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IMMUNOLOGICAL MECHANISMS OF
INTESTINAL GRAFT-VERSUS-HOST REACTIONS

© Michelle Victoria Felstein

A thesis submitted for the degree of Doctor of
Philosophy to the Faculty of Medicine at the
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SUMMARY

The work in this thesis developed three separate models of acute graft-versus-host reaction in irradiated adult and one day old neonatal (CBA x BALB/c) F_1 mice and (C57Bl/10 x DBA/2) F_1 mice given C57Bl/10 cells. All these models were associated with weight loss and mortality as well as an enteropathy characterised by villus atrophy. Furthermore, these different hosts consistently developed a very similar biphasic pattern of systemic and intestinal Gvhr. An initial proliferative phase was characterised by the development of splenomegaly which coincided with an increase in intraepithelial lymphocytes and crypt cell production rate, in the small intestine, as well as a transient increase in splenic NK cell activity. These early changes were similar to those described previously in unirradiated, adult (CBA x BALB/c) F_1 mice with Gvhr, but mice with acute Gvhr subsequently developed weight loss and villus atrophy, which were accompanied by specific CTL activity, features not observed in previous work on unirradiated (CBA x BALB/c) F_1 hosts.

The evolution of acute Gvhr was shown most clearly in irradiated (CBA x BALB/c) F_1 hosts where the proliferative disease rapidly transformed into a lethal disorder with severe villus atrophy and ultimately mucosal necrosis. This was also accompanied by a reduction in the CCPR and the number of IEL. The systemic and intestinal consequences of a Gvhr in neonatal (CBA x BALB/c) F_1 mice depended upon the age of the host, with one day old hosts developing an

acute Gvhr, whereas seven day old animals merely showed a proliferative disorder with no weight loss, CTL activity or villus atrophy. Five day old mice were intermediate hosts, where some animals developed a lethal Gvhr while others developed a proliferative form of the disease. However even in those mice which developed villus atrophy, no evidence of specific CTL was found. These differences were not influenced by the number of donor cells used but it was found that spleen cells from one day old mice were defective at inducing allospecific delayed-type hypersensitivity and no anti-host DTH response developed during a Gvhr in one day old mice. Furthermore, one day old mice appeared to have a T-cell defect as shown by an inability of their lymphoid cells to induce a local Gvhr. In contrast to these findings spleen cells from 7 day old mice induced similar levels of allospecific DTH and developed an anti-host DTH response during Gvhr which was similar to that found in adult mice of the same strain.

An acute destructive Gvhr also occurred in adult (C57Bl/10 x DBA/2) F_1 mice given B10 spleen cells, whereas BDF $_1$ mice given DBA/2 cells had very little evidence of systemic or intestinal Gvhr, apart from some splenomegaly. In parallel, specific CTL were only found in hosts given B10 spleen cells and these mice also had evidence of active immunosuppression. The more potent ability of B10 donor cells to induce Gvhr was consistent with the observation that B10 cells responded better in vitro to both adult and

neonatal BDF₁ stimulators. Both B10 and DBA/2 donor cells could induce an acute Gvhr in one day old BDF₁ mice, while 5 day old hosts showed a similar pattern to adult mice. These results suggest that DBA/2 cells are merely defective at inducing Gvhr and do not induce a qualitatively different disorder.

Villus atrophy was also found in a fourth model of Gvhr which occurred in adult athymic (CBA x BALB/c)F₁ mice given CBA donor cells. Compared with euthymic hosts of the same strain these mice developed more intense splenomegaly and crypt hyperplasia while only athymic hosts had villus atrophy, CTL activity and reduced NK activity. These findings suggested that one reason for the more severe Gvhr found in irradiated and neonatal (CBA x BALB/c)F₁ mice was an absence of host T-cells which normally regulate the severity of the Gvhr in intact hosts.

The immunopathogenesis of the different forms of intestinal Gvhr was investigated by examining the nature of the donor T-cells and the region of the MHC necessary for the induction of Gvhr. Both Lyt 2⁺ and L3T4⁺ T-cells were required for the development of full splenomegaly and intestinal alterations in unirradiated adult (CBA x BALB/c)F₁ mice with Gvhr. Similar results were found in irradiated (CBA x BALB/c)F₁ hosts, although Lyt 2⁻ cells alone were capable of inducing a lethal Gvhr in this case. In contrast, L3T4⁻ cells were able to induce an acute lethal Gvhr in one day old (CBA x BALB/c)F₁ hosts and

these mice also developed some splenomegaly, crypt lengthening and villus atrophy. Lyt 2⁻ cells produced no clinical Gvhr in these hosts and neither L3T4⁻ nor Lyt 2⁻ cells produced any systemic or intestinal changes in 5 day old F₁ hosts. An interesting and consistent observation was that compared with unseparated donor cells both L3T4⁻ and Lyt 2⁻ donor cell populations produced more enhancement of NK cell activity during a Gvhr.

Although these results using sub-populations of donor T-cells were inconclusive induction of a Gvhr across a Class II MHC incompatibility in (ATH x ATL)F₁ mice produced an acute Gvhr with weight loss, splenomegaly and crypt hyperplasia. These alterations developed in the absence of specific CTL and NK activity and the onset of clinical Gvhr occurred about a week later than normally found using a full major histocompatibility difference. No systemic disease was found in a Gvhr induced across an H-2D or H-2K Class I MHC incompatibility and although transient NK cell activation and crypt lengthening occurred across an H-2K disparity, this was minor and was not found during an H-2D disparate Gvhr.

NK cell activation was a consistent feature of most models of Gvhr and the role of NK cells in systemic and intestinal Gvhr was examined in adult (CBA x BALB/c)F₁ mice treated with anti-asialo G_{M1} antibody. This depleted host NK cell activity and also reduced the density of IEL in control animals. In addition, anti-asialo treated mice

with Gvhr had less splenomegaly than normal Gvhr mice and did not develop any intestinal alterations of Gvhr. These findings were complimented by experiments in which neonatal (CBA x BALB/c) F_1 mice were treated with polyinosinic: polycytidylic acid. Poly I:C augmented NK activity in these mice, and this was associated with more severe weight loss; crypt hyperplasia and villus atrophy during Gvhr. These results indicate that NK cells may act as non-specific effector cells in both the proliferative and destructive phases of a Gvhr and do not protect the host animal, as others have suggested.

Active immunosuppression occurred in several models of acute, destructive Gvhr and the final experiments examined the role of suppressor T-cells in acute Gvhr. Host mice were depleted of Ts by treatment with 2-deoxyguanosine which significantly reduced the mortality due to Gvhr in adult BDF $_1$ mice given B10 spleen cells and delayed the development of villus atrophy in these mice. dGuo treatment of neonatal (CBA x BALB/c) F_1 with acute Gvhr had less effects on systemic Gvhr but, it prevented the development of villus atrophy and produced more intense crypt hyperplasia. The role of Ts was also investigated by inducing a Gvhr across an incompatibility at the I-J locus in (B10.A3R x B10.A5R) F_1 mice. Although these mice do not develop any systemic Gvhr and had no immunological alterations, transient villus atrophy was found. However, this was associated with crypt shortening and an absence of local lymphoid cells,

rather than the hypertrophy and inflammation normally found in Gvhr.

The overall conclusion from this thesis is that intestinal Gvhr provides an excellent means of studying enteropathy which is associated with a local T-cell mediated immune response. Both proliferative and destructive forms of enteropathy can be induced and it appears that the destruction represents a continuation of an earlier, proliferative phase. Although CTL were often associated with destructive Gvhr, this was not always the case and therefore other mechanisms such as NK cells or Ts may also be involved. The onset of acute Gvhr may represent progression of the DTH response thought to be responsible for proliferative enteropathy.

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To my Grandfather, Hyman Silver, with
sadness that he was unable to see my
thesis completed.

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Declaration

These studies represent original work carried out by the author, and have not been submitted in any form to any other University. Where use has been made of material provided by others, due acknowledgement has been made.

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Michelle Victoria Felstein

PUBLICATIONS

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I. Delayed type hypersensitivity is responsible for the proliferative and destructive enteropathy in irradiated mice with graft-versus-host reaction.

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Abbreviations used in the text

Anti AsG _{M1}	antiasialoG _{M1}
APC	antigen presenting cell
B cell	bone marrow derived lymphocyte
B10	C57B1/10
BDF ₁	(C57B1/10 x DBA/2)F ₁
BMT	bone marrow transplant
c	complement
CCPR	crypt cell production rate
CMI	cell mediated immunity
ConA	concanavalin A
cpm	counts per minute
⁵¹ Cr	sodium chromate ⁵¹
CTL	cytotoxic T-lymphocyte
dGuo	2-deoxyguanosine
DTH	delayed type hypersensitivity
F ₁	first generation
FCS	foetal calf serum
FSE	food sensitive enteropathy
Gvhd	graft-versus-host disease
Gvhr	graft-versus-host reaction
H-2	murine major histocompatibility complex
H & E	haematoxylin and eosin
³ H-Tdr	tritiated thymidine
I-A	subregion of murine H-2
Ia	immune associated antigen
IEL	intraepithelial lymphocyte
IL-1	interleukin 1
IL-2	interleukin 2

I-J	subregion of murine H-2
ip	intraperitoneal
iv	intravenous
LGL	large granular lymphocyte
L-Glu	glutamine
Lyt	T-lymphocyte associated antigen (mouse)
LPS	lipopolysaccharide
2-ME	2-mercaptoethanol
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
NCS	newborn calf serum
NK	natural killer cell
NRS	normal rabbit serum
ρ	probability
pen/strep	penicillin/streptomycin
PLN	popliteal lymph node
poly I:c	polyinosinic polycytidylic acid
s.d.	standard deviation
SLE	systemic lupus erythematosus
T-cell	thymus derived lymphocyte
T _{DTH}	delayed type hypersensitivity T-lymphocyte
T _h	helper T-lymphocyte
T _s	suppressor T-lymphocyte
<u>Length</u>	
cm	centimetre
mm	millimetre
μ m	micrometre
nm	nanometre

Volume

l	litre
ml	millilitre
μ l	microlitre

Weight

kg	kilogram
g	gram
mg	milligram
μ g	microgram
ng	nanogram

time

hr	hour
min	minute

Concentration

m	molar
N	normal

Symbols

<	less than
>	greater than
=	equal to
/	per

CHAPTER 1
INTRODUCTION

Cell mediated immunity and the small intestine

The small intestine is constantly bombarded with foreign particles or antigens and it is not surprising that this important tissue has developed a complex immune system to protect itself against potential pathogens. However, activation of potent effector mechanisms during initially protective responses may also be associated with intestinal damage (Mowat, 1984).

One important group of diseases caused by a local immune response are the enteropathies associated with active hypersensitivity to dietary proteins. These disorders are common causes of infantile morbidity and may reflect a breakdown in the immune tolerance which normally occurs after feeding proteins to naive animals (Mowat 1984). The commonest form of food sensitive enteropathy is coeliac disease, which is due to an intolerance to the wheat protein given, but similar conditions are associated with hypersensitivity to soya, fish, rice, chicken and milk (Ament et al., 1972; Kuitunen et al., 1975; Vitoria et al., 1982). These food sensitive enteropathies are characterised by diarrhoea, malabsorption and a failure to thrive, normally within the first 2 years of life. The pathological changes are restricted mainly to the upper jejunum and duodenum and consist of villus atrophy and crypt hyperplasia (Mike & Asquith, 1987). These alterations in mucosal architecture are accompanied by a chronic inflammatory cell infiltrate (Rubin et al., 1960), which includes increased numbers of plasma cells and mucosal mast cells (Strobel et al., 1983;

Mowat, 1987a) in the lamina propria as well as an increased density of IEL (Ferguson & Murray, 1971; Holmes et al., 1974). In coeliac disease, many IEL have an activated morphology and have an increased mitotic rate, as well as showing evidence of increased movement in and out of the epithelium (Marsh, 1987). A similar pattern of villus atrophy, crypt hyperplasia and increased lymphocytic infiltration of the epithelium is also found in several parasitic infections of the small intestine, as well as tropical sprue and the active lesions of Crohn's disease (Cooke & Holmes, 1984; Miller, 1987). Therefore it seems likely that this group of disorders share a common pathogenesis and it would be important to elucidate the mechanisms involved.

There is considerable circumstantial evidence from clinical studies that FSE is associated with local and systemic immunity to dietary antigens (Mowat, 1984; Mike & Asquith, 1987). Furthermore, coeliac disease shows a close genetic linkage with certain genes coded by the class II region of the human MHC, including HLADW3, DR7 and DQW2 (Keuning et al., 1976; Beutel et al., 1980). Together with the chronic inflammatory cell infiltrate, these features suggest that this group of enteropathies are due to a local immune response with the intestine. Nevertheless, many different types of immunity to food proteins have been detected in patients with FSE and the immunopathogenesis of the intestinal damage remains unclear. As it is difficult to investigate these issues in diseased patients under clinical conditions, animal models have been sought which

will reproduce the appropriate intestinal pathology in association with defined immune responses.

Experimental models of food sensitive enteropathy

1. Naturally occurring disease

There are no naturally occurring models of FSE in inbred laboratory animals. Nevertheless, post weaning diarrhoea in piglets is characterised by villus atrophy crypt hyperplasia and an increased rate of crypt cell proliferation (Kenworthy & Allen, 1966(a)(b); Kenworthy, 1970) and recent evidence suggests that this is associated with a DTH response to antigens present in the weaning diet (Stokes, 1984). However, the experimental use of this model is limited by the outbred nature and availability of the host animal and by claims that enteropathogenic E.coli are primarily responsible for the intestinal damage (Kenworthy, 1970).

2. Local CMI responses to fed protein antigens

Induction of an active immune response to fed proteins would be the most appropriate experimental model of clinical FSE. Although antibodies to food proteins causing the enteropathy are present in a majority of patients, they are probably not important, as antibodies are also present in normal children (Ferguson & Carswell, 1972; Savilahti, 1973; Eastham & Walker, 1977) and in patients with intestinal damage due to diseases not associated with food hypersensitivity (Ferguson, 1976). Furthermore, the induction of antibodies

which react with the small intestine does not produce the appropriate histologic changes (Rabin & Rogers, 1976). Finally, although mucosal mast cell numbers are increased in coeliac disease, there are no alterations in mucosal IgE, plasma cell numbers and local anaphylactic reactions do not produce crypt hyperplasia (Strobel et al., 1983). This direct approach of inducing immunity to dietary antigens has also proved very difficult, because the usual result of feeding soluble antigens to naive animals is the induction of profound systemic tolerance (Mowat, 1987b). If this induction of oral tolerance can be prevented after feeding mice ovalbumin, subsequent oral challenge with antigen will produce a CMI response in the intestinal mucosa and lymphoid tissues (Mowat, 1987b). This local hypersensitivity reaction is associated with crypt hyperplasia and increased IEL counts (Mowat & Ferguson, 1981a; Mowat, 1986) and so has some similarities to the natural disorders.

In addition recent studies indicate that immune responsiveness to dietary ova may be controlled in part by MHC genes (Lamont et al., 1988; Mowat et al., 1987). Together, these studies suggest that clinical FSE may reflect a local CMI response which occurs due to a genetic defect in the induction of oral tolerance to dietary antigens. However, this experimental model of food hypersensitivity is limited by the fact that the intestinal damage is generally mild and, thus far, it has proved impossible to induce villus atrophy. Therefore, it seems likely that the study of immune responses to dietary antigens in mice will provide

information on the induction phase of FSE and it will be necessary to examine the pathogenic effector mechanisms in other models.

3. Experimental parasitic infections

The mucosal lesions seen during FSE are reproduced by certain experimental parasitic infections including Nippostrongylus brasiliensis, Trichinella spiralis and Giardia muris (Miller, 1987; Roberts-Thomson, 1987). The intestinal damage caused by N. brasiliensis is preceded by an influx of T-lymphoblasts into the mucosa and does not occur in thymus-deprived, B rats (Ferguson & Jarrett, 1975). Furthermore, both the mucosal pathology and associated hyperplasia of mucosal mast cells can be adoptively transferred by immune T-cells (Manson-Smith et al., 1979). The enteropathy found in mice with T. spiralis infection is also preceded by increased localization of T-lymphoblasts in the small intestine (Manson-Smith et al., 1979) and immune T-cells are required for the expulsion of the parasite from the intestine (Wakelin & Wilson, 1979).

T-cell immunity has also been implicated in murine giardiasis (Roberts-Thomson, 1987), which is accompanied by villus atrophy, crypt hyperplasia and an increase in IEL (MacDonald & Ferguson, 1978; Ferguson et al., 1981).

Although these studies suggest that activated T-cells are responsible for the intestinal pathology in parasite infections, others have shown that although nude mice had a delayed clearance of T. spiralis, these mice still developed

a full range of pathology (Ruitenberg et al., 1977).

Furthermore, it has also been suggested that antibody mediated immunity may be of most importance in giardiasis (Ridley & Ridley, 1976). In general, the complexity of parasite infections limits their usefulness as models of specific immune-mediated tissue damage.

4. Rejection of intestinal allografts

Several years ago, it was shown that rejecting allografts of canine jejunum developed villus shortening and crypt hyperplasia followed by crypt necrosis (Holmes et al., 1971). Later studies in mice by Ferguson & Parrott (1972, 1973) showed that rejection of allografts of foetal intestine implanted under the kidney capsule was associated with villus atrophy and crypt hyperplasia. These features were thymus-dependent and did not correlate with the development of the humoral immune response (Elves & Ferguson, 1975).

More detailed analysis of mucosal architecture and epithelial cell kinetics in this model showed that the earliest sign of rejection was an increased IEL count, and that villus atrophy was preceded by an increased CCPR (MacDonald & Ferguson, 1976). These studies were the first clear demonstration that a local T-cell response could produce this pattern of intestinal damage. In addition, they established sensitive methods which could be used to measure intestinal immune responses of this kind.

5. Intestinal phase of Graft-versus-host reaction

Another form of alloreactivity in vivo is graft-versus-host reaction and it has long been known that Gvhr in man and experimental animals is associated with damage to the small intestine (Billingham, 1967; Glucksberg et al., 1974; Woodruff et al., 1976; Slavin & Santos, 1973; Bril & Benner, 1985). As will be described below, the intestinal features include villus atrophy, crypt hyperplasia as well as increased lymphocytic infiltration of the epithelium and so mimic closely those of naturally occurring enteropathies (MacDonald & Ferguson, 1977; Mowat & Ferguson, 1981b, 1982). One further advantage of Gvhr as an experimental model is that the pathological consequences can be manipulated, depending on the donor-host combination used and many workers have found the Gvhr to be a flexible means of studying T-cell mediated immunopathology (Gleichmann et al., 1984; Bril & Benner, 1985). The aim of this thesis was to investigate further whether murine Gvhr provided a suitable means of elucidating the pathogenesis of FSE.

Graft-versus host reactions

Gvhr is the syndrome which occurs after the transfer of immunocompetent lymphocytes into genetically incompatible, immunoincompetent hosts (Billingham, 1967). The first formal description of Graft-versus-host disease was applied by Billingham, Brent & Medawar (1955) to the runting disease which occurred in some neonatal mice after the induction of tolerance to allogeneic cells.

Around the same time, Simonsen (1957) reported that newly hatched chicks given allogeneic lymphoid cells developed hepatosplenomegaly, as well as haemolytic anaemia. Splenomegaly also occurred in newborn mice inoculated with spleen cells from homologous adult donors (Simonsen, 1962) and, subsequently Gowans (1962) demonstrated that the small lymphocyte was important in evoking what were termed "Gvh" reactions. Later studies showed that T-lymphocytes were responsible for the induction of Gvhr (Argyris, 1974; Grebe & Streinlein, 1976; Vallera et al., 1981) and since then Gvhr has received considerable attention from both clinicians and experimental immunologists.

Pathological features of Gvhr

Gvh disease remains one of the most important complications of human bone marrow transplantation although it is now less common due to the use of T-cell depleted donor bone marrow (Waldman et al., 1984; Prentice et al., 1984). Although pathological changes may occur in a wide variety of different tissues, the principal target organs are usually the small intestine, skin and liver (Elkins, 1971; Bril & Benner, 1985). The skin normally shows the first signs of damage, with an erythematous rash which may progress to formation of bullae and desquamation. Histological examination reveals degeneration of the epidermis or mononuclear cell inflammatory infiltration and necrosis (Woodruff et al., 1976; Bril & Benner, 1985). Intestinal involvement occurs shortly after the skin lesions and presents as diarrhoea and vomiting.

Macroscopic examination shows ulcerative lesions in the small intestine (Elkins, 1971) and, as the disease progresses, histological sections exhibit degeneration of the mucosa with vacuolated crypts, flattened villi and a mononuclear cell infiltrate (Bril & Benner, 1985). These features may be complicated by infection (Santos et al., 1985) and are less marked in patients treated with barrier nursing. The liver damage which occurs varies according to the form of Gvhd (Bernuau et al., 1980; Bril & Benner, 1985), but commonly involves lymphoid infiltration at sinusoids and portal tracts, destruction of the bile ducts followed by hepatocellular necrosis (Suitters & Lampert, 1984). In all these tissues, induction of Class II MHC antigens on tissue cells is a prominent feature of Gvhd, but its relationship to the pathological damage is unknown.

Two forms of human Gvhd have been described; acute and chronic (Tsoi, 1982; Santos et al., 1985; Gale et al., 1985). Acute Gvhd normally begins within 30 days post-transplant and may be accompanied by an early enhancement of antibody production and DTH (Santos et al., 1985). Thereafter non-specific suppressor T-cells may appear (Hess et al., 1982; Witherspoon et al., 1984) and these probably account for the high incidence of bacterial and viral infections in these patients (Witherspoon et al., 1984). Acute Gvhd is also associated with epithelial cell damage in many tissues (de Gast et al., 1987) and in the gut this results in necrosis of individual enterocytes, followed by degeneration and loss of crypts (Sloane & Dilly, 1988).

Chronic Gvhd begins >100 days after BMT and may develop after acute Gvhd or can occur de novo. It has many characteristics of collagen vascular disorders, autoimmune diseases, such as SLE and scleroderma (Tsoi et al., 1978; Graze and Gale, 1979; Shulman et al., 1980; Santos et al., 1985). Severe immunodeficiency is a further feature of chronic Gvhd and this may lead to recurrent and sometimes fatal infections. Infection is predisposed by the profound immunosuppression which is a characteristic feature of most patients with chronic Gvhd. This may be due to reduced activity of helper T-cells, B cells or antigen presenting cells, while elevated levels of non-specific suppressor T-cell activity may also be present (Tsoi et al., 1982). In contrast, long term stable chimeras, without Gvhd are specifically tolerant of host antigens, but lack anti-host cellular responses and do not have non-specific Ts or abnormal B and T helper cell activities. The maintenance of long term chimerism appears to be dependent upon specific suppressor cells (Tsoi et al., 1981).

Many studies of Gvhr in animals have also found evidence of immune suppression which may affect both cellular (Lapp et al., 1974; Shand, 1976) and humoral immunity (Pickel & Hoffman, 1977). Although, later work showed that this immunosuppression may be attributed to suppressor T-cells (Ishikawa et al., 1982) as in man, several different mechanisms have been implicated in this phenomenon. These include defects in the function of T cells, B cells and macrophages (Seddik et al., 1984, 1985, 1986a,b). Interleukin-2 and

interleukin-1 production has been shown to be reduced (Joseph et al., 1985; Mendes et al., 1985) and it has been suggested that the T-cell deficiency is due to damage to thymic epithelium (Seddik et al., 1979). In addition many groups have identified specific donor Ts in mice with Gvhr (Lapp et al., 1974; Pickel & Hoffman, 1977; Pollison & Shearer, 1980; Gleichmann et al., 1984), while others have shown the generation of non-specific "natural suppressor" cells (Maier et al., 1986). Interestingly, there is some evidence that the appearance of suppressor cell activity may be preceded by an early phase of enhanced immune responsiveness (Byfield et al., 1973), illustrating the complex abnormalities of immunoregulation which may occur in Gvhr.

Different patterns of pathology have also been reported in animal models although these rarely reproduce the clinical forms described above. Many workers have shown that the consequences of a Gvhr depend on several different factors, including the age of the host, its immune status and the donor-host combination as well as the dose and nature of the donor cells. One of the best characterised of these models is that using adult, unirradiated BDF₁ mice, where it has been shown that B10 donor cells produce an acute, often lethal Gvhr which is associated with anaemia, hypoplasia of the lympho-haemopoietic system and hypogammaglobulinaemia (Gleichmann et al., 1984). In contrast, DBA/2 cells produce a chronic proliferative disorder with hypergammaglobulinaemia

leading to the formation of autoantibodies characteristic of SLE, immune complex glomerulonephritis and symptoms characteristic of other collagen vascular diseases (Gleichmann et al., 1984). As will be discussed below, these forms of Gvhr appear to be due to different immune effector mechanisms, illustrating the potential usefulness of experimental Gvhr in studying immunopathology.

Cellular and genetic basis of Gvhr

The outcome of a Gvhr reflects a complex interaction between donor and host elements and depends on the donor cells used, the genetic differences involved and the nature of the host animal. T-lymphocytes have been shown to be essential for the induction of a Gvhr (Argyris, 1974; Grebe & Streilin, 1976) and this has been confirmed by studies where depletion of T cells from the donor inoculum prevents the development of Gvhd in man (Prentice et al., 1984; Waldmann et al., 1984) and mice (Cantor, 1972; Korngold & Sprent, 1978). T-cells are also required for the rejection of allografts, as animals made T-cell deficient by adult thymectomy, lethal irradiation and bone marrow reconstitution are unable to reject skin allografts (Loveland et al., 1981; Dallman et al., 1982).

Although T-lymphocytes are clearly required for the induction of Gvhr, the T cell system displays a broad spectrum of functions, including helper and suppressor T cell activity, cell mediated cytotoxicity and delayed type hypersensitivity (Sprent et al., 1986b). Therefore, considerable effort has been made to attempt to determine which of these activities

is required for the induction of Gvhr. Particular attention has centred on the relative roles of CTL and T_{DTH} but these studies should be interpreted carefully in the light of the fact that phenotype does not necessarily predict the function of a T-cell.

Early studies using Lyt 1 and Lyt 2 as markers of mouse helper and cytotoxic T-cells showed that although both Lyt 1⁺ and Lyt 2⁺ T cells were required for the induction of Gvhd (Kisielow et al., 1975) only depletion of Lyt 1⁺ cells prevented the induction of a lethal Gvhr. Later studies in rats also showed that helper/inducer T cells appeared to be essential for a lethal Gvhr (Mason, 1981), these cells were also shown to be MRC OX 22⁺ (Spickett et al., 1983) and together, these results were interpreted as support for the role of DTH in Gvhr. However, further work suggested that both Lyt 1⁺ and Lyt 2⁺ T-cells were required for the induction of anti-host DTH in Gvhr (Wolters & Benner, 1981), as well as for the development of lethal Gvhr in unirradiated BDF₁ mice (Rolink et al., 1983; Rolink & Gleichmann, 1983). Korngold & Sprent[†] (1982) also provided evidence in irradiated mice that Lyt 1⁺ 2⁺ cells were required to induce a lethal Gvhd across minor histocompatibility antigens.

In addition, further experiments in rats showed that radioresistant CD8⁺ cells were still present in the host animals in whom Gvhr appeared to be due entirely to helper/inducer T-cells. Furthermore, the MRC OX 22⁺ T cells which were required to induce a local Gvhr in rats were found to assist the generation of CTL as well as mediating DTH (Spickett et al., 1983; Arthur & Mason, 1986).

The interpretation of initial studies in mice was also complicated by the demonstration that Lyt 1 was expressed on both T_H and CTL (Ledbetter et al., 1980) and more recent work in mice has used L3T4 and Lyt 2 as non-overlapping markers on Class II restricted and Class I MHC restricted T cells (Dialynas et al., 1983; Sprent et al., 1986b). These studies have shown that both $L3T4^+$ and $Lyt\ 2^+$ T cells alone can cause systemic Gvhr, providing they are stimulated by the appropriate MHC antigen (Cobbold et al., 1986). In contrast it has been shown that the induction of Gvhr associated immune deficiency across a combined Class I and Class II MHC disparity is dependent upon both $L3T4^+$ and $Lyt\ 2^+$ cells whereas only $L3T4^+$ cells are required to induce immune deficiency across an isolated Class II difference (Moser et al., 1985). There is also some evidence that different T cell subsets may be responsible for different components of the Gvhr. Thus, while both $Lyt\ 2^+$ and $Lyt\ 2^-$ cells are required for an acute lethal Gvhr in BDF_1 mice, $Lyt\ 2^-$ cells alone induce the chronic proliferative form of Gvhr. In contrast, $Lyt\ 2^+$ cells alone could not induce any form of Gvhr (Rolink & Gleichmann, 1983).

The earliest studies showed that generation of a Gvhr requires genetic differences between the host and donor (Billingham, 1967). Subsequent work has found that these differences can be either MHC gene products (Elkins, 1976; Klein & Chiang, 1976; Gleichmann et al., 1984) or minor histocompatibility differences (Hamilton et al., 1981; Korngold and Sprent, 1982; Hamilton, 1984; Maier et al., 1985).

In mice, the H-2 complex is the most potent inducer of systemic Gvhr and, in congenic mice Class II MHC loci have been shown to be of most importance in the development of lethal Gvhr (Klein & Chiang, 1976; Elkins, 1976; Rolink et al., 1983), splenomegaly (Klein, 1977; Mowat et al., 1986), and anti-host DTH (Wolters & Benner, 1979). The I-A region has also been shown to be the most important stimulus for intestinal damage in irradiated (Piguet, 1985) and unirradiated mice (Mowat et al., 1986) with Gvhr. In contrast, isolated Class I MHC differences in congenic mice have little or no ability to induce systemic changes of Gvhr (Klein & Chiang, 1976) and do not cause intestinal Gvhr (Mowat et al., 1986). However, the development of a fully lethal Gvhr in unirradiated BDF₁ mice has been shown to require both Class I and Class II MHC differences (Rolink et al., 1983; Rolink & Gleichmann, 1983).

Recent work using MHC mutant mice has shown that both Class I and Class II MHC differences can induce systemic Gvhr under appropriate circumstances (Sprent et al., 1986a). However, there is some evidence that these incompatibilities may cause different forms of Gvhr associated pathology (Rolink et al., 1983; Sprent et al., 1986b). Together, these results suggest that both Class I and II restricted T cells can induce Gvhr in experimental animals and as these T cell subsets are both capable of CTL and DTH (Sprent & Webb, 1987), the functional nature of the inducer T cell remains unclear. However, it should be noted that most studies emphasise the role of Class II restricted T cells and therefore DTH mediated reactions may be of most importance. Nevertheless, different MHC classes may result in different pathology.

Role of host factors in the outcome of Gvhr

Although Gvhd necessitates recognition of host allo-antigens by donor T-lymphocytes, the pathogenesis of Gvhd is greatly influenced by host factors, including the age, sex and microbiological and immune status of the host animal (Billingham, 1967; Brill & Benner, 1985). The potential importance of host mechanisms is underlined by the severity of the Gvhr which occurs in hosts which have received chemotherapy, whole body irradiation or which are developmentally immature (Grebe & Streilin, 1976). The reasons for host dependent regulation are complex, but it has been shown that certain strains of allogeneic and F₁ hybrid mice can reject lymphocytes (Fox & Howard, 1963; Cudkowicz & Stimpfling, 1964; Nakamura & Cudkowicz, 1979; Elkins & Quant, 1981) or bone marrow cells from fully or semi-allogeneic donors (Kiessling et al., 1977; Dennert et al., 1985). F₁ hybrid resistance in mice is controlled by MHC-linked haemopoietic histocompatibility loci (Cudkowicz & Nakamura, 1983) which appear to be non-codominantly expressed by homozygous, parental mice, but not by F₁ animals (Daley & Nakamura, 1984). In many cases, host NK cells seem to indicate resistance to BM cells (Cudkowicz & Stimpfling, 1964; Elkins & Quant, 1981; Kiessling et al., 1977; Murphy et al., 1987) and this may involve ADCC (Warner & Dennert, 1985). However, recent studies suggest that Class I restricted CTL may also be required for resistance to fully allogeneic BM in mice (Harper et al., 1987). In addition, anti-idiotypic host T-lymphocytes with CTL activity against donor lymphoblasts can suppress a Gvhr mediated by parental lymphocytes in rats (Bellgrau & Wilson, 1978; Kimura & Wilson, 1984; Kimura et al., 1984).

One particular host factor which has profound effects on the outcome of the Gvhr and which is important for many of the results described in this thesis, is the age of the host. Neonatal animals undergo a much more severe form of Gvhr than adults of the same strain given the same donor cells. Neonatal animals have a functionally immature immune system (Murgita & Wigzell, 1981). Although neonatal splenic T cells reach 75% of adult numbers within the first week (Murgita & Wigzell, 1981), there is evidence that neonatal T-cell function is reduced until about 4 weeks of age (Spear et al., 1973). However, the evidence is conflicting as some T-cell functions have developed by 7-8 days of age (Spear & Edelman, 1974; Pilarski, 1977; Schwartz & Doherty, 1981). Furthermore, by 4-5 days old neonates will reject allogeneic transplants (Billingham & Brent, 1959). NK cell activity is also defective in neonatal mice (Kiessling et al., 1975; Roder et al., 1981; Koo et al., 1982) and this may involve both a lack of precursors in very young mice, followed by functional immaturity of precursors which arise at later times (Roder et al., 1981; Koo et al., 1982). NK activity appears rapidly around the time of weaning, at 3 weeks (Kiessling et al., 1975) reaching a peak at 6-8 weeks of age (Murgita & Wigzell, 1981).

In addition to these effector cell defects, neonatal mice have high levels of non-specific suppressor cell activity which may be due to Ts (Mosier & Johnson, 1975; Ptak & Skowron-Cendrzak, 1977; Argyris, 1978; 1984) or large granular lymphocytes with natural suppressor activity (Strober, 1984; Maier et al., 1986). These suppressor cells may affect both

B and T cell functions (Mosier & Cohen, 1975; Ptak & Skowron-Cendrzak, 1977) and may also account for the poor production of IL-2 in the neonate (Ishizaka & Stuttman, 1983; Argyris et al., 1985). Finally, a lack of macrophages and dendritic cells (Steinman et al., 1974; Steinman et al., 1979; Lu et al., 1979) may also contribute to the defective immunity in neonates (Snyder et al., 1982). Studies in neonatal rats have also shown the absence of Class II expression in the gut epithelium (Mayrhofer et al., 1983).

Effector mechanisms in Gvhr

The effector mechanisms responsible for tissue damage in Gvhr are controversial and their complex nature can be imagined from the preceding discussion of the induction phase. For many years it was assumed that specific CTL activity was responsible for the tissue damage which occurs during allograft rejection and Gvhr (Cerrottini et al., 1971; Cantor & Asofsky, 1972; Elkins, 1978). In support of this, specific anti-host CTL activity was described in early studies of experimental Gvhr in irradiated hosts (Elkins, 1978) as well as in patients with clinical Gvhr (Tsoi, 1982). In addition, immunohistochemical studies show that a large proportion of lymphocytes infiltrating the skin, gut and liver lesions of acute Gvhr have the suppressor/cytotoxic phenotype (Janossy et al., 1982). Although these findings support those from studies of allograft rejection they do not prove that CTL are the pathogenic effector cells. As noted above, Class II MHC restricted T-cells seem to be of most importance in the

induction of Gvhr and recent work has indicated that the presence of anti-host CTL activity did not correlate with the development of acute Gvhd in irradiated mice, across either full MHC differences (Jadus & Peck, 1983) or minor histocompatibility differences (Hamilton, 1984).

Furthermore, specific anti-host CTL activity frequently cannot be detected in unirradiated mice with Gvhr (Borland et al., 1983) and does not predict the outcome of the Gvhr when it does occur (Van Elven et al., 1981; Kubota et al., 1983). In parallel, the signs of acute Gvhd in irradiated mice are preceded by a specific anti-host DTH response (Benner et al., 1985).

There are also several reports that non-specific effector mechanisms are activated during a Gvhr (Singh et al., 1971; 1972). Early studies showed that macrophages were activated during a Gvhr in unirradiated mice (Ptak et al., 1975; Fung & Sabbadini, 1976) and macrophages from mice with Gvhr had enhanced microbicidal activity (Blanden, 1969; Rhodes et al., 1979; Zinkernagel, 1980; Anthony et al., 1984) and antigen presenting cell activity (Strobel et al., 1985). There is also evidence for the recruitment of mast cells in Gvhr (Mowat & Ferguson, 1982; Schrader et al., 1983), while many workers have shown that there is a generalised increase in NK activity during a Gvhr in unirradiated F₁ rats (Clancy, 1984) and mice (Roy et al., 1982; Borland et al., 1983; Kubota et al., 1983; Mowat et al., 1985; Varkila & Hurme, 1985). This enhanced NK cell activity develops in parallel with the evolution of other proliferative features of the Gvhr

(Borland et al., 1983) involves both donor and host NK cells and is preceded by the anti-host DTH response (Ghayur et al., 1985; Mowat et al., 1985). These findings suggest that NK cells may be one component of the effector mechanisms responsible for the tissue damage in Gvhr (Mowat et al., 1983) as has been suggested in allograft rejection (Mason & Morris, 1986). Alternatively, host derived NK cells could be responsible for a form of F_1 resistance against the parental lymphoid cells, as described earlier.

Suppressor T cells have already been discussed with respect to the disordered immunoregulation found in Gvhr. However, Gleichmann and colleagues have suggested that allospecific Ts may also account for the anaemia, lymphoid atrophy and weight loss which occurs in unirradiated BDF_1 mice given B10 spleen cells (Gleichmann et al., 1984). This acute Gvhr does not appear to correlate with anti-host CTL activity but is associated with the appearance of donor-derived Ts which inhibit the function of F_1 lymphoid cells (Van Elven et al., 1981). Activation of allospecific Ts occurs subsequent to proliferation of donor anti-host T and requires both Class I and Class II MHC incompatibilities (Rolink et al., 1983; Rolink & Gleichmann, 1983). In contrast, Gleichmann has also presented evidence that the chronic proliferative SLE-like Gvhr in BDF_1 hosts given DBA/2 cells is due to continued activation of Class II MHC restricted allospecific T_H . These findings suggest that abnormalities in immunoregulation may contribute to organ damage in Gvhr and highlight the possibility that manipulating the immune

response in Gvhr may produce predictable changes in tissue pathology.

Intestinal Gvhr

Intestinal damage was noted in the earliest studies of Gvhr in mice (Gorer & Boyse, 1959) and severe enteropathy was first detailed in work on newborn BDF₁ mice (Reilly & Kirsner, 1965). With the development of runt disease, the intestinal pathology in these mice was most pronounced in the ileum and was characterised by clubbing of the villi, with enlargement and hyperplasia of the crypts (Wall et al., 1971). In parallel there was a decreased number of goblet cells (Reilly & Kirsner, 1965). The same authors showed that more severe intestinal damage was observed if the neonates had been irradiated, indicating that the Gvhr and the irradiation might act synergistically.

Later studies on 10 day old mice with runt disease, confirmed the histological changes noted above and showed they were associated with a deficiency of brush border disaccharidases (Hedberg et al., 1968), malabsorption (Palmer & Reilly, 1971) and protein losing enteropathy (Cornelius, 1970). Degeneration and necrosis of the mucosa, flattening of the villi and infiltration of the mucosa by lymphocytes and other mononuclear cells is also a prominent feature of Gvhr after clinical BMT and is a frequent cause of morbidity and death in these patients (Slavin & Santos, 1973; Glucksberg et al., 1974; Woodruff et al., 1976). Although some workers have shown that bacterial antigens may influence

the severity of the intestinal lesions during Gvhr (Van Bekkum & Knaan, 1977), similar mucosal alterations occur in sterile antigen free intestine implanted under the kidney capsule of mice with Gvhr (Ferguson & Parrott, 1971; 1972). Therefore, it seems unlikely that the lesions are due to direct infection of the mucosa (Elson et al., 1977; Mowat & Ferguson, 1981b), although a role for bacterial products from the host intestinal flora cannot be excluded (Van Bekkum & Knaan, 1977).

As in other work on Gvhr, the villus atrophy and crypt necrosis were originally assumed to be due to specific CTL. However, the studies by MacDonald and Ferguson (1977) in 5 day old (CBA x BALB/c) F_1 mice showed that the villus atrophy was preceded by an increased crypt cell production rate and crypt hypertrophy (MacDonald & Ferguson, 1977). Therefore, it was suggested that the primary cause of the enteropathy was the production of stimulatory lymphokines during a local DTH response (MacDonald & Ferguson, 1977). Further experiments in adult or one week old, unirradiated F_1 hybrid mice supported these conclusions, by showing that the principal mucosal alterations were increased in IEL, CCPR and crypt depth (Mowat & Ferguson, 1981, 1982). These features appeared within 24 hours of cell transfer and evolved in parallel with the proliferative phase of the Gvhr, as measured by splenomegaly (Mowat & Ferguson, 1981; 1982). There was no evidence of specific CTL in the gut or elsewhere (Borland et al., 1983), and the mucosal changes also occurred as a bystander effect in grafts of foetal gut

which were syngeneic to the donor cells (Elson et al., 1977). More recent work in semi-allogeneic unirradiated mice shows that the intestinal Gvhr was due principally to Lyt-2 T cells recognising Class II alloantigens present on bone marrow derived cells (Piguet, 1985; Mowat, 1986; Mowat et al., 1986). Together, these results suggest strongly that the mucosal alterations in this model of Gvhr occur as a result of a DTH reaction. This idea is supported by the findings that the intestinal Gvhr is associated with recruitment of mucosal mast cells (Mowat & Ferguson, 1982) and enhancement of NK cell activity by IEL (Borland et al., 1983; Mowat et al., 1985). Thus activation of IEL NK cell activity parallels the other changes in the gut, as well as the development of splenomegaly, and was preceded by an anti-host DTH response (Borland et al., 1983; Mowat et al., 1983).

The crypt hyperplasia and increased numbers of IEL and MMC found during a Gvhr in unirradiated mice are similar to those seen in clinical FSE. Therefore, the features discussed above are consistent with the hypothesis that a local DTH reaction is the principal mechanism responsible for this pattern of pathology in FSE. However a major defect in these earlier studies has been that the villus atrophy which characterises FSE, was never found (Mowat & Ferguson, 1981; 1982). Thus it is possible that the development of villus atrophy may require effector mechanisms which differ from those which induce crypt hyperplasia.

The principal aims of this thesis were therefore to develop models of acute Gvhr in mice which would produce villus atrophy and to elucidate the effector mechanisms which were responsible. In addition, I have attempted to define the conditions which influence the outcome of the Gvhr and have investigated the role of selected effector mechanisms in more detail.

CHAPTER 2

MATERIALS AND METHODS

Animals

CBA/Ca (H-2^k), BALB/C (H-2^d) C57B1/10 (H-2^b), DBA/2 (H-2^d) and BALB/B (H-2^b) mice were obtained from departmental stocks, unless stated otherwise. ATH (H-2^{to}), ATL (H-2^{t2}), B10.A (H-2^d), B10.AQR (H-2^{y1}), B10.AKM (H-2^m), B10.A3R (H-2ⁱ³) and B10.A5R (H-2^{is}) mice were purchased from Harlan Olac Ltd. (Bicester, Oxon.). (A list of these mice and their haplotypes is found in Table 1). (CBA x BALB/C)_{F₁}, (C57B1/10 x DBA/2)_{F₁}, (ATH x ATL)_{F₁}, (BALB/B x DBA/2)_{F₁}, (B10.A x B10.AQR)_{F₁}, (B10.A x B10.AKM)_{F₁} and (B10.A3R x B10.A5R)_{F₁} hybrid mice were bred in the Department from the appropriate parental strains, the maternal strain being the first named of each pair. Adult mice were first used at 6-10 weeks of age, except when 12-16 week old mice were used for irradiation experiments. In experiments using neonatal mice, age-matched neonates from individual mothers were mixed immediately after birth to obtain larger groups and were then maintained with their mothers throughout the course of the experiment. Male and female (CBA x BALB/C)_{F₁} nu/nu mice were obtained from Mrs S. Jenks, National Institute for Medical Research, Mill Hill, London. Control mice in these experiments were age and sex-matched euthymic (CBA x BALB/C)_{F₁} mice. Athymic mice were kept for 3 weeks before use to enable stabilization of their intestinal flora, and all animals were maintained under standard conditions.

All animals were maintained on a standard rodent diet (Labsure Maintenance Diet) and had access to tap water ad libitum.

Anaesthesia and sacrifice of animals

Procedures such as footpad or intravenous injection via the tail vein were carried out under light ether anaesthesia. Mice were sacrificed by ether inhalation followed by cervical dislocation.

Irradiation of mice

Mice received 950 Rads irradiation from a ^{60}Co cobalt source of γ rays at a distance of 100 cm and a dose rate of 250 Rad/min. This was performed by courtesy of the staff at Belvidere Hospital, Glasgow.

After irradiation, mice received 100 mg/l Neomycin (Neomycin Sulphate; Sigma) in their drinking water.

Measurement of haematocrit

The circulating packed red cell volume was assessed by measuring the haematocrit. Under ether anaesthesia, blood was collected from the heart, into heparinised capillary tubes (Hawksley, England). These were sealed, centrifuged at 3,000g for 10 mins and the haematocrit calculated by measuring the relative proportion of red blood cells and plasma in the total blood volume.

Body and organ weights

Mice were weighed using an Oertling JC 12 single pan balance (accurate to 0.01g), while individual organs were dissected free of surrounding tissues and weighed to the nearest mg on a fine twin pan balance (accurate to 0.2 mg) (Gallenkamp).

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Histology

Tissues were removed immediately after sacrifice and placed in fixative. For conventional histology, the jejunum and liver were fixed in 10% phosphate buffered formalin. Tissues were embedded in paraffin wax and sections 4 μm thick cut for staining with Haematoxylin and Eosin.

All histological specimens were examined using a Leitz Ortholux II microscope and processing was carried out by Mr Hector Cairns and Mrs Margaret Hardie.

Intraepithelial lymphocyte counts

Intraepithelial lymphocytes were counted by the method of Ferguson and Murray (1971) on H & E stained sections of jejunum taken 10 cm from the pylorus. Specimens were examined under x 400 magnification and only sections with a single layer were counted. Differential cell counts were performed by counting epithelial and lymphoid cell nuclei lying above the basement membrane and ignoring goblet cells. A total of 600 epithelial cells was counted in each specimen and results were expressed as IEL/100 epithelial cells.

Measurement of mucosal architecture by microdissection

The method of Clarke (1970) was employed to assess mucosal architecture. Mice were injected with 7.5 mg/kg Colchicine (Sigma) i.p. and sacrificed at intervals of 20-100 mins, thereafter 1 cm^2 pieces of jejunum were removed 10 cm from the pylorus, opened and placed villus surface

upwards on pieces of card for fixation in 75% Ethanol/25% acetic acid for 6-24 hours. Tissues were stored in 75% Ethanol before staining in bulk by the modified Feulgen reaction as follows: the pieces of gut were first immersed in 50% Ethanol for 10 mins, followed by 10 mins in tap water and 7 minutes in 1M HCl at 60°C. After a further 10 minutes in tap water, the specimens were flushed x 3 with fresh tap water, stained with Schiff reagent (Difco Ltd.) for 30-40 minutes at room temperature and kept in tap water before microdissection. Under the dissecting microscope, the muscularis mucosae was first stripped off, and thin slices of mucosa, one villus thick and each containing a few villi with their surrounding crypts, were then cut from the edge by dissection with a cataract knife (Weiss Ltd.). The fragments were then placed on a microscope slide in 45% acetic acid, covered with a coverslip and villus and crypt lengths measured by conventional microscopy, using a previously calibrated eyepiece micrometer. The pieces were then gently squashed under the coverslip and the number of metaphases per crypt counted. In each specimen, 10 villi and crypts were counted and the crypt cell production rate was obtained by correlating the mean number of metaphases/crypt with the time after Colchicine. The pairs of results were plotted by linear regression analysis and having established linearity, the CCPR was calculated from the gradient of the line of best fit calculated by the method of least squares.

2' Deoxyguanosine

2' Deoxyguanosine (Sigma, Poole, Dorset) was dissolved in warm distilled water at 5 mg/ml before use. Adult mice were injected with 1 mg in 0.2 ml daily by the intraperitoneal route, while neonatal mice received a weight adjusted dose of 50 µg/g in 0.05 ml daily i.p. This dose was increased every 3rd day according to the weight of the mice. Control mice received an appropriate amount of distilled water i.p.

Polyinosinic : polycytidylic acid

PolyI:C (Sigma) was dissolved in 0.01M NaOH for use. Adult mice received 100 µg polyI:C in 0.2 ml NaOH i.p., while neonatal mice received a weight related dose of 5 µg/g polyI:C in 0.05 ml NaOH i.p. Control mice received the appropriate control volume of 0.01M NaOH.

Monoclonal antibodies

The GK1.5 hybridoma (a kind gift of Professor N.A. Mitchison) was maintained in RPMI 1640 in 5% foetal calf serum, 5% newborn calf serum (Gibco), Penicillin (100 µ/ml)/ Streptomycin (100 µg/ml) (Gibco) and 2 mM glutamine (Gibco), and the supernatant used as a source of anti-L3T4 antibody at a concentration of 1:10.

In later experiments rat monoclonal anti-L3T4 ascites was obtained from Serotec Ltd. and used at a final dilution of 1:500.

Monoclonal rat anti-Lyt2 ascites was obtained from Serotec Ltd. and used at 1:400.

Treatment with anti-asialo G_{M1} anti-serum

Mice were injected intravenously with 0.2 ml rabbit anti-asialo G_{M1} antiserum (a kind gift from Dr N. Hanna SK & F Research, Philadelphia, PA) diluted 1:50 in saline, 3 days before the induction of Gvhr and at 3-4 day intervals thereafter (Charley et al., 1983). Control mice were injected with 1:50 normal rabbit serum.

Preparation of cell suspensions

Spleens, thymuses and lymph nodes were removed immediately after sacrifice. After washing in RPMI 1640, they were dissected free of surrounding material and sliced with scissors. The pieces were then gently passed through a fine gauge wire mesh using the plunger of 25 ml syringe (BD Ltd.) or teased apart using a scalpel. After repeated passage through a glass pasteur pipette to break up any clumps, the cells were allowed to stand for a few minutes at room temperature to allow debris to settle out, and washed three times at 400g in RPMI 1640. Cells which were to be injected intravenously were further passed through 1-2 ml glass wool (May & Baker), loosely packed into a 10 ml syringe, to remove any clumps, before being washed once more. After counting in a haemocytometer (Neubauer) the final cell pellet was made up to the required concentration in RPMI 1640 for use. Cell viability was assessed by phase contrast microscopy and was normally greater than 90%.

Purification of lymphocytes by nylon wool columns

The method of Julius et al. (1973) was followed. 0.6g nylon wool (Fennal Labs) which had been washed previously, in distilled water for one week at 37°C was packed loosely into a 10 ml syringe and the column washed through with RPMI 1640, supplemented with 5% FCS. The column was drained and incubated at 37°C for 1 hour before use. Spleen cell suspensions in RPMI 1640/5% FCS were passed over a glass wool column to remove debris and dead cells and, after washing in fresh medium, were allowed to pass into the nylon wool column which was then incubated for 45 minutes at 37°C. Non-adherent cells were then flushed out with warm RPMI 1640/5% FCS, washed once and resuspended for use.

Titration of monoclonal antibodies

100 µl of a suspension containing 10^6 spleen or popliteal lymph node cells were added to 100 µl aliquots of serial dilutions of anti-L3T4 or anti-Lyt2 in 96 well u-bottomed microtitre plates (Titertek Flow Labs) and incubated for 40 minutes at 4°C. Fresh guinea pig serum, as a source of complement, was then added to each well to a final concentration of 1:10, and the plate incubated for a further 30 mins at 37°C. Control wells contained cells in the presence of antibody or complement alone. The efficiency of the depletion was assessed by counting the proportion of dead cells by phase contrast microscopy.

Bulk depletion of T-cell subsets with monoclonal antibodies

10^7 Spleen cells/ml in RPMI 1640/5% NCS, were incubated with anti-L3T4 or anti Lyt2 for 40 minutes at 4°C. The cells were then centrifuged at 300g for 5 mins, the antibody containing supernatant removed and stored at 4°C. The cells were resuspended in the same volume RPMI 1640/5% NCS containing a final concentration of 1:10 guinea pig serum as a source of complement, for 30 mins at 37°C. The cells were then centrifuged and the complement containing supernatant removed and stored at 37°C. The cells were then resuspended in antibody and the depletion procedure repeated. Before use, the depleted cells were washed three times in RPMI 1640 and resuspended at the required concentration, without correcting for the number of cells lost.

Induction of local Gvhr

The ability of spleen cells to induce a local Gvhr was assessed by the popliteal lymph node assay as described by Ford et al. (1970).

F₁ mice were injected into the rear left footpad with 10^7 parental spleen cells in a volume of 0.05 ml RPMI 1640. One week later the mice were sacrificed, the draining popliteal lymph nodes removed and weighed. The intensity of the local Gvhr was assessed by comparing weights of the popliteal lymph nodes draining footpads injected with parental cells with the weights of the lymph nodes draining the opposite footpads which had been injected with medium alone.

Induction of systemic graft-versus-host reactions

1. Adult unirradiated mice

A graft-versus host reaction was induced in adult unirradiated (CBA x BALB/c) mice by injecting 6×10^7 viable CBA spleen cells in 0.2 ml RPMI 1640 intraperitoneally, while (C57Bl/10 x DBA/2) F_1 mice received 10^8 B10 or DBA12 spleen cells in 0.3 ml RPMI 1640 intravenously. In each case, age and sex-matched controls received medium alone.

2. Adult irradiated hosts

Within 24 hours of receiving 900 Rad γ -irradiation, (CBA x BALB/c) F_1 mice received $1 - 4 \times 10^7$ CBA spleen cells, i.v., in a volume of 0.2 ml or 0.3 ml RPMI 1640. Control mice received appropriate numbers of syngeneic spleen cells or were left unreconstituted.

3. Neonatal hosts

A Gvhr was induced in neonatal F_1 hybrid mice of various ages, by injecting 10^7 spleen cells of maternal origin in 0.05 ml RPMI 1640 i.p. All injections were performed through the musculature of the lower inguinal region with a 30G needle to prevent leakage and to minimize trauma.

Initially age and sex-matched littermate controls received 0.05 ml RPMI i.p., but a comparison of uninjected controls and medium injected controls proved it was unnecessary to inject control mice, and in most experiments, control littermates received no injection.

Assessment of systemic graft-versus-host reaction

The development of an acute/lethal Gvhr was assessed by weighing all the mice at frequent intervals and noting the appearance of clinical disease, such as hunched posture, ruffled fur. In the case of neonatal mice, the late development of body hair or opening of the eyes was also noted. In addition the time of death of the mice was noted and mortality curves constructed.

The spleen weight assay of Simonsen (Simonsen, 1962) was used to assess the proliferative response during the Gvhr. On the day of sacrifice, mice were weighed, the spleens removed and weighed, and the relative spleen weight expressed as mg/10g body weight.

The spleen index was then given as:

Relative spleen weight in individual mice with Gvhr

Mean relative spleen weight in control mice.

Assessment of allospecific systemic delayed-type hypersensitivity

Mice were immunised into the rear left footpad with 2×10^7 allogeneic spleen cells and 5 days later, the systemic DTH response was assessed by measuring the increment in footpad thickness, 24 hours after intradermal injection of 10^7 stimulator spleen cells into the opposite rear footpad, using a pair of skinfold calipers (Carobronze). Antigen specific DTH responses were calculated by subtracting the increment in footpad thickness after challenge of unimmunized mice.

Anti-host DTH in mice with Gvhr

Spleens were pooled from 3-4 F₁ mice with Gvhr and 10⁷ viable cells injected into one rear footpad of recipients, which were syngeneic to the parent strain used to induce the Gvhr. The adoptive transfer of DTH was assessed by the increment in footpad thickness measured 24 hours after injection. The opposite footpads were injected with appropriate numbers of control F₁ spleen cells, and the specific DTH activity of the Gvhr was obtained by subtracting the increment in control footpads from that in footpads injected with Gvhr cells.

Tumour cell lines

The following cell lines were originally purchased from Flow Labs. and used as target cells in cytotoxicity assays. YAC-1, a Moloney virus, induced T-cell leukaemia of A strain mice (H-2^a) was used as a natural killer cell sensitive target. P815 (H-2^d) a methylcholanthrene induced mastocytoma of DBA/2 mice and EL-4 (H-2^b), a C57B1/6 thymoma cell line were used in assays of specific CTL activity. These cell lines were maintained at 37°C in 5% CO₂ in air, in RPMI 1640 supplemented with 10% FCS (YAC-1) or 5% FCS and 5% NCS (P815; EL-4); Penicillin/Streptomycin (Gibco); L-Glutamine and 1.25 µg/ml Fungizone (Gibco) in 275 ml plastic tissue culture flasks (Costar). The cells were subcultured at 1:10 ratios every second day and, whenever possible, 24 hours before use.

Preparation of lymphoblast target cells

Lymphoblasts for use in cytotoxicity assays were prepared by culturing 4×10^6 spleen cells/ml with 2 mg/ml concanavalin A (Sigma) for 3 days or with 10 μ g/ml LPS for 2 days in RPMI 1640 supplemented with 10% FCS; Penicillin/Streptomycin, L-Glutamine and 5×10^{-5} M 2-mercaptoethanol (Sigma) in a total volume of 15-20 ml in a 50 ml plastic tissue culture flask (Lux) at 37°C in 5% CO₂ in air in a humidified incubator (Flow Labs. Digital CO₂ incubator 220). After culture surviving lymphoblasts were washed x2 in RPMI 1640/5% NCS and counted before use in microcytotoxicity assays.

Measurement of specific and non-specific cell mediated cytotoxicity

The cytotoxic activity of lymphoid cell populations was measured in a microcytotoxicity assay as described by Davies and Parrott (1980).

5×10^6 tumour or lymphoblast target cells/ml were labelled with 50 μ Ci Sodium⁵¹ Chromate (Amersham) for 45 min at 37°C and washed five times in RPMI 1640 with 5% NCS before being resuspended at 2×10^5 cells/ml immediately before use. 100 ml lymphoid effector cells at different concentrations were added to the wells of v-bottomed microtitre plates (Flow Labs, Irvine) and 2×10^4 target cells in 100 ml added to give effector:target (E:T) ratios of 50:1, 25:1 and 12.5:1.

NK cell assays were incubated for 4 hours at 37°C in 5% CO₂ in air, while specific CTL assays were incubated for 2 hours at 37°C in 5% CO₂ in air, followed by 1 hour at 45°C. After culture, 100 µl of supernatant were removed carefully and the ⁵¹Cr-specific radioactivity assessed in a gamma counter (LKB Compugamma). The percentage lysis was determined as follows:-

Natural cytotoxicity:-

$$\% \text{ Cytotoxicity} = \frac{(\text{release with effector cells} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100\%$$

Specific cytotoxicity:-

$$\% \text{ Specific Cytotoxicity} = \frac{(\text{release with experimental cells} - \text{release with control cells})}{(\text{maximum release} - \text{control release})} \times 100$$

In NK assays, appropriate numbers of NK inactive thymocytes were used to obtain spontaneous release, while control spleen cells were used to obtain spontaneous release ^{in CTL assays}. In some experiments NK results are expressed as a ratio to controls, because of low and variable results in the controls.

Maximum release in all assays was obtained by incubating target cells with 10% Triton X. All assays were performed in quadruplicate and variability was normally <10% between wells.

Mixed lymphocyte reactions

Spleen cells to be used as stimulator cells were treated with 60 μg mitomycin C/ 10^7 cells/ml in RPMI/5% NCS for 30 mins at 37°C before being washed x 4 in RPMI 1640/5% NCS and resuspended at 2×10^7 cells/ml in RPMI 1640/5% NCS.

Responder cell populations were made up to a final concentration of 2×10^6 /cells/ml in RPMI 1640 + 5% FCS, pen/strep, L-Glu and 2 ME and 100 μl aliquots added to the wells of flat-bottomed microtitre plates (Linbro: Flow Labs). 2×10^5 Stimulator cells were then added in 10 μl to give a stimulator: responder ratio of 1:1 and the cells were cultured in a total volume of 200 μl for 96 hours at 37°C in 5% CO_2 in air. Cultures were pulsed with 1 μCi ^3H -Thymidine (Amersham International) for the last 18 hours of culture and the cell bound radioactivity was harvested with water using a Skatron multi-well cell harvester. After drying, the filters were added to 2 ml scintillation fluid (Ecoscint) and counted on a Packard liquid scintillation counter.

In all cultures, control wells were included which contained either responder or stimulator cells alone and the viability of responder cells was confirmed by culturing one aliquot in the presence of 20 $\mu\text{g}/\text{ml}$ ConA. All assays were performed in quadruplicate and variability between wells was normally $<15\%$. The stimulation index was given by the response of wells in the presence of stimulators divided by the mean value for the unstimulated responder cells.

Proliferative responses to Concanavain A in vitro

2×10^5 Spleen cells in RPMI 1640 supplemented with 10% FCS, L-Glu; pen/strep and 2-ME were cultured in a final volume of 100 μ l in flat-bottomed 96 well microtitre plates in the presence of 20 μ g/ml ConA. Cultures were performed at 37°C in 5% CO₂/air in a humidified incubator for 4 days.

In assays using mixtures of responder and "suppressor" cells, the responder stimulator ratio was maintained at 1:1 and different volumes of suppressor cells added to give a final volume of 200 μ l.

Statistics

Results are expressed as means \pm 1 standard deviation, unless otherwise stated. Student's t-test was used to compare differences between groups in most cases. In experiments where non-parametric distributions were observed such as mortality assays, results were compared by Wilcoxon's Rank Sum test.

Crypt cell production rates, calculated by linear regression were compared by covariance analysis to detect differences between the slopes. Calculations were performed on Casio PB-100 and 180P calculators.

Fixatives

<u>Clarkes</u>	1 litre
Ethanol	750 ml
Glacial acetic acid	250 ml
<u>10% Buffered formalin</u>	1 litre
Formaldehyde	100 ml
Distilled water	900 ml
NaH ₂ PO ₄ ·2H ₂ O	4 g
Na ₂ HPO ₄	6.5 g

Strain	Haplotype	K	A	E	D	"I-J"
CBA	k	k	k	k	k	k
BALB/c	d	d	d	d	d	d
C57B1/10	b	b	b	b	b	b
DBA/2	d	d	d	d	d	d
ATH	t ₂	s	s	s	d	s
ATL	t ₁	s	k	k	d	k
B10.A	a	k	k	k	d	k
B10.AKM	m	k	k	k	q	k
B10.AQR	y ₁	q	k	k	d	k
B10.A3R	i ₃	b	b	b	d	b
B10.A5R	i ₅	b	b	b	d	k

Table 1 H-2 haplotypes of mouse strains used in this thesis.

CHAPTER 3

INVESTIGATION OF THE MECHANISMS RESPONSIBLE FOR THE
ENTEROPATHY IN IRRADIATED MICE WITH GRAFT-VERSUS-
HOST REACTION

Introduction

The previous work which has been performed using intestinal Gvhr as a model of naturally occurring enteropathies has concentrated on the Gvhr in unirradiated adult or older neonatal mice. Under these circumstances, the principal intestinal features consist of crypt hyperplasia and increased IEL counts (Mowat & Ferguson, 1981b, 1982), both of which are found in clinical enteropathy (Mowat, 1984). Although these studies have indicated that a local DTH response is responsible for the intestinal features, this model of Gvhr is limited, as it does not reproduce the villus atrophy which is a major component of these clinical disorders. Therefore it is not clear whether the same immune mechanism causes villus atrophy.

It is well known that an acute lethal Gvhr in irradiated hosts does produce villus atrophy (Reilly & Kirsner, 1965), but the intestinal pathology in this model has not been investigated in detail. Furthermore, the immunological mechanisms responsible for the intestinal damage are unknown. Therefore the intestinal phase of Gvhr in irradiated mice was chosen as the first means of studying the immunopathology of an acute destructive enteropathy.

Experimental design

Adult (CBA x BALB/c) F_1 mice received 950 Rads irradiation and a Gvhr was induced by injection of 4×10^7 parental spleen cells i.v. At daily intervals, mice were sacrificed and the development of intestinal Gvhr was assessed by

measurements of mucosal architecture and IEL count. In parallel, anti-host CTL activity was assayed in the spleen as a measure of specific anti-host cell mediated effector activity using ^{51}Cr labelled P815^(H-2d) target cells. Splenic NK activity against ^{51}Cr labelled YAC-1 labelled target cells was also measured as an index of non-specific cell mediated functions which are recruited by anti-host DTH. In experiments examining cytotoxicity, control mice were either unreconstituted F_1 mice or irradiated CBA mice given CBA spleen cells to assess the normal behaviour of host and donor NK cells under these conditions.

Results

Progress of Gvhr in irradiated (CBA x BALB/c) F_1 mice

Irradiated (CBA x BALB/c) F_1 mice given 4×10^7 CBA spleen cells developed clinical signs of Gvhr within 4-5 days of cell transfer, including ruffled fur, diarrhoea and runting and all animals died on days 6-7. Irradiated CBA or F_1 mice given 4×10^7 syngeneic spleen cells as controls, survived indefinitely, while most irradiated F_1 mice which were left unreconstituted died within 20 days of irradiation.

Specific and non-specific cytotoxicity in irradiated (CBA x BALB/c) F_1 mice with Gvhr

The splenic NK activity of F_1 mice was virtually eliminated by the dose of irradiation used and remained very low throughout the 7 days of study (Fig. 1). Spleen NK activity was also absent in CBA hosts reconstituted with syngeneic CBA spleen cells, until 2-3 days after cell transfer, when a progressive recovery began. In contrast, significant

NK levels were directly apparent in Gvhr mice on day 1 and rose to a peak on days 2-3, when syngeneically reconstituted mice still had little or no NK activity. Thereafter, NK activity in Gvhr mice fell rapidly to zero after day 4.

Specific anti-host CTL activity did not appear in mice with Gvhr until day 3 and peaked on day 4, when NK activity was already markedly depressed (Fig. 1). CTL activity then declined as the clinical condition of the mice deteriorated. Thus, enhanced NK activity occurs very soon after inducing a Gvhr in irradiated mice and a marked decrease in NK levels coincides with the development of specific CTL activity as well as the onset of overt clinical disease.

Intestinal phase of Gvhr

The jejunum of syngeneically reconstituted F_1 mice showed few histological abnormalities after irradiation, other than the expected depletion of lymphoid cells. Mice with Gvhr had evidence of inflammatory cell infiltration by days 1-2 and after day 5, diffuse mucosal necrosis and complete villus atrophy precluded morphometric analysis in many specimens.

Intraepithelial lymphocyte counts

(CBA x BALB/c) F_1 mice reconstituted with syngeneic spleen cells showed a gradual rise in IEL count throughout the course of the experiment, reflecting the recovery of the mucosal lymphocyte population after irradiation (Fig. 2). F_1 mice with Gvhr had a significantly increased IEL count

compared with these controls by day 1 (12.8 ± 2.2 vs 7.9 ± 2.0 $\rho < 0.01$ and this rose to a maximum of 21.2 ± 2.8 vs 9.61 ± 2.90 in controls $\rho < 0.02$). Thereafter, the IEL counts in mice with Gvhr fell and, at later times, became difficult to estimate due to the evolving mucosal damage.

Mucosal architecture

The cytostatic effect of the irradiation was evidenced by the very low CCPR seen in syngeneically reconstituted F_1 mice, one day after irradiation (Fig. 3) (1.02 ± 0.2). Thereafter the CCPR in these mice recovered rapidly and this was paralleled by a progressive increase in crypt length (Fig. 4). Mice with Gvhr had a significantly increased CCPR on day 1 (Fig. 3) (5.8 ± 0.5 $\rho < 0.005$) and this rose to an extremely high level of 42.9 ± 4.0 on day 3 (vs 14.7 ± 3.3 for $F_1 \rightarrow F_1$ controls, $\rho < 0.02$). Thereafter, the deteriorating morphology made estimation of CCPR difficult in many samples. Nevertheless it could be shown that epithelial cell turnover ceased abruptly on day 4, when no normal metaphases were detectable at any time after colchicine. The Gvhr specimens suitable for analysis on day 5 also showed a very low CCPR which was significantly less than in control mice. In parallel with these changes in CCPR, mice with Gvhr had significant crypt lengthening on days 1 to 3 reaching a maximum of $207.8 \pm 16.0 \mu\text{m}$ on day 3 (Fig. 4) vs. $188.3 \pm 11.5 \mu\text{m}$ for $F_1 \rightarrow F_1$ controls, but had significantly lower crypt lengths than controls by day 4 ($108.2 \pm 10 \mu\text{m}$ vs $101.6 \pm 4.5 \mu\text{m}$, $\rho < 0.001$). Villus lengths remained virtually constant in

syngeneically reconstituted mice and in unreconstituted F_1 mice, while villus lengths in mice with Gvhr were similar to those in controls until day 5, when significant villus atrophy was observed (Fig. 5). Interestingly, this occurred after the sudden cessation of CCPR and no significant villus alterations were found before the initial increase in CCPR.

Therefore the intestinal phase of Gvhr in irradiated mice is characterised by an early, proliferative stage of crypt hyperplasia and increased numbers of IEL which precedes the fully developed picture of villus atrophy and mucosal destruction.

Active suppression of NK cell activity late in Gvhr

Earlier studies in unirradiated (CBA x BALB/c) F_1 mice with Gvhr showed that the majority of NK cell activity was due to cells of host origin (Mowat et al., 1985). Therefore it was possible that the loss of NK activity at later times in Gvhr mice was due to direct killing of F_1 NK cells by the coincidental appearance of anti-host CTL. Alternatively, the depressed NK activity could reflect an active, non-specific suppressor mechanism, as found in other models of Gvhr (Pickel & Hoffman, 1977; Hurtenbach & Shearer, 1983). I therefore performed a preliminary experiment to examine the ability of spleen cells from Gvhr mice to suppress NK cells of both CBA and F_1 origin.

Spleen cells from (CBA x BALB/c) F_1 mice on day 5 of Gvhr were mixed 1:1 with normal F_1 spleen cells and assayed

against YAC-1 target cells at a total E:T ratio of 50:1. As shown in Table 1, the NK activity of this mixture was considerably less than would be anticipated if the cytotoxicity obtained with either population alone at 25:1 had been added (7.8% vs. 18.4%). Suppression of normal NK activity was also found when Gvhr spleen cells were mixed with normal CBA spleen cells (8.5% vs. 13.4%). These results suggest that the depression of NK activity was not merely due to anti-F₁ CTL activity, but was due to a non-specific suppressor mechanism.

Summary and conclusions

The results presented here show that a Gvhr in irradiated mice produces a biphasic enteropathy, with each stage being associated with different forms of cell mediated immune effector activity.

The mucosal alterations which occurred in the first 2-3 days of Gvhr were characterised by increases in IEL and CCPR and were associated with enhanced splenic NK activity, but occurred in the absence of significant specific CTL activity. After the initial phase of crypt hyperplasia, a sudden inhibition of crypt cell production rate preceded the development of villus atrophy, which was paralleled by the appearance of specific anti-host CTL activity and by the clinical deterioration of the mice.

Therefore the induction of a Gvhr in irradiated hosts provides an experimental model of destructive enteropathy which is characterised by villus atrophy. The early stages of this enteropathy are similar to the changes found in

unirradiated hosts, with Gvhr, suggesting that a similar pathogenic mechanism may be responsible. Nevertheless, the results also show that the appearance of villus atrophy is associated with the generation of CTL. Although this could indicate that villus atrophy is due to a distinct mechanism, irradiated hosts develop specific CTL activity particularly readily in Gvhr and irradiation itself can have significant effects on the gut (Wiernik, 1966; Brill & Benner, 1985). Therefore, it would be important to confirm these findings in other models of Gvhr, which produce villus atrophy.

Cell source	% NK Activity		% Suppression (a)
	50:1	25:1	
Gvhr	4.9	4.6	
Control F ₁	18.9	13.8	
Gvhr + F ₁	7.8	-	58%
Control CBA	15.2	8.8	
Gvhr + CBA	8.5	-	37%

Table 1 Active suppression of NK cell activity in later stages of Gvhr in irradiated (CBA x BALB/c) F₁ mice.

Spleen cells from mice with Gvhr were assayed alone against YAC + target cells on day 5 of Gvhr or mixed with spleen cells from normal, unirradiated CBA or (CBA x BALB/c)F₁ mice. Results shown were obtained from cells pooled from 4-5 individual mice and are from one of three separate experiments with similar findings.

(a) Mixed populations contained each population at an effector: target cell ratio of 25:1 and % suppression was calculated by comparing the lysis obtained from the mixed population with that which would have been expected by summing the activity of each population alone at 25:1 E:T ratio.

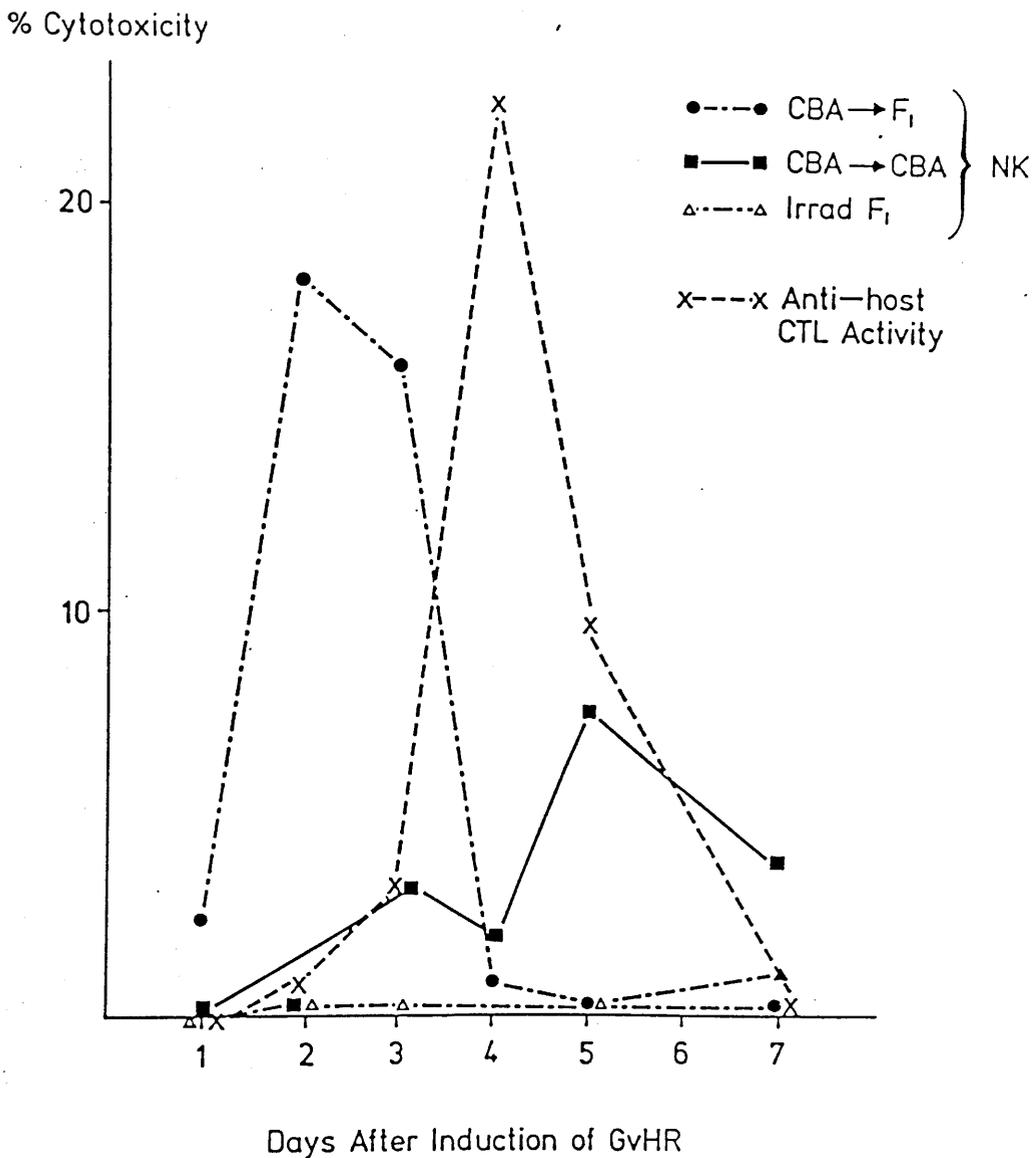


Fig. 1: Natural and specific cell mediated cytotoxicity during a Gvhr in irradiated (CBA x BALB/c)F₁ mice induced by 4 x 10⁷ CBA spleen cells. Spleen cells were pooled from 5-6 mice with Gvhr and assayed at 50:1 Effector:Target cell ratios against YAC-1 (NK cells) or P815 (anti-H^{-2D} CTL) target cells. In NK assays, control spleen cells were from 5-6 irradiated, un-reconstituted F₁ mice or syngeneically reconstituted CBA mice.

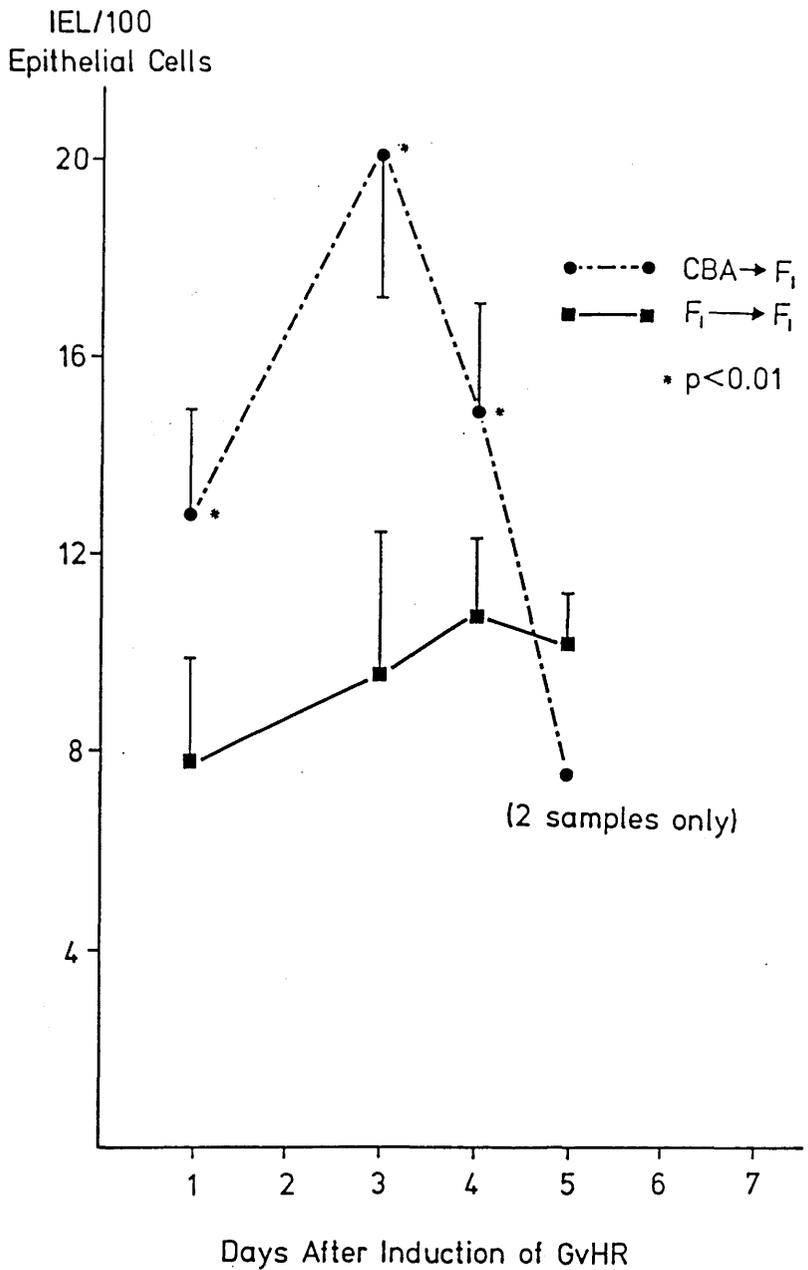


Fig. 2: Intestinal phase of Gvhr in irradiated (CBA x BALB/c)F₁ mice. Intraepithelial lymphocyte counts in the jejunum at intervals after transfer of 4×10^7 CBA or F₁ spleen cells. Results shown are mean IEL/100 epithelial cells \pm 1 standard deviation for 4-5 mice/group, unless otherwise stated.

Crypt Cell
Production Rate
(/crypt/hr)

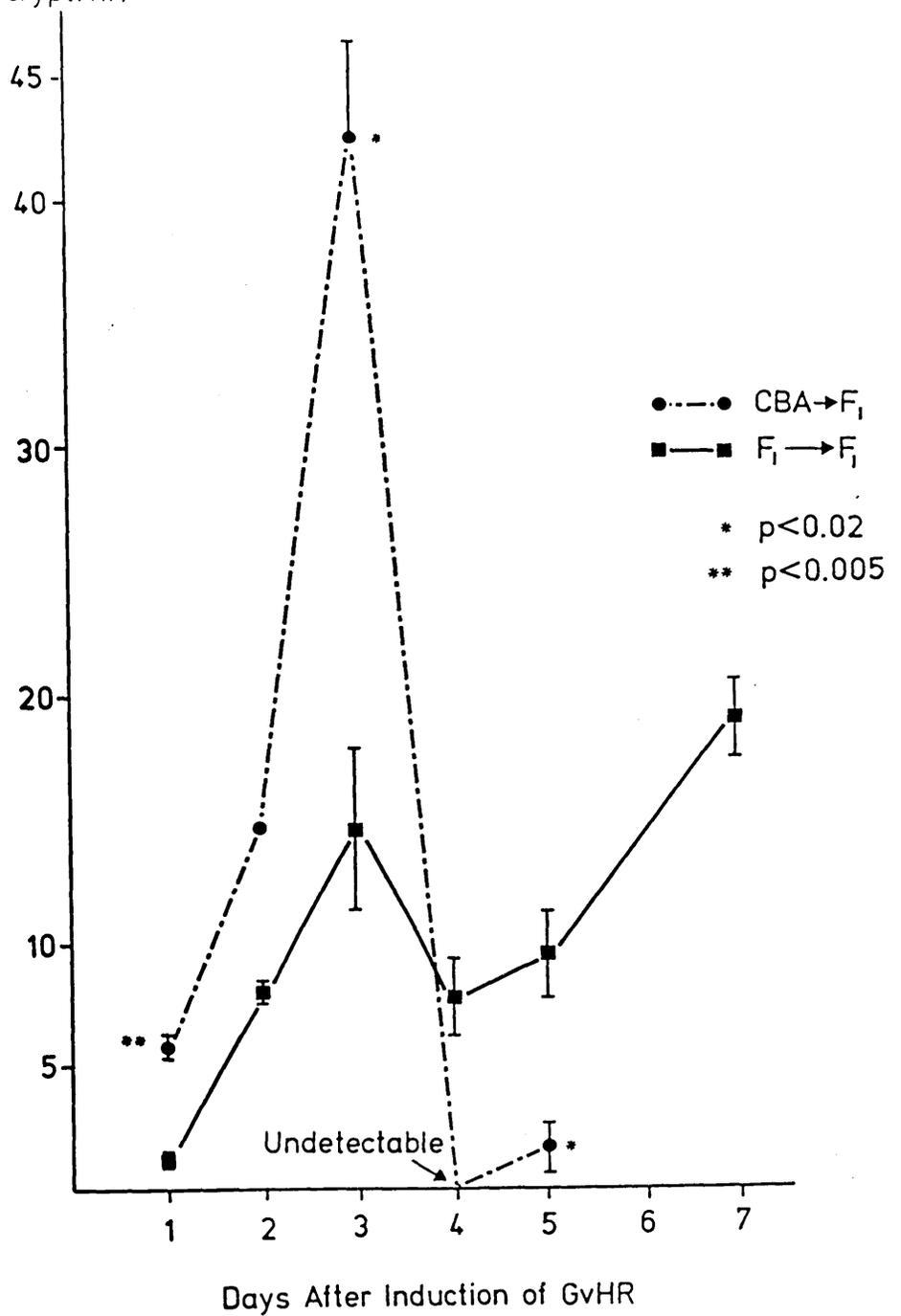


Fig. 3: Intestinal phase of Gvhr in irradiated (CBA x BALB/c)F₁ mice. Crypt cell production rates in the jejunum of mice with Gvhr and in syngeneically reconstituted F₁ mice. Results shown are means ± 1 standard deviation for 3-5 mice/group.

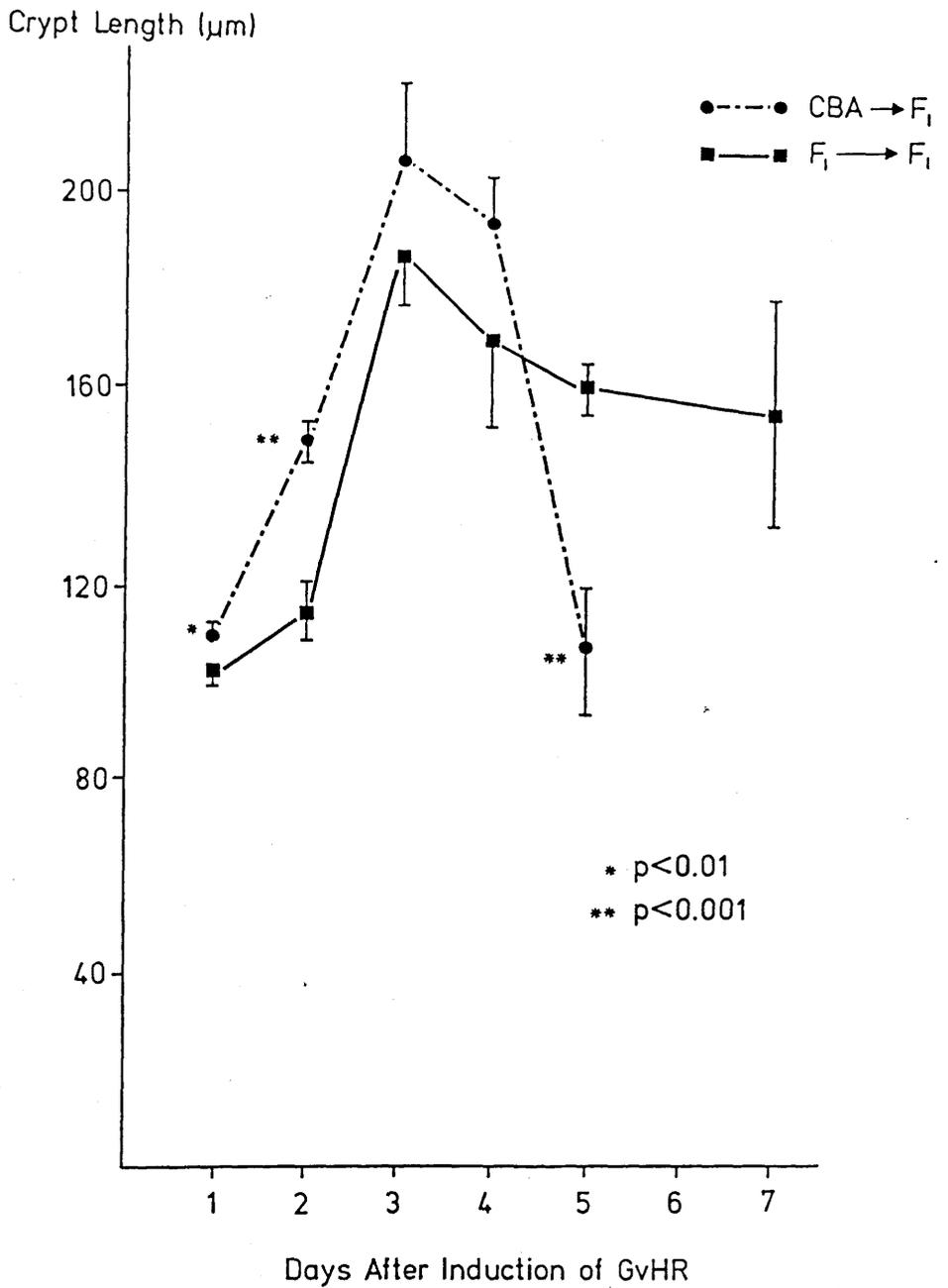


Fig. 4: Intestinal phase of Gvhr in irradiated (CBA x BALB/c)F₁ mice. Crypt lengths in the jejunum of F₁ mice with Gvhr and in syngeneically reconstituted F₁ mice. Results shown are mean lengths \pm 1 standard deviation for 3-5 mice/group.

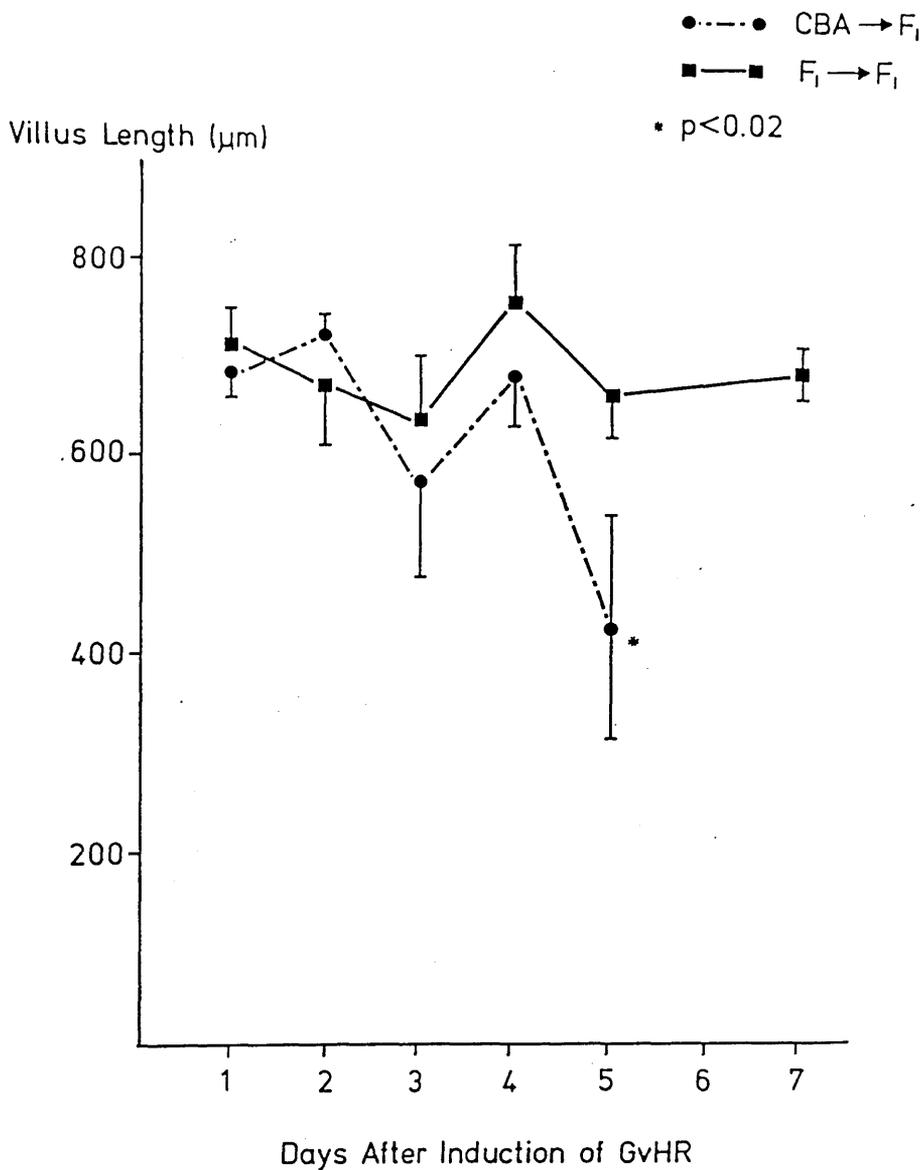


Fig. 5: Intestinal phase of Gvhr in irradiated (CBA x BALB/c)F₁ mice. Villus lengths in the jejunum of mice with Gvhr and in syngeneically reconstituted F₁ mice. Results shown are mean lengths ± 1 standard deviation for 3-5 mice/group.

CHAPTER 4

CORRELATION BETWEEN IMMUNE EFFECTOR MECHANISMS
AND THE TYPE OF ENTEROPATHY DURING A GVHR IN
NEONATAL MICE OF DIFFERENT AGES

Introduction

The results in the previous chapter showed that an acute, destructive Gvhr in lethally irradiated mice was associated with villus atrophy and the parallel development of specific CTL activity. However, in view of the effects irradiation has on the small intestine it was possible that the severe enteropathy was an artefact of the use of irradiation. Furthermore, irradiated hosts do not provide a flexible model in which a spectrum of intestinal damage and immune effector responses can be produced.

Earlier reports have shown that a Gvhr in neonatal mice can produce an enteropathy which may or may not include villus atrophy, apparently depending on the age of the host animals (MacDonald & Ferguson, 1977; Mowat & Ferguson, 1982). However, the conditions which influence the type of enteropathy have not been defined and the cell mediated effector mechanisms have not been studied. Therefore, the aim of this chapter was to test the hypothesis that a Gvhr in neonatal mice of different ages would produce different forms of intestinal pathology in association with different types of CMI. In this way, it would be possible to generate varying severities of enteropathy in the same mouse strain and using the same donor cell inoculum.

Experimental design

A Gvhr was induced in neonatal (CBA x BALB/c) F_1 mice within the first 48 hours of life or at 5 or 7 days of age

by injection with 10^7 CBA parental spleen cells. The development of systemic Gvhr was assessed by regular weighing, noting developmental changes, such as the appearance of fur and eye-opening and by measuring the haematocrit as an assay for anaemia.

The immunological changes were assessed by measuring splenomegaly, as well as specific and non-specific cytotoxicity and measurement of immune responsiveness in Gvhr mice. In parallel, anti-host DTH and mucosal architecture was also studied.

Results

The relationship between intestinal pathology and immune effector mechanisms was examined by inducing a Gvhr in (CBA x BALB/c) F_1 mice aged 48 hours or less, or on the 5th or 7th day of life. Up to four individual experiments were conducted on these 3 groups of neonatal mice with essentially identical findings within each group. Therefore, for simplicity, only the results of one typical experiment from each series are presented.

Progress of systemic Gvhr in neonatal (CBA x BALB/c) F_1 mice

(CBA x BALB/c) F_1 mice given 10^7 CBA spleen cells before 48 hours of age developed an acute Gvhr, which was characterised by runting, diarrhoea and ruffled fur. These mice matured less rapidly than littermate controls with delayed appearance of body fur and later opening of the eyes. Gvhr mice began to lose weight rapidly after day 8 and in most experiments all died within 21 days of inducing the

Gvhr (Fig. 1A). In contrast, littermate controls grew continuously throughout the study and deaths were rare. These mice with Gvhr had significant splenomegaly from day 8 until day 18 (Fig. 1B), and this was particularly notable in view of the marked weight loss which occurred at the later stages of the Gvhr. However, a frequent finding in some of the most severely runted mice was the development of an involuted, shrunken spleen immediately before death. This phenomenon is evidenced by the large variation in spleen index seen at later times in this experiment. In parallel, these mice became anaemic by day 12 (Table 1) (haematocrits: 29.7 ± 2.4 vs 36.2 ± 4.7 in controls $p < 0.05$) and this became more severe, as mice continued to runt.

Finally, most of these mice developed macroscopic evidence of liver damage and showed lymphocytic portal infiltrates followed by diffuse necrotic changes on histological examination.

In contrast to these findings, mice injected with parental spleen cells at 7 days of age did not develop a lethal Gvhr. There was no evidence of weight loss or runting and both control and Gvhr mice developed at the same rate (Fig. 2A). However, these Gvhr mice developed intense splenomegaly which first appeared on day 9 with a maximum spleen index of 3.40 ± 0.90 on day 13, falling to 1.34 ± 0.67 by 30 days (Fig. 2B). No anaemia (Table 1) or macroscopic evidence of liver necrosis was found in these mice, although histological sections showed occasional portal infiltrates.

The Gvhr in 5 day old mice was a more variable disease, whose outcome was usually midway between the acute, lethal disease found in 1-2 day old mice and the entirely proliferative disease in 7 day old animals. Most 5 day old neonates with Gvhr developed normally until day 14 with little or no evidence of weight loss compared with controls (Fig. 3A). Thereafter, some animals showed evidence of clinical runting, weight loss and diarrhoea and a small proportion of these mice died, usually between days 17 and 24 of the Gvhr. Nevertheless, other mice in the same group continued to grow normally with no evidence of runting. Five day old mice with Gvhr developed moderate splenomegaly which peaked around day 13 (Fig. 3B). Interestingly, by day 18, mice which developed signs of runting had less splenomegaly than their non-runting littermates with Gvhr, although by day 24, the spleen index in both groups had fallen. As in 1 day old hosts, severely runted mice usually had splenic atrophy.

After day 18 in experiments using 5 day old hosts, samples from mice which lost weight were processed separately from those of non-runting littermates and the results are presented separately in subsequent sections.

Thus, there were clear differences between the systemic Gvhr which could be induced in neonatal mice during the first 7 days of life and I next investigated whether these differences reflected different immune effector mechanisms.

Development of specific and non-specific cytotoxicity
in neonatal hosts with Gvhr

In the experiment using 1-2 day old mice, control animals showed little or no splenic NK activity throughout the course of the study, confirming other studies of NK cell maturation (Murgita & Wigzell, 1981; Koo et al., 1982). In contrast, mice with Gvhr had a small but consistent peak of NK activity on day 5, which declined over the next few days, although detectable levels were present until day 11 (Fig. 1B). Coinciding with this loss of NK activity, specific anti-host cytotoxicity appeared at 8-9 days and increased rapidly to a peak of 13.5% by day 11 (Fig. 1B). Thereafter, as the clinical condition of the Gvhr mice deteriorated in the third week, only very low levels of specific CTL activity were found.

In the studies of Gvhr induced in 7 day old mice, spleen NK activity appeared in controls by days 10-11 (17-18 days of age) and continued to increase steadily thereafter (Fig. 2B). By day 5, mice with Gvhr already had detectable NK activity and had consistently higher NK activity than controls until day 18. By day 30, adult levels of NK activity had developed in both the Gvhr and control mice. Little or no specific CTL activity could be detected at any time during the Gvhr in these mice (Fig. 2B).

When 5 day old mice were used as hosts for Gvhr, NK activity appeared in controls around days 10-11, while in mice with Gvhr, some NK activity was detectable by day 5 and was markedly increased compared with controls by day 10 (12.2% vs. 5.4%). (Fig. 3B). Thereafter the pattern of

NK activity in Gvhr mice depended on whether mice developed overt runting. Thus, NK activity in Gvhr mice which did not lose weight developed in parallel with that in controls while in mice which runted, NK activity became less than controls (day 18: 2.5% vs. 6.6%) and eventually disappeared completely. Interestingly, only a low level of specific CTL activity was present in either group of mice on day 10 of the Gvhr and was never found thereafter (Fig. 3B).

Thus, the development of acute lethal Gvhr in 1-2 day old mice is associated with a transient enhancement of NK activity, followed by the development of marked specific CTL activity which parallels the onset of clinical disease. In contrast, 7 day old mice with Gvhr have prolonged activation of NK cell activity, but no specific CTL are detectable. The intermediate form of Gvhr in 5 day old mice showed some features in common with both acute and proliferative Gvhr, although specific CTL activity was not a prominent feature.

Induction of anti-host DTH in adult and neonatal mice with Gvhr

These findings indicate that early neonatal mice suffer a much more severe systemic Gvhr than mice even a few days older. As this was also associated with marked differences in the development of cell mediated cytotoxicity in host mice, it was of interest to examine another form of anti-host immune response in these hosts.

Anti-host DTH activity in Gvhr can be assayed by measuring the increment in footpad thickness 24 hours after local transfer of spleen cells from mice with Gvhr into mice syngeneic with the donor of parental cells (Mowat et al., 1985). Therefore, I used this approach to assess the presence of anti-host DTH during a Gvhr in 1 day, 6 day and adult (CBA x BALB/c) F_1 mice.

10^7 spleen cells taken from adult mice 1 day after induction of a Gvhr with 6×10^7 CBA spleen cells, produced a significantly greater increase in footpad thickness, in CBA mice than that found after transfer of control F_1 cells (Fig. 4) (specific increase: 0.25 ± 0.08 mm vs. 0.07 ± 0.04 mm, $\rho < 0.01$). A similar, but smaller anti-host DTH response was still present in the spleens of adult mice on day 7 of the Gvhr (0.12 ± 0.05 mm vs. $0.02 \pm .0$ mm, $\rho < 0.005$). In contrast, spleen cells taken from 1 day old mice were unable to transfer an anti-host DTH response on either day 1 or day 7 of Gvhr, with similar footpad swellings found in CBA mice after injection of Gvhr and control spleen cells (Fig. 4).

A separate experiment was performed to compare the DTH response during a Gvhr in 6 day old and adult F_1 mice. Once again, adult cells taken on day 1 of Gvhr induced significantly greater increases in footpad thickness in CBA mice, compared with that found after transfer of control F_1 cells (Fig. 4) (0.27 ± 0.06 mm vs. 0.20 ± 0.3 mm, $\rho < 0.05$). Spleen cells from 6 day old mice with Gvhr also transferred

a significant anti-host DTH response, although this was less than that found with adult cells (Fig. 4).

Thus 1 day old neonatal mice do not induce the anti-host DTH response found during a Gvhr in more mature hosts, despite the more aggressive disease which occurs in 1 day old hosts. Interestingly, the ability to induce an anti-host DTH response seems to appear around 6 days, the time at which the nature of the Gvhr also altered.

Ability of Gvhr cells to proliferate in response to fully allogeneic spleen cells in vitro

Gleichmann has suggested that the development of an acute Gvhr is associated with the generation of Ts activity (Gleichmann et al., 1984). Therefore it was of interest to determine whether neonatal mice with Gvhr had reduced immune responses and if this was due to active suppression.

In this preliminary study, a Gvhr was induced in 1-2 day old (CBA x BALB/c) F_1 hosts and spleen cells were taken from control and Gvhr neonatal mice on days 8 and 15 of the experiment and tested for their ability to proliferate in response to 3rd party allogeneic stimulator cells. At both times, Gvhr cells responded less well than control cells to mitomycin C treated C57Bl/10 stimulator cells (Fig. 5). In addition, if Gvhr and control cells were mixed (at a ratio of 1:1), the response of the mixed cultures was less than that of control cells alone (Fig. 5).

These results are preliminary evidence that mice with an acute Gvhr induced at 1-2 days old have evidence of active immunosuppression. Unfortunately, lack of time prevented me confirming these studies or extending them to older mice with Gvhr.

Mucosal phase of Gvhr in neonatal hosts of different ages

I next examined whether the differences in systemic disease and immune responsiveness during a Gvhr in neonatal mice of different ages would be reflected in different forms of intestinal Gvhr.

Intestinal Gvhr in 1-2 day old hosts

Villus atrophy was a consistent feature of the Gvhr in 1-2 day old neonates and was already present when the mice first began to runt on day 9 (Fig. 1C) ($409.4 \pm 16 \mu\text{m}$ vs. $563.3 \pm 11 \mu\text{m}$ for controls, $p < 0.001$).

This became even more marked by day 15, when all the mice with Gvhr were extremely ill and had significant CTL activity. At both times, mice with Gvhr had significantly longer crypts than controls (Fig. 1C) ($90.6 \pm 6 \mu\text{m}$ vs. $57.2 \pm 6 \mu\text{m}$, $p < 0.001$ on day 9 and $162.5 \pm 35 \mu\text{m}$ vs. $80.5 \pm 6 \mu\text{m}$, $p < 0.005$ on day 15). In parallel, mice with Gvhr had a moderate, but statistically insignificant increase in crypt cell mitotic activity at the earliest time examined (Table 2) (7.50 ± 1.15 vs. 4.2 ± 0.5 for controls), but thereafter, Gvhr mice had levels of CCPR which were roughly half those in control mice.

Intestinal Gvhr in 7 day old hosts

No evidence of villus atrophy was found at any time during the Gvhr induced in 7 day old mice (Fig. 2C+H) However these mice had significant crypt lengthening compared with controls from day 9 (Fig. 2C) ($85.6 \pm 11 \mu\text{m}$ vs. $44.8 \pm 3.8 \mu\text{m}$ for controls $p < 0.01$) until day 30 ($152.5 \pm 10 \mu\text{m}$ vs. $113.9 \pm 9 \mu\text{m}$ for controls $p < 0.02$). In addition these animals had a significantly enhanced CCPR compared with controls during the same period (Table 2).

Intestinal Gvhr in 5 day old hosts

This group of mice developed a form of intestinal Gvhr which had features in common with both the proliferative and destructive enteropathies described above. On day 13, the fact that some Gvhr animals would runt was already reflected in a slight overall decrease in villus length compared with controls (Fig. 3C) ($400.6 \pm 27 \mu\text{m}$ vs. $466.6 \pm 11.5 \mu\text{m}$, $p < 0.05$). Thereafter, runting mice developed progressive villus atrophy which became extremely marked by day 24 ($346.3 \pm 10 \mu\text{m}$ vs. $625.4 \pm 44 \mu\text{m}$ for controls $p < 0.001$). Mice which did not runt had no evidence of villus atrophy until day 24, when, although their villus lengths were significantly shorter than controls, these were still significantly greater than their runting littermates ($453.0 \pm 32 \mu\text{m}$, $p < 0.02$). All Gvhr mice had crypt lengthening on day 13 and this was maintained in non-runting mice until day 24 (Fig. 3C) ($153.2 \pm 50 \mu\text{m}$ vs. $128.7 \pm 23 \mu\text{m}$ for controls). In contrast, on day 18, crypt lengths in

runting mice were no greater than those of controls and by day 24, crypt lengths in runting mice were shorter than control values ($93.6 \pm 5.5 \mu\text{m}$ vs. $128.68 \pm 23 \mu\text{m}$ for controls). In parallel, all mice with Gvhr had an increased CCPR on days 5 and 10 (Table 2) and, in non-runting mice, this became even more marked by day 24 (35.0 ± 0 vs. 9.5 ± 1.73 , $p < 0.02$). At this time crypt cell mitotic activity was absent in runting mice with Gvhr.

Intraepithelial lymphocyte counts in neonatal mice with Gvhr

Mice which had a Gvhr induced at one day old had slightly higher IEL counts than controls of a similar age on days 5 and 11, and this was also present in the only surviving mouse examined on day 15 (Table 3) (6.1 vs. 3.6 in controls).

It should be noted that at all times very few IEL were counted in these mice. In addition, these differences were not statistically significant and were small compared with previous reports (Mowat & Ferguson, 1982). Nevertheless, similar results were obtained in mice which received donor cells at 5 days of age where Gvhr mice had increased IEL counts on days 10 (Table 3) (3.8 ± 0.3 vs. 2.0 ± 0.4 , $p < 0.01$) and 18 (4.1 ± 0.2 vs. 3.7 ± 0.3). Mice with a Gvhr induced on day 7 also had increased IEL counts on days 5, 13 and 17, although again the differences were small (Table 3).

Therefore the acute systemic Gvhr in very young neonatal mice was paralleled by severe intestinal damage which was characterised by both crypt hyperplasia and villus atrophy.

In contrast, the 7 day old mice with Gvhr which did not die, developed only the proliferative enteropathy which has been described previously in adult mice, with crypt hyperplasia, but no villus atrophy. Interestingly, 5 day old mice developed a Gvhr with some of the systemic and intestinal features of both the other groups of mice, and therefore seem to represent an intermediate group.

Effect of donor cell number on the outcome of Gvhr in neonatal hosts

The experiments described above used an arbitrary number of donor cells, which were not corrected for body weight. Therefore one explanation for the more severe Gvhr in one day old mice could be that these mice effectively received a larger dose of donor cells. Therefore, I examined whether the nature of the Gvhr in 1 day old mice would be different if they received a lower number of donor cells.

At one day old, mice weighed about 1.5 g while 7 day old mice weighed about 3 g. Therefore, to examine the importance of donor cell numbers, I compared the Gvhr which occurred in one day old mice given either 5×10^6 or 10^7 donor cells.

Progress of systemic Gvhr

One day old mice which received 0.5×10^7 or 10^7 donor spleen cells began to lose weight and develop clinical signs of Gvhr at a similar rate (Fig. 6A) and there was no difference in their mortality rate. Both groups of mice with Gvhr had significant splenomegaly by day 11, with spleen indices of

2.53 ± 0.34 and 2.75 ± 0.50 in recipients of 1×10^7 and 0.5×10^7 cells respectively (Fig. 6B).

Specific cytotoxicity was found in all Gvhr mice on day 11, although the CTL activity in recipients of 10^7 donor cells was double that of mice given 0.5×10^7 cells (Fig. 7A). A small amount of NK activity was also found in all Gvhr mice at this time, but this had disappeared by day 15 (Fig. 7A). Levels of specific cytotoxicity, although lower than on day 11, were still found at the later time.

Mucosal phase of Gvhr

One group of these mice was killed on day 15 for assessment of intestinal Gvhr, when both Gvhr groups had significant crypt lengthening and villus atrophy compared with controls. Interestingly, despite the higher levels of CTL activity in recipients of 10^7 cells, there was no significant differences in the villus or crypt lengths in mice given 10^7 or 0.5×10^7 cells (Fig. 7B).

Together these results show that halving the dose of donor cells does not alter substantially the consequences of Gvhr in neonatal mice and so indicate that the different forms of Gvhr in 1 or 7 day old hosts is not merely because one day old mice received a larger number of cells.

Therefore these differences in Gvhr must be a consequence of the relative maturity of neonatal mice of different ages. The next experiments were undertaken to investigate some of the other differences in the neonatal immune system which could account for this maturational effect.

Comparison of allospecific DTH responses induced by neonatal and adult spleen cells

The ability of neonatal mice to induce an anti-host DTH response appeared to correlate with the different outcome of Gvhr and suggested that one difference between mice of different ages might be their ability to activate certain types of donor effector cells. Therefore in the first experiments on the maturity of the neonatal immune system, I examined the ability of neonatal and adult F_1 cells to provoke an allospecific DTH response in CBA mice.

In the first experiment, CBA mice were primed intradermally with either adult or neonatal (< 5 days) (CBA x BABL/c) F_1 spleen cells and 5 days later mice were challenged into opposite footpad with 10^7 adult or neonatal F_1 spleen cells. Mice primed and challenged with adult F_1 cells showed a specific increase in footpad thickness of 0.47 ± 0.10 mm ($p < 0.001$) compared with that found after challenge of immunised mice (Fig. 8). In contrast, mice primed with neonatal spleen cells only showed a specific increase of 0.22 ± 0.10 mm after challenge with neonatal spleen cells ($p < 0.02$ vs. adults).

The second experiment was designed to establish whether neonatal cells were deficient at inducing either the afferent or efferent limb of the DTH response. This was particularly important in view of reports that the neonatal spleen contains a large number of non-specific suppressor cells which could potentially inhibit the recall phase of the DTH response when used as a challenge inoculum.

Thus, mice were again primed with either adult or neonatal spleen cells, but on this occasion, all mice were challenged with adult F_1 spleen cells. Under these conditions, the DTH response in mice primed with neonatal cells was greater than that shown above, but was still significantly reduced compared with that found in mice primed with adult cells (Fig. 8) (0.35 ± 0.10 mm vs. 0.53 ± 0.12 mm for adults $p < 0.05$). These results indicate that the poor ability of newborn spleen cells to induce a DTH response is primarily due to a failure to sensitise parental T_{DTH} and are consistent with the poor anti-host DTH response found during a Gvhr in these mice.

Influence of age on the ability of donor spleen cells to induce a local Gvhr

The next series of experiments were performed to establish the degree of immunocompetence of neonatal mouse cells in vivo. This was addressed by inducing a local Gvhr in (CBA x BALB/c) F_1 mice by footpad injection of 2×10^7 spleen cells from either adult or neonatal CBA mice. The degree of local Gvhr was then measured by weighing the draining PLN 8 days later.

Spleen cells from < 5 day old CBA mice induced only a small increase in popliteal lymph node weight compared with that found after injection of medium alone (Fig. 9) (7 ± 10 mg vs. 3 ± 1.4 mg). In contrast, adult CBA cells caused a very marked enlargement of PLN (46.9 ± 16 mg vs. 9.6 ± 3 mg, $p < 0.001$) in a separate experiment spleen cells

from 8 day old mice induced an increase in PLN weight that was similar to that found with adult spleen cells (46.8 ± 11 mg and 46.4 ± 13 mg respectively).

These results indicate that one day old mice have markedly deficient allospecific T-cell activity and as host T-cells have been reported to be one component of resistance against Gvhr, I performed a preliminary study to examine whether T-cell deficiency was responsible for the severe Gvhr in one day old neonates.

Effect of reconstituting one day old neonatal mice with adult spleen cells

A Gvhr was induced in one day old (CBA x BALB/c) F_1 mice with 10^7 CBA spleen cells as before, but in this experiment, two groups of Gvhr mice were given either 10^7 unpurified or T cell enriched spleen cells from syngeneic F_1 adults, at the time of inducing the Gvhr.

All the Gvhr mice began to lose weight after day 8 of the Gvhr and although this was slower in the mice which had received adult T-cells enriched spleen cells, the differences were not significant (Fig. 10). By day 14 all of the mice with Gvhr had developed clinical signs of Gvhr and runting, and one mouse with Gvhr, which received no adult cells had died. By day 20 of the experiment, a similar proportion of the mice in all the groups of Gvhr mice had died (Fig. 10).

The results of this experiment do not show any clear difference in the severity of Gvhr in mice given adult syngeneic spleen cells.

Summary

This chapter has shown that the systemic and intestinal consequences of a Gvhr in neonatal mice depend on the age of the host used. Neonatal (CBA x BALB/c) F_1 mice given 10^7 CBA spleen cells at less than 48 hours old, developed an acute destructive Gvhr, typified by runting and severe intestinal damage, which included villus atrophy. These animals also developed marked specific anti-host CTL activity and invariably died. Preliminary studies indicated that these mice also had active suppression of immune responses. In contrast, 7 day old F_1 mice given the same number of donor cells developed a proliferative Gvhr, characterised by intense splenomegaly, prolonged NK cell activation and crypt hyperplasia. These mice did not lose weight, had no villus atrophy or specific CTL activity and all recovered. A similar proliferative phase of splenomegaly, transient NK cell activation and crypt hyperplasia preceded the established destructive Gvhr in 1-2 day old hosts. Induction of a Gvhr in 5 day old hosts produced a disease which had some characteristics of both the proliferative and destructive forms of Gvhr. Thus, all mice had early proliferative features but some mice then lost weight and developed villus atrophy. In contrast, others did not lose weight or develop villus atrophy, but had prolonged crypt hyperplasia and NK cell activation. However, an important feature was that very little specific CTL activity was found in any of these animals, irrespective of whether they had a destructive Gvhr or not.

The differences in the outcome of Gvhr were not due to an effect of relative donor cell numbers, as weight related adjustment of the donor cell inoculum only produced some increase in specific CTL activity, but had no significant effect on the systemic or intestinal consequences of the Gvhr.

Subsequent studies showed that, in comparison with adult cells, spleen cells from 1 day old F_1 mice could not induce an efficient allospecific DTH response in vivo. Furthermore, an anti-host DTH response did not occur in one day old F_1 mice after induction of a Gvhr with parental cells, whereas 6 day old F_1 mice were capable of inducing almost as much anti-host DTH as adult hosts. These results suggest that very young neonatal mice may activate different forms of effector T-cell than those induced in older hosts.

2-3 day old mice also had defective allospecific T-cell function as measured by the ability of their spleen cells to mediate a local Gvhr in F_1 hosts. In contrast, cells from 8 day old mice induced a local Gvhr which was equal to that caused by adult cells. However, it proved impossible to prevent the severe Gvhr in very young neonatal mice by reconstituting with syngeneic adult spleen cells.

Conclusions

This chapter confirms that a destructive enteropathy which includes villus atrophy can be reproduced by a Gvhr in neonatal F₁ mice, providing the hosts are less than 6-7 days of age. As in irradiated mice with Gvhr, specific CTL activity was frequently associated with the development of an acute intestinal Gvhr. However, villus atrophy occurred in the absence of CTL in 5 day old mice. These findings indicate that CTL may not be essential for the development of villus atrophy. It would be important to confirm these findings in a model of acute Gvhr which uses intact mature hosts. Finally, this study has highlighted the potential importance of host immunocompetence in modifying the Gvhr and this issue will be addressed further in Chapter 6.

Age of host (days)	Day of Gvhr	Haematocrit	
		control	Gvhr
1	8	41.8 \pm 1.3	41.9 \pm 4.9
	12	36.2 \pm 4.7	29.7 \pm 2.4*
	16	37.8 \pm 3.7	25.8 \pm 0.5**
7	7	36.8 \pm 1.6	34.8 \pm 2.1
	20	45.8 \pm 2,4	47.3 \pm 4,2

Table 1: Assessment of haematocrit in neonatal (CBA x BALB/c) F_1 mice with Gvhr and in littermate controls. Results shown are means \pm 1 standard deviation for 5 mice per group, in each experiment.
* p < 0.05; ** p < 0.005.

Age of host (days)	Day of Gvhr	Crypt Cell Production Rate	
		Control	Gvhr
1-2	5	4.12 ± 0.50	7.50 ± 1.15
	7	5.80 ± 0.52	11.81 ± 2.80**
	9	8.80 ± 4.10	4.38 ± 1.32
	15	12.73 ± 2.50	6.15 ± 4.04**
5	5	0.69 ± 0.43	5.25 ± 0.64**
	10	3.30 ± 1.12	7.81 ± 1.78
	24	15.10 ± 1.73	35.25 ± 0
7	9	3.24 ± 1.0	8.65 ± 0.99**
	18	6.98 ± 1.50	13.20 ± 1.66*
	24	11.80 ± 0	18.22 ± 2.08

Table 2: Crypt cell production rate in the jejunum of neonatal (CBA x BALB/c)F₁ mice with Gvhr, and in littermate controls. Results shown are means ± 1 standard deviation for four mice per group.
 * p < 0.05; ** p < 0.02; *** p < 0.001.

Age of host (days)	Day of Gvhr	IEL/100 Epithelial Cells	
		Control	Gvhr
1-2	5	2.6 ± 0.5	3.2 ± 0.4
	11	1.5 ± 0.2	3.3 ± 1.2
	15	3.6	6.1
5	4	2.9 ± 0.3	2.8 ± 0.9
	10	2.0 ± 0.4	3.8 ± 0.3***
	18	3.7 ± 0.3	4.1 ± 0.2
	24	8.4	7.6
7	5	2.5 ± 0.5	3.8 ± 0.5
	13	6.0 ± 0.6	7.1 ± 0.7
	18	5.6 ± 0.4	7.2 ± 0.4**
	30	8.6 ± 0.8	6.7 ± 0.3*

Table 3: IEL counts in the jejunum of neonatal (CBA x BALB/c)_F₁ mice with Gvhr, and in littermate controls. Results shown are means ± 1 standard deviation for either 3 or 4 mice per group.
 * p < 0.05; p < 0.025; *** p < 0.01.

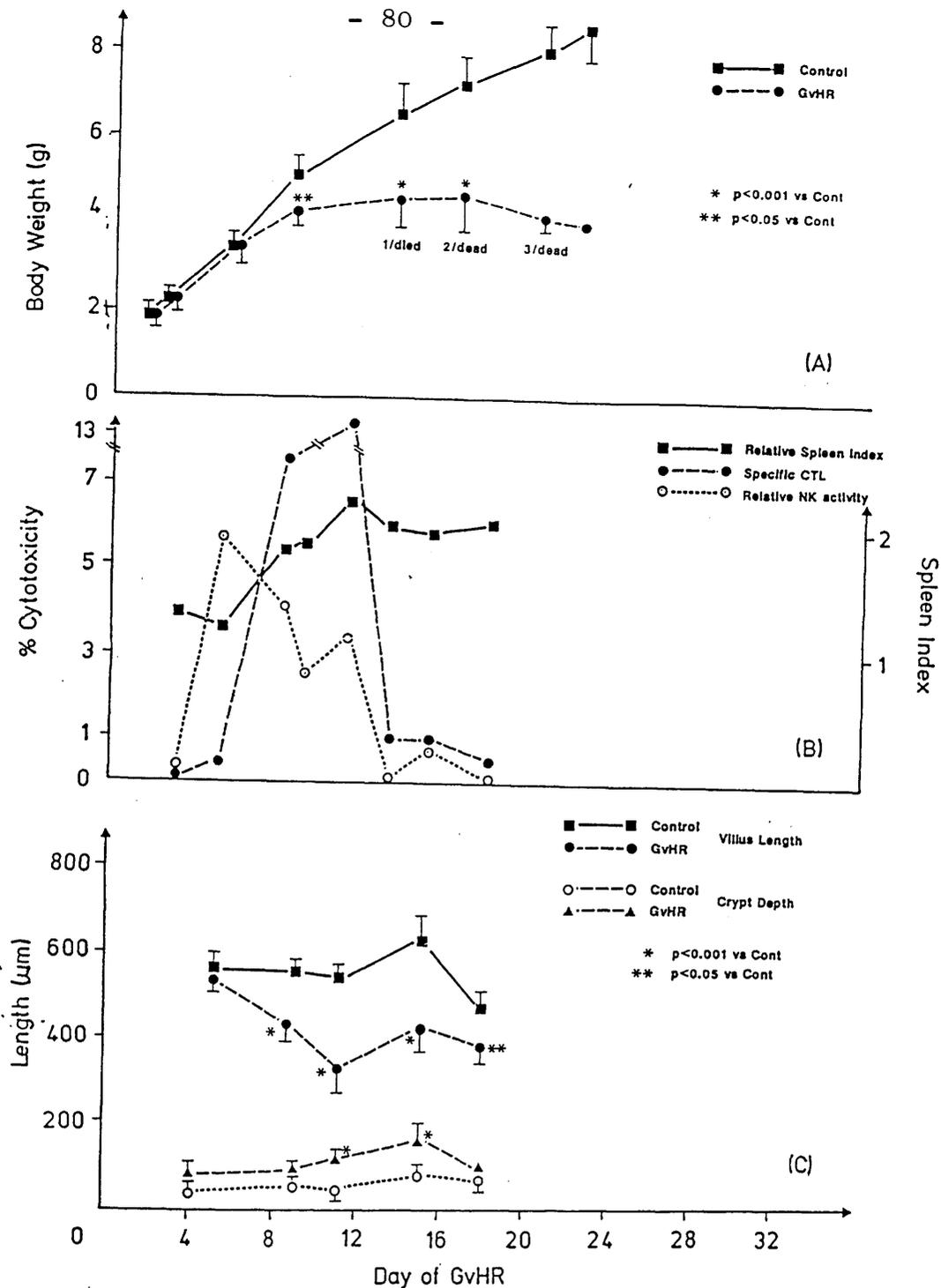


Fig. 1: Development of Gvhr in 1-2 day old neonatal (CBA x BALB/c) F_1 mice. A) Growth rate of neonates with Gvhr compared with littermates controls. B) Spleen index, NK activity and specific CTL activity against YAC and P815 target cells respectively. C) Mucosal architecture in jejunum of mice with Gvhr and in controls. Results shown are means \pm 1 s.d. for 3 mice/group. Specific CTL activity is shown as the % lysis, while NK activity in Gvhr mice is expressed as a ratio to control values. All cytotoxicity results are 50:1 E:T for spleen cells pooled from 3 mice/group.

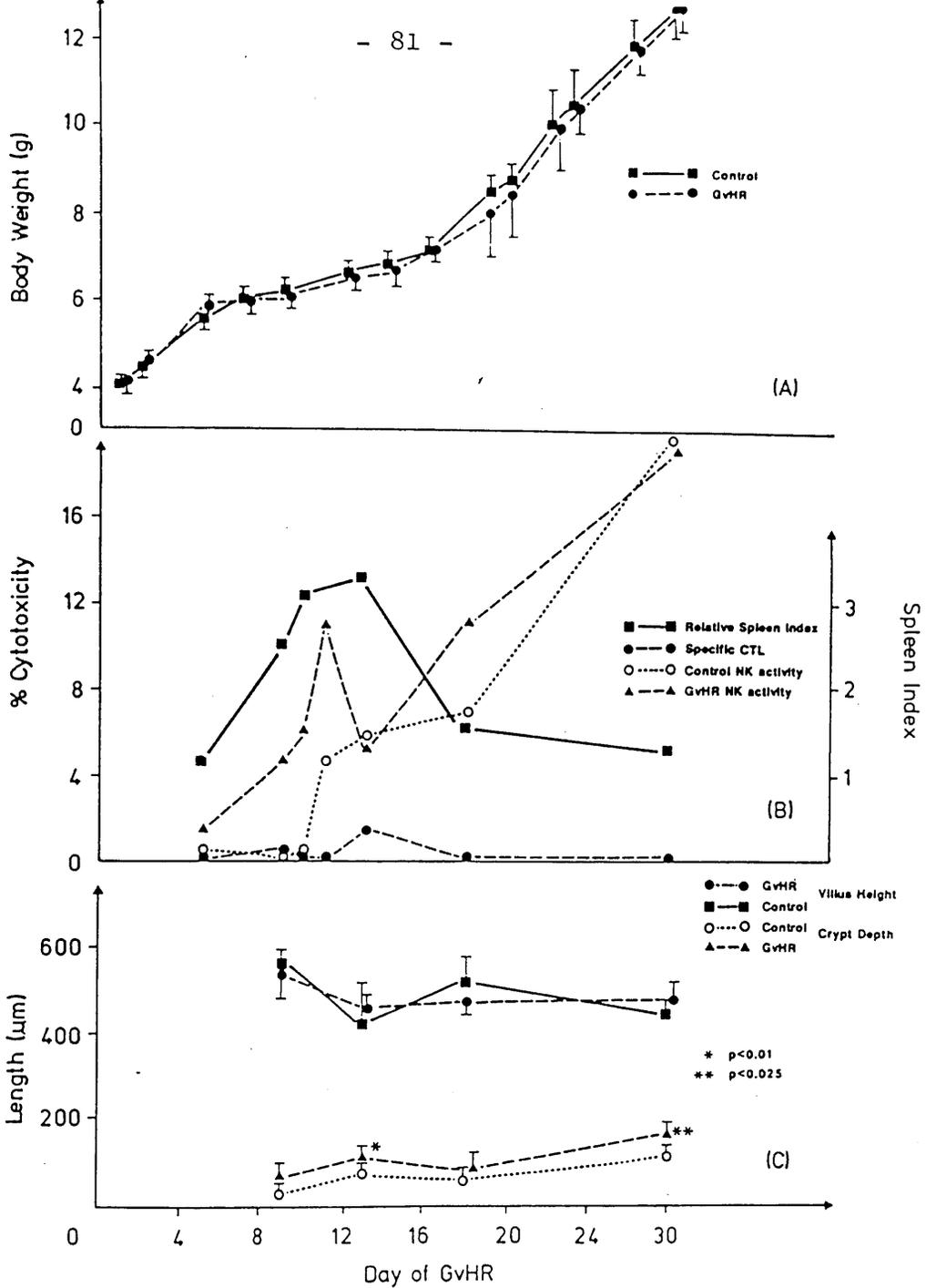


Fig. 2: As for Fig. 1 - except NK cytotoxicity results are shown as the lysis 50:1 E:T for spleen cells from control and Gvhr mice.

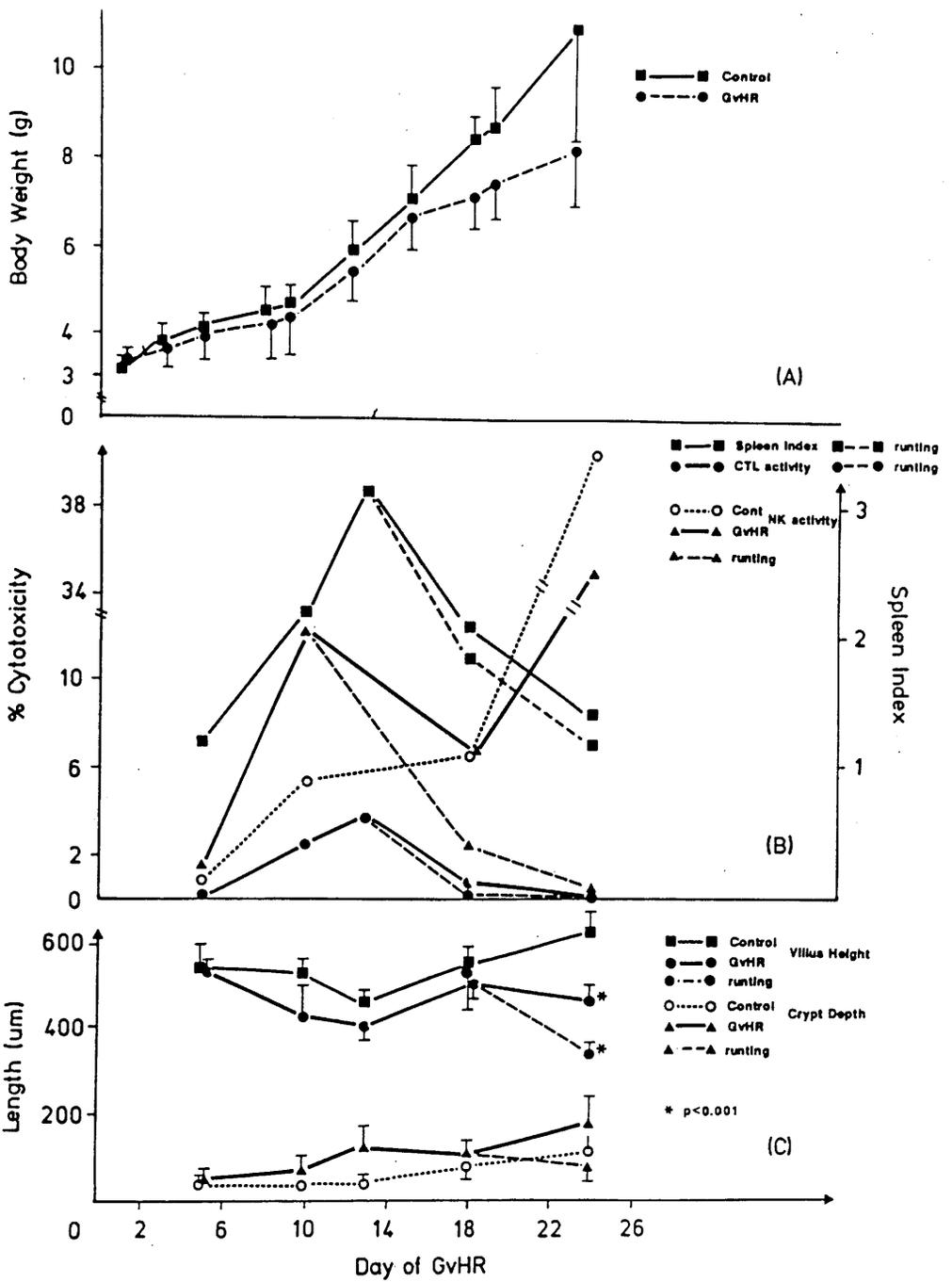
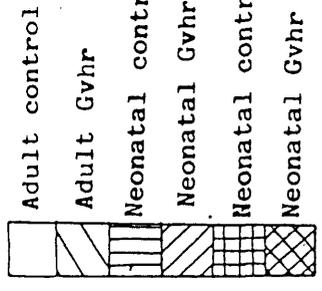


Fig. 3: As Figure 2. After day 18 results are shown for running and non-running Gvhr mice.

Experiment 2



Experiment 1

** p<0.01
 * p<0.05
 *** p<0.005

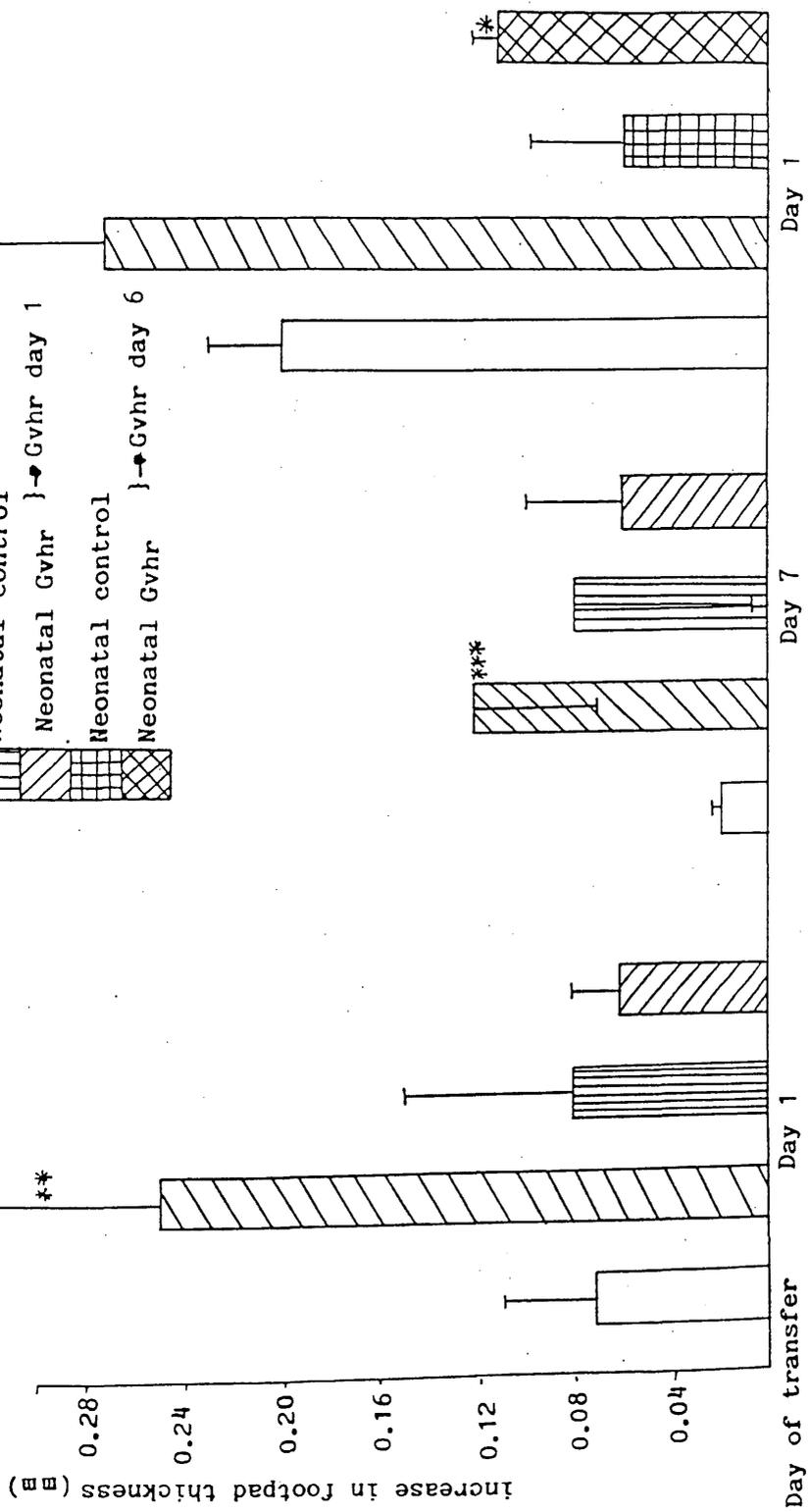


Fig. 4: Induction of anti-host DTH in adult and neonatal (CBA x BALB/c)_{F1} mice with Gvhr. 10 spleen cells from adult, one day old (Exp 1) or 6 day old (Exp 2) neonatal mice with Gvhr were transferred into the footpad of adult CBA mice on day 1 or 7 of the Gvhr. Control footpads received spleen cells from normal mice of the appropriate age. Results shown are the mean thickness 24 hours after challenge ± 1 s.d. for 6 mice/group.

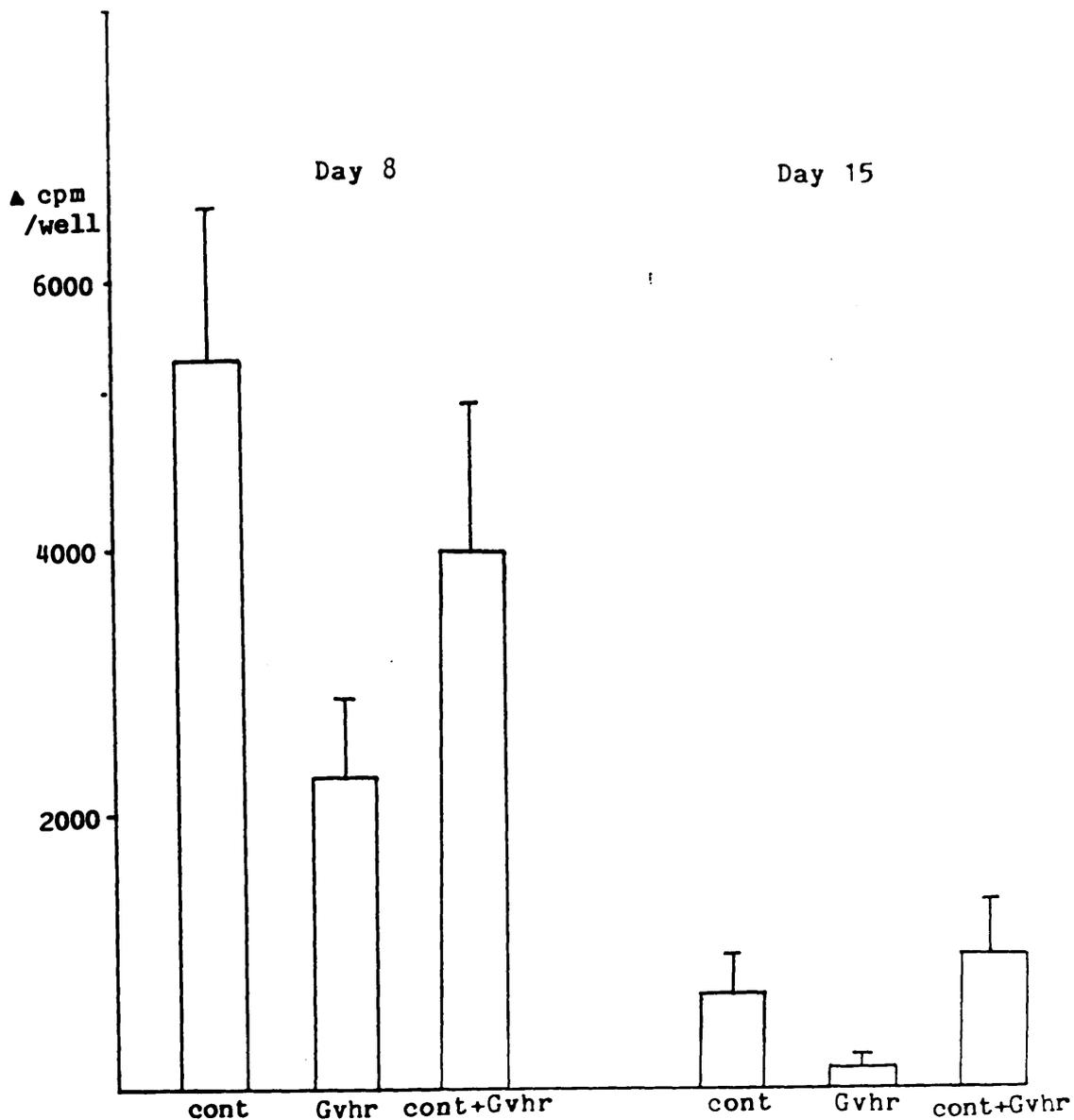


Fig. 5: Development of immunosuppression during a Gvhr in 1-2 day old (CBA x BALB/c) F_1 mice. Spleen cells were pooled from 4-5 mice on days 8 and 15 of Gvhr and from control mice and were stimulated with mitomycin c treated B10 spleen cells for 4 days. Results shown are mean cpm/well \pm 1 s.d. for quadruplicate assays.

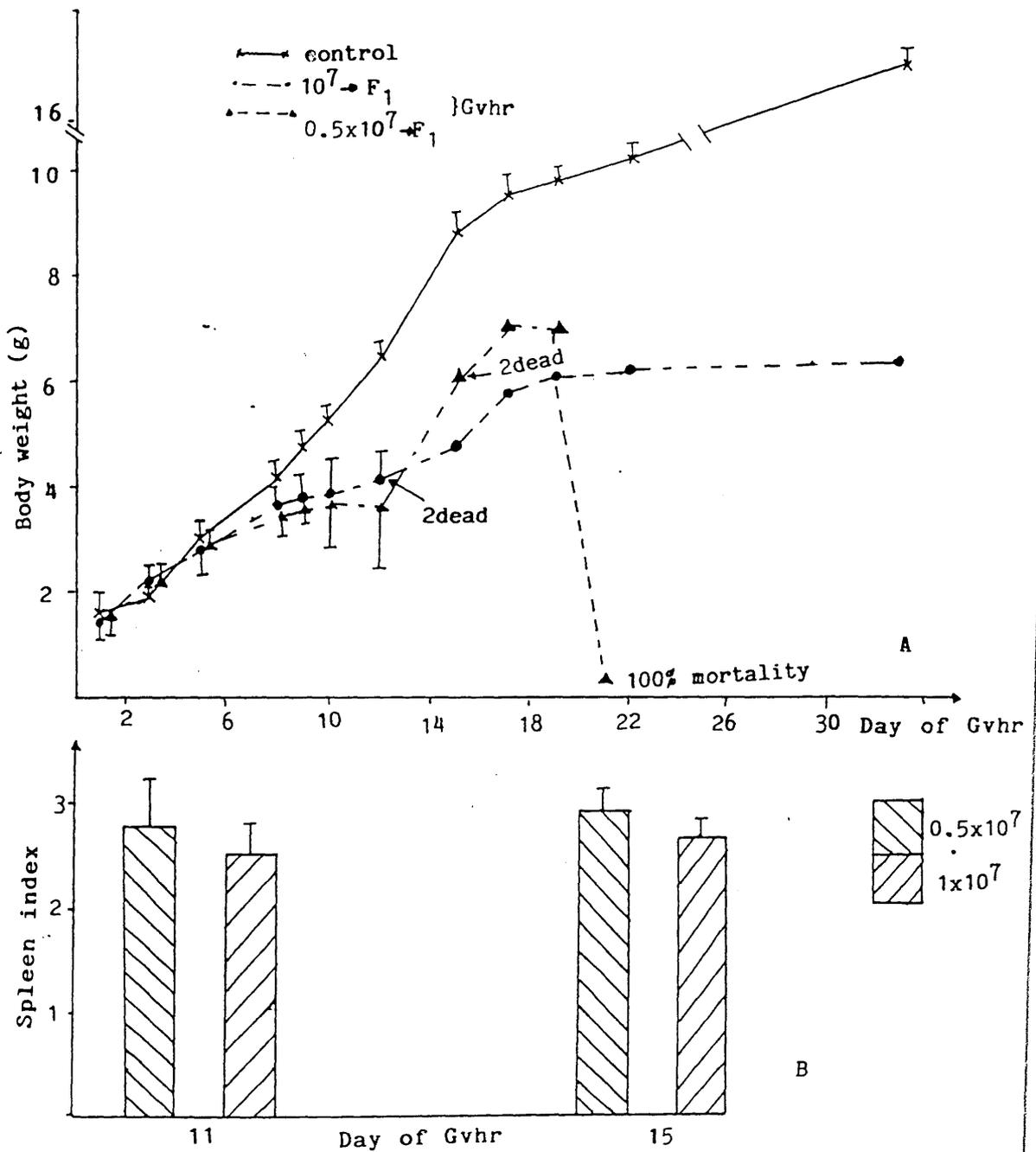


Fig. 6: The effect of donor cell number on the outcome of Gvhr in one day old (CBA x BALB/C)_{F1} mice. A) Growth rate of neonates with Gvhr induced with 0.5×10^7 or 10^7 CBA spleen cells compared with littermate controls. B) Spleen indices in neonatal (CBA x BALB/C)_{F1} mice with Gvhr. Results shown are means \pm 1 s.d. for 3 mice/group.

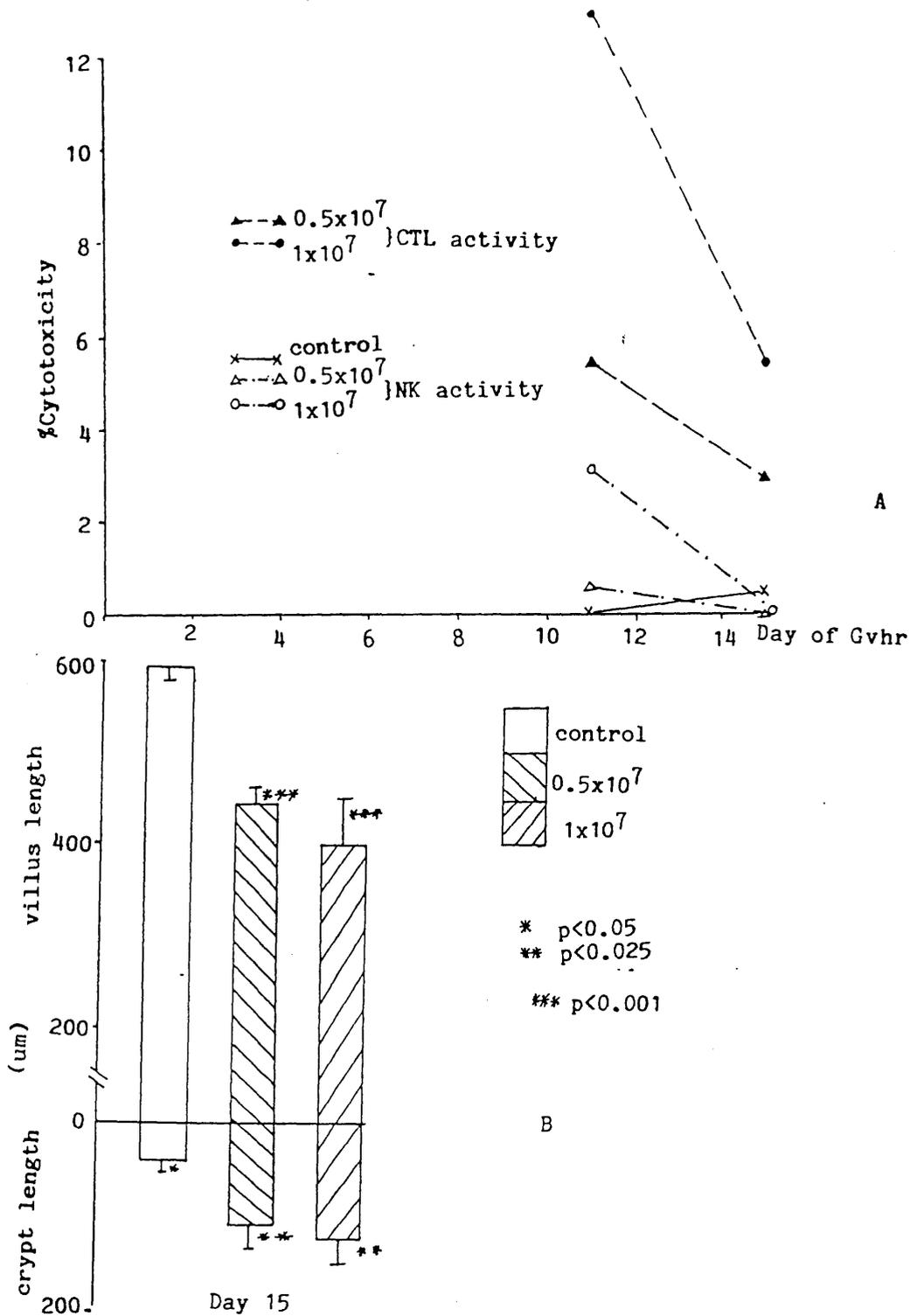


Fig. 7: As for Figure 6. A) NK and specific CTL activity against YAC-1 and P815 target cells respectively at 50:1 E:T using spleen cells pooled from 3 mice/group. B) Mucosal architecture in jejunum of mice on day 15 of Gvhr and in controls. Results shown are means \pm 1 s.d. for 4 mice/group.

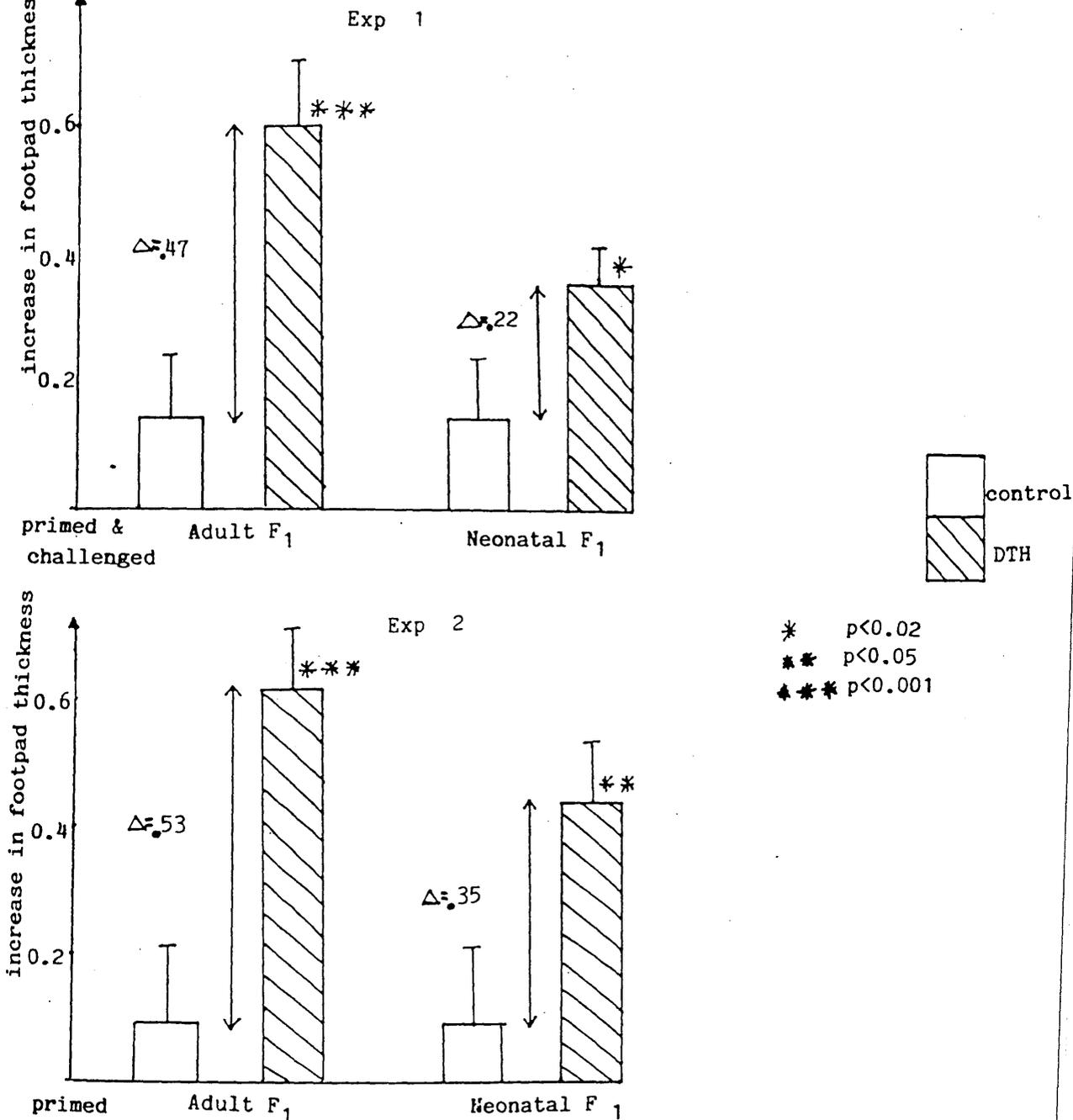


Fig. 8: Ability of adult or neonatal (CBA x BALB/c)_{F₁} spleen cells to induce an allospecific DTH response in adult CBA mice. In Experiment 1, host mice were primed and challenged 5 days later, with adult or neonatal _{F₁} spleen cells, while in Experiment 2, hosts were primed with adult or neonatal _{F₁} spleen cells and challenged with adult _{F₁} cells. Results shown are the mean increase in footpad thickness 24 hours after challenge of immunized or control mice ± 1 s.d. for 6 mice/group.

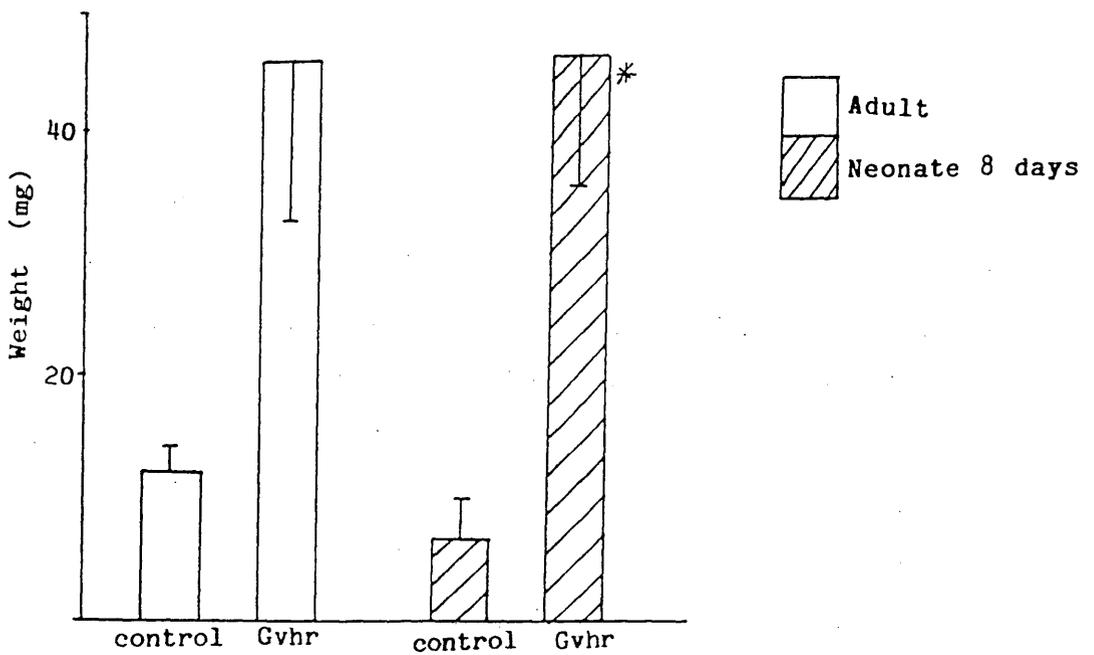
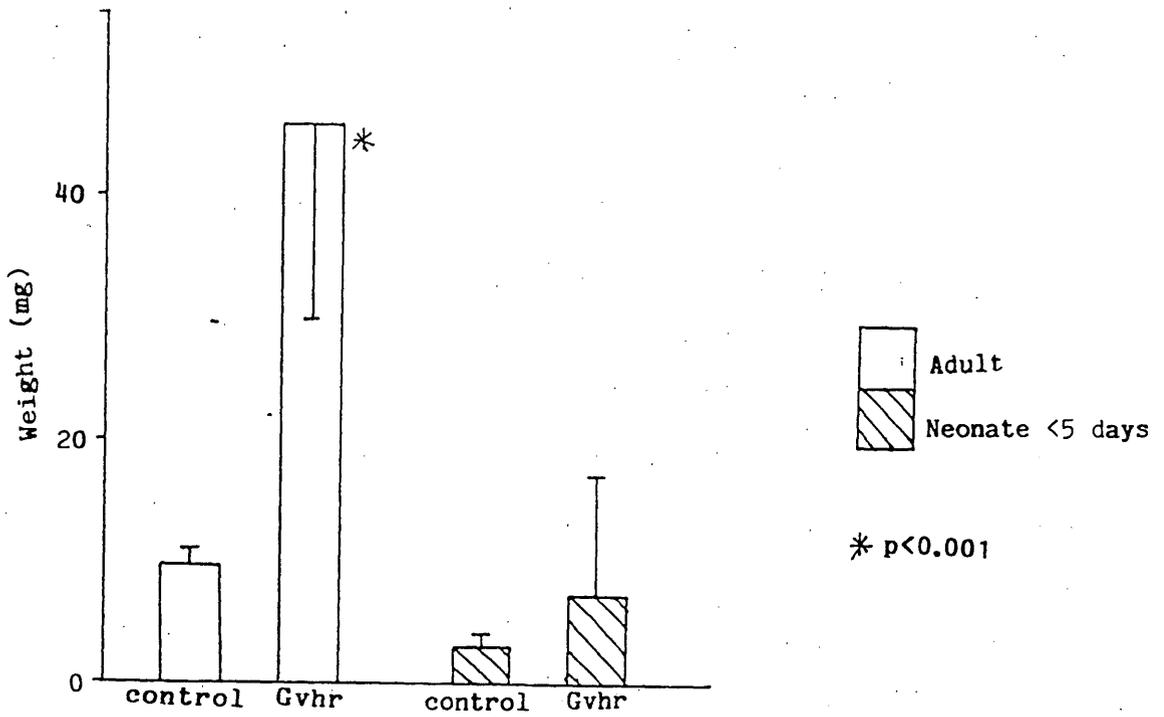


Fig. 9: Influence of age on the ability of parental spleen cells to induce a local Gvhr in F_1 hosts. Popliteal lymph node weights 7 days after footpad transfer of 10^7 spleen cells from adult, 8 day old or less than 5 day old CBA mice. Control footpads were injected with medium. Results shown are means \pm 1 s.d. for 5 mice/group.

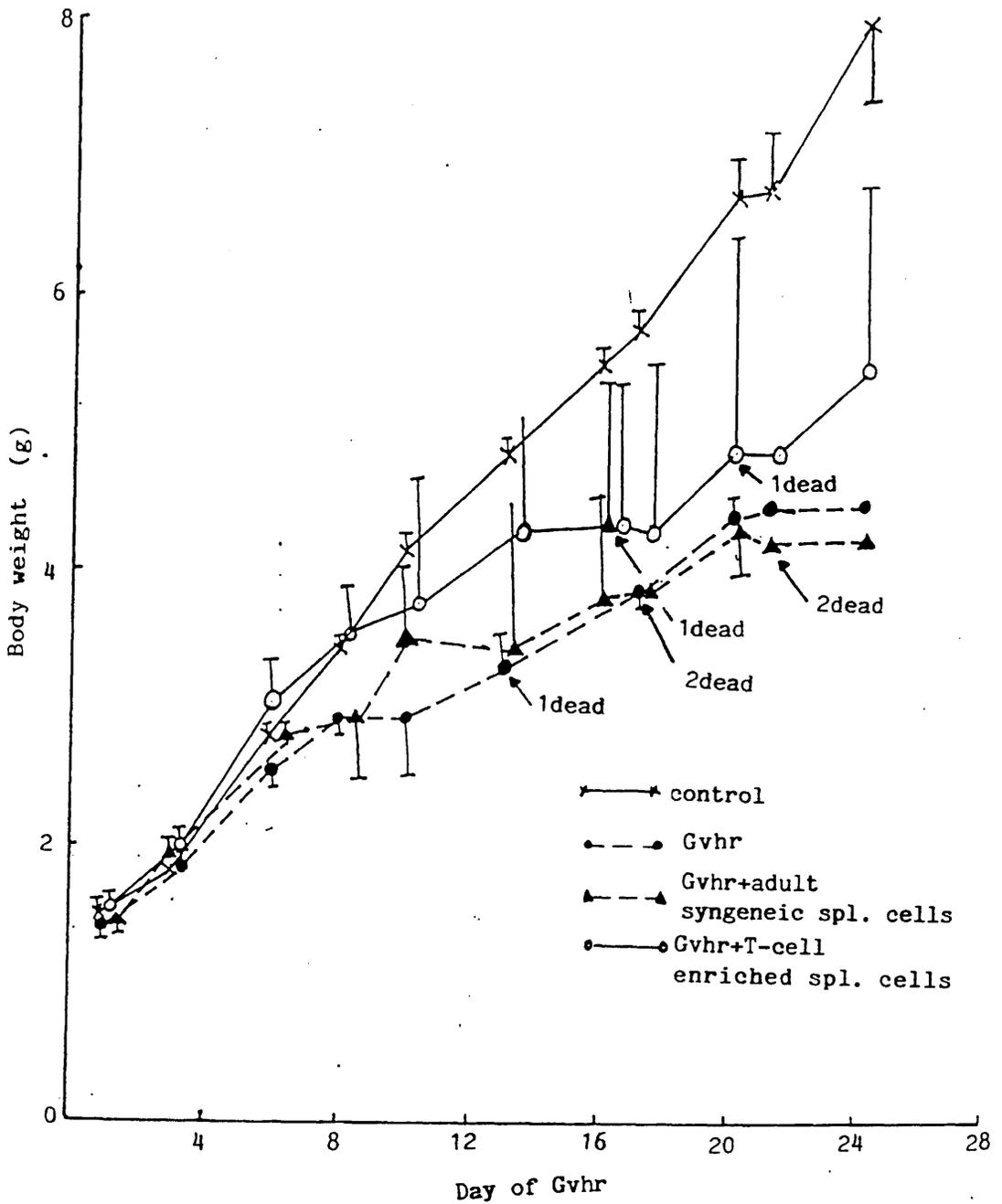


Fig. 10: Effect of reconstituting one day old neonatal (CBA x BALB/c) F_1 mice with adult spleen cells on the systemic Gvhr induced by 10^7 CBA spleen cells. Growth rate of neonatal mice with Gvhr given 2.5×10^7 syngeneic spleen cells or T-cell enriched spleen cells on day 1. Results shown are means \pm 1 s.d. for 4 mice/group.



A) Microscopic appearance of a microdissected section of jejunum from a 1 day old control (CBA x BALB/c) F_1 mouse. Note the short crypts and tall slender villi (Schiff x 400)



B) Microscopic appearance of the jejunum on day 11 of Gvhr induced on day 1 of life. There is marked crypt hypertrophy, as well as shortening and thickening of the villi (Schiff x 400)

Fig. 11: Effects of Gvhr on mucosal architecture of neonatal (CBA x BALB/c) F_1 mice.



Legend as
Fig. 11 B, but
shows crypt
hypertrophy
in the absence
of villus
damage.

CHAPTER 5

INVESTIGATION OF THE MECHANISMS

INVOLVED IN THE ENTEROPATHY CAUSED

BY A GVHR IN BDF₁ MICE

Introduction

The previous chapters have shown that it is possible to modify the outcome of systemic and intestinal Gvhr by inducing different forms of immune response in different types of (CBA x BALB/c)_{F₁} hosts. These studies showed that a destructive enteropathy involving villus atrophy, occurred when mice developed an acute lethal form of Gvhr. However, this was only found in hosts, whose immune systems had been altered by either immaturity or irradiation and it was possible that the pathology was an artefact of using these abnormal hosts. Furthermore, the low numbers of cells obtainable from the lymphoid tissues of irradiated or neonatal hosts made it difficult to examine immune effector mechanisms in detail. Therefore I decided it would be important to be able to confirm the findings by examining the immune mechanisms responsible for the different forms of pathology during a Gvhr in unmanipulated, preferably adult hosts.

One possible approach to this was suggested by the findings that adult BDF₁ mice developed an acute lethal Gvhr after injection of C57Bl/10 parental spleen cells, while a milder more chronic form of Gvhr induced by T-cells from the opposite parent (Gleichmann et al., 1984). In parallel, mice given B10 cells developed anaemia and destruction of lymphoid tissues while hosts receiving DBA/2 cells only showed late signs of autoimmune disease and lymphoid cell proliferation. Interestingly, it has also been shown that specific CTL only develop in B10 → BDF₁ mice (Kubota et al., 1983) and so this model might provide

a further means of assessing the effector mechanisms responsible for different forms of immunologically mediated enteropathy.

Experimental design

A Gvhr was induced in adult BDF₁ mice by i.v. injection of 10⁸ spleen cells from either B10 or DBA/2 parents while neonatal BDF₁ mice were given 10⁷ parental spleen cells i.p. at various times after birth. The progress of the Gvhr was assessed at intervals by the usual means of weighing mice, measurement of splenomegaly, splenic NK activity and specific anti-host CTL activity as well as by examining the ability of Gvhr spleen cells to suppress the mitogenic responses of normal lymphocytes. In parallel, the intestinal phase of Gvhr was also measured. (see Appendix)

Results

Graft-versus-host reaction in adult (C57Bl/10 x DBA/2)F₁ mice

The first experiments were designed to determine whether the different forms of systemic Gvhr which had been described in adult BDF₁ mice would be reflected in the intestinal pathology.

Progress of systemic Gvhr

Ten days after the induction of Gvhr, mice receiving B10 parental spleen cells were already losing weight compared with control mice (Fig. 1A) and this continued until day 24 when the weights of the B10 → BDF₁ mice had fallen to 18.2g ± 1.6g compared with controls 25.9 ± 1.2g (p < 0.001)

and two of the Gvhr group had died (Fig. 1B). Thereafter, the majority of these mice began to recover and the mean weight of the group increased gradually. However, two more mice died on days 64 and 70 of the Gvhr. In contrast, recipients of DBA/2 spleen cells showed no signs of clinical Gvhr throughout the study and none of these mice lost weight or died (Fig. 1A and B).

BDF₁ mice given B10 parental spleen cells had significant splenomegaly by day 4 (Fig. 2) and by day 8 this had risen to give a spleen index of 4.8 ± 0.5 . This declined thereafter and had disappeared by day 20. Although mice given DBA/2 spleen cells had much less intense splenomegaly, with a maximum spleen index of 2.04 ± 0.15 on day 8, a similar level of splenomegaly was maintained until day 41 (Fig. 2).

When specific anti-host CTL activity was measured in Gvhr mice, it was found that B10 \rightarrow F₁ mice developed consistently high levels of specific cytotoxicity against P815 cells, which reached a peak of 30.8% on day 8 before falling to 11% by day 25 and being absent on day 41 (Fig. 3). In contrast, only very low levels of specific cytotoxicity against EL-4 were found in DBA/2 \rightarrow F₁ mice at any time during the Gvhr. Furthermore, in view of the enhanced NK activity in these mice (see below), it was not clear whether this cytotoxicity reflected true CTL activity, as, unlike P815, EL-4 cells are sensitive to NK lysis (Harmon et al., 1977).

Control mice had variable levels of NK activity during this experiment (Fig. 4A) and to clarify the level of NK activity in Gvhr mice, this is also expressed as a ratio of that in appropriate controls (Fig. 4B). Mice given B10 cells had normal levels of NK activity on day 3, after which NK activity fell rapidly and was undetectable after day 25. In contrast, mice given DBA/2 cells had increased NK activity compared with controls from day 3 onwards and this peaked on day 8 (24.4% vs. 6.1% in controls). Thereafter, NK activity in these mice fell but was never lower than that in controls.

Suppression of lymphocyte proliferation in BDF₁ mice with Gvhr

The studies by Gleichmann et al. (1984) showed that the acute Gvhr in BDF₁ mice given B10 spleen cells was accompanied by suppressor T cell activity and I attempted to confirm this phenomenon in my own model. In the first experiment, I examined the ability of Gvhr spleen cells to generate a MLR response against mitomycin C treated CBA (H-2^k) spleen cells on days, 3, 10 and 18 of the Gvhr. Throughout the experiment control BDF₁ spleen cells showed a healthy proliferation to CBA spleen cells, while mice given B10 spleen cells always showed little or no MLR response and these were always markedly smaller than those of control cells (Fig. 5).

The proliferative response of DBA/2 → F₁ mice to CBA spleen cells was also less than control levels on day 3 and 10 (548.4 ± 79 cpm and 677.05 ± 184 cpm vs. 1672.5 ± 535 cpm and 1882.5 ± 163 cpm in controls), but by day 18

spleen cells from DBA/2 → F₁ mice responded markedly better than control cells (2665.7 ± 235 cpm vs. 1340.3 ± 407 cpm in controls, $p < 0.005$). Furthermore, these Gvhr mice always had better MLR responses than those of B10 → F₁ mice.

The next experiments were designed to examine whether this defect in lymphocyte proliferation in Gvhr mice was associated with the presence of active suppression. Spleen cells were taken on days 3, 17 and 25 of the Gvhr and mixed at different ratios with control BDF₁ spleen cells which were stimulated with ConA.

On day 3 spleen cells from both B10 → BDF₁ mice and DBA/2 → BDF₁ mice suppressed the ConA response of control cells, especially when mixed at high or very low ratios. However cells from DBA/2 F₁ mice were generally less suppressive than those from B10 → BDF₁ mice (Fig. 6). On day 17, B10 → BDF₁ cells produced a similar pattern of suppression which was always < 30% and became more marked at higher numbers of Gvhr cells. In contrast, cells from DBA/2 → BDF₁ mice always exhibited less suppression than B10 → BDF₁ cells and, at lower ratios, little or no suppression was seen with these cells. By day 25, neither Gvhr population suppressed control responses at low cell numbers, but addition of larger numbers of B10 → F₁ cells produced dose dependent suppression which was virtually complete at the highest cell numbers. In contrast, the same numbers of DBA/2 → BDF₁ cells led to a dose-dependent enhancement of ConA responses. These results confirm those

of Gleichmann et al. and are preliminary evidence that B10 donor cells are much more efficient than DBA/2 donor cells at inducing Gvhr mediated suppression.

Development of auto-antibodies in BDF₁ mice with Gvhr

A further index of systemic immunity which is reported to distinguish the Gvhr induced by B10 or DBA/2 parental cells in BDF₁ mice is production of autoantibodies during the later stages of Gvhr in DBA/2 → BDF₁ mice (Rolink & Gleichmann, 1983). Therefore, I looked for the presence of serum antibodies to autologous red blood cells during the 20th week of the Gvhr in both groups of Gvhr mice using a standard haemagglutination assay. However, despite the fact that DBA/2 → F₁ mice still had significant splenomegaly at this time (1.48 ± 0.52), anti-red cell autoantibodies were not detectable in any mice (Data not shown).

The results of this series of experiments confirm and extend those of other workers showing that B10 and DBA/2 parental spleen cells induce markedly different forms of systemic Gvhr in unirradiated BDF₁ mice. Furthermore, these models of Gvhr are accompanied by different forms of cell mediated effector and regulatory cell activity. In the next experiments I went on to investigate whether those differences were accompanied by different types of intestinal pathology.

Intestinal pathology during Gvhr in adult, unirradiated BDF₁ mice

The intestinal pathology was examined on days 4-128 of Gvhr. Mice given B10 spleen cells had no evidence of villus damage until day 28, when significant villus atrophy

was found (Fig. 7) ($335.5 \pm 59.8 \mu\text{m}$ vs. $509.5 \pm 36 \mu\text{m}$ in controls, $p < 0.005$). At this time these mice also had significant weight loss. Some of these mice survived until day 128 and there was still some evidence of villus atrophy at that time, although this did not reach statistical significance ($530.9 \pm 122.6 \mu\text{m}$ vs $589.5 \pm 20.5 \mu\text{m}$ in controls). In parallel, these Gvhr mice had significant crypt lengthening by 8 of Gvhr (Fig. 7) ($199.9 \pm 13.4 \mu\text{m}$ vs. $116.3 \pm 10.7 \mu\text{m}$ in controls, $p < 0.001$) and this was maintained until the end of the experiment in surviving mice (day 128: $185.5 \pm 22.3 \mu\text{m}$ vs. $119.2 \pm 12.7 \mu\text{m}$ in controls, $p < 0.001$).

This group also had increased crypt cell turnover which was particularly marked on day 8 ^(Fig 8A) (40.4 ± 6.5 vs. 10.5 in controls) and persisted until day 128 (26.5 ± 8.6 vs. 9.1 ± 0.5 , $p < 0.02$). Interestingly, B10 \rightarrow BDF₁ mice had significantly lower IEL counts than controls on day 3 (Fig. 8B) (5.71 ± 1.15 vs. 9.67 ± 1.84 in controls, $p < 0.02$) and although IEL counts had recovered to normal levels by day 8, very low IEL counts were again found on days 17 and 28. Indeed, by day 28, virtually no IEL were seen in the epithelium of Gvhr mice (1.42 ± 1.09 vs. 11.38 ± 1.46 in controls, $p < 0.001$). On day 128, surviving mice had normal levels of IEL.

Mice given DBA/2 spleen cells had surprisingly few intestinal changes, despite the presence of persistent splenomegaly and enhanced NK activity shown earlier. There was no villus atrophy at any time during the Gvhr and there were only small increases in crypt length and CCPR on days 8 and 17 respectively (Fig. 7 & 8A). Similarly, no change in IEL numbers occurred at any time during the Gvhr in these mice (Fig. 8B).

Graft-versus-host reaction in neonatal (C57B1/10 x DBA/2)_F₁ mice

The results described above showed that although B10 and DBA/2 parental spleen cells induced markedly different forms of systemic Gvhr in adult hosts, only B10 cells caused significant intestinal pathology. Therefore, it proved impossible to correlate intestinal alterations with systemic immune responses. The results in Chapter 4 show that mature mice are more resistant to Gvhr than 1-2 day old mice. Therefore, it seemed possible that intestinal pathology might occur after injection of DBA/2 cells if neonatal BDF₁ hosts were used and I carried out a series of experiments examining the Gvhr induced by B10 or DBA/2 cells in one day old neonatal BDF₁ hosts. As a control this Gvhr was compared with that occurring in 5 day old hosts.

Progress of systemic Gvhr in neonatal BDF₁ mice

One day old neonatal BDF₁ mice which received either B10 or DBA/2 parental spleen cells developed normally until about day 8, when both groups began to lose weight. At the same time, Gvhr mice developed other clinical signs of runting, and deaths began to occur after day 18 (Fig. 9A). Interestingly, recipients of DBA/2 spleen cells lost weight at a slightly slower rate than B10 → F₁ Gvhr mice (Day 16: 5.22 ± 0.8g, $p < 0.05$ and 3.72 ± 0.7g, $p < 0.001$ vs. 6.56 ± 0.39 in controls). DBA/2 → BDF₁ mice also had a slower mortality rate and the first mouse of this group had not died until day 23, while 60% of B10 → BDF₁ mice had died.

by day 19. However DBA/2 cells were undoubtedly capable of causing an acute Gvhr in these neonatal hosts as the overall mortality and weight loss was similar to recipients of B10 cells.

B10 → BDF₁ mice had significant splenomegaly by day 8, (S.I. = 1.52 ± 0.3) and this rose to 1.53 ± 0.32 on day 12, before falling rapidly to control levels thereafter. In contrast, recipients of DBA/2 cells had no splenomegaly on day 8, but then developed marked splenomegaly which reached a maximum of 2.35 ± 0.3 on day 13 and was still present on day 18 (1.48 ± 0.17) when none was present in B10 → F₁ mice.

Neonatal mice given B10 or DBA/2 parental spleen cells at 5 days old showed a markedly different pattern of systemic Gvhr. Mice given B10 spleen cells began to lose weight and developed the characteristic features of runting by about day 12 and all the mice died by day 24 (Fig. 12A). In contrast, recipients of DBA/2 cells grew at the same rate as controls throughout the experiment and none of these animals died. In this experiment, mice given B10 spleen cells always had greater splenomegaly than DBA/2 → F₁ mice, with a spleen index of around 2 throughout the course of the experiment (Fig. 12B). In contrast, DBA/2 → F₁ mice never had significant splenomegaly.

Development of cytotoxicity in neonatal BDF₁ mice with Gvhr

Mice given B10 spleen cells on day 1 of life had a low level of specific CTL activity, which peaked on day 13

and had disappeared by day 18. One day old recipients of DBA/2 spleen cells also had some specific CTL activity on days 12 and 13, but this was much less than in B10 → BDF₁ mice (3% vs 15%) (Fig.10A). Five day old mice given B10 spleen cells also showed specific CTL activity, which was maximal on day 12 (Fig.13A) and fell thereafter. However, no significant specific CTL activity was found in mice given DBA/2 cells on day 5 of life.

When a Gvhr was induced in 1 day old mice, both groups of Gvhr mice had an early rise in splenic NK cell activity on day 8, but this was small and short-lived on day 12 and thereafter, all Gvhr mice had NK levels less than 1%. Control mice in these experiments had no NK activity at all until day 18, when levels of 7% were reached (Fig.10B). Both groups of 5 day old mice with Gvhr also had a small rise in NK activity on day 12, but this was more apparent in mice receiving B10 cells than in recipients of DBA/2 cells (Fig.13B) (6.8% and 2.5% respectively). Thereafter, NK activity in B10 → BDF₁ mice declined to be undetectable by day 17, and was much less than in control mice in animals surviving until day 24 (9.5% vs. 31%). Although recipients of DBA/2 cells had little evidence of NK cell activation on days 12 and 17, these animals had NK cell activity which was equal to control levels on day 24 of the Gvhr.

These results are similar to those described earlier in neonatal (CBA x BALB/c)F₁ hosts and confirm in a different strain that the outcome of a systemic Gvhr is dependent on the age of the host animal. Interestingly, although

DBA/2 cells could not induce an acute, lethal Gvhr in 5 day old BDF₁ mice in the same way as B10 cells, the use of one day old neonates did allow DBA/2 cells to produce an acute Gvhr with similar features to that found using B10 cells. I next examined the intestinal phase of Gvhr induced by the two parental cells in neonatal hosts.

Intestinal pathology during a Gvhr in neonatal BDF₁ mice

Mice given B10 parental spleen cells on day 1 of life had a significant increase in crypt depth on day 7 (Fig. 11) ($62.4 \pm 6 \mu\text{m}$ vs. $35.1 \pm 6 \mu\text{m}$ in controls, $p < 0.02$) and this became more marked by day 14. Mice given DBA/2 cells also had some evidence of crypt lengthening at both times, but this was only significant on day 14, and was less than that found in B10 \rightarrow BDF₁ mice ($76.3 \pm 9.9 \mu\text{m}$ vs. $98.4 \pm 4.6 \mu\text{m}$, $p < 0.05$). At this time, both groups had a significantly increased CCPR compared with controls which was slightly more apparent in recipients of DBA/2 cells (18.9 ± 2.2 vs. 15.9 ± 1.8 for B10 \rightarrow BDF₁ mice and 9.12 ± 1.4 for controls, both $p < 0.05$). No villus atrophy was found in Gvhr mice on day 7, but on day 14, mice given B10 cells had significant villus shortening compared with controls. Mice given DBA/2 cells also had shorter villi than controls on day 14, but this was not statistically significant.

Mice given B10 spleen cells on day 5 of life had marked intestinal changes at all times examined. Clear villus atrophy and crypt lengthening were directly present on day 12 in these mice, although the small number of animals in

the group prevented statistical analysis (Fig. 14). Crypt hypertrophy became more marked in these mice on day 17 ($145.2 \pm 44 \mu\text{m}$ vs. $74.5 \pm 14 \mu\text{m}$ in controls, $p < 0.05$) but had disappeared on day 24. Severe villus atrophy was still present at these times and on day 24 Gvhr mice had villus lengths of $399.4 \pm 100 \mu\text{m}$ compared with $624 \pm 59 \mu\text{m}$ for controls ($p < 0.05$). On day 17, B10 \rightarrow BDF₁ mice also had an increased CCPR compared with controls (17.1 ± 7.5 vs 4.65 ± 0.9). In comparison 5 day old mice given DBA/2 spleen cells exhibited little evidence of intestinal Gvhr, with the only alterations being a small but insignificant rise in CCPR on day 17 (9.9 ± 1.13) and some crypt lengthening on day 24 ($126.1 \pm 35 \mu\text{m}$ vs. $109.4 \pm 1.4 \mu\text{m}$). No villus atrophy was found at any time.

Therefore, if one day old neonates are used as hosts for the Gvhr, DBA/2 spleen cells are capable of inducing a destructive enteropathy which has an identical pattern to that produced by B10 parental cells. However, as in adult BDF₁ mice, this was slightly less severe, and DBA/2 cells could not induce an acute, intestinal Gvhr in 5 day old neonates, despite the fact that B10 cells caused an acute Gvhr in both types of neonate.

Ability of B10 and DBA/2 parental mice to mediate systemic DTH to BDF₁ cells

The most consistent finding from this Chapter was that DBA/2 cells failed to induce severe systemic or intestinal Gvhr in mature BDF₁ hosts. As intestinal Gvhr has been associated with the development of anti-host DTH, I was

interested to determine if the differences in Gvhr generated by C57Bl/10 or DBA/2 spleen cells were reflected in the DTH response of the parental strains to F_1 spleen cells. B10 and DBA/2 mice were immunized with 10^7 BDF_1 spleen cells and 5 days later, both parental strains exhibited virtually identical DTH responses, as measured by the specific 24 hour increase in footpad thickness after challenge with BDF_1 cells (Fig.15A) (0.28 ± 0.12 mm and 0.29 ± 0.07 mm in B10 and DBA/2 mice respectively). This experiment was repeated three times with essentially identical results. Therefore, B10 and DBA/2 mice do not generate different levels of DTH to F_1 alloantigens.

Proliferative response of B10 and DBA/2 spleen cells to adult or neonatal BDF_1 cells

In view of the fact that B10 and DBA/2 mice made equivalent DTH responses to BDF_1 cells, it was of interest to determine whether an MLR would reveal differences in the ability of B10 and DBA/2 cells to proliferate in response to BDF_1 stimulators. This approach also allowed me to examine whether the different types of Gvhr which developed in neonatal mice under 5 days old and in adult mice reflected differences in the ability of adult or neonatal BDF_1 cells to stimulate an MLR by either type of parental cells.

B10 and DBA/2 parental spleen cells both responded well to adult BDF_1 stimulator cells in vitro, but B10 cells responded markedly better than DBA/2 cells (Fig.15B) S.I. 27 vs 11). Therefore, these results are consistent with the more severe Gvhr induced by B10 cells in vitro.

Neonatal BDF₁ cells also induced an MLR response by parental cells, but this was considerably less than that obtained with the same cells stimulated by adult BDF₁ cells (Fig. 15B). As before, B10 parental cells proliferated markedly better than DBA/2 responder cells (S.I. 8 vs 2). These results show that neonatal cells are poor stimulators of an MLR.

Summary and conclusion

The results in this Chapter confirm and extend earlier work showing that B10 and DBA/2 donor cells have markedly different capacities to induce Gvhr in BDF₁ mice. Injection of adult BDF₁ mice with B10 parental spleen cells produced an acute lethal Gvhr which was characterised by weight loss, runting and a mortality rate of up to 80%. These features were preceded by a marked splenomegaly and an early transient activation of NK cells, but the subsequent development of specific anti-host CTL activity was accompanied by a decline of NK cell activity to levels below those in controls. At the same time, these mice developed evidence of active immunosuppression. BDF₁ mice given B10 cells also had evidence of a severe intestinal Gvhr, with an early and intense crypt hyperplasia which was maintained until several weeks after induction. In addition, villus atrophy became a major feature of this model of Gvhr. The evolution of mucosal pathology was accompanied by an early fall in IEL count, which recovered for a time, only to decline to very low levels later.

In contrast to this severe Gvhr adult BDF₁ mice given DBA/2 spleen cells showed no clinical signs of Gvhr, did not lose weight and no mice died. Furthermore, there was prolonged splenomegaly and enhancement of NK activity in this model of Gvhr, but there was no evidence of significant specific CTL activity and immunosuppression was not marked. BDF₁ mice given DBA/2 cells also showed few features of intestinal Gvhr, with only mild and transient crypt hyperplasia and no villus atrophy or alterations in IEL count. In contrast to previous work (Gleichmann et al., 1984), my preliminary studies found no evidence of autoantibody production in DBA/2 → BDF₁ mice.

A similar pattern of Gvhr was observed in 5 day old BDF₁ neonates, where B10 parental cells induced an acute, lethal Gvhr associated with intense splenomegaly, specific CTL activity and loss of NK cell activity as well as a severe enteropathy which included villus atrophy. In contrast, DBA/2 cells induced little splenomegaly or changes in villus and crypt measurements, and none of the mice lost weight or died. In parallel, there was some enhancement of NK activity but no CTL activity and no loss of NK cell function. However, an important finding from these studies was that it proved possible to induce an acute Gvhr by injecting one day old BDF₁ mice with either B10 or DBA/2 spleen cells. Under these circumstances both parental cells produced a similar type of disorder with weight loss, runting and death. They also developed similar levels of NK activity and crypt hyperplasia. Although significant CTL activity was only

found in recipients of B10 cells, both groups of Gvhr mice developed villus atrophy. However, DBA/2 cells were less efficient than B10 cells at causing all these lesions.

The different abilities of B10 and DBA/2 cells to induce a Gvhr in BDF₁ mice was not reflected by differences in the ability of the parental strains to generate a DTH response to adult BDF₁ cells in vivo. However, B10 cells responded much better to both adult and neonatal BDF₁ stimulator cells in a MLR in vitro. The contrasting types of Gvhr found in adult or older neonatal BDF₁ mice given B10 or DBA/2 parental cells is consistent with the results of Gleichmann, who suggested that these differences reflected the generation of different forms of anti-host immune response. Nevertheless, my finding that DBA/2 cells were able to induce a similar, if less severe form of Gvhr in one day old neonates suggests that DBA/2 cells are merely less efficient at inducing the same type of alloreactivity in vivo and that this can be revealed in an immunoincompetent host.

Together, these results in unmanipulated adult hosts support these from earlier chapters by confirming that the development of specific CTL activity and a severe enteropathy involving villus atrophy are features of a more severe progressive Gvhr and that these are preceded by proliferative alterations such as activation of NK cells and crypt hyperplasia. In addition, these results further support the temporal association between the development of villus atrophy and cytotoxic T-cells. This phenomenon will be investigated further in the following chapters.

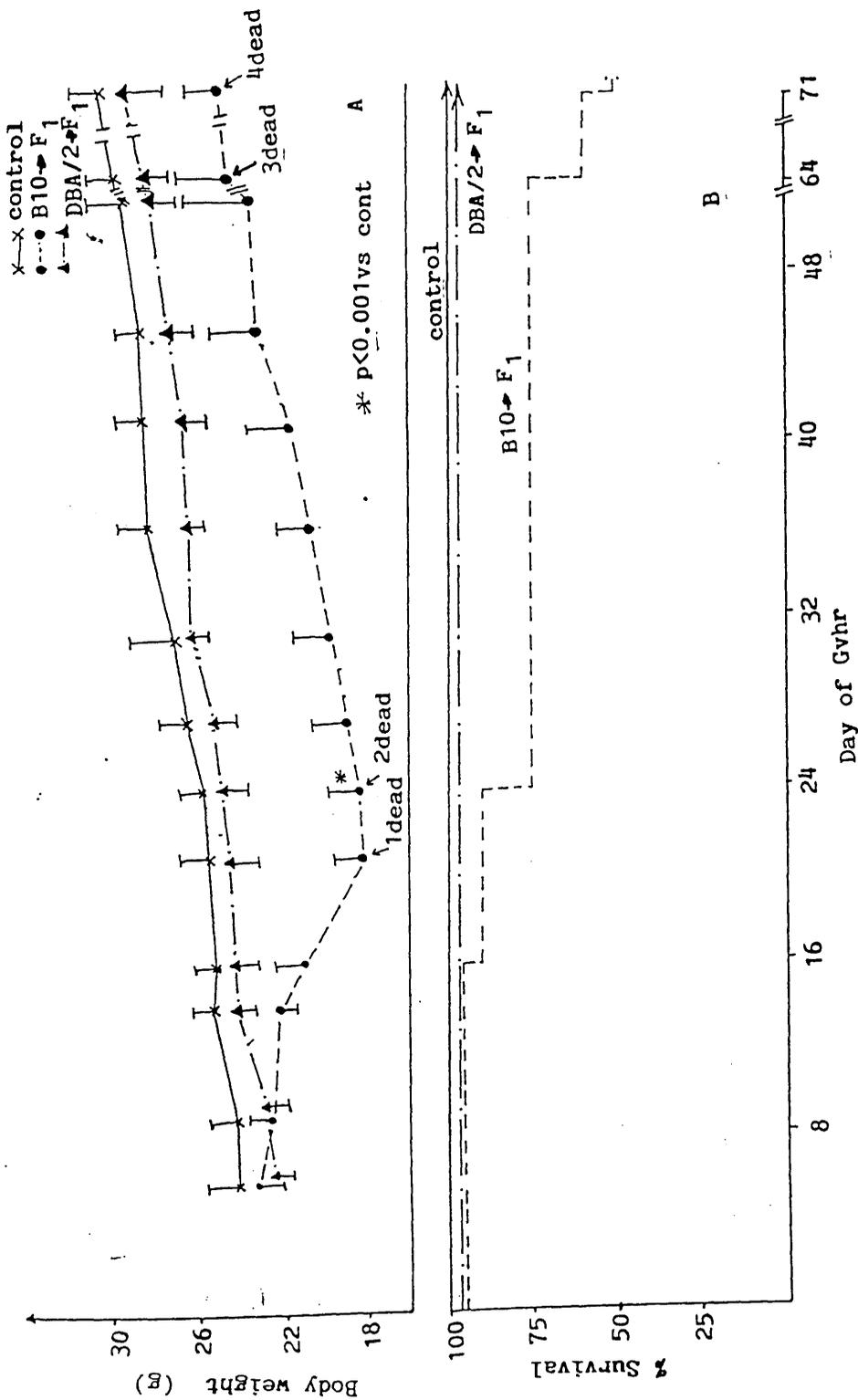


Fig. 1: Induction of systemic Gvhr in adult, unirradiated BDF₁ mice with 10⁸ B10 or DBA/2 spleen cells. A) Growth rate of mice with Gvhr and controls. B) Mortality rates of mice with Gvhr. Results shown are means ± 1 s.d. for 8 mice/group.

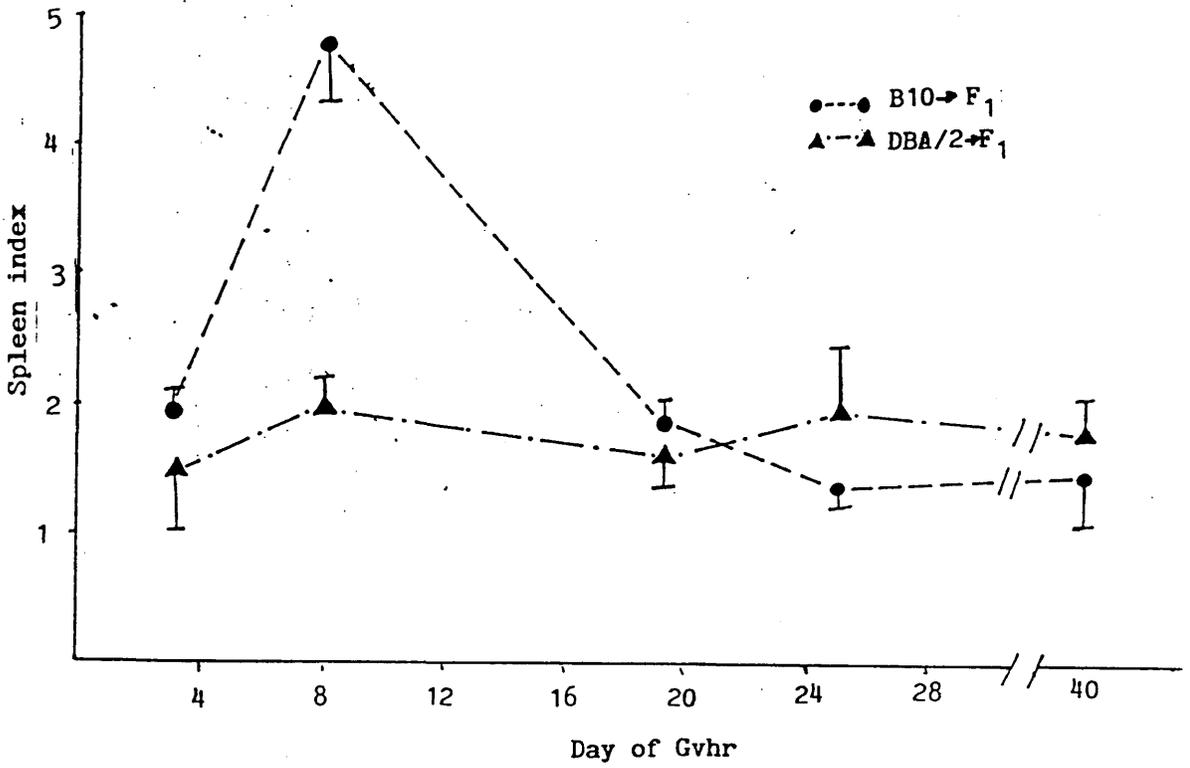


Fig. 2: Induction of systemic Gvhr in adult unirradiated BDF₁ mice with 10⁸ B10 or DBA/2 spleen cells. Development of splenomegaly in mice with Gvhr. Results are expressed as mean spleen indices ± 1 s.d. for 3 mice/group.

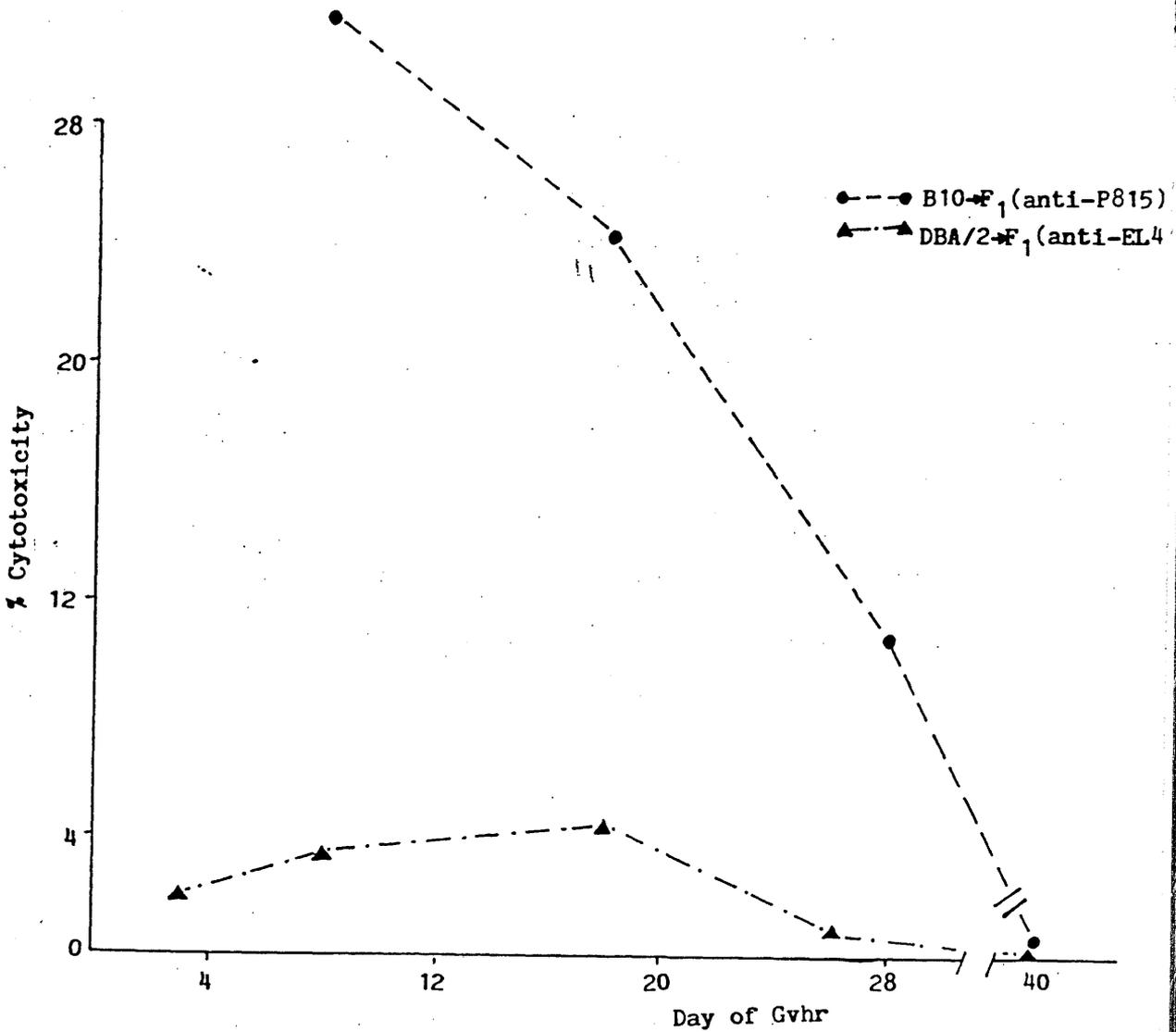


Fig. 3: Induction of systemic Gvhr in adult unirradiated BDF₁ mice with 10⁸ B10 or DBA/2 spleen cells. Specific CTL activity in B10 → BDF₁ and DBA/2 → BDF₁ mice, measured against P815 and EL-4 target cells respectively. Results shown are % cytotoxicity at 50:1 E:T using spleen cells pooled from 3 mice/group.

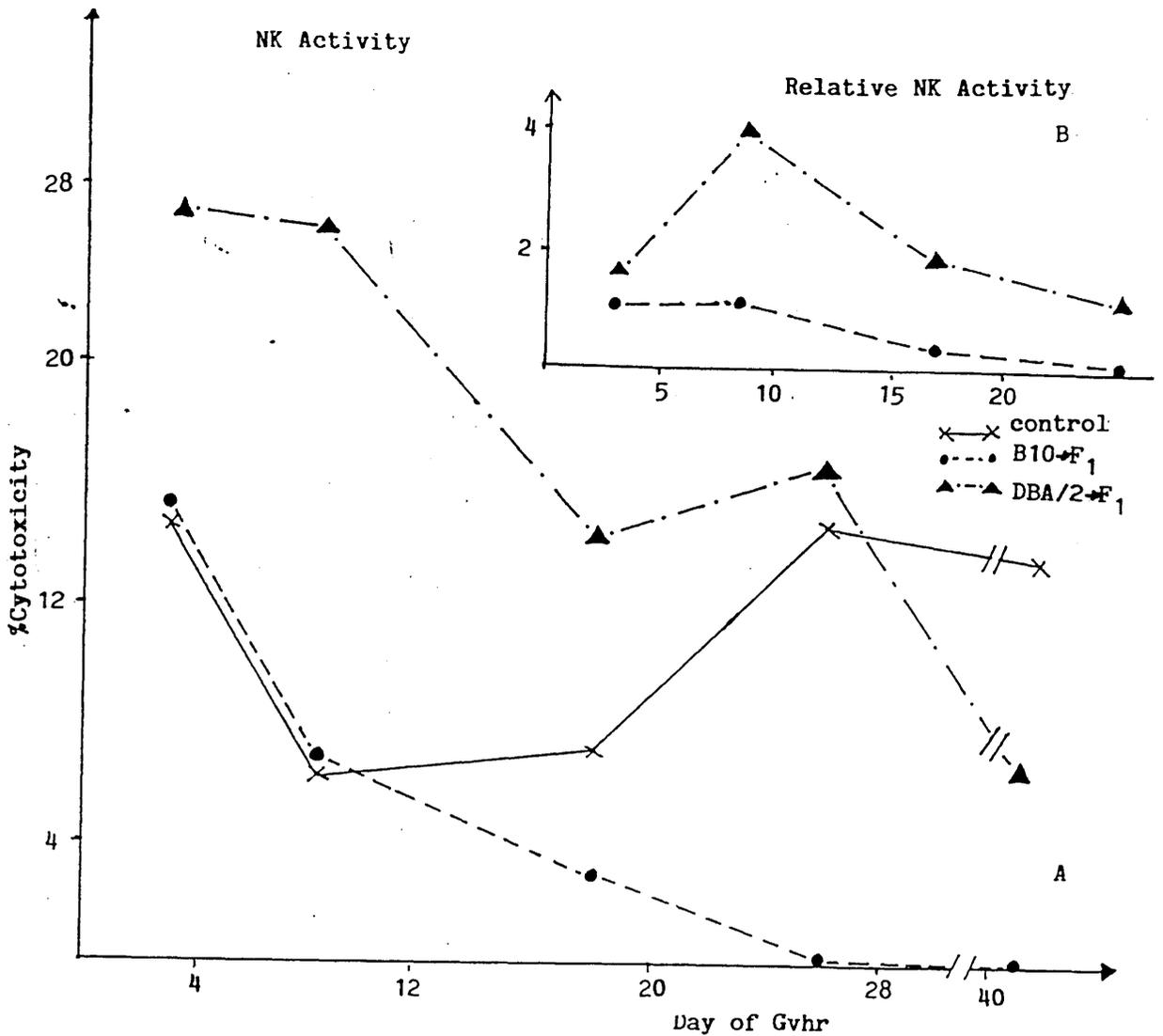


Fig. 4: Induction of systemic Gvhr in adult, unirradiated BDF₁ mice with 10⁸ B10 or DBA/2 spleen cells. A) Main graph shows absolute levels of NK activity in Gvhr and control mice while the inset B) shows the NK activity in mice with Gvhr expressed as a ratio to control levels. Results shown are % cytotoxicity at 50:1 E:T using spleen cells pooled from 3 mice/group.

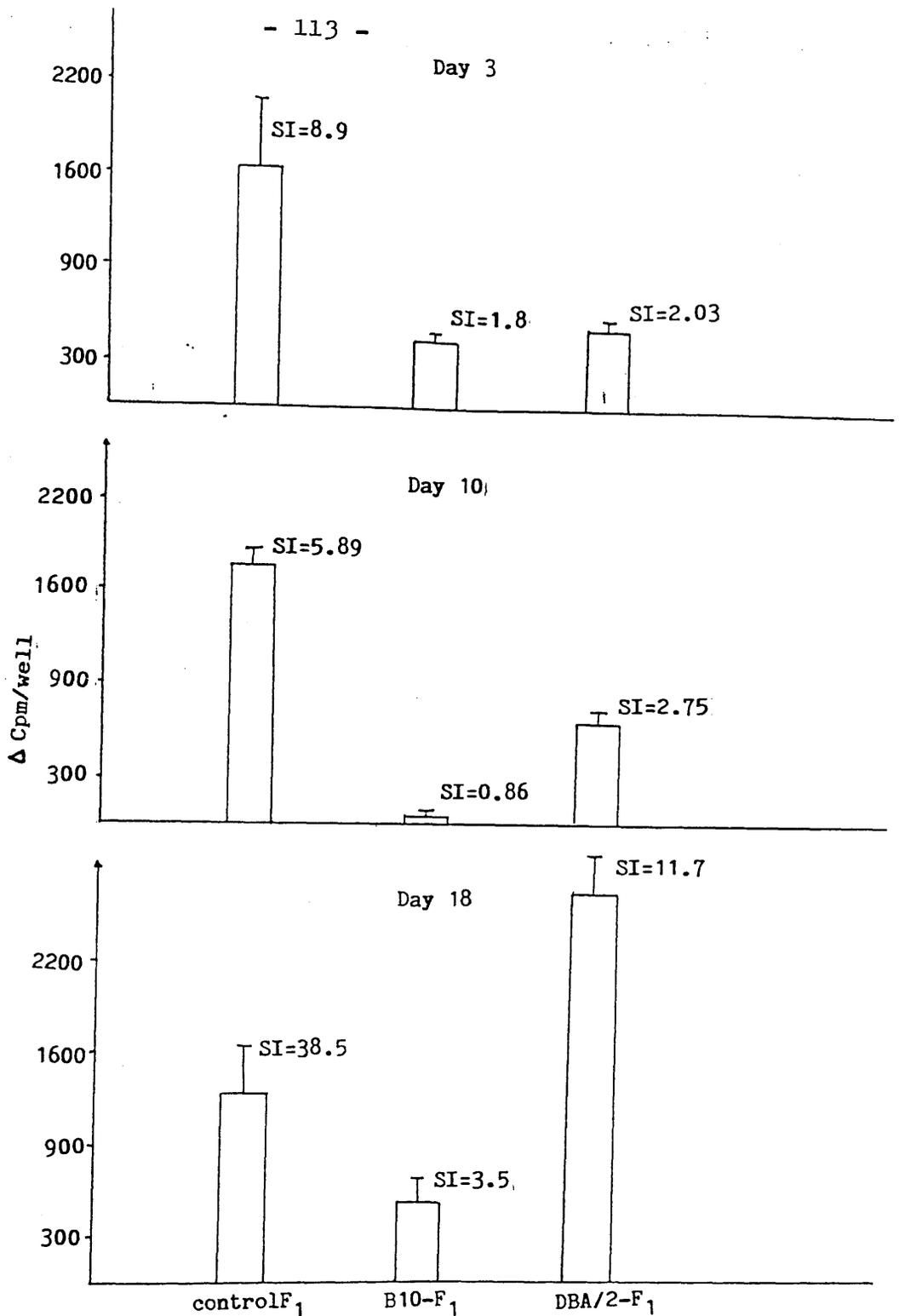


Fig. 5: Mixed lymphocyte responses of BDF₁ mice with Gvhr induced by B10 or DBA/2¹ spleen cells. Spleen cells were pooled for 4/5 mice/group on days 3, 10 and 18 of the Gvhr or from controls and were stimulated with mitomycin c treated CBA spleen cells for 4 days. Results shown are mean cpm/well ± 1 s.d. and stimulation induces (SI) for quadruplicate assays.

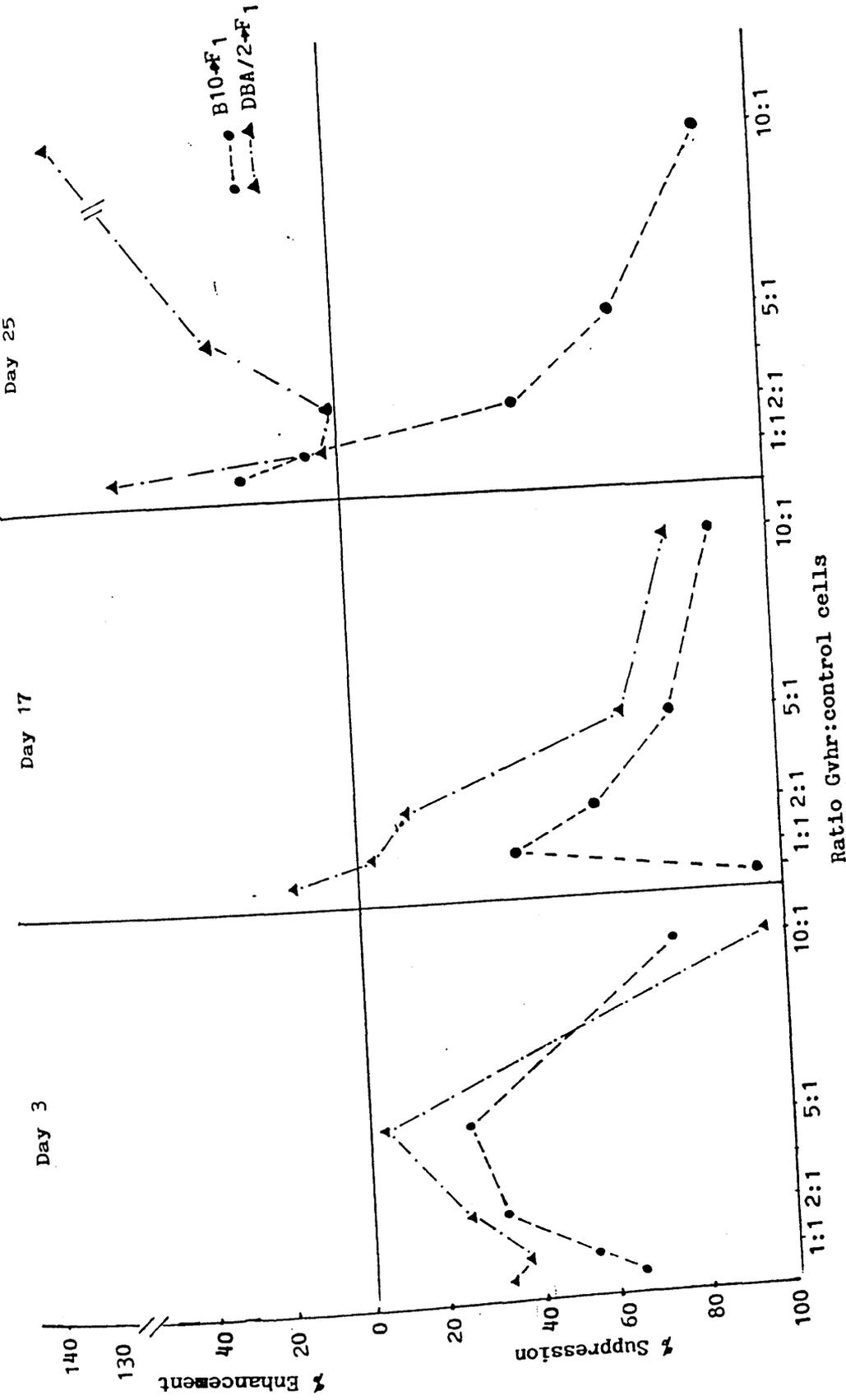


Fig. 6: Development of immunosuppression during a Gvhr in adult BDF₁ mice induced with B10 or DBA/2 spleen cells. Spleen cells were pooled from 3-4 mice/group with Gvhr on days 3, 17 and 15 and were mixed with control BDF₁ spleen cells at different ratios, before culture with Con A for 3 days. Results are expressed as % enhancement/suppression of the response of control cells to Con A using quadruplicate assays.

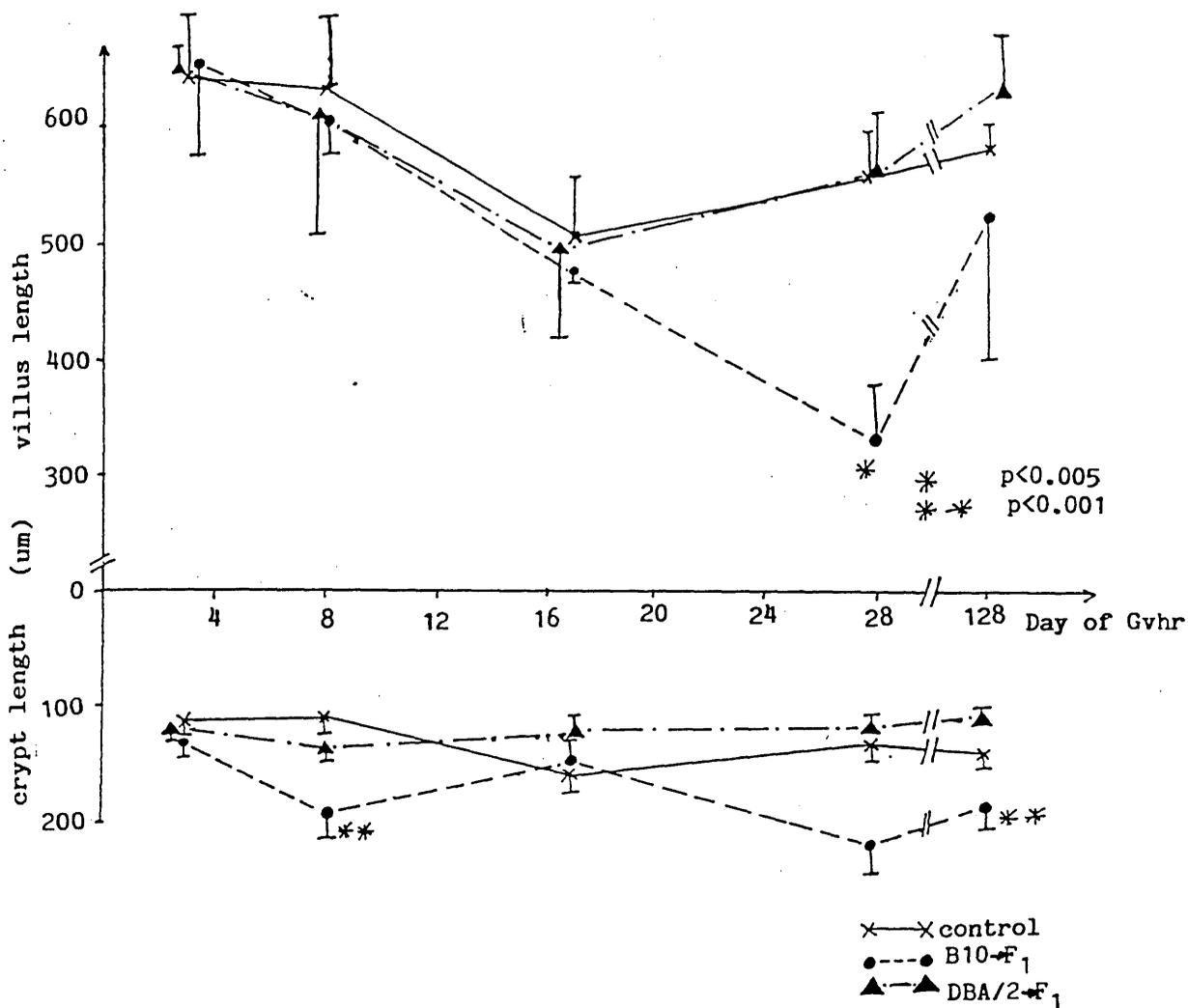


Fig. 7: Induction of intestinal Gvhr in adult, unirradiated BDF₁ mice with 10⁸ B10 or DBA/2 spleen cells. Villus and crypt lengths in the jejunum of mice with Gvhr and in controls. Results shown are means ± 1 s.d. for 4-5 mice/group.

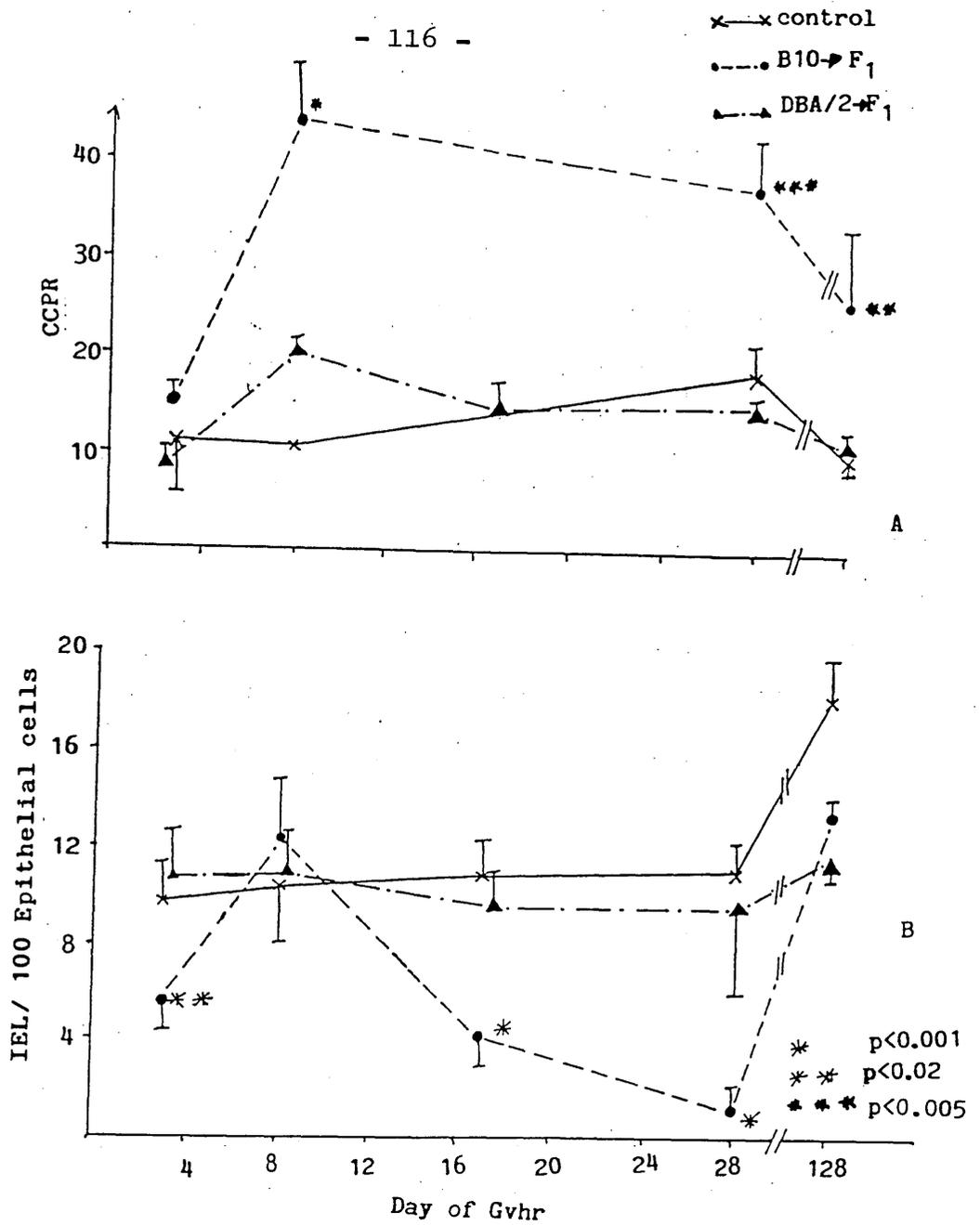


Fig. 8: Induction of intestinal Gvhr in adult, unirradiated BDF₁ mice with 10⁸ B10 or DBA/2 spleen cells. A) CCPR and B) IEL counts in the jejunum of mice with Gvhr and controls. Results shown are the CCPR or mean numbers of IEL/100 epithelial cells ± 1 s.d. for 4-5 mice/group.

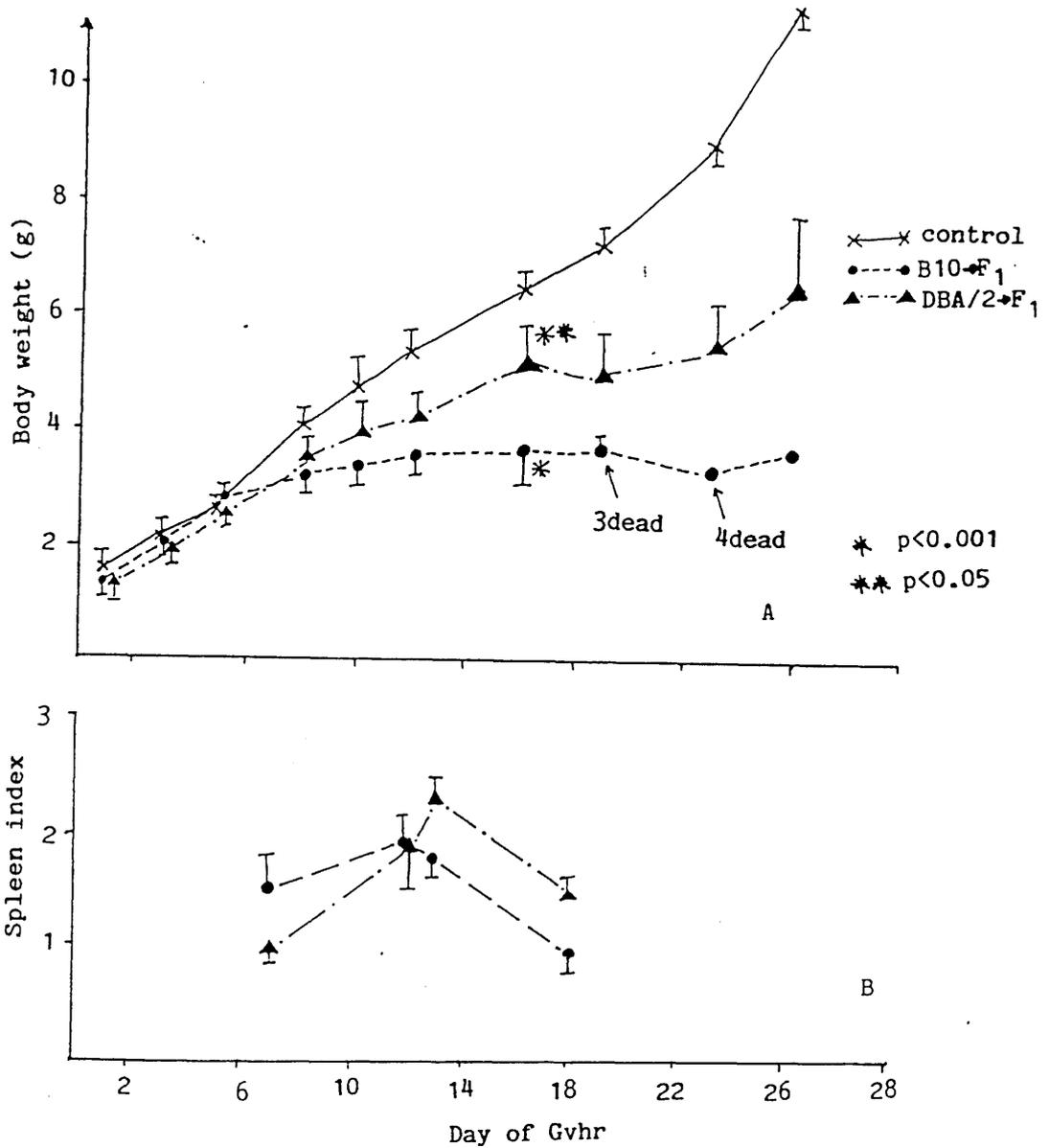


Fig. 9: Induction of systemic Gvhr in one day old BDF₁ mice with 10⁷ B10 or DBA/2 spleen cells. A) Growth rates of neonates with Gvhr compared with littermate controls. B) Development of splenomegaly in Gvhr. Results shown are means ± 1 s.d. for 6-7 mice/group for growth rate and 3 mice/group for spleen index.

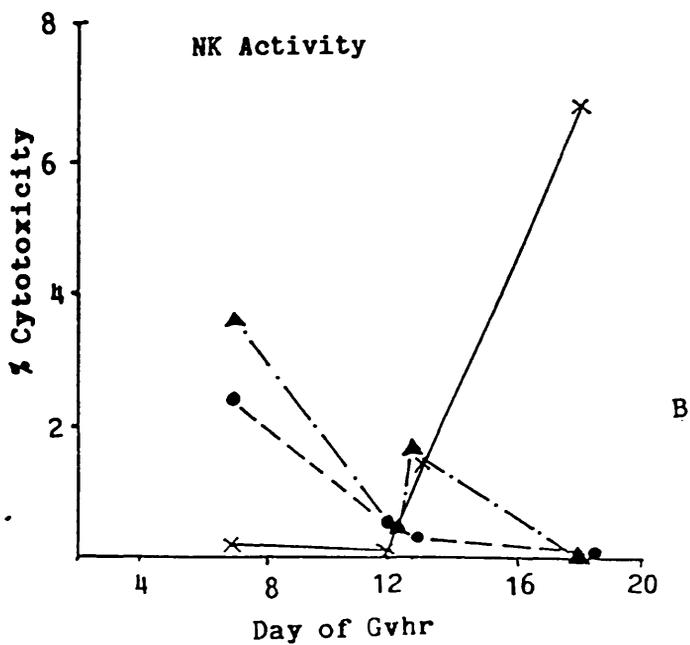
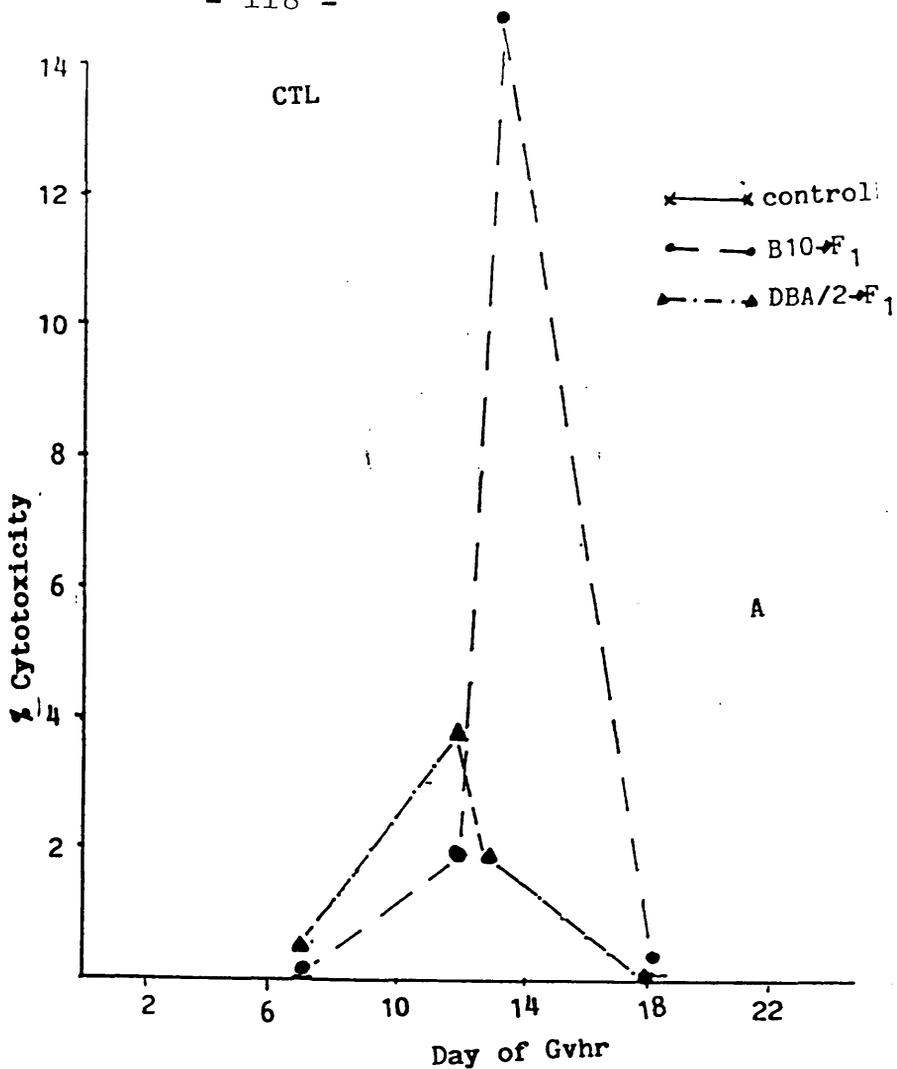


Fig. 10: Induction of systemic Gvhr in one day old BDF₁ mice with 10⁷ B10 or DBA/2 spleen cells. A) Specific CTL activity and B) NK cell activity. Results shown are % cytotoxicity at 50:1 E:T for spleen cells pooled from 3-4 mice/group.

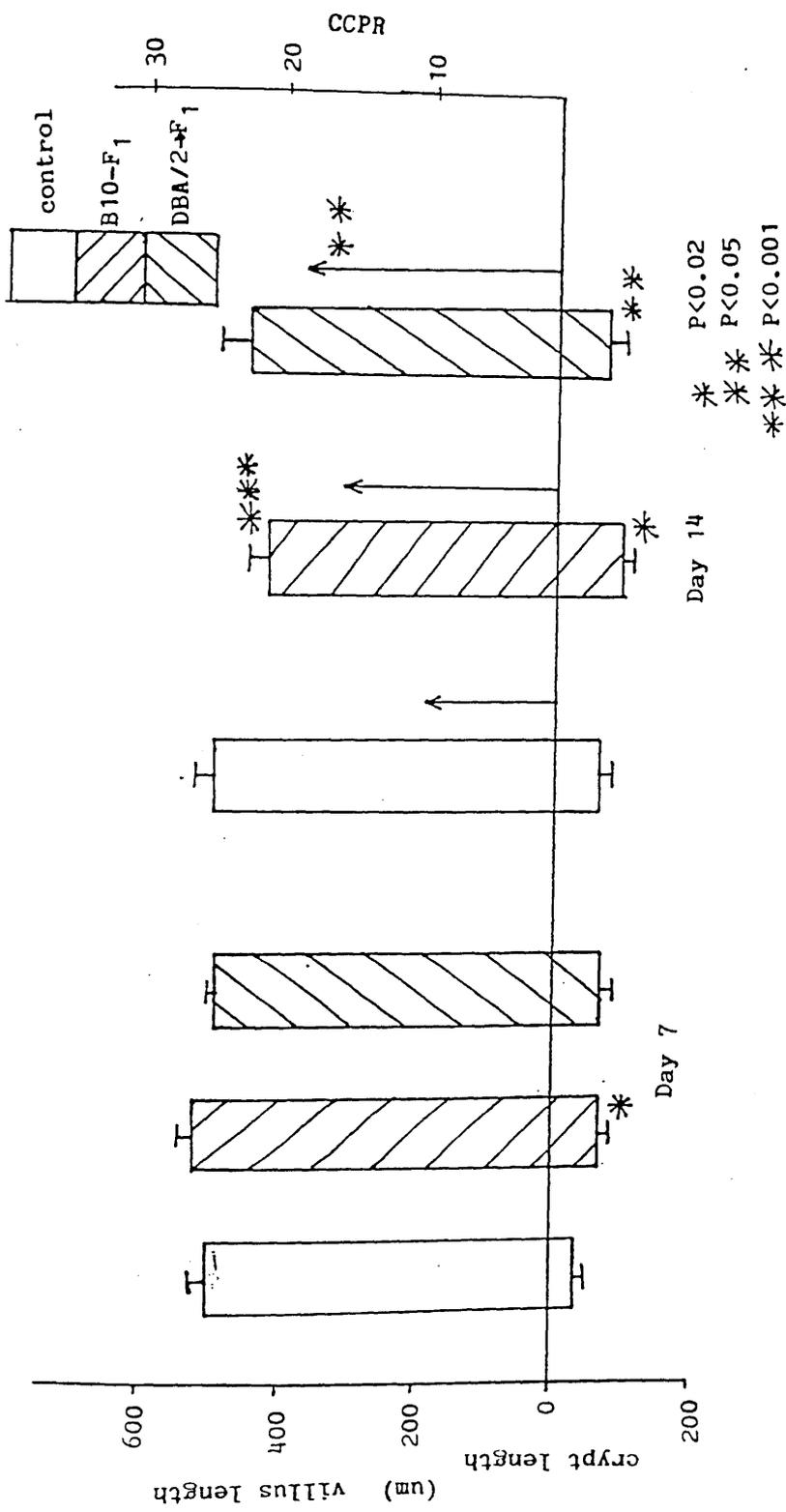


Fig. 11: Induction of intestinal Gvhr in one day old BDF₁ mice with 10⁷ B10 or DBA/2 spleen cells. Mucosal architecture in jejunum of mice on days 7 and 14 of Gvhr and in controls. Bars represent mean villus and crypt lengths ± 1 s.d. and arrows show CCPR for 3-4 mice/group.

Day 14
 * P<0.02
 ** P<0.05
 *** P<0.001

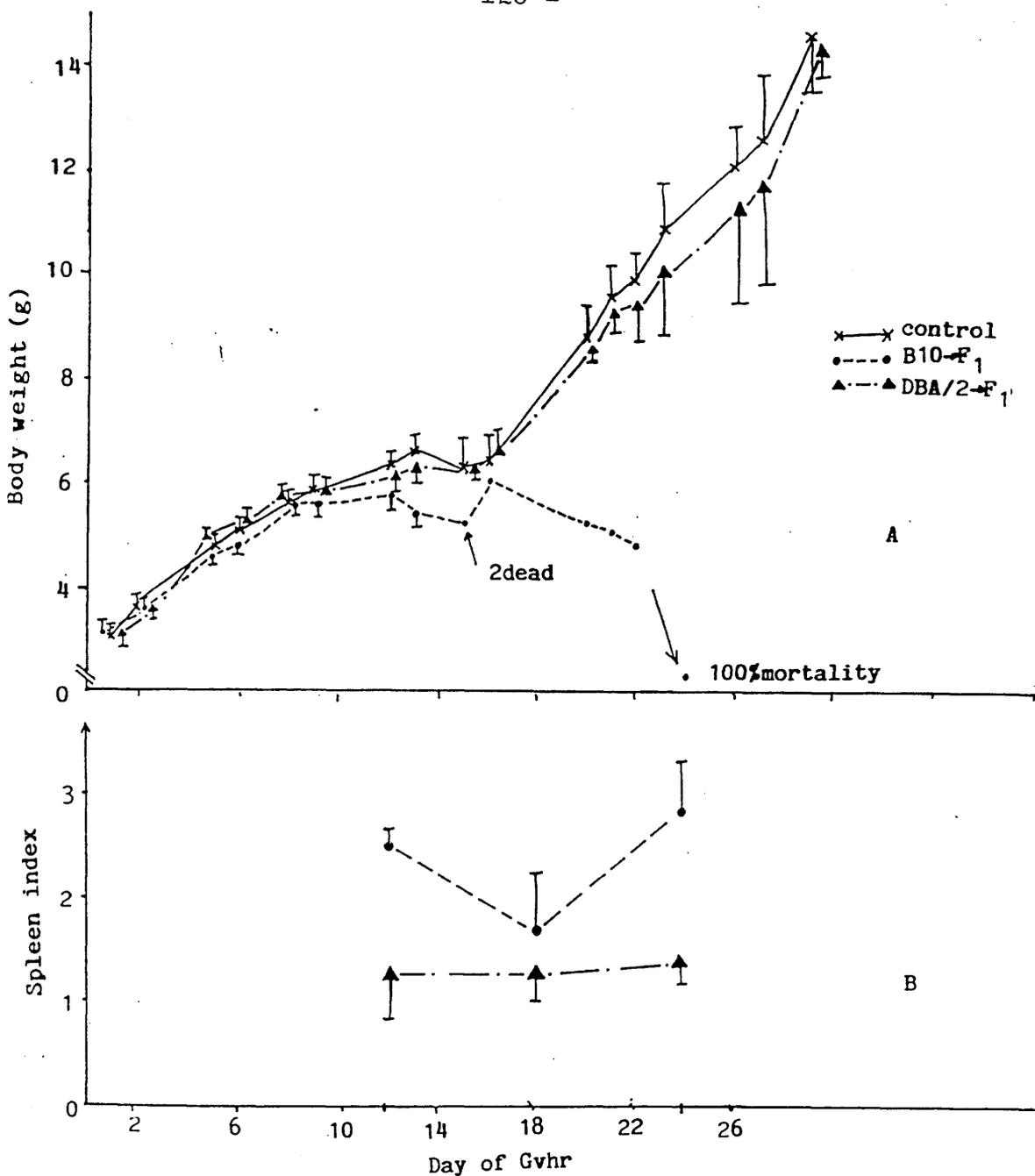


Fig. 12: Induction of systemic Gvhr in 5 day old BDF₁ mice with 10⁷ B10 or DBA/2 spleen cells. A) Growth rates of neonates with Gvhr compared with littermate controls. B) Development of splenomegaly in Gvhr. Results shown are means ± 1 s.d. for 3-4 mice/group.

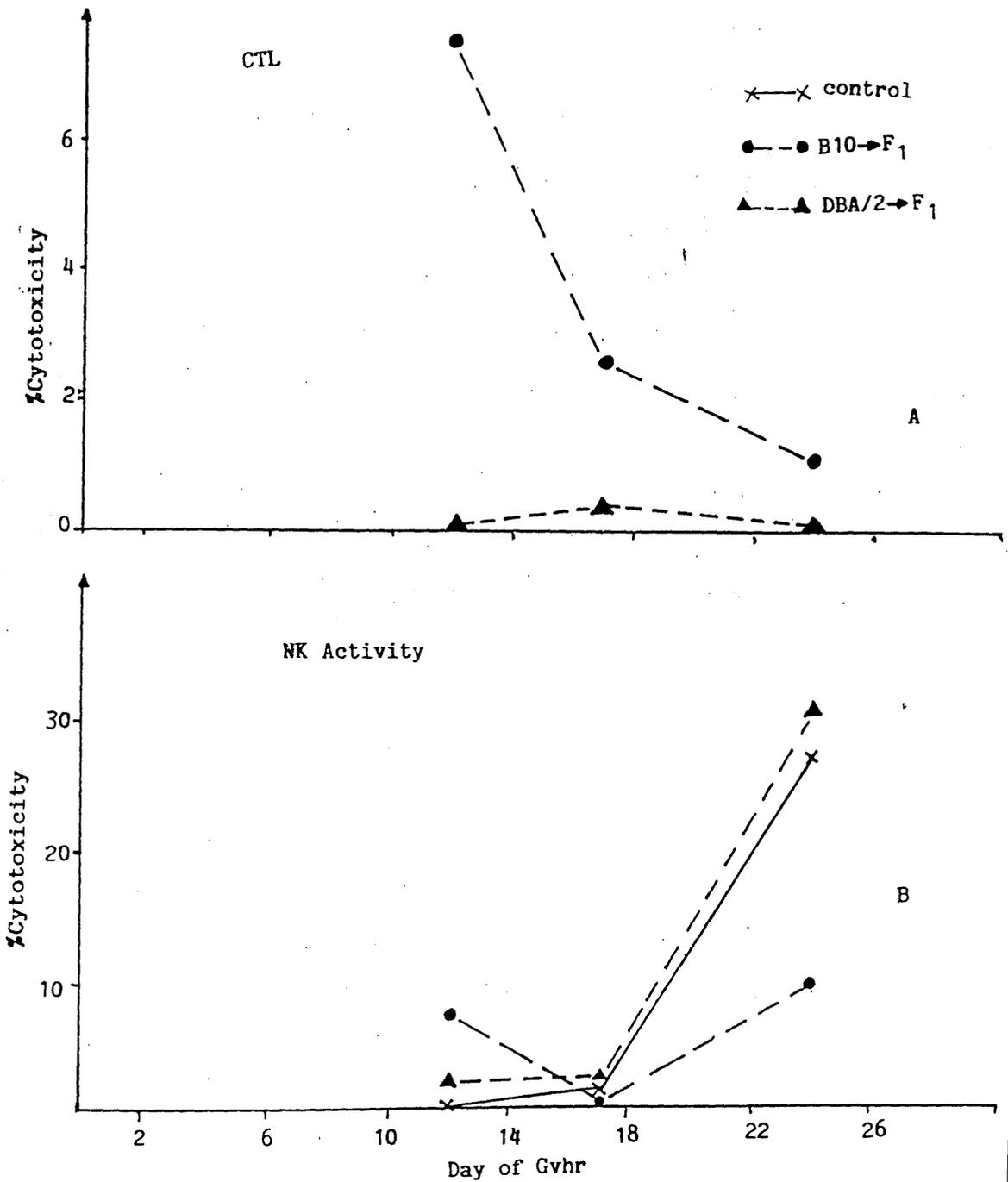


Fig. 13: Induction of systemic Gvhr in 5 day old BDF₁ mice with 10⁷ B10 or DBA/2 spleen cells. A) Specific CTL activity. B) NK cell activity. Results shown are % cytotoxicity at 50:1 E:T for spleen cells pooled from 3-4 mice/group.

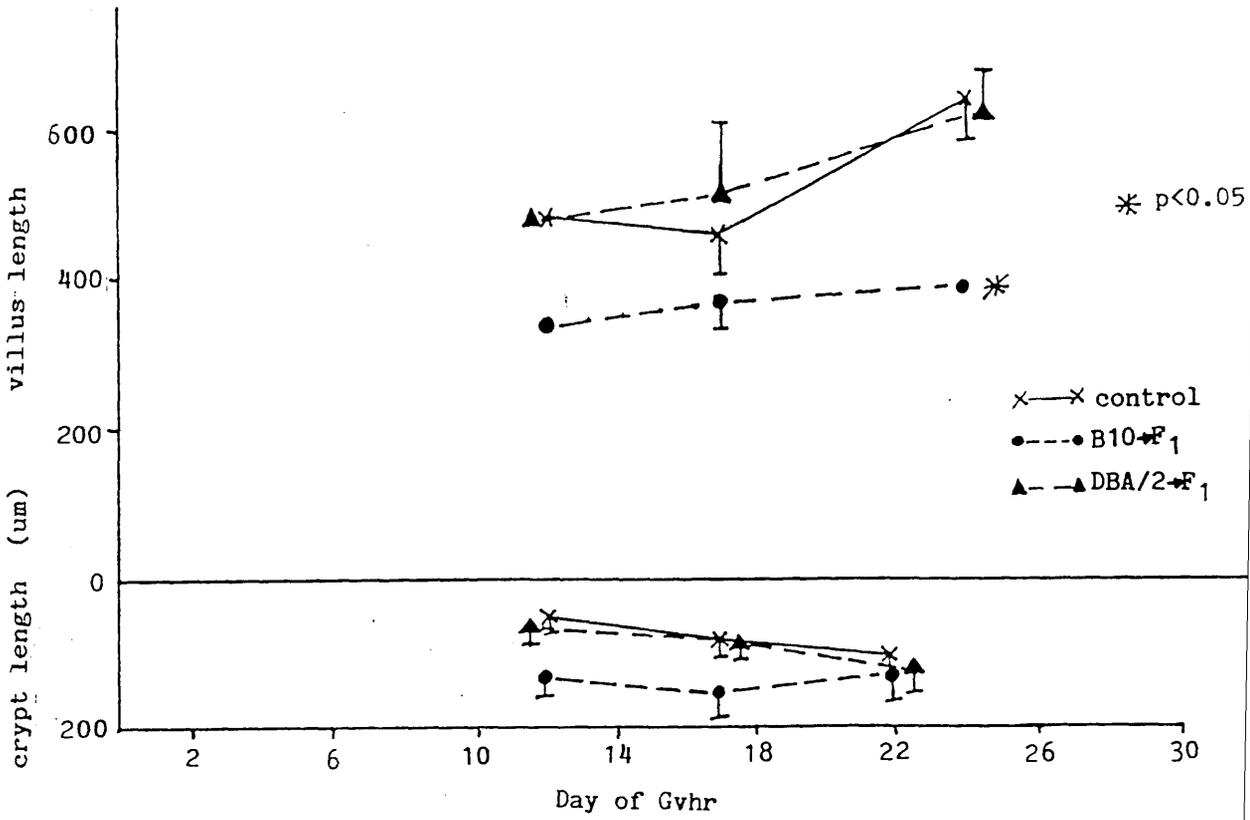


Fig. 14: Induction of intestinal Gvhr in 5 day old BDF₁ mice with 10⁷ B10 or DBA/2 spleen cells. Villus and crypt lengths in the jejunum of mice with Gvhr and in controls. Results shown are means ± 1 s.d. for 4-5 mice/group.

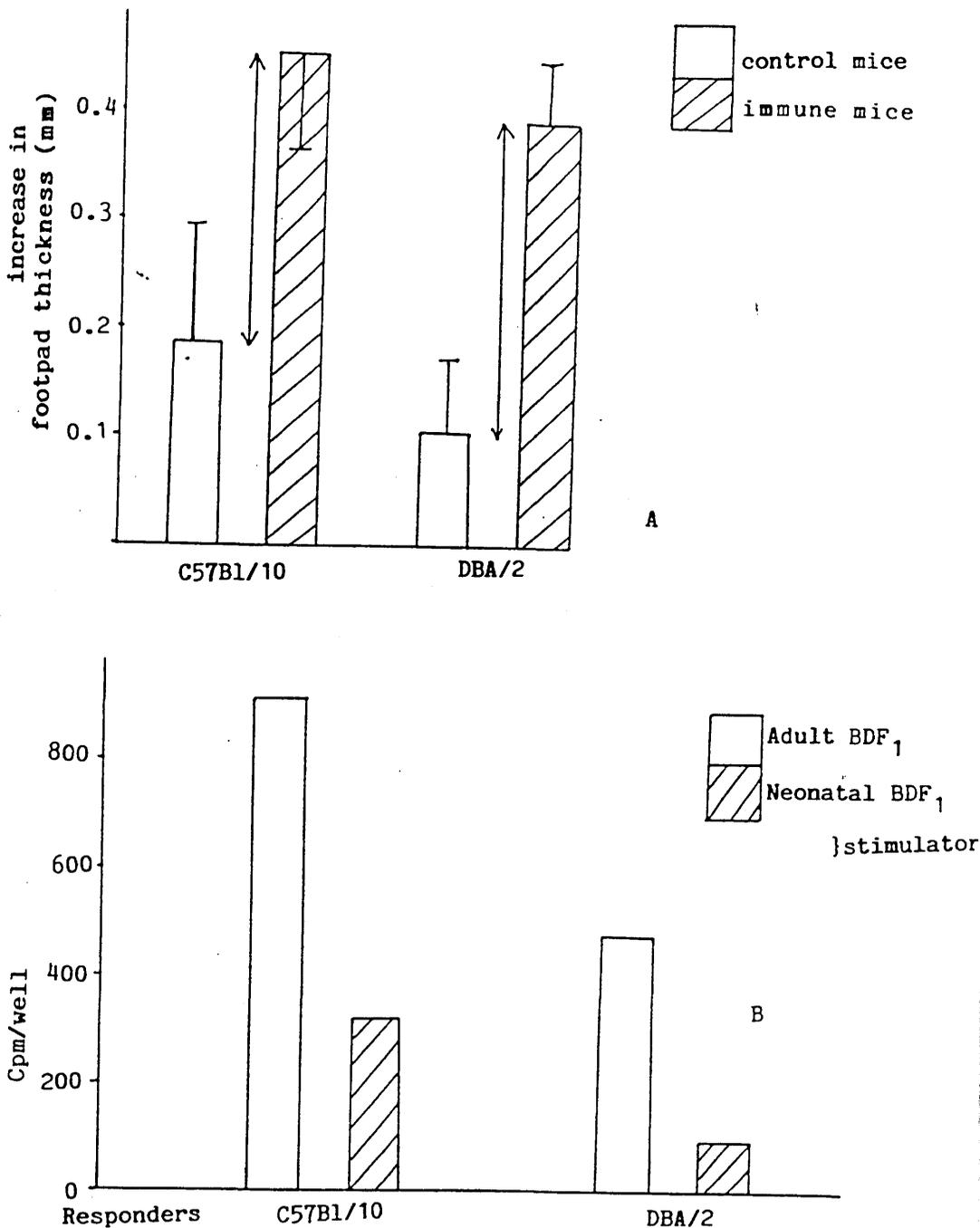


Fig. 15: A) Ability of B10 and DBA/2 mice to generate systemic DTH to BDF₁ spleen cells. DTH responses 7 days after immunization with 2×10^7 F₁ spleen cells and in controls. Results shown are increases in footpad thickness 24 hours after challenge with 10^7 BDF₁ spleen cells, for 6 mice/group. B) Mixed lymphocyte responses of B10 and DBA/2 spleen cells to mitomycin c treated spleen cells from adult or neonatal BDF₁ mice. Stimulator and responder cells were mixed at a 1:1 ratio and results shown are mean incorporation of ³H TdR (cpm/well) for triplicate assays.

CHAPTER 6

ROLE OF HOST MECHANISMS

IN THE ENTEROPATHY OF GVHR

Introduction

The results in Chapters 3 and 4 showed that irradiated or very young (CBA x BALB/c) F_1 mice developed much more severe systemic and intestinal Gvhr than that found in intact adults of the same strain. These findings underline the importance of host immunocompetence in determining the outcome of a Gvhr and the aim of the following experiments was to try and identify some of the host factors involved. Both NK cells (Kiessling et al., 1977; Dennert et al., 1985) and T-lymphocytes (Bellgrau & Wilson, 1978; Kimura & Wilson, 1984) have been implicated in protecting F_1 hybrid animals against parental lymphoid cells. As the function of both NK cells and T-cells is deficient in neonatal and irradiated mice, I decided to examine the effect of depleting NK cells and T-cells, on a Gvhr in adult, unirradiated (CBA x BALB/c) F_1 mice.

The importance of host T-cells was investigated by inducing a Gvhr in athymic (CBA x BALB/c) F_1 mice, while NK cells were depleted from normal (CBA x BALB/c) F_1 mice by in vivo treatment with anti-asialo G_{M1} antibody (Kasai et al., 1980; Habu et al., 1981; Beck et al., 1982).

The possible role of NK cells in Gvhr was examined further by augmenting NK cell activity in neonatal F_1 mice by treatment with polyinosinic:polycytidylic acid.

Experimental design

The role of host NK cells was examined in adult (CBA x BALB/c) F_1 hosts by injecting 0.2 ml 1:50 rabbit anti-asialo G_{M1} antiserum, 3 days before inducing a Gvhr with 6×10^7 CBA spleen cells and then at 3-4 day intervals thereafter. Control mice were injected with 1:50 normal rabbit serum and all groups were examined for both systemic and intestinal Gvhr.

In studies of the role of NK cells was also examined in neonatal (CBA x BALB/c) F_1 mice which had been given 10^7 parental spleen cells at 2 days of age and were then treated with 5 μ g/g poly I:C i.p. on days 7 and 10 of the experiment. These mice were examined for systemic and intestinal Gvhr on days 9 and 12.

The role of host T-cells was examined by inducing a Gvhr in athymic nude (CBA x BALB/c) F_1 mice with 6×10^7 CBA spleen cells, and these mice were sacrificed on days 4, 11, 17 and 27. The Gvhr was then compared with that found in control hosts given the same inoculum of donor cells.

Results

Induction of a Gvhr in NK cell depleted adult (CBA x BALB/c) F_1 mice

Progress of systemic Gvhr

The effects of anti-asialo G_{M1} on Gvhr were studied in four separate experiments and none of the mice with Gvhr showed any clinical evidence of weight loss, skin disease or diarrhoea. In the experiment shown NRS-treated hosts

with Gvhr had marked splenomegaly by day 4 and this rose to a peak of 2.84 ± 0.6 on day 8 (Fig. 1A) before declining thereafter. Although anti-asialo treated mice with Gvhr showed a similar pattern of splenomegaly, those animals had lower spleen indices throughout the Gvhr, with a maximum of 1.70 ± 0.50 on day 8 ($p < 0.05$ vs intact Gvhr).

As in previous studies, normal hosts with Gvhr had enhanced spleen NK cell activity compared with appropriate controls (Fig. 1B). The enhanced NK cell activity paralleled the development of splenomegaly and peaked on day 8 (50:1:48% vs 33% in controls). The efficacy of the anti-asialo G_{M1} treatment was substantiated by the markedly reduced NK activity in control mice treated with the antibody, which was always less than that of normal controls. Induction of a Gvhr in these mice also produced an increase in NK activity which peaked on day 11. However, these levels were considerably less than in intact mice with Gvhr and did not even attain those found in normal control (CBA x BALB/c) F_1 mice. Specific anti-host CTL activity was never found in any Gvhr mouse. (Results not shown).

Intestinal phase of Gvhr in NK cell-depleted hosts

The intestinal phase of Gvhr was assessed on day 11 by counts of IEL and by measurements of mucosal architecture in sections of jejunum.

As has been found previously in this model (Mowat & Ferguson, 1981b), NRS treated (CBA x BALB/c) F_1 mice with Gvhr had a significant increase in IEL count compared with

controls (Fig. 2) (19.4 ± 2.2 vs 13.5 ± 1.7 , $\rho < 0.05$). Although control mice given anti-asialo G_{M1} had a slight, but significant, reduction in IEL counts compared with normal controls (10.2 ± 2.1 vs. 13.5 ± 1.7 , $\rho < 0.05$), induction of a Gvhr in anti-asialo treated mice still led to an increase in IEL counts approaching that found in normal mice with Gvhr (18.0 ± 2.8).

NRS-treated (CBA x BALB/c) F_1 mice with Gvhr also had significant increases in both CCPR (Fig. 3) (22.9 ± 4.5 vs. 10.3 ± 1.1 , $\rho < 0.025$) and in crypt length ($141.9 \pm 5.8 \mu\text{m}$ vs. $111.1 \pm 5.7 \mu\text{m}$, $\rho < 0.001$) compared with controls. Villus atrophy was not found in these adult mice with Gvhr. In contrast to these findings, anti-asialo treated mice with Gvhr had CCPR and crypt lengths which were identical to those found in appropriate controls. Again no villus atrophy was found. It was also of interest to note that both groups of mice which were treated with anti-asialo antibody had significantly longer crypts than normal controls ($126.6 \pm 3.8 \mu\text{m}$ vs. $111.1 \pm 5.7 \mu\text{m}$, $\rho < 0.025$), as well as a slightly increased CCPR.

Thus, treatment of mice with anti-asialo G_{M1} does not prevent the increased infiltration by IEL which occurs during a Gvhr in unirradiated (CBA x BALB/c) F_1 mice, but abolishes the crypt hyperplasia which usually characterises the intestinal lesions.

Effects of anti-asialo G_{M1} on specific CTL and DTH activity

Although repeated injections of anti-asialo G_{M1} produced clear depression of NK activity in vivo, it was also necessary to exclude the possibility that the prevention of intestinal and systemic Gvhr was due to effects on other aspects of cellular immunity. In the first experiment, the effects of anti-asialo on CTL activity was examined in CBA mice which had been immunised with 3×10^7 P815 (H-2^d) cells i.p. Eleven days after immunisation, normal CBA mice had significant levels of splenic CTL activity, but this was much reduced in mice which had been treated with anti-asialo G_{M1} antibody (Fig. 4A). Allospecific DTH activity was examined by immunizing CBA mice with (CBA x BALB/c)_{F1} spleen cells into one footpad. When these mice were challenged 5 days after immunization, there was no difference between the DTH responses generated in NRS or anti-asialo treated mice (Fig. 4B). Thus anti-asialo G_{M1} inhibits generation of CTL in vivo, but has no effect on DTH responses.

The results of these experiments show that depletion of NK cells decreases the severity of Gvhr. Together with the enhanced NK cell activity found in mice with Gvhr, these results suggest that NK cells may act as effector cells in the intestinal phase of a Gvhr, in unirradiated adult F₁ mice. However the anti-asialo G_{M1} treatment had a similar inhibitory effect on CTL activity and so it was important to study the potential role of NK cells in more detail. Therefore, I examined whether selectively boosting NK activity during a Gvhr would induce a more severe intestinal

Gvhr. Poly I:C is a potent activator of NK cells in vivo, by virtue of its ability to induce interferon production (Djeu et al., 1979) and this was chosen as a means of examining further the role of NK cells in Gvhr.

Influence of poly I:C on a Gvhr in neonatal (CBA x BALB/c)_{F₁} mice

Neonatal mice were chosen as hosts in these experiments, partly because of the small amount of poly I:C available and partly because it was anticipated that they would be more sensitive to small changes in the effector cell function.

Preliminary experiments were first conducted in adult (CBA x BALB/c)_{F₁} mice to establish the pattern of NK cell activation which occurred after injection of poly I:C. These studies showed that mice given 100 µg poly I:C had elevated levels of NK activity within 24 hours and these continued to increase at 48 and 72 hours before declining (Data not shown). Therefore in the experiments examining a Gvhr in neonatal hosts, mice were treated with poly I:C 24 hours before inducing a Gvhr and then on days 7 and 10 of the experiment around the time when the onset of a destructive Gvhr was anticipated.

Two day old (CBA x BALB/c)_{F₁} mice were injected with 10^7 CBA spleen cells and both groups of mice with Gvhr developed weight loss and runting about day 9. Most of the mice with Gvhr died by day 18 and there was no difference in the mortality rate (Fig. 5A). Nevertheless, mice treated with

polyI:C had consistently more weight loss than untreated littermates at all times after day 8 of the Gvhr, although this did not reach statistical significance. There was no significant difference in weight between polyI:C treated control mice and untreated controls, and no polyI:C treated mice died. Both groups of Gvhr mice had significant splenomegaly on days 9 and 12 of the Gvhr and there was no significant difference between the spleen indices in the two groups of mice at either time (Fig. 5B).

Anti-host specific cytotoxicity was virtually undetectable on day 9 but by day 12 untreated mice with Gvhr had moderate levels of CTL activity, which was greater than that found in mice which had received polyI:C (Fig. 6) (8.5% vs. 2.7%). As expected, virtually no NK activity was found in normal control mice at either time. However, some NK activity was detectable on day 9 in control mice given poly I:C (Fig. 6) (2.8% vs. 0.4% in controls) and similar levels were still present on day 12 (3.6% vs. 1.2% in controls). Gvhr mice treated with polyI:C had markedly increased NK cell activity on day 9 (10.9%) and there was still some NK cell activation on day 12 (3.6%). In comparison, although untreated mice with Gvhr had increased NK cell activity compared with appropriate controls, at both times these levels were less than those in polyI:C treated Gvhr mice (1.4% and 3%).

Thus poly I:C increased the NK cell activation found in Gvhr and there was some evidence that it also produced a more severe systemic disease. However, the generation of CTL activity in Gvhr was decreased by poly I:C.

Effects of poly I:C on the development of intestinal Gvhr

By day 9 of the experiment untreated mice with Gvhr had developed significant villus atrophy ($493.4 \pm 13 \mu\text{m}$ vs. $565.3 \pm 1 \mu\text{m}$ in controls, $p < 0.001$) and crypt hypertrophy ($90.6 \pm 6 \mu\text{m}$ vs. $57.2 \pm 6 \mu\text{m}$ in controls, $p < 0.001$) (Fig. 7). Similar features were found in Gvhr mice given poly I:C, but these mice had more severe intestinal damage with significantly shorter villi and longer crypts than untreated mice with Gvhr ($384.9 \pm 11 \mu\text{m}$, $p < 0.001$ and $137.0 \pm 16 \mu\text{m}$, respectively, $p < 0.001$). Furthermore, whereas untreated Gvhr mice had a lower CCPR than controls at this time (Fig. 7) (4.61 ± 0.30 vs. 8.8 ± 4.1). Poly I:C treated mice with Gvhr had a very marked increase in CCPR (26.4 ± 3.02 vs. 4.75 ± 1.65 , $p < 0.001$). Poly I:C itself had no marked effects on mucosal architecture, although poly I:C treated controls appeared to have slightly shorter villi and longer crypts than untreated controls. A similar pattern of results was found on day 12, with more severe villus atrophy and crypt hypertrophy in poly I:C treated mice with Gvhr (Fig. 7). Insufficient mice remained at this stage of the experiment to examine CCPR.

Effects of activated adult NK cells on syngeneic neonatal mice

These results suggested that poly I:C exacerbated the intestinal phase of Gvhr in neonatal (CBA x BALB/c) F_1 mice and this was not accompanied by an increase in specific CTL activity. Therefore, the most likely interpretation was that non-specific activation of NK cells was responsible for the intestinal damage. If this were the case, it might be anticipated

that poly I:C activated NK cells themselves might produce some intestinal pathology. Although this idea was supported by the alterations in mucosal architecture found in some poly I:C treated control neonates, these were minor and inconsistent and so I examined the possible role of activated NK cells in more detail by examining the intestine of adult (CBA x BALB/c) F_1 mice in whom poly I:C had induced a large rise in NK activity. In addition, I investigated whether spleen cells from poly I:C treated mice would cause intestinal changes in syngeneic neonatal recipients.

Adult (CBA x BALB/c) F_1 mice were given two daily doses of 100 μ g poly I:C i.p. and sacrificed 48 hours after the second dose. At this time, the splenic NK activity of these mice was 58.4% compared with 9.2% in untreated controls (Fig. 8A). Although these mice had minor villus shortening (Fig. 8B) ($579.0 \pm 4.0 \mu\text{m}$ vs. $616.5 \pm 21 \mu\text{m}$) and longer crypts ($106.3 \pm 13 \mu\text{m}$ vs. $84.7 \pm 9 \mu\text{m}$) compared with control mice, these differences were not significant and poly I:C had no effect on the CCPR.

The effect of poly I:C treated donor cells was then examined by transferring 2.5×10^7 spleen cells from these adult donors into syngeneic 2 day old mice. When these hosts were sacrificed 48 hours later, they showed a small increase in CCPR compared with that in mice given normal adult spleen cells (Fig. 8C). However, the CCPR was very low in all groups and this difference was not significant. No other changes in mucosal architecture were noted and together, these experiments provided little conclusive evidence that NK cells, themselves, could induce intestinal pathology.

Induction of Gvhr in athymic (CBA x BALB/c)_F₁ mice

Progress of systemic Gvhr

Two separate experiments were performed to compare the Gvhr in normal and athymic hosts. All the mice remained well throughout, with no evidence of runting or diarrhoea. The progress of systemic Gvhr was assessed in the first experiment and the results are shown in Figure 9. The Gvhr in euthymic hosts followed a prolonged course with moderately intense splenomegaly from days 4-27 and, at each time after day 4, athymic mice with Gvhr had more severe splenomegaly than the corresponding euthymic hosts. The small number of athymic mice available, normally precluded statistical analysis, but in the groups studied on day 11, athymic mice had significantly greater splenomegaly than intact hosts (2.55 ± 0.45 vs. 1.30 ± 0.47 in controls, $p < 0.02$).

Splenic NK and CTL activity was measured throughout the Gvhr. As in other experiments using this model, an elevated level of NK activity was found during the first two weeks of Gvhr in euthymic (CBA x BALB/c)_F₁ mice and this peaked on day 11 of the experiment before returning to control levels thereafter (Fig.10A). Compared with normal (CBA x BALB/c)_F₁ mice control nude mice had extremely high levels of spleen NK activity, confirming earlier studies (Mowat et al., 1983). However, in contrast with enhanced NK activity found in euthymic hosts, nude mice with Gvhr had lower levels of NK activity than athymic controls at all times of the Gvhr. This suppression was most marked on day 15 (Fig.10A: 12.8% vs. 53% in nude controls). In parallel,

nude mice with Gvhr, had low, but consistent levels of specific anti-host CTL on days 11-27, while intact mice with Gvhr never showed significant CTL activity (Fig. 10B). CTL activity in nude mice with Gvhr reached a peak of 7.5% on day 11 and as found in previous chapters this seemed to be associated with the marked loss of NK activity in these mice.

Intestinal phase of Gvhr in athymic (CBA x BALB/c)_F₁ mice

In the second experiment, intestinal pathology was examined in euthymic and athymic (CBA x BALB/c)_F₁ mice on day 11 of the Gvhr, as this was around the time of maximal systemic Gvhr in the nude mice used in experiment 1.

At this time, intact mice with Gvhr had an increased IEL count compared with controls (Fig. 11) (21.3 ± 1.2 vs. 16.8 ± 2.0 , $\rho < 0.02$). As reported previously (Ferguson & Parrott, 1972), control nude mice had very low IEL counts compared with intact controls (5.0 ± 0.7 , $\rho < 0.005$) but a Gvhr led to a significantly increased IEL count in athymic mice with Gvhr (9.8 ± 1.4 , $\rho < 0.001$ vs. nude controls). However, the IEL count in these mice did not even approach the levels found in control euthymic mice.

In this experiment, fixation difficulties prevented measurement of the CCPR in euthymic _F₁ mice, but intact Gvhr mice had a significant increase in crypt length compared with controls (Fig. 12) ($141.5 \pm 5.5 \mu\text{m}$ vs. $123.3 \pm 8.8 \mu\text{m}$, $\rho < 0.005$) and previous studies indicate that there would also be an increase in CCPR under these conditions. As usual there was no evidence of villus atrophy in this model of

Gvhr. In contrast, athymic F_1 mice with Gvhr not only had very large increases in both CCPR (27.1 ± 0.7 , $p < 0.001$) and crypt length ($180.9 \pm 9.8 \mu\text{m}$, $p < 0.001$) compared with athymic controls (7.6 ± 0.6 and $101.7 \pm 1.8 \mu\text{m}$ respectively) but they also had significant villus atrophy ($659.4 \pm 75.0 \mu\text{m}$ vs. $804.3 \pm 10.2 \mu\text{m}$, $p < 0.001$). It was also interesting to note that athymic control mice had significantly reduced crypt lengths compared with normal controls ($p < 0.005$).

Thus athymic mice not only develop more severe splenomegaly and proliferative intestinal pathology during a Gvhr but also have evidence of a more destructive disease as shown by the development of villus atrophy and CTL activity and suppression of NK cell activity. Interestingly, the acute Gvhr in adult, athymic mice had the same features of the destructive disease described in earlier chapters.

Summary and conclusions

These results confirm that the systemic and intestinal consequences of Gvhr can be altered by manipulating the immunocompetence of host mice.

Treatment of adult (CBA x BALB/c) F_1 mice with anti-asialo G_{M1} reduced their NK activity and inhibited the development of the splenomegaly and NK cell activation found in normal F_1 mice with Gvhr. Furthermore, anti-asialo-treated mice had slightly lower IEL counts than controls and although these rose during Gvhrs, anti-asialo-treated mice did not develop any of the alterations in intestinal architecture normally associated with Gvhr. Control experiments also showed that treatment of CBA mice with anti-asialo G_{M1} antibody

reduced the generation of allospecific CTL, but had no effect on the generation of DTH responses to F_1 cells. These findings show clearly that NK cells do not protect the host from Gvhr induced intestinal lesions, but rather may play a pathogenic role in the Gvhr.

Complementary studies in neonatal (CBA x BALB/c) F_1 provided some support for this idea by showing that augmentation of NK activity with poly I:C produced more severe intestinal damage in mice with Gvhr as well as a slightly more severe systemic disease. This effect of poly I:C was paralleled by a greater enhancement of NK activity than that found in untreated Gvhr mice, but poly I:C reduced the generation of anti-host CTL activity.

Some evidence was also obtained that poly I:C itself produced minor degrees of villus atrophy and crypt hyperplasia in normal adult and neonatal mice. However, it proved impossible to induce intestinal damage by transferring activated NK cells from poly I:C treated mice and the pathogenic role of NK cells requires further confirmation.

The effect of host T-lymphocyte depletion was investigated using athymic adult (CBA x BALB/c) F_1 mice. In comparison with intact (CBA x BALB/c) F_1 hosts athymic mice developed a much more severe Gvhr, with more intense splenomegaly and crypt hyperplasia. In addition, nude mice with Gvhr had suppressed NK cell activity, which contrasted with the NK cell activation found in normal hosts. In parallel, only athymic hosts developed specific CTL activity as well as villus atrophy in Gvhr. These features are therefore similar

to those found during an acute Gvhr in early neonatal (CBA x BALB/c) F_1 mice and suggest that host T cells normally protect intact adults from the most severe consequences of a Gvhr.

In conclusion, the results in this chapter suggest that host T-cells protect recipient mice against Gvhr, while the non-specific activation of host NK cells plays a pathogenic rather than a protective role.

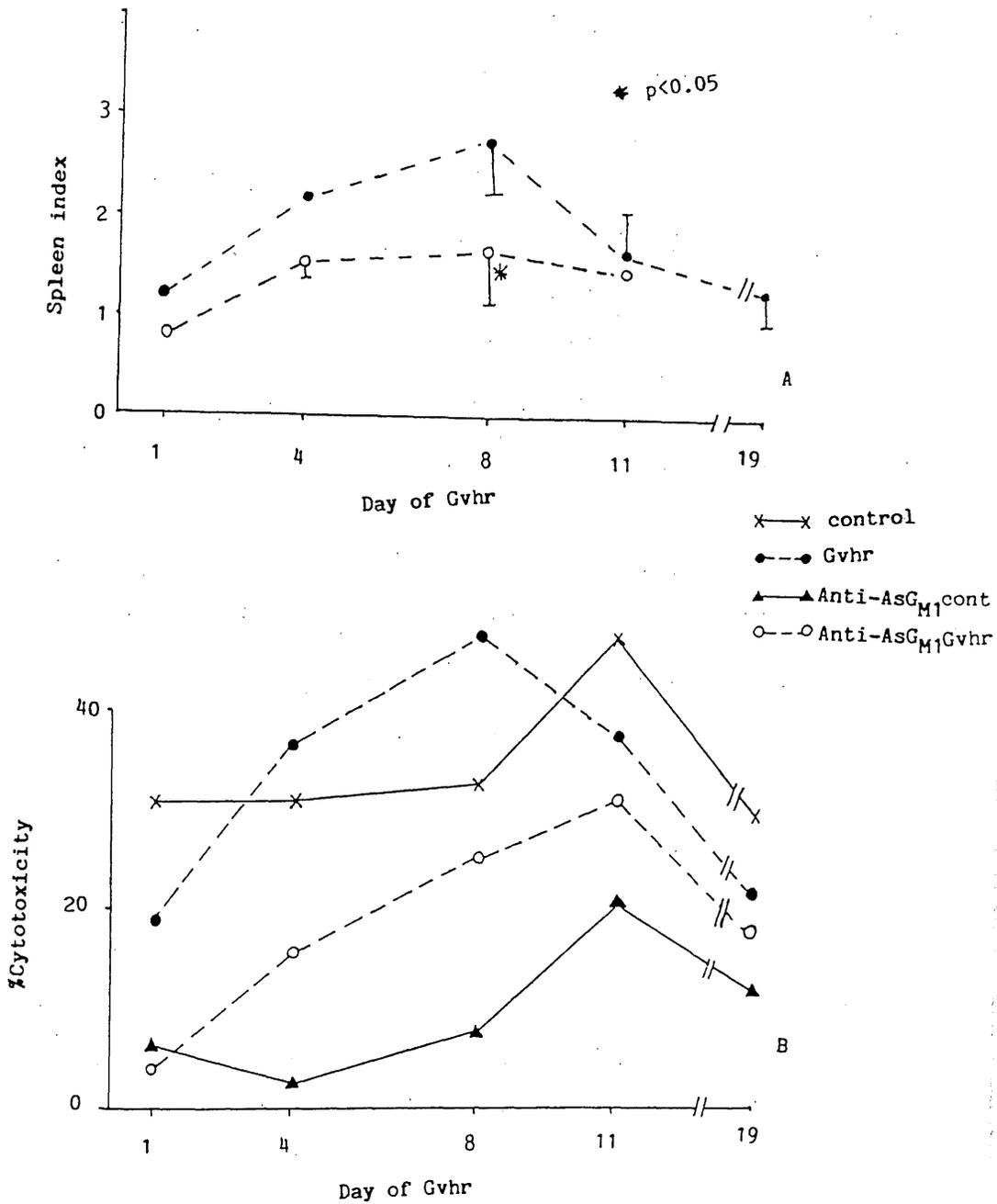


Fig. 1: Effect of anti-asialo G_{M1} on NK cell activity during a Gvhr in adult unirradiated (CBA x BALB/c)_{F1} mice. A) Assessment of spleen index in anti-As G_{M1} and intact Gvhr mice. B) NK Activity in anti-AsG_{M1} and intact Gvhr mice, compared with appropriate controls. Results shown are means ± 1 s.d. for 3 mice/group. Cytotoxicity results are 50:1 E:T for spleen cells pooled from 3 mice/group.

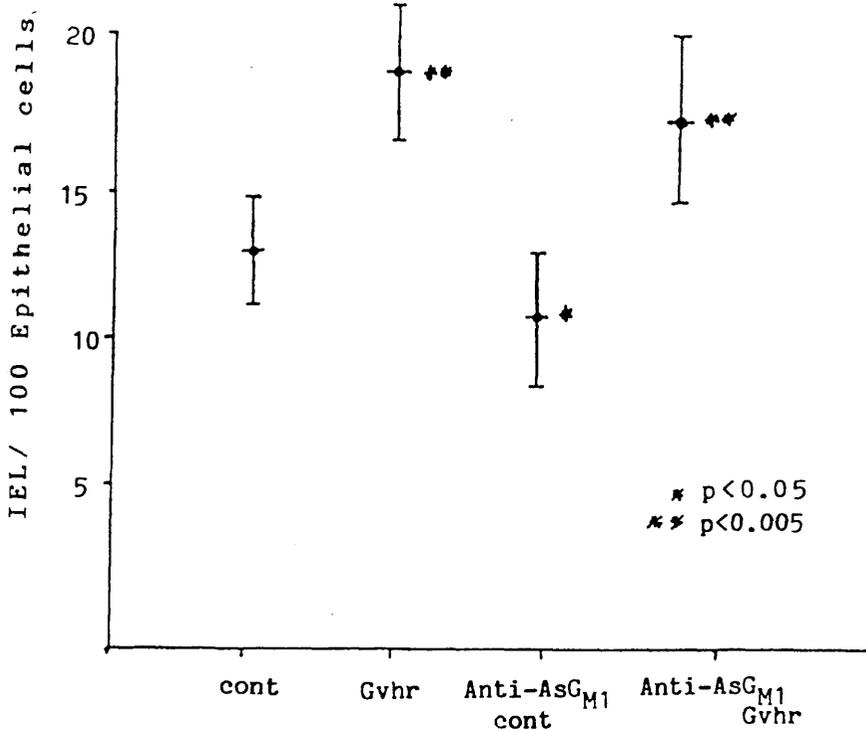


Fig. 2: Effect of anti-asialo G_{M1} on the intestinal phase of Gvhr in unirradiated adult (CBA x BALB/c)F₁ mice. IEL counts in the jejunum of NRS -₁ or anti-AsG_{M1} treated mice on day 11 of the Gvhr and in appropriate controls. Results shown are the mean numbers of IEL/100 epithelial cells \pm 1 s.d. for 4-5 mice/group.

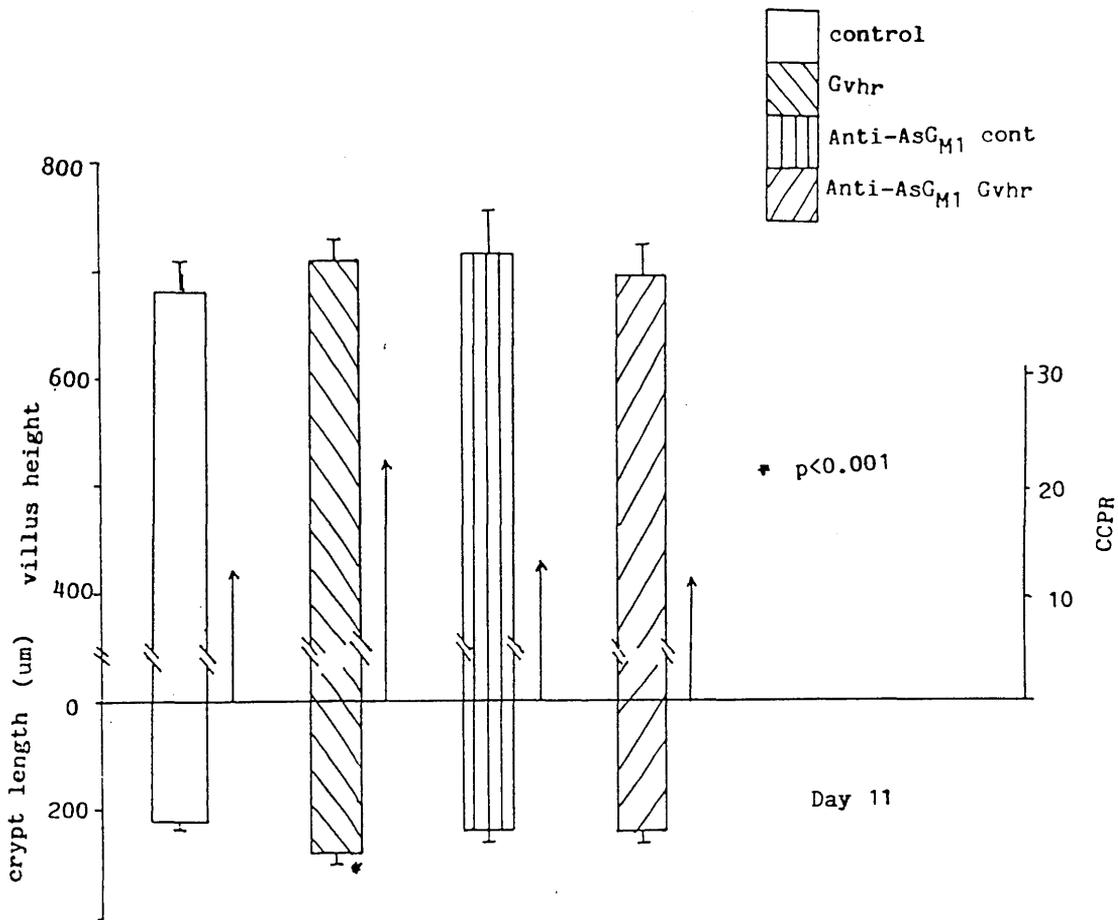


Fig. 3: Effect of anti-asialo G_{M1} on the intestinal phase of Gvhr in unirradiated adult (CBA x BALB/c) F_1 mice. Mucosal architecture in the jejunum of NRS or anti-As G_{M1} treated mice on day 11 of the Gvhr, and in appropriate controls. Bars represent mean villus and crypt lengths \pm 1 s.d., and arrows show CCPR for 4-5 mice/group.

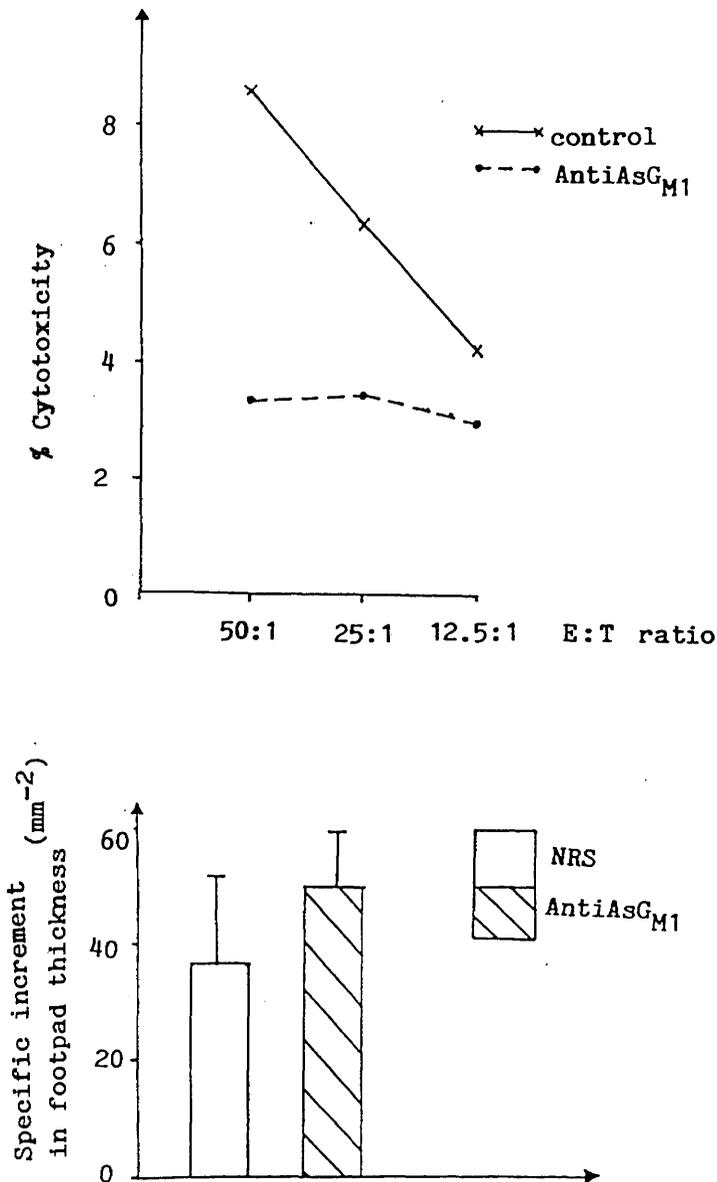


Fig. 4: Effect of anti-asialo G_{M1} on allospecific CTL and DTH responses. A) Specific anti-H-2^d CTL activity in spleens of NRS or anti-AsG_{M1} treated mice, 11 days after immunization with 10⁷ P815 (H-2^d) tumour cells i.p. Results are the percentage lysis of P815 targets at different E:T ratios, using spleen cells pooled from 3 mice/group. B) Anti-H-2^d specific DTH responses in NRS or anti As G_{M1} treated CBA mice, measured 7 days after immunization with (CBA x BALB/c)F₁ spleen cells. Results shown are mean specific increments in footpad thickness \pm 1 standard deviation, 24 hr after intradermal challenge with 10⁷ F₁ spleen cells for 6 mice/group.

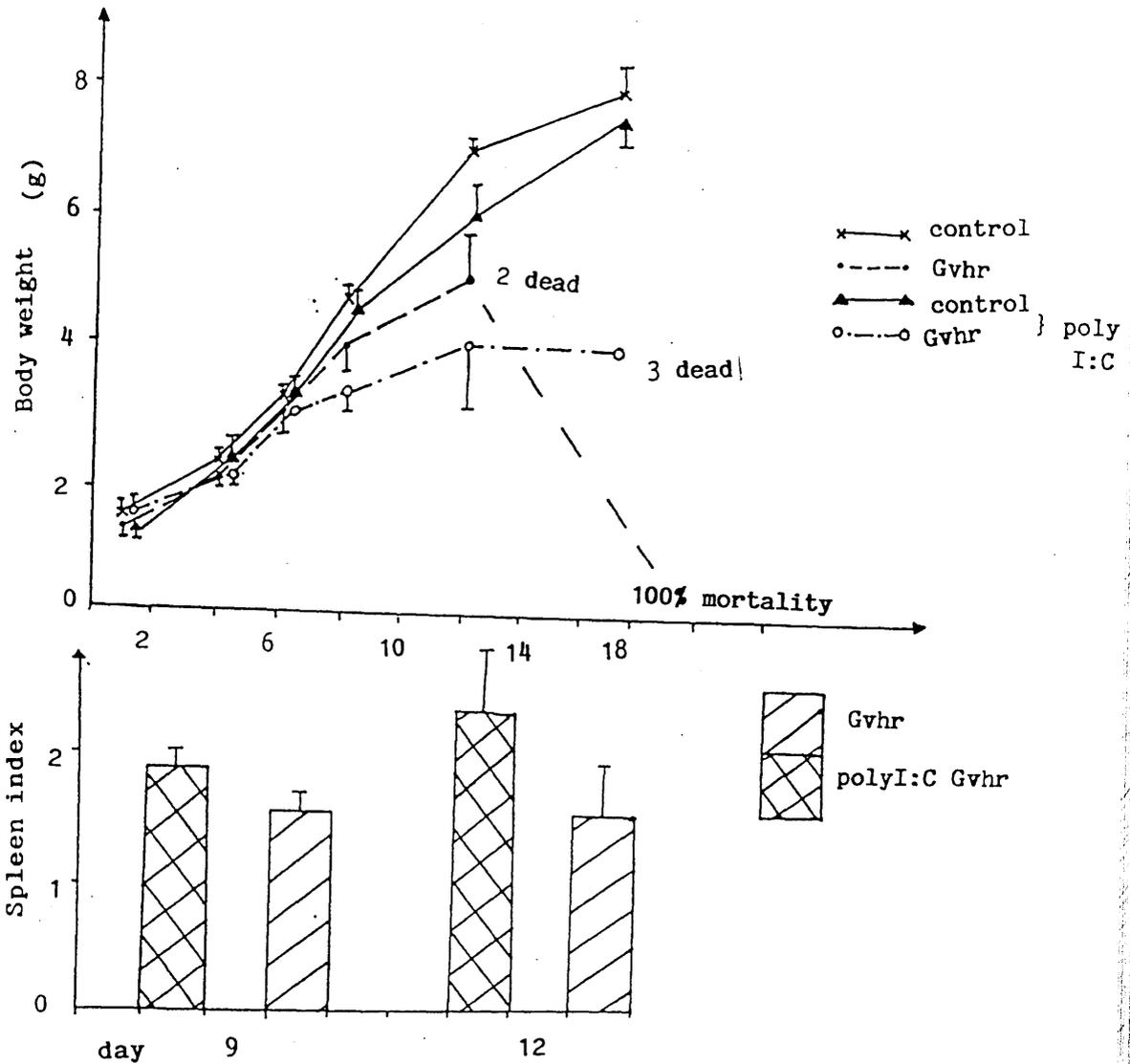


Fig. 5: Effect of 5 µg/g poly I:C on systemic Gvhr in 1-2 day old neonatal (CBA x BALB/c)F₁ mice. A) Growth rates of Gvhr mice which had been treated with poly I:C or NaOH on days 7 and 10, and of appropriate controls. B) Development of splenomegaly in poly I:C and NaOH treated mice with Gvhr. Results shown are means ± 1 s.d. for 5 mice/group for body weight and 3 mice/group for spleen index.

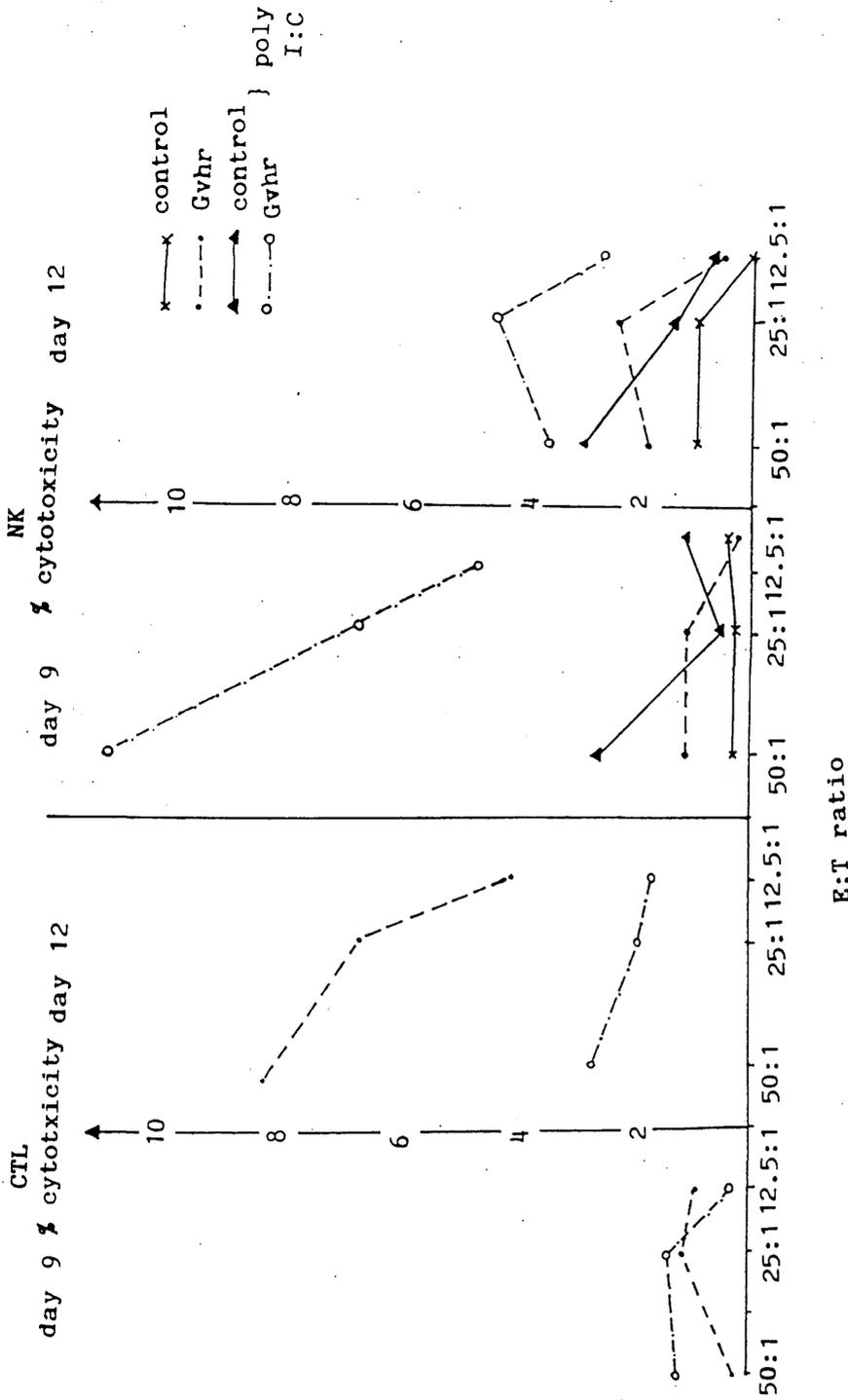


Fig. 6: Effects of poly I:C on systemic Gvhr in 1-2 day old neonatal (CBA x BALB/c)_{F₁} mice. Specific CTL and NK activity in poly I:C and NaOH treated mice with Gvhr and in appropriate controls. Results shown are % cytotoxicity at different E:T ratios, using spleen cells pooled from 3 mice/group.

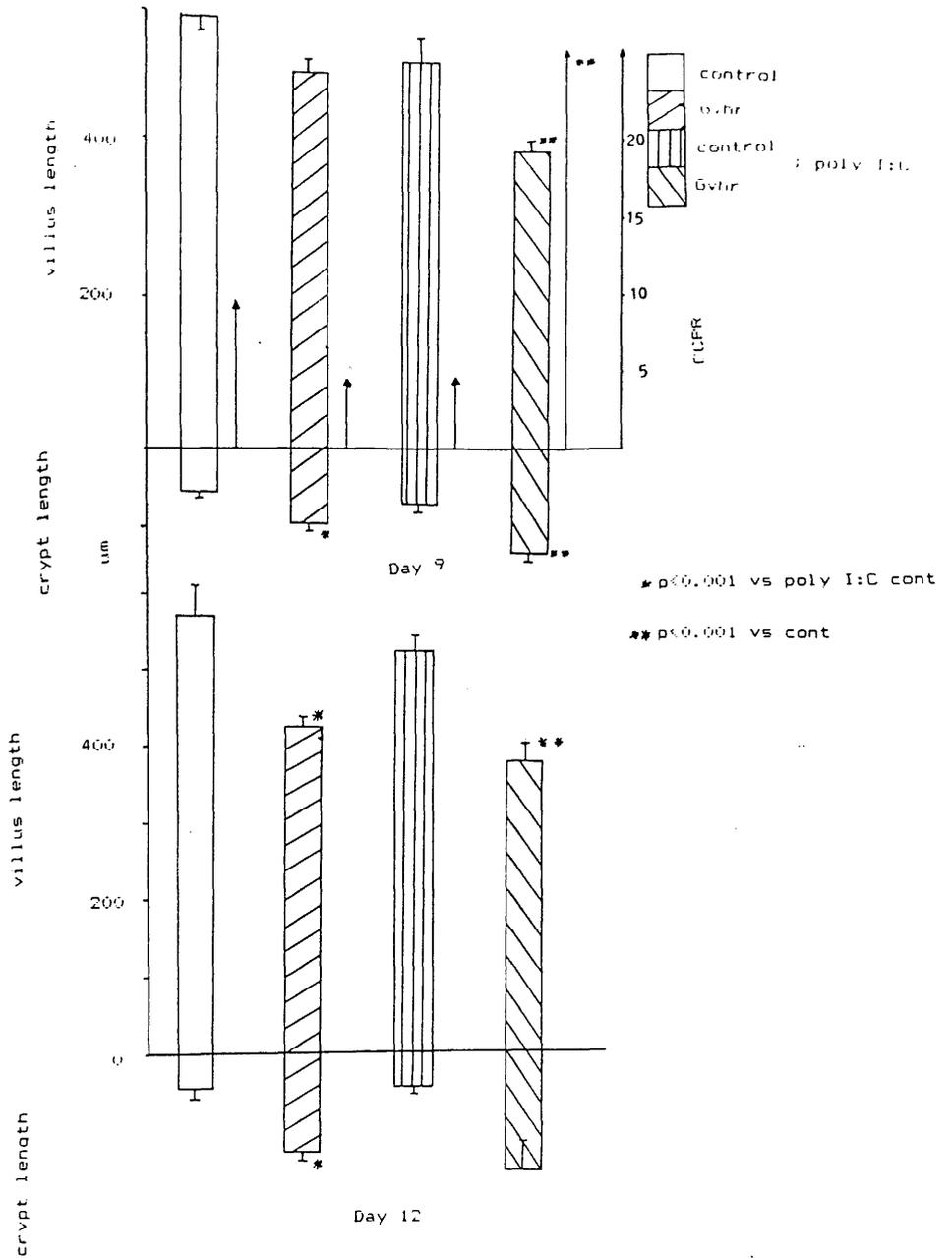


Fig. 7: Effects of poly I:C on the intestinal phase of Gvhr in 1-2 day old neonatal (CBA x BALB/c) F_1 mice. Mucosal architecture in the jejunum of poly I:C or NaOH treated mice on days 9 and 12 of the Gvhr, and in appropriate controls. Bars represent mean villus and crypt lengths \pm 1 s.d. and arrows show CCPR for 4 mice/group.

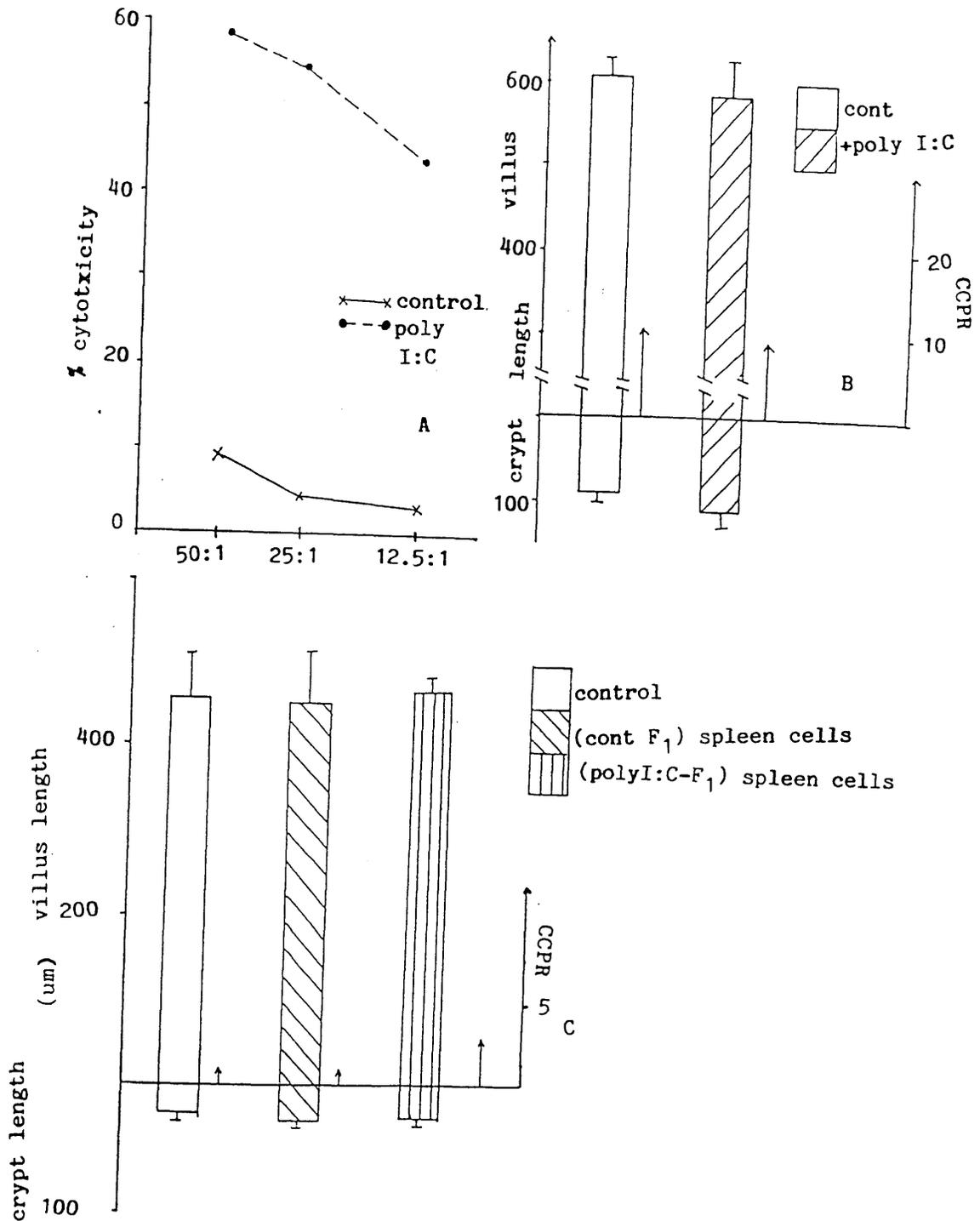


Fig. 8: Effects of poly I:C activated adult NK cells on mucosal architecture in syngeneic neonatal mice. A) NK activity in adult (CBA x BALB/c)F₁ mice, which were sacrificed 48 hours after the second daily dose of poly I:C, and in appropriate controls. B) Mucosal architecture in adult (CBA x BALB/c)F₁ mice as 8A). C) Mucosal architecture in the jejunum of 2 day old neonatal (CBA x BALB/c)F₁ mice given control or poly I:C treated syngeneic adult NK cells, and in appropriate controls. Bars represent mean villus and crypt lengths \pm 1 s.d., and arrows show CCPR for 4-5 mice/group. Cytotoxicity results are shown at different E:T ratios using spleen cells pooled from 5 mice/group.

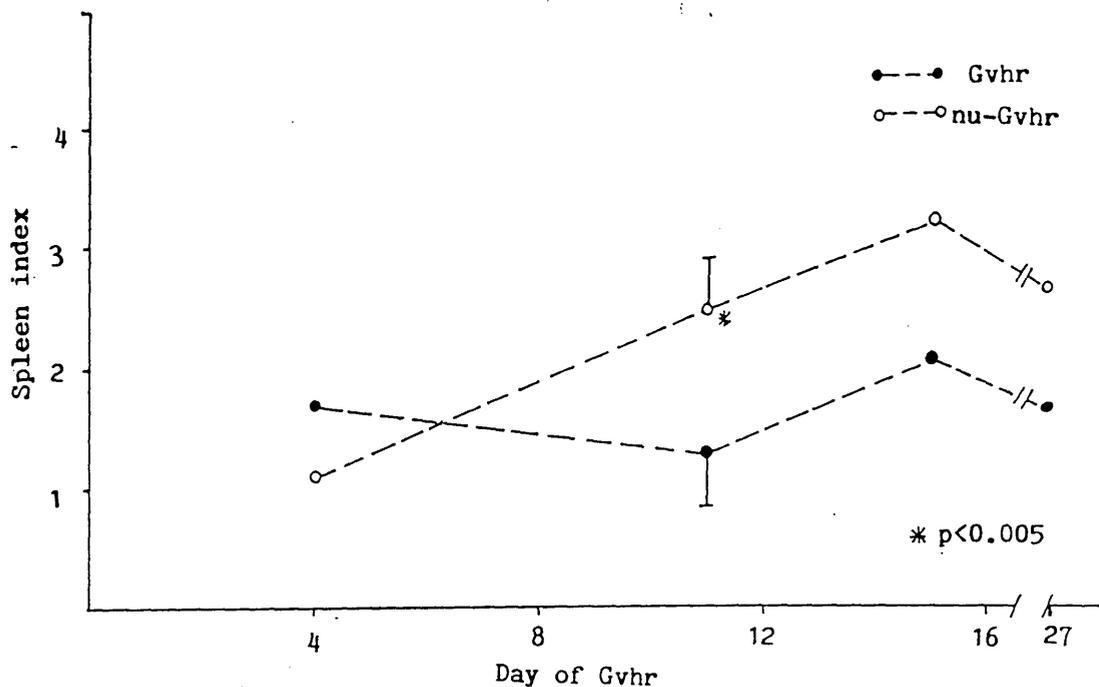


Fig. 9: Progress of systemic Gvhr in athymic (nu/nu) and intact (CBA x BALB/c)_{F1} mice. Results shown are mean spleen index for two mice/group at intervals after transfer of 6×10^7 CBA spleen cells i.p. (intact Gvhr, 6 mice/group).

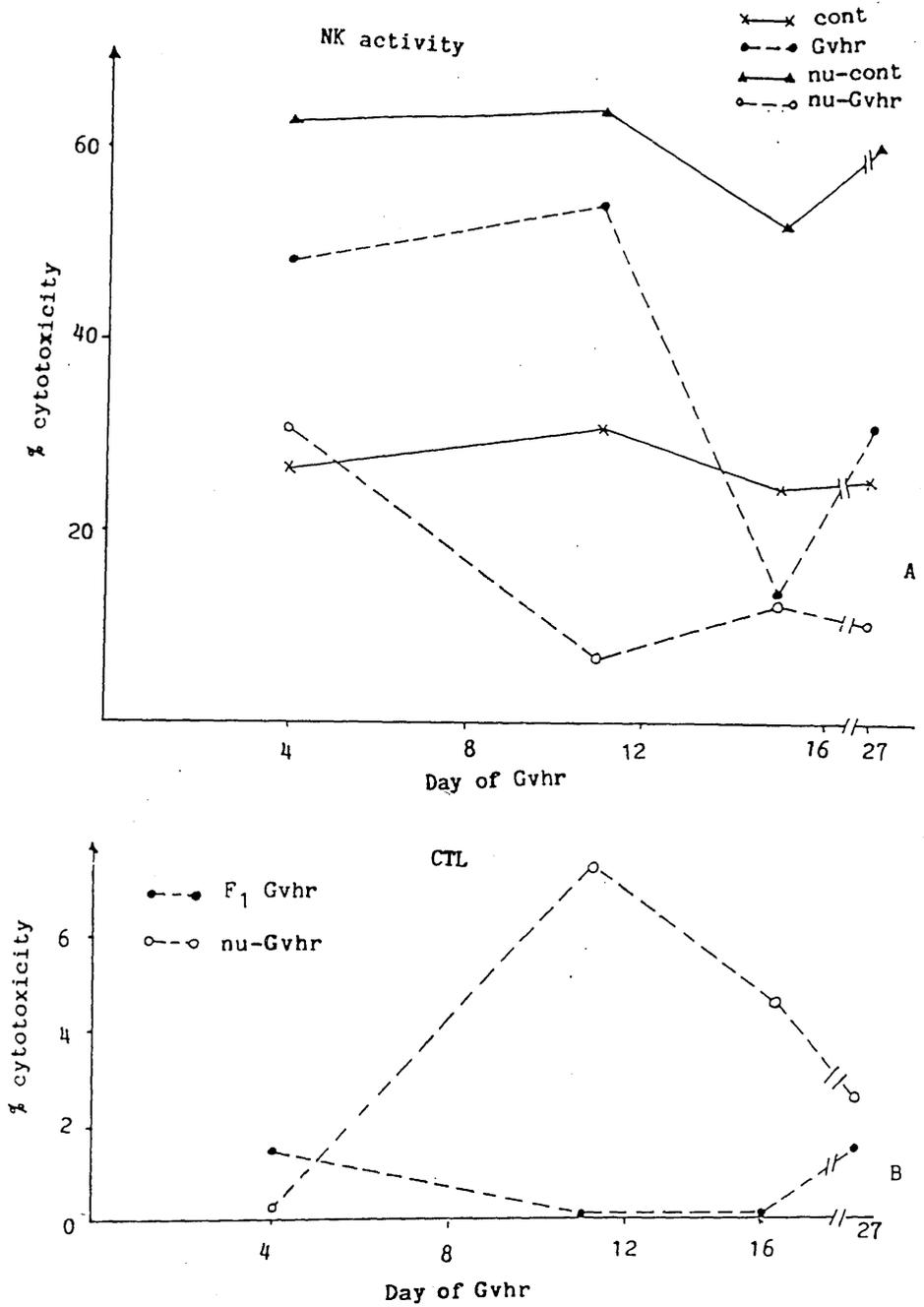


Fig. 10: Specific and non-specific cytotoxicity in athymic (nu/nu) and intact (CBA x BALB/c)F₁ mice with Gvhr. A) NK activity and B) CTL activity against YAC-1 and P815 target cells respectively. Cytotoxicity results are 50:1 E:T for spleen cells pooled from 3 mice/group.

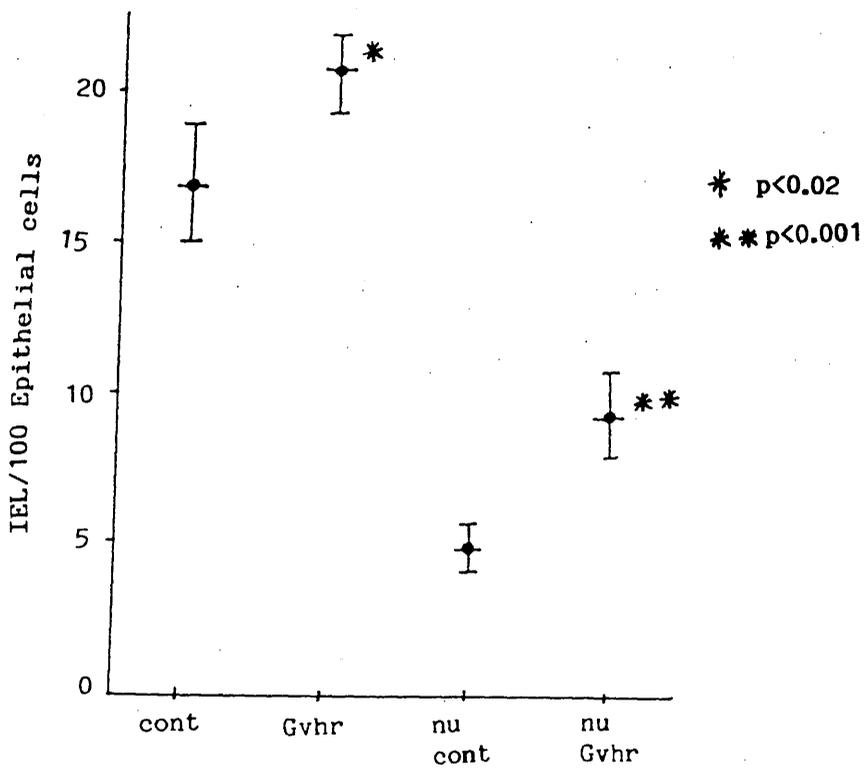


Fig. 11: Intestinal phase of Gvhr in athymic (nu/nu) and intact (CBA x BALB/c)_{F1} mice. IEL counts in jejunum on day 11 of Gvhr, expressed as mean IEL/100 epithelial cells \pm 1 s.d. for 4-5 mice/group.

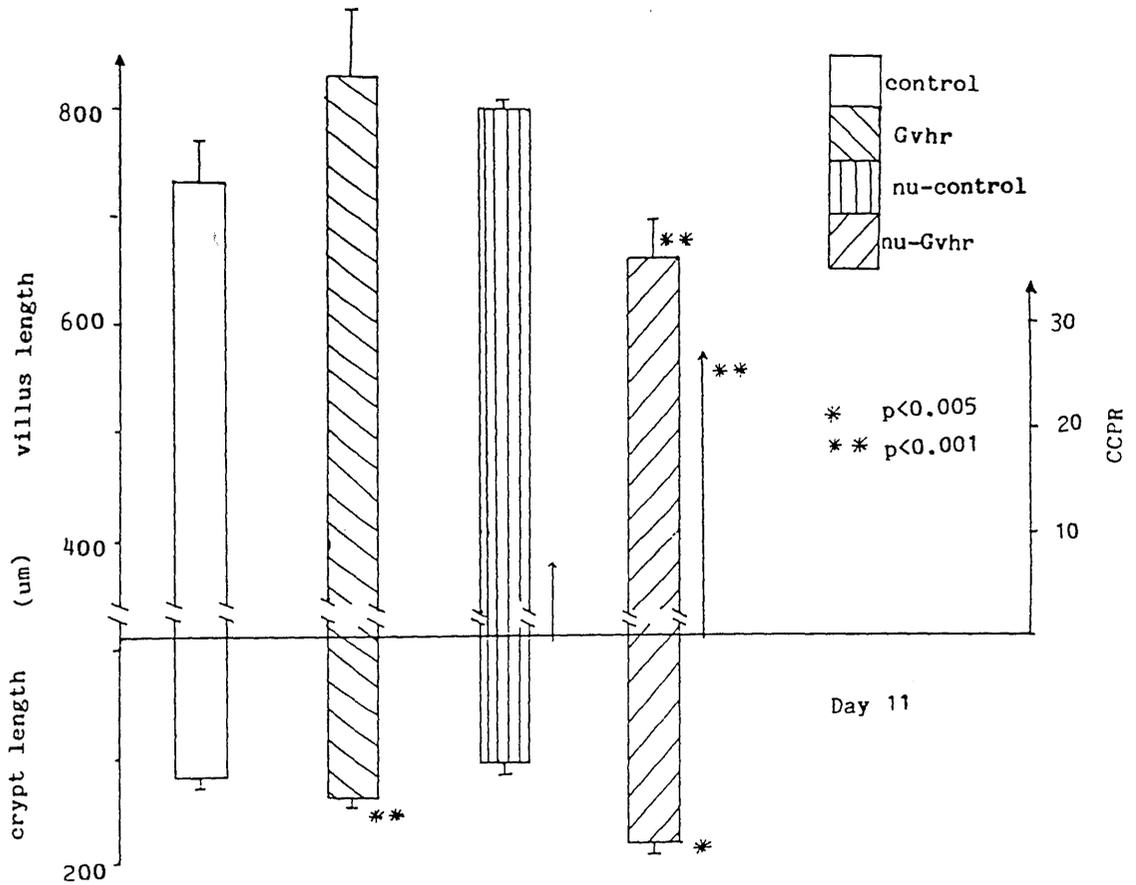


Fig. 12: Intestinal phase of Gvhr in athymic (nu/nu) and intact (CBA x BALB/c) F_1 mice. Mucosal architecture in jejunum on Day 11 of Gvhr. Bars represent villus and crypt lengths \pm 1 s.d., while arrows represent CCPR for 4-5 mice/group.

CHAPTER 7
GENETIC AND CELLULAR BASIS
OF INTESTINAL GVHR

Introduction

The results in the preceding chapters have shown that there are two forms of intestinal Gvhr, and these may be associated with different effector mechanisms. One possible means of investigating these mechanisms further would be to study the phenotype of the donor T cells responsible for inducing the intestinal Gvhr and to examine its genetic basis. This concept is supported by the work of Gleichmann et al. on systemic Gvhr which showed that an SLE-like proliferative Gvhr is mediated by $\text{Lyt } 1^{+}2^{-}$ -T-cells and is stimulated by class II MHC antigens, whereas an acute, lethal Gvhr required activation of both $\text{Lyt } 1^{+}2^{+}$ and $\text{Lyt } 1^{+}2^{-}$ T cells by a combination of class I and class II alloantigens. (Rolink & Gleichmann, 1983). Although these workers did not examine the intestine, earlier studies in our own laboratory showed that a proliferative enteropathy in unirradiated mice is due mainly to $\text{Lyt } 1^{+}2^{-}$ T-cells and was entirely class II MHC restricted (Mowat et al., 1986).

However, these latter studies did not investigate a Gvhr which involved villus atrophy. Furthermore it is now known that the Lyt 1 antigen is expressed on virtually all mouse T-lymphocytes (Ledbetter et al., 1980), and is therefore not a reliable marker of functionally distinct subsets. This difficulty can be overcome using antibodies to the L3T4 antigen, which is expressed only on class II restricted T-cells and defines a population of T-cells which does not overlap with that carrying the Lyt 2 antigen (Dialynas et al., 1983).

Thus, the experiments in this chapter used anti-Lyt 2 or L3T4 monoclonal antibodies and MHC congenic mice to examine the cellular and genetic basis of proliferative and destructive intestinal Gvhr.

Experimental design

The ability of T-cell subpopulations to induce different forms of Gvhr was investigated by depleting donor spleen cells of L3T4⁺ or Lyt 2⁺ T-cells using monoclonal antibodies and complement. These cells were then used to induce a Gvhr in irradiated or unirradiated adult (CBA x BALB/c)F₁ mice, or in neonatal (CBA x BALB/c)F₁ mice of different ages. As usual the development of systemic and intestinal Gvhr was then followed by measurements of splenomegaly, NK and CTL activity and of mucosal architecture.

In the second part of this Chapter, the genetic basis of acute intestinal Gvhr was studied in 1 day old neonatal F₁ mice which had been bred from parents which differed at selected regions of the MHC.

Results

Induction of systemic Gvhr in adult unirradiated (CBA x BALB/c)F₁ mice with T-cell sub-populations

In the first experiments using different T-cell subpopulations a proliferative Gvhr was induced in adult, unirradiated (CBA x BALB/c)F₁ mice with 6×10^7 CBA spleen cells. Before transfer, the donor spleen cells were treated with either anti-Lyt 2 or anti-L3T4 (GK1.5) antibodies plus complement, or were left untreated.

Progress of systemic Gvhr

Mice were sacrificed on days 5 and 10 of Gvhr, when recipients of untreated spleen cells had significant splenomegaly (Table 1) (Spleen Indices: 1.46 ± 0.13 and 1.31 ± 0.15 on days 5 and 10 respectively). Recipients of $L3T4^-$ cells had greater splenomegaly than that in control Gvhr mice on day 5 (1.68 ± 0.12), but by day 10, this had fallen to 1.10 ± 0.09 . $Lyt\ 2^-$ donor cells induced splenomegaly which was similar to that in recipients of untreated cells on day 5 (1.48 ± 0.08), but by day 10, this was much less than in intact Gvhr mice.

As anticipated, recipients of untreated spleen cells had enhanced splenic NK activity compared with controls on day 5 (Fig. 1) (34.4% vs. 26.1% 50:1 E:T), but this had fallen almost to control levels by day 10. Interestingly, recipients of either $L3T4^-$ or $Lyt\ 2^-$ donor cells had NK levels which were considerably higher than those in untreated Gvhr mice on both days 5 and 10. This was most marked in recipients of $Lyt\ 2^-$ cells, whose splenic NK activity was extremely high compared with all other groups at both times. None of the animals with Gvhr developed specific CTL activity during the Gvhr (data not shown).

Development of intestinal pathology

The intestinal phase of Gvhr was also examined in the same groups of mice given untreated or T-cell depleted spleen cells. Mice given untreated donor cells had significantly longer crypts than controls on both day 5 and day 10 of the Gvhr as well as marked increases in CCPR

(Table 2). Although mice which received L3T4⁻ cells also had significantly longer crypts and a higher CCPR than controls on day 5, these changes were less than those found in recipients of untreated cells, and had returned to control values by day 10. Mice given Lyt 2⁻ cells had only minor and insignificant increases in crypt lengths and CCPR, compared with controls on day 5. Nevertheless by day 10, these mice with Gvhr had increases in both crypt length and CCPR, which were similar to those in recipients of intact donor cells.

In parallel, recipients of intact donor cells had a moderate increase in IEL count on day 10 compared with controls (Table 3) (18.25 ± 4.8 vs. 13.4 ± 0). In contrast, mice receiving L3T4⁻ or Lyt 2⁻ donor cells did not have a significant increase in IEL counts at this time of the Gvhr. None of the Gvhr mice had increased IEL counts on day 5 of Gvhr.

The results obtained from these experiments indicate that both L3T4⁺ and Lyt 2⁺ cells are required to induce the intestinal changes normally seen in irradiated mice with Gvhr. However, both L3T4⁻ and Lyt 2⁻ populations induce some systemic changes during Gvhr, and both induced very high levels of NK cell activation.

Induction of Gvhr in irradiated (CBA x BALB/c)_F₁ mice with T-cell subpopulations

In the next experiment, the ability of T-cell subsets to induce an acute destructive, Gvhr was studied by injecting irradiated (CBA x BALB/c)_F₁ mice with 2×10^7 untreated, L3T4⁻ or Lyt 2⁻ CBA spleen cells.

Progress of systemic Gvhr

Mice which received untreated CBA spleen cells rapidly developed Gvhr and all died within 8 days after cell transfer (Fig. 2). Recipients of Lyt 2⁻ cells also lost weight and runted rapidly and the majority had died by day 10. Only a small proportion of this group survived indefinitely. In contrast, most mice receiving L3T4⁻ cells showed few signs of acute Gvhr and 40% of these mice survived beyond day 36 of the experiment. Thus, depletion of L3T4⁺ cells significantly reduced the capacity of donor spleen cells to induce a lethal Gvhr in irradiated F₁ mice. Insufficient numbers of mice were available in this experiment to obtain enough cells to study the generation of cytotoxic effector cells.

Development of intestinal pathology

Small intestinal changes were examined on day 6 of Gvhr, before recipients of untreated donor cells showed overt signs of clinical Gvhd. In this way, it was hoped to be able to examine the intestinal Gvhr when components of both proliferative and destructive enteropathy would be apparent.

At this time, mice which received untreated donor cells had significant villus atrophy (Fig. 3) ($316.9 \pm 58.7 \mu\text{m}$ vs. $664.1 \pm 60.1 \mu\text{m}$ in controls, $p < 0.001$) and crypt lengthening ($142.8 \pm 13.1 \mu\text{m}$ vs. $111.0 \pm 6.56 \mu\text{m}$ in controls, $p < 0.05$). However, as was found at later stages of the Gvhr in irradiated mice in Chapter 3, mice with Gvhr in the present experiments had a CCPR which was slightly less than control values. In

contrast, no significant change in villus height was found in mice which received either L3T4⁻ or Lyt 2⁻ spleen cells, but both these had significant crypt lengthening compared with controls (143.8 ± 8.7 , $p < 0.005$ and 154.0 ± 28.1 , $p < 0.05$ respectively). Recipients of both L3T4⁻ and Lyt 2⁻ cells did not have suppressed CCPR, but actually had crypt cell mitotic activity which was similar to, or greater than that found in controls.

These results suggest that both L3T4 and Lyt 2 cells are required to induce destructive intestinal pathology during a Gvhr across a full MHC incompatibility in irradiated mice.

Induction of Gvhr in neonatal mice of different ages with T-cell subpopulations

The findings described above indicate that both L3T4⁺ and Lyt 2⁺ T-cells are required to induce an intestinal Gvhr which involves villus atrophy. However, the mortality due to Gvhr appeared to be predominantly due to L3T4⁺ cells and so I thought it was important to confirm these results in another model. Furthermore, it would be of interest to examine whether depleted populations of donor T cells induced different forms of cell mediated effector functions. Therefore the experiments described below investigated the role of T-cell subpopulations in inducing systemic and intestinal Gvhr in neonatal mice of different ages. This approach allowed me to compare directly the cellular basis of proliferative and destructive Gvhr in the same model.

Progress of systemic Gvhr in one day old (CBA x BALB/c)_{F1} hosts

As described in previous chapters, (CBA x BALB/c)_{F1} mice given 10^7 untreated CBA spleen cells on day 1 of life began to runt by day 8 (Fig. 4A) and by day 9 two of the mice had died. In this experiment, 4 mice from each group were sacrificed on day 11, and therefore very few mice could be followed in later stages of the Gvhr. However, the one remaining mouse in the group given untreated spleen cells died on day 15. Mice receiving L3T4⁻ cells also lost weight and had a similar runting syndrome to that found in recipients of untreated cells. This was particularly noticeable in the remaining mouse which remained in this group after day 11. In contrast, recipients of Lyt 2⁻ spleen cells grew and developed at an identical rate to control mice and showed no evidence of runting disease.

Mice from all these groups were assessed for splenomegaly on day 11 of Gvhr, when recipients of untreated spleen cells had a spleen index of 1.38 ± 0.27 . Although recipients of L3T4⁻ cells also had splenomegaly at this time (S.I. 1.30 ± 0.14) recipients of Lyt 2⁻ cells had no splenomegaly compared with controls (S.I. 0.90 ± 0.10). Thus, Lyt 2⁺ cells appear to be essential for systemic Gvhr in 1 day old neonates.

As before, neonatal hosts of this age given untreated spleen cells, developed low, but consistent CTL activity on day 11 of the Gvhr (4.2%). Depletion of either L3T4⁺ or Lyt 2⁺ T cells abolished the ability of donor spleen cells to induce CTL activity in Gvhr mice (Data not shown). Thus both Lyt 2⁺ and L3T4⁺ cells were required to induce anti-host

CTL, despite the fact that only Lyt 2⁺ cells were required for systemic disease.

When NK activity was examined in these mice on day 11 very low levels were found in control mice as usual. Increased levels of NK activity were found in mice given untreated donor cells (Fig. 4B) (4.8% vs. 0.6% at 50:1 E:T). As seen in adult unirradiated mice, very high levels of NK activity were found in mice given either L3T4⁻ or Lyt 2⁻ cells (16.6% and 15.3% respectively at 50:1 E:T).

Development of intestinal pathology in one day old mice with Gvhr

As anticipated, the mice given untreated spleen cells had markedly longer crypts and shorter villi than controls on day 11 of Gvhr (Fig. 5) ($134.9 \pm 23.0 \mu\text{m}$ and $325.0 \pm 14.1 \mu\text{m}$ respectively vs. $47.5 \pm 8 \mu\text{m}$ and $622.4 \pm 36.0 \mu\text{m}$ in controls). In contrast, no significant changes in villus or crypt lengths were found in recipients of L3T4⁻ or Lyt 2⁻ donor cells, although there was some evidence of a minor degree of crypt lengthening in both groups, as well as villus atrophy in mice given L3T4⁻ cells (Fig. 5). Insufficient mice were available for estimation of CCPR in these groups.

Only very low numbers of IEL were found in the very young mice used in this experiment and none of the group with Gvhr had a significant increase in IEL count on day 11 of the Gvhr (Table 3).

Induction of Gvhr in 5 day old (CBA x BALB/c)_F₁ mice with T-cell subpopulations

Progress of systemic Gvhr

Neonatal mice given parental cells at 5 days of age showed no significant differences in growth rate compared with control mice, irrespective of the nature of the donor cell population (Fig. 6A). However, as found in previous experiments, recipients of untreated spleen cells showed a minor degree of weight loss by the time the experiment was ended on day 11.

Recipients of untreated spleen cells had marked splenomegaly on day 11 with a spleen index of 2.78 ± 0.50 . This was significantly greater than that found in mice given either L3T4⁻ (1.45 ± 0.51 , $p < 0.02$) or Lyt 2⁻ cells (1.36 ± 0.33 , $p < 0.01$).

Although none of the Gvhr mice had marked CTL activity some was detectable in untreated recipients with Gvhr (2.4% 50:1 E:T) while recipients of L3T4⁻ or Lyt 2⁻ cells had none at all. In this experiment control mice also had low levels of NK activity by day 11, and this was slightly increased in mice given intact spleen cells (Fig. 6B) (7.2% vs. 5.3% in controls 50:1 E:T). Once again, much higher levels of NK activity were found in recipients of either L3T4⁻ or Lyt 2⁻ donor cells (14.6% and 17.6% respectively). Therefore, both L3T4⁺ and Lyt 2⁺ T cells were required for splenomegaly and CTL activity in 5 day old mice with Gvhr.

Development of intestinal pathology

Mucosal architecture was examined on day 11 of Gvhr and the results were similar to those in one day old hosts. Mice receiving untreated donor cells had significantly longer crypts than controls (Fig. 7) ($196.6 \pm 26.0 \mu\text{m}$ vs. $74.0 \pm 11.6 \mu\text{m}$, $p < 0.001$) as well as significant villus shortening ($373.6 \pm 45 \mu\text{m}$ vs. $480.3 \pm 50.2 \mu\text{m}$, $p < 0.05$). These Gvhr mice also had a significantly increased CCPR compared with controls (19.8 ± 0.05 vs. 8.6 ± 0.04 , $p < 0.001$). In contrast, mice which received L3T4^- or $\text{Lyt } 2^-$ donor spleen cells showed no significant changes in mucosal architecture compared with littermate controls.

Once again, control mice in these experiments had very low numbers of IEL, but in this case, recipients of untreated donor cells had significantly higher IEL counts than controls (Table 3) (3.8 ± 0.3 vs. 2.0 ± 0.4 , $p < 0.01$). In contrast, mice receiving L3T4^- or $\text{Lyt } 2^-$ donor cells had IEL counts which were the same, or even less than control levels.

These results show that both L3T4^+ and $\text{Lyt } 2^+$ cells are required for proliferative and destructive enteropathy in 1 or 5 day old mice.

Genetic basis of intestinal Gvhr in neonatal mice

The second group of experiments in this Chapter examined the genetic requirements for the induction of systemic and intestinal Gvhr in F_1 mice, using 1 day old neonates bred from parents differing at defined regions of the MHC.

Induction of Gvhr across a class II MHC difference in neonatal (ATH x ATL)_{F1} mice

Progress of systemic Gvhr

A Gvhr was induced across a class II MHC difference in one day old (ATH x ATL)_{F1} mice with 10^7 ATH spleen cells. Mice with Gvhr developed normally until day 16 (Fig. 8A), when weight loss and other symptoms of clinical Gvhr began. These features were most marked on day 24, but were never as severe as those found in previous studies using a full MHC incompatibility. Furthermore, mortalities did not occur until the fourth week of the Gvhr. On day 14, mice with Gvhr had marked splenomegaly, with a spleen index of 2.42 ± 0.21 and, although this fell sharply thereafter, some residual splenomegaly remained until at least day 21 (Fig. 8B).

Specific CTL activity was measured using LPS induced lymphoblasts from ATL mice, but no specific cytotoxicity was found at any time during the Gvhr (data not shown). In addition no significant NK activity was found in either Gvhr or control mice. Thus, there is no activation of either specific or non-specific cytotoxicity in a Gvhr induced by a class II MHC difference, despite the fact that an acute systemic Gvhr is present.

Development of intestinal pathology in neonatal (ATH x ATL)_{F1} mice with a Gvhr

There was no evidence of significant villus atrophy throughout the course of the Gvhr. Although the fall in villus height which occurred in all mice, on day 21,

appeared to be somewhat greater in mice with Gvhr, the small number of control samples at this time precluded statistical analysis. Mice with Gvhr had longer crypts than controls on day 14 (Fig. 9) ($78.3 \pm 18 \mu\text{m}$ vs. $52.5 \pm 40 \mu\text{m}$, $p < 0.05$) but, thereafter, crypt lengths in Gvhr animals were similar to control values throughout the course of the experiment. In parallel, an increased CCPR was found in Gvhr mice on day 14 (8.81 ± 2.36 vs. 4.09 ± 0.66) although this was not statistically significant and had disappeared by day 26 (11.4 ± 2.7 vs. 9.8 ± 1.13 in controls).

Thus induction of a Gvhr across a class II MHC incompatibility in 1 day old mice produces many of the systemic and intestinal features of an acute Gvhr, but these are generally milder than those found using a full MHC incompatibility.

Induction of Gvhr across an isolated class I MHC incompatibility at H-2^k

In this experiment a Gvhr was induced across an isolated H-2 K^q disparity by injecting newborn (B10.A x B10.AQR)_{F₁} mice with 10^7 B10.A spleen cells.

Progress of systemic Gvhr

Gvhr mice gained weight at the same rate as control mice throughout the experiment and no clinical signs of Gvhr were evident at any stage. Mice with Gvhr never had marked splenomegaly although a maximum spleen index of 1.38 ± 0.40 was found on day 22 of the Gvhr (Fig. 10A). Virtually no specific anti-host cytotoxicity was found against ConA

lymphoblasts from B10.AQR mice at any time during the Gvhr, but a low level was found on day 22 (Fig. 10B) (2.4%).

Interestingly, mice with Gvhr did have a minor and transient enhancement of NK activity on day 11 (Fig. 10B) (3.5% vs. 0.6% at 50:1 E:T in controls).. NK activity in Gvhr mice then fell to control levels. Therefore an isolated class I difference produced little evidence of systemic Gvhr.

Development of intestinal pathology in neonatal (B10.A x B10.AQR) F_1 mice with Gvhr

Mice with Gvhr across H-2K^q had few intestinal changes. Although Gvhr mice had significantly larger crypts than controls on day 17 (Fig. 11) ($76.71 \pm 14 \mu\text{m}$ vs. $49.16 \pm 4.82 \mu\text{m}$, $p < 0.05$), no significant changes in crypt length were found at any other time and there was never any villus atrophy. Indeed, the crypt lengthening on day 17 was accompanied by a significant increase in villus length ($545.43 \pm 11.2 \mu\text{m}$ vs. $450.10 \pm 26.31 \mu\text{m}$ for controls, $p < 0.03$). Mice with Gvhr also had a small increase in CCPR on day 9 (3.06 ± 1.49 vs. 1.16 ± 0.43), but this was minor in comparison with that found in other models of Gvhr and was not significant.

Induction of a Gvhr across an isolated class I MHC incompatibility at H-2D

In view of the fact that the changes which occurred during a Gvhr across an isolated H-2K incompatibility were minor and inconsistent, I thought it important to examine a Gvhr induced by another class I incompatibility. Therefore, in this experiment, I induced a Gvhr across H-2D^q by injecting 1 day old (B10.A x B10.AKM) F_1 with 10^7 B10.A spleen cells.

Progress of systemic Gvhr

As in the Gvhr across H-2K^q, mice with a Gvhr across H-2D^q developed at the same rate as their littermate controls and showed no signs of clinical Gvhr (Fig. 12). Furthermore, these mice never developed marked splenomegaly with a maximum spleen index reaching 1.30 ± 0.07 on day 11, before disappearing by day 24 (Data not shown).

No specific cytotoxicity was found against ConA lymphoblasts of B10.AKM origin in Gvhr mice and both control and Gvhr mice had identical, virtually undetectable levels of NK activity at all times (Data not shown).

Development of intestinal pathology in neonatal (B10.A x B10.AKM)F₁ mice with Gvhr

No evidence of intestinal damage was found in this model of Gvhr, with identical crypt lengths being found in mice with Gvhr and in controls, both at the peak of splenomegaly on day 11 and at the later time of day 24 (Fig. 12B). There were no alterations in villus or crypt length.

Thus the transient increases in villus and crypt length seen in the previous experiment using a Gvhr across H-2K^q could not be confirmed using an H-2D^q incompatibility. Together, these findings indicate that an isolated class I incompatibility produces only minor systemic and intestinal Gvhr and these alterations are transient and inconsistent.

Summary and conclusions

The first series of experiments in this Chapter examined the T cell subsets responsible for inducing systemic and intestinal Gvhr in a variety of models.

When unirradiated adult (CBA x BALB/c) F_1 mice were used as hosts, Lyt 2⁺ or L3T4⁺ T cells were both required for the development of full splenomegaly. However, both Lyt 2⁺ and L3T4⁺ cells were alone capable of inducing some splenomegaly. Only recipients of untreated donor cells developed consistent and marked intestinal pathology but a transient, early increase in CCPR was seen in recipients of L3T4⁻ cells and some crypt hyperplasia also occurred at later times in recipients of Lyt 2⁻ T-cells. However, the changes due to depleted donor cells were generally minor and only mice given untreated donor cells had increased IEL counts. No Gvhr mice had villus atrophy or specific CTL activity.

The development of a destructive enteropathy in irradiated (CBA x BALB/c) F_1 mice also required both Lyt 2⁺ and L3T4⁺ T cells and, although both subpopulations alone did produce some crypt hypertrophy, this did not progress to villus atrophy. However, Lyt 2⁻ donor cells were capable of inducing a lethal Gvhr in irradiated mice, whereas depletion of L3T4⁺ cells resulted in milder systemic disease, in which only a few delayed deaths occurred.

In contrast to these findings in irradiated adults, L3T4⁻ cells were alone able to induce an acute lethal Gvhr in 1 day old (CBA x BALB/c) F_1 hosts and also produced significant splenomegaly as well as some evidence of both

crypt hyperplasia and villus damage. However, all these features were less severe than those found in recipients of untreated donor cells. In contrast, Lyt 2⁻ donor cells produced no clinical Gvhr, no splenomegaly and very little evidence of mucosal pathology. Interestingly, only mice given untreated donor spleen cells had any specific anti-host CTL activity, despite the fact that L3T4⁻ T cells could induce some systemic and intestinal consequences of acute Gvhr.

As had been found previously, the Gvhr in five day old (CBA x BALB/c)F₁ mice had some of the features both of the disease found in 1 day old neonates and of that in adult hosts, with some mice developing weight loss and villus atrophy, despite the absence of significant CTL activity. However, in these 5 day old mice, neither L3T4⁻ nor Lyt 2⁻ donor cells alone induced any clinical Gvhr and produced none of the alterations in crypt length, villus length or IEL counts, which were normally present in Gvhr mice.

An interesting feature of these experiments was the effect of depleting T-cell subpopulations on the activation of NK cells during the Gvhr. As anticipated, NK cell activation was a characteristic feature of a Gvhr in intact hosts, but this was always much more marked in mice given donor cells which had been depleted of either L3T4⁺ or Lyt 2⁺ cells. This feature seemed more apparent using Lyt 2⁻ cells and was in marked contrast to the absence of clinical and intestinal Gvhd in recipients of L3T4⁻ or Lyt 2⁻ cells.

Together, these results indicate that both $L3T4^+$ and $Lyt\ 2^+$ T cells are required to induce the full systemic and intestinal consequences of Gvhr. However, a potential pathogenic role for the individual subsets was revealed in immunoincompetent hosts, with $L3T4^+$ cells being capable of inducing a lethal Gvhr in irradiated hosts and $Lyt\ 2^+$ cells causing mortality in 1 day old neonates. Furthermore, $Lyt\ 2^+$ cells seemed to be able to induce a mild, intestinal Gvhr in both these hosts. These results highlight the complexity of the cells involved in the induction of acute Gvhr and also suggest that the intestinal and systemic consequences of a Gvhr may not be due to the same mechanisms.

The second part of this Chapter showed that a class II MHC incompatibility was sufficient to produce an acute, lethal Gvhr in 1 day old neonatal mice. This was associated with many of the clinical signs found during the acute Gvhr described in earlier chapters, as well as prolonged splenomegaly and some evidence of crypt hyperplasia and villus atrophy. However, the onset of severe, lethal disease occurred about one week later than was found across a full MHC incompatibility in mice of the same age. Furthermore, only about half of the $(ATH \times ATL)F_1$ mice with Gvhr died, whereas a full MHC incompatibility would normally produce 100% mortality. In parallel, no CTL or enhanced NK activity were found in the class II MHC restricted Gvhr and the intestinal changes were somewhat less marked than was normally found in a Gvhr across a full MHC incompatibility.

In contrast, a Gvhr induced across an isolated class I difference at either H-2 K or D did not produce an acute Gvhr associated with weight loss, death or CTL activity, although a minor degree of splenomegaly was found occasionally. In a Gvhr across H-2K^q, there was also some enhancement of NK cell activity and minor crypt lengthening. However, these changes were transient and were not present during a Gvhr induced by an incompatibility at H-2D^q. Villus atrophy was never found in a Gvhr induced across a class I MHC incompatibility.

Thus a class II MHC incompatibility is alone sufficient to induce many of the systemic and intestinal consequences of an acute Gvhr in neonatal mice, whereas isolated class I differences have little or no ability to induce these alterations. However, the class II MHC restricted Gvhr is delayed and milder than that found across a full MHC incompatibility. Together with the results obtained using T-cell subpopulations, those findings indicate that both class I and II MHC restricted T cells are required to induce all the systemic and intestinal consequences of Gvhr. Interestingly, this feature applies to both proliferative and destructive Gvhr, suggesting that these disorders have a common cellular basis.

Day of Gvhr	Donor Cell Populations		
	Untreated	L3T4 ⁻	Lyt 2 ⁻
5	1.46 ± 0.13	1.68 ± 0.12	1.48 ± 0.08
10	1.31 ± 0.15	1.10 ± 0.09	1.09 ± 0.11

Table 1: Development of splenomegaly in adult unirradiated (CBA x BALB/c)_{F₁} mice with Gvhr induced with 6×10^7 untreated L3T4⁻ or Lyt 2⁻ CBA spleen cells. Results shown are mean spleen indices \pm 1 s.d. 3 mice/group.

	Day of Gvhr	Donor Cell Populations			
		None	Untreated	L3T4 ⁻	Lyt 2 ⁻
villus length (μm)	5	644.0±17.0	599.8±68.5	609.5±67.0	605.4±23.9
	10	557.8±10.0	587.9±12.0	617.4±9.0	619.8±35.0
crypt length (μm)	5	103.9±10.0	135.2±9.4*	126.0±16.0	111.5±7.0
	10	97.9± 3.0	147.3±23.0**	101.6±5.8	117.1±9.5**
CCPR	5	7.7± 1.2	21.9±1.8***	16.7±4.3	12.2±1.9
	10	11.7± 2.3	17.5±3.9	8.4±0.7	17.1±1.5

Table 2: Intestinal pathology in adult unirradiated (CBA x BALB/c)F₁ mice with Gvhr induced with 6 x 10⁷ untreated, L3T4⁻ or Lyt 2⁻ CBA spleen cells. Villus and crypt lengths and CCPR in jejunum of mice with Gvhr and in controls. Results shown are means ± 1 s.d. for 4 mice/group.

* p < 0.025
 ** p < 0.02
 *** p < 0.005 vs controls.

Host	Day of Gvhr	Donor Cell Population			
		None	Untreated	L3T4 ⁻	Lyt 2 ⁻
Adult	5	14.4 [±] 1.1	10.1 [±] 2.1	14.3 [±] 2.1	15.5 [±] 0.2
	10	13.4 [±] 0.4	18.3 [±] 4.8	15.7 [±] 0.7	12.9 [±] 0.2
1-2 day old neonate	11	3.8 [±] 1.1	4.10 [±] 0.9	2.9 [±] 0.8	1.8 [±] 0.5
5 day old neonate	10	2.0 [±] 0.4	3.8 [±] 0.3*	1.2 [±] 0.4	1.6 [±] 0.4

Table 3: Intestinal pathology in unirradiated adult or neonatal (CBA x BALB/c)_{F1} mice with Gvhr induced with untreated L3T4⁻ or ¹ Lyt 2⁻ CBA spleen cells. Intraepithelial lymphocyte counts in the jejunum of mice with Gvhr and in controls. Results shown are the mean IEL/100 epithelial cells [±] 1 s.d. for 4 mice/group.

* $p < 0.01$ vs controls.

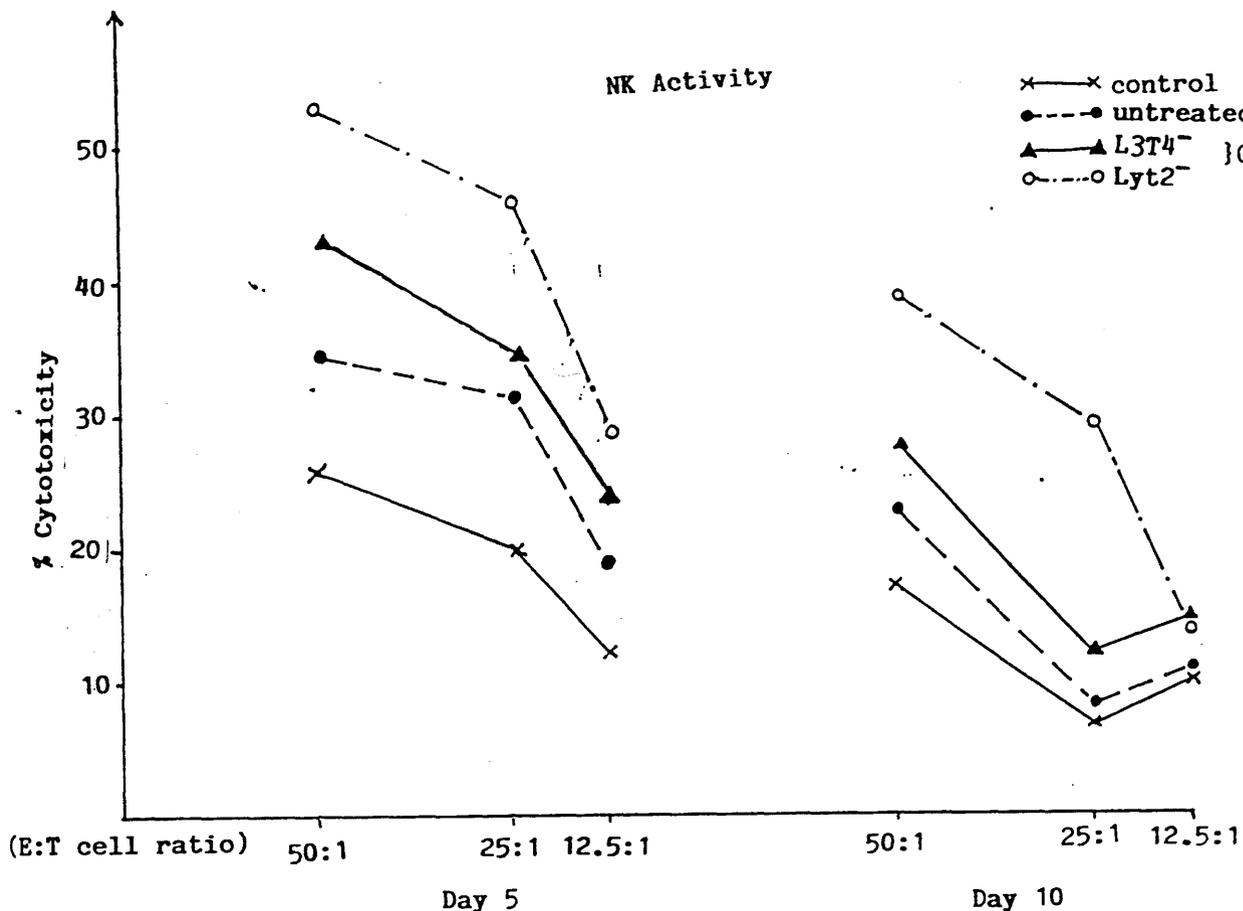


Fig. 1: Induction of systemic Gvhr in adult unirradiated (CBA x BALB/c)_{F₁} mice with 6 x 10⁷ untreated, L3T4⁻ or Lyt 2⁻ CBA spleen cells. NK activity in mice with Gvhr and in controls measured against YAC-1 target cells. Results shown are % cytotoxicity at different E:T ratios using spleen cells pooled from 3 mice/group.

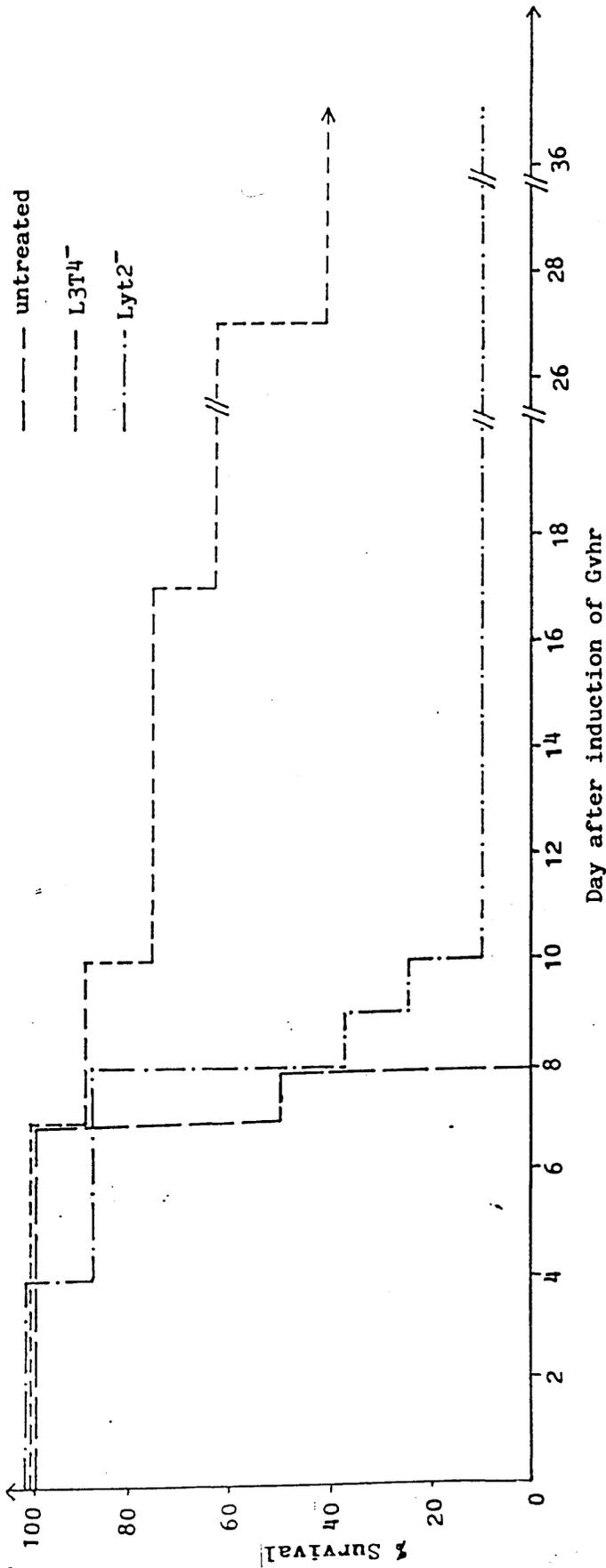


Fig. 2: Development of systemic Gvhr in adult irradiated (CBA x BALB/c)F₁ mice induced with 2 x 10⁷ untreated L3T4⁻ or Lyt 2⁻ CBA spleen cells. Mortality rates of mice with Gvhr at intervals after induction of Gvhr in 8 mice/group.

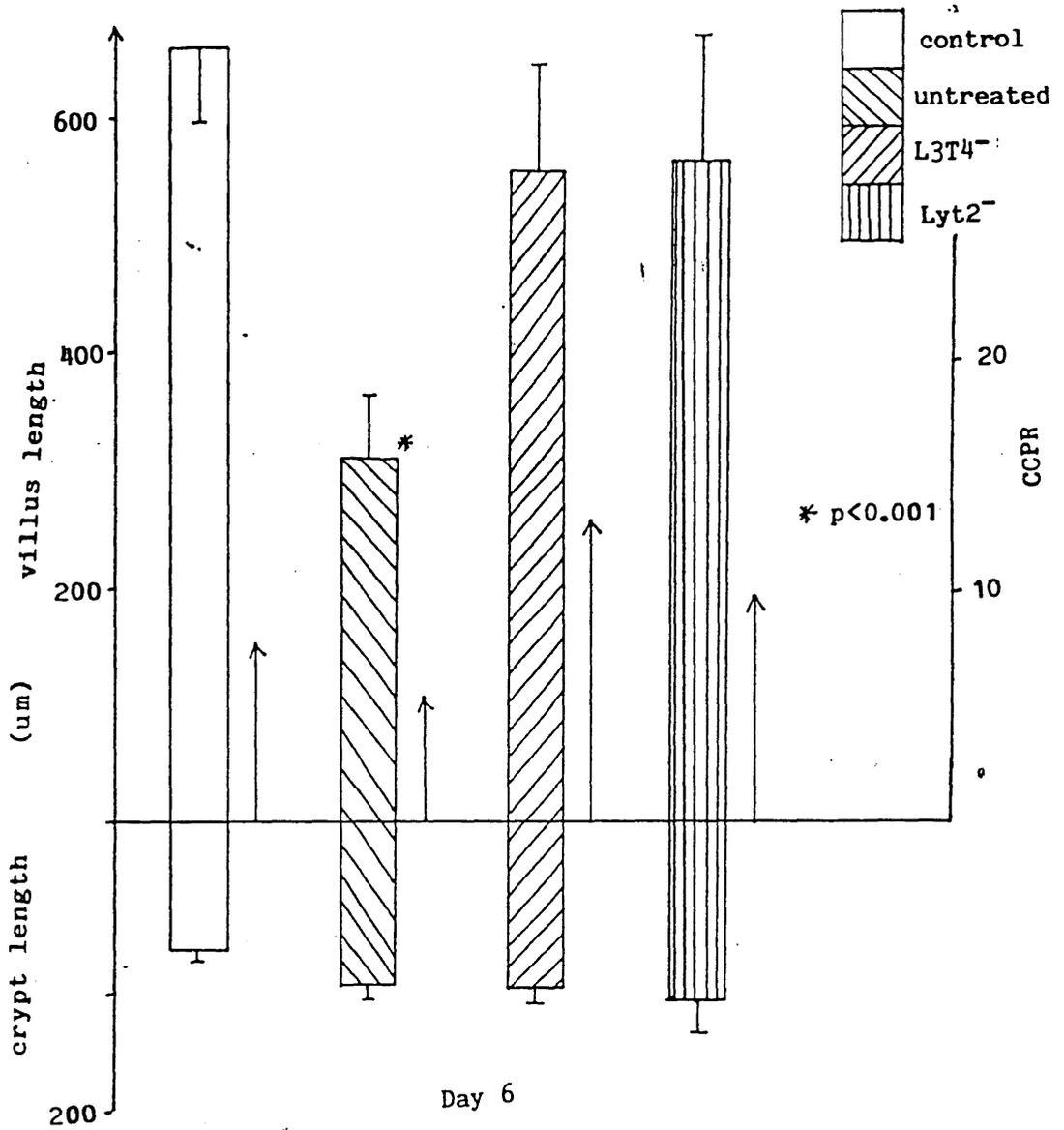


Fig. 3: Induction of intestinal Gvhr in adult irradiated (CBA x BALB/c)_{F₁} mice with 2×10^7 untreated L3T4⁻ or Lyt 2⁻ CBA spleen cells. Mucosal architecture in the jejunum of Gvhr and control mice on day 6 of Gvhr. Bars represent mean villus and crypt lengths \pm 1 standard deviation and arrows show CCPR for 4-5 mice/group.

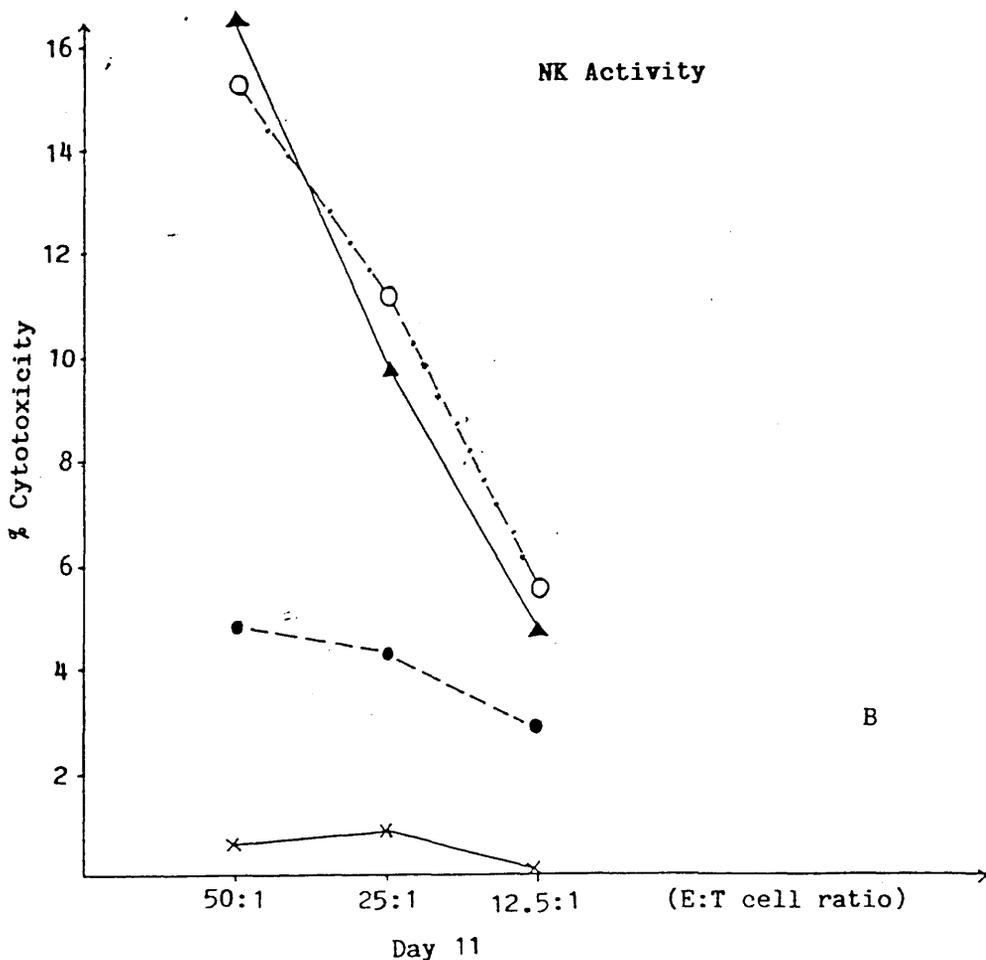
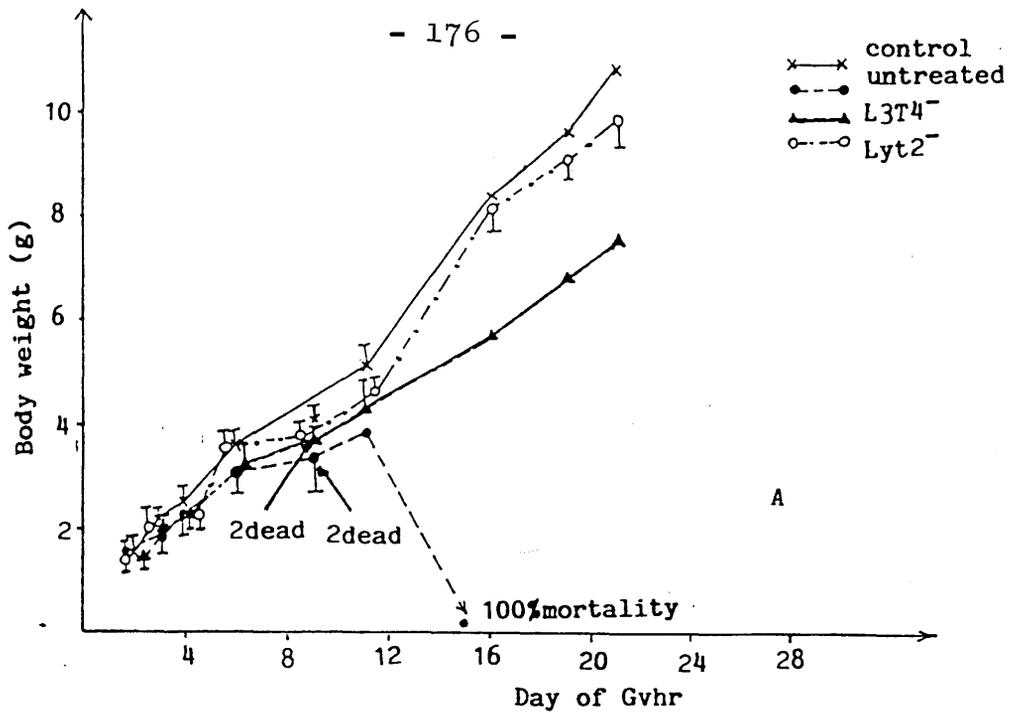


Fig. 4: Development of systemic Gvhr in one day old (CBA x BALB/c)_{F₁} mice induced with 10⁷ untreated; L3T4⁻ or Lyt 2⁻ CBA spleen cells. A) Growth rate of neonates with Gvhr compared with littermate controls 3-4 mice/group. B) NK cell activity against YAC-1 target cells on day 11 of Gvhr using spleen cells pooled from 3-4 mice/group.

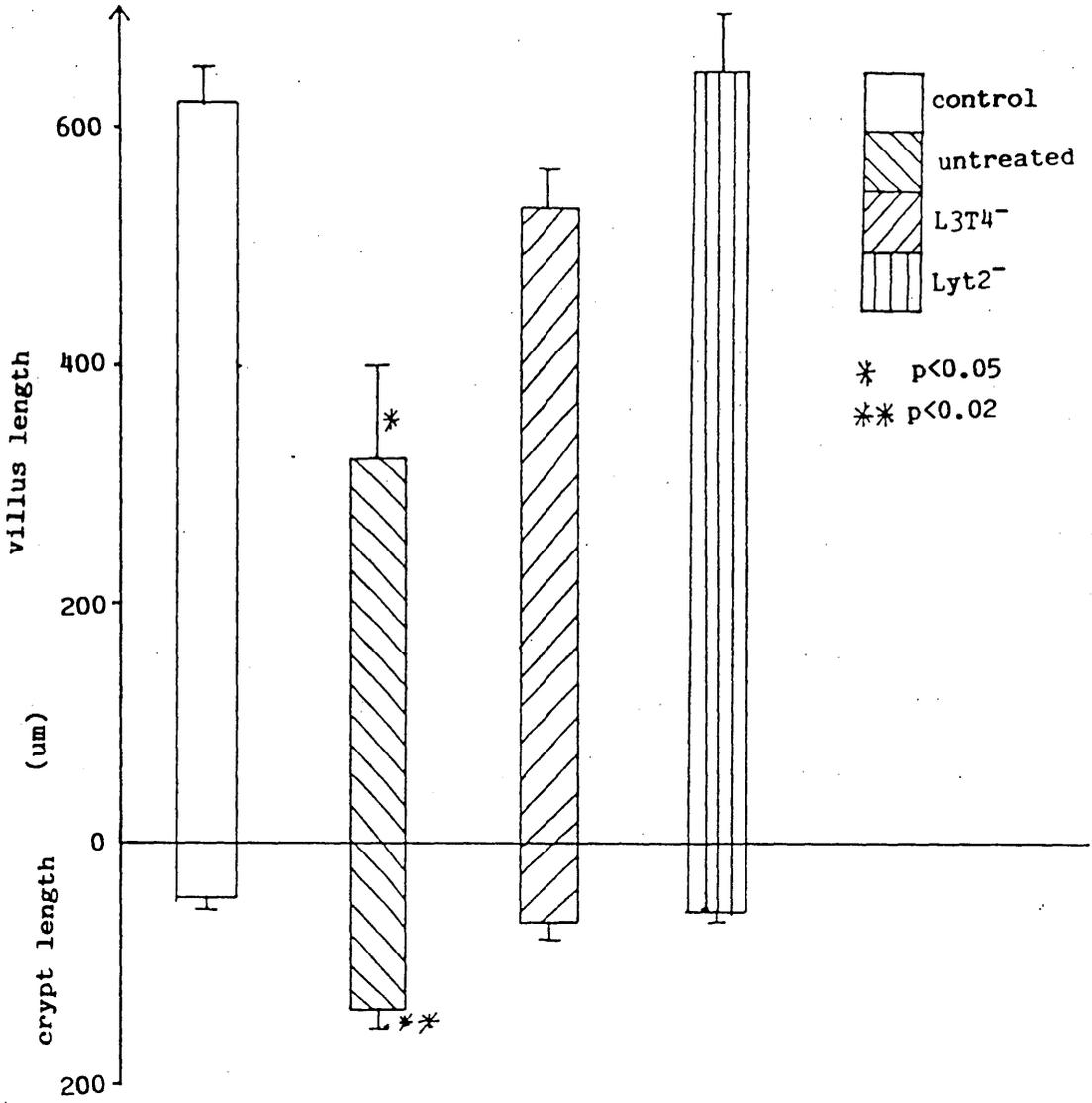


Fig. 5: Induction of intestinal Gvhr in one day old (CBA x BALB/c)_{F1} mice, 10⁷ untreated, L3T4⁻ or Lyt 2⁻ CBA spleen cells. Mucosal architecture in the jejunum of Gvhr and control mice on day 11 of Gvhr. Bars represent mean villus and crypt lengths ± 1 standard deviation for 3-4 mice/group.

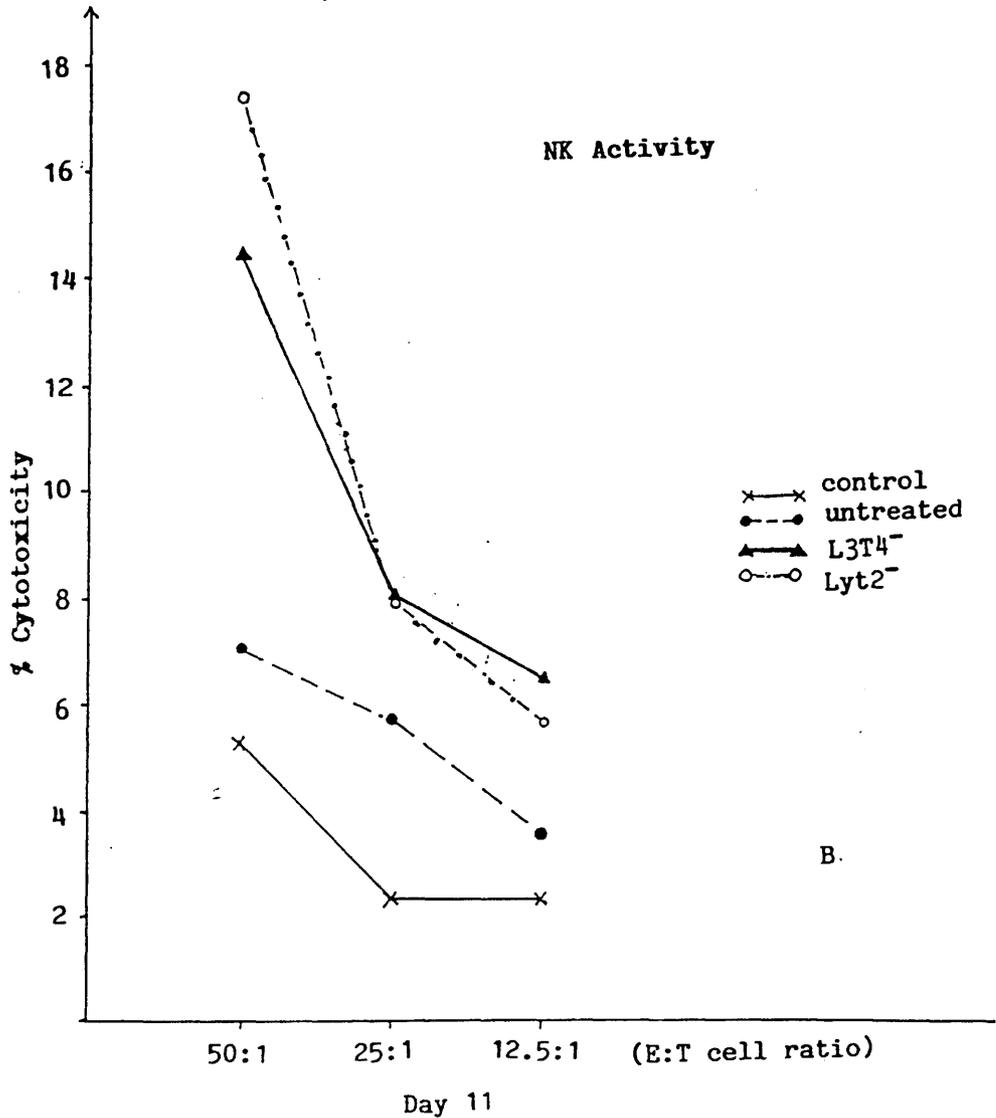
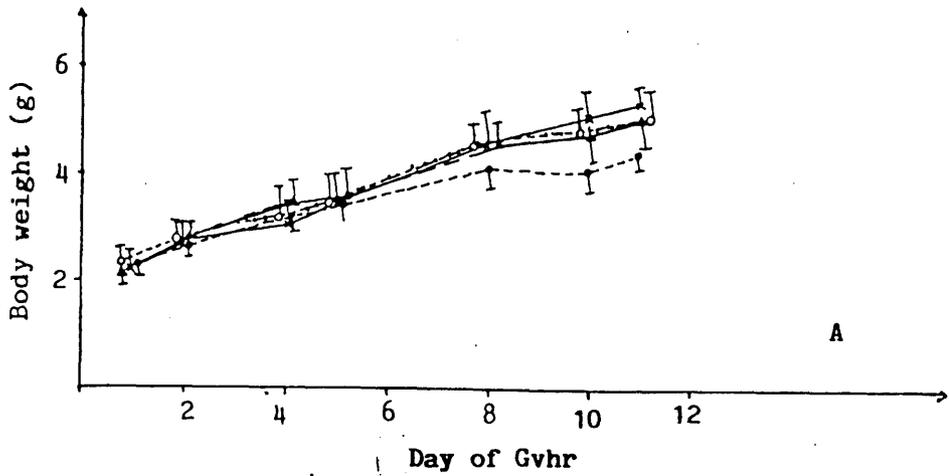


Fig. 6: Development of systemic Gvhr in 5 day old (CBA x BALB/c)_{F₁} mice induced with 2.5×10^7 untreated, L3T4⁺ or Lyt 2⁻ CBA spleen cells. A) Growth rate of neonates with Gvhr compared with littermate controls using 5 mice/group. B) NK activity on day 11 of Gvhr against YAC-1 target cells, using spleen cells pooled from 3 mice/group.

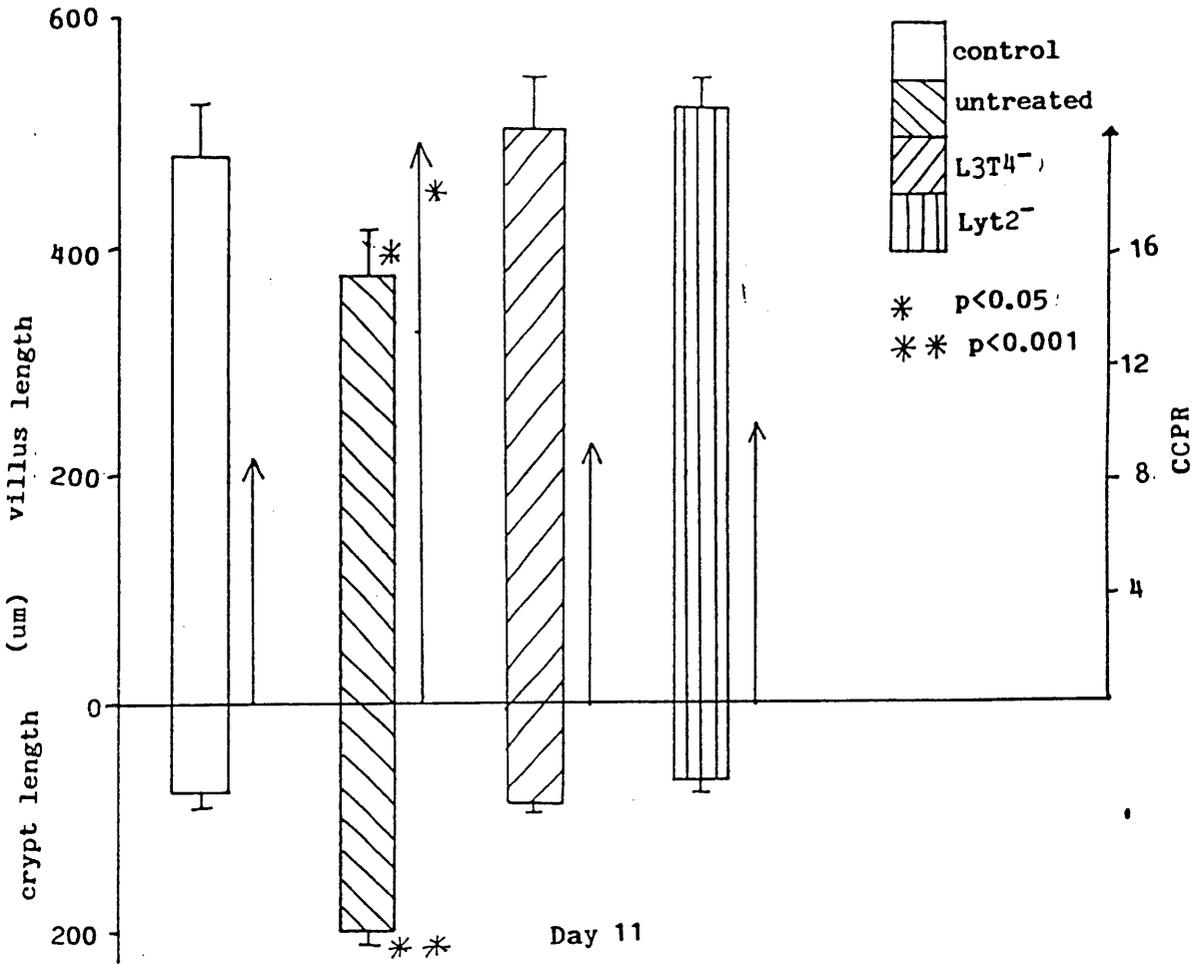


Fig. 7: Induction of intestinal Gvhr in 5 day old (CBA x BALB/c)F₁ mice by 2.5 x 10⁷ untreated, L3T4⁻ or Lyt 2⁻ CBA spleen cells. Mucosal architecture in the jejunum of Gvhr and control mice on day 11 of Gvhr. Bars represent mean villus and crypt lengths ± 1 s.d. and arrows show CCPR for 4 mice/group.

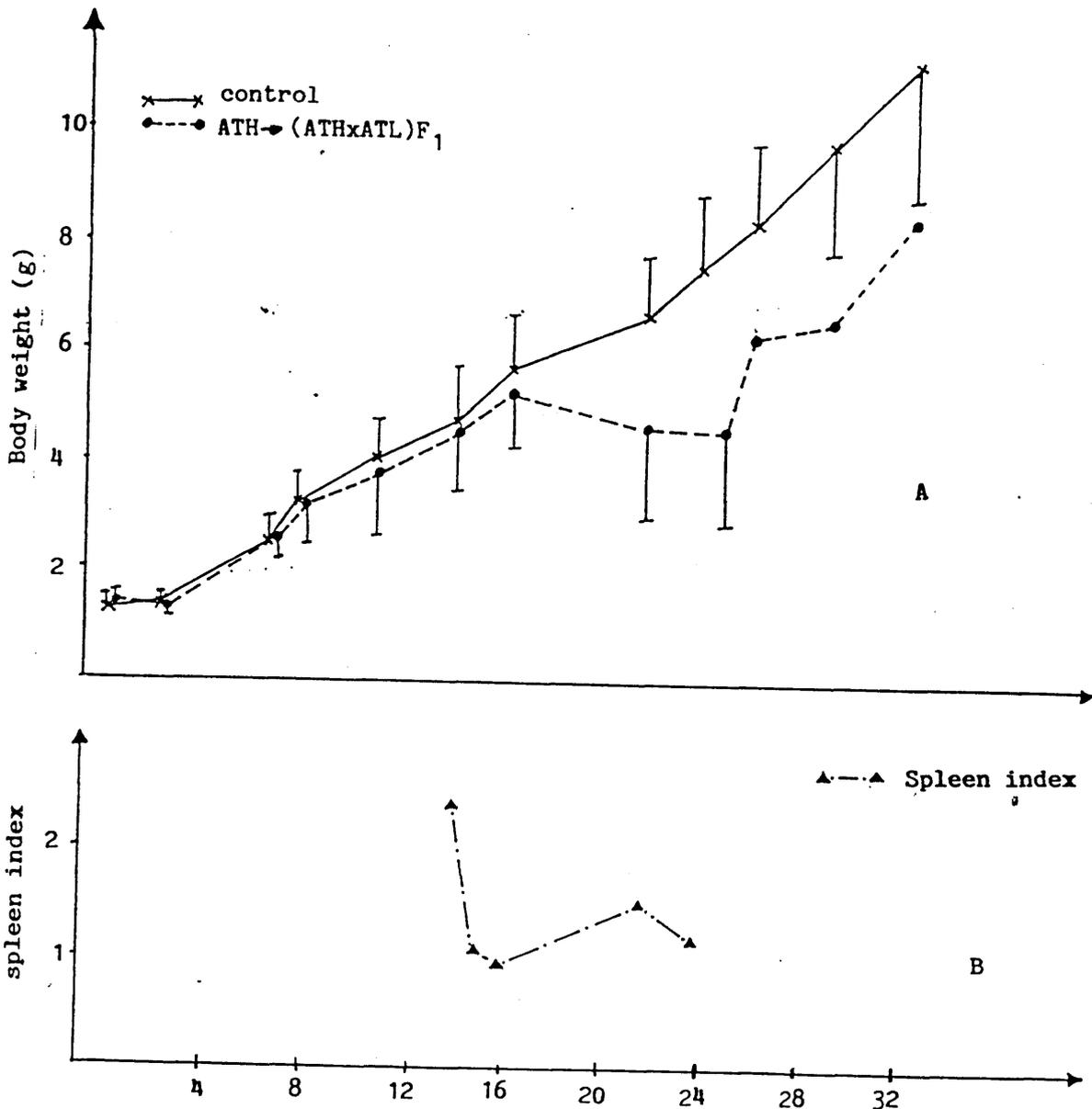


Fig. 8: Development of systemic Gvhr across a Class II MHC disparity in 1 day old (ATH x ATL)F₁ mice. A) Growth rates of neonates with Gvhr compared with littermate controls. B) Spleen index. Results shown are means \pm 1 s.d. for 3 mice/group for spleen index and 5 mice/group for growth rate.

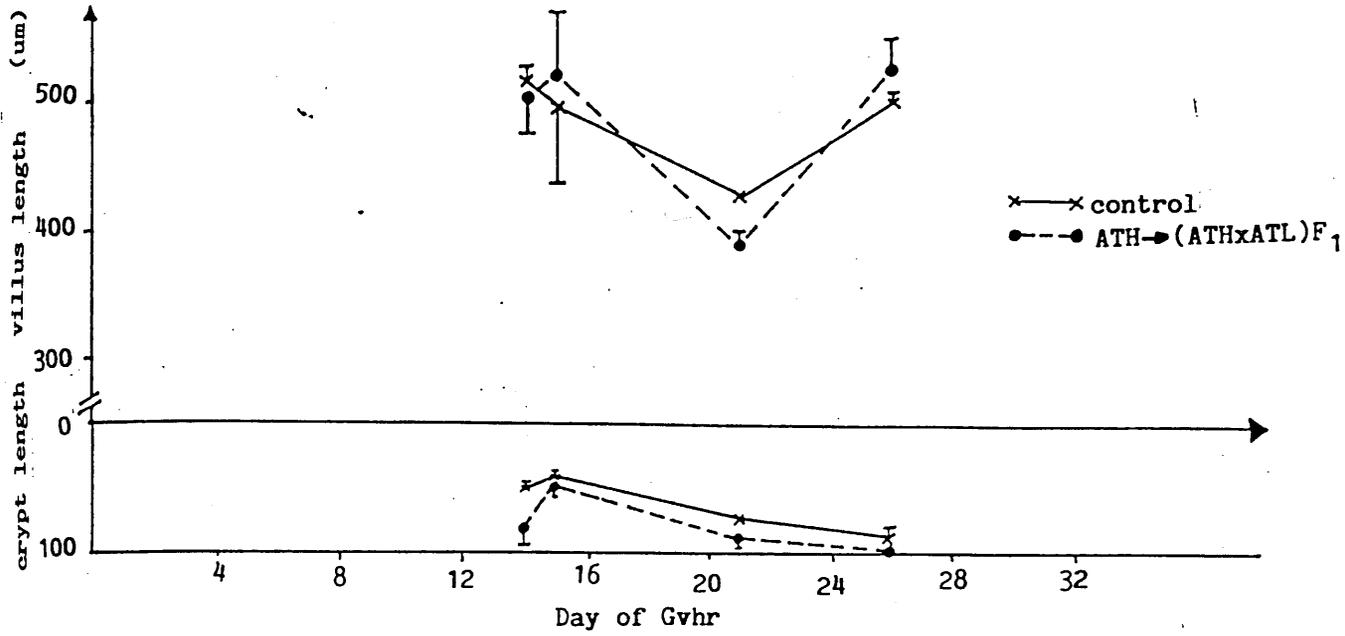


Fig. 9: Development of intestinal Gvhr in 1 day old (ATH x ATL)F₁ mice. Villus and crypt lengths in the jejunum of Gvhr and control mice. Results shown are the means ± 1 s.d. for 3-4 mice/group.

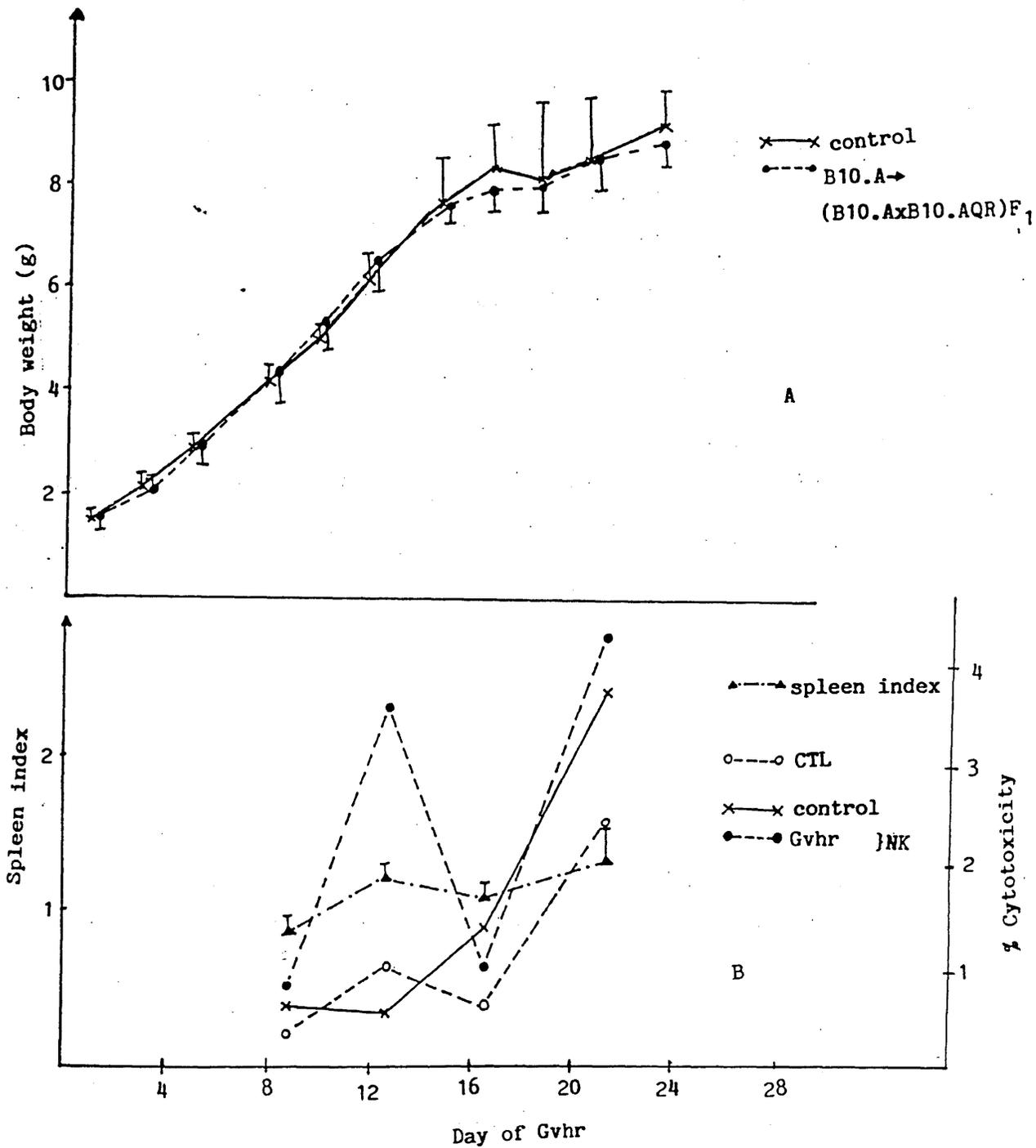


Fig. 10: Development of systemic Gvhr across an H-2K Class I MHC disparity in 1 day old (B10.A x B10.AQR)F₁ mice. A) Growth rates of neonates with Gvhr¹ compared with littermate controls. B) Spleen index, NK activity and specific CTL activity, against YAC-1 and P815 targets respectively. Results shown are means \pm 1 s.d. for 4 mice/group. Cytotoxicity is shown as the lysis at 50:1 E:T for spleen cells pooled from 4 mice/group.

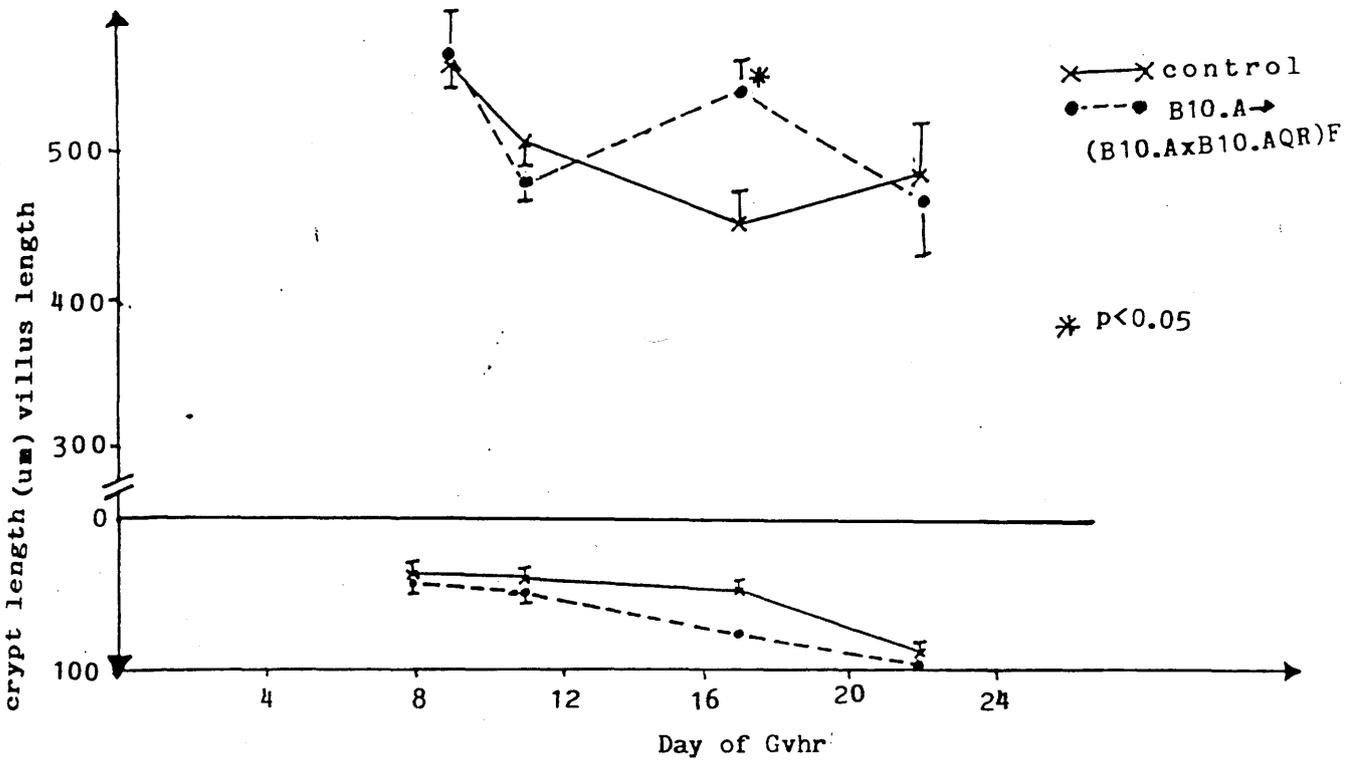


Fig. 11: Development of intestinal Gvhr across an H-2K Class I MHC disparity in 1 day old (B10.A x B10.AQR)_{F1} mice villus and crypt lengths in the jejunum of Gvhr and control mice. Results shown are the means \pm 1 s.d. for 4 mice/group.

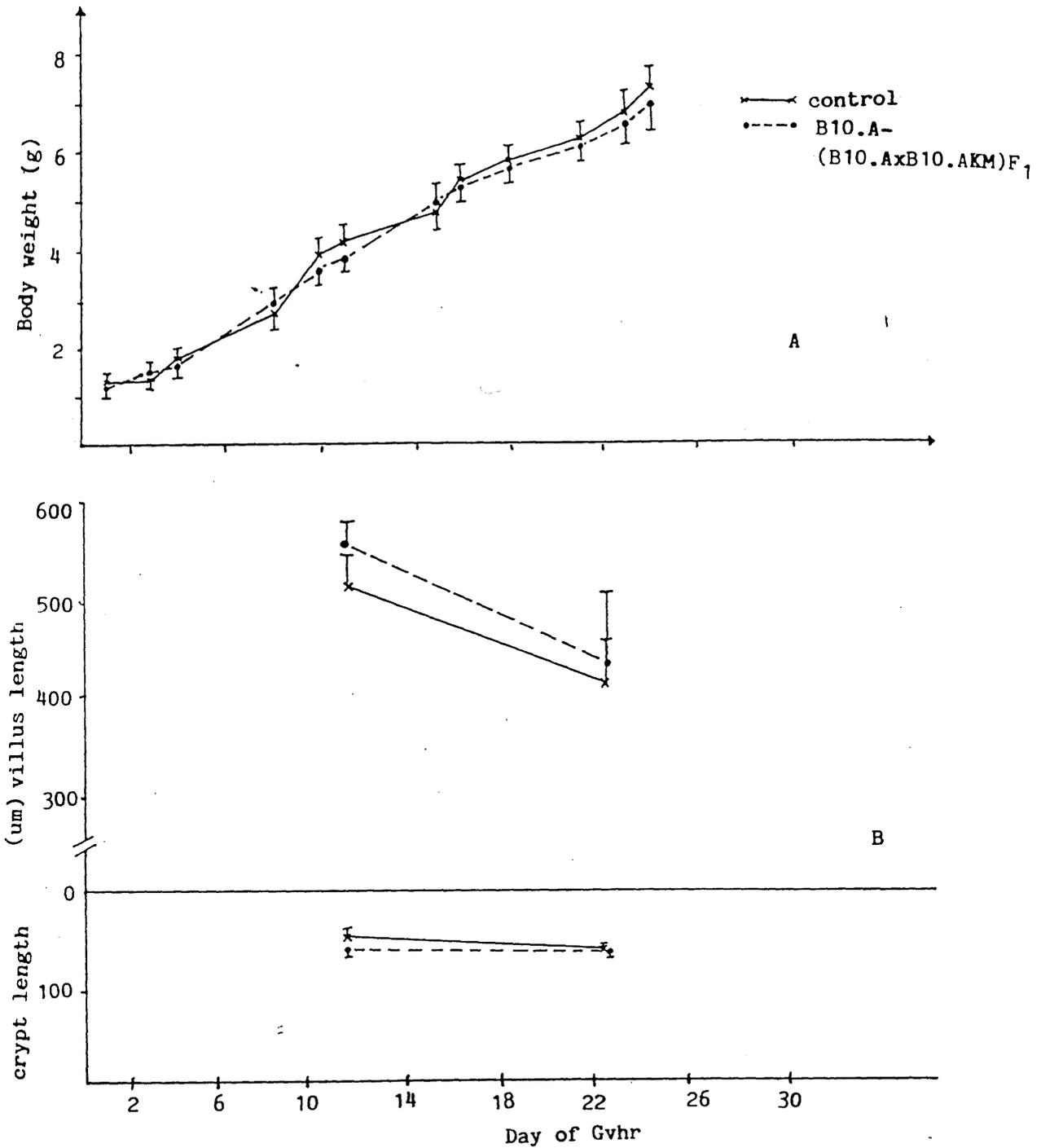


Fig. 12: Development of Gvhr across an H-2D Class I MHC disparity in (B10.A x B10.AKM)F₁ mice. A) Growth rates of neonates with Gvhr compared with littermate controls. B) Villus and crypt lengths in the jejunum of Gvhr and control mice. Results shown are the means \pm 1 s.d. for 4 mice/group.

CHAPTER 8

EFFECTS OF DEOXYGUANOSINE ON THE DEVELOPMENT
OF SYSTEMIC AND INTESTINAL CHANGES IN ACUTE

GVHR

Introduction

This thesis has concentrated on models of Gvhr which produce a destructive enteropathy involving villus atrophy. In many experiments the development of villus atrophy coincided with the appearance of specific anti-host CTL. Nevertheless, this was not always the case suggesting that another immune effector mechanism might be responsible for this type of intestinal damage. One of the models characterised by villus atrophy was the Gvhr which occurred in BDF₁ mice given B10 parental cells, and others have suggested that allospecific Ts may be responsible for the pathological consequences of this Gvhr (Pals et al., 1984). In support of this idea, I obtained evidence that suppressor cells were present during a destructive Gvhr in both BDF₁ and neonatal (CBA x BALB/c)F₁ mice.

Therefore, in this chapter I examined the effect of modifying Ts activity in vivo on the development of an acute destructive Gvhr. My first approach to this was to treat host mice with the purine metabolite 2'deoxyguanosine using a regime that has been shown to prevent the generation of Ts in vivo, but which does not affect effector T-cell functions (Dosch et al., 1980; Bril et al., 1985). In addition, I examined the effect of activating Ts in vivo, by inducing a Gvhr across an incompatibility at the I-J locus.

Effects of 2' deoxyguanosine on acute Gvhr

Experimental design

The models of acute Gvhr which were studied were those occurring in adult BDF₁ mice given 10⁸ B10 spleen cells and in one day old (CBA x BALB/c)F₁ mice given 10⁷ CBA spleen cells. Both these models had been examined in detail earlier and both provided evidence of a destructive enteropathy in association with the presence of active suppression. In each experiment, one group of Gvhr and control mice received daily injections of 40 µg/g dGuo intraperitoneally from day 5 onwards, while untreated mice received daily injections of saline. The development of systemic Gvhr was assessed by regular weighing and, at intervals mice were sacrificed for measurement of splenomegaly, cytotoxic cell activity and of mucosal architecture.

Effects of 2' deoxyguanosine on cell mediated immunity in vivo

Although others have found that dGuo has a selective ability to eliminate Ts activity in vivo (Dosch et al., 1980; Brill et al., 1984, 1985), I considered it important to ensure that the regime I used did not also alter cell mediated effector responses. Therefore the first studies examined the effect of treating mice with dGuo on the generation of DTH responses as well as specific and non-specific cytotoxicity in alloimmunized mice.

Effect of dGuo on allospecific DTH responses

Allospecific DTH responses were generated by immunizing adult CBA mice with 2×10^7 (CBA x BALB/c) F_1 spleen cells into the footpad and the effect of dGuo was examined by treating host mice with 1 mg/day dGuo for the 5 days between priming and footpad challenge with (CBA x BALB/c) F_1 spleen cells. As shown in Figure 1A, mice given dGuo mounted an allospecific DTH response which was identical to that found in control mice treated with saline daily (0.49 ± 0.11 mm vs. 0.43 ± 0.17 mm).

Effect of dGuo on the generation of CTL and NK cell activity in alloimmune mice

In the second experiment, CBA mice were immunized with 2×10^7 P815 tumour cells i.p. and were given 1 mg dGuo daily before being sacrificed on days 8, 11 and 14 for assessment of specific CTL and NK cell activity in the spleen. Specific CTL activity was found at each time, with peak levels occurring on day 11. At all times, mice given dGuo had higher levels of specific CTL activity than saline treated mice (Fig. 1B).

dGuo and saline treated mice also had similar degrees of NK cell activation after immunisation with P815, although this occurred earlier in dGuo treated mice (day 11: 21.1% vs. 12% for controls) than in saline treated mice (day 14: 27.5% vs. 14.5% in controls). dGuo had no significant effect on the NK cell activity of unimmunized controls.

Thus, dGuo does not inhibit the generation of allo-specific DTH or of natural and specific cytotoxicity in alloimmunized mice. These results indicate that dGuo should not interfere with cell mediated effector mechanisms during a Gvhr.

Effects of dGuo on the development of systemic Gvhr in adult BDF₁ mice

In the first experiment examining the effects of dGuo on Gvhr, an acute Gvhr was induced in adult BDF₁ mice by injection of B10 spleen cells. As anticipated, saline treated mice with Gvhr lost weight rapidly from day 8, until day 21, by which time they were significantly lighter than controls (Fig. 2) (17.4 ± 2.3 g vs. 27.9 ± 1.7 g, $p < 0.001$) and 4 of the 8 mice had died (Fig. 3). Thereafter, the remaining Gvhr mice gradually increased in weight although one further mouse died on day 49, and the last mouse died on day 54. (Two mice from this group of 8 had been sacrificed on day 21). Gvhr mice given dGuo also lost weight, although this was somewhat delayed in comparison with saline treated mice with Gvhr and the minimum weight did not occur until day 25 (18.2 ± 2.1 vs. 26.4 ± 2.7 , $p < 0.01$). Furthermore, there were only 2 deaths in this group and most of these mice continued to full recovery (MST < 100 days vs. 19 days for saline Gvhr, $p < 0.001$).

Additional mice from this experiment were sacrificed on days 8 and 21 of the Gvhr for assessment of splenomegaly.

As found in earlier studies in this strain combination, significant splenomegaly was found in Gvhr mice on day 8 of the Gvhr and there was no difference between dGuo and saline treated mice (Fig. 4). By day 21, the splenomegaly had virtually disappeared in both groups.

Effects of dGuo on the development of cell mediated cytotoxicity in adult BDF₁ mice with Gvhr

CTL and NK activity were examined on days 8 and 21 and, as found previously, specific anti-host cytotoxicity was present in Gvhr mice on day 8. This was greater in Gvhr mice given dGuo than in saline treated mice (Fig. 5A) (34.7% vs. 25.7% 50:1 E:T). By day 21, control Gvhr mice had no detectable CTL activity, but low levels remained in dGuo treated mice. On day 8, both groups of Gvhr mice had markedly lower NK activity than that of appropriate controls (Fig. 5B), but there was more NK activity in Gvhr mice given dGuo than in control Gvhr mice (8.8% vs. 6.8% 50:1 E:T ratio). On day 21, difficulties in labelling the target cells resulted in unusually low NK activity in all mice. However, the results showed that saline treated mice with Gvhr had no detectable NK activity, whereas dGuo treated mice with Gvhr had levels which were actually greater than those in dGuo treated controls (Fig. 5B). Interestingly, at both times, dGuo treated control mice had slightly higher levels of NK activity than untreated controls.

Thus, treatment of mice with dGuo ameliorates the systemic consequences of an acute Gvhr in adult BDF₁ mice, despite the fact that the cell mediated effector mechanisms are spared or even enhanced.

Effects of dGuo on the intestinal phase of acute Gvhr
in adult BDF₁ mice

Pieces of jejunum were examined on days 8 and 21 for mucosal damage. By day 8 minor, but insignificant villus atrophy was found in mice with Gvhr (Fig. 6) ($553.2 \pm 2.7 \mu\text{m}$ vs. $645.4 \pm 72.5 \mu\text{m}$), which increased in severity until day 21, when villus lengths were almost half those in control mice ($314.9 \pm 59.2 \mu\text{m}$ vs. $635.6 \pm 67 \mu\text{m}$ in controls, $p < 0.001$). Although dGuo treated mice with Gvhr had villus lengths identical to those of appropriate controls on day 8, by day 21 these mice had evidence of severe villus atrophy, which was comparable to that seen in untreated mice with Gvhr ($345.1 \pm 65.1 \mu\text{m}$ vs $557.6 \pm 49.6 \mu\text{m}$ in dGuo controls, $p < 0.005$).

Saline treated mice with Gvhr had a small but insignificant increase in crypt length compared with controls on day 8 (Fig. 6) ($156.9 \pm 14.3 \mu\text{m}$ vs. $123.6 \pm 4.8 \mu\text{m}$) but mice surviving until day 21 had marked crypt lengthening ($290.2 \mu\text{m} \pm 28.7$ vs. $126.3 \pm 6.8 \mu\text{m}$, $p < 0.001$). At both times, Gvhr mice also had significant increases in CCPR compared with controls (Table 1). Gvhr mice treated with dGuo already had crypt lengthening on day 8 which was significantly greater than that found in saline treated Gvhr mice ($198.0 \pm 23.1 \mu\text{m}$ $p < 0.05$) and had a very large increase in CCPR (49.8 ± 3.8 $p < 0.001$ versus saline Gvhr). However, by day 21 dGuo treated mice had the same degree of crypt lengthening as that found in saline treated Gvhr mice, and had a smaller increase in CCPR. dGuo itself had no effect on the mucosal architecture of control mice.

Therefore, dGuo appeared to delay the development of a destructive enteropathy in Gvhr, producing a concomitant increase in the early proliferative changes. However, this effect was not as marked as found with systemic disease and was overcome at later stages.

Effect of dGuo on the development of suppression during a Gvhr in BDF₁ mice

In view of the ability of dGuo to delay the Gvhr, I conducted a preliminary investigation of the effects of dGuo on the generation of suppressor cells during Gvhr.

A Gvhr was induced in a separate group of BDF₁ mice and spleen cells taken on days 8 and 22 were examined for their ability to suppress the response of control BDF₁ spleen cells to ConA. On day 8 of this experiment, addition of different concentrations of spleen cells from both saline and dGuo treated mice with Gvhr led to an enhanced response to ConA, except when the highest number of control Gvhr cells was used, when some suppression was detectable (Fig. 7). This suppression was not found using any concentration of dGuo treated Gvhr cells.

On day 22 of the Gvhr, the splenic atrophy present in saline treated mice prevented the use of a full range of concentrations of Gvhr cells and there was no consistent evidence of suppression using the low ratios of Gvhr cells which were possible. However the results from the experiment described in Chapter 5 would suggest that higher concentrations of Gvhr cells would produce active suppression at this time.

In the present study the responses of cell mixes were always less when saline treated Gvhr cells were present than when dGuo treated Gvhr cells were used. Cells from Gvhr mice treated with dGuo did not suppress the ConA response of control cells at any ratio. These results provide preliminary evidence that dGuo may interfere with the development of suppressor cells during an acute Gvhr in BDF₁ mice.

Effect of dGuo on a Gvhr in one day old (CBA x BALB/c)F₁ mice

As dGuo had only a rather transient effect on the long term Gvhr in adult BDF₁ mice, I thought it was of interest to confirm its ability to inhibit another model of acute Gvhr using one day old neonatal (CBA x BALB/c)F₁ mice.

As usual, saline treated mice of this age, given CBA spleen cells, developed an acute Gvhr, with a markedly delayed growth rate compared with controls after day 7. Although dGuo had no significant effects on the development of systemic Gvhd, there was some evidence that dGuo did prevent the most severe consequences. This was shown by the fact that, although Gvhr mice given dGuo undoubtedly lost weight during the experiment, the difference in weight between this group and their dGuo treated controls, was always less than that found with saline treated Gvhr and control mice (Fig. 8A). In addition, 3 of the 4 remaining mice in the dGuo Gvhr group appeared to be healthier and heavier than the remaining Gvhr mouse and three of the saline treated Gvhr mice had died at earlier times. Although dGuo

treated control mice were always lighter than saline-treated controls, these mice developed at a similar rate throughout the study, suggesting that dGuo itself was not influencing the growth of neonatal mice.

All Gvhr mice developed significant splenomegaly which peaked on day 11 (Fig. 8B). At this time, saline treated mice had slightly greater splenomegaly than dGuo treated Gvhr mice (2.85 ± 0.26 vs. 2.51 ± 0.14), but virtually identical splenomegaly was found at all other times in the two groups (Fig. 8B).

Effects of dGuo on the development of cell mediated cytotoxicity in neonatal mice with Gvhr

Specific anti-host cytotoxicity appeared in both groups of Gvhr mice on day 9, when dGuo treated mice had slightly more CTL activity than saline treated mice. CTL activity peaked in both groups on day 11 (Fig. 9) (15.6% and 10.5% in saline and dGuo treated mice respectively) and had almost disappeared by day 18. Thus dGuo had no effect on the development of CTL.

As in previous experiments using one day old (CBA x BALB/c) F_1 mice a transient and minor enhancement of NK activity appeared in saline treated mice with Gvhr on day 11, at a time when there was no NK activity in control mice (Fig. 9). By day 18, saline treated mice with Gvhr had no detectable NK activity, despite the appearance of some NK activity in controls. In contrast, dGuo treated mice with Gvhr had no activation of NK cells on day 11, but at the later time,

these mice had NK cell activity which was actually higher than that of either control group. These results provide some evidence that dGuo has converted a destructive Gvhr into a more proliferative form.

Effect of dGuo on intestinal Gvhr in neonatal (CBA x BALB/c)_{F1} mice

Significant villus atrophy was already present by day 9 in saline treated mice with Gvhr (Fig. 10) ($442.7 \pm 9.7 \mu\text{m}$ vs $473.5 \pm 12.5 \mu\text{m}$ in controls, $p < 0.05$). This became more severe by day 11 ($364.0 \pm 2.6 \mu\text{m}$ vs. $546.2 \pm 36 \mu\text{m}$ in controls, $p < 0.01$) and was still present in mice surviving until day 18. In contrast, dGuo treated mice with Gvhr never had villus atrophy and indeed had significantly longer villi than appropriate controls on both days 9 and 18. Both groups of Gvhr mice had significantly longer crypts than appropriate controls at all times of the Gvhr with maximum values being attained on day 11. As in adult mice, dGuo itself had no significant effects on mucosal architecture.

Thus, dGuo prevents the development of a destructive enteropathy as typified by villus atrophy in neonatal mice with Gvhr, but does not inhibit the associated crypt hyperplasia.

These findings provide some evidence that dGuo-sensitive suppressor cells may play a role in the pathogenesis of acute Gvhr. I decided to investigate this further by examining a model of Gvhr which has been shown to activate Ts, selectively, namely, the Gvhr induced across an I-J incompatibility in (B10.A3R x B10.A5R)_{F1} mice.

Induction of a Gvhr across an I-J incompatibility

A Gvhr across an I-J alloantigen difference has been shown to induce suppressor T-cells (Liew, 1981) and has been reported to suppress some of the indices of mucosal Gvhr in adult mice (Mowat et al., 1986). Therefore, it was of interest to examine whether the changes which develop during a Gvhr induced across an I-J incompatibility would produce any of the features of an acute systemic and intestinal Gvhr in neonatal mice. This was investigated by inducing a Gvhr in 1 day old (B10.A3R x B10.A5R) F_1 mice with B10A3R spleen cells.

Progress of systemic Gvhr

No evidence of clinical Gvhr was found in this model of Gvhr, with Gvhr mice gaining weight at the same rate as controls throughout the period examined (Fig. 11A). Furthermore, none of these mice developed any splenomegaly during the course of the Gvhr (Fig. 11B). Mice with a Gvhr across I-J never showed any CTL activity against LPS induced lymphoblasts of B10.A5R origin and no NK activity was detectable in either control or Gvhr mice until day 26, when identical levels appeared in both groups (Data not shown).

Development of intestinal pathology during a Gvhr in neonatal

(B10.A3R x B10.A5R) F_1 mice

Despite the absence of features associated with systemic Gvhr (B10.A3R x B10.A5R) F_1 mice given B10.A3R spleen cells had villus atrophy on both days 11 (Fig. 12) ($506.4 \pm 40.1 \mu\text{m}$ vs. $584.7 \pm 31.2 \mu\text{m}$ in controls, $p < 0.05$) and 16 of the

Gvhr ($498.4 \pm 47.1 \mu\text{m}$ vs. $540.8 \mu\text{m}$ in controls). By day 24 villus lengths in Gvhr mice had returned to normal.

Interestingly, the shorter villi found in Gvhr mice on days 11 and 16 were thin and lacked an inflammatory cell infiltrate, and so were completely unlike the inflamed, stunted villi which characterised the enteropathy found in other models of destructive Gvhr. Furthermore, these alterations in villus length were not associated with crypt lengthening. Indeed, crypt lengths in Gvhr mice were consistently shorter than control crypts, although this was only significant on day 16 ($40.5 \pm 3.7 \mu\text{m}$ vs. $48.7 \pm 0.4 \mu\text{m}$ in controls, $p < 0.05$).

Interestingly, crypt lengths began to recover after this time and, on day 24, Gvhr mice had a moderate but statistically insignificant increase in CCPR compared with controls (11.3 ± 1.72 vs. 6.48 ± 2.77). This perhaps reflected a homeostatic response to the earlier mucosal atrophy.

Thus the induction of a Gvhr across an I-J incompatibility had no effects on systemic immunity and produces no clinical disease, but was associated with some evidence of an atrophic enteropathy.

Summary and conclusions

The experiments in this Chapter show that daily administration of 2'deoxyguanosine substantially reduced the mortality due to an acute lethal Gvhr in adult BDF₁ mice and delayed the associated weight loss. In parallel, dGuo appeared to delay slightly the onset of villus atrophy in Gvhr and prevented the loss of NK cell activity which normally characterises the later stages of Gvhr in these mice. dGuo treated mice with Gvhr also had more intense crypt cell

proliferation during the early stages of the Gvhr.

Although dGuo had less effects on the systemic consequence of acute Gvhd in one day old neonatal (CBA x BALB/c) F_1 mice, there was some evidence that dGuo treated mice with Gvhr had less severe weight loss and eventually showed some signs of recovery at later times. In addition dGuo treated mice did not have the early enhancement of NK cell activity found in control Gvhr mice and dGuo prevented the usual loss of NK activity at later times. dGuo had profound effects on the intestinal Gvhr in neonatal mice, no villus atrophy being found in dGuo treated mice with Gvhr. In parallel, these mice appeared to have a higher level of crypt proliferation than untreated Gvhr animals. dGuo had no effect on the development of splenomegaly or anti-host CTL activity in either model of Gvhr and also did not influence either the growth or mucosal architecture of control mice. Furthermore, control experiments showed that dGuo did not affect the generation of DTH, CTL or NK cell activation in alloimmunized mice. However, experiments in adult BDF $_1$ mice with Gvhr provided some preliminary evidence that dGuo interfered with the suppressor cell activity found in this model of acute Gvhr.

The possible involvement of I-J restricted Ts in Gvhr was examined by inducing a Gvhr in (B10.A3R x B10.A5R) F_1 mice. No clinical Gvhr occurred in these mice and there was no splenomegaly, anti-host CTL or increase in NK cell activity. Despite these findings, a Gvhr across I-J did produce villus atrophy. However, unlike other models of destructive Gvhr,

this villus atrophy was accompanied by crypt shortening rather than hyperplasia and there was no mucosal inflammation. Interestingly, some evidence was obtained to suggest that the mucosa was attempting to recover from the atrophy at later times of this Gvhr.

These results indicate that dGuo may reduce the destructive consequences of an acute Gvhr, and together with the findings in mice with a Gvhr induced across an I-J difference, provide some support for a role for suppressor T cells in the pathogenesis of Gvhr.

Day of Gvhr	Control	Gvhr	dGuo control	dGuo Gvhr
8	8.88 \pm 4.58	31.16 \pm 2.79*	15.60 \pm 1.80	49.81 \pm 3.89**
21	15.40 \pm 1.91	50.62 \pm 11.94**	11.22 \pm 1.72	23.61 \pm 7.61

Table 1: Effects of dGuo on intestinal Gvhr in adult BDF₁ mice given 10⁸ B10 spleen cells. CCPR in the jejunum of mice with Gvhr which had been treated with saline or dGuo daily from day 5 and in appropriate controls. The results shown are means \pm 1 s.d. for 4 mice/group.

* $\rho < 0.01$

** $\rho < 0.001$ vs. appropriate controls.

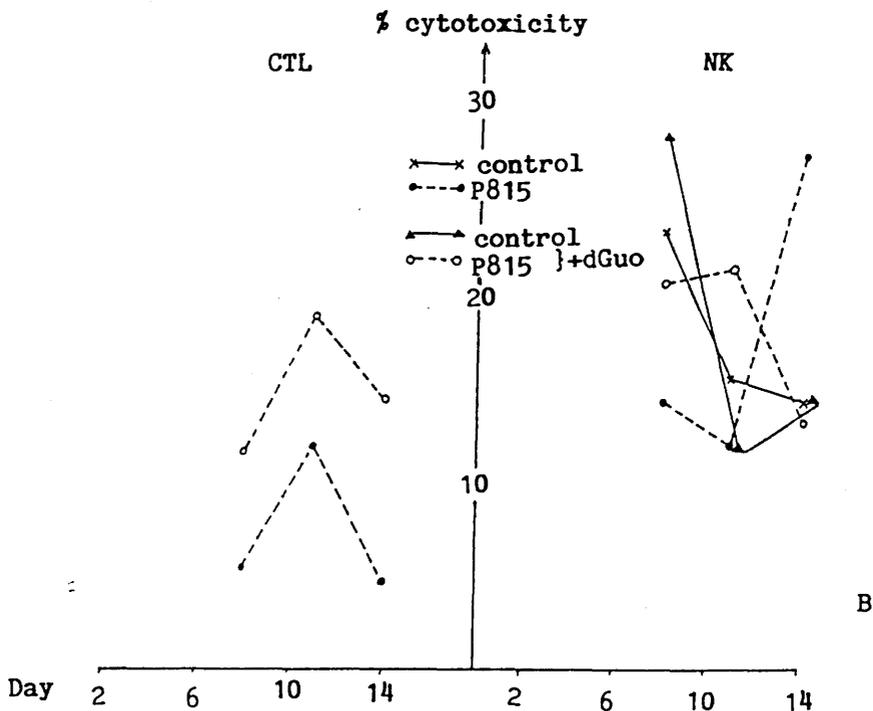
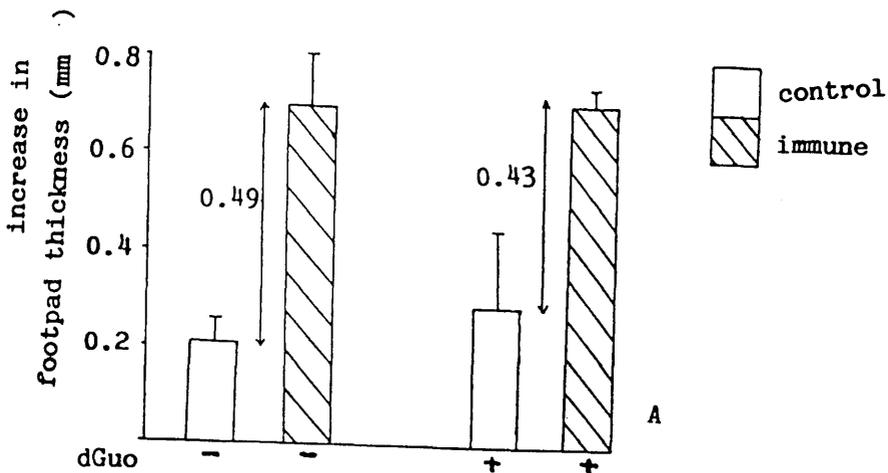


Fig. 1: Effects of 1 mg/day dGuo on the generation of allospecific DTH and cytotoxicity in mice treated with dGuo (ip) daily. A) DTH responses in adult CBA mice immunized with 2×10^7 (CBA x BALB/c) F_1 spleen cells and in controls. Results shown are increases in footpad thickness 24 hours after challenge with 10^7 F_1 spleen cells in 6 mice/group. B) Specific CTL and NK activity in CBA mice immunized with 2×10^7 P815 tumour cells and in controls. Results shown are % cytotoxicity vs P815 and YAC-1 at 50:1 E:T in 3 mice/group.

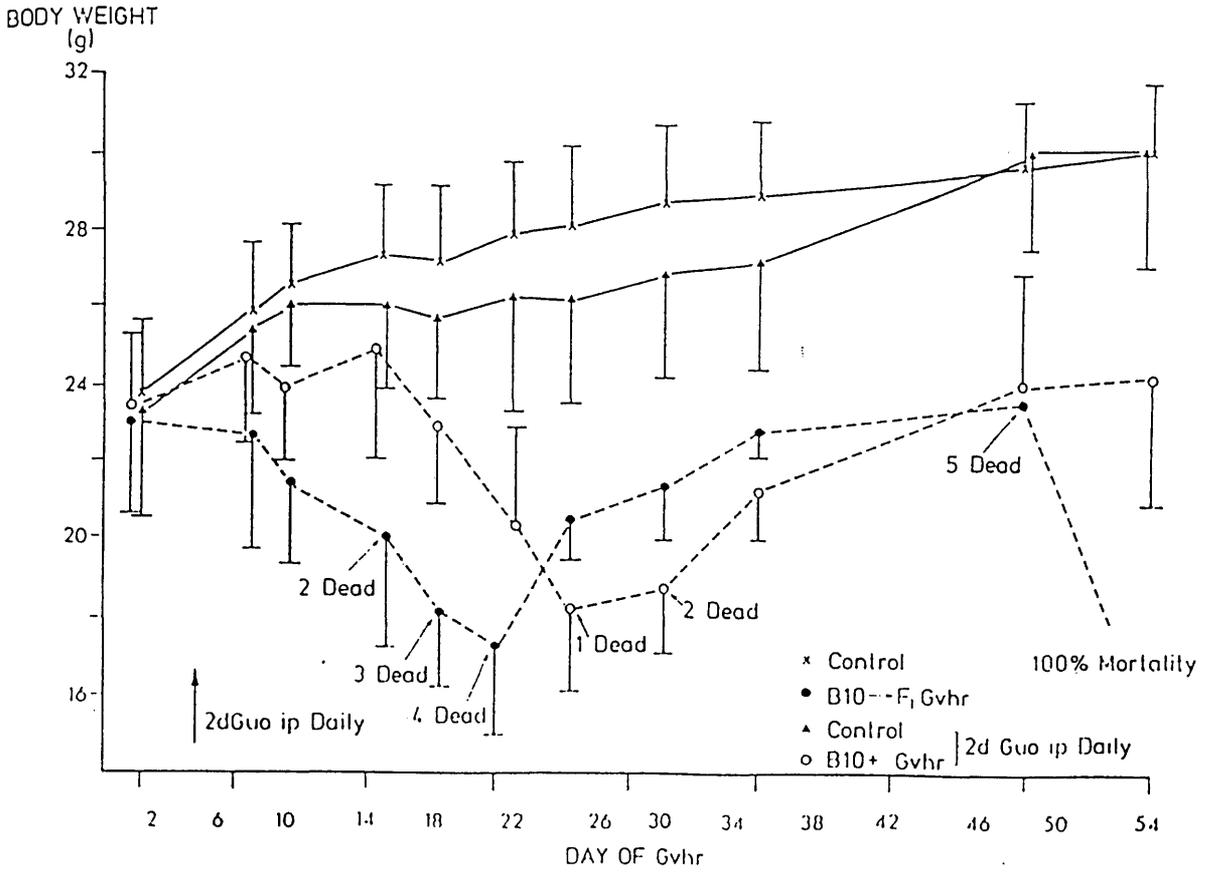


Fig. 2: Effects of dGuo on systemic Gvhr in adult BDF₁ mice given 10⁸ B10 spleen cells. Body weights in control and Gvhr mice treated with dGuo or saline i.p. daily from day 5 onwards. Results shown are means ± 1 s.d. for 8 mice/group.

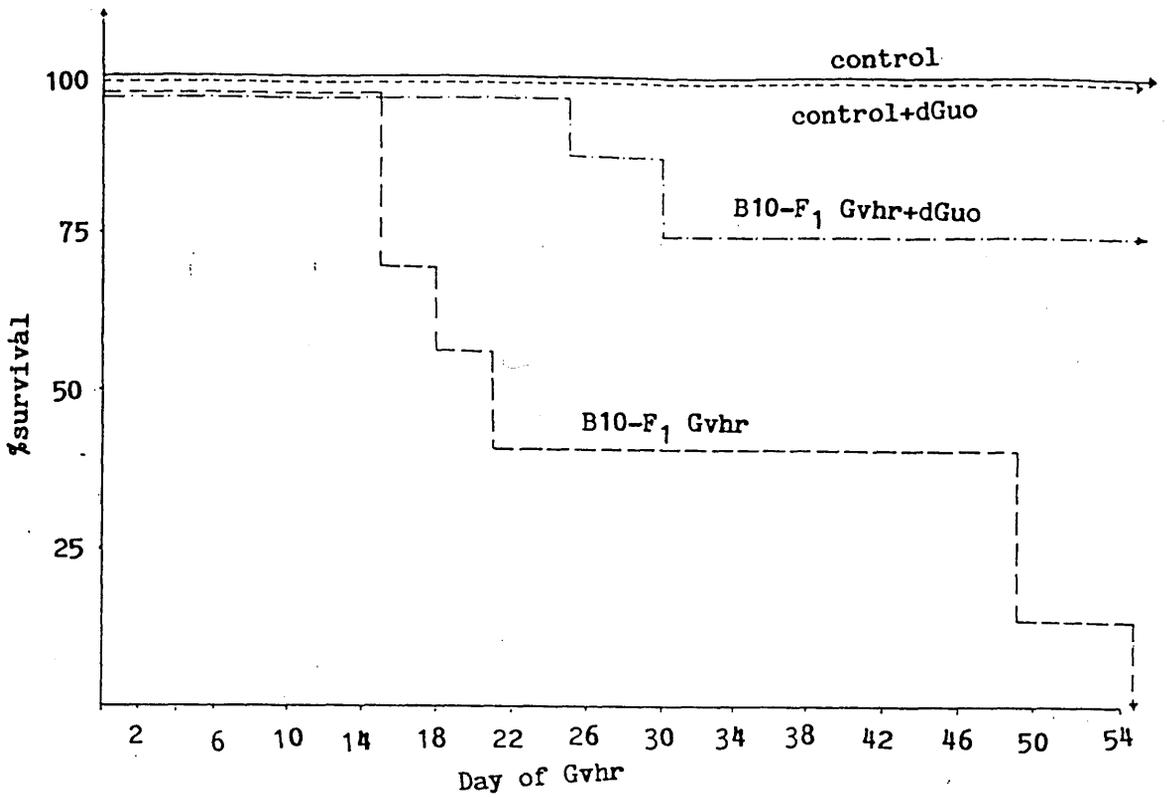


Fig. 3: Effects of dGuo on systemic Gvhr in adult BDF₁ mice mortality rates of control and Gvhr mice which were treated with dGuo or saline i.p. daily from day 5 onwards.

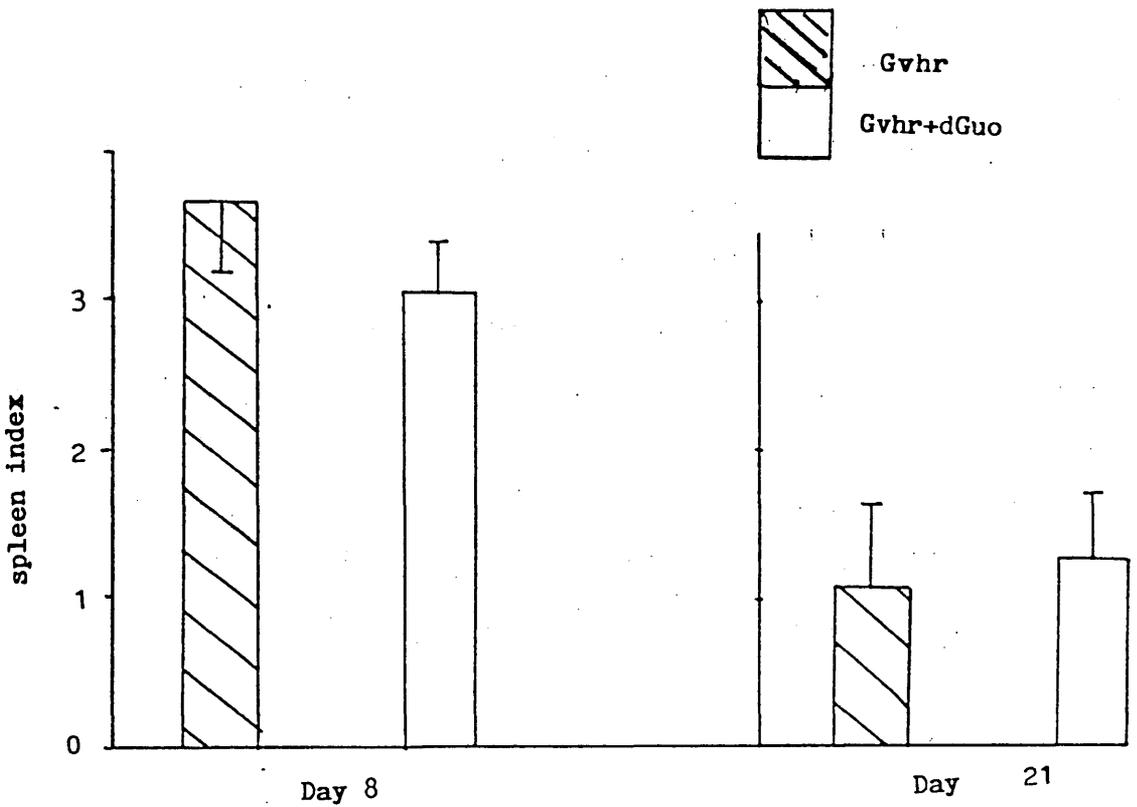
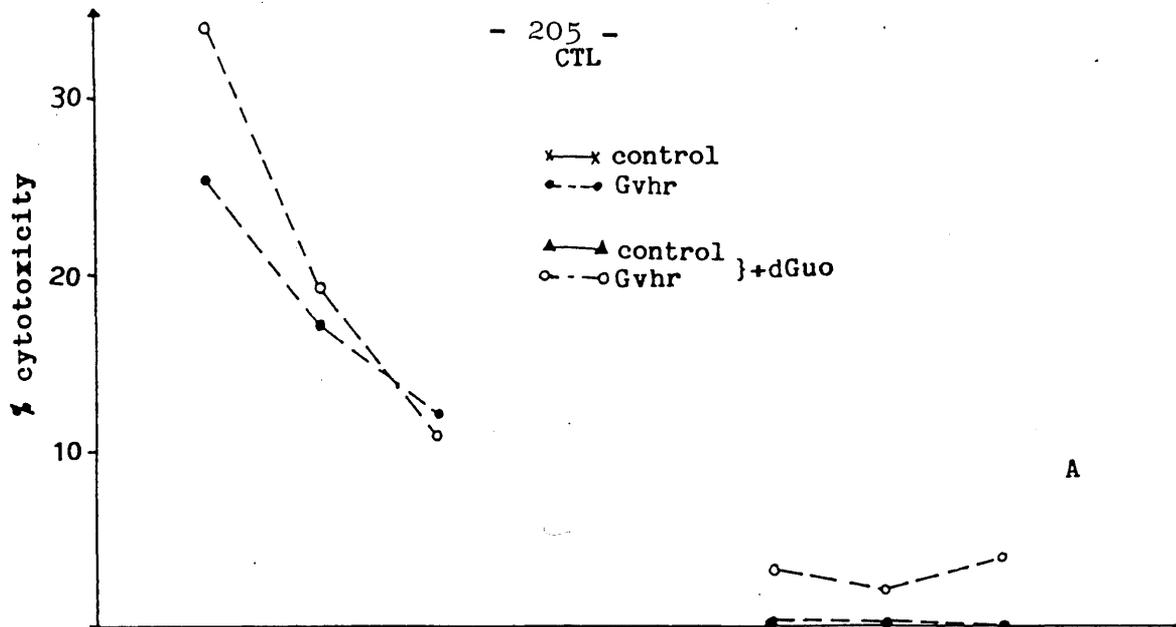
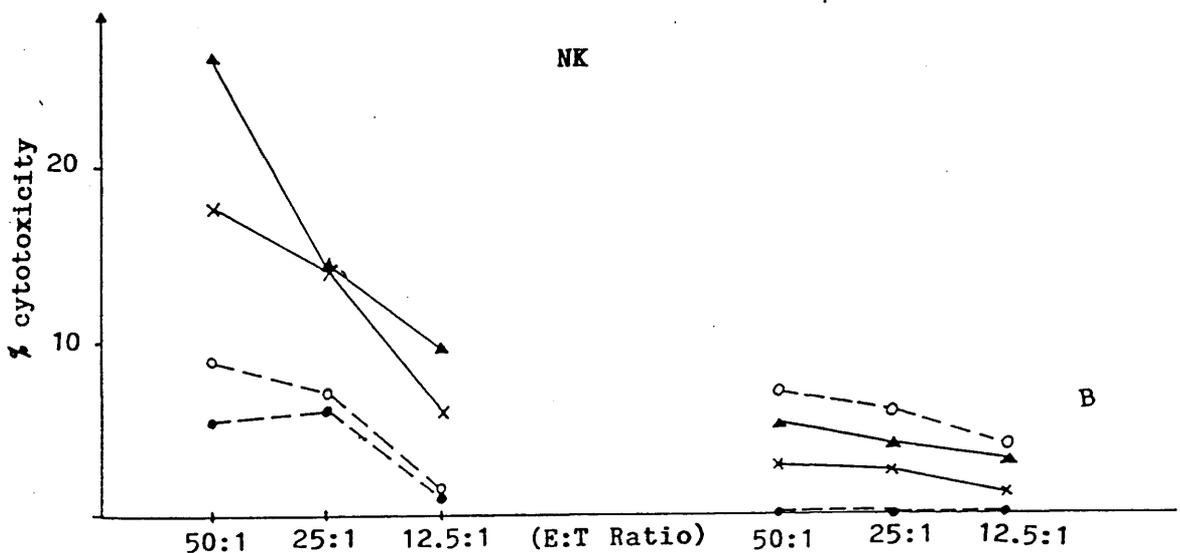


Fig. 4: Effects of dGuo on systemic Gvhr in adult BDF₁ mice. Development of splenomegaly in dGuo and saline treated mice with Gvhr. Results shown are mean spleen indices ± 1 s.d. for 5 mice/group.



A

NK



B

Fig. 5: Effects of dGuo on systemic Gvhr in adult BDF₁ mice. Development of A) specific CTL and B) NK activity in dGuo and saline treated mice with Gvhr on days 8 and 21 in appropriate controls. Results shown are % cytotoxicity at different E:T ratios using spleen cells pooled from 3 mice/group.

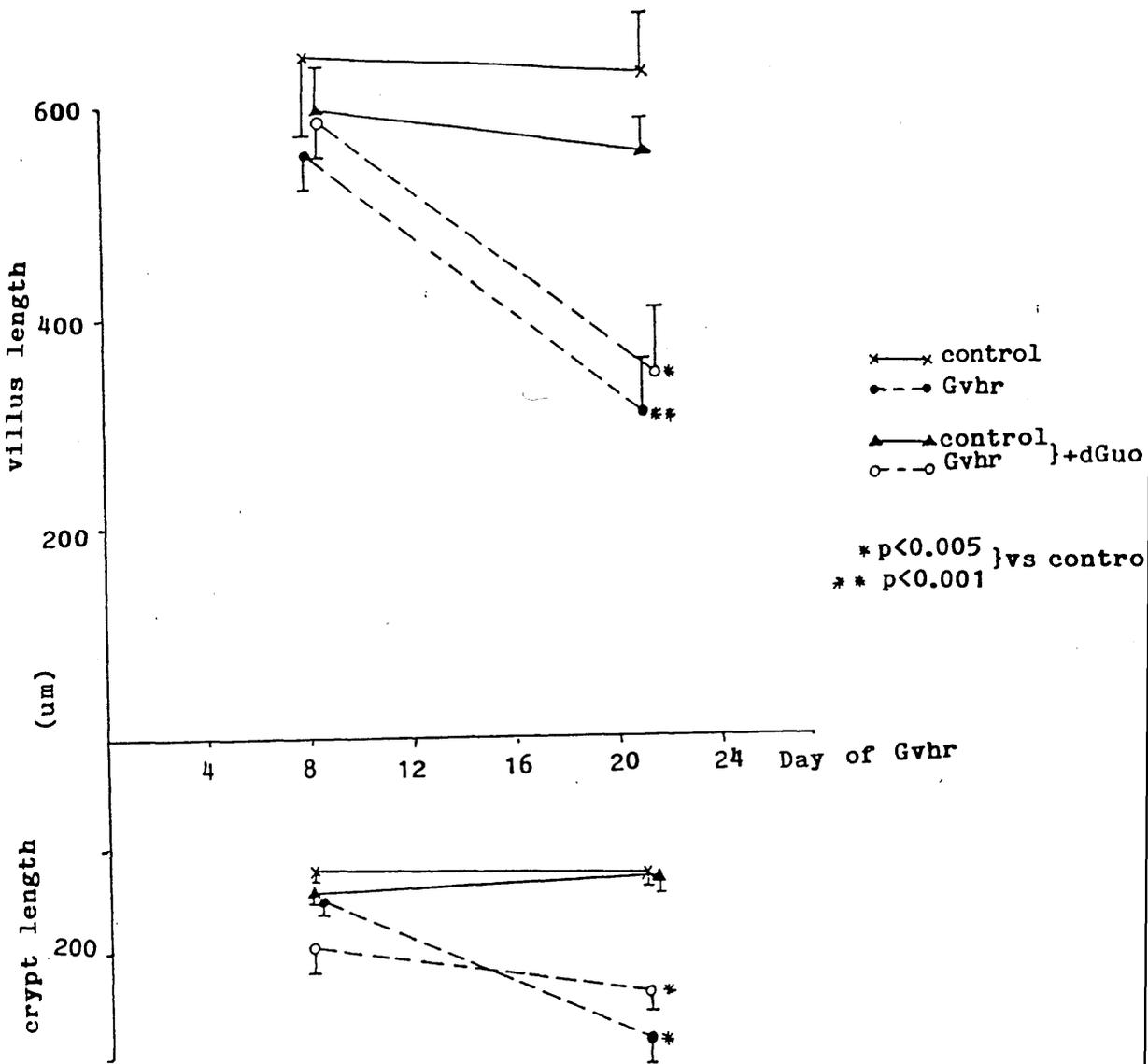


Fig. 6: Effects of dGuo on intestinal Gvhr in adult BDF₁ mice. Villus and crypt lengths in dGuo and saline treated mice with Gvhr, and in appropriate controls. Results shown are the means \pm 1 s.d. for 5 mice/group.

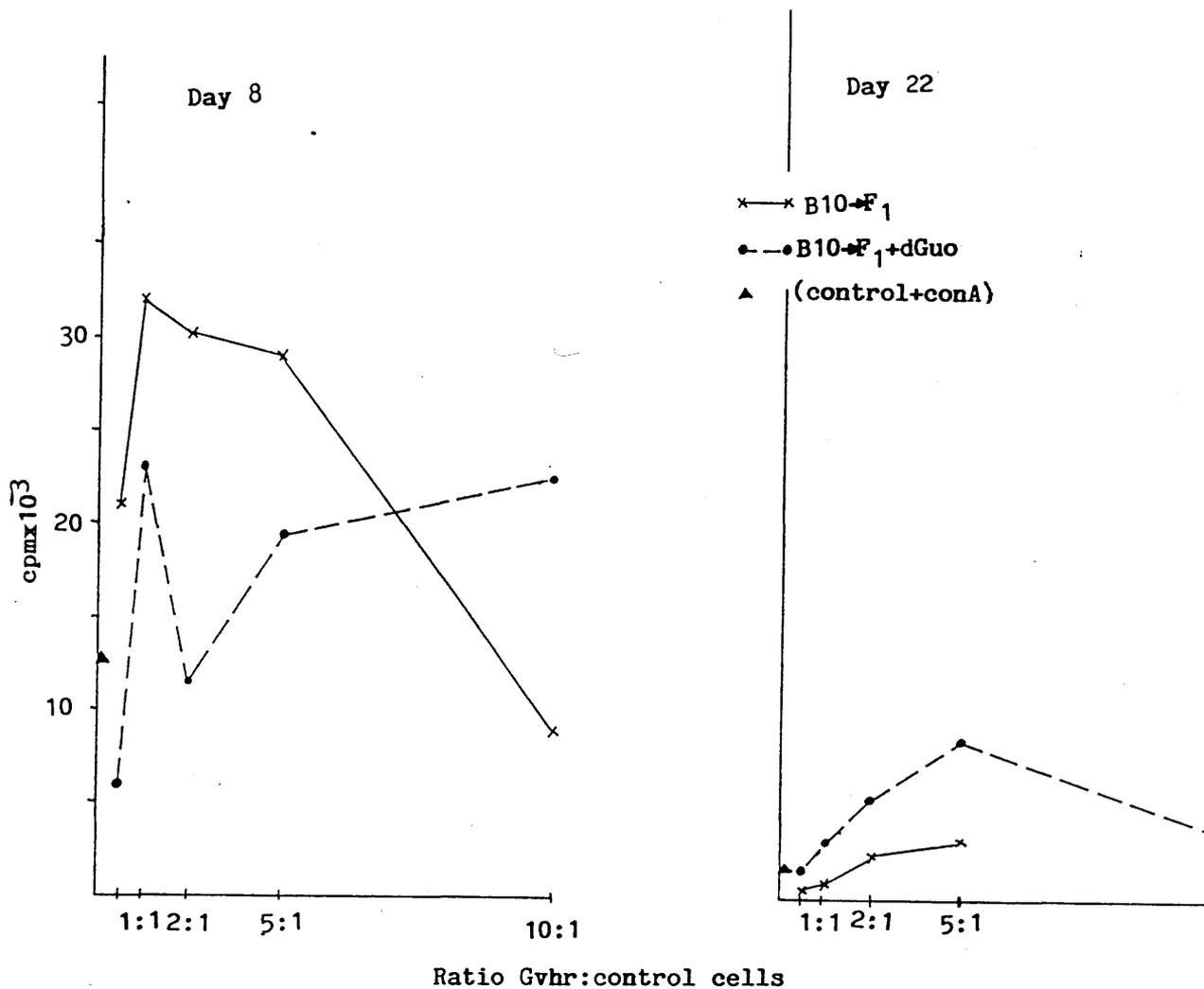
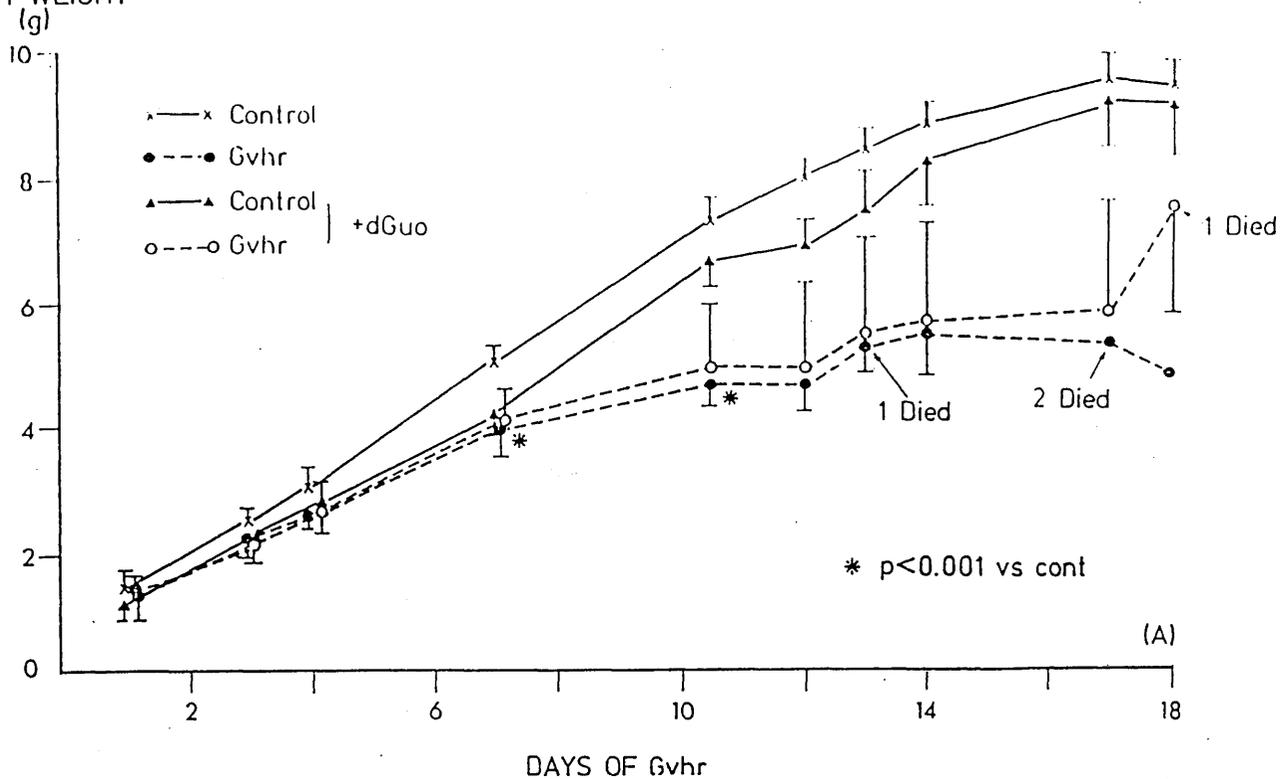


Fig. 7: Effects of dGuo on the development of suppression during Gvhr in adult BDF₁ mice. Spleen cells from dGuo and saline treated mice with Gvhr were mixed with control BDF₁ spleen cells at different ratios and cultured with ConA for 3 days. Results shown are mean incorporation of ^3H TdR (cpm/well) for triplicate assays.

BODY WEIGHT



SPLEEN INDEX

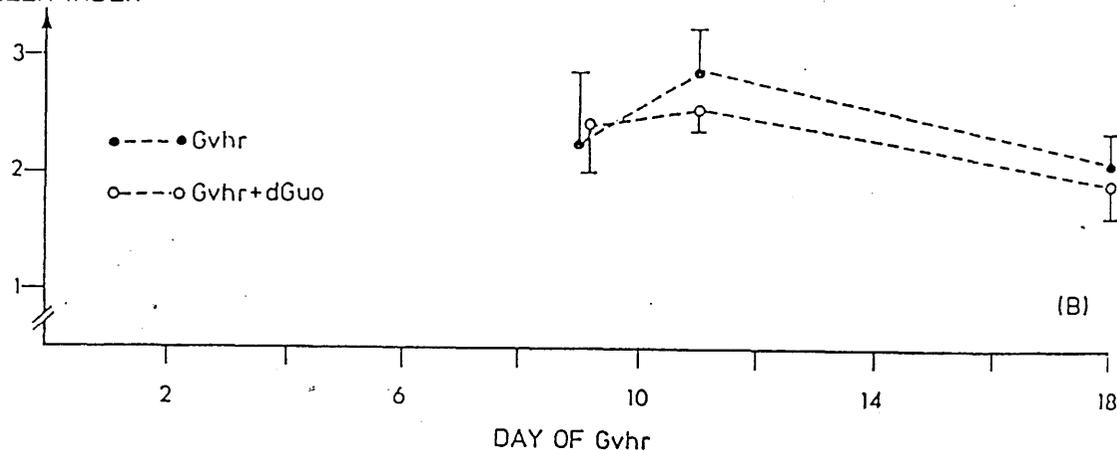


Fig. 8: Effects of dGuo on systemic Gvhr in 1 day old (CBA x BALB/c)_{F1} mice. A) Growth rates of Gvhr mice which had been treated with dGuo or saline daily from day 1 and appropriate controls. B) Development of splenomegaly in dGuo and saline treated mice with Gvhr. Results shown are means \pm 1 s.d. for 5 mice/group for body weight, and 3 mice/group for spleen index.

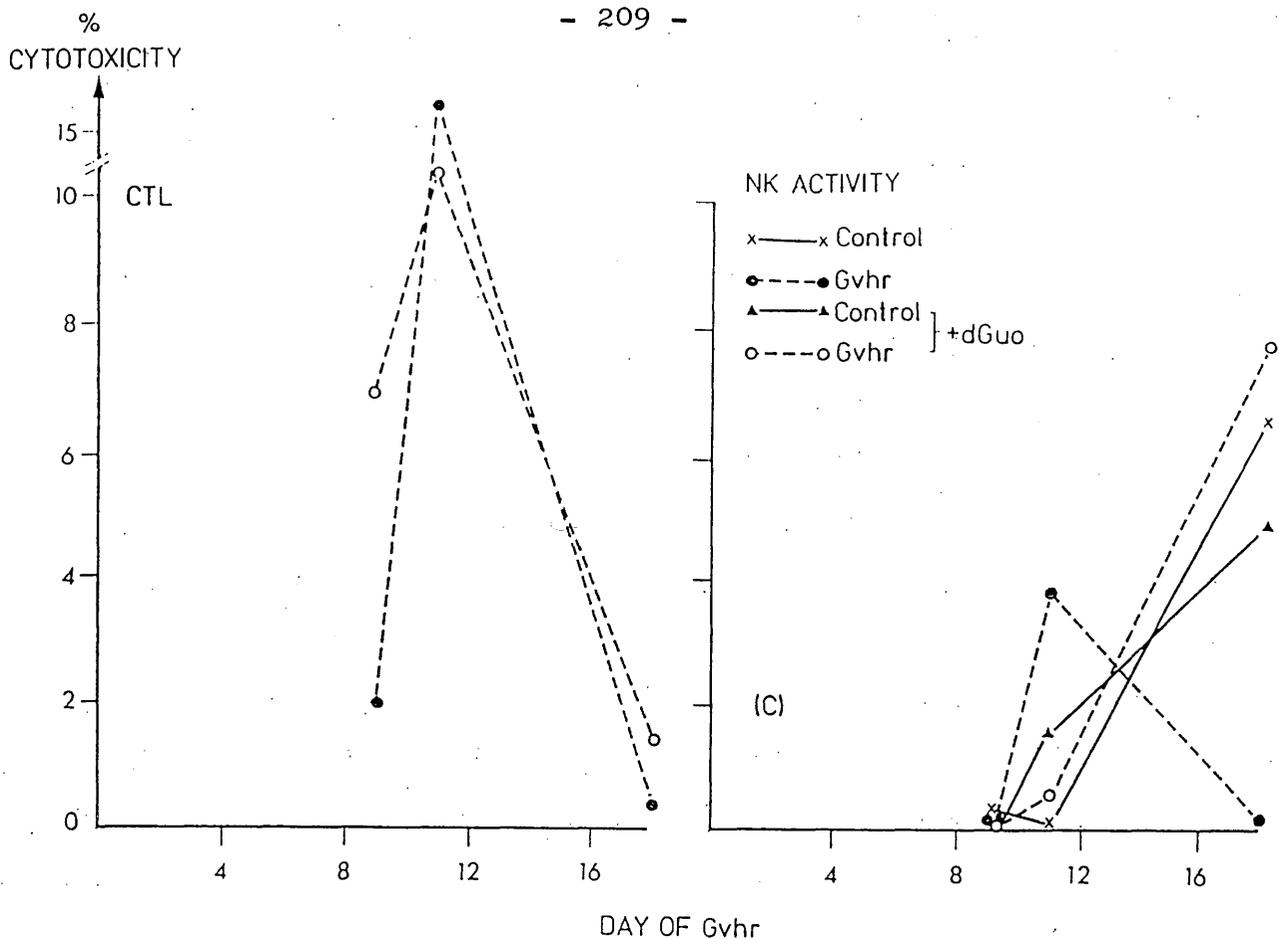


Fig. 9: Effects of dGuo on systemic Gvhr in 1 day old (CBA x BALB/c)_{F1} mice. Development of NK and specific CTL activity in dGuo and saline treated mice with Gvhr, and in appropriate controls. Results shown are % cytotoxicity using spleen cells pooled from 3 mice/group.

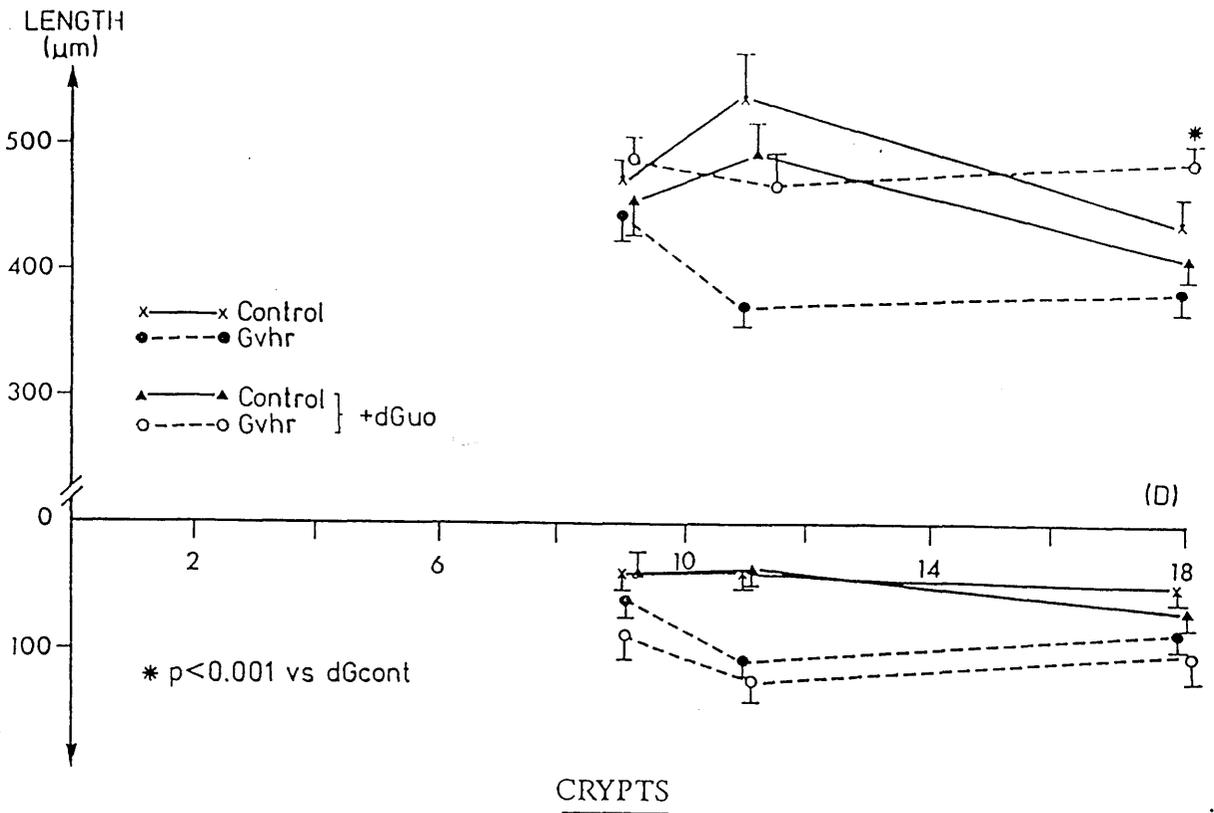


Fig. 10: Effects of dGuo on intestinal Gvhr in 1 day old (CBA x BALB/c)_{F1} mice. Villus and crypt lengths in dGuo and saline treated mice with Gvhr, and in appropriate controls. Results shown are the means ± 1 s.d. for 4 mice/group.

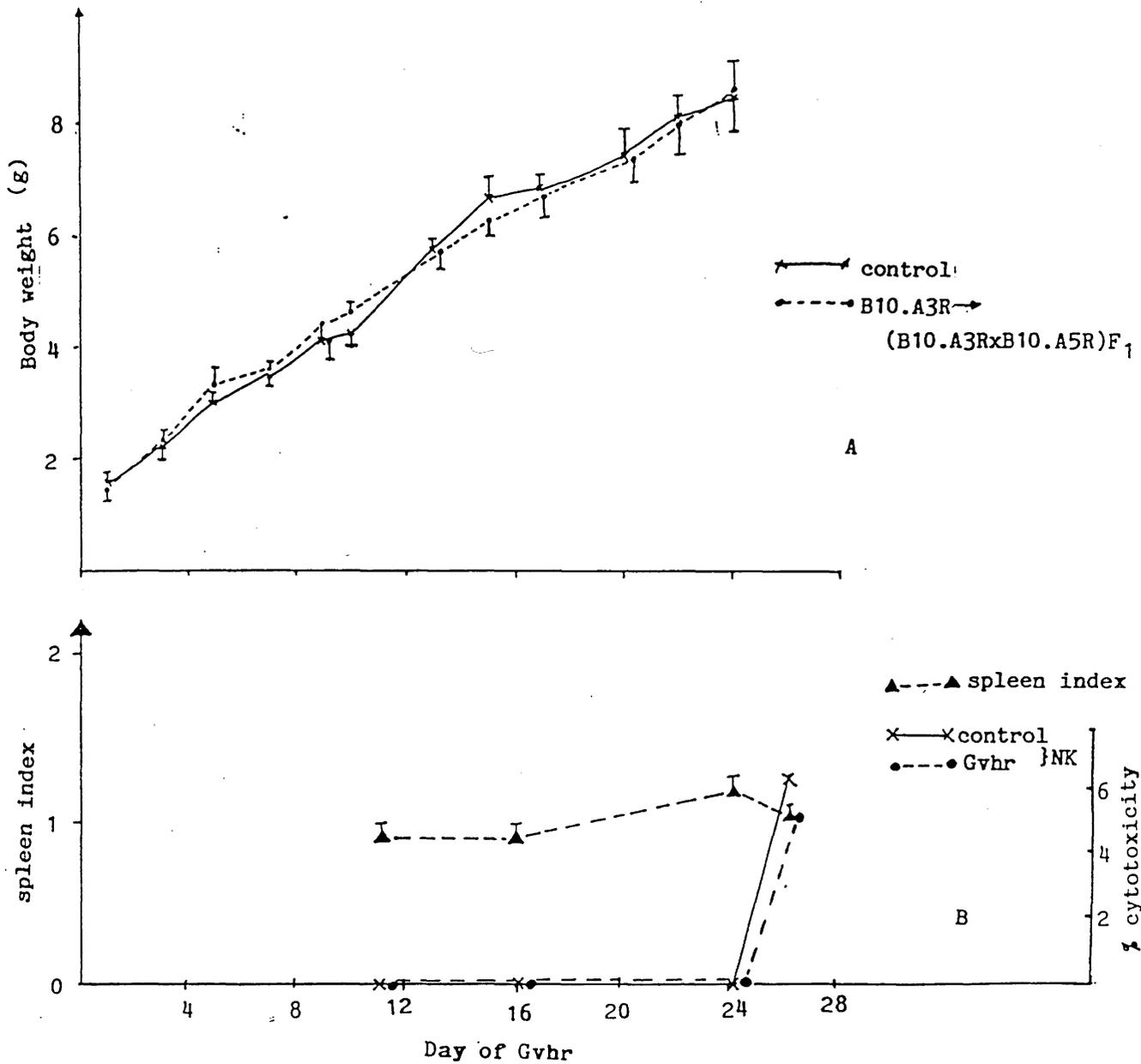


Fig. 11: Development of Gvhr in 1 day old (B10.A3R x B10.A5R) F_1 mice. A) Growth rates of neonates with Gvhr compared with littermate controls. B) Spleen index. Results shown are means \pm 1 s.d. for 3 mice per group.

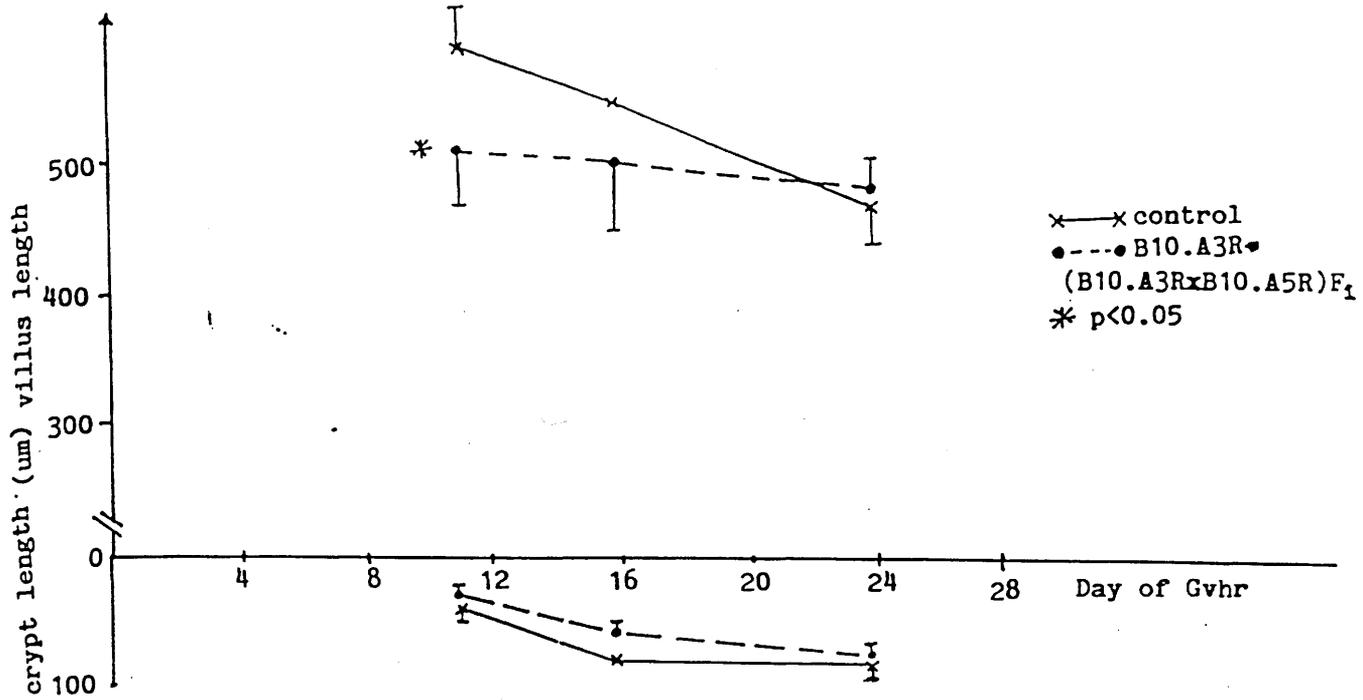


Fig. 12: As for Figure 11. Villus and crypt lengths in the jejunum of mice with Gvhr and in controls. Results shown are means \pm 1 s.d. for 3 mice/group.

CHAPTER 9

GENERAL

DISCUSSION

The principal aim of this thesis was to extend previous work on intestinal Gvhr in unirradiated mice, by establishing models of Gvhr which were associated with villus atrophy in the small intestine. My intention was then to investigate the effector mechanisms responsible for this type of intestinal damage.

In Chapters 3, 4 and 5 I have shown that an acute Gvhr developed in irradiated adult (CBA x BALB/c) F_1 mice, in neonatal (CBA x BALB/c) F_1 mice and in both adult and neonatal BDF $_1$ mice given B10 parental cells. All these models of acute Gvhr were characterised by destructive intestinal lesions, including villus atrophy. It was then possible to compare the mucosal pathology and immune effector mechanisms found in those models, with the features described earlier during a non-lethal, proliferative Gvhr. The results from these experiments on acute Gvhr will be discussed first before going on to the studies which attempted to alter the intestinal Gvhr by modifying either the host or the donor cell population.

Models of acute Gvhr

The development of acute Gvhr was studied in three different models and the results of these will be discussed separately before assessing the overall significance of the results.

The first model chosen was the Gvhr which occurred in heavily irradiated (CBA x BALB/c) F_1 mice given CBA spleen cells, as earlier studies had identified villus atrophy and mucosal necrosis in this form of Gvhr (Reilly & Kirsner, 1965;

Hedberg et al., 1968). However, the intestinal changes had not been quantified previously and had not been correlated with immune effector mechanisms. All the mice in my experiments developed a rapidly lethal disease, but there was also an initial, proliferative phase of intestinal Gvhr which was associated with a marked enhancement of splenic non-specific cytotoxicity. The onset of overt clinical disease was then accompanied by the appearance of specific anti-host CTL and by villus atrophy in the small intestine. Little attention has been given, previously, to the proliferative alterations which I found to precede the established phase of acute Gvhr in irradiated hosts. These comprised elevated NK activity, increased numbers of IEL as well as increases in CCPR and crypt length, and thus were identical to the features which characterise the entire Gvhr in unirradiated (CBA x BALB/c) F_1 mice (Mowat & Ferguson, 1981b; 1982; Borland et al., 1983). However a major difference is that villus atrophy and anti-host CTL only develop during a Gvhr in these hosts if they have been irradiated.

Interestingly, the appearance of these latter features was preceded by a sudden loss of NK cell activity and a parallel cessation of crypt cell mitotic activity. The reasons for this are unclear, but an obvious possibility is that host derived NK or crypt cells are being lysed by donor CTL. Although NK cell precursors are relatively radioresistant and increased NK activity occurred in irradiated

mice with Gvhr (Charley et al., 1983; Varkila & Hurme, 1985), it is unclear whether this is due to cells of host or donor origin.

However, there is some evidence from studies of Gvhr in unirradiated mice that both host and donor NK cells may be recruited non-specifically during a Gvhr in unirradiated mice (Roy et al., 1982; Borland et al., 1983; Kubota et al., 1983; Mowat et al., 1985; Ghayur et al., 1987). Thus the loss of NK activity may be due to a direct attack on host cells by donor CTL. Alternatively, it has been suggested that NK cells can suppress CTL activity (Schaaf-Lafontaine et al., 1984; Gilbertson et al., 1986) and therefore it is possible that CTL appear as a result of the cessation of NK activity. Finally, the possibility that the abrupt disappearance of NK cell function may not reflect the killing of host derived NK cells by developing CTL, is supported by the finding of an active suppressor mechanism which inhibits NK cells of both donor and host origin develops in irradiated mice with Gvhr.

These studies showed that an acute Gvhr in irradiated hosts led to villus atrophy which was associated with specific CTL activity. Although one feature of this study was the surprisingly minor effects which irradiation itself had on the intestine, irradiation did alter epithelial cell renewal markedly and others have shown that irradiation and Gvhr may synergise in the development of a destructive enteropathy in neonatal mice (Reilly & Kirsner, 1965). Therefore, I thought it important to confirm the findings in irradiated mice, using a model which did not require radiation to

produce an acute Gvhr. A different approach was also considered necessary as the progression of the Gvhr in irradiated host was too rapid to investigate fully the effector mechanisms responsible for the intestinal lesions.

The nature and intensity of systemic and intestinal Gvhr in neonatal (CBA x BALB/c) F_1 mice depended on the age of the host. Thus, seven day old mice developed an entirely proliferative disease, characterised by intense splenomegaly, NK cell activation and crypt hyperplasia, identical to that described previously in adult mice of this strain (Mowat & Ferguson, 1981b, 1982; Borland et al., 1983). In contrast, one or two day old mice developed an acute lethal Gvhr, associated with weight loss, specific CTL activity and villus atrophy with only transient activation of NK cells. In all these experiments, weight loss began about day 8 and death normally occurred within 3 weeks. In parallel, the splenomegaly found in the early stages of Gvhr disappeared and severely runted mice had shrunken spleens, anaemia and liver necrosis. Finally, as described previously in acute Gvhr (Pickel & Hoffman, 1977; Kubota et al., 1983; Pals et al., 1984) there was also some evidence of active immunosuppression during the later stages of Gvhr.

As in irradiated mice, the development of acute destructive Gvhr in 1-2 day old neonates was preceded by an early proliferative phase of NK cell activation and crypt hyperplasia. Therefore this biphasic pattern of enteropathy was not merely an artefact induced by irradiation, but probably reflects a rapidly progressive immune response in immunologically

deficient hosts. One difference between the models was that there was only a very small increase in NK activity during the early stages of Gvhr in 1-2 day old neonates. Although this probably reflects the absence of NK cell activity in mice of this age (Kiessling et al., 1975; Roder et al., 1981; Koo et al., 1982), rather than a difference in the mechanisms involved. It should also be noted that there was also no evidence of a reduction in CCPR or IEL numbers in neonates with Gvhr.

My findings confirm older observations that the incidence of runt disease in neonatal mice decreases with time after birth (Nisbet & Heslop, 1962). Together, these features provide an explanation for the fact that previous studies of Gvhr in neonatal (CBA x BALB/c) F_1 mice found villus atrophy in 5 day old, but not 6-8 day old hosts and indicate these differences were due to inherent differences in the mice used rather than to experimental variation (MacDonald & Ferguson, 1977; Mowat & Ferguson, 1982). Furthermore, an experiment using a weight related adjustment of donor cells did not alter the systemic or intestinal consequences of the Gvhr, therefore the differences I observed were not due to a cell dose effect.

One other difference between the intestinal Gvhr I observed in neonatal mice and that described previously in adult hosts, was that an increased IEL count was not a prominent feature of the present studies. In addition, when an increased IEL count occurred, this tended to be found later than had been noted in other studies. However, this

discrepancy is perhaps not surprising in view of the fact that mice of this age have very few IEL (Ferguson, 1977; Strobel & Shields, 1987). Performing IEL counts in these animals also presented considerable technical difficulties. One constant feature of the acute Gvhr in irradiated and 1 day old mice was the association between the appearance of specific CTL and villus atrophy. However, this was not confirmed in experiments using 5 day old neonates. Those hosts developed an extremely varied form of Gvhr which ranged from an acute, lethal disorder to an entirely proliferative disease, and so was somewhere between that found in 1 and 7 day old mice. All 5 day old mice had an early proliferative phase of Gvhr with crypt hyperplasia and NK cell activation, which was maintained in some mice, and these animals did not runt. In contrast, some 5 day old mice with Gvhr lost weight and eventually died. As in irradiated hosts, this was associated with villus atrophy, the disappearance of NK cell activity, decreased crypt cell turnover and crypt shortening. However the transition from proliferative to destructive Gvhr in 5 day old neonates was accompanied by very little of the specific CTL activity found in other models of acute Gvhr. These findings suggest that CTL may not be essential for villus atrophy and systemic runting.

Thus, the acute Gvhr in neonatal or irradiated (CBA x BALB/c) mice provided very similar findings. However, both these models used hosts which were inherently abnormal, either because of irradiation or immaturity. Therefore, it was possible that the ability to induce an acute intestinal disease

was an artefact of using abnormal hosts. Furthermore, the nature of the host mice meant it was difficult to obtain sufficient lymphoid cells to perform a comprehensive range of functional assays during the Gvhr. Therefore, I was concerned to produce an acute, destructive Gvhr in unmanipulated adult hosts.

Although an acute systemic Gvhr had been detailed in adult, unirradiated BDF₁ mice given B10 spleen cells, the development of intestinal pathology had not been described previously. The results in Chapter 5 confirmed that an acute lethal Gvhr could be induced in adult, unirradiated BDF₁ mice by transfer of 10⁸ B10 parental spleen cells. As reported previously mice developed weight loss (Van Elven et al., 1981; Pals et al., 1984) from around day 8, and a proportion of these mice died. In parallel, specific anti-host CTL activity appeared as mice began to runt and villus atrophy developed in the small intestine. Therefore, this Gvhr was very similar to the acute Gvhr in neonatal and irradiated mice. This was emphasised further by the fact that the early stages of the acute Gvhr in BDF₁ hosts were also characterised by proliferative changes such as splenomegaly and crypt hyperplasia. Although, others have also reported enhanced NK cell activity in this model of Gvhr (Kubota et al., 1983), I did not find this feature, possibly because it occurred before I sacrificed the first group of mice on day 3. As in other models of acute Gvhr, the transition from the early proliferative phase to an acute, destructive disorder was accompanied by the loss of NK activity and reduced numbers of IEL, as well as declining splenomegaly.

The consistent finding of specific CTL activity in BDF₁ mice with acute Gvhr contrasts with initial studies in BDF₁ mice given B10 spleen cells which suggested that this model of Gvhr was not due to specific CTL activity. Nevertheless, this earlier work did not measure CTL activity directly in unirradiated BDF₁ hosts (Van Elven et al., 1981) and others have confirmed the presence of CTL in B10 → BDF₁ mice (Kubota et al., 1983). Interestingly, these workers also found that activation of NK cells preceded the development of CTL and that a sudden loss of NK activity paralleled the appearance of specific CTL. Suppressed NK activity has been noted in other studies of Gvhr in BDF₁ mice given B10 donor cells (Pattengale et al., 1983) and as discussed above, it seems possible that this may be due to elimination of host NK cells by CTL.

IEL are a further population of cells which declined during the most severe phase of acute intestinal Gvhr. Indeed, IEL had virtually disappeared by day 28, of the Gvhr in B10 → BDF₁ mice, although animals which survived to day 128 had normal levels of IEL. A similar reduction in IEL was seen in irradiated mice with acute Gvhr. The reasons for the disappearance of IEL at the peak of the acute phase of the Gvhr are unclear. Although the accompanying appearance of specific CTL could indicate that infiltrating IEL of host origin are being killed by donor CTL, recent work shows most IEL in irradiated mice with Gvhr are donor derived (Guy-Grand & Vassalli, 1986). Furthermore, a similar decrease in the absolute number of IEL has been reported in the

enteropathy of coeliac disease (Marsh, 1987) and it seems unlikely that this could be due to classical CTL. Therefore, the decline in IEL may merely reflect severe mucosal damage or a generalised defect in immune regulation. It would be important to investigate the origin of infiltrating IEL during different models of Gvhr.

My findings in BDF₁ mice given B10 spleen cells confirm that an acute intestinal Gvhr can be induced in intact adult animals. In addition, my results in this model are very similar to those described by Gleichmann et al. (1984) and indicate that an acute destructive enteropathy accompanies the systemic disease. However, Gleichmann's experiments had also suggested that BDF₁ mice given DBA/2 cells would develop a different form of systemic Gvhr. Therefore, I had been interested to examine whether this strain combination would produce an enteropathy which was associated with a different pattern of anti-host immune responsiveness. This proved not to be the case, as my studies in adult BDF₁ recipients of DBA/2 cells showed virtually no evidence of the chronic, proliferative Gvhr described previously. Although these mice had persistent splenomegaly and elevated NK activity, there were few intestinal lesions with little evidence of crypt hyperplasia and no alterations in IEL counts, or villus atrophy. Furthermore, I was unable to detect autoantibodies against mouse red blood cells, despite previous work suggesting that this model of Gvhr was associated with a SLE-like pattern of auto-antibody production (Gleichmann et al., 1984).

Although these features might suggest that I had merely been unable to induce any Gvhr at all in this model, this possibility was ruled out by the consistent findings of splenomegaly and NK cell activation. The ability of DBA/2 cells to respond to BDF₁ alloantigens in vivo was confirmed by the fact that DBA/2 and B10 donor cells produced a very similar pattern of Gvhr in one day old BDF₁ neonates, although the disease induced by DBA/2 cells was milder, with a delayed onset of runting and less severe villus atrophy. Furthermore, as in one day old (CBA x BALB/c)F₁ mice, both DBA/2 and B10 donor cells induced a small, transient peak of NK activity in neonatal BDF₁ hosts, and this was followed by low levels of specific cytotoxicity, crypt hyperplasia and eventually villus atrophy. In contrast, 7 day old neonatal BDF₁ mice given DBA/2 donor cells revealed the same pattern of defective Gvhr seen in adult mice, despite the ability of B10 cells to induce an acute, lethal Gvhr. Mice of this age given DBA/2 cells had no splenomegaly or elevated NK activity, and the only sign of intestinal Gvhr was a small amount of crypt lengthening. Therefore DBA/2 cells seem to be capable of inducing most features of acute Gvhr, but this requires the use of immunoincompetent hosts.

Together these results suggest that DBA/2 cells do not cause a qualitatively different form of Gvhr, but merely have a generalised defect in their ability to induce a Gvhr. Although this is supported by the fact that B10 cells responded better than DBA/2 cells to BDF₁ stimulator cells in an in vitro MLR, there is also evidence that the defect

in DBA/2 cells may affect some immune functions more than others. Whereas I found that both donor cell types appeared to induce similar levels of anti-host DTH in Gvhr, anti-host CTL activity could be generated in BDF₁ mice given B10, but not DBA/2 donor cells. Recently, it has been shown that the failure of DBA/2 cells to induce a lethal Gvhr in adult BDF₁ mice is due to an absolute reduction in Lyt 2⁺ cells in DBA/2 mice (Via et al., 1987). Furthermore, Sprent has not only confirmed that DBA/2 mice have fewer Lyt 2⁺ cells than B10 mice but has also shown that B10 cells make a much stronger proliferative response to Class I MHC alloantigens than DBA/2 cells (Sprent, unpublished observations). These features suggest that the ability of donor cells to induce an acute Gvhr may depend on their response to Class I MHC, and this will be discussed in more detail below.

A final point of interest from my experiments in BDF₁ mice was that B10 cells produced more severe Gvhr and reconstitute BDF₁ hosts very efficiently, despite the theoretical problem that H-2^b cells should be resisted by F₁ mice (Cudkowicz & Stimpfling, 1964; Shearer & Polisson, 1980, 1981). These findings suggest that F₁ resistance may not be of major importance in the outcome of Gvhr.

In conclusion, these experiments in irradiated or neonatal (CBA x BALB/c)F₁ mice and in adult, unirradiated BDF₁ mice given B10 donor cells show that an acute enteropathy involving villus atrophy can occur in appropriate models of Gvhr. In each case, the destructive intestinal pathology was accompanied

by a systemic runting disease and frequently by the appearance of anti-host CTL activity. These features appeared to reflect a severe Gvhr which was allowed to progress due to host immunoincompetence or because of particularly potent donor cells. The destructive phase was always preceded by a proliferative stage of Gvhr, which was characterised by increased NK activity, as well as crypt hyperplasia and occasionally by increased numbers of IEL. As these initial alterations are essentially identical to the features which constitute the entire Gvhr in adult, unirradiated mice, it seems reasonable to propose that similar mechanisms may be responsible. Nevertheless, the onset of runting and villus atrophy was associated with a sudden transition both in immune effector functions and pathology, with an abrupt loss of NK activity, disappearance of IEL and sometimes cessation of crypt cell mitotic activity. In parallel, there was a concomitant appearance of specific anti-host CTL and active immunosuppression suggesting that different effector mechanisms cause the two aspects of acute Gvhr. The next sections will address some of the factors which determine whether an acute rather than a proliferative Gvhr occurs and will then discuss the potential effector mechanisms in acute enteropathy.

Role of host factors in the outcome of Gvhr - Ability of hosts to resist Gvhr

The importance of the host in determining the consequences of Gvhr is highlighted by the fact that irradiated or 1-2 day old (CBA x BALB/c) F_1 mice developed an acute, destructive Gvhr, whereas older, intact mice of this strain showed only a

proliferative disorder. These features suggested that intact animals possess active mechanisms which normally resist the progress of Gvhr and I examined the role of two particular mechanisms of this type. First, NK cells have been implicated in rejection of parental bone marrow and lymphoid cells by F₁ mice (Kiessling et al., 1977; Warner & Dennert, 1982; Dennert et al., 1985; Waterfall et al., 1987). That NK cells may protect intact hosts from severe Gvhr is supported by the fact that prolonged enhancement of NK cell activity is associated with mild proliferative forms of Gvhr, whereas loss of NK cell activity is one of the first signs of the transition to an acute destructive disorder. Furthermore, resting NK cell activity is absent in both neonatal (Chapter 4; Kiessling et al., 1975; Roder et al., 1981, Koo et al., 1982) and irradiated mice (Cudkowicz & Hochman, 1979), which proved highly susceptible to acute Gvhr. Nevertheless, certain features argue against an essential role for NK cells in protection against acute Gvhr. First, resting NK activity does not become apparent in neonatal mice until the third week of life (Chapter 4; Kiessling et al., 1975; Koo et al., 1982) and this was long after the ability to resist the development of an acute Gvhr develops. The best evidence that NK cells do not protect mice from Gvhr was my demonstration that depletion of NK cells by anti-ASG_{MI} antibody reduced, rather than enhanced the systemic and intestinal Gvhr in adult (CBA x BALB/c)F₁ mice.

The second immune mechanism which may account for the ability of intact hosts to resist Gvhr is host T-cells. Anti-idiotypic host T-lymphocytes can suppress a Gvhr in both rats (Bellgrau & Wilson, 1978; Kimura & Wilson, 1984) and mice (Kosmatopoulous, 1987) and both neonatal and irradiated hosts are markedly defective in T-cell functions. The development of neonatal T-cell activity remains somewhat controversial, but I found some evidence that T-cells from neonatal mice under 5 days old were immunoincompetent, as measured by their ability to induce a local Gvhr in the popliteal lymph nodes of F_1 hosts. This confirms earlier work showing that peripheral lymphocytes from mice less than 3-4 days old have deficient allospecific T-cell activity (Adler et al., 1971; Goldstein et al., 1971). In addition, neonatal mice do not develop the ability to develop T-cell tolerance after feeding antigen until after 4 days of age (Hanson, 1981), while seeding of peripheral lymphoid tissues with thymic T-cells also occurs around this time (Joel et al., 1972). Time did not permit me to perform detailed studies of the ontogeny of T-cell function in neonatal mice, but I did attempt to reconstitute 1 day old neonatal mice with adult spleen cells prior to the induction of a Gvhr. Although, neonatal mice given T-cell enriched adult spleen cells lost weight slightly more slowly than normal mice with Gvhr, this effect was minor and was not observed with unseparated adult cells. However, others have shown that syngeneic adult cells can prevent the induction of a Gvhr in neonatal mice (Billingham, 1967) and it would be important to identify the

cell responsible for these protective effects.

Strong evidence that host T-cells can regulate Gvhr came from the experiments using athymic (CBA x BALB/c) F_1 mice as hosts. Compared with intact (CBA x BALB/c) F_1 mice, nude animals developed a more severe Gvhr, with more intense splenomegaly and crypt hyperplasia. In addition, only the athymic mice showed any evidence of villus atrophy or specific CTL activity and also had suppression rather than enhancement of NK cell activity. Therefore the absence of host T-cells converts a normally proliferative Gvhr into a disease with many of the features of the acute Gvhr which was found in neonatal or irradiated mice of the same strain. This work extends earlier studies which showed that athymic hosts developed an unusually severe systemic Gvhr (Piguet & Vassalli, 1983; Klein et al., 1984) and indicates that a Gvhr in intact unirradiated mice is normally limited by a T-cell mediated host resistance mechanism. More recently, work by Moser et al. (1987) suggests that host Lyt 2⁺ cells may play a role in preventing the development of an acute Gvhr. However, it is unclear whether this is mediated by cytotoxic or suppressor T-cells.

Nature of the anti-host response induced by different types of host

These findings show that a lack of host resistance may be one factor which allows an acute Gvhr to develop in certain types of host. However one further possible explanation for the varying types of Gvhr in different hosts could be that distinct immune mechanisms are activated under those

circumstances. Some evidence for this idea was provided by experiments which showed that one day old neonate (CBA x BALB/c) F_1 mice were deficient in inducing the anti-host DTH reaction normally found in this strain combination. The inability of 1 day old neonatal mice to stimulate an effective anti-host DTH reaction during Gvhr was also reflected by the poor ability of their spleen cells to induce a systemic DTH response in semi-allogeneic adult mice. In contrast, 6 day old neonates, which developed less severe Gvhr induced an anti-host DTH reaction similar to that seen with adult spleen cells. Furthermore, compared with adult spleen cells, 1 day old neonatal BDF $_1$ spleen cells stimulated a smaller MLR response by either DBA/2 or B10 parental cells.

Therefore, the development of a severe, acute Gvhr in neonatal mice appears to be associated with a maturational defect in the ability of host cells to stimulate an effective anti-host DTH response. This seems rather surprising in view of the evidence that a donor cell mediated anti-host DTH response is an important early event in a Gvhr in both irradiated and unirradiated mice (Wolters & Benner, 1978; Mowat et al., 1985). Therefore, the acute Gvhr in 1 day old mice may be due to a different mechanism to the DTH response that is thought to cause a proliferative Gvhr in intact adults (Mowat & Ferguson, 1981b). One explanation for the poor induction of DTH and MLR by neonatal stimulator cells could be low expression of Class II MHC antigens by tissues of neonatal rodents (Mayrhofer et al., 1983; Guy-Grand & Vassalli, 1986). As a result, the acute Gvhr in these animals

could reflect a predominantly Class I MHC restricted reaction which does not induce DTH.

As noted above, an acute Gvhr in adult BDF₁ mice is dependent on the presence of Lyt 2⁺ donor cells and the general implications of these findings will be discussed below.

Influence of tissue susceptibility on the outcome of Gvhr

One final factor which could influence the nature of the Gvhr in different types of host may be the susceptibility of tissues to the pathogenic mechanisms of the Gvhr. Many of the characteristic target organs in Gvhr are those with a high rate of cell renewal, such as the lymphohaemopoietic tissues, skin and gut. Irradiation has profound effects on cell renewal while the neonatal small intestine has an immature pattern of epithelial cell turnover (O'Conner, 1966; Al-Nafussi & Wright, 1982). Therefore, it is possible that these factors combine with the direct consequences of Gvhr to produce an unusually severe Gvhr. However, it should be noted that the time course of maturation of the intestine does not correlate with the age-dependent changes in susceptibility to Gvhr which I found (O'Conner, 1966; Al-Nafussi & Wright, 1982). Furthermore, the fact that a similar pattern of acute Gvhr could be produced in intact, adult BDF₁ mice argues against tissue susceptibility being more than a contributory factor to the outcome of the Gvhr.

Immunological effector mechanisms in acute and proliferative Gvhr
Cellular and genetic basis of different models of Gvhr

Investigations of the immunological basis of allograft rejection and Gvhr have concentrated on determining whether CTL and/or DTH are responsible. Previous work suggested that the proliferative enteropathy found during a Gvhr in unirradiated adult (CBA x BALB/c) F_1 mice was due to DTH (Mowat & Ferguson, 1981b). Therefore, the experiments in Chapter 7 were designed to investigate whether the induction of destructive and proliferative forms of enteropathy in Gvhr required different subsets of donor T-cells or recognition of different MHC alloantigens. In this way it was hoped to identify some of the mechanisms involved. However, my results showed that both $L3T4^+$ and $Lyt\ 2^+$ T-cells were usually required to induce the systemic and intestinal alterations seen in a Gvhr in both irradiated and unirradiated adult mice, as well as in neonatal (CBA x BALB/c) F_1 hosts. Nevertheless, there was some evidence that individual subsets could have some effects alone, under certain circumstances. Thus, both $Lyt\ 2^+$ and $L3T4^+$ T-cells induced some splenomegaly in unirradiated adult (CBA x BALB/c) F_1 recipients, while $Lyt\ 2^-$ donor cells alone were capable of inducing a lethal Gvhr in irradiated mice. In contrast, $L3T4^-$ cells were able to induce a lethal Gvhr in 1 day old neonatal (CBA x BALB/c) F_1 mice and this was accompanied by some splenomegaly and intestinal lesions.

The findings that both $L3T4^+$ and $Lyt\ 2^+$ donor cells were usually required to induce most of the lesions of Gvhr

contrast with earlier studies in rats which showed that $W3/25^+$ ($CD4^+$) T-cells are able to mediate both local and systemic Gvhr reactions (White et al., 1978; Mason, 1981). In addition, previous work in this laboratory showed that $Lyt\ 1^+2^-$ cells were almost entirely responsible for inducing the intestinal phase of Gvhr in adult unirradiated (CBA x BALB/c) F_1 mice (Mowat et al., 1986). Nevertheless, this work also indicated a role for $Lyt\ 2^+$ T-cells in the development of the full crypt alterations, while others have shown that both $Lyt\ 2^+$ and $Lyt\ 2^-$ cells are required to induce mortality (Vallera et al., 1982; Rolink & Gleichmann, 1983) and splenomegaly (Kisielow et al., 1975; Mage et al., 1981) during a systemic Gvhr in unirradiated hosts. One possible reason for discrepancies between different studies is that earlier work in mice employed $Lyt\ 1$ as a marker for helper T-cells. As it is now clear that $Lyt\ 1$ may be expressed on all mouse T-cells (Ledbetter et al., 1981), it is difficult to interpret work using $Lyt\ 1$ -depleted T-cells and more recent studies now employ the $Lyt\ 2$ and $L3T4$ antigens as non-overlapping markers of Class I and Class II restricted T-cells in mice (Dialynas et al., 1983).

Using this approach, more recent studies have shown that both $L3T4^+$ and $Lyt\ 2^+$ T-cells are required for systemic Gvhr across a fully allogeneic disparity (Cobbold et al., 1986), while both T-cells subsets can mediate Gvhr across appropriate MHC loci or minor histocompatibility differences (Korngold & Sprent, 1985; 1987; Sprent et al., 1986a,b). There is only one other study on gut Gvhr using $L3T4$ and

Lyt 2 depleted T-cells. This showed that although both L3T4⁺ and Lyt 2⁺ cells can alone elicit lymphocytic infiltration, crypt hyperplasia and villus damage in irradiated or unirradiated adult or neonatal (C3H x DBA/2)_F₁ mice (Guy-Grand & Vassalli, 1986), far fewer L3T4⁺ cells were required to induce the enteropathy. In contrast, I found that although L3T4⁻ cells were able to induce a lethal Gvhr across a fully mis-matched MHC in one day old neonatal mice, these mice had only minor crypt and villus damage.

My studies did not support previous suggestions that isolated populations of L3T4⁺ and Lyt 2⁺ T-cells could cause distinct forms of Gvhr (Gleichmann et al., 1984; Moser et al., 1987). Nevertheless, a consistent observation was that both L3T4⁻ and Lyt 2⁻ T-cells induced a much greater enhancement of NK cell activity in Gvhr than that found using unseparated donor cells. This was particularly noticeable using Lyt 2⁻ cells and has been noted previously in this laboratory (unpublished observations). The reasons for this are unclear, but could reflect the possibility that recruitment of host NK cells is normally limited by an anti-host mechanism which requires both Lyt 2⁺ and L3T4⁺ donor cells.

The experiments using T-cell depleted populations of donor cells were complimented by examination of the genetic basis of the intestinal and systemic Gvhr in one day old neonatal mice. A Class II MHC difference in (ATH x ATL)_F₁ mice produced an acute lethal Gvhr with marked splenomegaly and crypt lengthening, and some villus shortening but there was no activation of NK cells or CTL. In addition, the onset

of clinical Gvhr was delayed by around 1 week compared with the Gvhr seen across a full MHC incompatibility in neonatal (CBA x BALB/c) F_1 mice, perhaps reflecting the delayed appearance of Ia in mice of this age (Guy-Grand & Vassalli, 1986). Induction of a Gvhr across an isolated Class I disparity did not result in a lethal Gvhr and there was no weight loss or significant splenomegaly. Although the Gvhr across H-2K^q in neonatal (B10.A x B10.AQR) F_1 mice did produce minor enhancement of NK activity and some crypt lengthening, these changes were minor and transient and were not confirmed in the H-2D^q disparate combination. Therefore Class I and II antigens do not induce different forms of Gvhr and indeed Class I alone has virtually no effect.

These findings support previous work in congenic mice, showing that Class II MHC differences are most important for several different aspects of systemic Gvhr (Klein & Chiang, 1976; Klein, 1977; Zinkernagel, 1980; Rolink & Gleichmann, 1983). Furthermore, a Class II MHC incompatibility has been shown to be alone sufficient and necessary for intestinal Gvhr in both irradiated (Piguet, 1985) and unirradiated adult mice (Mowat et al., 1986). Therefore the intestinal phase of Gvhr in both acute and proliferative Gvhr is primarily Class II restricted. Nevertheless, these results contrast with the fact that both Lyt 2⁺ and L3T4⁺ T-cells were required to induce intestinal Gvhr in (CBA x BALB/c) F_1 mice. Indeed L3T4⁻ cells alone induced a lethal Gvhr in one day old (CBA x BALB/c) F_1 mice. This indication that Class I MHC alloantigens may provide the stimulus for Gvhr under certain

circumstances is supported by recent experiments in mice carrying mutations in specified MHC molecules.

These studies have shown that both Class I and Class II MHC restricted T-cells can cause MLR, allograft rejection and systemic Gvhr provided these are stimulated with an appropriate mutant MHC molecule (Sprent & Schaefer, 1985; Sprent et al., 1986a). Very recent studies in MHC mutant mice have shown that Class I and Class II MHC restricted T-cells can also cause intestinal Gvhr under appropriate circumstances, although Class II restricted T-cells were consistently more potent (Mowat & Sprent, manuscript in preparation). Therefore, both Class I and Class II MHC antigens may be inherently capable of inducing similar forms of Gvhr and as both Class I and II restricted T-cells can mediate CTL activity or DTH (Golding & Singer, 1985; Mizouchi et al., 1985; Rosenberg et al., 1986; Sprent & Webb, 1987), it is probably impossible to define different pathogenic mechanisms purely on the basis of the phenotype or MHC restriction of the T-cells responsible.

Therefore, the remaining parts of the thesis were designed to investigate the role of different effector mechanisms which were present in animals undergoing a Gvhr and to correlate these with the intestinal pathology.

Effector mechanisms in proliferative and destructive Gvhr

Previous studies had shown that anti-host CTL could never be detected during the early proliferative Gvhr in adult, unirradiated (CBA x BALB/c) F_1 mice, supporting the

view that a DTH response was responsible for the resulting crypt hyperplasia (Borland et al., 1983). However, my experiments have provided evidence that two distinct phases of enteropathy can be induced in certain models of Gvhr and these were associated with different cell mediated effector mechanisms. Specific CTL were not detectable during the entirely proliferative Gvhr which occurred in older neonates or during the early proliferative phase which characterised all models of acute Gvhr. However, CTL activity was present when an acute Gvhr developed in one day old or irradiated (CBA x BALB/c)_{F₁} mice or in adult BDF₁ mice given B10 donor cells. Therefore, the simplest interpretation of these findings is that while a DTH response may be responsible for proliferative alterations such as splenomegaly, crypt hyperplasia and NK cell activation, specific CTL are required to produce the more severe features of Gvhr, including villus atrophy. Nevertheless, others have shown that the presence of CTL does not necessarily correlate with the development of a lethal Gvhr (Van Elven et al., 1981; Jadus & Peck, 1983; Hamilton, 1984), while I found that villus atrophy and runting occurred in 5 day old neonatal (CBA x BALB/c)_{F₁} mice with Gvhr, despite the absence of CTL. Furthermore, as discussed below, the ability of both poly I:C and dGuo to modify the systemic and intestinal consequences of Gvhr did not correlate with their effects on CTL activity. Finally, it has been shown recently that during a Gvhr in irradiated hosts, villus atrophy occurs in grafts of foetal small intestine which are syngeneic to the donor cells (Mowat et al.,

1988) indicate that specific anti-host CTL are not essential for the development of villus atrophy. Thus, although it is impossible to formally exclude some pathogenic role for CTL in intestinal Gvhr, other mechanisms must be considered.

Activation of NK cells was found in virtually all my experiments and has been noted in many previous studies of Gvhr (Dokhelar et al., 1981; Roy et al., 1982; Borland et al., 1983; Kubota et al., 1983; Varkila & Hurme, 1985). I therefore examined the role of NK cells in Gvhr by depleting host mice of NK cells by treatment in vivo with anti-asialo G_{M1} antibody. As shown by others, this protocol resulted in profound depletion of resting NK cell activity in control mice (Habu et al., 1981; Charley et al., 1983; Stitz et al., 1986). Although a Gvhr produced some increase in NK activity in these mice, the NK levels did not even approach those found in control untreated mice. Furthermore, in comparison with normal (CBA x BALB/c)F₁ mice, anti-asialo treated mice with Gvhr had consistently less splenomegaly and did not develop the crypt lengthening and increased CCPR that characterize the intestinal lesions of this model of Gvhr. These findings confirm and extend other reports that anti-AsG_{M1} treatment prevents the development of systemic, lethal Gvhr across minor and major histocompatibility differences in irradiated mice (Charley et al., 1983; Varkila, 1987). In addition, my results suggest that AsG_{M1}+ cells are an essential component of the local immune response which causes the intestinal crypt hyperplasia in Gvhr. Interestingly, although a Gvhr in anti-asialo treated hosts still induced an increase in IEL counts,

control mice given anti-AsG_{M1} had a small, but significant reduction in IEL count, which was roughly proportional to the small number of IEL which have been shown to be AsG_{M1}⁺ (Flexman et al., 1983; Carman et al., 1986). It is tempting to speculate that AsG_{M1}⁺ IEL are involved in inducing a proliferative enteropathy during Gvhr.

Those experiments were interpreted on the basis of previous findings that anti-AsG_{M1} antibody has a selective effect on NK cells (Kasai et al., 1980) and I showed that anti-AsG_{M1} treated mice generated normal allospecific DTH responses. However, AsG_{M1} has also been found to be expressed on other lymphoid cells including T-lymphocytes (Stein et al., 1978; Nakano et al., 1980; Akagawa et al., 1981; Beck et al., 1982; Suttles et al., 1986) and the majority of Lyt 2⁺ CTL clones (Ting et al., 1986). Furthermore, my findings that anti-AsG_{M1} treatment prevented the generation of specific CTL in alloimmunised mice have been confirmed both in virus-infected mice (Stitz et al., 1986) and during a Gvhr in irradiated mice (Varkila, 1987). Although it is unclear whether effector CTL themselves express AsG_{M1}, it is difficult to completely exclude the possibility that the ability of anti AsG_{M1} to inhibit Gvhr may not be due to an effect on CTL activity. However, it should be noted that the model of Gvhr which I used was chosen because it does not involve CTL. Furthermore, AsG_{M1} is expressed in much higher concentrations on NK cells than on T-cells and this antibody eliminates NK cells more readily in vivo and in vitro (Kasai et al., 1980; Suttles et al., 1986; Stitz et al., 1986).

Although it would be important to confirm the effects of treating mice with anti-AsG_{M1}, using NK cell specific antibodies such as anti-NK.1, my studies using poly I:C supported the view that NK cells may be important effector cells in Gvhr. Because of its ability to induce production of IFN (Gresser et al., 1978; Korngold et al., 1983), poly I:C is a potent augmentor of NK activity (Field et al., 1967; Oehler et al., 1978; Oehler & Herberman, 1978; Djeu et al., 1979). This was confirmed by the fact that treatment of one day old neonatal (CBA x BALB/c)_{F1} mice with poly I:C induced the appearance of some NK activity which did not normally appear in these very young mice until much later. Furthermore, poly I:C led to more marked enhancement of NK cell activity in one day old mice with Gvhr and, compared with untreated mice with Gvhr, poly I:C treated animals developed more marked weight loss and more severe intestinal damage, which included both villus atrophy and crypt hyperplasia. In parallel, poly I:C treated mice actually generated lower levels of specific anti-host cytotoxicity during a Gvhr, confirming other work that poly I:C does not activate CTL in vivo (Biron et al., 1987).

Poly I:C had no significant effect on the growth of control mice, but some evidence was obtained that poly I:C could produce a degree of crypt lengthening and villus shortening. These findings are consistent with the idea that non-specific activation of NK cells can result in intestinal damage. Although this could not be confirmed by transferring poly I:C activated adult spleen cells into syngeneic neonatal mice,

this may have reflected the inability of NK cells to migrate to the small intestine (Rolstad et al., 1986). The possibility that poly I:C activated effector mechanisms may be important in the pathogenesis of Gvhr are supported by the findings that increased levels of serum α/β interferon correlate well with the development of lethal Gvhd in irradiated mice (Reyes & Kimpel, 1987). Nevertheless, it has been reported that poly I:C does not affect the outcome of a Gvhr in unirradiated (CBA x C57Bl/6) F_1 mice (Varkila & Hurme, 1985) while others have found that poly I:C actually inhibited the development of immunosuppression and tissue damage during a Gvhr in (B6 x A) F_1 mice (Peres et al., 1986a). The discrepancies between this result and my own may reflect the fact that the other study used B6 donor cells, which are highly susceptible to F_1 resistance (Fox & Howard, 1963; Shearer & Polisson, 1980, 1981). Under these circumstances, activation of host NK cells might be expected to increase the ability of F_1 hosts to resist the B6 donor cells (Peres et al., 1986b). In contrast, (CBA x BALB/c) F_1 mice show no resistance to CBA cells (Mowat et al., 1985) and so in my experiments, NK cell activation may only play an effector role. Nevertheless, those findings underline the need to confirm the effects of poly I:C on several different models of Gvhr. In addition, the exact effects of poly I:C and its mediators require extensive investigation, as it has been suggested that both poly I:C and α/β interferon may affect other limbs of the immune system, including antibody production and DTH (Abruzzo & Rowley, 1983; Shah et al., 1985).

If confirmed, these results with anti AsG_{M1} and poly I:C provide some evidence that NK cells may be involved as effector cells in damage to the intestine and other tissues during a Gvhr. These findings are supported by the presence of NK cell infiltrates in rejecting allografts (Nemlander et al., 1986) and by the fact that depletion of NK cells inhibits the induction of autoimmune diabetes in rats (Like et al., 1986).

The nature of the NK cell involved in intestinal Gvhr and the mechanisms by which NK cells could damage the intestine are unclear. Previous studies have shown that the activation of NK cells in unirradiated (CBA x BALB/c)F₁ mice with Gvhr is preceded by the anti-host DTH response (Borland et al., 1983; Mowat et al., 1985). Under these circumstances, the NK activity of intestinal IEL is also enhanced and this parallels the other features of intestinal pathology. As noted above, the ability of anti-AsG_{M1} to prevent intestinal Gvhr is associated with a small decrease in IEL count and together, these features suggest that activation of epithelial NK cells by a local DTH response might contribute to the intestinal pathology in Gvhr. The pathogenic role of NK cells could of course involve nonspecific cytotoxic damage to the intestine. Nevertheless, NK cells are capable of a wide range of other immunological functions, including production of lymphokines such as IL-2 and γ IFN and these activities could also contribute to the mucosal lesions of Gvhr (Handa et al., 1983; Kashara et al., 1983).

One point which argues against an essential role for NK cells in Gvhr, was my finding that although recipients of L3T4⁻ or Lyt 2⁻ cells had significantly elevated levels of NK activity, this occurred in the absence of significant intestinal pathology. Furthermore, the evidence linking NK cells to intestinal Gvhr mainly concerns the proliferative form of enteropathy, and my experiments showed clearly that the transition to a destructive disease was accompanied by a loss of NK cell activity. Therefore, it is necessary to consider the possibility that other mechanisms may be responsible for the more severe intestinal alterations such as villus atrophy.

Previous studies of the acute Gvhr found in BDF₁ mice given B10 cells concluded that specific CTL were not responsible, but suggested that allospecific T_s caused the pathological lesions in the lymphohaemopoetic system (Gleichmann et al., 1984). As this model of Gvhr was one which produced villus atrophy, it was of interest to consider a possible role for T_s in intestinal Gvhr. This idea was supported by the presence of active suppressor cell activity in the spleen of adult BDF₁ and neonatal (CBA x BALB/c)F₁ mice with acute Gvhr. Furthermore, there was evidence of suppression of NK activity and CCPR in the transition from a proliferative to a destructive Gvhr. Although this phenomenon requires more detailed investigation and confirmation in more models of Gvhr, it provides circumstantial evidence that an acute Gvhr is associated with T_s.

This idea is also supported by experiments which examined the effects of dGuo on the outcome of an acute Gvhr. Previous studies have shown that treatment of mice with dGuo prevents the generation of specific Ts, without affecting effector or helper T-cell development (Dosch et al., 1980; Varey et al., 1983; Brill et al., 1984, 1985). My own experiments also showed that dGuo did not alter the generation of allospecific effector functions such as CTL or DTH. However, I obtained preliminary evidence that dGuo did prevent the activation of suppressor cells which normally occurred in BDF₁ mice given B10 cells. In parallel, dGuo reduced the mortality and runting during an acute Gvhr in adult BDF₁ mice. Although dGuo did not prevent the development of villus atrophy in this model, it did delay the onset of this feature. In contrast, dGuo had few effects on systemic Gvhr in one day old neonatal (CBA x BALB/c)F₁ mice, but prevented the development of villus atrophy found in untreated littermates with Gvhr. In parallel, dGuo appeared to increase the proliferative changes of Gvhr, as measured by crypt hyperplasia. dGuo had no effects on the generation of either NK cell or CTL activity in Gvhr and did not itself produce any intestinal damage. Thus, although dGuo had somewhat different effects on these two models of Gvhr, each model gave some support to the idea that T_s play a role in the development of acute systemic and intestinal Gvhr.

The reasons for the different effects of dGuo on the Gvhr in adult and neonatal mice are not clear, but it could be because T_s play different roles in these models of acute

Gvhr. Alternatively, I may not have adjusted the dose of dGuo correctly for adult and neonatal mice, or there may be age-dependent differences in susceptibility to dGuo.

Therefore, the effects of dGuo on each of these models requires further examination and it would also be important to examine in more detail, the effects of dGuo on immune cells other than T_s . Nevertheless, in subsequent experiments, dGuo has always had clear effects on the development of acute Gvhr (personal observation) and dGuo has never been shown to interfere with immune effector cells in the doses used here.

Finally, the ability of T_s to affect the intestinal mucosa was supported by experiments in neonatal mice with a Gvhr induced across the I-J locus. Despite the current controversy over the nature of the I-J gene and its product (Murphy, 1987), induction of a Gvhr across a difference at I-J, has been shown previously to activate T_s in vivo (Zinkernagel, 1980; Bromberg et al., 1981). In my studies, neonatal (B10.A3R x B10.A5R) F_1 mice given B10.A3R donor cells had no evidence of clinical Gvhr or splenomegaly, but these mice did develop some villus atrophy. These findings are supported by the suppressed IEL counts, crypt lengths and CCPR during a Gvhr across I-J in adult, unirradiated mice (Mowat et al., 1986) and indicate the potential ability of T_s to modulate epithelial cell renewal in vivo. However, it should be noted that the villus atrophy found in neonatal mice with a I-J restricted Gvhr was associated with crypt atrophy and an absence of the inflammatory cell infiltrate normally found when villus atrophy is present in Gvhr. Therefore,

this form of villus damage may be due to different mechanisms. Nevertheless, it is tempting to speculate that activation of T_s during a severe, progressive Gvhr contributes to the inhibition of CCPR and NK cell activation seen during the transition from a proliferative Gvhr to a destructive disorder. Modulation of epithelial cell renewal in this way might then contribute to the villus damage.

Conclusions

The work in this thesis has used three separate models of acute Gvhr to try and define the mechanisms responsible for the development of villus atrophy. Previous work in unirradiated mice suggested that DTH is responsible for the crypt hyperplasia and NK cell activation (Mowat & Ferguson, 1981b, 1982; Borland et al., 1983) and the work here showed that similar features preceded the development of destructive enteropathy. This was found in models of Gvhr when immunoincompetent hosts or very potent donor cells were used. Therefore it is possible that villus atrophy is the result of a more progressive form of DTH response, as has been suggested previously (MacDonald & Ferguson, 1977). Furthermore, this is supported by the incomplete correlation of CTL activity with villus atrophy and recently, it has been shown that the development of mucosal damage in mice with acute Gvhd can be prevented by treatment with anti-tumour necrosis factor (Piguet et al., 1987) and anti- γ interferon (Mowat, unpublished observations). Therefore villus atrophy could be due to the prolonged release of high levels of lymphokines,

which in smaller amounts cause crypt hyperplasia. This is supported by the fact that γ -interferon may be stimulatory as well as inhibitory (Trinchieri & Perussia, 1985).

Alternatively, this could be due to the cumulative effects of different lymphokines being produced at various stages of the Gvhr.

It is clear that much more work examining these problems is required and this may be pursued by measuring the production and level of lymphokines during Gvhr induced enteropathy or by the direct effects on the small intestine of in vivo treatment with lymphokines. Examination of the phenotype of T-cells responsible for the mucosal damage did not provide any conclusive information, but agents which are said to discriminate effector cell functions, for example leucyl-leucine methyl ester (Thiele et al., 1987), may be more useful. Finally, the most direct approach may be to study the effects of purified lymphokines and, or effector cells on intestinal organ cultures or epithelial cells in vitro.

The clinical relevance of this work is that the villus atrophy, crypt hyperplasia and lymphocytic infiltration reproduces many of the mucosal alterations found during FSE. Therefore, it is important to pursue these in vivo studies of the evolution of enteropathy, although the relevance of Gvhr as a suitable model may require further examination. This is highlighted by the apparent reduction in CCPR and loss of IEL seen during the destructive phase of Gvhr, which are not found in FSE. Nevertheless, it would be useful to be able to make use of some of the features of my work on Gvhr, to attempt to produce a similar, acute enteropathy in animals fed protein antigens.

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Appendix:

As the two models of adult unirradiated mice which developed different forms of Gvhr, received donor spleen cells by different routes, it was necessary to examine whether this alone may contribute to the differences observed.

A Gvhr was induced in adult unirradiated (CBAxBALB/c) F_1 mice with 6×10^7 CBA spleen cells, which were given either intravenously or intraperitoneally. Differences in mortality, splenomegaly and cytotoxicity were measured at intervals thereafter.

Results

Progress of systemic Gvhr in adult (CBAXBALB/c)F₁ mice

Both groups of (CBAXBALB/c)F₁ mice with Gvhr were inspected at regular intervals and no clinical signs of Gvhr were seen at any time and none of the mice died.

Mice from each group were sacrificed on days 1,4,8,12 and 26 and a significant spleen index was present in the Gvhr mice which received donor cells intravenously, by day 4 (Table 1 APP), and by day 8 significant splenomegaly had developed in both groups of mice with Gvhr (1.34±0.10 and 1.63±0.14 in ip and iv Gvhr mice respectively). Significant splenomegaly was found in both groups of mice with Gvhr until day 26 but this was always greater in mice given donor cells iv.

Development of specific and non-specific cytotoxicity

Only very low levels of specific cytotoxicity were found in (CBAXBALB/c)F₁ mice with Gvhr at all times, and there was no difference between the two groups (Table 1 APP). In contrast, increased levels of NK cell activity were found in mice with Gvhr. These were relatively modest in the ip injected group, but the increase appeared sooner and was noticeably greater in mice given donor cells intravenously. Nevertheless, by day 12 NK cell activity in both groups of Gvhr mice were similar (31% and 32% in ip and iv mice respectively vs 18% in controls).

Although the induction of a Gvhr in (CBAXBALB/c)F₁ mice with 6×10^7 CBA spleen cells iv, produced more severe systemic proliferative changes than ip injected mice, these did not correlate with the development of an acute lethal Gvhr. Therefore the difference in the type of Gvhr which developed in adult unirradiated (CBAXBALB/c)F₁ mice and adult unirradiated BDF₁ mice was not due to the method of inoculating cells.

	<u>Day of Gvhr</u>	<u>Control</u>	<u>Gvhr</u>	
			<u>intraperitoneal</u>	<u>intravenous</u>
Spleen index	1	-	1.03±0.06	1.15±0.15
	4	-	1.18±0.08	1.76±0.36
	8	-	1.34±0.10	1.63±0.14
	12	-	1.30±0.02	2.12±0.5
	26	-	1.37±0.3	1.58±0.1
CTL	1	-	0%	0%
	4	-	4%	1.2%
	8	-	4.2%	3.6%
	12	-	0%	2.6%
	26	-	0%	0%
NK	1	32%	30%	51%
	4	14%	20%	29%
	8	42.4%	44%	82%
	12	18%	31%	32%
	26	9.4%	16.7%	14%

Table 1 APP: Assessment of systemic Gvhr in adult (CBAxBALB/c)_{F₁} mice given 6×10^7 CBA spleen cells, either ip or iv. Development of splenomegaly and cytotoxicity in mice with Gvhr and in controls. Results shown are means (\pm 1 s.d. for spleen indices) for 3 mice/group.

