostered.

The mice in Group D had a positive antibody response to BLG, comparable to the control Group A, despite having been fed sheep BLG. There was no statistical difference between Groups C and D.

A statistical comparison between Groups B and D (both groups having received sheep BLG in milk from transgenic females) revealed that the antibody response in Group B was significantly lower ($P<0.05$) than in Group D. The difference between the groups was that mice in Group B were born of transgenic mothers; therefore up to 50% of the offspring would have received a copy of the sheep BLG gene from the mothers.

DTH response

No statistical difference between the groups was observed, indicating that the presence of BLG in the milk ingested in the first weeks of life did not suppress the DTH response. The milk is known to contain a wide spectrum of BLG concentrations and this would certainly have an effect on the DTH response.

Comments

The results obtained from this investigation show that normal mice fed sheep BLG from the milk of transgenic females were not tolerant to this antigen on subsequent systemic challenge, neither were they primed, as observed by Strobel and Ferguson, (1984).

In those animals born of transgenic mothers, and possibly possessing a copy of the gene, there was a wide variation in the antibody response regardless of whether or not these animals had been previously fed BLG; suggesting that possession of the sheep gene may alter the outcome of the antibody response.

Under certain conditions, the foetus and the newborn are believed to express milk (Witch's milk). It is therefore possible that those mice carrying the sheep BLG gene expressed sheep BLG while in the uterus; thus influencing the humoral and DTH responses in later life. In this respect serum collected from nonimmunised, transgenic females, after expression of the gene product, did not contain detectable IgG anti-BLG antibodies (data not shown).

The transgenic mouse colony was outbred, while all other oral tolerance experiments have been carried out in inbred strains. This alone may explain the wide variation in results.

The transgenic mouse was initially expected to be a good, practical model of studying tolerance induction to BLG in neonates. However, these preliminary experiments have shown

that there are many considerations that have to be taken into account.

In summary, the results from this chapter show that feeding 50mgs of BLG to adult BDF_1 mice 7 days before immunisation suppresses both the humoral and DTH responses to that antigen. However, the DTH response in positive controls fed saline and immunised with BLG, was low which may effect the interpretation of oral tolerance to this antigen.

Feeding the same dose to aged BDF_1 mice did not induce oral tolerance.

The transgenic mice proved to be a useful model of antigen delivery to neonatal mice, and the feeding of normal mice on transgenic lactating females, did not produce systemic hyporesponsiveness to BLG when challenged in adult life. However, the initial evidence gained from this study, suggests that possession of the sheep BLG gene, and the possible expression of the gene product, influences the systemic antibody response to BLG.

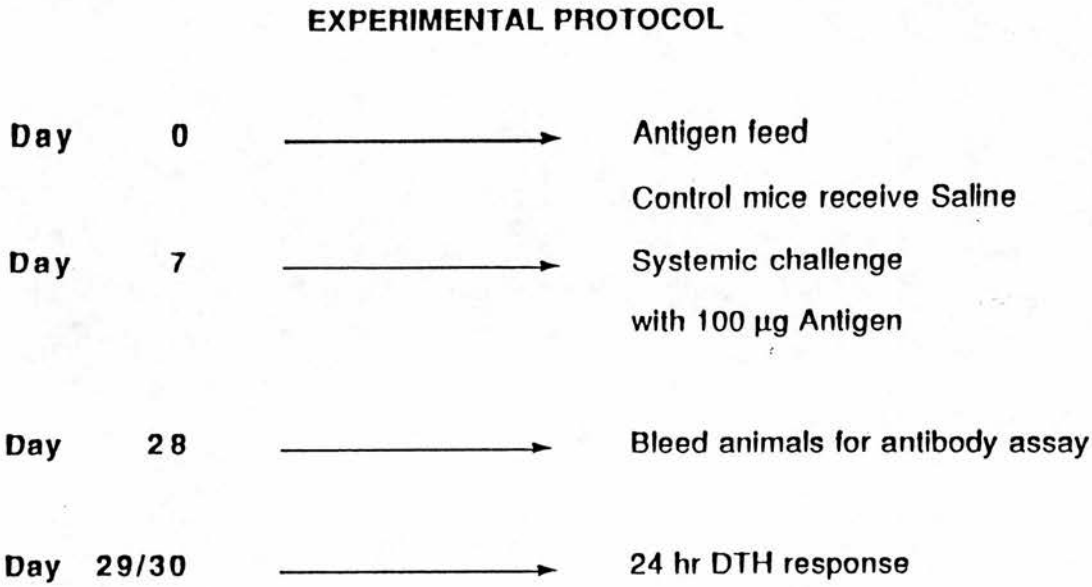


FIGURE 10:1 Experimental protocol of oral tolerance induction in mice.

Group FED / IMM	IgG anti-OVA OD 405 nm	DTH Response 24hr footpad increment (mm)
SAL / OVA	0.860 ± 0.05	0.26 ± 0.05
OVA/ OVA	0.560 ± 0.09*	0.04 ± 0.02* (85%)

* P<0.01

TABLE 10:1 Oral tolerance to OVA in adult BDF1 mice. The figures represent the mean±SEM of both the IgG antibody and DTH responses after prior feeding of 25mgs of OVA.

Group FED/IMM	IgG anti-BLG OD 405 nm	DTH Response 24hr footpad increment (mm)
SAL / BLG	1.167 ± 0.05	0.06 ± 0.02
1 mg BLG / BLG	1.146 ± 0.02	0.14 ± 0.02*
10 mg BLG / BLG	1.070 ± 0.05	0.08 ± 0.01
25 mg BLG / BLG	0.906 ± 0.03	0.07 ± 0.01
50 mg BLG / BLG	0.763 ± 0.06*	-0.02 ± 0.007** (133%)

* P<0.05
** P<0.005

TABLE 10:2 Oral tolerance to BLG in adult BDF1 mice. The figures represent the mean±SEM of both the IgG antibody and DTH responses after prior feeding of various doses of BLG.

Group FED/IMM	IgG anti-BLG OD 405 nm	DTH Response 24hr footpad increment (mm)
SAL / BLG	0.810 ± 0.04	0.13 ± 0.01
10 µg BLG / BLG	0.890 ± 0.03	0.08 ± 0.01* (38%)
50 µg BLG / BLG	0.814 ± 0.03	0.10 ± 0.02
100 µg BLG / BLG	0.900 ± 0.05	0.12 ± 0.03
250 µg BLG / BLG	0.949 ± 0.04	0.13 ± 0.02

* $P < 0.05$

TABLE 10:3 Oral tolerance to BLG in adult BDF1 mice. The figures represent the mean±SEM of both the IgG antibody and DTH responses after prior feeding of various doses of BLG.

Group FED/IMM	IgG anti-BLG OD 405 nm	DTH Response 24hr footpad increment (mm)
SAL / BLG	1.016 ± 0.05	0.05 ± 0.007
500 µg BLG / BLG	0.950 ± 0.05	0.06 ± 0.01
1 mg BLG / BLG	0.999 ± 0.05	0.07 ± 0.02
25 mg BLG / BLG	0.703 ± 0.08*	0.06 ± 0.02
50 mg BLG / BLG	0.527 ± 0.05*	-0.02 ± 0.007** (140%)

* P <0.01
** P <0.001

TABLE 10:4 Oral tolerance to BLG in adult BDF1 mice
The figures represent the mean±SEM of both the IgG antibody and DTH responses after prior feeding of various doses of BLG.

Group FED/IMM	IgG anti-BLG OD 405 nm	DTH Response 24hr footpad increment (mm)
SAL / BLG	0.822 ± 0.03	0.07 ± 0.01
1 µg BLG / BLG	0.766 ± 0.08	0.06 ± 0.02
5 µg BLG / BLG	0.901 ± 0.04	0.06 ± 0.01
10 µg BLG / BLG	0.748 ± 0.11	0.08 ± 0.02
75 mgs BLG / BLG	0.342 ± 0.05*	0.07 ± 0.01

* $P<0.01$

TABLE 10:5 Oral tolerance to BLG in adult BDF1 mice. The figures represent the mean±SEM of both the IgG antibody and DTH responses after prior feeding of various doses of BLG.

Group FED / IMM	IgG anti-BLG OD 405 nm	IgG anti-OVA OD 405 nm	DTH Response to BLG (mm)	DTH Response to OVA (mm)
A: Sal / BLG	0.350 ± 0.08	negative	0.08 ± 0.01	ND
B: BLG/BLG	0.120 ± 0.04*	negative	0 ± 0.01**(100%)	ND
C: Sal/OVA	negative	0.710 ± 0.10	ND	0.20 ± 0.03
D: BLG/OVA	negative	0.720 ± 0.05	ND	0.23 ± 0.02

* P<0.01

** P<0.001

ND Not Done

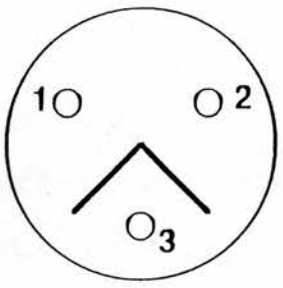
TABLE 10:6 Antigen specificity of oral tolerance induction to 50mgs of BLG.
The figures represent the mean±SEM of both the humoral and DTH responses.

	Group FED/IMM	IgG anti-BLG OD 405 nm	DTH Response 24hr footpad increment (mm)
A	6-8 wk old SAL/BLG	1.198 ± 0.03	0.08 ± 0.01
B	6-8 wk old BLG/BLG	0.249 ± 0.07*	-0.01 ± 0.01** (112%)
C	18 mth old SAL/BLG	1.205 ± 0.03	0.20 ± 0.04*
D	18 mth old BLG/BLG	1.087 ± 0.08	0.25 ± 0.03

* $P<0.05$
** $P<0.01$

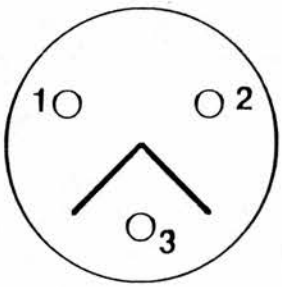
TABLE 10:7 Oral tolerance to BLG in aged BDF1 mice. The figures represent the mean±SEM of both the IgG antibody and DTH responses after prior feeding of 50mgs of BLG.

Plate 1



Wells 1, 2	**Normal mouse serum**
Well 3	**Rabbit anti-mouse immunoglobulin**

Plate 2



Well 1	**Ovine BLG**
Well 2	**Bovine BLG**
Well 3	**Mouse serum containing IgG anti-bovine BLG antibodies**

FIGURE 10:2 Diagrammatic representation of the cross-reactivity between ovine and bovine BLG.
Plate 1 represents the control plate, and plate 2 depicts the complete identity between ovine and bovine BLG.

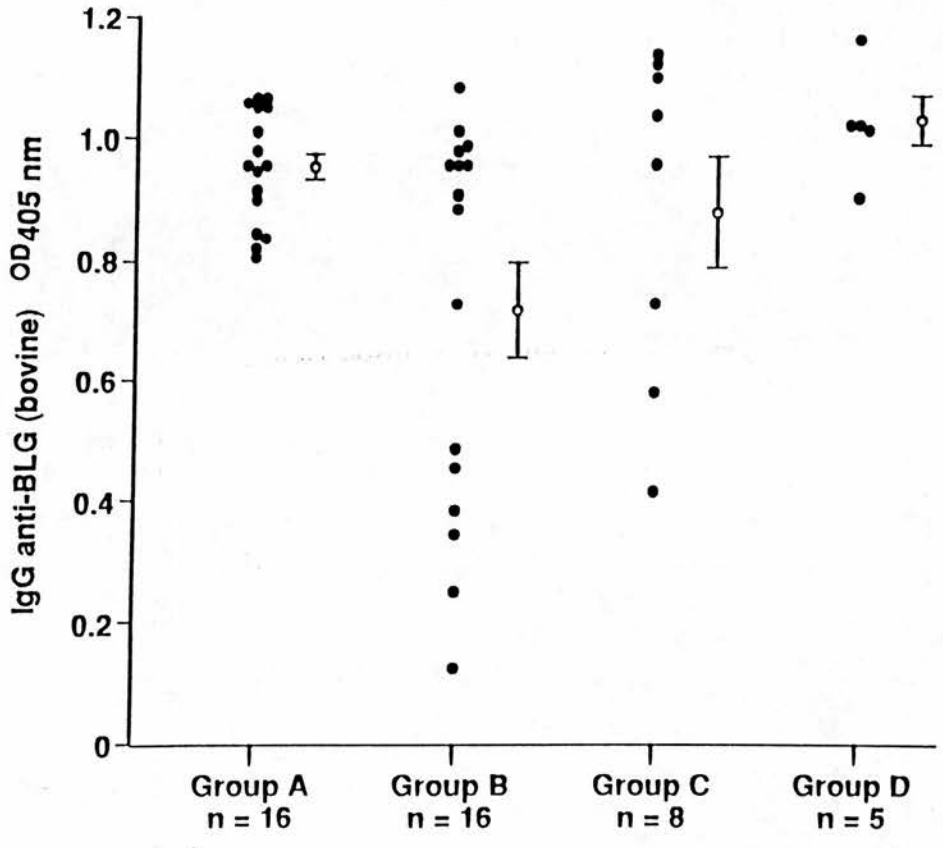


FIGURE 10:3 Serum IgG antibodies to Bovine BLG in transgenic mice.
The mean±SEM is given for each group. The description of each group is given in the text.

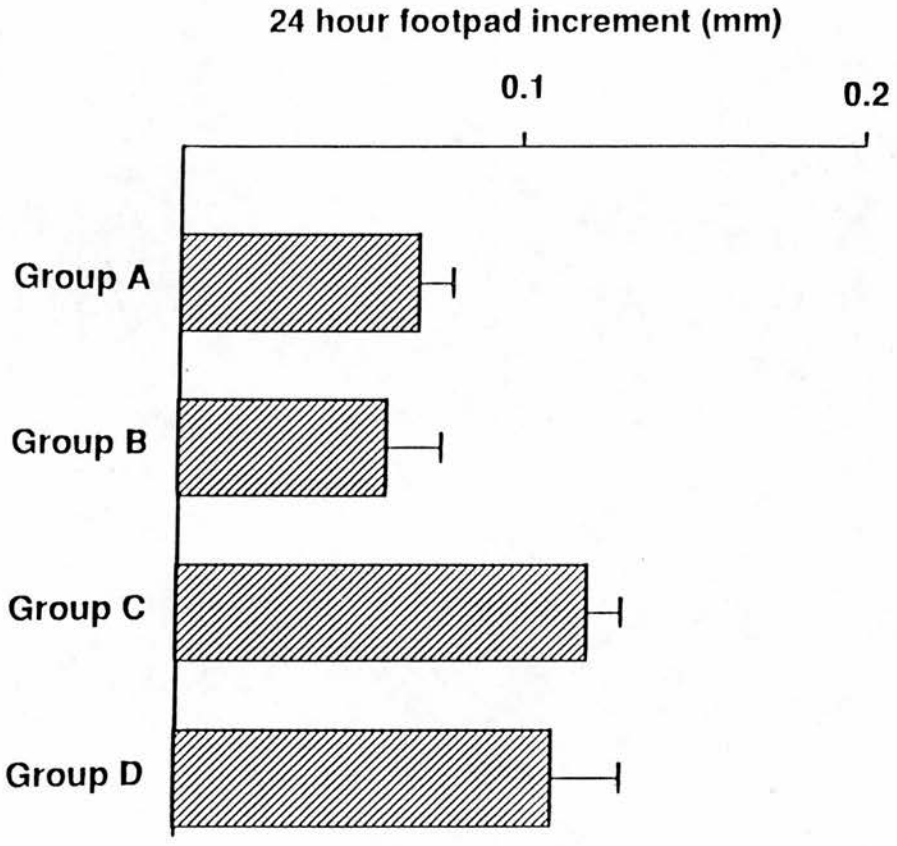


FIGURE 10:4 The DTH response to Bovine BLG in transgenic mice.
Figures represent the mean±SEM of each group. The description of each group is in the text.

CHAPTER ELEVEN

THE SYSTEMIC AND MUCOSAL IMMUNE RESPONSES TO ENTERICALLY PRESENTED ANTIGENS; A STUDY OF THE RELATIONSHIP BETWEEN ORAL TOLERANCE AND MUCOSAL IMMUNITY

INTRODUCTION

The presentation of antigen via the gut is generally considered to induce systemic hyporesponsiveness; thus protecting the body from the potentially harmful effects of a systemic immune reaction. There are conflicting reports, however, as to whether systemic tolerance is accompanied by a mucosal antibody response or not (chapter 1). While Challacombe and Tomasi (1980), have demonstrated a salivary IgA response occurring simultaneously with oral tolerance, Elson and co-workers (1984ab) have shown, using the potent immunogen CT together with KLH, that the intestinal IgA response and systemic tolerance are reciprocally linked and do not occur simultaneously. Thus the abrogation of oral tolerance to KLH, by feeding with CT, was accompanied by a mucosal IgA anti-KLH response, detectable in the gut lumen. Other studies of oral tolerance abrogation, such as by depletion of suppressor T cells, have demonstrated an antigen-specific active DTH response in the intestinal mucosa after feeding of OVA (Mowat and Ferguson, 1981b; Mowat, 1986), however humoral immunity was not assessed. Previous work from our laboratory has shown that feeding OVA during the initial phase of a GvHR prevents oral tolerance induction and enhances antigen presentation (Strobel, Mowat and Ferguson, 1985). In addition, the findings of this work, i.e. the immunostimulatory effects of the GvHR on immunoglobulin production, both in the mucosa (chapter 6) and in the circulation (chapter 7) and the evolving DTH response in the mucosa (chapter 4),

suggest that the GvHR may be a possible model for studying the relationship between mucosal IgA production and oral tolerance after feeding a dietary antigen.

The aim of the investigations described in this chapter was to assess the mucosal IgA antibody response to dietary antigens (OVA and BLG) fed during a GvHR, and to determine if the immunostimulatory nature of the GvHR causes an immunopotentiation of the IgA antibody response in a similar manner as that described with CT. Further to this, the co-existence of systemic hyporesponsiveness and mucosal immunity was investigated.

THE MUCOSAL IGA RESPONSE TO DIETARY ANTIGENS FED DURING A GVHR

The mucosal IgA antibody response to OVA and BLG was studied in gut washings collected from mice fed antigen at various times throughout the GvHR, and comparisons were made to the IgA antibody response when CT was fed with the antigen and when antigen was fed alone.

General protocol

The basic experimental design of the investigation was as follows:-

Three groups of adult BDF_1 mice (n=8-10) were studied. The details of the timings of antigen feeds are given with the descriptions of each experiment.

Group A: A semi-allogeneic GvHR was induced in nonirradiated BDF_1 mice (chapter 3) on day 0 of the investigation, and the animals were fed OVA or BLG at a dose known to induce tolerance.

Group B: Adult, normal BDF_1 mice were fed antigen (OVA or BLG) concomitant with an immunogenic dose of CT (10ug) (Elson and Ealding, 1984ab; Lycke and Holmgren, 1986). This group acted as the positive control as CT is known to have an immunopotentiating effect on the mucosal IgA antibody response, both to itself and unrelated antigens fed at the same time (chapter 1).

Group C: Adult, normal BDF_1 mice were fed antigen (OVA or BLG) alone.

Gut washings were taken (chapter 3) 7 days after the last feed of antigen. Total IgA concentrations and IgA antibodies to OVA and BLG were determined by ELISA (chapter 3; appendix A:2 and A:6).

Presentation of results for the levels of IgA antibodies to OVA and BLG in gut washings.

For each experiment the OD 405nm values (mean±SEM) for IgA antibodies to either OVA or BLG are given. In addition, the Units of IgA antibodies/ug total IgA are also submitted.

Determination of Units of IgA antibodies.

The standard curves for the OD 405nm values of IgA anti-OVA (Fig 11:1) and IgA anti-BLG (Fig 11:2) were produced by incubating doubling dilutions of serum, collected from mice immunised with either OVA or BLG (chapter 3), in the respective ELISAs.

Units were assigned to the dilutions of positive serum, as illustrated on the graphs, with the lowest OD 405nm value still on the linear part of the curve given a Unit value of 1. For IgA anti-OVA this was a 1:64 dilution and for IgA anti-BLG a 1:512 dilution. The Unit values were doubled as the concentration of positive serum doubled (see Figs 11:1 and 11:2).

Using a Casio fx-180P scientific calculator, each standard curve was assessed by linear regression analysis and the Unit value corresponding to the OD 405nm value of each sample read off the standard curve. The Units of IgA antibody were then expressed per ug total IgA, and the mean±SEM calculated for each group.

A: IgA anti-OVA antibody response in gut washings after 2 feeds of OVA.

Experimental design

On day 0, a GvHR was induced in 8-10 BDF_1 mice (Group A). On days 3 and 11 of the experiment (corresponding to days 3 and 11 of the GvHR) animals in Groups A and C received 25mgs OVA i.g., and mice in Group B received 25mgs of OVA

plus 10ug CT. Gut washings were collected on day 19.

Results

The results are given in Table 11:1.
There was no significant difference in total IgA concentrations (ng/ml) between the three groups.
IgA anti-OVA antibodies were detected in the gut washings collected from mice with GvHR (Group A) and in comparable levels to those found in mice which had been fed CT with OVA (Group B). No IgA anti-OVA antibodies were found in mice fed OVA alone (Group C).

B: IgA anti-OVA antibody response in gut washings after 4 feeds of OVA.

Experimental protocol

On day 0 a GvHR was induced in 8-10 BDF_1 mice (Group A). On days 6, 7, 12 and 13, Groups A and C received 25mgs OVA i.g. Animals in Group B received 25mgs OVA plus 10ug CT. Gut washings were collected on day 20.

Results

The results are given in Table 11:2.
The concentrations of total IgA (ng/ml), although lower than had been found in the previous experiment (Table 11:1), were not significantly different between groups.

The OD 405nm values of IgA anti-OVA in Groups A and B were higher than had been observed in the previous experiment (Table 11:1) reflecting the increase in antigen load.
IgA anti-OVA antibodies were detectable in mice fed OVA alone (Group C), and in levels within the same range as detected in the gut washings of Groups A and B.

Comments

The results showed that feeding 2 doses of OVA during the initial phase of the GvHR produced detectable levels of IgA anti-OVA antibodies in the gut washings, whereas feeding 2 doses of OVA to normal mice did not produce an IgA antibody response. The levels of antibodies detected in animals with GvHR were in the same range as those found in normal mice fed OVA plus CT. Further investigation, however, revealed that feeding 4 doses of OVA produced an IgA anti-OVA response in all three groups, which suggests that the induction of an IgA antibody response is dependent on the number of times the gut encounters the antigen. The enhanced antigen presentation that occurs during the initial phase of GvHR would therefore account for the IgA antibody response after only 2 doses of antigen.

C: IgA anti-BLG antibody response in gut washings after 3 feeds of BLG.

Experimental protocol

On day 0 a GvHR was induced in 8-10 BDF_1 mice (Group A). On

days 3, 11 and 19, animals in Groups A and C received 50mgs BLG i.g., and mice in Group B received 50mgs BLG plus 10ug CT. Gut washings were collected on day 26.

Results

The results are given in Table 11:3.
The concentrations of total IgA (ng/ml) were significantly increased in both Group A ($P<0.005$) and Group B ($P<0.05$) as compared to the controls (Group C), and there was no significant difference between Groups A and B.
All three groups had detectable levels of IgA anti-BLG antibodies in the gut washings. Statistical analysis of the OD 405nm values showed that there was no significant difference in antibody levels between groups. However, when the results were expressed as Units IgA anti-BLG/ug total IgA, the levels were significantly greater in animals fed CT plus BLG (Group B) as compared to both the GvHR group (Group A; $P<0.05$) and the controls (Group C; $P<0.05$). There was no significant difference between Groups A and C.

D: IgA anti-BLG antibody response in gut washings after 5 feeds of BLG.

Experimental protocol

On day 0 a GvHR was induced in 8-10 BDF_1 mice (Group A). On days 6, 7, 12, 13, and 24 the animals in Groups A and C received 50mgs BLG i.g., and animals in Group B received

50mgs BLG plus 10ug CT. Gut washings were collected on day 31.

At this point the increased concentrations of total IgA and IgM in gut washings collected on day 31 of the GvHR had been discovered (chapter 6) and so total IgM and IgM anti-BLG antibodies were analysed as well as IgA in this particular experiment.

Results

Total IgA and IgA anti-BLG antibody levels in gut washings.

There was no significant difference in total IgA concentrations (ng/ml) in the gut washings collected from Groups A, B and C (Table 11:4).

Mice fed 5 doses of BLG plus CT (Group B) had significantly higher levels of IgA anti-BLG antibodies than both Group A ($P<0.005$) and Group C ($P<0.005$). Interestingly, mice fed BLG alone (Group C) had significantly greater antibody levels ($P<0.05$) than mice fed during a GvHR (Group A).

Total IgM and IgM anti-BLG antibodies in gut washings after 5 feeds of BLG.

The concentrations (ng/ml) of total IgM (Table 11:5) were significantly increased in mice with GvHR (Group A; $P<0.05$) as compared to both Groups B and C.

IgM anti-BLG antibodies were not detectable in any of the groups.

Comments

An IgA anti-BLG response was observed in all three groups after feeding either 3 or 5 doses of BLG.

Feeding 5 doses of CT and BLG (Group B) gave a greater IgA anti-BLG response (OD 405nm) than after 3 feeds, which indicates that with increased feeds of CT the levels of antibodies to the unrelated antigen (BLG) are also increased. Similarly, feeding 5 doses of BLG alone produced a greater IgA antibody response (OD 405nm) than feeding 3 doses, although the response was not as great as that observed when CT was fed concomitantly. In contrast this effect was not observed in mice fed during a GvHR which suggests that antibody production at this phase of the GvHR may be depressed.

However, when the results were expressed as Units of IgA antibodies, the levels of IgA anti-BLG antibodies/ug total IgA were lower in both mice fed during a GvHR and fed alone (Groups A and C), when 5 doses of antigen were given (Table 11:4) as compared to 3 doses (Table 11:3).

An interesting observation was the complete absence of detectable IgM antibodies in all three groups, even in the mice with GvHR (Group A) which had significantly increased concentrations of total IgM.

In conclusion, the GvHR appeared to potentiate the IgA anti-OVA response after 2 feeds, however this effect was not found when mice were fed more than 2 doses of antigen.

The interesting finding of this investigation was the

production of small amounts of IgA antibodies to fed antigens in the gut washings of normal mice. Oral tolerance induction was therefore studied in these animals to determine if both a mucosal immune response and oral tolerance did co-exist. Oral tolerance experiments were also performed on mice fed antigen plus CT, as in this situation oral tolerance to the unrelated antigen is known to be abrogated.

ORAL TOLERANCE INDUCTION AFTER MULTIPLE FEEDS OF DIETARY ANTIGEN

Oral tolerance induction after 4 feeds of OVA

Experimental protocol

Three groups of adult BDF_1 mice were used (n=16). The animals were fed at intervals corresponding to those described in the studies of the mucosal IgA response. On days 0, 1, 6 and 7, Group A (controls) received 0.2mls of saline, Group B received 10ug CT plus 25mgs OVA and Group C received 25mgs OVA. 7 days after the last feed of antigen, 8 mice from each group were sacrificed, gut washings collected and analysed. The remaining 8 mice/group were immunised with 100ug OVA/CFA. 3 weeks post-immunisation serum was collected and the antibody and DTH responses assessed (chapter 10).

Results

IgA anti-OVA antibody response in gut washings

An IgA anti-OVA antibody response was detected in animals fed OVA alone or concomitantly with CT. The results were similar to those described in Table 11:2, and are thus not presented here.

Oral tolerance to OVA

The results are presented in Table 11:6.
Mice fed 4 doses of OVA alone (Group C) had both a significantly suppressed systemic IgG anti-OVA antibody response ($P<0.05$) and DTH response ($P<0.01$; 80% suppression) as compared to the saline fed controls.
Mice fed CT with OVA did not have a suppressed serum IgG anti-OVA antibody response although the DTH response was significantly lower than the saline fed controls ($P<0.01$; 55% suppression).

Oral tolerance induction after 5 feeds of BLG

Experimental protocol

Three groups of BDF_1 mice (n=16) were used. The groups were the same as described above. On days 0, 1, 6, 7 and 17, Group A were fed 0.2mls of saline, Group B received 50mgs BLG plus 10ug CT, and Group C received 50mgs BLG alone. 7 days after the last feed, 8 mice/group were sacrificed and

the gut washings analysed. The remaining 8 mice/group were immunised with 100ug BLG/CFA. 3 weeks post-immunisation, serum was collected and the antibody and DTH responses assessed (chapter 10).

Results

IgA anti-BLG antibodies in gut washings

IgA antibodies to BLG were found in Groups B and C in levels similar to those presented in Table 11:4.

Oral tolerance induction to BLG

Mice fed 5 doses of BLG alone (Group C) (Table 11:7) had significantly suppressed serum IgG anti-BLG antibodies ($P<0.05$) as compared to the saline fed controls (Group A), however the DTH response was not suppressed. As described in Chapter 10, the DTH response to BLG in positive control mice was lower than expected which may complicate the analyses of the data. Mice fed CT with the BLG (Group B) did not have a suppressed systemic response to BLG.

Comments

Feeding 4 doses of OVA alone produced the following response:-

i) a mucosal IgA anti-OVA antibody response

ii) systemic hyporesponsiveness.

In contrast, mice receiving 4 doses OVA plus CT had:-

i) a mucosal IgA anti-OVA antibody response

ii) no suppression of the systemic IgG anti-OVA response
iii) suppression of the DTH response.

Animals fed 5 doses of BLG alone produced:-
i) a small but detectable mucosal IgA anti-BLG antibody response
ii) a suppressed systemic IgG antibody response
iii) no suppression of the DTH response.

Feeding 5 doses of CT with the BLG:-
i) completely abrogated the induction of oral tolerance
ii) induced a high mucosal IgA anti-BLG antibody response.

General comments

The results presented in this chapter demonstrate that an IgA antibody response to enterically presented dietary antigens (OVA and BLG) can be detected in the gut lumen. The initial experiments showed that feeding 2 doses of OVA, on days 3 and 11 after the induction of GvHR, produced an IgA antibody response not found in animals without GvHR. However, when the number of doses given was increased to 4, an IgA anti-OVA antibody response was observed in mice without GvHR, and in both cases the results were comparable to those found when CT was fed concomitantly. These results suggest that repeated feeding of antigen is required to induce an IgA response, and that the enhanced antigen presentation that is known to occur during the initial phase of a GvHR, is responsible for the IgA response after only 2 doses of OVA.

Feeding CT with OVA or BLG produced an IgA antibody response to the dietary antigen and the OD 405nm readings of IgA anti-BLG increased with the number of feeds given. This effect was observed to a lesser extent when antigen was fed alone, but not at all when a GvHR was in progress. In fact, after 5 doses of BLG the Units of IgA anti-BLG/ug total IgA were lower in both normals and mice with GvHR than after 3 doses.

Expression of the IgA antibody results per total IgA may be a more accurate indicator of the proportion of antibodies to the total IgA produced, however this figure is very much dependent on other endogenous factors which may be stimulating IgA production at the time of the investigation. Similarly, technical aspects such as fluid secretion into the gut lumen and the volume of gut washings collected may influence the total IgA concentrations. Therefore, expressing the IgA antibody results as both OD values and Units of IgA anti-BLG/ug total IgA is valuable in describing the results.

The study of the systemic response to multiple feeds of OVA and BLG in normal animals revealed that oral tolerance and a mucosal IgA response co-existed after 4 feeds of OVA. However, when 5 feeds of BLG were given, an IgA anti-BLG response and a suppressed systemic IgG anti-BLG response were detected, but the DTH response was not suppressed.

Oral tolerance to 5 doses BLG was completely abrogated when CT was given as well, and a very high IgA anti-BLG response was observed, which suggests that when a high IgA antibody response is "switched on" oral tolerance is abrogated, but

small levels of "background" IgA antibodies can be produced simultaneously with systemic hyporesponsiveness.

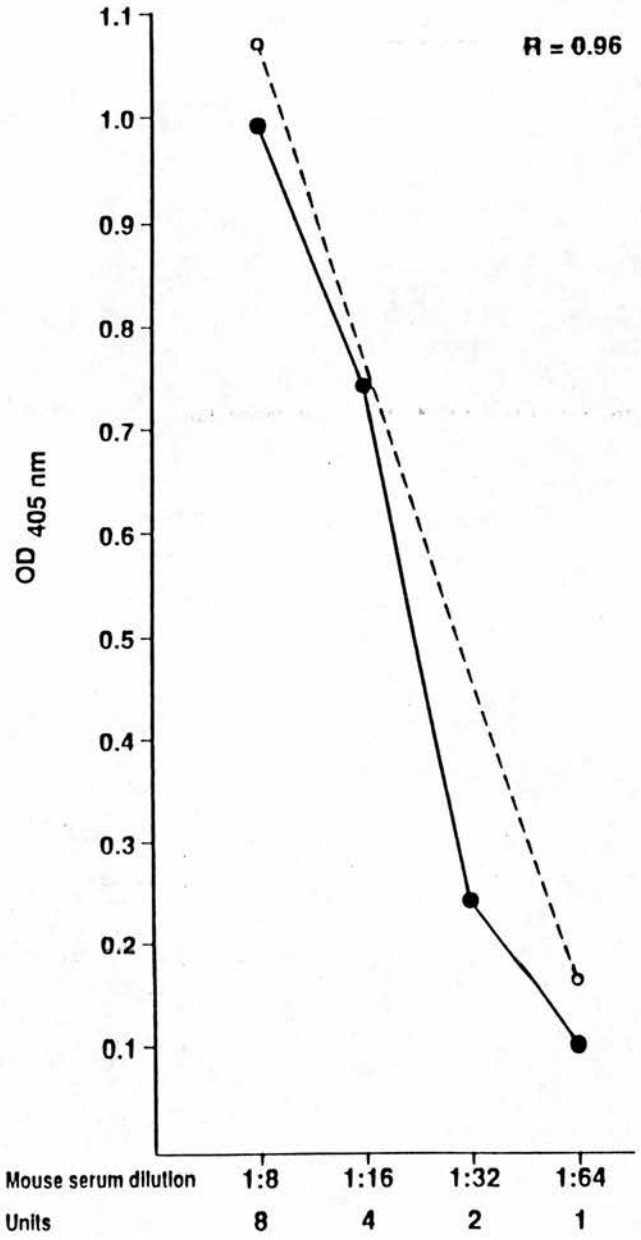


FIGURE 11:1 IgA anti-OVA antibody ELISA standard curve. Both the OD 405nm values (solid line) and the best fitting line (broken line), as determined by linear regression analysis, are shown.

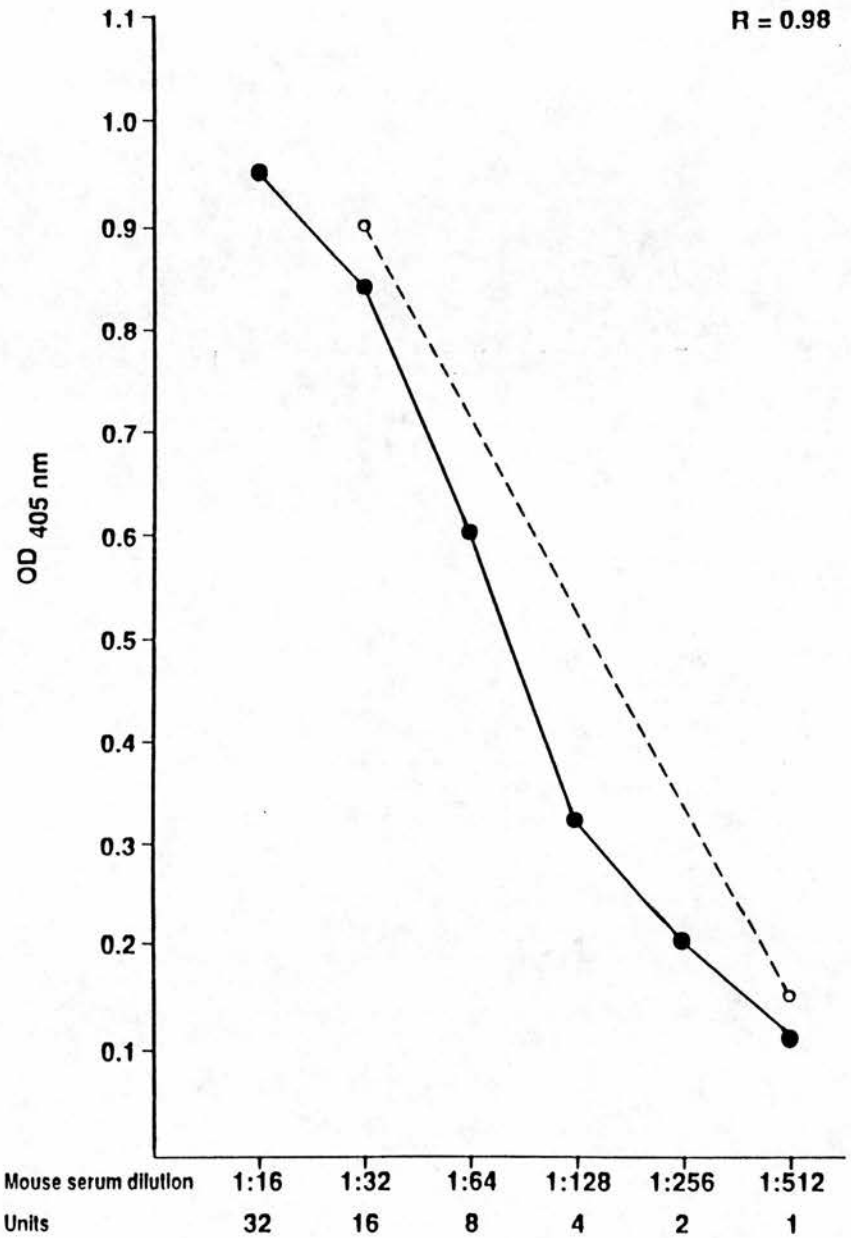


FIGURE 11:2 IgA anti-BLG antibody ELISA standard curve. Both the OD 405nm values (solid line) and the best fitting line (broken line), as determined by linear regression analysis, are shown.

Group	Total IgA ng/ml	IgA anti-OVA OD 405 nm	Units IgA anti OVA/ µg Total IgA
A	164.80 ± 12.22	0.251 ± 0.04	10.59 ± 2.49
B	248.77 ± 67.19	0.290 ± 0.03	10.45 ± 2.34
C	173.50 ± 38.99	ND	ND
Analysis of variance	NS	NS	NS

ND - Not Detectable

TABLE 11:1 Total IgA and IgA anti-OVA antibody responses in gut washings after 2 feeds of OVA.

Group A: mice fed OVA during a GvHR.
Group B: control mice fed CT and OVA.
Group C: control mice fed OVA alone.

Group	Total IgA ng/ml	IgA anti-OVA OD 405 nm	Units IgA anti OVA/ µg Total IgA
A	89.04 ± 7.99	0.497 ± 0.120	29.20 ± 3.59
B	99.11 ± 13.33	0.333 ± 0.196	33.26 ± 16.90
C	78.60 ± 7.32	0.360 ± 0.140	45.48 ± 17.99
Analysis of variance	NS	NS	NS

TABLE 11:2 Total IgA and IgA anti-OVA antibody responses in gut washings after 4 feeds of OVA.

Group A: mice fed OVA during a GvHR.
Group B: control mice fed CT and OVA.
Group C: contol mice fed OVA alone.

Group	Total IgA ng/ml	IgA anti-BLG OD 405 nm	Units IgA anti-BLG/ µg Total IgA
A	202.01 ± 47.50	0.168 ± 0.03	7.01 ± 2.48
B	143.50 ± 49.17	0.294 ± 0.09	30.64 ± 8.54
C	54.73 ± 6.27	0.105 ± 0.02	18.97 ± 4.34
Analysis of variance	P = 0.046	NS	P=0.046

TABLE 11:3 Total IgA and IgA anti-BLG antibody responses in gut washings after 3 feeds of BLG.

Group A: mice fed BLG during a GvHR.
Group B: control mice fed CT and BLG.
Group C: control mice fed BLG alone.

Group	Total IgA ng/ml	IgA anti-BLG OD 405 nm	Units IgA anti-BLG/ µg Total IgA
A	498.23 ± 115.27	0.165 ± 0.04	2.78 ± 0.50
B	515.22 ± 149.44	0.949 ± 0.09	47.60 ± 9.99
C	465.17 ± 127.66	0.305 ± 0.06	7.79 ± 2.04
Analysis of variance	NS	P = 0.0001	P=0.0001

TABLE 11:4 Total IgA and IgA anti-BLG antibody responses in gut washings after 5 feeds of BLG.

Group A: mice fed BLG during a GvHR.
Group B: control mice fed CT and BLG.
Group C: control mice fed BLG alone.

Group	Total IgM ng/ml	IgM anti-BLG OD 405 nm
A	32.81 ±10.58	ND
B	3.87 ± 0.76	ND
C	5.47 ± 1.40	ND
Analysis of variance	P = 0.005	

ND - Not Detectable

TABLE 11:5 Total IgM and IgM anti-BLG antibody responses in gut washings after 5 feeds of BLG.

Group A: mice fed BLG during a GvHR.
Group B: control mice fed CT and BLG.
Croup C: control mice fed BLG alone.

Group FED/IMM	IgG anti-OVA OD 405 nm	DTH Response 24hr footpad increment (mm)
SAL / OVA	0.710 ± 0.108	0.20 ± 0.03
10 µg CT / 25 mgs OVA / OVA	0.748 ± 0.08	0.09 ± 0.01** (55%)
25 mgs OVA/OVA	0.459 ± 0.07*	0.04 ± 0.003** (80%)

* P <0.05
** P <0.01

TABLE 11:6 Oral tolerance to OVA after 4 feeds of OVA. Systemic IgG anti-OVA antibody response (mean±SEM) and DTH response (mean±SEM) in mice fed OVA alone and in mice fed CT and OVA.

Group FED/IMM	IgG anti-BLG OD 405 nm	DTH Response 24hr footpad increment (mm)
SAL / BLG	1.337 ± 0.04	0.10 ± 0.02
10 µg CT / 50 mgs BLG / BLG	1.392 ± 0.07	0.06 ± 0.02
50 mgs BLG/BLG	0.858 ± 0.09*	0.09 ± 0.06

* $P < 0.05$

TABLE 11:7 Oral tolerance to BLG after 5 feeds of BLG. Systemic IgG anti-BLG antibody response (mean±SEM) and DTH response (mean±SEM) in mice fed BLG alone and in mice fed CT and BLG.

CHAPTER TWELVE

DISCUSSION

INTRODUCTION

The semi-allogeneic GvHR is known to produce an immunostimulation of the immune response, and alloactivation of T cells is a recognised feature. A major site affected by the presence of activated T DTH effector cells is the gut. The murine GvHR is thus an excellent model for the study of mucosal immune responses both of the humoral and cell mediated type.

The aim of this thesis was to define the effects of the GvHR on the architecture of the small intestine and the mucosal humoral response.

The progression of both systemic and intestinal reactions was studied, and subsequent to developing a method for collecting gut washings, a definitive study of mucosal immunoglobulin concentrations was performed in adult, neonatal and aged mice with GvHR.

Striking effects on the levels of IgA and IgM were found and in order to investigate possible mechanisms to explain this feature, studies on the dissemination of the response to distant mucosal sites were performed. In addition, the effect of the GvHR on Ig-SC numbers in the SP, MLN and PP was analysed.

Finally, oral tolerance to BLG, both in normal mice and in a transgenic mouse model was examined and the possible relationship between mucosal immunity and systemic hyporesponsiveness investigated.

The results obtained were discussed in each separate chapter, and in this discussion I shall bring together

these results in order to delineate the possible mechanisms underlying the effects of the GvHR on the mucosal immune response.

THE ONSET AND PROGRESSION OF A SEMI-ALLOGENEIC GVHR IN NONIRRADIATED F1 MICE

In most animal models of GvHR the onset of the reaction is recognised by a proliferation of cells in the organised lymphoid tissue, particularly the SP; splenomegaly being a measure of the progression of the reaction (Simonsen, 1962). In the semi-allogeneic GvHR in nonirradiated F_1 hosts, donor cell proliferation is not necessarily required, however upon stimulation by alloantigens, nondividing donor cells are believed to release soluble factors that induce host cells to proliferate (Scollay, Hofman and Globerson, 1974).

The results presented in chapter 9 demonstrate that the induction of a GvHR in BDF_1 mice produced an increase in the total cell count of PP, MLN and SP on day 7 of the GvHR, indicating cell proliferation and the onset of the reaction. Similarly, a significant infiltration of IEL into the small intestinal epithelium was found by day 7 of the GvHR (chapter 4) indicating that the intestinal reaction was underway.

The progression of the systemic GvHR was monitored by the spleen index. Gleichmann *et al* (1984), delineated two phases which occur during the acute semi-allogeneic GvHR in

nonirradiated mice. Initially there is a brief phase of alloactivation of T helper cells (lasting approximately 14 days), resulting in DTH reactivity and T cell-dependent B cell stimulation. This is followed by alloactivation of T suppressor/cytotoxic cells and reduced activity. In this particular model of GvHR, a chronic immunostimulatory form may follow. A consistent finding of my work was an elevated SI between days 7 and 11 (chapter 4), followed by a phase of low SI. However, on day 32 of the reaction a striking increase in SI was observed. This increase was reflected in total cell counts in the SP, but not in the mucosal-associated lymph nodes.

These investigations confirm that the induction of a semi-allogeneic GvHR in BDF_1 mice, induces an increase in cell numbers in the PP, MLN and SP during the initial immunostimulatory phase of the acute reaction. A second increase in cell numbers occurs on day 32, in the SP only, and I suggest that this reflects the onset of the chronic form of the GvHR. Interestingly, 18 months after the induction of the GvHR (chapter 8) the SI was still elevated.

THE INTESTINAL GVHR

Previous investigations have described the effects of intestinal GvHR, both at single time points and throughout the reaction, using a variety of animal strain combinations, and within a wide range of ages. Effects on the structure of the gut have been noted in irradiated

hosts (Piguet, 1985; Mowat *et al*, 1988), nonirradiated, neonatal mice (MacDonald and Ferguson, 1977; Mowat and Ferguson, 1982; Felstein and Mowat, 1988) and nonirradiated, adult mice (Mowat *et al*, 1988; Mowat, 1989). From these reports two distinct forms of enteropathy, a proliferative and a destructive form, have been described. The investigations described in chapter 4 show that while both recognised forms of enteropathy can be determined in a semi-allogeneic GvHR in nonirradiated adult BDF_1 mice, the reaction actually manifests in an evolution of architectural alterations.

The mice remained healthy throughout the time of study, up to 5 weeks post-GvHR induction, with no significant weight loss and no deaths. The absence of any clinical signs of GvHR, especially during the phase of a destructive enteropathy (between days 19 and 27) is in contrast to the significant weight loss, ruffled fur, abnormal gait and hunched posture and mortality observed by other workers (Mowat, 1989; Mowat and Felstein, 1989).

An important factor in the severity of the systemic GvHR is the cleanliness of the animal house in which the experiments are performed, as bacterial products can influence the level of systemic anti-host immune responsiveness, without affecting the gut (chapter 2). The BDF_1 mice I used were an inbred strain, bred in the Animal Unit at the Western General Hospital, Edinburgh. The male parents (DBA/2) were obtained from a stock colony, while the female parents (C57Bl/6J) were obtained from commercial

animal breeders (chapter 3). The BDF_1 mice used in each experiment were therefore born and raised in the environs of the Animal Unit. In contrast, the BDF_1 mice used by Mowat and co-workers (Mowat, 1989; Mowat and Felstein, 1989) were purchased from commercial breeders and brought into a new environment. The GvHR was thus studied in animals which were possibly reacting to novel bacterial antigens; which may explain the exaggerated systemic manifestations.

The intestinal GvHR was assessed by measuring villus and crypt lengths and IEL counts; parameters known to signal a local DTH reaction (Mowat and Ferguson, 1982). At least 7 phases of change to both the mucosal architecture and cellular infiltration were determined, and assessed in conjunction with the progression of the systemic reaction (Fig 4:12).

The infiltration of increased numbers of IEL into the epithelium during the initial phase of the GvHR, is a well recognised feature of the proliferative phase of the intestinal reaction (Mowat and Ferguson, 1982; Mowat and Felstein, 1989).

A significant reduction in the numbers of IEL/100 epithelial cells was subsequently observed between days 19 and 24. A decreased IEL count has been previously described, but only in irradiated hosts (Mowat *et al*, 1988). In both animal models (i.e. nonirradiated and irradiated hosts), the decreased IEL count coincided with a period of significantly reduced villus length. Studies are

now in progress in our laboratory, to determine, by image-analysis, the relationship between the fall in IEL counts and the absolute numbers of IEL, and the relationship between the fall in IEL counts and reduction in villus epithelial cell populations, in terms of time.

A spectrum of alterations to the mucosal architecture was observed throughout the GvHR.

During the initial phase of the reaction, the prominent feature was crypt hyperplasia. A reduced villus length was observed on day 11 of the GvHR, however, the villi maintained their long, finger-like structure.

Between days 19 and 27 of the GvHR, a phase of intestinal damage occurred which resulted in flattening of the villi. At the onset of this villus atrophy, the crypts were of normal length, however, by day 24 the crypts were hyperplastic. By day 27, a severe destruction of the mucosal structure was observed and both the villi and crypts were atrophic.

Villus atrophy and crypt hyperplasia are recognised features of the destructive-type lesion observed in neonatal mice with GvHR (MacDonald and Ferguson, 1977; Felstein and Mowat, 1988), and in irradiated mice with GvHR (Mowat *et al*, 1988). Similarly, villus atrophy and crypt hypoplasia have been described in irradiated mice with GvHR (Mowat *et al*, 1988), and in a protein-deprived murine model (Lamont, 1986).

Under normal conditions, a steady state is maintained within the villus/crypt unit. The enterocytes move up the

villi in continuous sheets from their origin in the crypts, where a self-renewing population of stem cells balances the loss of enterocytes from the villus tip. Consequently, any effect on the crypt cell kinetics will eventually manifest in alterations to the villus length and to the maturity of the enterocyte.
The GvHR is believed to directly affect the cell kinetics within the crypt (reviewed Mowat and Felstein, 1989).

The results presented here emphasize, that in any form of intestinal damage, it is important that the changes to the villus and crypt lengths are not considered as separate, singular events, but rather as a spectrum or evolution of alterations resulting from changes in the dynamic equilibrium of the complete villus/crypt unit.

The mechanisms underlying the intestinal GvHR are not yet fully understood. However, as reviewed in chapter 2, the evidence so far suggests that both the proliferative and the destructive forms of enteropathy are the result of a "bystander phenomenon" which is class II MHC-restricted, and most probably the result of a local DTH response rather than classical CTL activity. In addition, there is gathering evidence to substantiate the original hypothesis of Ferguson and co-workers that enteropathic lymphokines play a major role in the intestinal GvHR (Ferguson and Parrott, 1973; MacDonald and Ferguson, 1976; 1977).

Mucosal lymphocytes isolated from mice with acute GvHR are able to produce IFN-*gamma*, IL-2 and IL-3 (Guy-Grand and

Vassalli, 1986) and IFN-*gamma* and IFN-*beta* have been demonstrated, by immunofluorescence, in the spleens of irradiated mice with GvHR (Cleveland, Annable and Klimpel, 1988). Similarly, increased levels of serum TNF have been demonstrated in mice with acute GvHR (Bayston, Faulkner and Cohen, 1988).

More importantly, treatment of mice with antibodies against IFN-*gamma* prevents the proliferative intestinal manifestations in nonirradiated hosts (Mowat, 1989), while treatment with antibodies to TNF-*alpha* prevents the destructive lesion found in irradiated hosts (Piguet *et al*, 1987).

The role of IFN-*gamma* in the intestinal GvHR is pertinent when one considers the effects of this cytokine on cells involved in the inflammatory response. IFN-*gamma* has been shown to induce the migration of lymphocytes out of circulation, into the site of inflammation, during a DTH response (Isselkutz *et al*, 1988). In addition, IFN-*gamma* is believed to be a potent inducer of both class II (Ia) expression on gut epithelial cells (Cerf-Bensussan *et al*, 1984; MacDonald *et al*, 1988) and macrophage activation (Schultz and Kleinschmidt, 1983); all of which are features of the intestinal GvHR (Bland, 1988; Barclay and Mason, 1982).

Continuous treatment of mice with anti-IFN-*gamma* during a GvHR prevents the increase in IEL count normally observed at the onset of the intestinal reaction (Mowat, 1989). If the infiltration of IEL into the gut epithelium is a direct result of locally released IFN-*gamma* then it is possible

that the decrease in IEL counts observed on day 13 is indicative of a cessation of IFN-*gamma* release. This coincides with the entry of the systemic GvHR into a phase of reduced activity (Gleichmann *et al*, 1984). In consequence, it is possible that the destructive-type lesion observed between days 19 and 24, is not a direct result of IFN-*gamma* release, but perhaps a secondary effect due to the IFN-*gamma*-induced activation of macrophages, and the enhanced class II (Ia) expression on the enterocytes.

Mowat (1989), did show that continuous treatment of mice with anti-IFN-*gamma* prevented the destructive enteropathy, however, the anti-IFN-*gamma* was administered from 1 day prior to the induction of the GvHR, until the end of the study. The results therefore demonstrate that IFN-*gamma* is involved in the manifestation of the destructive lesion, however, they do not dissociate between the presence of IFN-*gamma* and the function of cells activated by IFN-*gamma*.

Piguet *et al* (1987), described the elimination of the destructive lesion in irradiated mice after the continuous injection of anti-TNF-*alpha*. TNF-*alpha* is produced mainly from activated macrophages, it is therefore interesting to consider the possibility that the destructive lesion, observed between days 19 and 24, is a result of the action of TNF-*alpha* released from macrophages, previously activated by IFN-*gamma*.

On day 27 of the GvHR, a rise in IEL count and an elevated SI was observed. The presence of villus atrophy and crypt atrophy at this point is very interesting. Lee *et al* (1984), described a synergistic effect of IFN-*gamma* and

human lymphotoxin on neoplastic cells, which resulted in an inhibition of cell proliferation. It is therefore possible that the combined release of IFN-*gamma* and TNF-*alpha* during the GvHR may result in the arrest of crypt cell proliferation, the end result being crypt atrophy and villus atrophy.

MUCOSAL IMMUNOGLOBULIN PRODUCTION DURING A GVHR

Having established a gut washing technique for the collection of gut luminal contents (chapter 5), I was able to define the effects of the GvHR on mucosal immunoglobulin concentrations (chapter 6).

A pattern of increased immunoglobulin production, similar to the pattern of systemic progression of the GvHR occurred, in that increased levels of IgA and IgM were found in gut washings taken during the initial immunostimulatory phase of the GvHR, and at the onset of the chronic GvHR. Of great interest was the striking isotype variation in duration and magnitude of the response. While total IgM levels were elevated from day 11 up to and including day 14 of the reaction, total IgA levels were raised on day 12 only. This effect was reflected in the plasma cell counts in the LP. IgG concentrations were not analysed in the gut washings as very few IgG^+ plasma cells were found in the LP of either normal or GvHR mice.

In the immunoregulatory pathway of mucosal B cells, there

are two stages at which T cells (or their soluble mediators) are believed to play a specific role. The first is at isotype-switching from $sIgM^+$ B cells to $sIgA^+$ B cells within the PP, and the second at terminal differentiation within the mucosal sites (chapter 1).

The finding of a transient increase in IgA accompanied by an increase in IgM lasting 4 days, initially suggested that the GvHR was affecting mucosal B cells early in their maturation pathway. The possibility existed that activation of non-specific T helper cells at the onset of GvHR was driving B cells, within the PP, into proliferation without a switch in surface isotype expression. The predominant isotype would therefore be $sIgM^+$. Migration of this population of $sIgM^+$ B cells into the LP would, upon receiving a signal inducing terminal differentiation, result in increased IgM levels, in gut washings, over a longer period of time.

The studies described in chapter 7 and 9 refute this hypothesis and, although no direct studies on cell migration were performed, oppose the original idea that migration of committed plasmablasts into the LP was occurring. Prior to entering the LP, a population of mucosa-seeking plasmablasts would be expected in the SP. Therefore, had the GvHR influenced the migration of plasmablasts into the LP, one would have expected a large number of cells to have migrated from the SP into the LP. However, as early as day 4 after the induction of GvHR, large numbers of IgM^+-SC were detectable in the SP (chapter 9), and yet no increase in IgM^+ plasma cells was observed

in the LP, until day 11. Similarly, a significant increase in SP IgG^+-SC numbers was observed on day 7, and at no time was there an increase in IgG^+ plasma cells in the LP. In addition, no increase in IgA concentrations were observed in either milk or saliva (chapter 7), which again suggests that there was no general migration of plasmablasts to distant mucosal sites. Hence, it is most likely that the GvHR exerts its effect at the later stage of terminal differentiation.

Support for GvHR-induced terminal differentiation of B cells was given by Lindholm *et al* (1973). The authors demonstrated that axillary LN, taken from nonirradiated mice with GvHR, contained large numbers of plasma cells in the medullary areas, a development not found in control mice.

The role of T helper cells in the terminal differentiation of $sIgA^+$ B cells into IgA synthesizing cells has been well described (Elson *et al*, 1979; Kawanishi, Saltzman and Strober, 1983b; Kawanishi and Strober, 1983). Systemically it is now well appreciated that B cell differentiation is regulated, in part, by lymphokines released from activated T cells (Kishimoto and Hirano, 1988). The lymphokines involved in this process are IL-4 (B cell activation), IL-5 (B cell proliferation) and IL-6 (B cell differentiation). These lymphokines are now being studied with regard to their influence on B cells within the GALT, however their roles in mucosal B cell differentiation are not yet completely understood.

The LP contains T cells predominantly of the helper phenotype and LP T cell help for Ig synthesis has been demonstrated (Elson *et al*, 1983). The majority of published work, however, on T cell regulation of IgA synthesis has been acquired from *in vitro* studies using PP derived cells, and although it is not entirely representative of the effector site of the LP, the results do give information on the effect of soluble mediators on mucosal B cells.

The information gained from this thesis suggests that during the GvHR, activated T helper cells present in the gut, induce LP plasmablasts to differentiate into Ig synthesizing plasma cells which release Ig into the gut lumen, and as with the local DTH response, it is possible that soluble mediators play a major role.

Several studies have shown that murine IL-5 can selectively enhance IgA synthesis *in vitro* (Murray *et al*, 1987; Bond *et al*, 1987; Kunimoto, Harriman and Strober, 1988; Harriman *et al*, 1988; Beagley *et al*, 1988; Maghazachi and Phillips-Quagliata, 1988; Matsumoto *et al*, 1989).
IL-5, produced by murine PP T cells, added to LPS-driven B cell cultures has been shown to enhance IgA production; an effect further enhanced by the addition of IL-4, although IL-4 itself, has no effect on IgA production (Murray *et al*, 1987). IL-5 is believed to affect PP B cells at the stage of terminal differentiation, and not isotype-switching (Harriman *et al*, 1988), although a role for IL-4 in isotype-switching has been suggested (Kunimoto *et al*, 1988).

A perhaps more pertinent study by Beagley *et al* (1988), has shown that rIL-5 can induce PP B cells to enter into high rate IgA synthesis with no requirement for exogenous LPS. Interestingly, the increase in IgA synthesis observed by Beagley *et al* (1988), was not accompanied by an increase in absolute numbers of IgA^+-SC which infers that the effect of rIL-5 is to increase IgA production by each plasma cell.
It is now recognised that IL-6 can induce a significant increase in IgA secretion in PP B cell cultures, to greater levels than observed with IL-5 (Beagley *et al*, 1989). As with IL-5, IL-6 exerts its influence on $sIgA^+$ B cells, and is thus not involved in isotype-switching. Similarly cell proliferation does not necessarily occur, thus an increase in IgA synthesis per plasma cell is observed (Beagley *et al*, 1989; McGhee *et al*, 1989).
The information on the role of lymphokines in IgM synthesis at the gut level is not as extensive as that of IgA, however, IL-5 has been shown to induce polyclonal IgM secretion from SP B cells in the absence of a stimulating antigen (Murakimi *et al*, 1988). Similarly IL-6 is able to induce terminal differentiation and high rate IgM synthesis from B lymphoblastoid cell lines (Kishimoto and Hirano, 1988).

The intriguing question is that if both IL-5 and IL-6 are equally capable of inducing terminal differentiation of both IgA^+ and IgM^+ B cells, why then was the increase in IgA levels in the gut, transient?
The interesting possibility is that IgA production or

secretion is in some way down-regulated. Aicher *et al* (1989), have recently demonstrated an IEL population in Balb/c mice which express both mRNA for IL-5, and an IgA Fc*alpha*R. Binding of IgA to this receptor resulted in the down-regulation of IL-5 production which in turn down-regulated the synthesis of IgA. In this respect, it is interesting to note that at the time of the transient production of IgA, during the GvHR, there were a large number of IEL present in the epithelium.

As discussed previously, the increased IEL infiltration observed at certain points during the GvHR, may indicate the release of IFN-*gamma*. At both points of increased IEL counts, i.e. during the acute immunostimulatory phase and at the onset of the chronic form, increased levels of immunoglobulins were observed. IFN-*gamma* has been shown to enhance the expression of secretory component in a human colonic adenocarcinoma cell line (HT-29) (Sollid *et al*, 1987; Kvale, Brandtzaeg and Lovhaug, 1988), which although not directly transposable to the situation of the murine GvHR, does offer the interesting possibility of a direct relationship between the effects of the DTH response and increased Ig levels in the gut lumen.

SYSTEMIC IMMUNOGLOBULIN PRODUCTION DURING A GVHR

One of the many effects of the systemic, immunostimulatory semi-allogeneic GvHR in nonirradiated F_1 mice is B cell hyperplasia and B cell activation; a phenomenon triggered

by the alloactivation of T helper cells (Gleichmann *et al*, 1984). Increased levels of host serum Igs have been described in F_1 mice during the initial phase of the acute reaction (Lindholm *et al*, 1973), and in BMT patients with acute GvHD (Ringden *et al*, 1987). However, the majority of research, on B cell responses during the GvHR, has concentrated on antibody and autoantibody formation in the chronic reaction (van Rappard van der Veen *et al*, 1984; Gleichmann *et al*, 1982; Gleichmann *et al*, 1984).
The results from chapters 7 and 8 demonstrate, in detail, the effects of both the acute and chronic forms of the semi-allogeneic GvHR on IgG, IgM and IgA levels in serum.

The initial brief phase of immunostimulation occurring during the acute reaction produced elevated levels of IgM and IgA. While IgA levels remained at a consistently high level until day 12, IgM concentrations were higher than controls from day 4 until day 11, with a second burst of production on days 12 and 13. In contrast, serum IgG levels did not become elevated until day 13. By day 24 of the GvHR, the levels of all three isotypes were similar to controls, or in the case of IgM, significantly lower than the controls. This drop in Ig concentrations coincided with a reduction in activity of the GvHR.
A significant rise in Ig levels was observed on day 32, but only in IgA and IgG concentrations; coinciding with the onset of the chronic GvHR. Indeed, when serum Ig were measured 18 months after the induction of the GvHR (chapter 8), when the chronic GvHR is well established, both IgG and

IgA levels were very much higher than age-matched controls, while IgM was significantly lower than controls.

In the initial days of a chronic GvHR in BDF_1 mice injected with DBA/2 parental spleen cells, an increase in SP IgM^+- and IgG^+-SC numbers has been described, and although the IgM^+-SC numbers returned to normal the IgG^+-SC numbers remained elevated (Van Rappard van der Veen *et al*, 1984). A similar pattern was observed in the initial, acute reaction studied in chapter 9.

The T cell-B cell collaborations occurring during the initial immunostimulatory phase of the systemic GvHR are believed to be between host B cells and alloactivated donor T cells (Gleichmann *et al*, 1984). Hence, activated B cells undergo polyclonal stimulation under the influence of alloreactive T helper cells and their soluble mediators. Terminal differentiation occurs and Igs are secreted.

The majority of circulating B cells express sIgM, and a population of activated B cells committed to IgG production would not be expected in nonimmunised mice; hence the initial increase in IgM. The elevated levels of circulating IgA suggest that pre-committed IgA^+ activated B cells, some of which may be mucosa-seeking, exist in the circulation.

The second burst of IgM production and the onset of elevated IgG levels occurred after the increase in total cell count in the SP (chapter 9). Previous studies have suggested that after the initial random, polyclonal stimulation of IgM, the subsequent increase in circulating Igs is not random, but due to the activation of

autoreactive B cells by "self-antigens" of a particular linear structure (van Rappard van der Veen *et al*, 1984; Gleichmann *et al*, 1984). Thus in the murine model of chronic GvHR, autoantibodies to double-stranded DNA and RBC are detectable. In this respect, what is interesting about the relationship between the increased production of IgM and IgG is its similarity to that found in the primary and secondary antibody responses.

At the onset of the chronic GvHR, and in the established chronic reaction, levels of IgG and IgA were elevated, however, IgM production was significantly depressed. Hypergammaglobulinaemia is a recognised feature of chronic GvHR, and is due to isotype-switching of B cells from IgM to IgG expression by the continuous presentation and binding of "self-antigens" (Gleichmann *et al*, 1984). The results from chapter 8 demonstrate that not only is there an isotype-switch from $sIgM^+$ to $sIgG^+$, but also to $sIgA^+$ expression. The switch from IgM to IgA occurs only systemically, as no such change was observed in the gut washings of these animals (chapter 8).

As already stated, the majority of published work describes the effect of the chronic stimulatory GvHR on antibody production. It would therefore be of great interest to determine the antigen specificities of the Igs produced, both systemically and in the mucosa, during the acute and chronic forms of this murine model of GvHR.

MUCOSAL AND SYSTEMIC IMMUNE RESPONSES TO ENTERICALLY PRESENTED ANTIGENS

The final investigation of this work was to study the mucosal humoral immune response to enterically presented antigens, and to determine the relationship between oral tolerance and mucosal immunity.

The initial investigations into the mucosal antibody response to OVA and BLG (chapter 11), demonstrated that unlike the dose-dependent immunopotentiating effect of feeding CT concomitantly with antigen, on the mucosal IgA anti-OVA and IgA anti-BLG responses, feeding antigen during the GvHR did not enhance IgA antibody production.

An interesting observation was that feeding 2 doses of OVA during the initial phase of the GvHR, produced an IgA anti-OVA response; an effect not observed in normal mice.

Enhanced presentation of OVA has been described in mice fed OVA during the initial phase of the GvHR (Strobel *et al*, 1985), which may account for the increased IgA antibody response. Indeed, when 3 or more doses of antigen were fed to normal mice, a small but detectable IgA antibody response was observed in the gut, which suggests that the amount of antigen encountered by the GALT is important in the IgA response.

However, when 5 doses of antigen were fed to mice on days 6, 7, 12, 13 and 24 of the GvHR, the IgA antibody response, as measured by OD values, was lower than that found in normal mice. This seemingly suppressed mucosal, humoral response is most probably not due to the numbers of feeds

encountered by the gut, but the stage in the GvHR when the antigen was administered.

Investigations into the humoral response to systemically presented antigen, have demonstrated that a potentiated antibody response occurs when the T-independent antigen SIII is administered immediately after the induction of the GvHR. However, when the antigen was given at a later stage of the GvHR (day 6), a suppression of the antibody response was observed (Byfield *et al*, 1973).
A similar finding was described by Treiber and Lapp (1976), in which systemic sensitization of mice with SRBC in CFA on days 13, 20 and 24 of the GvHR, produced no detectable humoral response, although a DTH response was stimulated.
Once again the effects of the GvHR fall into a biphasic pattern of immunostimulation followed by reduced activity. This interesting dichotomous effect on the humoral and DTH responses was suggested by Treiber and Lapp (1976), to indicate the possible existence of two distinct T helper cells and/or soluble mediators, and during the GvHR, the T helper cells involved in the humoral response were more easily suppressed than those involved in the DTH response.

The complicated effects of feeding and immunising mice during the GvHR, and the lack of immunopotentiation on the IgA response, demonstrated that the GvHR model was not a suitable model for studying the relationship between mucosal and systemic immunity, as had been hypothesized. This study was therefore confined to normal mice.

The finding of small, but detectable levels of IgA antibody in the gut washings simultaneous with oral tolerance, after intra-gastric presentation of antigen, reinforces the findings of Challacombe and Tomasi (1980). The existence of antigen-specific T helper cell activity for IgA, and antigen-specific T suppressor cell activity for IgG in the PP (Richman *et al*, 1981), or the possible existence of a contrasuppressor cell network (Green *et al*, 1981; Ernst *et al*, 1988) may be important in the co-existence of oral tolerance and mucosal immunity.

Repeated feeding of antigen did not enhance the mucosal IgA antibody response as observed when CT was fed concomitantly, which suggests that under normal conditions of dietary antigen presentation, the simultaneous suppression of the systemic response and isotype-specific help for the IgA response is under strict regulatory control. Once again, the interesting possibility exists that Fc*alpha*R^{+} IEL within the epithelium (Aicher *et al*, 1989) may play an important role in the immunoregulation of the mucosal IgA antibody response.

When 5 doses of BLG were fed simultaneously with CT, oral tolerance to BLG was completely abrogated and a very high IgA anti-BLG response detected in the gut. This is in keeping with the findings of Elson and Ealding (1984ab), however, it would appear from my results that CT up-regulates the production of IgA rather than "switches on" its production, as suggested by Elson and Ealding (1984b).

The mode of action of CT on the immunoregulatory network is

as yet not fully understood. *In vitro* studies have shown that CT can enhance the production of IL-1 from APC and the proliferation of both Con A-stimulated SP T cells and LPS-stimulated SP B cells, although this effect is dependent on the concentration of CT present, and the length of incubation (Lycke *et al*, 1989).

It is possible therefore, that, in direct contrast to other dietary antigens presented via the gut, CT is able to exert an immunostimulatory effect on the mucosal immune response by down-regulation of the suppressor network. Lange, Lindholm and Holmgren (1978), found that SP cells taken from donor mice injected intra-venously with CT 1-3 days earlier, gave a significantly stronger GvHR when injected into nonirradiated F_1 recipients. The authors suggested that parenterally administered CT inhibits a cell population in the SP which normally exerts a suppressive, regulatory influence on the developing GvHR.

CT enters the gut by binding to the monosialoganglioside GM_1 receptor present on the enterocyte, and subsequently interacts with the adenylate cyclase complex on the basal membrane (reviewed by Stephen and Pietrowski, 1981). Increased levels of cyclic adenosine monophosphate (cAMP) may thus affect cell populations within the LP or PP. Indeed, T suppressor/cytotoxic cells have been found to be more susceptible to inhibition by cAMP than T helper cells and B cells (Tsuro *et al*, 1982; Gilbert and Hoffman, 1985). Entry of CT, via the villus enterocyte, may thus inhibit the suggested down-regulatory influence of IEL on IgA synthesis. This interesting hypothesis requires

investigation.

In conclusion, a small mucosal IgA antibody response does co-exist with systemic hyporesponsiveness, although the IgA antibody response is under strict regulation. Feeding CT concomitantly with OVA or BLG abrogates oral tolerance and up-regulates the mucosal IgA antibody response to the unrelated antigen.

An important model for future studies of oral tolerance and mucosal immunity may be the transgenic mouse model described in chapter 10.

The investigations demonstrated that normal mice fostered onto transgenic females, which secrete sheep BLG in their milk, were not tolerant to BLG on subsequent systemic challenge.
Previous reports have shown that when OVA is fed to mice within the first week of life, systemic priming for both the humoral and CMI responses occurs. The initial priming of the humoral response can be partially suppressed if the mice are fed OVA 2 weeks after the initial dose, and completely suppressed if fed 4 weeks after the initial dose. Retolerization of the CMI response is not accomplished before 14 weeks after the initial encounter with OVA (Strobel, 1983; Strobel and Ferguson, 1984).

A more detailed analysis of the systemic response to BLG, fed during the neonatal period to normal mice, would be achieved if the neonates were fostered onto transgenic

females at various times throughout the preweaning period. The transgenic female would therefore be an excellent delivery system of native BLG, presented in the environment of maternal milk, to immunologically immature mice.
An important consideration however, which must be taken into account in future studies, is the concentration of sheep BLG present in the milk of lactating transgenic females; a range of BLG concentrations between 3mg/ml and 23mg/ml was described by Simons *et al*, (1987). Antigen concentration has been shown to be important to the immunological outcome after enteric presentation, as low doses of OVA do not induce oral tolerance but do induce an antigen-specific local DTH response (Lamont *et al*, 1989).

The study of oral tolerance in mice born of transgenic mothers, was complicated by the possible possession of a copy of the sheep BLG gene and expression of the gene product. With the correct genetic analysis of the offspring, it would be possible to determine the effects of possessing and expressing the gene on the eventual immune response of the animal. Indeed, the transgenic mouse model may prove important to the elucidation of the cellular basis of tolerance to "self antigens"; a phenomenon not yet fully understood.

CONCLUDING REMARKS

Throughout this discussion, I have concentrated on the possible role of lymphokines in the intestinal GvHR, both

with regard to the changes in mucosal architecture and immunoglobulin concentrations in the gut lumen.

Recently, it has been demonstrated that mouse $CD4^+$ T helper cell clones and cell lines can be divided into two subsets, designated T_{H1} and T_{H2}, based on their lymphokine profiles (Mosmann *et al*, 1986; Mosmann and Coffman, 1987).
T_{H1} cells produce IL-2, IFN-*gamma* and lymphotoxin in response to activation by foreign antigen, alloantigen or Con A. T_{H2} cells produce IL-4, IL-5 and possibly IL-6 in response to stimuli (Mosmann and Coffman, 1989).

Murine T_{H1} cells are capable of producing an antigen-specific and MHC-restricted DTH response (Cher and Mosmann, 1987), and although it is unclear if they are responsible for the initial activation signal of the DTH reaction, they are involved in the effector stage. The effector stage results in the recruitment and activation of monocytes and granulocytes that mediate the subsequent stages of the reaction. IFN-*gamma* has been shown to play a major role in the T_{H1}-mediated DTH reaction (Mosmann and Coffman, 1989).

Murine T_{H2} cells are generally agreed to be excellent helpers in both antigen specific and polyclonal B cell responses. However, unlike T_{H1} cells, T_{H2} cells are found as precursors that require priming before they can be induced to secrete their lymphokines, and consequently it is several days before T_{H2} cells develop their effector functions (Swain *et al*, 1988). Unlike resting B cells, large B cells do not require contact with the T_{H2} cells and

thus can proliferate and differentiate in response to the lymphokines alone (Mosmann and Coffman, 1989).
A study of $CD4^+$ T cells from mucosal sites has shown that a large number of the cells are IL-5-secreting cells and thus akin to T_{H2} cells.

During the immunostimulatory phase of the semi-allogeneic GvHR, it is possible that alloactivation of both T_{H1}- and T_{H2}-type cells occurs. The release of lymphokines such as IFN-*gamma*, may thus induce the infiltration of IEL into the epithelium and the proliferative form of enteropathy. The increase in total IgA and IgM concentrations in the gut lumen, and the increase in plasma cell counts, may indicate the effect of lymphokines such as IL-4, IL-5 and IL-6, released from activated T_{H2} cells.
The GvHR subsequently enters a period of reduced activity, during which T suppressor/cytotoxic cells are activated (Gleichmann *et al*, 1984). In the class II MHC-restricted semi-allogeneic GvHR, both the proliferative and destructive forms of intestinal GvHR can occur in the absence of CTL activity (Borland *et al*, 1983; Felstein and Mowat, 1988), and I suggest that the changes in the mucosal architecture observed during this phase are due to the IFN-*gamma*-induced activation of effector cells such as macrophages, and the subsequent release of cytostatic cytokines such as TNF-*alpha*.
As the GvHR enters the chronic form of T helper cell activation, it is possible that the combination of TNF-*alpha* and IFN-*gamma* released from activated T_{H1} cells

produces the villus and crypt atrophy.

The increased levels of total IgA and IgM observed at this time, without a concomitant increase in plasma cell counts, may reflect the activation of T_{H2} cells, or perhaps an IFN-*gamma*-induced increase in the expression of the secretory component on villus enterocytes.

Treiber and Lapp (1976), suggested that T helper cells, involved in the humoral response, were suppressed in the later stages of the GvHR. However, Gleichmann *et al* (1984), have suggested that T cell help is present for B cells during the systemic, chronic reaction. The inability of mice with GvHR to produce a humoral response to exogenous antigen, lies in the physical dissociation of the antigens seen by T cells and those seen by B cells. Hence, only autoreactive B cell clones are activated.

The concept of two T helper cell subsets, T_{H1} and T_{H2}, their lymphokine profiles, and their separate regulatory functions within the immune system, will not only influence the direction of research in experimental gut injury, but will clearly be relevant to future theories on the pathogenesis of diseases of the immune system.

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