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ROLE OF ENDOGENOUS GLUCOCORTICOIDS DURING MURINE GRAFT-VERSUS-HOST DISEASE

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of Ph.D.

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ABBREVIATIONS

ACTH:	adrenocorticotropin hormone
ADX:	adrenalectomized
ASGM1:	asialoGM1
BMT:	bone marrow transplantation
CRH:	corticotropin releasing hormone
GVHD:	graft-versus-host disease
GVHR:	graft-versus-host reaction
HLA:	human leucocyte antigens
HPA:	hypothalamus-pituitary-adrenal
IFN-γ:	interferon-γ
IL-:	interleukin-
LN:	lymph nodes
LPS:	lipopolysaccharide
MHC:	major histocompatibility complex
NDV:	Newcastle Disease Virus
non-ADX:	non-adrenalectomized
PLC:	parental lymphoid cells
POMC:	proopiomelanocortin
TCDBM:	T cell depleted bone marrow
TNF-α:	tumour necrosis factor-α

ABSTRACT

The studies presented in this thesis investigated the mechanism responsible for glucococorticoid secretion during graft-versus-host disease (GVHD), and the role of endogenous glucocorticoids on the outcome of the disease. GVHD was induced in unirradiated F1 hybrid mice by an intravenous injection of parental lymphoid cells. Our results demonstrated that the secretion of glucocorticoids during GVHD was independent of pituitary adrenocorticotropin hormone (ACTH). However, adrenal hyperactivity was associated with increased expression of proopiomelanocortin (POMC) mRNA in the adrenal glands of GVHD mice. Expression of adrenal POMC transcripts was not due to mononuclear infiltrates. The transcripts for interleukin-12, a cytokine produced by activated macrophages, were also upregulated in GVHD adrenals. Since macrophages have been shown to reside in the adrenal glands and produce ACTH, it appeared that resident adrenal macrophages were activated during GVHD to produce local ACTH that stimulated the secretion of glucocorticoids, independent of pituitary ACTH.

We next investigated the role of endogenous glucocorticoids on the outcome of GVHD by adrenalectomizing the F1 recipient mice before GVHD induction in order to deplete the source of glucocorticoids. Our results showed that adrenalectomized (ADX), but not non-ADX, F1 recipients

injected with parental lymphoid cells recovered rapidly from symptoms characteristic of GVHD, after a two week manifestation of the disease. Recovery from GVHD was attributed to the induction of a glucocorticoid sensitive, asialoGM1$^+$ and/or CD8$^+$, but not NK1.1$^+$, F1-anti-parental effector cell that rejected or eliminated the parental graft, after an initial period of engraftment. In addition, the effector was not dependent on a mature thymus and was not renewed after anti-asialoGM1 treatment, but was renewed after glucocorticoid treatment.

We further demonstrated that high levels of glucocorticoids during GVHD caused severe deficiency of host T cell populations in the lymph nodes and contributed to the suppression of lymph nodes T cells. Taken together these studies suggest that endogenous glucocorticoids play a central role in the pathogenesis of GVHD.

RÉSUMÉ

Les études présentées dans cette thèse traitent du mécanisme responsable de la sécrétion de glucocorticoïdes durant la maladie du greffon contre l' hôte (MGCH), ainsi que de l' impact qu' ont les glucocorticoïdes endogènes sur la maladie. La MGCH a été induite chez des souris hybrides F1 non-irradiées par injection intraveineuse de cellules lymphoïdes parentales. Nos résultats ont démontré que la sécrétion de glucocorticoïdes pendant la MGCH a été indépendante de la corticotrophine hypophysaire. Cependant, l' hyperactivité surrénale a été associée à l' augmentation de l' expression d' acide ribonucléique messager (ARNm) de la pro-opiomélanocortine (POMC) dans les glandes surrénales des souris atteintes de la MGCH. L' expression des transcripts surrénaux de POMC n' a pas été due à des infiltrats mononucléaires. Les transcripts de l' interleukine-12, une cytokine produite par des macrophages activés, ont aussi été trouvé en quantité plus abondante dans les surrénales de souris malades de la MGCH. Puisque la présence de macrophages résidents dans les glandes surrénales pouvant engendrer de la corticostimuline a été démontrée, il apparaît que ces derniers aient été activés pendant la MGCH pour produire localement de la corticotrophine stimulant ainsi la sécrétion de glucocorticoïdes, indépendemment de la corticostimuline hypophysaire.

Nous avons ensuite étudié le rôle des glucocorticoïdes endogènes dans

la finalité de la MGCH en surrénalectomisant les souris receveuses F1 avant l' induction de la maladie, afin d' éliminer la source de glucocorticoïdes. Nos résultats ont montré que les receveurs F1 surrénalectomisés (SRX) mais non les non-SRX, injectés avec des cellules parentales lymphoïdes, ont recouvré plus rapidement des symptômes caractéristiques de la MGCH, après les deux premières semaines de la maladie. La récupération lors de la MGCH a été attribuée à l' induction d' une cellule effectrice sensible aux glucocorticoïdes, asialoGM1$^+$, et/ou CD8$^+$, mais non NK1.1$^+$, qui rejette ou élimine la greffe parentale, après une période initiale de prise de la greffe. En plus, l' effecteur n' a pas été dépendant d' un thymus mature et n' a pas été renouvelé après un traitement à l' anti-asialoGM1, mais a été renouvelé après traitement aux glucocorticoïdes.

Nous avons ensuite démontré que de hauts niveaux de glucocorticoïdes durant le MGCH ont causé une sévère déficience des populations de cellules T de l' hôte dans les ganglions lymphatiques et ont contribué à la suppression des cellules T ganglionnaires. Dans leur ensemble, ces études suggèrent que les glucocorticoïdes endogènes jouent un rôle central dans la pathogenèse de la MGCH.

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To

my parents

for their courage and sacrifices

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STATEMENTS OF CONTRIBUTION TO ORIGINAL KNOWLEDGE

1. Persistent elevated levels of plasma glucocorticoids during GVHD is independent of pituitary ACTH.

2. Increased levels of plasma glucocorticoids correlate with increased expression of POMC transcripts in adrenal glands of GVHD mice.

3. The nucleotide sequence of the adrenal POMC transcripts is identical to the pituitary POMC.

4. Increased expression of POMC transcripts in GVHD adrenals is not due to mononuclear infiltrates.

5. Transcripts for IL-12, a cytokine produced by activated macrophages, are also upregulated in adrenals of GVHD mice.

6. ADX, but not non-ADX, B6AF1 mice injected with $20x10^6$ A strain PLC recover rapidly from GVHD symptoms, after an initial two week manifestation of GVHD.

7. Recovery from GVHD is mediated by the induction of an F1-anti-parental mechanism that rejects or eliminates the parental graft, after an initial period of engraftment.

8. The F1-anti-parental mechanism is mediated by an effector cell of host origin that is sensitive to glucocorticoids.

9. The F1-anti-parental effector cell is $ASGM1^+$ and/or $CD8^+$, but not $NK1.1^+$.

10. The F1-anti-parental effector cell is not dependent on a mature thymus and is non-renewable when treated with anti-ASGM1 antibody, but is renewable after glucocorticoid treatment.

11. High levels of endogenous glucocorticoids during GVHD are responsible for the severe deficiency of host T cell populations in the LN of GVHD mice since adrenalectomy before GVHD induction protects against the severe loss of host LN T cell populations.

12. High levels of endogenous glucocorticoids play an important role in suppressing T cell functions in the LN of GVHD mice since adrenalectomy improves LN T cell functions.

CHAPTER 1.

INTRODUCTION

A. GRAFT-VERSUS-HOST DISEASE

1. Brief history of graft-versus-host reaction

The phenomenon of the graft-versus-host reaction (GVHR) was first observed by Murphy in 1916 when he transferred adult chicken splenic cells into chick embryos and observed a marked enlargement of the spleen (ie. splenomegaly) in the newborn chicks 10 days after the transplant (1). In retrospect, splenomegaly became one of the hallmarks of GVHR where it is mediated by a GVHR as a result of the immunocompetent graft reacting against the immature recipients. However, splenomegaly was dismissed by Murphy as a phenomenon of organ-specific growth, a concept endorsed at that time (1).

The concept of GVHR was first introduced in early 1950's by Simonsen (2) and Dempster (3). Both investigators observed independently cells infiltrating the cortex of allorenal grafts and mistakenly interpreted the infiltrates as host cells that entered the cortex and were immunologically attacked by donor cells residing in the renal graft. This immunological reaction was erroneously described as a GVHR, although the true interpretation of the results was a standard host-versus-graft reaction where host lymphoid cells were attacking the renal allograft. In 1957,

Simonsen inoculated adult chicken blood cells into outbred embryonic chicks to induce tolerance, but instead induced a lethal disease in many chicks (4). At the same time, Billingham and Brent also observed high mortality in some strains of newborn mice that were previously injected with adult allogeneic splenic cells (5, 6). Both groups of investigators independently suggested that the recipients died from an immunological disease inflicted by the grafted cells reacting against the hosts; hence the concept of GVHR emerged. The disease was termed "runt disease" because the recipients experience symptoms of growth retardation (ie. severely underweight and markedly undersized) and diarrhea.

Similar characteristics of a GVHR were described by other investigators using different animal models. Mice rescued with allogeneic bone marrow after lethal irradiation developped a wasting syndrome that was lethal (7-9). It was postulated that this syndrome was due to a GVHR and was called "secondary disease" or "homologous disease". In addition, injection of parental lymphoid cells into non-irradiated adult F1 hybrid recipients also led to a lethal wasting disease that was termed "F1 hybrid disease" (10-12). The latter disease was mediated by the parental graft reacting against histocompatibility antigens of the other parent haplotype of the F1 hybrid recipient.

Although all of the diseases described above are induced in different animal models, they are all caused by a GVHR as a consequence of the graft reacting against the recipient. In 1962 Simonsen defined three criteria that are necessary to induce a GVHR (13). These are [1] the graft contains immunocompetent cells, [2] antigen disparity between the graft and the host, and [3] the host is incapable of rejecting the graft. GVHR in animal models is characterized by many symptoms including splenomegaly (14, 15), severe immunosuppression of T and B cells (16-18), histopathological injury to many lymphoid and non-lymphoid organs (19-22), weight loss (10-12), diarrhea (10-12), morbidity and mortality (23, 24). The severity of GVHR and the extent of the symptoms depend on several factors: [1] donor cell dose, [2] age of the host, and [3] degree of antigen disparity between donor and host. The symptoms of GVHR are commonly observed after human bone marrow transplantation (BMT), particularly, after allogenic BMT, and clinical GVHR is termed graft-versus-host disease (GVHD) (25-27). Over the years GVHD has also been employed to describe GVHR in animal models. Despite the wealth of knowledge that has been accumulated over 30 years, GVHD still remains a major complication of BMT, and the cellular and molecular mechanisms responsible for the disease have not been fully elucidated.

2. Experimental models of GVHD

The availability of inbred mice has become an invaluable tool for immunologists to study GVHD. Two principal murine models have been studied to provide insight into the mechanisms of GVHD: an irradiated and non-irradiated model.

In the first model, the recipient is irradiated which destroys the immune system prior to reconstitution with either allogenic or semiallogenic bone marrow (28, 29). This model mimics the clinical setting where the recipient is also irradiated to eliminate the malignant cells, however, at the cost of compromising all hematopoiesis including the immune system that requires reconstitution with bone marrow (30). Irradiation of the recipient prior to BMT renders the host immunoincompetent and thus the recipient is unable to reject the graft that immunologically reacts against the recipient's foreign antigens (ie. histocompatibility antigens) to cause GVHD.

Although the irradiated model has been extensively employed by many investigators, the effects of the irradiation alone complicate the underlying mechanisms of GVHD. For these reasons, other investigators and our own laboratory have employed another model that excludes irradiation. This model is known as the Parental into F1 (P $\rightarrow$ F1) hybrid system. This

system consists of injecting parental lymphoid cells (ie. spleen and lymph node cells) into a non-irradiated F1 hybrid recipient (10-12). Because histocompatibility antigens of the F1 hybrid recipient are co-dominantly expressed (31), lymphoid cells from either parent injected into the F1 recipient are normally accepted as self. However, the parental lymphoid graft recognizes histocompatibility antigens of the other parental haplotype as foreign (ie. non-self) and mounts an immunological reaction, leading to GVHD. In our laboratory, the P → F1 model is employed to study strictly the mechanisms underlying GVHD without the complications of irradiation. Manifestation of GVHD in the P → F1 model parallels those of the clinical disease (16-21, 25-27). Furthermore, the severity of GVHD can be controlled by the genetic disparity between the parental graft and F1 recipient, parental cell dose, and age of the recipient. Induction of GVHD in this model can be acute with symptoms of severe immunosuppression, histopathological lesions to lymphoid and non-lymphoid organs, weight loss, diarrhea, hunched posture, piloerection, followed by mortality (16-21). A less severe form of acute GVHD can also be induced where the animals survived but displayed tissue injury and long term immunosuppression (18, 20, 21, 24, 26). Lastly, in certain parental into F1 combinations, chronic or autoimmune GVHD is induced as characterized by B cell hyperactivity and autoreactive antibodies (32, 33).

3. Pathogenesis of GVHD

3.1. Role of T cells in initiating GVHD

Mature T cells are normally identified based on the expression of cell surface molecules, CD4 and CD8 (34). $CD4^+$ cells, also known as T-helper (Th) cells, have further been distinguished as Th1 or Th2, based on the secretion of specific cytokines (35, 36). Th1 cells secrete predominantly interferon-γ (IFN-γ) and interleukin-2 (IL-2), in contrast to IL-4, IL-5 and IL-10 secretion by Th2 cells (35, 36). $CD8^+$ cells normally display cytotoxic or suppressor functions (37), but they may also secrete IL-2 (38, 39). T cells recognize antigen in association with self-major histocompatibility complex (ie. H-2) molecules (34, 40). $CD8^+$ cells recognize antigen in the context of H-2 class I molecules, whereas, $CD4^+$ cells recognize antigen in association with H-2 class II molecules. In addition, $CD8^+$ and $CD4^+$ cells respond to allogenic class I and II molecules, respectively.

There is no doubt that mature T cells present in the bone marrow or lymphoid cell graft are critical in inducing GVHD since elimination of mature T cells from the graft prevents GVHD (41, 42). The exact T cell subset(s) involved in inducing GVHD depends largely on the histocompatibility antigen disparity between the donor and recipient. For

transplants made across a full MHC (ie. class I and II), donor $CD4^+$ and $CD8^+$ T cells are each capable of causing GVHD, and both subsets together synergize to induce a more severe disease (43, 44). GVHD induced across MHC class II differences only requires donor $CD4^+$ cells (45). Clones isolated from MHC class II disparate mixed lymphocyte reaction and injected into recipient expressing the appropriate class II haplotype have been shown to cause GVHD (46, 47). In donor and recipient combinations of MHC class I differences, donor $CD8^+$ cells induce GVHD without the participation of $CD4^+$ cells since purified donor $CD8^+$ cells, in the absence of donor $CD4^+$ cells, are able to cause GVHD (48, 49). However, the intensity of the disease is increased when donor $CD4^+$ cells are present in the graft to potentiate the function of donor $CD8^+$ cells, despite no differences in MHC class II molecules (48, 49). Furthermore, triggering of endogenous viruses such as herpes simplex, cytomegalovirus, and varicella zoster during GVHD (50-52) can activate host $CD4^+$ cells which in turn potentiate the function of donor $CD8^+$ cells, resulting in a more severe GVHD (53). GVHD across minor histocompatibility differences is induced mostly by donor $CD8^+$ cells (54), although $CD4^+$ cells do play a role (55).

3.2. Lymphoproliferative phase of GVHD

The injection of bone marrow or lymphoid cells containing mature T cells into an allogenic or semiallogenic recipient leads to the activation and proliferation of donor T cells. Since alloreactive T cells are normally present at a relatively high frequency (56), recognition of the H-2 alloantigens expressed on the recipient's tissue causes clonal expansion and proliferation of the alloreactive T cells. Within two days after engraftment, donor T cells in the spleen undergo massive proliferation, peaking by the second week of GVHD induction (57-59). At the same time host T cells are also increased (57). Although the mechanism of host T cell proliferation has not been determined, it is postulated that activation of endogenous viruses during GVHD and cytokine production by activated donor T cells may contribute to increased host T cell proliferation (50-52, 57). The marked increase of both donor and host T cells can account for up to 50% of the total cells in the spleen (57). In the lymph nodes (LN), early activation of donor $CD4^+$ cells leads to a massive expansion of host B cells which is largely responsible for the enlargement of the LN during early GVHD (60, 61). It is postulated that donor $CD4^+$ cells interact with the MHC class II alloantigens presented by the host B cells, and thus are activated to produce growth factors that cause proliferation of host B cells in the LN. Following the brief period of lymphoproliferation, both donor and host T cell numbers decrease dramatically and GVHD is accompanied by severe T and B cell immunosuppression (16-18) and severe hypoplasia

of the entire lympho-hematopoietic tissue (58, 62, 63).

3.3. Cytokine production during GVHD

In response to alloantigens donor $CD4^+$ T cells are activated to secrete marked increased levels of IL-2 (57, 64). IL-2 functions both as an autocrine and paracrine factor to further activate donor T cells. Macrophages are recruited and activated during GVHD, leading to upregulation and increased production of IL-1 transcripts and protein (65-68). IL-1 mRNA levels during GVHD, particularly in the skin, have been observed to be increased several hundred fold (69). IFN-γ, another $CD4^+$ cytokine, is also upregulated early during GVHD. Lymphocytes derived from mice undergoing GVHD have increased expression of IFN-γ transcripts and produce elevated levels of IFN-γ (65, 70). In fact, serum levels of IFN-γ are increased in patients experiencing GVHD following allogenic BMT (71). IFN-γ can be detected in GVHD mice and patients even in the period of marked reduction of $CD4^+$ cell number and function, suggesting that NK cells may be the source of the IFN-γ. One of the functions of IFN-γ is to activate macrophages to secrete tumour necrosis factor-α (TNF-α) (72, 73). Indeed, serum levels of TNF-α are increased during GVHD (74), and many organs that are targeted during GVHD contain cells expressing high levels

of TNF-α mRNA (75).

3.4. GVHD-induced immunosuppression

After the brief period of lymphoproliferation (57-59), GVHD causes severe immunosuppression of T and B cell function. The immunosuppression results in many defective functions including suppressed proliferation to mitogens, marked decreased responses to third-party antigens and TNP-modified self, and depressed plaque-forming response to sheep red blood cells (44, 76-78). Suppression of T and B cell functions occurs as early as one week after disease induction, despite normal or increased numbers of T and B cells. In the first week of GVHD, B cell numbers in the spleen remain normal (76) and splenic T cell numbers are markedly increased (57). During this period, prostaglandin E and nitric oxide production by macrophages is increased, resulting in non-specific immunosuppression which is reversed by indomethacin, a prostaglandin synthesis inhibitor, and N^G-monomethyl-L-arginine, a competitive inhibitor of nitric oxide (79, 80). Recent evidence suggests that elevated levels of glucocorticoids also contribute to early immunosuppression by downregulating the levels of $p56^{lck}$ and $p59^{fyn}$, two src family protein tyrosine kinases involved in T cell activation (81). Activation of donor derived $CD8^+$ suppressor cells specific

against the host's alloantigens also contributes to early GVHD-induced immunosuppression (82-84). High production of IFN-γ during GVHD has profound immunosuppressive effects since anti-IFN-γ antibodies added *in vitro* to GVHD-derived splenic cells markedly improved mitogen-induced proliferation (85-87). Evidence suggests that the suppressive activity of IFN-γ is associated with non-specific null cells (ie. non-T and non-NK cell) that is sensitive to L-leucyl methyl ester, but not to anti-Thy1, treatment (86, 88). As GVHD progresses beyond the second week, a cytotoxic phase ensues resulting in severe deficiency of host T and B cells in the periphery, further exacerbating the immunosuppressive state (57, 56). The loss of host T and B cells is attributed in part by the direct attack of activated specific donor-anti-host $CD8^+$ cytotoxic cells (89-91).

Mice that survive the lethal effects of GVHD experience long-term immmunodeficiency that persists over 5-6 months after disease induction. This state of long-term immunosuppression is mediated by defective $CD4^+$ cells as a result of a maturation defect in the GVHD-induced dysplastic thymus (93, 94). The dysplastic thymus is characterized by the effacement of the cortico-medullary junction, loss of Hassall's corpuscles, and injury to medullary epithelial cells (93). Since Hassall's corpuscles and the medullary epithelial cells are thought to play an important role in thymocyte maturation (95, 96), $CD4^+$ thymocytes immigrating from the

dysplastic thymus are immunoincompetent; they are unable to produce IL-2 and respond to IL-1 (97). However, the function of $CD4^+$ cells recovers gradually, but only after the thymic medulla has regenerated (98). Given the pivotal role of $CD4^+$ cells in the immune system, it is not surprising that defective $CD4^+$ cells can cause long-term immunodeficiency.

3.5. Histopathological lesion during GVHD

Another profound effect of GVHD is extensive injury to tissues rich in epithelial cells. These include the skin, liver, intestine, thymus, salivary glands, and pancreas (93, 99-102). There is no doubt that donor T cells are important for triggering GVHD (41-49). It is however questionable whether donor T cells directly mediate histopathological tissue lesions. Activated donor $CD4^+$ cells have been identified in many targeted organs early during GVHD and are believed to recruit other effector cells responsible for mediating histopathological lesions (103). All nucleated cells express MHC class I antigens and are therefore susceptible to attack by donor $CD8^+$ cells. However, evidence suggests that donor $CD8^+$ cells are not necessary for inducing tissue lesions, although they can contribute to the injury (104, 105). In addition, histological examination reveals very few donor $CD8^+$ cells among the infiltrating mononuclear cells (106), and tissue lesions occur

at a period of severe T cell immunosuppression (104, 105, 108). Instead, morphological analysis shows that the infiltrates are large granular cells expressing NK markers such as asialoGM1 (ASGM1) (109). Animal studies demonstrate that moderate to severe lesions in the pancreas and liver of GVHD mice is associated with an early and rapid increase in splenic NK cell activity (108). Furthermore, moderate to severe thymic dysplasia is observed in GVHD mice only when NK cell activity in the thymus is augmented early and rapidly (110). Intestinal injury during GVHD also correlates with increased intestinal NK cell activity (105). The studies cited above suggest that histopathological tissue injury during GVHD is mediated by effector cells with NK cell activity. The most convincing evidence supporting the role of these NK-like effector cells is derived from studies using beige mice that display impaired NK cell activity (111). Lymphoid cells of beige mice injected into the recipient failed to induce moderate to severe lesions in the liver and pancreas, although GVHD-induced splenomegaly, a T-cell mediated event was induced (112). Further studies showed that the effector cell was an inducible ASGM1$^+$ cell with NK cell activity that required activation before it caused tissue injury. In addition, elimination of the ASGM1$^+$ inducible effector cell with anti-ASGM1 antibody prevented tissue injury without affecting GVHD-induced splenomegaly (113). However, the effector cell was not a classical NK cell despite its ability to lyse NK targets (ie. YAC cells) since it displayed

allospecificity (114). Evidence suggests that the effector cell may be a γδ T cell because lymphoid cells derived from αβ transgenic mice fail to induce GVHD (115) and anti-γδ antibody eliminates the inducible ASGM1$^+$ cell (Gartner J. G., personal communication). Thus an inducible ASGM1$^+$ NK-like cell appears to play an important role in mediating histopathological lesions during GVHD.

The tissue antigens targeted by the infiltrates remain elusive. It is postulated that upregulated MHC class I and II antigens, mediated by inflammatory cytokines during GVHD, may serve as target antigens (116-118). Increased expression of adhesion molecules such as ICAM-1, ELAM-1, AND VACM-1, may also facilitate the entry of infiltrates into the GVHD-targeted organs and contribute to tissue injury (119-120, 124-126).

The role of inflammatory cytokines has also been implicated in mediating tissue lesions during GVHD. IL-1, IFN-γ and TNF-α transcripts are elevated in many organs targeted during GVHD, including, the skin, lung, intestine, thymus, and salivary glands (69, 75, Kichian *et al.*, manuscript in preparation). In fact, mice and patients experiencing GVHD display elevated levels of IL-1, IFN-γ and TNF-α in the serum (68, 74, 71). Tissue injury can be neutralized or markedly ameliorated when the recipients are treated with antibodies against these inflammatory cytokines or their

receptors, providing strong evidence for the role of IL-1, IFN-γ and TNF-α in inducing tissue lesions during GVHD (67, 121-123).

3.6. Role of endotoxin and macrophages during GVHD

Although T and B cells are severely suppressed during GVHD, macrophages are activated as demonstrated by an increase in their phagocytic and bactericidal activity (124), and the production of pathological amounts of TNF-α that results in cachexia, septic shock and death (125). Two important triggering stimuli, IFN-γ and lipopolysaccharide (LPS, endotoxin), are believed to activate macrophages to secrete TNF-α during GVHD. Macrophages are primed by IFN-γ and are therefore more readily activated to secrete TNF-α when exposed to LPS (126, 127). Thus priming of macrophages by IFN-γ results in a significant reduction in the amount of LPS needed to trigger macrophage-production of TNF-α (126-128). Early during GVHD donor Th1, $CD4^+$, cells are activated to produce high levels of IFN-γ which primes macrophages (125). However, macrophages remain primed at the time of severe T cell immunosuppression suggesting that NK cells are maintaining the source of IFN-γ (129; Kichian *et al.*, manuscript in preparation). Indeed, increased levels of IFN-γ in the serum of BMT patients correlate with the severity of GVHD (71). During the course of

GVHD the gastrointestinal tract is injured, most likely mediated by inducible ASGM1$^+$ NK-like cells that infiltrate the gut since elimination of these cells prevents tissue injury and GVHD-induced mortality (108, 112, 113). Hence, E. coli residing in the gut is translocated across the injured gastrointestinal tract and enters the circulation to provide a source of endogenous LPS which activates the prime macrophages to secrete pathological amounts of TNF-α that is responsible for the septic shock-like symptoms of acute GVHD (125, 129). In fact, an increase in LPS is detected in the serum of GVHD mice at the onset of GVHD-induced mortality (125, 129, Price *et al.*, manuscript submitted), and elimination of the gastrointestinal microflora, the source of gut LPS, in mice and patients markedly reduces the severity of GVHD or prevents the disease (130-135). Interestingly, mice raised in a germ-free environment do not develop GVHD (131), and GVHD is less severe in patients kept in a protective laminar flow environment (135). These studies strongly suggest that gut LPS entering the systemic circulation via the injured gut plays an important role in the pathogenesis of GVHD, particularly, in activating primed macrophages to produce pathogenic amounts of TNF-α; the final mediator of mortality in GVHD since anti-TNF-α antibodies prevent GVHD-induced septic shock (123) and ameliorates the symptoms of GVHD (122).

4. Bone marrow transplantation

Studies of BMT were first carried out in animal models, mainly in mice and dogs. At the end of World War II following the use of atomic bombs, there was a great interest in the effects of irradiation on living organisms. In the 1950's, experiments on mice showed that bone marrow was very sensitive to irradiation and that marrow failure due to irradiation resulted in death (136-143). However, protection against irradiation was achieved if the recipient was infused with marrow cells (138-141). It was concluded from these studies that the transfer of living bone marrow cells provided the protection against irradiation. In 1961, landmark studies by Till and McCulloch provided evidence that the bone marrow contained stem cells which repopulated the entire hematopoietic system and was the basis for the protection against irradiation (144-146).

Chemotherapy and/or irradiation are used extensively to treat hematological malignancy, however, they also destroy the bone marrow and the hematopoietic system. Thus BMT has become the treatment of choice for a number of malignant and nonmalignant blood disorders and more recently for some solid tumours (147). BMT is also the only treatment for accidental irradiation injury (148). Unlike solid organs which contains limited number of cells with immunological properties, bone marrow

contains large number of cells capable of mounting an immunological reaction. In solid organ transplantation, prevention of graft rejection is the main concern, in contrast, to BMT where prevention of GVHD is the principal goal along with successful engraftment. Clinical allogenic BMT meets the classical requirements for GVHD put forth by Simonsen; that is, [1] the recipient is immunoincompetent due to irradiation and is therefore unable to reject the graft, [2] the graft contains immunocompetent cells, and [3] antigen disparity exists between the recipient and the graft.

Similar to experimental GVHD, clinical GVHD is also induced by mature T cells contaminating the bone marrow. The major factor governing the severity and incidence of GVHD is the genetic disparity between the recipient and the donor. The highest incidence of GVHD and most severe forms occurs in patients transplanted with allogenic BMT. For this reason, optimal matching of the major histocompatibility antigens (Human Leukocytes Antigens, HLA) using HLA-identical siblings is preferred in order to reduce the incidence of GVHD. Unfortunately, only 20-25% of the BMT patients will receive HLA-identical bone marrow (149, 150). Gender differences between the donor and recipient can account for up to a three fold increase risk of GVHD, especially in situations where the graft is of female origin and the recipient is male (151, 152). Occurrence of GVHD in the female → male combination is attributed to the female graft reacting

against the male H-Y antigens (151, 152). Age is another key factor associated with the development of acute GVHD (153, 154). Increasing age of the recipient correlates with a higher incidence of GVHD (a two-fold risk) (153, 154). Based on these risk factors, the incidence of GVHD is between 40-60% for HLA-matched BMT and up to 90% for HLA-mismatched BMT (155).

GVHD remains one of the major complications to a successful BMT. Clinical GVHD results in injury to many organs, in particular the skin, gut and liver (156-162). GVHD patients also develop severe immunodeficiency that results in lethal sepsis and pneumonia (163, 164). Many strategies are currently used to control the incidence and severity of GVHD. One approach is the use of immunosuppressive drugs that target T cells since T cells contaminating the bone marrow graft induce GVHD. Corticosteroids, methotrexate and cyclosporin A are employed in different combinations to downregulate donor T cell activation and hence decrease the risk and severity of GVHD (165-171). A second approach is to deplete mature T cells present in the bone marrow in order to prevent GVHD. Animal studies have shown that mature T cells contaminating the bone marrow are responsible for inducing GVHD (172, 173) and that depletion of these T cells leads to the prevention of GVHD (174-176). As predicted from the animal studies, transplanting bone marrow depleted of T cells into

human patients markedly decreases the risk and severity of GVHD, however, there is a greater incidence of graft failure (177-187). The incidence of graft failure increases 10-fold (10-50%) in patients transplanted with bone marrow depleted of T cells as compared to patients with unmodified marrow graft (181-187). TNF-α has been shown to increase in the serum of patients undergoing GVHD and is associated with the severity of the disease (74, 188, 189). Thus another approach to control GVHD is to block TNF-α production or function by employing anti-TNF-α antibodies (122, 123), pentoxyfilline (190-193) and thalidomide (194-198). Although immunosuppressive drugs are routinely employed to manage GVHD, they also cause unwanted side-effects and further suppress the recipient, often contributing to complications of infection that result in mortality. Despite the wealth of knowledge on the pathogenesis and management of GVHD, this disease still remains a serious clinical problem of BMT, especially in cases of allogenic BMT.

B. NEUROENDOCRINE-IMMUNE INTERACTION

1. Hypothalamic-pituitary-adrenal axis

Organisms are constantly exposed to external stimuli or stressors which

can be detrimental for their survival. Fortunately, multiple internal homeostatic mechanisms are triggered to allow the host to adapt to the stress. One of the best characterized homeostatic neuroendocrine systems in humans and in rodents is the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis plays an important role in maintaining physiological homeostasis in response to stress. Stressor signals activate paraventricular nuclei in the hypothalamus to secrete corticotropin releasing hormone (CRH) into the portal circulation that connects the hypothalamus to the pituitary gland (199, 200). The CRH then stimulates corticotrophs in the anterior pituitary to produce biologically inactive proopiomelanocortin, a precursor hormone of 31 kDa, that is cleaved at sites of dibasic amino acids by prohormone convertase 1 (201, 202). Cleavage of the POMC molecule results in biologically active adrenocorticotropin hormone (ACTH) and β-lipotropin (199-202). ACTH enters the circulation and triggers the adrenal cortex to secrete cortisol, the major glucocorticoid that is produced in the human adrenals (203, 204). In rodents, corticosterone is the major glucocorticoid secreted by the adrenals. In order to prevent excessive production of glucocorticoids, the HPA axis is under the negative feedback inhibition of glucocorticoids acting at both the hypothalamus and pituitary to downregulate the production of CRH and POMC, respectively (205-207).

1.1. Activation of the HPA axis by the immune system

The HPA axis is activated by numerous types of stressor signals, including physical and psychological stress. In 1975, Besedovsky and his colleagues were the first to report that an immune response triggered the release of glucocorticoids. They showed that the activation of the immune system against various antigens in rats caused increased levels of circulating corticosterone that were proportional to the magnitude of the immune response (208). These observations prompted further investigation of the mechanism(s) responsible for the secretion of glucocorticoids during an immune response. Mice injected with Newcastle Disease Virus (NDV) display increased levels of blood ACTH and corticosterone suggesting that the HPA axis is activated (209). Furthermore, supernatant obtained from NDV co-cultured with leucocytes also triggers the release of corticosterone when injected into naive mice (209, 210). Since cytokines are released during an immune response, it was postulated that IL-1 may play a role in activating the HPA axis. In fact, anti-IL-1 antibodies added to the supernatant of leucocytes co-cultured with NDV neutralized the increase in blood ACTH and corticosterone (209, 210). In addition, recombinant IL-1 injected into rats and mice causes elevated levels of plasma ACTH and corticosterone (209, 210). Other cytokines such as IL-2, TNF-α and IFN-γ failed to induce corticosterone secretion whereas recombinant IL-1 injected

into athymic mice gave the same increased levels of corticosterone as in euthymic counterparts (210).

Another potent stimulus most extensively studied to activate the HPA axis is LPS. Similar to NDV, LPS injected into mice and humans activates macrophages to release IL-1 which then triggers the HPA axis resulting in increased levels of blood ACTH and glucocorticoids (211-213). *In vivo* treatment with antibodies against the IL-1 receptor abrogates activation of the HPA axis by LPS, strongly supporting the role of IL-1 as the culprit (214, 215). It could be argued that other inflammatory cytokines such as TNF-α and/or IL-6 could trigger the HPA axis. However, studies have shown that TNF-α and IL-6 do not appear to activate the HPA axis since anti-TNF-α antibody treatment of LPS-injected mice fails to block corticosterone secretion (216, 217), and corticosterone secretion is not attenuated in IL-6 deficient mice infected with LPS (218). Taken together, these studies suggest that IL-1 production during an immune response against viruses or bacteria triggers the HPA axis and results in increased levels of blood ACTH and glucocorticoids.

1.2. Immunosuppressive effects of glucocorticoids

It is well documented that glucocorticoids exert multiple immunosuppressive actions. Glucocorticoids cause cell death of immature as well as mature lymphocytes by inducing apoptosis (219-221). Glucocorticoids activate genes that encode for endonucleases that fragment DNA into a "ladder-like" pattern and results in apoptotic cell death (222-225). IL-2 production, a critical cytokine, involved in T cell activation is inhibited by glucocorticoids which interfere with the cooperativity between NFAT and AP-1, two IL-2 transcriptional factors, thus impairing IL-2 transcription (226, 227). Glucocorticoids also suppress production of inflammatory cytokines, TNF-α, IL-6, and IL-1, by inhibiting transcription (228-230). Glucocorticoids impair several macrophage functions including phagocytosis and, antigen processing and presentation (231).

As discussed above, an immune response against bacteria or viruses is often associated with increased levels of circulating glucocorticoids. However, glucocorticoids return to basal levels within a few hours to a few days after an immune response (208, 209, 211, 212). It is postulated that the secretion of glucocorticoids acts to negatively regulate the potential lethal production of cytokines. This is supported by evidence showing that removing the source of endogenous glucocorticoids by adrenalectomy results

in mortality of mice injected with LPS as a consequence of production of lethal amounts of inflammatory cytokines, most notably TNF-α (229, 230, 232). Furthermore, injection of anti-CD3 antibodies or superantigens triggers the secretion of glucocorticoids (233, 234), however, when these agents are injected into adrenalectomized mice or mice treated with RU486, a glucocorticoid receptor antagonist, high mortality is observed as a result of overproduction of T cell cytokines (233). In light of these studies, activation of the HPA axis during an immune response appears to be beneficial to the host, conversely, chronic elevated levels of glucocorticoids is detrimental and leads to a state of immunosuppression.

1.3. Production of POMC-derived peptides by the immune system

Hormones were once thought to be produced by the classical endocrine organs. However, it is now well documented over the last decade and a half that many hormones are also produced by immune cells (235-238). The most extensively studied hormones produced and secreted by the immune system are the POMC-derived peptides. In 1980, Blalock and Smith were the first to report that human lymphocytes incubated with NDV *in vitro* produced ACTH-like peptides (239). Further studies by the same group demonstrated that hypophysectomized mice injected with NDV displayed

increased levels of corticosterone (240). Splenic cells derived from the infected hypophysectomized mice were positive for an antibody against ACTH. Since these mice lacked the pituitary gland, it was concluded that the secretion of glucocorticoids was not mediated by pituitary ACTH, but instead, was due to biologically active ACTH secreted by lymphocytes (240). At that time these results were met with great skepticism, however, they were soon confirmed by other investigators. In 1982, Lolait and his colleagues observed that a subpopulation of splenic macrophages contained immunoreactive ACTH and β-endorphin (241), and they later demonstrated that splenic macrophages infected with NDV express the POMC transcripts (242, 243). NDV is not the only stimulus to cause ACTH production by immune cells. LPS or CRH incubated with mouse splenic cells also induces increased expression of POMC transcripts and production of ACTH (244, 245). The nucleotide sequence of the POMC transcript is identical to that of mouse pituitary POMC (245). In addition, reverse-phase chromatography showed that the immune-derived ACTH eluted at the same time as standard synthetic ACTH (245). Human leucocytes incubated with viruses, LPS or CRH also express the POMC transcripts and produce biologically active ACTH (246-252).

The levels of ACTH produced by immune cells are low compared to that secreted by the pituitary and it is thus questionable whether immune-

derived ACTH can trigger glucocorticoid secretion. Patients with leukemia have been reported to display high levels of circulating ACTH and show symptoms of Cushing' s syndrome (253). Leukemic cells taken from these patients produce high amounts of ACTH *in vitro*, and there is no post-mortem evidence of ACTH or cortisol-secreting pituitary or adrenal adenomas (253). In another report, a patient with an inflammatory mass experienced high levels of circulating ACTH and cortisol which returned to normal levels after removal of the inflammatory mass containing only leucocytes and fat (254). Thus it appears that immune-derived ACTH can trigger glucocorticoid secretion under pathological conditions. It was also proposed that ACTH produced by immune cells triggers glucocorticoid secretion only when the immune cells are in close proximity to the adrenals. This is supported by studies demonstrating that LPS-activated splenic cells separated from adrenal cells by a semipermeable membrane secrete biologically active ACTH to stimulate adrenal cells to secrete glucocorticoids (255).

2. Stress response during GVHD

The induction of GVHD in mice triggers a stress response that results in glucocorticoid secretion by the adrenal glands. The role of endogenous

glucocorticoids during GVHD has been focused mainly on the thymus. Mice undergoing GVHD experience severe thymic involution (256-258) which is prevented by surgical adrenalectomy before GVHD induction (256, 257, 259), and the involuted thymus returns to normal size by adrenalectomy during the course of the disease (256, 257). The thymic atrophy is due to the elimination of the steroid sensitive immature $CD4^+8^+$ thymocyte population (260). These studies strongly suggest that GVHD induces a stress response that resulted in the secretion of excessive amounts of glucocorticoids. In fact, plasma corticosterone levels are elevated in mice and rats experiencing GVHD (261, 262). We have previously shown that mature $CD4^+$ thymocytes fail to acquire steroid resistance as they undergo maturation in a GVHD-induced dysplastic thymus, and are thus eliminated by high levels of circulating glucocorticoids (260). In addition, we have recently reported that in T cells derived from the LN, the levels of $p56^{lck}$ and $p59^{fyn}$, two src family protein tyrosine kinases involved in T cell activation (263-266), are markedly reduced as early as day 12 after GVHD induction, hence leading to suppression of mitogen-induced T cell proliferation (81). However, adrenalectomy prior to GVHD induction prevents the downregulation of these protein tyrosine kinases in LN T cells, thus restoring mitogen-induced T cell proliferation to near normal (81). These studies suggest that GVHD-induced high levels of circulating glucocorticoids eliminate the $CD4^+8^+$ and $CD4^+$ thymocyte populations, and

contribute to the suppression of T cells.

C. OBJECTIVES OF THIS THESIS

In the present thesis, we have investigated the mechanism responsible for the secretion of glucocorticoids in mice undergoing GVHD (Chapter 2). Furthermore, we have examined the effects of endogenous glucocorticoids on the outcome of GVHD (Chapter 3 & 4) and on host T cell populations in the peripheral lymphoid organs of GVHD mice (Chapter 5).

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CHAPTER 2 INCREASED EXPRESSION OF PROOPIOMELANOCORTIN mRNA IN ADRENAL GLANDS OF MICE UNDERGOING GRAFT-VERSUS-HOST DISEASE: ASSOCIATION WITH PERSISTENT ELEVATED PLASMA CORTICOSTERONE LEVELS[1].

The studies presented in this chapter investigate the mechanism of glucocorticoid secretion during GVHD.

The study was designed and all experiments were carried out by K. E. You-Ten except for detection of POMC transcripts in the adrenals by A. Itié in collaboration with and supervised by K. E. You-Ten; the histological analysis by T. A. Seemayer; and sequencing of the POMC transcript by R. G. Palfree. The research was carried out in collaboration with R. G. Palfree, and supervised by W. S. Lapp.

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Increased Expression of Proopiomelanocortin mRNA in Adrenal Glands of Mice Undergoing Graft-versus-Host Disease: Association with Persistent Elevated Plasma Corticosterone Levels.

Short Title: **Expression of POMC mRNA in the adrenals during GVHD**

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Key words: ACTH, adrenals, corticosterone, GVHD, POMC

SUMMARY

Graft-versus-host disease (GVHD) in animal models induces severe thymic atrophy as a result of prolonged secretion of high concentrations of adrenal glucocorticoids. In this report, we investigated the mechanism responsible for the persistent stimulation of the adrenal glands to secrete glucocorticoids in mice undergoing GVHD. GVHD was induced across the major and multiple minor histocompatibility antigen difference in unirradiated C57BL/6 x AF1 hybrid mice by the intravenous injection of A strain parental lymphoid cells. Our results showed plasma corticosterone (CS) levels were elevated in association with high concentrations of corticotropin (ACTH) in both the GVHD and control syngeneic (SYN) groups on day 9. By days 16 and 24, plasma CS and ACTH in the SYN mice returned to basal levels. In contrast, plasma CS levels remained elevated in the GVHD animals on days 16 and 24 despite decreasing concentrations of plasma ACTH. Reverse transcription-polymerase chain reaction (RT-PCR) showed several fold increase in proopiomelanocortin mRNA in the adrenal glands of GVHD mice compared to SYN animals. In addition, high mRNA levels for murine prohormone convertase 1, the enzyme that cleaves POMC into ACTH, were also detected in GVHD adrenals. Histological analysis of GVHD adrenals failed to show any sign of adrenalitis, and RT-PCR of GVHD adrenals also failed to detect mRNA for interferon-γ, a

cytokine expressed by activated T and NK cells. However, mRNA for interleukin-12, a cytokine produced by activated macrophages, was increased in GVHD adrenals suggesting that resident adrenal macrophages were activated during GVHD. Our findings suggest that persistent elevated levels of plasma glucocorticoids during GVHD could be mediated by intra-adrenal ACTH produced by resident adrenal macrophages activated as a consequence of GVHD.

INTRODUCTION

The injection of homozygous parental lymphoid cells into unirradiated F1 hybrid mice results in the development of graft-versus-host disease (GVHD) [1, 2]. GVHD occurs in this model as a consequence of lymphoid cells in the parental graft reacting against the alloantigens that are encoded by histocompatibility genes inherited from the other parental strain of the F1 hybrid. Mice undergoing GVHD display many abnormal features including severe immunosuppression of T and B cells, diarrhea, weight loss, pathologic lesions in lymphoid and non-lymphoid organs and increased macrophage activation [3-7].

Another prominent feature of GVHD in mice is stress-mediated severe thymic involution [8-10] resulting from the complete elimination of the steroid sensitive double positive $CD4^+8^+$ thymocyte population [11] and selective elimination of the mature single positive $CD4^+$ thymocyte population [11]. Thymic atrophy persists for as long as two months after disease induction [11, 12]. Adrenalectomy before or during GVHD induction prevents severe thymic involution [8-10] and maintains or restores normal levels of $CD4^+8^+$ and $CD4^+$ thymocyte populations [11]. These findings imply that the adrenal glands secrete excessive amounts of glucocorticoids during GVHD and indeed, plasma corticosterone (CS) concentrations have been shown to be elevated during GVHD [13]. In

addition, we have recently reported that increased endogenous glucocorticoids during GVHD cause the downregulation of $p56^{lck}$ and $p59^{fyn}$, two important src-like family protein tyrosine kinases implicated in T cell signalling, and thus resulting in T cell immunosuppression [14].

One of the most important physiological responses to stress is the activation of the hypothalamic-pituitary-adrenal (HPA) axis. The stressor stimulus induces the hypothalamus to secrete corticotropin releasing hormone (CRH) [15]. CRH in turn stimulates the anterior pituitary to produce proopiomelanocortin (POMC) [16] which is cleaved by prohormone convertase 1 (PC1) into biologically active corticotropin (ACTH) [17, 18]. The ACTH enters the circulation and triggers the adrenal cortex to release glucocorticoids [19]. The HPA axis is regulated by negative feedback inhibition by glucocorticoids acting at the level of the hypothalamus and pituitary to downregulate the production of CRH and ACTH respectively [16, 19].

In this report, we investigated the mechanism responsible for glucocorticoid secretion in mice undergoing GVHD. Our results demonstrate that GVHD mice displayed persistent elevated concentrations of plasma CS, despite low plasma ACTH levels. Using semiquantitative reverse transcription-polymerase chain reaction (RT-PCR), we detected a marked increase in mRNA for POMC in the adrenal glands of GVHD mice. In addition, mRNA for murine PC1 (mPC1) was present in these tissues.

Histological analysis of GVHD adrenals failed to show any sign of lymphoid infiltrates and mRNA for interferon-γ (IFN-γ), a cytokine specific for activated T and NK cells, was also not detected in the adrenals of GVHD mice. However, mRNA for the p40 subunit of interleukin-12 (IL-12), a cytokine specific for activated macrophages, was increased in GVHD adrenals. These findings suggest that the secretion of CS during GVHD is independent of pituitary ACTH and may be triggered by paracrine stimulation of local ACTH most likely produced by activated macrophages residing in the adrenals.

MATERIALS AND METHODS

Animals

Mice of strain A ($H2^a$), C57BL/6 ($H2^b$) (B6) and their F1 hybrids, B6xAF1 ($H2^{a,b}$) (B6AF1) were used. All mice were bred and maintained in our animal colony. Donor A animals were 6-12 months old. B6AF1 recipient mice were older than 3 months.

Induction of GVHD

GVHD was induced by an intravenous (i.v.) injection of $30x10^6$ or $50x10^6$ A strain parental lymphoid cells into B6AF1 hybrid mice as described previously [6]. The syngeneic control group consisted of B6AF1 mice injected with B6AF1 lymphoid cells.

Adrenalectomy

B6AF1 mice, at 9-10 weeks of age, were surgically adrenalectomized (ADX) under ether anesthesia as described previously [10, 11]. ADX mice were maintained on physiological saline in lieu of drinking water for the duration of the experiment. One month after the surgery ADX mice were injected with A strain parental lymphoid cells to induce GVHD.

Splenic mitogen assay

On different days after GVHD induction, splenic cell suspensions were prepared from B6AF1 recipients using RPMI media supplemented with 10% fetal calf serum (Flow Laboratories, Mississauga, Ontario, Canada). Briefly, $5x10^5$ splenic cells were cultured for 48 h in triplicate with or without mitogen in 96-well plates. T cell proliferative responses to phytohemagglutinin (PHA) (2.5 µg/ml) (Wellcome Research Reagent Limited, Dartford, UK), and B cell responses to lipopolysaccharide (LPS) (10 µg/ml) (Sigma, St. Louis, MO) were evaluated by [^{3}H] thymidine incorporation, as described previously [6].

Cortisone acetate treatment

Two days before the mice were sacrificed, ADX GVHD mice were injected intraperitoneally with 2.5 mg cortisone acetate (Merck Sharp & Dohme Canada, Quebec, Canada), in a total volume of 0.5 ml PBS. Control animals were treated with PBS only.

Corticosterone (CS) and corticotropin (ACTH) measurement

All mice were handled every day for the duration of the experiment. Cardiac puncture was performed between 0900 and 1000 am while the mice were under ether anesthesia to remove blood which was collected in tubes containing EDTA. Plasma was extracted from the blood and aliquots were frozen at -20°C until assayed. Plasma CS and ACTH levels were

determined using a radioimmunoassay kit for rats and mice (ICN Biomedicals, Costa Mesa, CA).

Histology of the liver and adrenal

The liver and left adrenal were carefully removed from each mouse and placed in 10% buffered formalin. The fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin-eosin, as previously described [10]. Stained tissues were examined by light microscopy.

Reverse transcription-polymerase chain reaction (RT-PCR) and Southern blotting

On different days after GVHD induction, the right adrenal gland was carefully removed from each mouse, snap-frozen in liquid nitrogen and stored at -70°C until processed. Six to eight adrenals were pooled from each group and total RNA was isolated using the Single-Step Method of RNA Isolation by Acid Guanidine Isothiocyanate-Phenol-Chloroform Extraction described in detail by Chomczynski and Sacchi [20]. The first strand cDNA synthesis was carried out with 6 µg total RNA in a total volume of 20 µl containing PCR buffer (Bio/Can Scientific, Mississauga, Ontario, Canada), 15 pmole of antisense primers, 2 mM of $MgCl_2$, 30 units of RNase inhibitor (Pharmacia, Uppsala, Sweden), and 12 units of Avian Moloney Virus reverse transcriptase (Pharmacia, Uppsala, Sweden). After the reaction

was completed, 10 µl of the cDNA synthesis product was subjected to PCR in a volume of 50 µl containing PCR buffer, 10 pmoles of sense primers, 2 mM of $MgCl_2$, and 1 unit of Taq DNA polymerase. The PCR was subjected to 30 cycles in a "Thermal Reactor". All reagents for PCR and the Thermal Reactor were purchased from Bio/Can Scientific (Mississauga, Ontario, Canada). 10 µl of the amplified PCR product was analyzed on 1% agarose gel stained with ethidium bromide. The gel was then blotted onto nylon membranes (Hybond-N, Amersham, Oakville, Ontario, Canada) and membranes were hybridized with a ^{32}P-labelled specific oligonucleotide ($5.0x10^6$ cpm). Hybridization products were detected by autoradiography and scanned with a densitometer (BioImage, Millipore, Mississauga, Ontario, Canada).

Nucleotide sequencing of amplified POMC DNA fragment from GVHD adrenals

Amplified POMC DNA fragments generated by RT-PCR from RNA of GVHD adrenals were ligated into the plasmid pCRII using the TA-cloning system and protocol from Invitrogen Corp. (San Diego, CA). Positive colonies were identified by colony hybridization with the same ^{32}P-end-labelled POMC probe used for Southern blot analysis (see below for probe sequence). Twelve of the positive colonies were picked for plasmid preparation, restriction, and sequence analysis. Double strand DNA

sequencing was performed using the Multi-Pol system (Clontech Labs. Inc., Palo Alto, CA).

Primer sequences used for RT-PCR

Antisense primers

5' TCATGAAGCCACCGTAACGC 3' : POMC

5' GTGTATCAGCTTGGTGGAAGG 3' : G6PDH (Glucose-6-phosphate dehydrogenase)

5' CTTCATCTGCAAGTTCTTCGGC 3' : p40 subunit of IL-12

5' AATCAGCAGCGACTCCTTTTCC 3' : IFN-γ

5' GGTCTCTGTGCAGTCATTGTGC 3' : mPC1

Sense primers

5' CAACCTGCTGGCTTGCATCC 3' : POMC

5' CTAAACTCAGAAAACATCATGGC 3' : G6PDH

5' CGTGCTCATGGCTGGTGCAAAG 3' : p40 subunit of IL-12

5' TGGCTGTTTCTGGCTGTTACTG 3' : IFN-γ

5' GGTGAAATTGCCATGCAAGC 3' : mPC1

Oligonucleotide probe sequences used for Southern blotting

5' CCTACCGGGTGGAGCACTTC 3' : POMC

5' GAGCAGGTGGCCCTGAGCCG 3' : G6PDH

5' TCTGTCTGCAGAGAAGGTCACA 3' : p40 subunit of IL-12

5' GGAGGAACTGGCAAAAGGATGG 3' : IFN-γ

5' GGCTACCTCCTACAGCAGTGG 3' : mPC1

RESULTS

Immunosuppression of T and B cell functions in mice undergoing GVHD

In this report, GVHD was induced across the major and multiple minor histocompatibility antigen differences in unirradiated B6AF1 hybrid mice by the i.v. injection of A strain parental lymphoid cells. One of the hallmarks of GVHD is marked immunosuppression of T and B cell functions [3]. To confirm that our mice were experiencing GVHD, immune function was assessed by the ability of splenic T and B cells to respond to PHA and LPS, respectively. Figure 1 shows that proliferation to PHA and LPS was decreased as early as day 7 and was completely suppressed by day 29. These results confirm that T and B cell functions were severely immunosuppressed in mice undergoing GVHD.

Dissociation between the levels of CS and ACTH in the plasma of GVHD mice

Previous studies have shown that GVHD causes severe thymic involution which is mediated by the secretion of excessive amounts of adrenal glucocorticoids [8-10, 12, 13]. Normally, secretion of glucocorticoids is triggered by pituitary ACTH resulting in elevated concentrations of plasma glucocorticoids in association with increased concentrations of plasma ACTH [14, 15, 18]. Thus to investigate whether glucocorticoid secretion

during GVHD was mediated by pituitary ACTH, radioimmunoassay was employed to measure the levels of ACTH and corticosterone in the plasma of mice undergoing GVHD. Figure 2 demonstrates that both the syngeneic (SYN) and GVHD mice displayed elevated concentrations of plasma CS on day 9, in association with high plasma ACTH. By days 16 and 24, CS and ACTH returned to basal levels in the SYN groups. In contrast, plasma CS concentrations in GVHD mice remained elevated on day 16 despite decreasing ACTH. On day 24, high CS levels persisted in GVHD animals with a further reduction in ACTH concentrations. To examine the possibility that the decreasing concentration of plasma ACTH in GVHD mice was attributed to the negative feedback inhibition of high circulating CS, plasma ACTH was measured in ADX GVHD mice treated with saline or cortisone acetate. Our results demonstrated that ADX GVHD mice treated with saline lacked the negative feedback inhibition of CS resulting in very high concentrations of plasma ACTH on day 16 and 24 (Fig. 3). However, plasma ACTH levels decreased dramatically two days after treatment with cortisone acetate, implying that the pituitary of GVHD mice was responding to the negative feedback inhibition of high circulating CS (Fig. 3). Similar results were observed in ADX SYN mice treated with saline or cortisone acetate (results not shown). Taken together, these results demonstrate a dissociation between CS and ACTH levels in the circulation of GVHD mice and suggest that persistent high plasma CS

during GVHD appeared to be independent of pituitary ACTH.

Increased expression of POMC mRNA in the adrenals of GVHD mice

Plasma ACTH levels appeared far too low to stimulate the high amounts of CS observed in GVHD mice. Thus we investigated whether the stimulus for excessive CS secretion during GVHD could come directly from an extra-pituitary source. We examined the adrenal gland as a potential source of ACTH production since local secretion of ACTH could act as a paracrine stimulation of CS secretion. Because ACTH is derived from the POMC precursor [15-18], production of ACTH within the adrenals was assessed by using semiquantitative RT-PCR to detect POMC mRNA and the amplified POMC fragment was identified by Southern blotting. The POMC fragment amplified by RT-PCR contained the nucleotide sequence which encodes the ACTH peptide. Weak signals for the POMC fragment were detected in the adrenals of SYN mice on all of the days assayed (Fig. 4A). In contrast, densitometry analysis showed that the amplified POMC fragment was three to eleven fold greater in the GVHD adrenals than in the respective SYN controls on days 7, 15, 22 and 29 (Fig. 4A). However, the control amplified fragment, G6PDH, was similar in both GVHD and SYN adrenals (Fig. 4A). These results were confirmed in another experiment where GVHD was induced by an i.v injection of $50x10^6$ A strain parental lymphoid cells into B6AF1 recipients. In this experiment, the amplified POMC fragment was

five and ten fold greater in GVHD adrenals than in the SYN counterparts on days 21 and 27, respectively (Fig. 4B). Again, no differences were observed between the GVHD and SYN adrenals for G6PDH (Fig. 4B). To confirm that we are detecting only fragments amplified from transcripts of the reported murine POMC gene [21], the POMC products of RT-PCR from GVHD adrenals were directly ligated into the vector, pCRII, and transformant E. coli were screened by colony hybridization with the same probe and stringency used for Southern blot analysis. Of 12 positive recombinants picked randomly for nucleotide sequencing, not one was false positive and all contained the expected POMC fragment sequence (results not shown). In addition, we detected high mRNA expression of mPC1, the enzyme that cleaves POMC precursor into biologically active ACTH [17, 18], in both GVHD and SYN groups (Fig. 4A, B). This is not surprising since the adrenals of normal mice contain relatively high levels of mPC1 mRNA [22] and the level of mPC1 does not appear to be regulated by POMC [23]. Collectively, these results showed a marked increased expression of POMC mRNA and high expression of mPC1 mRNA in the adrenals of GVHD mice, suggesting that there might be an increased production of adrenal POMC that is cleaved into biologically active ACTH.

Histological analysis of the adrenal glands and livers of GVHD mice

During GVHD many lymphoid and non-lymphoid organs are infiltrated

with mononuclear cells [5]. Cells of the immune systems are well documented to express POMC mRNA [24-27]. It is therefore possible that the increased amount of POMC mRNA observed in the adrenals of GVHD mice was a result of an infiltrate by immune cells. To test this possibility, histological analysis was performed on a total of 20 adrenals taken from GVHD mice on different days after GVHD induction. On all of the days assayed (days 7, 15, 22 and 29) adrenals from all GVHD mice failed to show any sign of immune cell infiltrates (results not shown). Conversely, massive infiltrates were observed in the livers of GVHD mice as early as day 9 and became progressively more severe by day 21 as we have previously reported [5] (results not shown). These results suggest that the increased expression of adrenal POMC mRNA was not due to cells infiltrating the adrenals of GVHD mice but rather to a resident population in the adrenals.

Increased mRNA expression of p40 subunit of IL-12 in the adrenals of GVHD mice

Results in Figure 1 showed a profound immunosuppression of T and B cell functions by day 15. However, we and others have previously demonstrated increased macrophage activity during GVHD [4, 28]. Although the adrenals of GVHD mice failed to show any sign of mononuclear cell infiltrate, resident macrophages have been identified in the adrenals of normal mice

[29]. In addition, activated macrophages have been shown to express increased levels of POMC mRNA and produce biologically active ACTH [25, 26, 30]. We therefore investigated whether there was evidence for resident adrenal macrophages to be activated during GVHD. Using RT-PCR and Southern blotting, we detected a marked increase in an amplified fragment of the p40 subunit of IL-12, a specific cytokine induced in activated macrophages, on days 22 and 29 in the adrenals of B6AF1 mice injected with $30x10^6$ A strain parental lymphoid cells (Fig. 5A). Similar results were observed on days 21 and 27 in the adrenals of B6AF1 animals injected with $50X10^6$ A strain parental lymphoid cells (Fig. 5B). Furthermore, RT-PCR failed to detect mRNA for IFN-γ, a cytokine produced by activated T and NK cells, in GVHD adrenals (results not shown). These data imply that macrophages residing in the adrenals of GVHD mice are activated and may be expressing increased levels of adrenal POMC mRNA since activated macrophages have been reported to produce POMC mRNA and POMC-derived peptides [25, 26, 30].

DISCUSSION

In this report, we investigated the mechanism responsible for the secretion of CS during GVHD. GVHD was induced across the major and multiple minor histocompatibility antigen differences in unirradiated B6AF1 mice by the injection of A strain parental lymphoid cells. Studies have shown that the secretion of CS is triggered by pituitary ACTH, leading to increased plasma CS concentrations in association with increased plasma ACTH levels [15, 16]. Hence, we measured the concentrations of both plasma ACTH and CS during GVHD in order to determine whether pituitary ACTH was stimulating the secretion of CS. Our results showed that the basal level of CS in the plasma of control SYN mice was associated with the basal level of plasma ACTH (Fig. 2). In contrast, plasma CS remained elevated during GVHD despite decreasing concentrations of plasma ACTH below basal levels (Fig. 2). A similar observation has been reported by Khairallah et al. using an irradiated model of GVHD induced across a minor histocompatibility difference [31]. These investigators also observed persistent high levels of CS in the presence of low plasma ACTH concentrations. The low levels of plasma ACTH could not account for the high plasma CS concentrations suggesting that secretion of excessive amounts of CS during GVHD was independent of pituitary ACTH. However, the mechanism responsible for the persistent high secretion of CS

during GVHD was not elucidated.

We therefore investigated whether changes occurring in the adrenal glands could be responsible for the persistent high levels of plasma CS during the course of GVHD. Using semiquantitative RT-PCR, we detected up to 11-fold increased expression of POMC mRNA in the adrenals of GVHD mice compared to SYN groups (Fig. 4). High expression of mRNA for mPC1 was also detected in GVHD adrenals (Fig. 4). Since the adrenals contain low amount of POMC mRNA and POMC protein [32-34], the increased POMC mRNA observed in GVHD adrenals strongly suggests that POMC could be produced locally. In addition, mRNA of mPC1, the enzyme that cleaves POMC to release biologically active ACTH [17, 18], was highly expressed in adrenals of GVHD mice, thus providing evidence that the intra-adrenal POMC could be cleaved into biologically active ACTH. This proposal is consistent with previous reports showing that the adrenals of other species contain POMC-derived peptides, including ACTH [32-35]. Thus it appears that increased local production of adrenal ACTH during GVHD could stimulate the adrenal cortex to secrete CS resulting in persistent elevated levels of CS independent of pituitary ACTH.

Increased POMC mRNA expression in the adrenals could result from either an infiltrate of POMC-producing cells or the activation of resident cells with the potential to produce POMC or both. It is well documented that T and B cells, and macrophages of mice express POMC mRNA and

secrete biologically active ACTH [30, 36-38]. However, since we failed to detect any sign of adrenalitis and were unable to detect any mRNA for IFN-γ in GVHD adrenals, it is highly unlikely that the increase in POMC mRNA observed in adrenals of GVHD mice resulted from immune cells infiltrating these tissues [5, 39]. Our histological findings are in accord with Gorer and Boyse who were also unable to demonstrate any sign of infiltrates in the adrenals of GVHD mice, although massive infiltrates were observed in the liver, pancreas, kidneys, lungs, spleen and lymph nodes [40]. Interestingly, we detected high expression of mRNA for the p40 subunit of IL-12, a cytokine produced primarily by activated macrophages [41], in the adrenals of GVHD mice (Fig. 5). We and others have shown that macrophages are activated during GVHD [4, 28] and moreover, resident macrophages have been identified in the adrenal cortex of normal mice [29]. Based on these findings, it would appear that resident adrenal macrophages were activated during GVHD and could have produced sufficient ACTH to maintain the high production of CS, thus disengaging control through the HPA axis.

We can only speculate at this time on the stimulus for the production of intra-adrenal ACTH. Endotoxin (lipopolysaccharide, LPS) has been reported to stimulate the production of p40 subunit of IL-12 [41, 42] and we have demonstrated a marked increase in systemic LPS during the course of GVHD [4, 43]. In addition, the adrenals can concentrate a higher

quantity of LPS per gram of tissue than any other organ [44]. Thus it is seems highly likely that, if LPS is present in the circulation during GVHD [4, 43], it would concentrate in the adrenals, and could stimulate resident macrophages to produce ACTH. In support of our hypothesis, macrophages incubated with LPS have been shown to release increasing amounts of biologically active ACTH that induced the in vitro secretion of CS from cultured adrenal cells [30]. The amount of ACTH produced by adrenal macrophages needs not be sufficient to affect blood levels in order to act effectively in local paracrine stimulation of CS secretion. Our findings support the hypothesis that immune-derived ACTH could stimulate the adrenals without affecting blood ACTH levels only if these immune cells are in close proximity to the target endocrine tissue [30, 45].

In conclusion, our study provides evidence that local production of intra-adrenal ACTH during GVHD may stimulate persistent secretion of adrenal glucocorticoids independent of pituitary ACTH. Although our study was performed in mice, it is possible that a similar mechanism could also be induced in human adrenal glands. Recently it was proposed that activated resident macrophages in human adrenals may play an important role in triggering the secretion of glucocorticoids [46, 47]. Thus ACTH produced by activated resident adrenal macrophages could constitute a potential paracrine stimulus for glucocorticoid secretion under physiological

and/or pathological conditions.

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Fig. 1. Immunosuppression of T and B cell functions in mice undergoing GVHD. GVHD was induced in B6AF1 hybrid mice by an i.v. injection of $30x10^6$ A strain parental lymphoid cells. Results are presented as mean net cpm ($x10^3$) ± SE of 8-10 mice per group. Net cpm was calculated as follows: cpm with mitogen stimulation - cpm without mitogen stimulation.

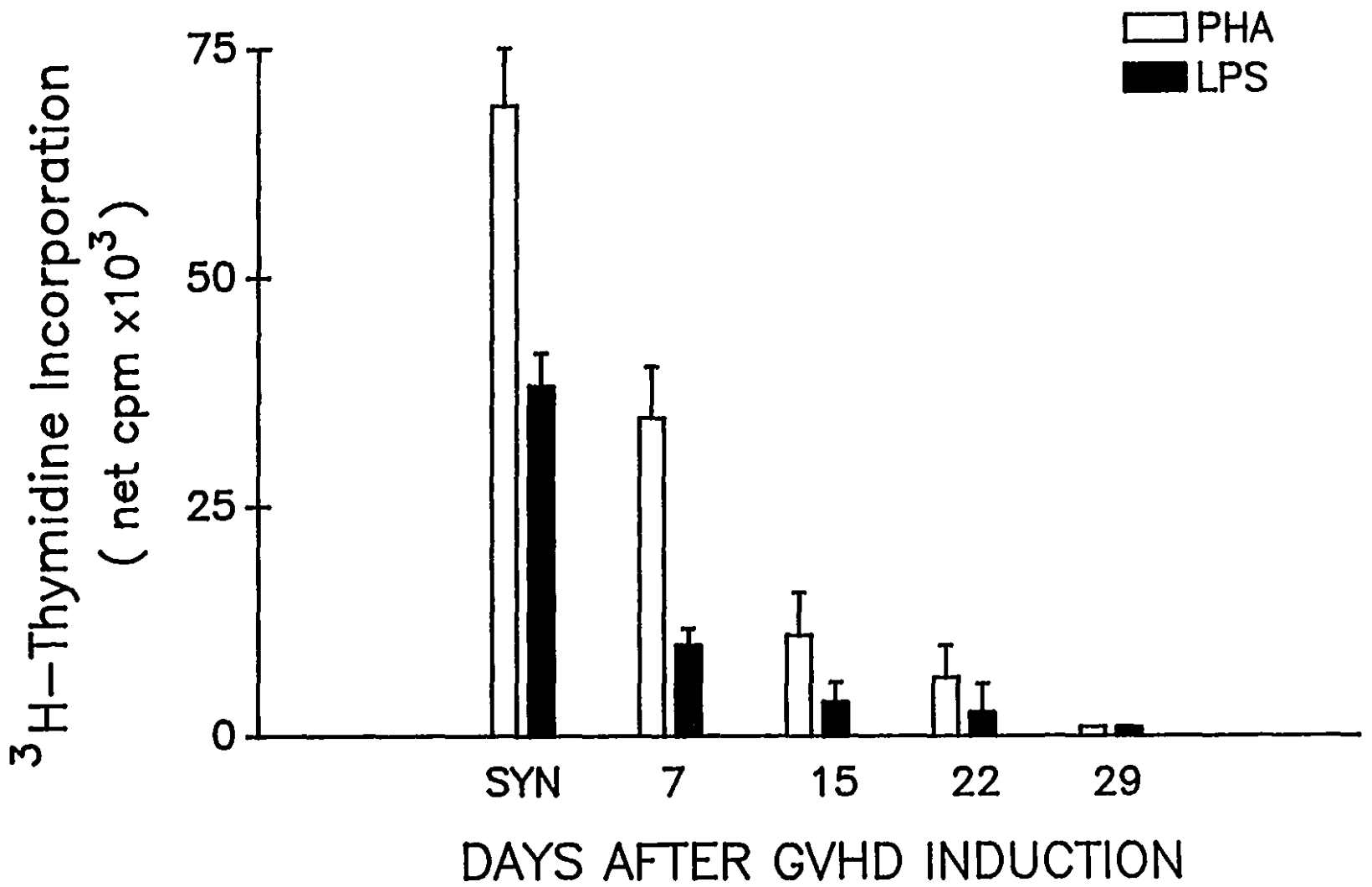

PHA
LPS
75
50
25
0
^{3}H–Thymidine Incorporation
(net cpm x10^3)
SYN
7
15
22
29
DAYS AFTER GVHD INDUCTION

Fig. 2. Dissociation between plasma CS and ACTH during GVHD. GVHD was induced in B6AF1 mice by an i.v. injection of $30x10^6$ A strain parental lymphoid cells. On different days after GVHD induction, plasma CS and ACTH were measured for each animal by radioimmunoassay. Results are presented as the mean ± SE of 8 mice in the GVHD groups and 5 mice in the SYN groups. CS levels were significantly reduced on days 16 and 24 compared to day 9 in the SYN groups ($P < 0.05$). No significant differences in CS levels were observed between the GVHD groups on days 9, 16 and 24. ACTH levels were significantly decreased on days 16 and 24 compared to day 9 in the SYN ($P < 0.1$) and GVHD ($P < 0.001$) groups. The student's *t* test was used to calculate the statistical significance.

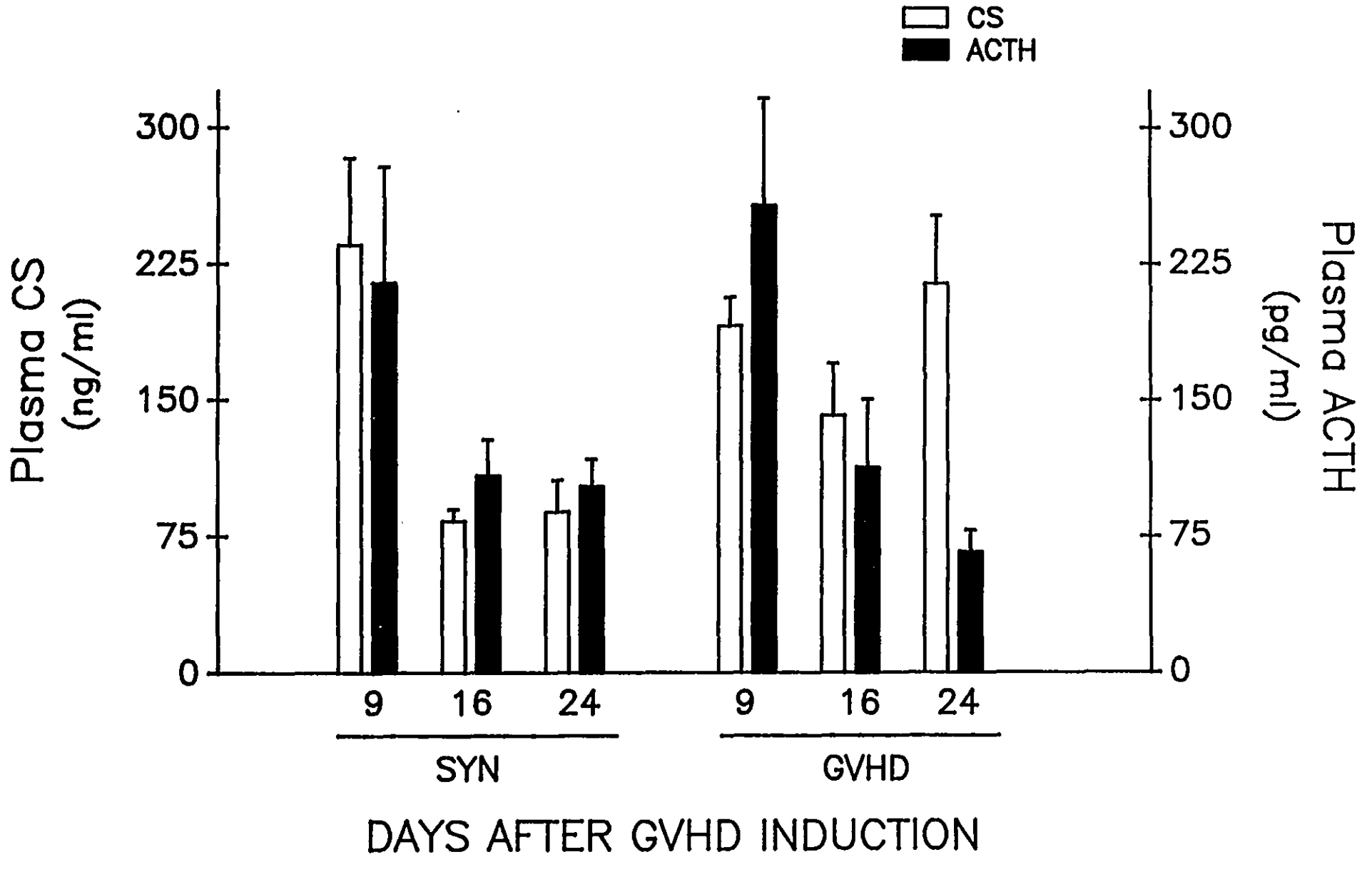

CS
ACTH
300
225
150
75
0
Plasma CS (ng/ml)
Plasma ACTH (pg/ml)
9
16
24
SYN
GVHD
DAYS AFTER GVHD INDUCTION

Fig. 3. Negative feedback inhibition of CS on the secretion of pituitary ACTH during GVHD. B6AF1 mice were adrenalectomized one month before an i.v. injection with $30x10^6$ A strain parental lymphoid cells. ADX GVHD mice were treated either with PBS or cortisone acetate (2.5 mg) two days before blood was collected by cardiac puncture, and plasma ACTH concentrations were measured by radioimmunoassay. The data are presented as mean ± SE of 5-6 mice per group.

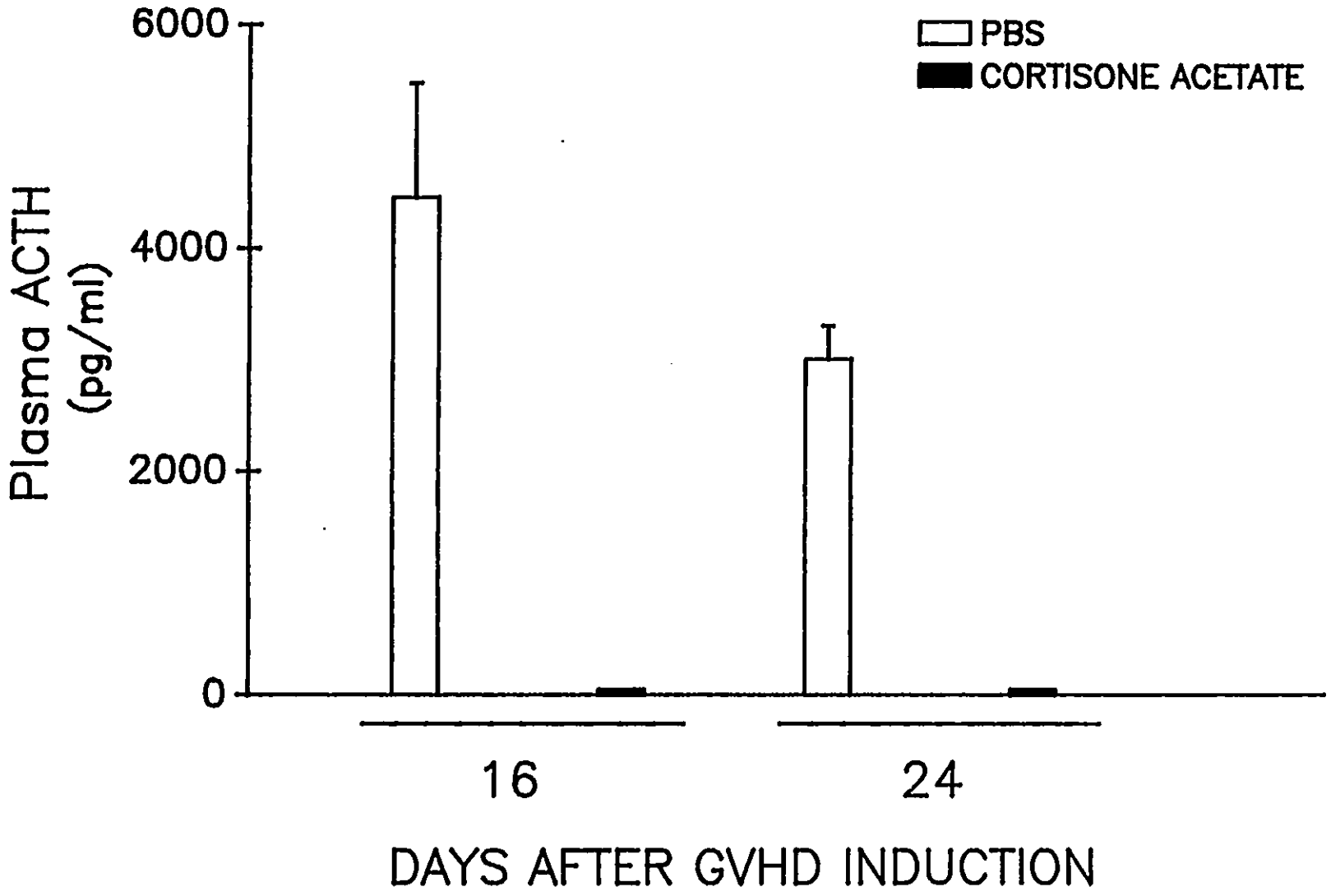

6000
4000
2000
0
Plasma ACTH
(pg/ml)
PBS
CORTISONE ACETATE
16
24
DAYS AFTER GVHD INDUCTION

Fig. 4. Increased expression of POMC mRNA in the adrenals of GVHD mice. GVHD was induced in B6AF1 mice injected with either (A) $30x10^6$ or (B) $50x10^6$ A strain parental lymphoid cells. SYN groups were B6AF1 mice injected with either $30x10^6$ or $50x10^6$ B6AF1 lymphoid cells. The data are shown as Southern blot analysis of POMC, mPC1 and G6PDH DNA fragments amplified by RT-PCR from mRNA extracted from adrenals of SYN (S) or GVHD (G) groups. The positive control (+) is mRNA extracted from pooled pituitary glands of normal mice and the amplified POMC fragment was diluted 20 fold for the Southern blotting. G6PDH was used as control mRNA expression. Each sample was amplified by RT-PCR three times and on each occasion similar results were obtained. RNA samples subjected to PCR without reverse transcription failed to amplify the POMC, mPC1 or G6PDH fragment.

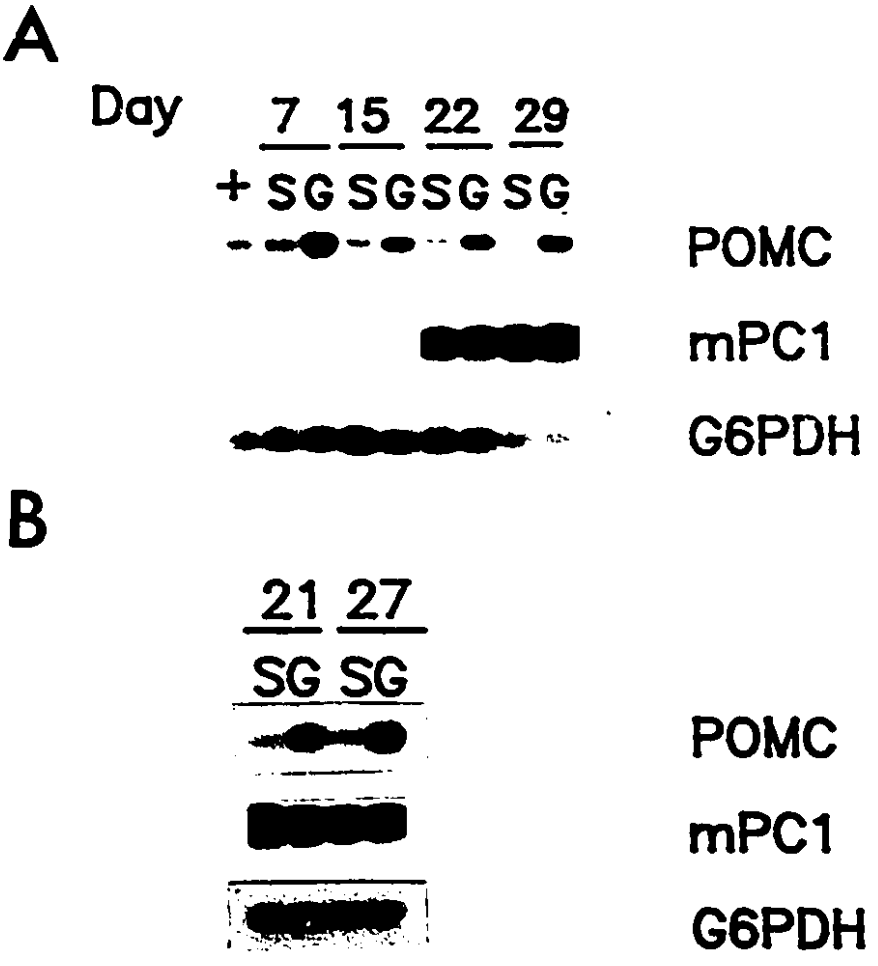
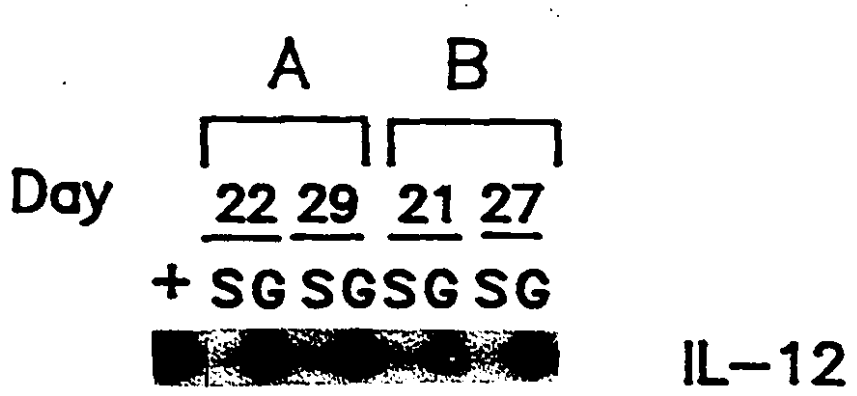

A
Day
7 15 22 29
+ SG SG SG SG
POMC
mPC1
G6PDH
B
21 27
SG SG
POMC
mPC1
G6PDH

Fig. 5. Increased mRNA expression of the p40 subunit of IL-12 in the adrenals of GVHD mice. GVHD was induced in B6AF1 mice injected with either (A) $30x10^6$ or (B) $50x10^6$ A strain parental lymphoid cells. The data are presented as Southern analysis of amplified p40 subunit of IL-12 DNA fragments obtained from adrenals of SYN (S) and GVHD (G) mice. Positive control for the p40 subunit of IL-12 is total RNA extracted from activated peritoneal macrophages of normal mice.


A
B
Day
22 29 21 27
+ SG SG SG SG
IL-12

CHAPTER 3 INDUCTION OF A GLUCOCORTICOID SENSITIVE F1-ANTI-PARENTAL MECHANISM THAT AFFECTS ENGRAFTMENT DURING GRAFT-VERSUS-HOST DISEASE[1]

The studies presented in this chapter examine the effects of endogenous glucocorticoids on the outcome of GVHD.

The study was designed and all experiments were carried out by K. E. You-Ten except for the histological analysis by T. A. Seemayer; Southern dot blot analysis by B. Wisse; and NK cell activity by F. M. N. Bertley in collaboration and supervised by K. E. You-Ten. The research project was supervised by W. S. Lapp.

[1]This manuscript is in press in the *Journal of Immunology*, 1995, 155: 172-180.

Induction of a Glucocorticoid Sensitive F1-anti-Parental Mechanism that Affects Engraftment during Graft-versus-Host Disease[1]

Running Title: **Induction of a Steroid Sensitive F1-anti-Parental Mechanism**

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Abstract

Studies have shown that graft-versus-host disease (GVHD) in animal models induces persistent elevated levels of circulating adrenal glucocorticoids. In this report, we investigated the effects of endogenous glucocorticoids on the outcome of GVHD by adrenalectomizing unirradiated (C57BL/6 X A)F1 (B6AF1) mice prior to GVHD induction. GVHD was induced by injection of $20x10^6$ A strain parental lymphoid cells into B6AF1 mice. Our results demonstrated that non-adrenalectomized (non-ADX) recipient mice experienced features characteristic of GVHD on day 13, which became progressively more severe by day 18-21. The GVHD features included severe immunosuppression, reversal in the host splenic $CD4^+/CD8^+$ ratio, histopathological lesions in different tissues, and high parental cell chimerism in the spleens and lymph nodes (LN). In contrast, ADX F1 recipient mice experienced GVHD features on day 13 similar to the non-ADX counterparts, however, ADX animals recovered rapidly from GVHD by day 18-21. Flow cytometry showed that although a relatively high frequency of parental cells was detected in the spleens and LN of ADX mice on day 13, nearly all of the parental cells in the peripheral lymphoid organs disappeared on day 18-21, the time of recovery from GVHD. The marked reduction of parental cells and recovery from GVHD were prevented by treating ADX F1 mice with either exogenous glucocorticoid, anti-asialoGM1

or anti-CD8, but not anti-NK1.1 antibody. These results suggest that a dramatic recovery from GVHD was induced by a cell-mediated steroid sensitive F1-anti-parental mechanism. The F1-anti-parental phenomenon described herein is different from classical hybrid resistance.

Homozygous parental lymphoid cells injected into an F1 hybrid are normally accepted as self since histocompatibility antigens are co-dominantly expressed by the F1 hybrid. T cells present in the parental graft recognize antigens of the other parental haplotype expressed by the F1 hybrid as foreign and as a consequence of such recognition, parental T cells initiate an immunological reaction that recruits other effector cells involved in the pathogenesis of graft-versus-host disease (GVHD). GVHD is a multi-factorial disease consisting of T and B cell immunosuppression, cachexia, injury to the lymphoid and non-lymphoid epithelial tissues, morbidity and mortality (1-7). In some instances, contrary to the basic immunogenetic laws of transplantation stated above, F1 hybrids prevent engraftment of certain parental grafts including bone marrow, tumour and lymphoid cells (8-12). This phenomenon is known as hybrid resistance and in such situations GVHD does not occur (13, 14).

There is increasing evidence that stimulation of the immune system induces a stress response that triggers the adrenal glands to secrete glucocorticoids. Injection of various antigens in rats causes an elevated level of plasma glucocorticoids, proportional to the magnitude of the immune response (15). The increase in circulating glucocorticoids is also observed in some viral infections (16), when T cells are activated with superantigens or anti-CD3 (17), and during an inflammatory response elicited by endotoxin or IL-1 (18, 19). Due to its immunosuppressive

properties, glucocorticoid secretion may represent a major effector end point of an immune response, consisting of an important feedback mechanism for regulating a potentially lethal hyperactivation of the immune system (16-19). In fact, surgical adrenalectomy or blocking the actions of glucocorticoids with an antagonist to its receptor enhances mortality of animals injected with inflammatory agents or T cell activators (17, 18). In addition, the susceptibility of certain animal strains to develop inflammatory diseases is linked to a defect in the secretion of adrenal glucocorticoids (20).

During the course of experimental GVHD blood glucocorticoid levels are elevated as a result of adrenal hyperactivity (21, 22). We have previously shown that GVHD-induced adrenal hyperactivity causes severe thymic involution and selective elimination of $CD4^+$ thymocytes (23, 24). In this study we further investigated the effects of endogenous glucocorticoids on the outcome of GVHD in mice. We postulated that elevated levels of blood glucocorticoids during GVHD may negatively regulate the immune reaction mediated by the donor cells in order to limit the severity of the disease, and that adrenalectomy may result in a more severe disease. Our results show the opposite. The injection of A strain parental lymphoid cells into B6AF1 recipients with intact adrenals induces GVHD which becomes progressively more severe with time. Adrenalectomized F1 recipients also experience GVHD symptoms that

persist for two weeks, but unexpectedly these animals display a rapid recovery from GVHD and disappearance of parental cells. Recovery from GVHD and the disappearance of parental cells are due to the induction of an F1-anti-parental response that is sensitive to glucocorticoids. The F1-anti-parental reactivity described herein displays unique properties which set it apart from the classical hybrid resistance phenomenon.

MATERIALS AND METHODS

Animals

Mice of the inbred strains A (H-2^a), C57BL/6 (B6) (H-2^b), and their F1 hybrids, B6xAF1 (H-$2^{b/a}$) (B6AF1) were used. All mice were bred and maintained in our animal colony. Donor A animals were 6-12 months old. B6AF1 recipients were older than 3 months. Both sexes were employed in the experiments.

Adrenalectomy

B6AF1 mice were adrenalectomized (ADX) at 9-10 weeks of age and were used at different times after adrenalectomy as indicated in the results section. Surgical adrenalectomy was performed under ether anesthesia as

described previously (25). ADX mice were maintained on physiological saline in lieu of drinking water for the duration of the experiment.

Thymectomy

B6AF1 mice at 7-8 weeks of age were surgically thymectomized. Two weeks after recovery from thymectomy the mice were adrenalectomized.

Induction of GVHD

GVHD was induced as previously described (5). Briefly, single cell suspensions were prepared from pooled spleens and lymph nodes by gently tamping them through a 50-mesh stainless steel screen. GVHD was induced with $20x10^6$ A strain parental lymphoid cells injected i.v. into age-matched unirradiated ADX and non-ADX B6AF1 hybrid recipients. B6AF1 mice injected with B6AF1 lymphoid cells were used as syngeneic controls. Normal B6AF1 mice received no injection.

Mitogen Assays

On different days after GVHD induction, the spleen was removed from individual B6AF1 recipient and made into a single cell suspension. T cell

proliferative responses to Con A (Pharmacia, Fine Chemicals, Uppsala, Sweden) and PHA (Wellcome Research Reagent Limited, Dartford, UK), and B cell responses to LPS (Sigma) were evaluated by [^{3}H] thymidine incorporation, as previously described (5). Briefly, $5x10^5$ splenic cells were cultured for 48 hrs in triplicate with or without mitogen in a 96 well titer plates. The plates were pulsed with 1 μCi/well of [^{3}H] thymidine for 16 hrs and then harvested with an automated cell harvester. [^{3}H] thymidine incorporation was quantitated on a beta counter (LKB Instruments, Turku, Finland).

Histology of the liver and lymph nodes (LN)

The liver was carefully removed from each mouse and fixed in 5% formalin. The fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin-eosin, as previously described (4). Stained tissues were then examined with a light microscope. The different intensities of injury in the liver were graded as normal, mild, moderate or severe. In a normal liver, there was no sign of mononuclear cell infiltrates. In livers with mild injury, some, but not all, portal tracts contained small number (up to 10) of mononuclear cells. With moderate injury all portal tracts contained an increasing number of infiltrates (up to 20). Severe hepatic lesions were characterized by a large number of mononuclear cells infiltrating all portal

tracts. In addition, the hepatic lobule often contained a modest diffuse mononuclear cell infiltrate and rare necrotic hepatocytes.

The axillary and brachial LN were carefully removed from each mouse and fixed in 5% formalin. The fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Stained tissues were then examined with a light microscope. The different intensities of injury in the LN were graded as normal, mild, moderate or severe. Mild injury in the LN featured slight nodal enlargement and mild immunoblastic/plasmacytic proliferation in paracortical regions. With moderate injury there was a greater immunoblastic proliferation and patchy nodal necrosis. Severe injury featured massive lymphoid cell necrosis/depletion and extensive macrophage replacement of nodal tissue.

Immunofluorescence Staining

On different days after GVHD induction, spleens were removed from B6AF1 recipients and single cell suspensions were made from each spleen. Staining was performed in 96 well plates. One million cells in a 50 ul volume of Hanks' balanced salt solution (GIBCO., Grand Island, NY) were incubated with 50 ul of the appropriate reagents on ice for 30 mins. After each incubation the plates were washed twice. Incubation and washing was repeated for the secondary reagent. Labelled samples were fixed in 1 ml

of 1% paraformaldehyde and stored at 4°C. The fixed cell samples were analyzed by a FACScan (Becton-Dickinson, Mountain View, CA) and 5000 flow cytometry events were counted per sample. A selective gate, excluding cell debris and erythrocytes, was set using forward light scatter.

Reagents for Flow Cytometry

FITC conjugated anti-$H2K^b$ (Clone AF6-88.5), phycoerythrin conjugated anti-CD4 (Clone YTS 169.4) and biotinylated anti-CD8 (Clone 53-6.7) mAbs were employed for triple immunofluorescence. All mAbs and the secondary reagent, strepavidin-cychrome, were purchased from Cedarlane, Hornby, Ontario, Canada. All reagents were diluted as recommended.

Southern Dot Blot Analysis of Male Parental Cells in Spleens of Female F1 recipients.

Non-ADX and ADX female B6AF1 hybrids were injected with $20x10^6$ male A strain splenic and LN cells. On day 21, the spleen was removed from each animal and DNA was extracted from each using standard procedures (26). Splenic chimerism was assayed by probing for male parental DNA in female F1 spleens, using a cDNA probe (pY2) specific for the male Y chromosome (a gift from Dr. E. Palmer) (27). Briefly, 5 ug samples of DNA

was blotted onto Zeta-Probe membranes (Bio-Rad). Membranes were hybridized with ^{32}P-labelled probe ($7.5x10^6$ cpm) and blots were exposed to Kodak XAR X-ray film, at -70°C for 1-2 days.

Dot blots were scanned by a densitometer (BioImage, Millipore, Mississauga, Ontario, Canada) and were compared with known values of male:female cells consisting of 0:100, 25:75, 50:50, 75:25 and 100:0. The densitometer reading was able to estimate the % of male cells within a 5-10% error.

Cortisone acetate treatment

B6AF1 mice were injected i.p. with a single dose of 2.5 mg of cortisone acetate (Merck Sharp & Dohme Canada., Quebec, Canada), in a total volume of 0.5 ml PBS. Control animals were treated with PBS only. Two days after treatment, the mice were injected i.v. with $20x10^6$ A strain parental lymphoid cells.

Anti-asialoGM1 (anti-ASGM1) treatment

B6AF1 mice were injected i.v. with a single dose (70 ug) of anti-ASGM1 antibody (Wako Chemicals, Dallas, Texas). Two days after the treatment, the animals were injected i.v. with $20x10^6$ A strain parental lymphoid cells.

Anti-NK1.1 and anti-CD8 treatment

Supernatants containing anti-NK1.1 (clone PK 136) or anti-CD8 (clone 2.43) antibodies were collected and concentrated with ammonium sulfate. B6AF1 mice were injected i.v. with either 0.5 ml of anti-NK1.1 or anti-CD8 concentrated supernatant three times at one day intervals. Two days after the last treatment, the mice were injected i.v. with $20x10^6$ A strain parental lymphoid cells.

RESULTS

Rapid recovery of immune function from GVHD-induced immunosuppression

To eliminate endogenous glucocorticoids, B6AF1 hybrids were surgically adrenalectomized two months before GVHD was induced with $20x10^6$ A strain parental lymphoid cells. One of the hallmarks of GVHD is profound suppression of T and B cell functions (1, 2). In this experiment, immune function was assessed by the ability of splenic T cells to respond to Con A and PHA, and B cells to LPS. Injection of A strain parental lymphoid cells into non-ADX F1 recipients led to GVHD-induced immunosuppression on days 13 and 18-21 as demonstrated in Figure 1. ADX F1 mice injected with parental lymphoid cells were also immunosuppressed on day 13, however, by day 18-21 most (8/10) of these animals had a near normal responses to mitogens. The mitogenic proliferative responses of ADX syngeneic mice were similar to the age-matched non-ADX counterparts (Fig. 1). Similar findings were observed on day 21 in recipients adrenalectomized for 4 or 12 months (results not shown). Such a rapid spontaneous immune recovery from GVHD-induced immunosuppression has not been observed before. In an earlier study, non-ADX B6AF1 recipients injected with $20x10^6$ A strain parental lymphoid cells regained immune function only 5-6 months after

GVHD induction (28).

Regression of tissue lesions in the livers and LN of ADX GVHD mice

Many lymphoid and non-lymphoid organs develop pathological lesions during GVHD (3-6). To further evaluate the symptoms of GVHD, we performed histological examinations on the livers and LN of B6AF1 recipients injected with $20x10^6$ A strain parental lymphoid cells. Table I shows that an increasing number of non-ADX GVHD mice were experiencing severe lesions in the livers and LN on day 21 compared to day 9 and 13 implying that tissue lesions in non-ADX GVHD mice worsened with disease progression. In contrast, none of the ADX GVHD mice exhibited severe hepatic and LN injury by day 21, although approximately half of the ADX GVHD animals experienced severe tissue lesions on day 13. Most (7/9) of the animals in the ADX GVHD group on day 21 displayed moderate lesions only and two mice had normal livers and LN, suggesting that histopathological injury to lymphoid and non-lymphoid organs of the ADX recipients was regressing.

Recovery of host splenic $CD4^+$ and $CD8^+$ T cell populations in ADX GVHD mice

In the first two weeks of GVHD, T lymphocytes, predominantly $CD8^+$ cells, undergo massive proliferation in the spleens leading to an inverted $CD4^+/CD8^+$ ratio (29). Following the proliferative phase, the third and fourth weeks are designated as "cytotoxic" and consist of a marked reduction in T cell numbers, in particular, a greater loss of $CD4^+$ cells and again the $CD4^+/CD8^+$ ratio is reversed (29). In this study, triple immunofluorescence was employed to identify splenic $CD4^+$ and $CD8^+$ cells of host (F1, $H2K^b$ positive) origin. Flow cytometry illustrated that host $CD4^+$ and $CD8^+$ T cells in the spleens of non-ADX GVHD mice underwent changes similar to those reported previously during the proliferative (Fig. 2E-F) and cytotoxic (Fig. 2I-J) phases. Conversely, after an initial "proliferative" phase on day 13 (Fig. 2G-H), ADX GVHD animals on day 18-21 (Fig. 2K-L) displayed a T cell profile similar to the ADX SYN group (Fig. 2C-D), concomitant with a near normal host $CD4^+/CD8^+$ T cell ratio. These results were reproducible in F1 recipients adrenalectomized for 4 or 12 months (data not shown). Collectively, the lack of endogenous glucocorticoids by surgical adrenalectomy allowed for a rapid recovery from symptoms characteristic of GVHD.

Marked reduction of parental lymphoid cells after an initial period of engraftment

Having established that adrenalectomy ameliorated GVHD, we next investigated whether the rapid recovery was associated with parental cell engraftment. Using an antibody against $H2K^b$ antigens, we distinguished unlabelled A ($H2^a$) strain parental lymphoid cells from labelled host F1 cells. On day 13, high absolute numbers of parental cells were detected in the spleens of both non-ADX GVHD ($31.1x10^6$) and ADX GVHD ($22.0x10^6$) mice (Table II). Parental T cells ($CD4^+$ and $CD8^+$ cells) constituted the majority of the parental cell population in both GVHD groups on day 13 (Fig. 2E-H & Table II). As the disease progressed to day 18-21, the absolute number of parental T cells was markedly reduced in both GVHD groups, however, the number of splenic parental non-T cells ($CD4^-8^-$ cells) in non-ADX GVHD animals increased three fold to $14.4x10^6$ (Table II). In contrast, parental non-T cells remained very low ($2.0x10^6$) in the ADX GVHD group (Fig. 2K-L & Table II). Absence of parental cells in the lymph nodes of ADX GVHD mice was also observed on day 18-21 (results not shown). Thus at the time of recovery from GVHD symptoms, ADX GVHD mice had very few parental cells in the peripheral lymphoid organs.

To further confirm the degree of splenic chimerism, male parental lymphoid cells were injected into ADX female F1 recipients and the male parental cells were identified using a cDNA probe (pY2) specific for the male Y chromosome (27). Southern dot blot analysis consistently showed that a high proportion (50-60%) of parental DNA was present in the spleens

of non-ADX GVHD animals on day 21 (Fig. 3). Conversely, the spleens of ADX GVHD mice contained very little (less than 5%) or near background levels of male DNA (Fig. 3), consistent with the results obtained from flow cytometry (Fig. 2I-L & Table II). Coincident with the recovery from GVHD, parental cells in the peripheral lymphoid organs of ADX GVHD mice were markedly decreased by day 18-21, after an initial period of engraftment that lasted for two weeks. It appears that the recovery from GVHD is mediated by the induction of an F1-anti-parental mechanism.

Abrogation of F1-anti-parental mechanism by cortisone treatment.

ADX, but not non-ADX, F1 recipients eliminated the parental lymphoid cells. Furthermore, non-ADX GVHD mice were shown to display persistent high levels of plasma corticosterone during the course of the disease (21, 22). We therefore tested whether the F1-anti-parental mechanism was sensitive to glucocorticoids by treating ADX F1 recipients with a single dose of cortisone acetate two days before GVHD induction. GVHD was assessed for immunosuppression and splenic chimerism. Cortisone acetate treatment abrogated the F1-anti-parental mechanism and resulted in lethal GVHD since all of these mice died by day 18 and prior to death, they showed the physical features of acute GVHD such as weight loss, diarrhea, hunched posture and piloerection. In addition, cortisone acetate-treated

ADX GVHD animals were severely immunosuppressed and displayed high splenic chimerism (26.0%) on day 13 (Table III). Immunosuppression in the cortisone acetate-treated ADX GVHD group on day 13 was not due to the treatment since splenic T and B cell mitogenic responses of syngeneic ADX mice treated with cortisone were similar to syngeneic ADX mice treated with saline (results not shown). These results strongly suggest that the F1-anti-parental mechanism is sensitive to glucocorticoids.

Phenotypic characterization of the effector cell mediating the F1-anti-parental response.

Studies were next carried out to determine the cellular basis for the F1-anti-parental response. In other models of F1-anti-parental reactivity in vivo, the effector cell is sensitive to anti-ASGM1 treatment (30). To test this possibility ADX F1 recipients were injected with anti-ASGM1 antibody two days prior to GVHD induction. As previously reported (31, 32), splenic NK cell activity against YAC-1 target cells was abrogated two days after treatment with anti-ASGM1 antibody (Table III). Furthermore, this treatment abolished the F1-anti-parental mechanism leading to severe immunosuppression and a high degree of splenic chimerism (17.4%) on day 18 (Table III). These studies imply that the F1-anti-parental mechanism is mediated by an ASGM1$^+$ effector cell. Since NK cells are sensitive to

anti-ASGM1 treatment we tested the possibility whether the F1-anti-parental effector cell was an NK cell. Because other cell types are also sensitive to anti-ASGM1 treatment (33, 34), we opted to use a more specific antibody against NK cells. As previously documented (35, 36), an antibody against the NK1.1 antigen specifically eliminates NK cell activity. Therefore, ADX F1 recipients were treated with anti-NK1.1 antibody (clone PK136) two days before injection of parental lymphoid cells. Two days after treatment with anti-NK1.1 antibody splenic NK cell activity against YAC-1 target cells was eliminated (Table IV), however, the F1-anti-parental mechanism was not affected. ADX F1 mice treated with anti-NK1.1 antibody and injected with parental lymphoid cells did not experience GVHD-induced immunosuppression and displayed an absence of parental chimerism on day 21 (Table IV). It could be argued that the anti-NK1.1 treatment affected the ability of the parental lymphoid cells to induce GVHD. This is not the case since non-ADX F1 recipients injected with anti-NK1.1 antibody were immunosuppressed and exhibited high splenic chimerism (26.8%) on day 21 (Table IV). Although the F1-anti-parental mechanism is sensitive to anti-ASGM1 treatment, it is not sensitive to anti-NK1.1 treatment thus, implicating the role of an ASGM1$^+$ effector that is not an NK cell.

Our laboratory recently reported a significant reduction in the incidence of CD8$^+$, but not CD4$^+$, T cells in the spleens of mice treated with

anti-ASGM1 antibody (37). Therefore, to further characterize the phenotype of the F1-anti-parental effector, ADX F1 recipients were thymectomized (TX) and treated with anti-CD8 antibody. Thymectomy was performed simply to prevent the possibility of new $CD8^+$ cells emerging from the thymus after treatment with anti-CD8 antibody. Flow cytometry showed that this treatment eliminates 95-97% of host $CD8^+$ T cells in the spleens (Table IV). Anti-CD8 treatment of TX ADX recipient mice abrogated the F1-anti-parental mechanism resulting in severe immunosuppression and high percent of splenic chimerism (23.3%) on day 21 (Table IV). Taken together, the F1-anti-parental mechanism appears to be mediated by an effector cell population(s) that is/are suppressed or deleted in the presence of high levels of glucocorticoids.

DISCUSSION

Previous reports demonstrated that animals undergoing GVHD secrete excessive amounts of adrenal glucocorticoids into the circulation (21, 22). In this report, the effects of endogenous adrenal glucocorticoids on the outcome of GVHD in mice was investigated. We showed that unirradiated non-ADX B6AF1 animals injected with $20x10^6$ A strain parental lymphoid

cells experienced symptoms characteristic of GVHD on day 13, which became progressively more severe on day 18-21. In contrast, ADX B6AF1 recipients injected with the same dose of A strain parental lymphoid cells displayed symptoms characteristic of GVHD in the first two weeks, however, the ADX recipients recovered rapidly from GVHD by day 18-21. Coincident with the recovery from GVHD was the disappearance of nearly all of the parental cells in the spleens and LN, after an initial two week period of engraftment as measured by flow cytometry and Southern dot blot analysis. The parental cells appeared to be markedly decreased by an F1-anti-parental mechanism that was sensitive to steroid treatment and treatment with either anti-ASGM1 or anti-CD8 antibody, but not anti-NK1.1 antibody.

In vivo F1 reactivity against lymphoid cells of certain parental strains is a well documented phenomenon known as hybrid resistance (8-12). However, F1-anti-parental reactivity described herein exhibits unique properties that sets it apart from hybrid resistance. First, ADX B6AF1 hybrids were able to eliminate A strain parental lymphoid cells, whereas hybrid resistance by the B6AF1 hybrids is directed against B6 (H-2^b), but not A (H-2^a), strain parental cells (38, 39). Second, the F1-anti-parental response in our model occurs three weeks after engraftment, in contrast, hybrid resistance occurs within 48 hours (10, 38, 39). Third, ADX F1 hybrids injected with parental lymphoid cells experienced symptoms of an

early GVHD, however, GVHD symptoms do not appear in hybrid resistance (13, 14). Fourth, the F1-anti-parental mechanism in our model was sensitive to corticosteroids, conversely, hybrid resistance is not (40). Fifth, the F1-anti-parental effector cell in our model was not sensitive to anti-NK1.1, whereas it is in hybrid resistance (35, 41). Therefore, it would appear that the F1-anti-parental phenomenon observed in this study has not been previously described and it is not mediated by the classical hybrid resistance mechanism.

Dennert et al. has implicated an effector T cell in the rejection of allogeneic marrow, however, the effector cell displayed unique features in that the precursor cell was $CD3^+CD8^-NK1^+$ which differentiated into a $CD3^+CD8^+NK1^-$ cell (42-44). However, since we were able to eliminate the precursor effector cell by anti-CD8, but not anti-NK1.1, antibody pretreatment, the precursor cell described by us was therefore $NK1^-CD8^+$. Thus, it seems highly unlikely that the F1-anti-parental effector cell described by us is the same as the one described by Dennert et al. We do not know from the present results whether we are dealing with a single effector cell or the interaction of two or more different cells. To our knowledge a cortisone sensitive $ASGM1^+CD8^+NK1.1^-$ cell has not been described previously in the periphery. Thus the F1-anti-parental mechanism might involve one cell that is $ASGM1^+$ and another that is $CD8^+$; either or both may be sensitive to glucocorticoids. It will now be

necessary to perform reconstitution experiments using purified cell populations in order to identify the different cells involved and their precise phenotype.

It is interesting that parental cells were markedly decreased after an initial period of engraftment and after induction of GVHD. This suggest that the F1-anti-parental mechanism was induced during the early GVHD (ie. first two weeks). The mechanism responsible for inducing the F1-anti-parental response is unknown. One possibility is that the F1-anti-parental effector cells ($CD8^+$) must reach a sufficiently high number in order to cause the reduction of parental cells by day 18-21. This critical number might be achieved as a result of F1-anti-parental effector cell proliferation in response to lymphokines, including IL-2, IL-3, IFN-γ and GM-CSF, secreted by activated parental T cells in the first two weeks of GVHD (45, 46). In fact, our results showed an increased number of F1 (host) $CD8^+$ cells on day 13 (Fig. 2H), consistent with the findings of other investigators who demonstrated that F1 (host) $CD8^+$ cells undergo massive proliferation in the second week of GVHD (29). It is thus possible that one function of the expanded F1 $CD8^+$ population is to regulate the immune reaction mediated by the parental cells. In the absence of adrenal glucocorticoids these $CD8^+$ cells become more reactive against the parental cells. Another possible mechanism in the induction of the F1-anti-parental response is activation of the parental cells to express surface molecules, which may serve as

target antigens recognized by the F1-anti-parental effector cell (47-49). Thus we speculate that a variety of lymphokines produced during early GVHD and/or target antigens expressed on activated parental cells might play a role in inducing the F1-anti-parental response resulting in a marked decrease of parental cells by day 18-21.

On examination of the different parental populations ($H2K^b$ negative) in the spleens of ADX GVHD mice compared to non-ADX counterparts, our results suggest that parental T cells did not appear to be targeted by the F1-anti-parental mechanism. An increase in parental T cells was observed up to day 13 followed by a rapid decrease in both GVHD groups by day 18-21 (Fig. 2I-L & Table II). Similar observations were described by Hakim et al. showing splenic parental T cells of non-ADX GVHD mice underwent proliferation in the first two weeks of GVHD, followed by a drastic loss of parental T cells in the third week of GVHD (29). Conversely, our results demonstrate that parental non-T cells ($CD4^-8^-$) were the parental population targeted by the F1-anti-parental effector cell, as demonstrated by the observations that very few parental non-T cells were detected in the spleens of ADX GVHD on day 18-21 (Fig. 2K-L & Table II), in comparison to a marked increase in the non-ADX GVHD group (Fig. 2I-J & Table II). This observation would argue against the veto phenomenon as being responsible for the elimination of parental cells since F1 veto cells target parental T cells (ie. CTL precursor) (50-52). The injection of parental spleen, but not

lymph node, cells into unirradiated F1 hybrids can give rise to long term stable chimerism, thus demonstrating that hematopoietic stem cells are present in the spleens of adult mice (53, 54). Since parental splenic cells were used to induce GVHD, the loss of parental non-T cells is likely to include stem cells present in the parental graft. This suggests that the F1-anti-parental effector cell may target stem cells and thus prevent long-term engraftment and chimerism.

An important question that needs to be addressed is whether the F1-anti-parental mechanism observed in ADX B6AF1 recipients also exists in non-ADX counterparts. When non-ADX F1 mice were treated with cortisone two days before injection of parental lymphoid cells they exhibited physical traits of a more severe GVHD as compared to PBS-treated animals (Table III). In addition, Ghayur et al. showed that non-ADX B6AF1 mice treated with cortisone two days before injection of B6 lymphoid cells experienced a more severe GVHD and an enhanced number of fluorescein-labelled B6 cells was recovered from the spleens of cortisone-treated animals (3). Taken together, our results and those reported by Ghayur et al. suggest that the F1-anti-parental effector cell is present in non-ADX F1 mice and is regulated by basal levels of glucocorticoids, however, situations in which the levels of endogenous glucocorticoids are elevated, the F1-anti-parental effector cell is deleted or suppressed. This is consistent with the findings that high splenic chimerism persisted on day 18-21 in non-ADX F1

recipients injected with parental lymphoid cells and that these non-ADX F1 recipients animals did not recover from GVHD. Elevated levels of glucocorticoids in the circulation of non-ADX F1 mice during GVHD (21, 22) would likely suppress or delete the F1-anti-parental effector cell resulting in GVHD and persistent chimerism on day 18-21. Hence, the F1-anti-parental effector cell appears to function in F1 mice with intact adrenals, but is regulated by endogenous glucocorticoid production.

It is of interest to note that an earlier report demonstrated that F1 mice adrenalectomized before injection of parental cells recovered rapidly from symptoms of an early GVHD (55). In addition, other investigators have shown that treatment with metyrapone, a drug that blocks glucocorticoids production, significantly reduced GVHD and prolonged survival of F1 recipients injected with parental cells, whereas, untreated F1 animals died from GVHD-induced mortality (56). Although the mechanism responsible for GVHD amelioration in these studies was not determined, our results would suggest that a steroid sensitive F1-anti-parental mechanism may have been induced and therefore allowed the F1 recipients to recover from GVHD.

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Footnotes

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[4]Abbreviations used in this paper: ADX, adrenalectomized; ASGM1, asialoGM1; GVHD, graft-versus-host disease; LN, lymph nodes; non-ADX, non-adrenalectomized; SYN, syngeneic.

Table I. Histological lesions in the liver and LN of non-ADX and ADX GVHD mice[a,b]

Groups	Days after GVHD induction	Organs examined	Intensity of lesions (frequency/total)			
			Normal (%)	Mild (%)	Moderate (%)	Severe (%)
Non-ADX SYN	—	Liver	3/3 (100)	0/3	0/3	0/3
		LN	3/3 (100)	0/3	0/3	0/3
ADX SYN	—	Liver	3/3 (100)	0/3	0/3	0/3
		LN	3/3 (100)	0/3	0/3	0/3
Non-ADX GVHD	9	Liver	0/6	2/6 (33.3)	3/6 (50.0)	1/6 (16.7)
		LN	1/6 (16.7)	1/6 (16.7)	3/6 (50.0)	1/6 (16.7)
ADX GVHD	9	Liver	0/7	1/7 (14.3)	5/7 (71.4)	1/7 (14.3)
		LN	0/7	0/7	6/7 (85.7)	1/7 (14.3)
Non-ADX GVHD	13	Liver	0/6	0/6	4/6 (66.7)	2/6 (33.3)
		LN	0/6	0/6	3/6 (50.0)	3/6 (50.0)
ADX GVHD	13	Liver	0/7	1/7 (14.3)	2/7 (28.6)	4/7 (57.1)
		LN	0/7	1/7 (14.3)	3/7 (42.9)	3/7 (42.9)
Non-ADX GVHD	21	Liver	0/7	1/7 (14.3)	2/7 (28.6)	4/7 (57.1)
		LN	0/7	1/7 (14.3)	1/7 (14.3)	5/7 (71.4)
ADX GVHD	21	Liver	2/9 (22.2)	0/9	7/9 (77.8)	0/9
		LN	2/9 (22.2)	0/9	7/9 (77.8)	0/9

[a]B6AF1 recipient mice were adrenalectomized 4 months before i.v. injection of either 20×10^6 A strain parental (GVHD) or B6AF1 (SYN) lymphoid cells.

[b]The different intensities of lesions in the liver and LN are described in greater detail in the *Materials and Methods* section.

Table II. Incidence and absolute numbers of donor T and non-T cell populations in the spleens of GVHD mice[a]

Groups	Days after GVHD induction	Splenic Cellularity mean x10^6 (SE)	Incidence of Donor Cells as % mean (SE)			Absolute Number of Donor Cells[c] x10^6 mean (SE)			
			$CD4^+$	$CD8^+$	$CD4^-8^-$[b]	$CD4^+$	$CD8^+$	$CD4^-8^-$	Total
Non-ADX (7)[d]	13	115.3 (11.9)	12.7 (0.8)	9.6 (0.5)	4.7 (0.7)	14.6 (1.6)	11.1 (1.6)	5.4 (0.8)	31.1
ADX[e] (7)	13	186.5 (18.7)	3.4 (0.5)	6.0 (1.2)	2.4 (0.0)	6.3 (1.2)	11.2 (2.9)	4.5 (0.0)	22.0
Non-ADX (10)	18-21	89.7 (26.0)	1.6 (0.6)	2.9 (1.0)	16.1 (3.3)	1.4 (0.5)	2.6 (1.0)	14.4 (1.4)	18.4
ADX (10)	18-21	249.3 (16.1)	0.3 (0.2)	0.2 (0.1)	0.8 (1.1)	0.7 (0.3)	0.5 (0.2)	2.0 (1.0)	3.2

[a]All B6AF1 mice were injected i.v. with 20x10^6 A strain parental lymphoid cells.
[b]The incidence of $CD4^-8^-$ cells was obtained as: % total number of donor cells - (% donor $CD4^+$ + % donor $CD8^+$).
[c]The absolute number of donor cells was calculated as: (Splenic cellularity X Incidence)/100
[d]Number of mice per group in parentheses.
[e]Mice were adrenalectomized two months before GVHD was induced.

Table III. Effects of cortisone acetate or anti-ASGM1 treatment on the F1-anti-parental mechanism[a,b,c]

Experimental group	Days after GVHD induction	Splenic mitogenic responses as % of normal (mean ± SE)			Splenic chimerism as % of parental cells (mean ± SE)
		Con A	PHA	LPS	
Saline (3)[d]	13	12.5 ± 8.5	3.8 ± 4.4	-8.9 ± 8.6	8.7 ± 1.3
Cortisone acetate (5)	13	-2.0 ± 0.1	-3.0 ± 0.5	-3.5 ± 1.1	26.0 ± 4.9
Anti-ASGM1 (3)	13	-2.0 ± 0.1	-2.0 ± 0.5	-3.0 ± 1.1	32.5 ± 2.8
Saline (6)	18	66.3 ± 10.8	65.0 ± 9.3	58.8 ± 10.0	3.1 ± 1.0
Cortisone acetate	18	NA[e]	NA	NA	NA
Anti-ASGM1 (6)	18	5.1 ± 5.2	5.4 ± 5.5	5.0 ± 1.9	17.4 ± 4.6

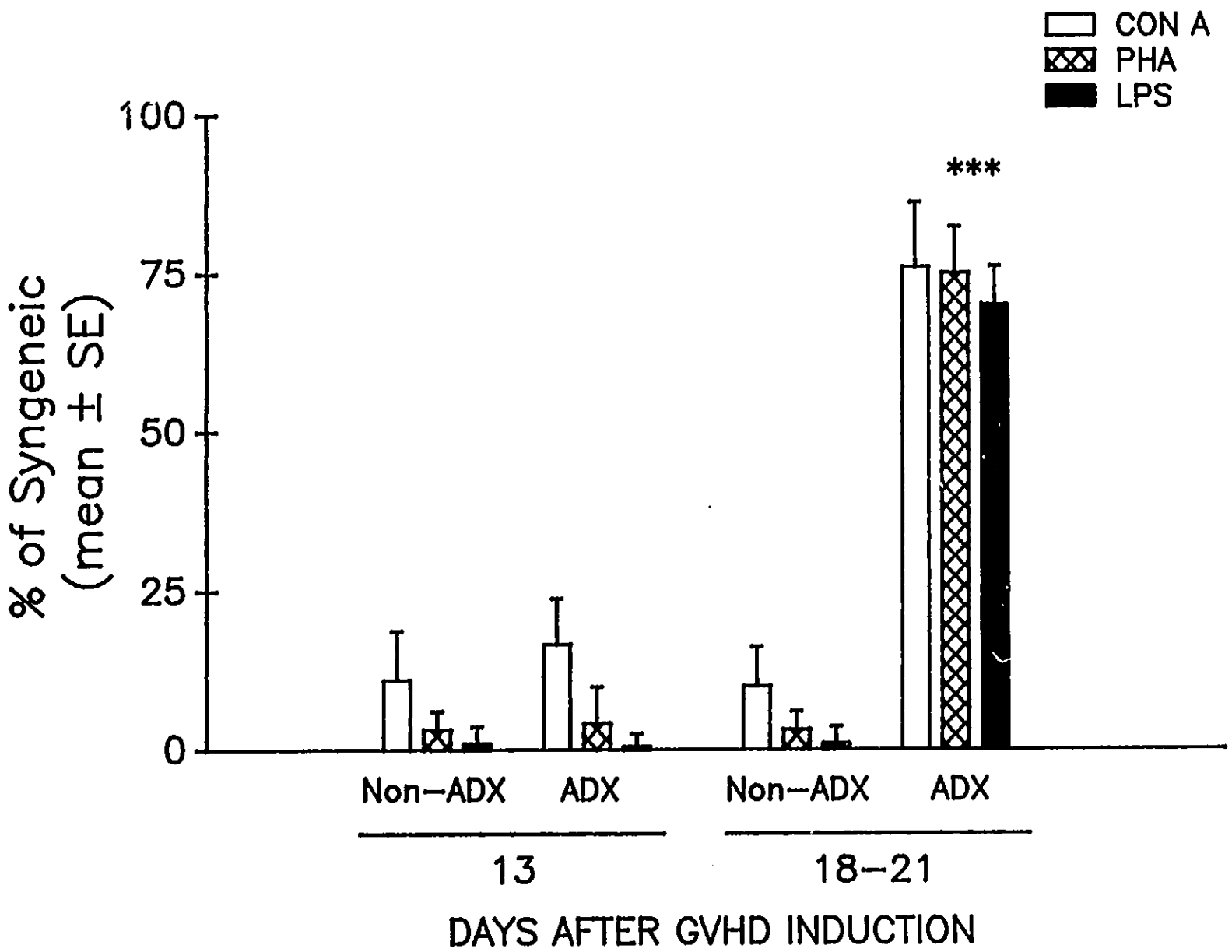
[a]All B6AF1 mice were adrenalectomized 2 months before GVHD induction with $20x10^6$ A strain parental lymphoid cells.
[b]Mice were treated either with cortisone acetate or anti-ASGM1 antibody two days before GVHD was induced.
[c]Two days after anti-ASGM1 treatment splenic NK cell activity against YAC-1 target cells was eliminated.
[d]Number of mice per group in parentheses.
[e]Not available due to mortality.

Table IV. Phenotypic characterization of the F1-anti-parental effector cell[a]

Experimental group	Treatment[b]	Splenic mitogenic responses as of % normal (mean ± SE)			Splenic chimerism as % of parental cells (mean ± SE)
		CON A	PHA	LPS	
Non-ADX (5)[c]	PBS	8.1 ± 3.0	1.7 ± 2.0	0.4 ± 1.5	16.4 ± 4.8
Non-ADX (5)	Anti-NK1.1[d]	11.1 ± 6	4.9 ± 4.0	3.0 ± 1.0	26.8 ± 5.6
TX[e] Non-ADX (5)	PBS	8.1 ± 3.4	1.7 ± 2.0	0.4 ± 1.5	15.0 ± 4.9
TX Non-ADX (4)	Anti-CD8[f]	3.1 ± 0.5	0.3 ± 0.3	4.9 ± 1.6	13.3 ± 5.1
ADX[g] (6)	PBS	73.5 ± 10.2	78.4 ± 14.6	81.8 ± 8.3	0
ADX (5)	Anti-NK1.1	75.5 ± 3.1	89.4 ± 9.6	50.9 ± 8.8	0
TX ADX (6)	PBS	73.6 ± 10.2	78.4 ± 14.7	81.8 ± 8.3	0
TX ADX (3)	Anti-CD8	5.7 ± 2.0	7.2 ± 3.0	11.0 ± 10.0	23.3 ± 6.1

[a]All B6AF1 mice were injected with $20x10^6$ A strain parental lymphoid cells to induce GVHD and were sacrificed 21 days later.
[b]Treatment was given two days before GVHD was induced.
[c]Number of mice in parentheses.
[d]Two days after anti-NK1.1 treatment splenic NK cell activity against YAC-1 target cells was eliminated.
[e]Thymectomy (TX) was performed two weeks before adrenalectomy.
[f]Flow cytometry showed that two days after anti-CD8 treatment 95-97% of the splenic $CD8^+$ T cell population was eliminated.
[g]Adrenalectomy was performed four months before GVHD induction.

Figure 1. Rapid recovery of immune function following GVHD-induced immunosuppression. The data are presented as the percent of the mean ± SE of the respective control groups injected with syngeneic cells. Each GVHD group had 7-10 mice. Mean mitogenic responses in cpm ($x10^4$) of non-adrenalectomized (non-ADX) B6AF1 mice injected with syngeneic cells were: Con A 16.3 ± 0.28, PHA 12.1 ± 0.12 and LPS 5.7 ± 0.03 and ADX B6AF1 mice injected with syngeneic cells were: Con A 13.3 ± 0.26, PHA 9.8 ± 0.15 and LPS 5.2 ± 0.06. The student's *t* test was used to calculate the statistical significance, (***) $p < 0.001$, of ADX GVHD mice compared to non-ADX GVHD animals on day 18-21.


CON A
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0
% of Syngeneic
(mean ± SE)
***
Non–ADX
ADX
Non–ADX
ADX
13
18–21
DAYS AFTER GVHD INDUCTION

Figure 2. Incidence of host $CD4^+$ and $CD8^+$ T cell populations in the spleens of non-ADX and ADX GVHD mice. T cells of F1 origin ($H\text{-}2^{b/a}$) are labelled for anti-$H2K^b$, in contrast, to unlabelled parental ($H\text{-}2^a$) cells. Representative FACScan profiles are illustrated as (A, B) Non-ADX SYN, (C, D) ADX SYN, (E, F) Non-ADX GVHD on day 13, (G, H) ADX GVHD on day 13, (I, J) Non-ADX GVHD on day 18 and (K, L) ADX GVHD on day 18. The percent of host $CD4^+$ or $CD8^+$ T cells is shown in the upper right quadrant of each profile. The host $CD4^+/CD8^+$ T cell ratio for each group is represented by the values to the right of the FACScan profiles.

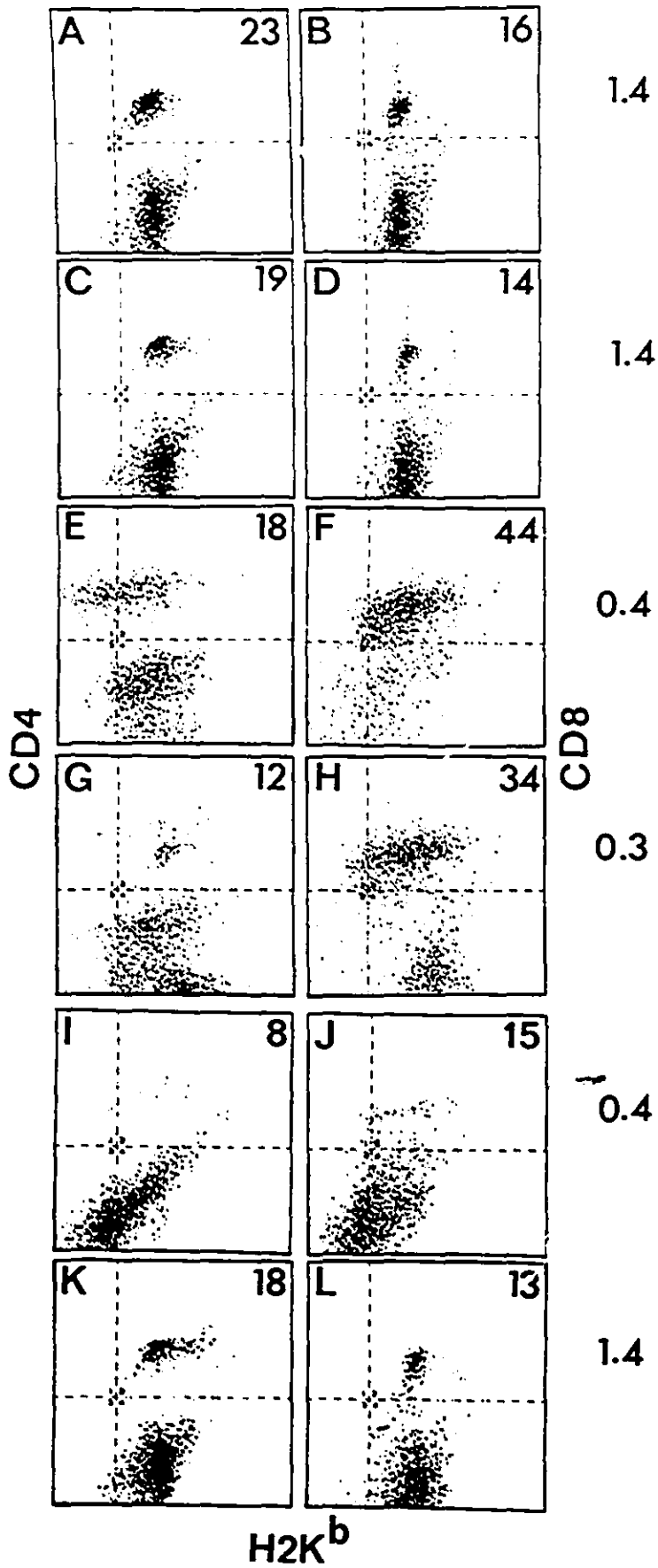
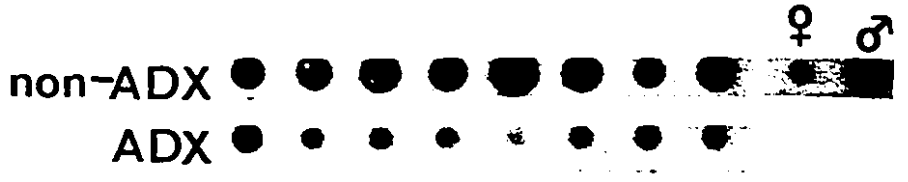

A 23
B 16
1.4
C 19
D 14
1.4
E 18
F 44
0.4
CD4
CD8
G 12
H 34
0.3
I 8
J 15
0.4
K 18
L 13
1.4
H2K^b

Figure 3. Detection of male chimerism in spleens of female B6AF1 recipients using a cDNA probe specific for the male Y chromosome. Age-matched female non-ADX and ADX B6AF1 recipients were injected i.v with $20x10^6$ male A strain parental lymphoid cells. On day 21 after GVHD induction DNA was extracted from the spleen of each mouse. Using Southern dot blot analysis parental male DNA was detected using a cDNA probe specific for the male Y chromosome. Each dot blot represents an individual animal. DNA extracted from the spleens of normal male (♂) and female (♀) B6AF1 mice were used as positive and negative controls respectively. A probe specific for B-actin was used as control and showed no significant differences between non-ADX and ADX GVHD groups (results not shown).


♀
♂
non-ADX
ADX

CHAPTER 4 NON-RENEWABLE EXTRATHYMIC DERIVED F1-ANTI-PARENTAL EFFECTOR CELL THAT CAUSES RECOVERY FROM MURINE GRAFT-VERSUS-HOST DISEASE[1]

The studies presented in this chapter is a follow up of Chapter 3 to further characterize the properties of the F1-anti-parental effector cell.

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Non-renewable Extrathymic Derived F1-Anti-Parental Effector Cell That Causes Recovery from Murine Graft-versus-Host Disease

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Running title: Extrathymic Effector Cell in Recovery from Murine GVHD

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Abstract

We have previously demonstrated that adrenalectomized (ADX), but not non-ADX, (C57BL/6 X A)F1 (B6AF1) mice recovered rapidly from graft-versus-host disease (GVHD) as a result of the induction of a glucocorticoid-sensitive F1-anti-parental mechanism that was mediated by an effector cell that eliminated the parental graft, after an initial period of engraftment. The F1-anti-parental effector cell was sensitive to anti-asialoGM1 (ASGM1) and anti-CD8, but not anti-NK1.1 treatment. Since the F1-anti-parental effector cell was $CD8^+$, we further investigated whether the effector cell was thymic derived. B6AF1 hybrid mice were thymectomized (TX) at 6-7 weeks of age and were treated with either cortisone acetate (CA) or anti-ASGM1 antibody two weeks later, followed by adrenalectomy two days after treatment with either CA or anti-ASGM1 antibody. Five months after adrenalectomy GVHD was induced with an injection of $20x10^6$ A strain parental lymphoid cells. Evidence for the induction of the F1-anti-parental mechanism was a lack of GVHD symptoms as characterized by the recovery from immunosuppression, normal number of host $CD4^+$ and $CD8^+$ T cell populations in the peripheral lymphoid organs, and near absence of donor cells by day 21 after GVHD induction. Our results demonstrate that the F1-anti-parental effector cell was renewed 5 months after CA treatment in TX ADX B6AF1 recipients and allowed recovery from GVHD. Conversely,

the effector cell was not renewed even 5 months after anti-ASGM1 treatment in either TX or sham-TX ADX B6AF1 recipients, thus resulting in GVHD. Taken together these results suggest that the F1-anti-parental effector cell was not dependent on a mature thymus and was renewed after glucocorticoids, but not after anti-ASGM1, treatment.

Introduction

The injection of parental lymphoid cells (PLC) into F1 hybrid mice normally results in graft-versus-host disease (GVHD). GVHD is a complex disease with multisymptoms including severe immunosuppression of T and B cell functions, injury to lymphoid and non-lymphoid tissues, alterations of the T cell populations in the lymphoid organs, cachexia, diarrhea, morbidity and mortality (1-8).

In a recent report, we demonstrated that adrenalectomized (ADX) (C57BL/6 X A)F1 (B6AF1) hybrid mice injected with $20x10^6$ A strain parental lymphoid cells (PLC) experienced GVHD symptoms on day 13, however, these animals recovered rapidly from GVHD by days 18-21 (9). In contrast, non-ADX B6AF1 mice injected with the same dose of PLC experienced GVHD symptoms on day 13 which progressively worsened by days 18-21. The rapid recovery from GVHD in ADX B6AF1 recipients was attributed to the induction of an F1-anti-parental mechanism that eliminated the parental graft, after an initial period of engraftment. The F1-anti-parental mechanism was mediated by an effector cell of host origin (ie. B6AF1) that was sensitive to glucocorticoids. In addition, the effector cell population was present in both ADX and non-ADX B6AF1 recipients, however, it was eliminated by high levels of circulating glucocorticoids during GVHD in non-ADX F1 recipients experiencing the disease (9). In

the absence of the adrenal glands, the F1-anti-parental mechanism was induced after an initial period of engraftment, resulting in the elimination of the parental graft and recovery from GVHD (9). Furthermore, treatment with various antibodies showed that the F1-anti-parental effector cell was sensitive to anti-asialoGM1 and anti-CD8, but not anti-NK1.1, treatment (9).

The in vivo rejection or elimination of certain parental lymphoid cells by the F1 hybrid recipient is a well documented phenomenon known as hybrid resistance (10, 11). However, the F1-anti-parental mechanism described in our recent report displays properties which sets it apart from the classical hybrid resistance. Hybrid resistance in B6AF1 recipients is demonstrated only against B6 ($H2^b$), but not against A ($H2^a$), strain PLC (12, 13), whereas, the F1-anti-parental mechanism we have recently described is against A strain PLC. In addition, hybrid resistance is not sensitive to glucocorticoids and is mediated by an effector cell that is $NK1.1^+$ (14-16). In contrast, the F1-anti-parental mechanism in our model is sensitive to glucocorticoids and is mediated by an $NK1.1^-$ effector cell. Lastly, elimination of the parental graft occurs within 48 hours after engraftment in hybrid resistance (10, 11, 17, 18), however, the parental graft in our system is eliminated in the third week after engraftment. Thus, the newly described F1-anti-parental mechanism is different from the classical hybrid resistance.

In our recent report the effector cell mediating the F1-anti-parental mechanism was $CD8^+$ (9). Hence, in the present study we investigated whether the F1-anti-parental effector cell was thymic derived. Our results demonstrated that the effector cell was not dependent on a mature thymus and was renewable after glucocorticoids, but not after anti-ASGM1, treatment.

Materials and Methods

Animals

Mice of the inbred strains A ($H2^a$), C57BL/6 (B6) ($H2^b$), and their F1 hybrids, B6xAF1 ($H2^{b/a}$) (B6AF1) were used. All mice were bred and maintained in our animal colony. Donor A strain animals were 6-12 months old. B6AF1 recipients were older than 6 weeks of age. Both sexes were employed in the experiments.

Experimental protocol

The following experimental protocol is illustrated in Fig. 1. B6AF1 mice were surgically thymectomized or sham-thymectomized at 6-7 weeks of age. Two weeks later, the mice were treated either with 2.5 mg of cortisone acetate (Merck Sharp & Dohme Canada., Quebec, Canada) or 70 ug anti-asialoGM1 antibody (Wako Chemicals, Dallas, Texas), followed two days later by surgical adrenalectomy. Adrenalectomized mice were maintained on physiological saline in lieu of drinking water for the duration of the experiment. Five months after adrenalectomy GVHD was induced with an i.v injection of $20x10^6$ A strain parental lymphoid cells (PLC) (ie. spleen and LN cells). B6AF1 mice injected with B6AF1 lymphoid cells were used as syngeneic controls. Surgical procedures were performed as previously described (7, 8).

Mitogen assays and flow cytometry

On day 21 after GVHD induction, the spleen was removed from individual B6AF1 recipient and made into a single cell suspension. Splenic T cell proliferative responses to phytohemagglutinin (PHA) (Wellcome Research Reagent Limited, Dartford, UK), and B cell responses to lipopolysaccharide (LPS) (Sigma) were evaluated by [^{3}H] thymidine incorporation, as previously described (5). In addition, 1x10^6 splenic cells were labeled with FITC conjugated anti-H2K^b (Clone AF6-88.5), phycoerythrin conjugated anti-CD4 (Clone YTS 169.4) and biotinylated anti-CD8 (Clone 53-6.7) mAbs as previously described (9). All mAbs and the secondary reagent, strepavidin-cychrome, were purchased from Cedarlane, Hornby, Ontario, Canada. Labeled samples were analyzed by a FACScan (Becton-Dickinson, Mountain View, CA) and 5000 flow cytometry events were counted per sample. A selective gate, excluding cell debris and erythrocytes, was set using forward light scatter.

Results

The F1-anti-parental effector cell was not thymic derived and non-renewable.

We have recently reported that an F1-anti-parental effector cell was induced in ADX B6AF1 recipients to cause recovery from GVHD by days 18-21 (9). In the present report, experiments were performed to determine if the F1-anti-parental effector cell was thymic derived since it was $CD8^+$. We first examined whether thymectomy alone had any effect on the F1-anti-parental effector cell. B6AF1 mice were thymectomized (TX) at 6-7 weeks of age, followed two weeks later by adrenalectomy. Five months after adrenalectomy GVHD was induced with $20x10^6$ A strain PLC. As we and others have previously demonstrated, GVHD causes severe immunosuppression, high splenic chimerism and marked reduction in the host T cell populations (9, 19). Thus, on day 21 after GVHD induction, GVHD symptoms were assessed by the ability of splenic T and B cells to proliferate to PHA and LPS, respectively. In addition, we have identified splenic T cell populations using mAbs against CD4 and CD8 antigens, and have employed an anti-$H2K^b$ mAb to distinguish host (ie. B6AF1, $H2^{a/b}$) cells from donor A ($H2^a$) cells. Results from Table 1 showed that TX ADX B6AF1 mice had a marked increased proliferation of splenic cells in response to PHA and LPS, displayed near normal numbers of host $CD4^+$

and $CD8^+$ T cell populations in the spleen and exhibited very low splenic chimerism. In contrast, injection of the same parental dose in TX B6AF1 mice with intact adrenals (ie. TX non-ADX) resulted in GVHD symptoms on day 21 as demonstrated by severe immunosuppression, severe deficiency of host $CD4^+$ T cell population in the spleen and high splenic chimerism (Table 1). These results suggest that thymectomy alone did not affect the F1-anti-parental effector cell and are in accord with our previous findings that adrenalectomy induces an F1-anti-parental mechanism which allows the recipients to recover from GVHD symptoms by day 21.

Having established that thymectomy alone did not affect the F1-anti-parental effector cell, we next investigated whether the effector cell required a mature thymus for renewal after depletion with either glucocorticoids or anti-ASGM1 treatment. To test this possibility, the following experimental protocol was designed (Fig. 1). B6AF1 mice were TX and two weeks later they were treated with either CA or anti-ASGM1 antibody in order to eliminate the F1-anti-parental effector cell. Two days after either CA or anti-ASGM1 treatment, adrenalectomy was performed, and 5 months after adrenalectomy GVHD was induced by an injection of $20x10^6$ A strain PLC. In agreement with our previous results, the F1-anti-parental effector cell was abrogated with CA two days before GVHD induction resulting in GVHD-induced immunosuppression on day 21 (results not shown) (9), however, it was renewed in most (6/8) TX ADX

animals 5 months after CA treatment, leading to recovery from GVHD symptoms on day 21. Recovery from GVHD was assessed by near normal responses to PHA and LPS, near normal numbers of splenic host T cell populations, particularly $CD4^+$ T cells, and low splenic chimerism (Table 2). The findings that the F1-anti-parental effector cell was renewable 5 months after being eliminated with CA treatment, even in the absence of a mature thymus, suggests that the effector cell was not dependent a mature thymus for renewal after glucocorticoid treatment.

In accord with our previous results, the F1-anti-parental effector cell was abolished with anti-ASGM1 treatment two days before GVHD induction and resulted in GVHD-induced immunosuppression on day 21 (results not shown) (9). In contrast to the CA-treated groups, the F1-anti-parental effector cell remained absent in both SHTX ADX and TX ADX recipients even 5 months after treatment with anti-ASGM1 antibody, resulting in GVHD symptoms on day 21 after disease induction (Table 2). GVHD symptoms were characterized as severe suppression of splenic T and B cells, severe deficiency of splenic host $CD4^+$ cells, and high splenic chimerism. These results suggest that the F1-anti-parental effector cell was not renewed 5 months after anti-ASGM1 treatment. Because the effector cell was not renewed in ADX recipients with an intact thymus (ie. SHTX ADX mice), this further implies that the effector cell was not thymic dependent. Taken together the above results suggest that the F1-anti-

parental effector cell was not dependent on a mature thymus and was renewable 5 months after glucocorticoids treatment, but not after anti-ASGM1 treatment.

Discussion

In our recent report, we demonstrated that ADX, but not non-ADX, B6AF1 mice injected with $20x10^6$ A strain PLC recovered rapidly from GVHD symptoms by days 18-21, after an initial two week manifestation of GVHD (9). The rapid recovery from GVHD symptoms was attributed to the elimination of the parental graft by an F1-anti-parental mechanism, after an initial period of engraftment (9). The F1-anti-parental mechanism was mediated at least in part by an effector cell that was sensitive to glucocorticoids, anti-ASGM1 and anti-CD8, but not anti-NK1.1, treatment (9).

In the present report, we investigated whether the F1-anti-parental effector cell was thymic derived since it was $CD8^+$ (9). Our results showed that the F1-anti-parental effector cell was eliminated two days after CA treatment, but was renewed 5 months after the treatment, even in the absence of the thymus. Conversely, the F1-anti-parental effector cell was

not renewed 5 months after anti-ASGM1 treatment despite the presence of the thymus. Thus the effector cell does not appear to be dependent on a mature thymus. This raises the possibility that the $CD8^+$ effector cell may be derived from an extrathymic environment. Numerous investigators have shown that $CD8^+$ cells are present in the spleen of athymic nude mice and that $CD8^+$ cells do not necessarily require a thymus to mature (20-23). Furthermore, precursors of $CD8^+$ cells derived from athymic mice can mature in an extrathymic environment and become functional $CD8^+$ cells with self-MHC restricted cytotoxicity (24-26). Based on these studies, it is quite possible that the $CD8^+$ effector cell could be derived from a precursor cell that matures in an extrathymic environment.

It is of interest to observe that the F1-anti-parental effector cell was not renewed even 5 months after anti-ASGM1 treatment. One possible interpretation of these results is that the effector cell is derived from a precursor cell that is $ASGM1^+$. This is supported by evidence showing that ASGM1 antigens are expressed on precursor cells such as precursor CTL (27-29). In addition, studies have shown that the $ASGM1^+$ precursor CTL give rise to mature effector CTLs and that elimination of the precursor CTL leads to a reduction in cytotoxic activity (27, 28). It is therefore conceivable that the F1-anti-parental $ASGM1^+$ precursor cell differentiates into the mature F1-anti-parental effector cell. Furthermore, our previous report revealed that the effector cell was also $ASGM1^+$ (9). Thus treatment with anti-ASGM1 antibody appears to eliminate both the effector and precursor

cell, leading to a permanent loss of the F1-anti-parental mechanism suggesting that the F1-anti-parental effector cell may be derived from a stem cell that is also ASGM1$^+$. However, studies have shown that anti-ASGM1 treatment does not result in a permanent elimination of precursor CTL since cytotoxicity activity returns to normal (27, 30, 31). It is thus possible that the F1-anti-parental effector cell is derived from a separate lineage than conventional CD8$^+$ CTL.

Unlike the anti-ASGM1 treated groups, our results showed that the F1-anti-parental effector cell was renewed 5 months after glucocorticoid treatment. Although we do not know the exact time when the F1-anti-parental effector cell is renewed after glucocorticoid treatment, studies have shown that the high doses of glucocorticoids injected into mice are cleared from the body within two weeks after the injection (32, 33). This would suggest that the F1-anti-parental cell could be renewed as early as two weeks after glucocorticoid treatment. A possible explanation for the renewable of the F1-anti-parental effector cell after glucocorticoid treatment is that the F1-anti-parental precursor cell is resistant to glucocorticoids whereas the effector cell is not. This is quite feasible since precursor B cells in the bone marrow have been shown to be glucocorticoid resistant, while mature B cells are glucocorticoid sensitive (34, 35). The F1-anti-parental precursor cell could differentiate into the effector cells if the effector cells are eliminated by glucocorticoids, hence renewing the pool of effector cells. The F1-anti-parental effector cell may therefore be regulated

by physiological levels of endogenous glucocorticoids where it is eliminated or suppressed by high levels of circulating glucocorticoids but is renewed when glucocorticoids return to basal levels.

In our previous report, we showed that the F1-anti-parental effector cell appears to target parental non-T cells (9). The injection of parental splenic cells into unirradiated F1 recipient can give rise to long term chimerism since the spleen contains haematopoietic stem cells (36, 37). Because parental splenic cells were employed to induce GVHD, elimination of the parental non-T cells is likely to include the stem cells in the parental graft. This implies that the F1-anti-parental effector cell may also target haematopoietic stem cells present in the bone marrow and may play a role in late rejection of T cell depleted bone marrow grafts after an initial period of engraftment (38-40). Studies have shown that late rejection of T cell depleted marrow grafts correlates strongly with an increase in circulating $CD8^+$ cells of host origin (41-43). Since T cell depletion of bone marrow grafts markedly reduces the incidence of GVHD, immunosuppressive drugs such as steroids are rarely used as posttransplant treatment in controlling GVHD (38-43). Interestingly, several groups of investigators observed that treatment of patients with steroids after transplantation with T cell depleted bone marrow causes the disappearance of host $CD8^+$ and reverses late graft rejection (44-46). It was proposed that the late graft failure may be reversed by treatment with steroids (44-46). Based on these studies, the $CD8^+$ F1-anti-parental effector cell described in our system may also be

involved in late rejection of T cell depleted bone marrow.

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TABLE I. Effect of thymectomy on the F1-anti-parental cell rejection

Groups[a]	Treatment[a] TX	ADX	Mitogen-induced proliferation of splenic cells (x10^3) [% SYN] PHA	LPS	Absolute number of splenic host T cell populations[b] (x10^6) CD4$^+$	CD8$^+$	Splenic chimerism (%)
SYN (2)	–	–	47.4 ± 8.3 [100]	41.8 ± 1.5 [100]	23.0 ± 4.6	20.0 ± 7.9	ND[c]
SYN (2)	+	–	65.0 ± 5.8 [100]	43.2 ± 0.3 [100]	21.2 ± 1.9	15.5 ± 4.4	ND
GVHD (6)	–	–	4.8 ± 2.6 [10.2 ± 5.5]	-1.7 ± 1.0 [-4.1 ± 2.4]	3.3 ± 0.9	13.9 ± 2.7	22.5 ± 5.4
GVHD (8)	+	–	5.3 ± 3.3 [8.2 ± 5.1]	2.3 ± 7.6 [5.3 ± 7.6]	5.1 ± 2.2	16.4 ± 3.3	18.5 ± 4.9
SYN (2)	–	+	88.0 ± 1.0 [100]	44.5 ± 4.1 [100]	26.4 ± 0.9	20.4 ± 3.0	ND
SYN (2)	+	+	80.1 ± 2.0 [100]	39.3 ± 10.4 [100]	21.7 ± 2.7	20.6 ± 3.7	ND
GVHD (6)	–	+	60.6 ± 13.5 [68.9 ± 15.3]	27.8 ± 7.3 [62.5 ± 16.4]	32.9 ± 8.1	37.8 ± 4.9	3.0 ± 1.7
GVHD (12)	+	+	44.7 ± 5.1 [59.9 ± 6.3]	23.2 ± 5.8 [59.0 ± 14.8]	18.3 ± 2.4	24.7 ± 2.6	0.8 ± 0.7

[a]B6AF1 mice were TX or SHTX at 6-7 weeks of age, followed two weeks later by adrenalectomy. Five months after adrenalectomy GVHD was induced with 20x10^6 A strain PLC and mice were sacrificed on day 21 after disease induction. Number of mice per group is in parenthesis. Data are presented as the mean ± SE.

[b]Absolute number was calculated as follows: (Splenic cellularity X Incidence)/100.

[c]ND: not done.

TABLE II. Long term effect of CA and anti-ASGM1 treatment on the F1-anti-parental cell rejection.

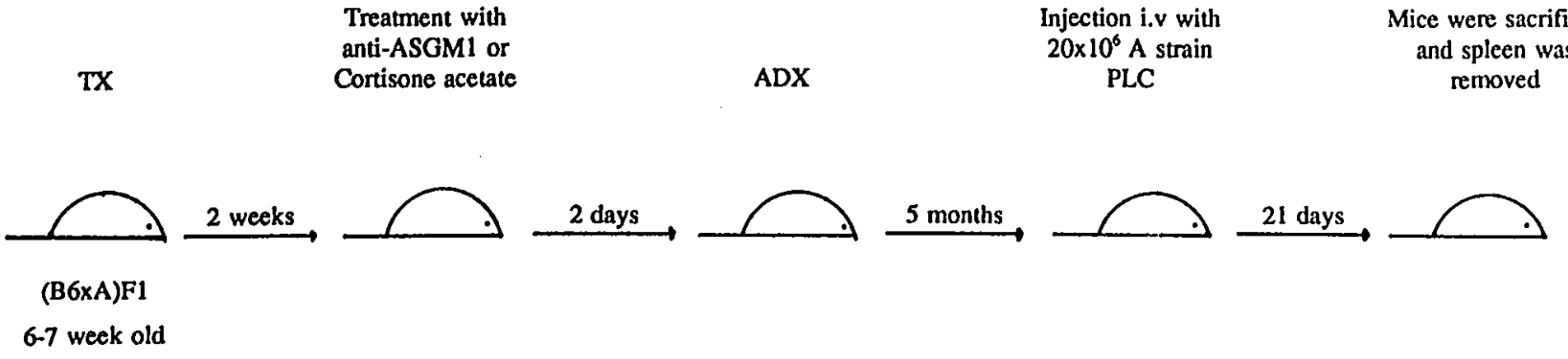
Groups[a]	TX[a]	Treatment[a]	Mitogen-induced proliferation of splenic cells ($x10^3$) [% SYN]		Absolute number of splenic host T cell populations[b] ($x10^6$)		Splenic chimerism (%)
			PHA	LPS	$CD4^+$	$CD8^+$	
SYN (2)	_	CA	110.4 ± 6.0 [100])	40.6 ± 4.2 [100]	38.1 ± 1.4	23.3 ± 5.3	ND[c]
SYN (2)	+	CA	47.9 ± 19.5 [100]	41.8 ± 6.1 [100]	10.0 ± 1.4	8.9 ± 4.5	ND
GVHD (5)	_	CA	42.6 ± 13.7 [38.5 ± 5.4]	20.7 ± 6.5 [50.9 ± 16.0]	27.1 ± 7.5	33.2 ± 3.2	3.7 ± 1.5
GVHD (6)	+	CA	34.7 ± 88.3 [88.3 ± 16.3]	36.3 ± 6.6 [86.8 ± 15.8]	22.0 ± 2.2	35.1 ± 4.9	0.8 ± 0.7
SYN (2)	_	ASGM1	86.9 ± 12.5 [100]	51.2 ± 2.1 [100]	17.4 ± 2.6	15.2 ± 3.1	ND
SYN (2)	+	ASGM1	60.9 ± 15.7 [100]	32.4 ± 5.9 [100]	25.3 ± 4.6	17.4 ± 1.9	ND
GVHD (7)	_	ASGM1	14.1 ± 5.5 [16.2 ± 6.4]	2.6 ± 3.7 [5.0 ± 7.3]	5.4 ± 2.6	20.0 ± 1.8	24.4 ± 6.2
GVHD (10)	+	ASGM1	4.1 ± 1.9 [6.8 ± 3.1]	-1.5 ± 0.8 [-2.5 ± 1.3]	3.8 ± 0.7	20.8 ± 3.1	11.2 ± 3.6

[a]B6AF1 mice were TX (+) or SHTX (-) at 6-7 weeks of age and treated with CA or anti-ASGM1 treatment two weeks later. All B6AF1 mice were ADX two days after either CA or anti-ASGM1 treatment and GVHD was induced 5 months later with $20x10^6$ A strain PLC (see Fig. 1). Mice were sacrificed on day 21 after GVHD induction. Number of mice per group is in parenthesis. Data were obtained from two separate experiments and are presented as the mean ± SE.

[b]Absolute number is calculated as follows: (Splenic cellularity X Incidence)/100.

[c]ND: not done.

Fig. 1. Experimental protocol. B6AF1 mice were surgically thymectomized (TX) at 6-7 weeks of age. Two weeks later, the mice were treated either with 2.5 mg of cortisone acetate (Merck Sharp & Dohme Canada., Quebec, Canada) or 70 ug anti-asialoGM1 (anti-ASGM1) antibody (Wako Chemicals, Dallas, Texas), followed two days later by surgical adrenalectomy. Adrenalectomized (ADX) mice were maintained on physiological saline in lieu of drinking water for the duration of the experiment. Five months after adrenalectomy GVHD was induced with an i.v injection of $20x10^6$ A strain parental lymphoid cells (PLC) (ie. spleen and LN cells). On day 21 after GVHD induction, the mice were sacrificed and the spleen was removed for assay.


TX
Treatment with anti-ASGM1 or Cortisone acetate
ADX
Injection i.v with 20x10^6 A strain PLC
Mice were sacrificed and spleen was removed
2 weeks
2 days
5 months
21 days
(B6xA)F1
6-7 week old

CHAPTER 5 THE ROLE OF ENDOGENOUS GLUCOCORTICOIDS ON HOST T CELL POPULATIONS IN THE PERIPHERAL LYMPHOID ORGANS OF MICE UNDERGOING GRAFT-VERSUS-HOST DISEASE[1]

The studies in this chapter investigate the effects of endogenous glucocorticoids on host T cell populations in the peripheral lymphoid organs during GVHD.

[1]This manuscript is in press in *Transplantation*, 1995.

THE ROLE OF ENDOGENOUS GLUCOCORTICOIDS ON HOST T CELL POPULATIONS IN THE PERIPHERAL LYMPHOID ORGANS OF MICE UNDERGOING GRAFT-VERSUS-HOST DISEASE[1]

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FOOTNOTES

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ABBREVIATIONS

ADX, adrenalectomized; non-ADX, non-adrenalectomized.

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ABSTRACT

We have previously demonstrated that immature $CD4^+8^+$ and mature $CD4^+$ thymocyte populations were selectively eliminated during murine graft-versus-host disease (GVHD) as a consequence of elevated levels of endogenous glucocorticoids. In this report, we investigated whether the marked reduction of $CD4^+8^+$ and $CD4^+$ thymocyte populations would affect host $CD4^+$ and $CD8^+$ T cell populations in the spleens and lymph nodes (LN) of mice undergoing GVHD. GVHD was induced in (C57BL/6xA)F1 (B6AF1) mice by injecting A strain parental lymphoid cells. Using an antibody against $H2K^b$ antigens, labelled host B6AF1 cells were distinguished from unlabelled donor A cells. Our results demonstrated a marked deficiency of host $CD4^+$ and $CD8^+$ T cells in the spleens and lymph nodes (LN) of GVHD mice on day 21 after GVHD induction. The severe reduction of host T cell populations in the peripheral lymphoid organs did not appear to result from the elimination of $CD4^+8^+$ and $CD4^+$ thymocyte populations. However, adrenalectomy before GVHD induction reversed the severe loss of both host $CD4^+$ and $CD8^+$ T cell populations in the LN of GVHD mice on day 21, whereas, cortisone treatment of adrenalectomized (ADX) GVHD mice resulted in reduction of host LN $CD4^+$ and $CD8^+$ T cell populations similar to that observed in non-adrenalectomized (non-ADX) GVHD animals on day 21. In addition, adrenalectomy markedly improved the proliferative response of LN T cells to mitogens when compared to

immunosuppressed T cells from the LN of non-ADX GVHD mice. In contrast, adrenalectomy did not reverse splenic T cell immunosuppression and the marked reduction of splenic host T cell populations during GVHD. These results suggest that high levels of endogenous glucocorticoids during GVHD play a central role in mediating severe deficiency of host T cell populations and inducing severe T cell immunosuppression in the LN, but not in the spleen, of GVHD mice.

INTRODUCTION

We have previously demonstrated that mice undergoing graft-versus-host disease (GVHD) experience severe thymic involution in association with thymic dysplasia (1-3). As a consequence of GVHD-induced stress, the adrenal glands secrete excessive amounts of glucocorticoids (4-6) which eliminate the steroid sensitive immature $CD4^+8^+$ thymocyte population, resulting in severe thymic involution (7). Surgical adrenalectomy prior to GVHD induction prevents thymic involution (7, 8), and adrenalectomy during the course of GVHD returns the involuted thymus to normal (9, 10). In addition, we have shown that mature $CD4^+$ thymocytes fail to acquire steroid resistance as they undergo maturation in a GVHD-induced dysplastic thymus (7). Consequently, the $CD4^+$ thymocytes are selectively eliminated in the presence of high levels of endogenous glucocorticoids during GVHD, and thus adrenalectomy before GVHD induction prevents the elimination of the $CD4^+$ thymocyte population (7).

In the present study, we investigated whether the elimination of $CD4^+8^+$ and $CD4^+$ thymocyte populations during GVHD would affect host $CD4^+$ and $CD8^+$ T cell populations in the spleens and lymph nodes (LN) of GVHD mice. GVHD was induced in unirradiated B6AF1 hybrid recipients by an intravenous injection of A strain parental lymphoid cells. Our results demonstrated that severe deficiency of host (ie. F1 origin) $CD4^+$ and $CD8^+$

T cell populations in the peripheral lymphoid organs during GVHD do not appear to result from the elimination of $CD4^+8^+$ and $CD4^+$ thymocyte populations. However, high levels of glucocorticoids during GVHD play a central role in suppressing T cell functions and in markedly decreasing the total number of host $CD4^+$ and $CD8^+$ T cell populations in the LN, but not in the spleen, of GVHD mice.

MATERIALS AND METHODS

Animals. Mice of the inbred strains A ($H2^a$), C57BL/6 (B6) ($H2^b$), and their F1 hybrids, B6xAF1 ($H2^{b/a}$) (B6AF1) were used. All mice were bred and maintained in our animal colony. Donor A animals were 6-12 months old. B6AF1 recipients were older than 3 months. Both sexes were employed in the experiments.

Thymectomy (TX) and Sham-thymectomy (SH-TX). B6AF1 mice were thymectomized at 12 weeks of age under ether anaesthesia as previously described (11). At the conclusion of the experiment each animal was autopsied to determine the adequacy of the surgical thymectomy. Sham-thymectomy was also performed on B6AF1 mice as control for the surgery.

Adrenalectomy. B6AF1 mice were adrenalectomized (ADX) at 9-10 weeks of age and were used 2-3 months after adrenalectomy. Surgical adrenalectomy was performed under ether anesthesia as described previously (7, 8). ADX mice were maintained on physiological saline in lieu of drinking water for the duration of the experiment.

Induction of GVHD. GVHD was induced as previously described (3, 4). Briefly, single cell suspensions were prepared from pooled spleens and lymph nodes by gently tamping them through a 50-mesh stainless steel screen. GVHD was induced with either 30, 35 or $50x10^6$ A strain parental lymphoid cells injected i.v. into age-matched unirradiated ADX and non-ADX B6AF1 hybrid recipients. B6AF1 mice injected with B6AF1 lymphoid cells were used as syngeneic controls. Normal B6AF1 mice received no injection.

Mitogen Assays. On different days after GVHD induction, the spleen was removed from individual B6AF1 recipient and made into a single cell suspension. T cell proliferative responses to Concanavalin A (Con A) (Pharmacia, Fine Chemicals, Uppsala, Sweden) and phytohemaglutinin (PHA) (Wellcome Research Reagent Limited, Dartford, UK) were evaluated by [^{3}H] thymidine incorporation, as previously described (3). Briefly, $5x10^5$ splenic cells were cultured for 48 hrs in triplicate with or without mitogen

in a 96 well titer plates. The plates were pulsed with 1 μCi/well of [^{3}H] thymidine for 16 hrs and then harvested with an automated cell harvester. [^{3}H] thymidine incorporation was quantitated on a beta counter (LKB Instruments, Turku, Finland).

Immunofluorescence Staining. On different days after GVHD induction, spleens and LN were removed from B6AF1 recipients and made into single cell suspensions. Staining was performed in 96 well plates. One million cells in a 50 ul volume of Hanks' balanced salt solution (GIBCO., Grand Island, NY) were incubated with 50 ul of the appropriate reagents on ice for 30 mins. After each incubation the plates were washed twice. Incubation and washing was repeated for the secondary reagent. After the final wash, the samples were fixed in 1 ml of 1% paraformaldehyde and stored at 4°C. The fixed cell samples were analyzed by a FACScan (Becton-Dickinson, Mountain View, CA) and 5000 flow cytometry events were counted per sample. A selective gate, excluding cell debris and erythrocytes, was set using forward light scatter.

Reagents for Flow Cytometry. FITC conjugated anti-H2K^b (Clone AF6-88.5), phycoerythrin conjugated anti-CD4 (Clone YTS 169.4) and biotinylated anti-CD8 (Clone 53-6.7) monoclonal antibodies (mAbs) were employed for triple immunofluorescence. All mAbs and the secondary reagent, strepavidin-

cychrome, were purchased from Cedarlane, Hornby, Ontario, Canada. All reagents were diluted as recommended.

Corticosterone (CS) Measurement. All mice were handled each day for the duration of the experiment. On day 21 after GVHD induction, cardiac puncture was performed while the mice were under ether anesthesia. Blood was collected in tubes containing EDTA between 0900 and 1100 hr. Plasma was extracted from the blood and aliquots were frozen at -20°C until assayed. Plasma corticosterone levels were determined by using a radioimmunoassay kit for rats and mice (ICN Biomedicals, CA) and the procedure was followed as instructed.

Cortisone Acetate (CA) Treatment. B6AF1 mice were injected i.p. with a single dose of 2.5 mg of cortisone acetate (Merck Sharp & Dohme Canada., Quebec, Canada), in a total volume of 0.5 ml saline.

RESULTS

Effect of endogenous glucocorticoids on thymocyte populations in mice undergoing GVHD. A prominent feature of GVHD is stress-mediated thymic involution (1, 7, 9, 10). In this report, GVHD was induced in unirradiated B6AF1 hybrid mice with an i.v. injection of $35x10^6$ A strain parental lymphoid cells. Our results showed that on day 9 after GVHD induction, the steroid sensitive immature $CD4^+8^+$ population was decreased, leading to a reduced thymic cellularity in non-ADX GVHD mice (Table 1). By day 24 a greater reduction of the $CD4^+8^+$ thymocyte population resulted in severe thymic involution. In addition, the mature $CD4^+$, but not $CD8^+$, thymocyte population was selectively eliminated by day 24, thus leading to a decrease of the $CD4^+/CD8^+$ thymocyte ratio (Table 1). Coincident with thymic involution, plasma corticosterone (CS) levels were elevated on days 9 and 24 (Table 1). Surgical adrenalectomy before GVHD induction markedly decreased the levels of plasma CS and abrogated thymic involution and maintained normal numbers of $CD4^+8^+$ and $CD4^+$ thymocytes (Table 1). These results are in accord with our previous reports (1, 7, 8) and confirm that GVHD induced in our mice causes the adrenal glands to secrete excessive amounts of glucocorticoids resulting in severe thymic involution in association with a marked reduction of $CD4^+8^+$ and $CD4^+$ thymocyte populations.

Marked reduction of host CD4⁺ and CD8⁺ T cell populations in the spleen and LN of mice undergoing GVHD. Having established that $CD4^+8^+$ and $CD4^+$ thymocyte populations were selectively eliminated in our GVHD mice (Table 1), we next investigated whether the host (ie. B6AF1 origin) T cell populations in the spleen and LN of GVHD mice would follow a similar fate. In this study, the peripheral T cell populations were identified using monoclonal antibodies (mAbs) against CD4 and CD8 antigens. In addition, an anti-$H2K^b$ mAb was employed to distinguish labelled host B6AF1 cells from unlabelled donor A cells, as previously reported by Hakim *et al.* (12). Our results showed that the absolute number of splenic host $CD4^+$ T cells was markedly reduced in the first two weeks of GVHD (ie. days 7 and 14), in contrast, to an increase of the host $CD8^+$ T cells (Table 2). Furthermore, the decrease of host $CD4^+$ T cells occurred as early as day 7 after GVHD induction (Table 2), which preceeded the elimination of $CD4^+$ thymocytes (Table 1). Similar changes were observed in the LN of GVHD mice where, after an early increase on day 7, host $CD4^+$ and $CD8^+$ T cells were decreased on day 14 (Table 2). By day 21, both host $CD4^+$ and $CD8^+$ T cell populations in the spleens and LN of GVHD mice were severely deficient. Owing to greater loss of host $CD4^+$ T cells than $CD8^+$ T cells, the host $CD4^+/CD8^+$ T cell ratio was reversed (ie. 0.2-0.4:1) (Table 2). These findings showed that host $CD4^+$ and $CD8^+$ T cell populations in the spleens and LN of GVHD mice were markedly deficient by the third week of GVHD.

Effect of thymectomy on host T cell populations in the spleen and LN of mice undergoing GVHD. We next investigated the possible mechanism(s) responsible for the severe deficiency of host T cell populations in the spleen and LN of GVHD mice . Table 1 demonstrated that GVHD mice experienced severe thymic involution resulting from the elimination of the $CD4^+8^+$ and $CD4^+$ thymocyte populations. We therefore explored the possibility that GVHD-induced thymic involution may cause a functional thymectomy which was responsible for the depletion of peripheral host T cell populations during GVHD. To test this possibility, B6AF1 mice were thymectomized (TX) and GVHD was induced with $20x10^6$ A strain parental lymphoid cells 5 months after thymectomy. Our results showed that TX GVHD mice displayed a severe loss of splenic host $CD4^+$ T cell population and an inverted host $CD4^+/CD8^+$ T cell ratio on day 21 (Table 3). In addition, thymectomy of normal mice after 5 months caused a non-specific decrease in the $CD4^+$ and $CD8^+$ T cell populations in the spleen, however, without altering the splenic $CD4^+/CD8^+$ T cell ratio (Table 3), implying that thymectomy alone could not cause reversal of the $CD4^+/CD8^+$ T cell ratio in the spleen. Taken together, these findings suggest that the marked decrease of host $CD4^+$ and $CD8^+$ T cell populations in the peripheral lymphoid organs of GVHD mice did not appear to result from the elimination of $CD4^+8^+$ and $CD4^+$ thymocytes.

Role of endogenous glucocorticoids on peripheral host T cell populations in GVHD mice. We next examined whether endogenous glucocorticoids may be involved in the marked reduction of host T cell populations in the spleen and LN of GVHD mice since the levels of plasma CS were elevated during GVHD (Table 1) (4-6). To study this possibility, B6AF1 mice were adrenalectomized (ADX) two months before GVHD induction. Although adrenalectomy prevented thymic involution and maintained normal levels of T cell subsets in the thymus (Table 1) (7), it did not prevent the severe reduction of host $CD4^+$ and $CD8^+$ T cells in the spleen of GVHD mice on day 21 (Table 4). In the first two weeks of GVHD, host $CD4^+$ and $CD8^+$ T cell populations in the spleens of ADX GVHD mice underwent changes similar to non-ADX GVHD animals. By day 21 host $CD4^+$ and $CD8^+$ T cells in the spleen of ADX GVHD mice were markedly reduced to values similar to non-ADX GVHD counterparts (Table 4). In contrast, adrenalectomy appeared to protect against the severe loss of host $CD4^+$ and $CD8^+$ T cell populations in the LN of GVHD mice (Table 4). In the first week of GVHD (ie. day 7), host $CD4^+$ and $CD8^+$ T cell populations in the LN of ADX GVHD mice (Table 4) increased to values similar to non-ADX GVHD animals (Table 3). However, by day 21 host $CD4^+$ and $CD8^+$ T cells in the LN of ADX GVHD mice decreased to values similar to ADX SYN control mice (Table 4), whereas, host LN T cells in non-ADX GVHD animals were markedly reduced when compared to non-ADX SYN control animals (Table 2).

Although the decrease of host LN $CD4^+$ and $CD8^+$ T cells in ADX GVHD mice on day 21 was significant, the reduction was, however, slight when compared to the marked reduction of splenic host T cell populations (Table 4). To reproduce the effects of high endogenous glucocorticoids, ADX GVHD mice were treated with a single high dose of cortisone acetate (CA). Our results showed that CA treatment of ADX GVHD caused a severe deficiency of host LN T cell populations on day 21 (Table 5) similar to the untreated non-ADX GVHD counterparts (Table 2). These results were reproducible in ADX B6AF1 recipients injected with 30 or $50x10^6$ A strain parental lymphoid cells (results not shown). Thus these findings suggest that the marked reduction of host $CD4^+$ and $CD8^+$ T cells in the LN, but not in the spleen, of GVHD mice was mediated largely by high levels of endogenous glucocorticoids.

Effect of endogenous glucocorticoids on T cell functions in the peripheral lymphoid organs. Studies were next conducted to determine the effects of endogenous glucocorticoids on T cell functions in the spleen and LN of GVHD mice, as assessed by in vitro thymidine incorporation in response to Con A and PHA T cell mitogens. Mitogen-induced proliferation of T cells in the spleen and LN of non-ADX GVHD mice was decreased as early as day 7 and was severely suppressed as the disease progressed to day 21 (Fig. 1A, B). Adrenalectomy failed to prevent severe T cell immunosuppression

in the spleen of ADX GVHD mice since T cell mitogenic responses were equally suppressed when compared to non-ADX GVHD counterparts (Fig. 1A). In contrast, adrenalectomy markedly improved the proliferative responses of LN T cells on days 14 and 21 (Fig. 1B). It appears that during the first three weeks of GVHD, high levels of endogenous glucocorticoids play a major role in suppressing T cell functions in the LN, but not in the spleen, of GVHD mice.

Degree of chimersim in the peripheral lymphoid organs of non-ADX and ADX GVHD mice. Several studies have shown that donor cells are actively involved in suppressing T cell functions and eliminating host T cells during GVHD (13-16). To examine whether the presence of donor cells in the spleen of GVHD mice may account for the dysregulation and dysfunction of host splenic T cell populations, we compared the degree of chimerism in the spleens and LN of GVHD mice by employing an anti-$H2K^b$ mAb to distinguish unlabelled A donor cells from labelled B6AF1 host cells. Our results demonstrated that high splenic chimerism was present in the spleen of both non-ADX and ADX GVHD mice on all of the days studied (Table 6). Conversely, low chimerism was detected in the LN of both GVHD groups. Thus the high degree of splenic chimerism may account for the marked reduction and suppression of host T cells in the spleen of GVHD mice.

DISCUSSION

We have previously demonstrated that GVHD mice experienced severe thymic involution and elimination of the $CD4^+8^+$ and $CD4^+$ thymocyte populations (1, 2, 7, 8). In this report, we examined whether the marked reduction of the $CD4^+8^+$ and $CD4^+$ thymocyte populations would affect host T cell populations in the spleens and LN of mice undergoing GVHD. GVHD was induced in B6AF1 mice by an i.v. injection of A strain parental lymphoid cells. Using a mAb against $H2K^b$ antigens, labelled host (ie. B6AF1) cells were distinguished from unlabelled donor A cells. Our findings showed that the marked reduction of host $CD4^+$ and $CD8^+$ T cell populations in the spleens and LN of GVHD mice on day 21 did not appear to result from GVHD-induced thymic atrophy since thymectomy had no effect on the depletion of these peripheral host T cell populations during GVHD (Table 3), and the decrease of host $CD4^+$ T cells in the spleen on day 7 (Table 2) preceeded the elimination of $CD4^+$ thymocytes (Tables 1). Interestingly, the severe defiency of host T cell populations in the LN, but not in the spleen, of GVHD mice appeared to be mediated by high levels of endogenous glucocorticoids since adrenalectomy prior to GVHD induction reversed the drastic decline of host LN $CD4^+$ and $CD8^+$ T cell populations (Table 4). Furthermore, CA treatment of ADX GVHD mice (Table 5) reduced the host LN T cell populations to values similar to those observed

in untreated non-ADX GVHD mice (Table 2).

The kinetics of host T cells in the LN of GVHD mice parallels the activation of peripheral T cells in response to superantigens (17-19). Injection of superantigens into normal mice causes a large number of peripheral $CD4^+$ and $CD8^+$ T cells to proliferate followed by a rapid decline in the responsive T cells as a consequence of cell death (20). The rapid decline of superantigen-activated T cells appears to be mediated by an increase secretion of glucocorticoids that causes apoptotic cell death (21, 22). In the present study and those reported by others, the levels of CS in the plasma of non-ADX GVHD mice were elevated as early after the first week and up to a month after GVHD induction (4-6). Thus persistent elevated levels of glucocorticoids during GVHD (Table 1) may induce apoptotic cell death of proliferative host T cells in the LN, resulting in severe deficiency of these cells by day 21.

We have recently demonstrated that adrenalectomy before GVHD induction prevents the glucocorticoid-mediated downregulation of $p56^{lck}$ and $p59^{fyn}$, two src family protein tyrosine kinases involved in T cell signalling (23-26), and thus restores mitogen-induced T cell proliferation to near normal (27). In this report, adrenalectomy reversed the severe suppression of mitogen-induced proliferation of T cell in the LN on days 14 and 21 (Fig. 1B). T cell immunosuppression in the LN of non-ADX GVHD mice, when compared to ADX GVHD animals, could not be due to a decrease in T cell

number since the incidence of T cells in the LN of non-ADX GVHD mice were similar in ADX GVHD counterparts (Table 4). It is most likely that downregulation of $p56^{lck}$ and $p59^{fyn}$ kinases mediated by high levels of endogenous glucocorticoids is responsible for the immunosuppression of T cells in the LN on days 14 and 21. We observed that adrenalectomy of GVHD mice markedly improved, but did not restore, the proliferative responses of T cells to normal values. This suggests that endogenous glucocorticoids are not the only factors involved in T cell immunosuppression. It has been demonstrated that interferon-γ (IFN-γ) and nitric oxide (NO) production during GVHD suppresses mitogen-induced proliferation of T cells, whereas, anti-IFN-γ antibody or L-N^G-monomethyl arginine, a specific inhibitor of nitric oxide synthesis, highly ameloriates T cell proliferation (28, 29). Although other factors are involved in T cell immunosuppression, our study suggest that elevated concentrations of circulating glucocorticoids during GVHD appear to play a central role in inducing T cell immunosuppression in the LN of GVHD mice.

In contrast to the LN, a marked decrease of splenic host T cells on day 21 (Table 2) was not entirely due to endogenous glucocorticoids since adrenalectomy did not reverse the severe deficiency of splenic host T cells (Table 4). It appears that host T cells in the LN, but not in the spleen, are sensitive to high levels of endogenous glucocorticoids during GVHD, suggesting that the LN and spleen may have different degrees of sensitivity

to glucocorticoids. Although the mechanism responsible for the differential degree of sensitivity of the spleen and LN to glucocoticoids during GVHD is unknown, it is possible that GVHD caused a specific marked reduction of heat shock protein 90 (hsp90) in the spleen. The actions of glucocorticoids are mediated by the glucocorticoid receptor, however, in its unliganded (ie. inactive) form the glucocorticoid receptor requires association with hsp90 to be functional (30, 31). Studies have shown that higher level of hsp90 in an organ is associated with increased sensitivity of that organ to glucocorticoids (32). Moreover, when hsp90 is markedly reduced glucocorticoid receptor function is deficient although glucocorticoid is bound to the receptor (33). Interestingly, Vamvakopoulos et al. demonstrated that chronic stress induces a specific marked reduction of hsp90 in the liver and spleen, but, not in the brain, thymus, testis or adrenal glands (34). In addition, these investigators showed that the specific marked decrease of hsp90 in the spleen and liver is not mediated by high levels of glucocorticoids and propose that the marked reduction of hsp90 in the spleen and liver could desensitize these organs to glucocorticoids (34). Based on these studies, we speculate that GVHD induces chronic stress which may cause a specific marked reduction of hsp90 in the spleen, but not in the LN, thereby rendering host splenic T cells less sensitive to glucocorticoids.

The mechanism(s) responsible for the severe decrease of splenic host

$CD4^+$ and $CD8^+$ T cell populations, however, remains unknown. Indirect evidence suggests that the induction of Fas antigen on T cells mediated by cytokines such as IFN-γ and tumour necrosis factor-α could lead to apoptic cell death (35). As early as the first week of GVHD, interleukin-2, IFN-γ and tumour necrosis factor-α are markedly upregulated (36, 37). Hence, these cytokines could stimulate host T cells to increase expression of Fas antigen, and may result in increased susceptibility of host T cells to apoptotic cell death.

Interestingly, a relatively high number of donor cells was detected in the spleens of both non-ADX and ADX GVHD mice throughout the three week period of GVHD, whereas the LN contained very few donor cells (Table 6). Parental-anti-F1 cytotoxic donor cells are generated in the spleen of GVHD mice and are involved in the elimination of host parental cells (15, 16, 38). In addition, T cell functions during the first three weeks of GVHD are actively suppressed by suppressor cells of donor origin (13, 14) and suppressor factors such as IFN-γ, nitric oxide and prostaglandin (28, 29, 39). Hence, high levels of donor cells in the spleens of both non-ADX and ADX GVHD mice could eliminate splenic host T cells by day 21 and could induce severe splenic T cell immunosuppression on days 14 and 21, without affecting LN host T cells.

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Table 1. Effect of endogenous glucocorticoids on the different thymocyte populations during GVHD[a]

Groups	Days after GVHD induction	Plasma CS levels (ng/ml)	Thymic cellularity ($x10^6$)	% Incidence of thymocyte populations			Ratio of $CD4^+/CD8^+$	Absolute number[b] of thymocyte populations ($x10^6$)		
				$CD4^+$	$CD8^+$	$CD4^+8^+$		$CD4^+$	$CD8^+$	$CD4^+8^+$
Non-ADX SYN[c]	____	83.4 ± 6.4	34.7 ± 1.6	12.7 ± 0.9	7.1 ± 0.6	75.0 ± 1.1	1.8 ± 0.1	4.4 ± 0.2	2.4 ± 0.1	26.0 ± 1.6
ADX SYN	____	27.9 ± 9.2	50.1 ± 13.4	11.1 ± 1.0	5.2 ± 1.5	78.3 ± 2.8	2.3 ± 0.3	5.6 ± 1.5	2.6 ± 0.8	39.4 ± 11.1
Non-ADX GVHD[d]	9	190.0 ± 15.8	23.3 ± 1.4	12.6 ± 2.6	9.7 ± 0.3	71.2 ± 3.4	1.3 ± 0.2	3.1 ± 0.7	2.3 ± 0.2	16.5 ± 0.9
ADX GVHD	9	25.1 ± 10.7	55.8 ± 6.1	13.9 ± 0.7	6.1 ± 0.3	75.0 ± 0.9	2.3 ± 0.1	7.8 ± 1.1	3.4 ± 0.3	41.9 ± 4.5
Non-ADX GVHD	24	378.0 ± 82.2	8.6 ± 4.0	27.8 ± 7.2	33.7 ± 3.9	44.2 ± 4.7	0.8 ± 0.2	2.4 ± 0.6	2.9 ± 0.3	3.8 ± 0.4
ADX GVHD	24	13.1 ± 5.6	40.6 ± 8.7	16.2 ± 1.3	7.6 ± 4.9	71.0 ± 8.4	2.1 ± 0.2	6.6 ± 0.5	3.1 ± 0.2	28.7 ± 8.6

[a]Data are present as the mean ± SE of 4-5 mice per group.
[b]Absolute number was calculated as (% Incidence x Thymic cellularity)/100.
[c]SYN groups are B6AF1 mice injected i.v. with $35x10^6$ B6AF1 strain lymphoid cells.
[d]GVHD groups are B6AF1 mice injected i.v. with $35x10^6$ A strain parental lymphoid cells.

Table 2. Host T cell populations in the spleen and LN of non-ADX GVHD mice on different days after GVHD induction[a]

Groups	Organ assayed	Days after GVHD induction	Cellularity ($x10^6$)	% Incidence of host T cell populations		Absolute number of host T cell populations ($x10^6$)	
				$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$
SYN	Spleen	___	97.8 ± 7.9	20.9 ± 1.1	12.7 ± 0.6	20.7 ± 2.3	12.5 ± 1.2
GVHD[b]	Spleen	7	161.0 ± 15.4	5.6 ± 0.2	16.5 ± 0.7	9.0 ± 1.6	26.0 ± 1.4
GVHD	Spleen	14	81.8 ± 11.5	10.6 ± 0.8	28.2 ± 2.0	8.6 ± 1.44	23.3 ± 1.4
GVHD	Spleen	21	45.0 ± 4.8	2.9 ± 0.3	15.5 ± 0.9	1.3 ± 0.1[c]	7.0 ± 0.9[c]
SYN	LN	___	28.6 ± 2.2	40.9 ± 1.4	28.4 ± 0.6	11.5 ± 0.9	8.0 ± 0.6
GVHD	LN	7	77.7 ± 5.0	24.6 ± 0.5	22.0 ± 0.7	19.2 ± 1.6	17.1 ± 1.2
GVHD	LN	14	22.8 ± 4.9	22.9 ± 1.7	34.0 ± 0.9	5.6 ± 1.6	7.7 ± 1.6
GVHD	LN	21	5.6 ± 1.5	14.2 ± 1.7	38.6 ± 2.0	0.9 ± 0.4[d]	2.1 ± 0.6[d]

[a]Data are presented as the mean ± SE of 4-5 mice per group.
[b]GVHD was induced in B6AF1 mice injected i.v with $35x10^6$ A strain parental lymphoid cells.
[c]Host splenic $CD4^+$ ($p < 0.001$) and $CD8^+$ ($p < 0.02$) T cell populations in non-ADX GVHD mice on day 21 were significantly decreased when compared to non-ADX SYN group. The student's *t* test was used to calculate the statistical significance.
[d]Host LN $CD4^+$ ($p < 0.001$) and $CD8^+$ ($p < 0.001$) T cell populations in non-ADX GVHD mice on day 21 were significantly decreased when compared to non-ADX SYN group. The student's *t* test was used to calculate the statistical significance.

Table 3. Effect of thymectomy on host T cell populations in the spleen of GVHD mice[a]

Groups[b]	Cellularity	% Incidence of host T cell populations		Absolute numbers of host T cell populations		Ratio of host $CD4^+/CD8^+$
		$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$	
SHTX[c] NORMAL	121.5 ± 8.5	18.8 ± 2.6	16.2 ± 5.4	23.1 ± 4.7	20.1 ± 7.9	1.3 ± 0.3
TX[c] NORMAL	108.0 ± 22.5	14.9 ± 1.0	11.6 ± 2.8	15.9 ± 2.3	11.9 ± 0.4	1.3 ± 0.3
TX GVHD[d]	57.5 ± 3.5	4.2 ± 0.3	19.8 ± 2.9	2.4 ± 0.3	11.2 ± 0.9	0.2 ± 0.0

[a]Data are presented as the mean ± SE of 4-5 mice per group.
[b]All mice used were of the B6AF1 strain.
[c]Sham-thymectomy (SHTX) and thymectomy (TX) were performed 5 months before the day of assay.
[d]GVHD was induced with $20x10^6$ A parental strain lymphoid cells and mice were sacrificed 21 days after GVHD induction.

Table 4. Effect of adrenalectomy on host T cell poulations in the spleen and LN of mice undergoing GVHD[a]

Group[b]	Organ assayed	Days after GVHD induction	Cellularity ($x10^6$)	% Incidence of host T cell populations		Absolute number of host T cell populations ($x10^6$)	
				$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$
SYN	Spleen	___	149.6 ± 14.0	21.0 ± 2.0	12.1 ± 1.7	31.6 ± 3.2	18.4 ± 2.9
GVHD[c]	Spleen	7	134.3 ± 9.8	6.3 ± 0.4	15.8 ± 1.2	8.5 ± 0.9	21.2 ± 2.2
GVHD	Spleen	14	87.0 ± 6.7	11.6 ± 0.7	33.3 ± 1.4	9.9 ± 0.5	28.8 ± 2.3
GVHD	Spleen	21	87.0 ± 8.5	3.6 ± 0.6	15.7 ± 2.4	2.7 ± 0.4[d]	7.2 ± 0.9[d]
SYN	LN	___	44.7 ± 3.2	35.7 ± 0.8	28.4 ± 0.7	15.9 ± 1.2	12.6 ± 0.9
GVHD	LN	7	82.3 ± 8.8	21.3 ± 0.9	20.5 ± 0.7	17.7 ± 2.2	17.0 ± 2.1
GVHD	LN	14	49.2 ± 7.7	25.5 ± 1.2	33.0 ± 1.2	12.9 ± 2.4	16.0 ± 2.3
GVHD	LN	21	33.7 ± 3.8	27.9 ± 1.4	25.5 ± 2.1	9.4 ± 1.1[e]	8.5 ± 1.1[e]

[a]Data are presented as the mean ± SE of 4-6 mice per group.
[b]All mice were adrenalectomized 2 months before GVHD induction.
[c]GVHD was induced in B6AF1 mice injected i.v. with $35x10^6$ A strain parental lymphoid cells.
[d]Host splenic $CD4^+$ ($p < 0.001$) and $CD8^+$ ($p < 0.01$) T cell populations in ADX GVHD mice on day 21 were significantly reduced when compared to ADX SYN animals. The student's *t* test was used to calculate the statistical significance.
[e]Host LN $CD4^+$ ($p < 0.05$) and $CD8^+$ ($p < 0.05$) T cell populations in ADX GVHD mice on day 21 were significantly decreased when compared to ADX SYN animals. The student's *t* test was used to calculate the statistical significance.

Table 5. Effect of cortisone treatment on host T cell numbers in the LN of ADX GVHD mice[a]

Groups[b]	Days after GVHD induction	Cellularity ($x10^6$)	% Incidence of host T cell populations		Absolute number of host T cell populations ($x10^6$)	
			$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$
SYN	—	3.4 ± 0.94	34.6 ± 1.9	32.9 ± 1.4	1.2 ± 0.3	1.1 ± 0.27
GVHD[c]	7	38.6 ± 5.7	18.3 ± 0.6	21.3 ± 1.0	7.2 ± 1.3	8.3 ± 1.5
GVHD	14	20.3 ± 6.4	22.9 ± 1.1	31.1 ± 1.6	4.6 ± 1.4	6.0 ± 1.7
GVHD	21	8.2 ± 2.8	24.6 ± 2.2	40.6 ± 1.7	1.9 ± 0.6	3.5 ± 1.3

[a]Data are presented as the mean ± SE of 4-6 mice per group.
[b]All ADX mice were treated i.p. with a single dose of 2.5 mg cortisone acetate 2 days before they were sacrificed.
[c]GVHD was induced in B6AF1 mice injected i.v. with $35x10^6$ A strain parental lymphoid cells.

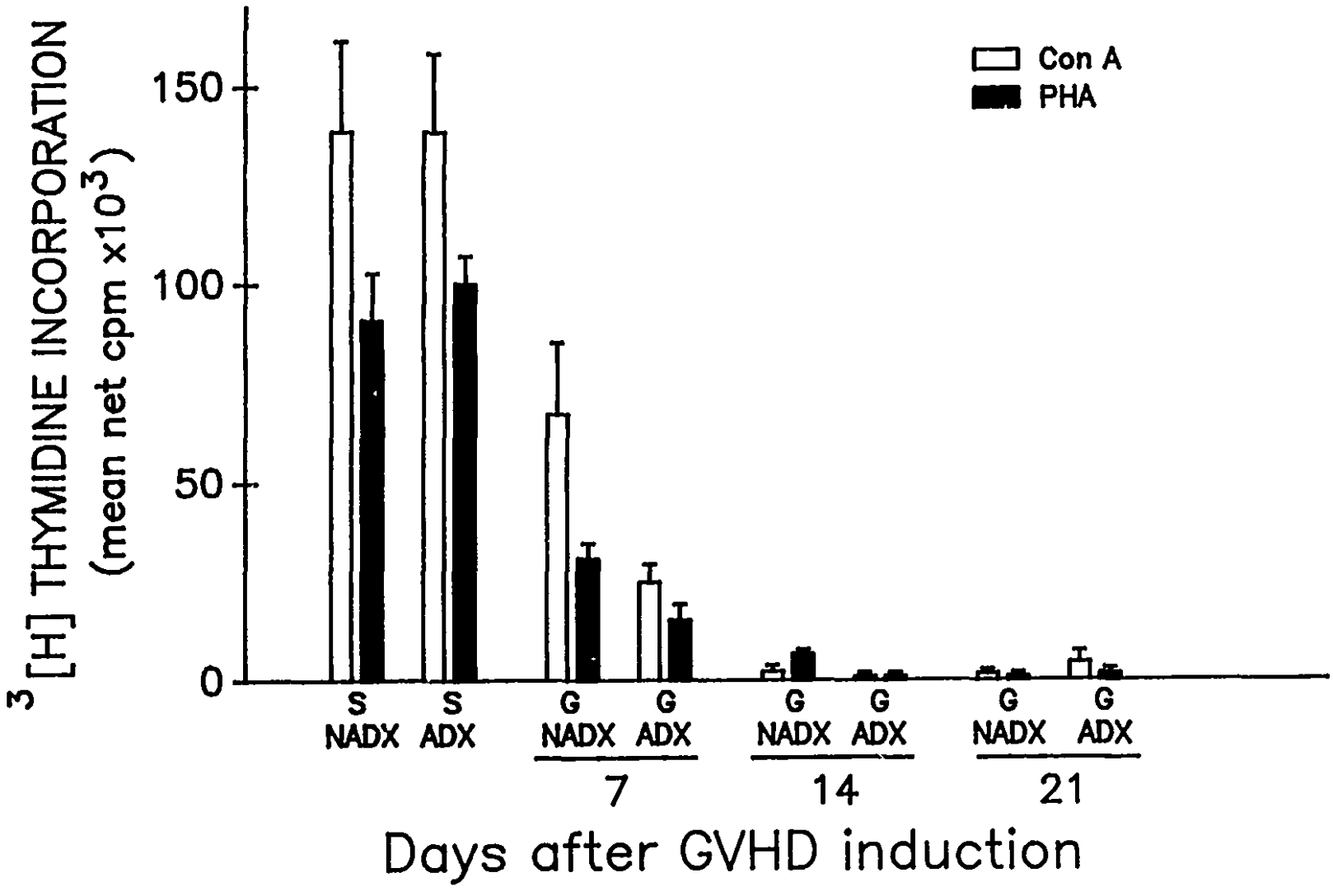
Table 6. Donor cell chimerism in the peripheral lymphoid organs of mice undergoing GVHD[a]

Groups[b]	Days after GVHD induction	Splenic chimerism		LN chimerism	
		Percent	Absolute number ($\times 10^6$)	Percent	Absolute number ($\times 10^6$)
Non-ADX	7	10.9 ± 0.7	17.5 ± 2.2	6.1 ± 0.3	4.7 ± 0.2
ADX[c]	7	7.0 ± 0.7	9.4 ± 2.2	3.9 ± 0.2	3.2 ± 0.2
Non-ADX	14	39.3 ± 3.6	31.9 ± 2.9	8.1 ± 2.0	3.3 ± 0.4
ADX	14	33.5 ± 1.5	29.1 ± 1.1	7.1 ± 1.1	4.9 ± 0.5
Non-ADX	21	24.4 ± 2.7	10.9 ± 2.6	5.0 ± 0.3	0.28 ± 0.1
ADX	21	34.3 ± 4.5	29.8 ± 2.3	3.4 ± 0.4	1.1 ± 0.1

[a]Data are presented as the mean ± SE of 4-6 mice per group.
[b]All groups were experiencing GVHD which was induced in B6AF1 mice injected i.v. 35×10^6 A strain parental lymphoid cells.
[c]Adrenalectomy was performed two months before GVHD induction.

Fig. 1. Effect of adrenalectomy on peripheral T cell function during GVHD. Data are presented as mitogen-induced proliferation of T cells derived from the spleens (Fig. 1A) and LN (Fig. 1B) of non-adrenalectomized (NADX) or adrenalectomized (ADX) SYN (S) and GVHD (G) mice. ADX and NADX mice were age-matched. Adrenalectomy was performed two months before GVHD induction. The student's *t* test was used to calculate the statistical significance of ADX GVHD mice compared to NADX GVHD animals on days 7 (* $p < 0.2$), 14 (** $p < 0.001$) and 21 (*** $p < 0.5$).

A.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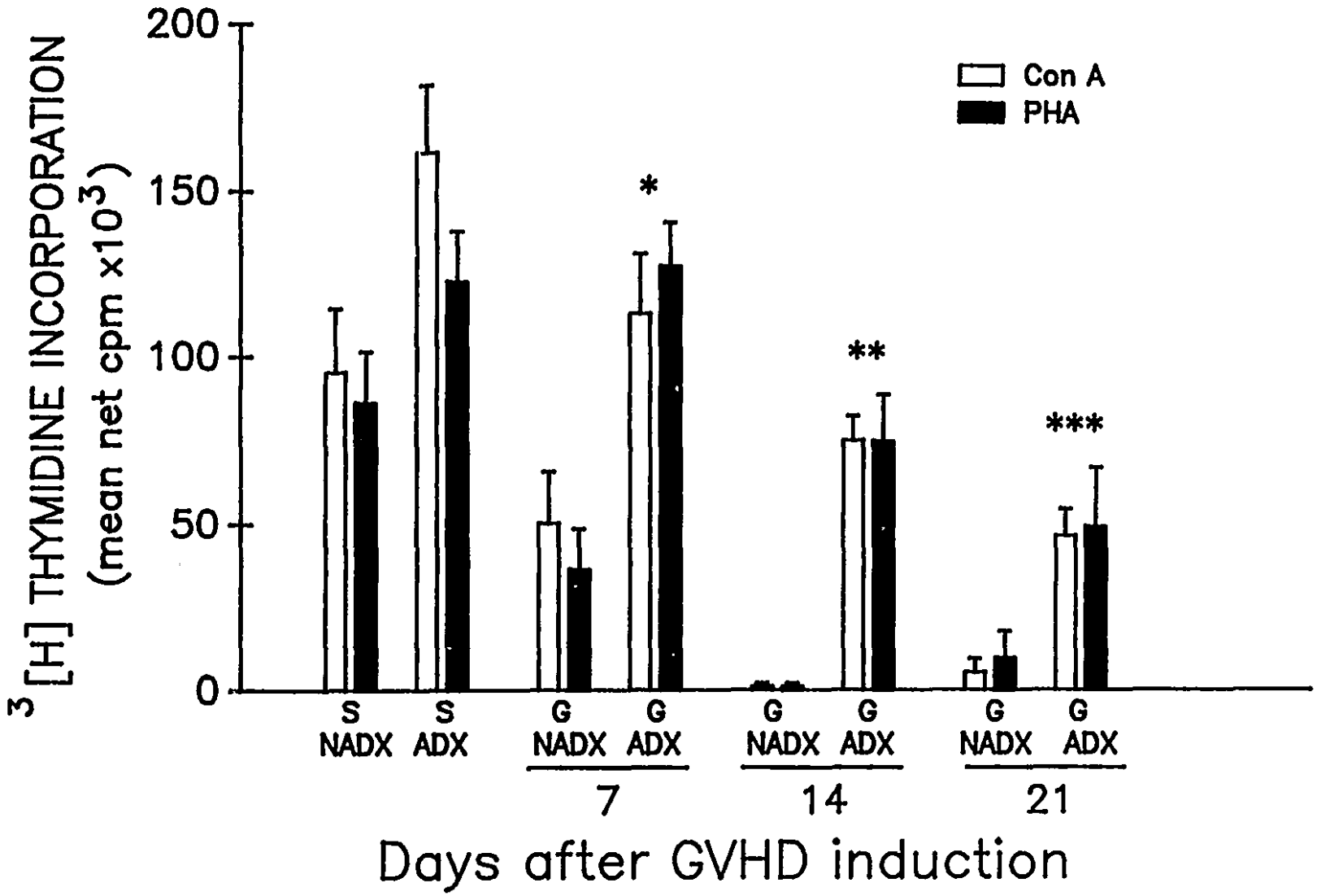
B.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CHAPTER 6

DISCUSSION

A. SUMMARY OF RESULTS

The results presented in this thesis examine the mechanism of glucocorticoid secretion during GVHD, and the role of endogenous glucocorticoids on the pathogenesis of the disease.

Radioimmunoassays show that persistent elevated plasma corticosterone levels during GVHD are independent of pituitary ACTH, however, plasma corticosterone levels coincide with marked increased expression of POMC mRNA in the adrenals of GVHD mice. Transcripts for IL-12, a cytokine produced by activated macrophages, are also upregulated in GVHD adrenals, without any sign of mononuclear infiltrates. In addition, the transcripts for mPC1, the enzyme that cleaves POMC into biologically active ACTH, are highly expressed in the GVHD adrenals. Macrophages have been shown to secrete biologically active ACTH (1, 2) and to reside in the adrenals of normal mice (3). Thus these results suggest that resident adrenal macrophages are activated during GVHD to produce local ACTH that stimulates glucocorticoid secretion.

Having established a possible mechanism of glucocorticoid secretion in GVHD mice, the role of endogenous glucocorticoids on the pathogenesis of the disease was investigated by adrenalectomizing B6AF1 recipients 2-5

months before GVHD induction with $20x10^6$ A strain PLC. Our results show that adrenalectomy allows the B6AF1 recipients to recover rapidly from symptoms characteristic of GVHD, after an initial two week manifestation of GVHD. The rapid recovery from GVHD is attributed to the induction of a glucocorticoid sensitive F1-anti-parental mechanism that eliminates or rejects the parental graft, after an initial period of engraftment. The effector cell mediating the F1-anti-parental mechanism is $ASGM1^+$ and/or $CD8^+$ but not $NK1.1^+$. Furthermore, the effector cell is not dependent on a mature thymus and is renewable 5 months after glucocorticoid treatment, but not after anti-ASGM1 treatment.

Further studies indicate that the glucocorticoid-sensitive F1-anti-parental mechanism can be overcome with increasing donor doses. ADX B6AF1 recipients injected with 35 or $50x10^6$ A strain PLC did not display the glucocorticoid sensitive F1-anti-parental mechanism and experience a more severe GVHD as observed by a greater mortality rate by day 14 in the ADX GVHD group when compared to non-ADX GVHD counterparts. In addition, our results show that levels of plasma glucocorticoids during GVHD play a central role in mediating severe deficiency of host T cell populations in the LN, but not in the spleen, of GVHD mice.

The results presented in this thesis have been discussed in the appropriate chapters. The following discussion will instead focus on the more speculative interpretations of the work from this thesis, in particularly, how endogenous glucocorticoids affect our understanding of the immune system and how they may play an important role in the pathogenesis of autoimmune diseases.

B. PROTECTIVE EFFECTS OF ENDOGENOUS GLUCOCORTICOIDS

Numerous reports have shown that the secretion of endogenous glucocorticoids during an immune response is beneficial to the host. Infection with bacteria, viruses or superantigens results in the production of a variety of cytokines, including IL-1, IL-2, IL-6, and TNF-α (4-6). The immune response is often associated with an increased secretion of glucocorticoids that returns to basal levels after a few hours to days (4, 5, 7-10). It is postulated that glucocorticoid secretion during an immune response acts to downregulate the production of cytokines by controlling transcriptional, translational and secretion (5, 9, 11-13), hence protecting the host from the lethal effects of cytokine overproduction. This is supported by the findings that LPS injected into ADX rodents induces the

production of lethal amounts of inflammatory cytokines, leading to septic shock (9, 14). Furthermore, injection of anti-CD3 antibodies and superantigens into ADX mice or mice treated with RU486, a glucocorticoid receptor antagonist, leads to mortality mediated by overproduction of T cell cytokines (5). In light of these studies, a similar scenario can explain the increased mortality observed in ADX B6AF1 recipients injected with 35 or $50x10^6$ A strain parental lymphoid cells (Chapter 5). More than 50% of the ADX GVHD mice died by day 14 compared to less than 10% of non-ADX GVHD animals. Prior to death the GVHD mice displayed physical traits characteristic of sepsis suggesting that mortality was mediated by GVHD-induced septic shock. In fact, TNF-α, the mediator of septic shock, is highly induced in many tissues and is increased in the serum during GVHD (15-17), whereas, treatment of GVHD recipients with anti-TNF-α markedly improves the symptoms of GVHD (18, 19). Plasma levels of glucocorticoids are elevated as early as day 5 after GVHD induction, the period when donor T cells are activated to induce the disease (20, 21). This increase in glucocorticoid secretion early during GVHD can negatively regulate donor T cell activation and cytokine production, thereby limiting the severity of the disease. In addition, glucocorticoid release may also downregulate the production of lethal amounts of inflammatory cytokines, most notably TNF-α. The depletion of endogenous glucocorticoids by surgical adrenalectomy therefore results in increased activation of donor T cells and production of

pathological amounts of cytokines that are lethal to the GVHD hosts.

Glucocorticoid secretion is normally triggered by pituitary ACTH, which in turn is stimulated by hypothalamic CRH (22, 23). Negative feedback inhibition by glucocorticoids acting at the anterior pituitary and hypothalamus ensures that the levels of glucocorticoids in the circulation are not chronically elevated. Our findings show that in GVHD mice the local immune-adrenal axis is activated to cause chronic secretion of glucocorticoids. In this case, as will be discussed below, the activation of the local immune-adrenal axis during GVHD and in other pathological situations can be detrimental to the host.

C. A MODEL OF IMMUNE-ADRENAL AXIS

1. Virus and LPS stimulation of the immune-adrenal axis

Chapter 2 provides evidence that a local immune-adrenal axis is induced during GVHD to trigger the release of glucocorticoids independent of pituitary ACTH. We propose that LPS (ie. gut E. coli) translocates across the injured gut into the circulation and activates resident adrenal macrophages to produce local ACTH which in turn stimulates the secretion

of glucocorticoids. Such a mechanism may also occur in other pathological situations where there is a dissociation between glucocorticoid secretion and pituitary ACTH. In patients with chronic endotoxemia plasma cortisol levels are persistently elevated (24-26), and in burned victims the levels of plasma cortisol are also increased (27, 28). However, the levels of plasma ACTH in these pathological conditions are normal or subnormal, suggesting that glucocorticoid secretion may be independent of pituitary ACTH. The trauma of the burn causes gut E. coli to translocate across the gut wall into the circulation, providing the source of LPS (29). Thus similar to patients with chronic endotoxemia, burned victims are chronically exposed to LPS. In light of our findings in chapter 2, the dissociation between plasma cortisol and ACTH concentrations in burned victims and in patients with chronic endotoxemia might also be attributed to LPS stimulating adrenal macrophages to produce ACTH which acts locally to trigger the secretion of cortisol. This hypothesis is supported by the observations that resident macrophages are present in human adrenals (30) and human immune cells are activated with LPS to produce biologically active ACTH (31-34).

Besides LPS, viruses are well documented to activate immune cells to produce POMC transcripts and to secrete biologically active ACTH (1, 2, 35-37). Numerous endogenous viruses are activated during GVHD (38-40) and can therefore provide another possible stimulus for local ACTH production

by adrenal macrophages. GVHD-induced endogenous viruses, such as HSV, EBV, and/or CMV, can activate adrenal macrophages to produce ACTH that triggers the release of glucocorticoids. Interestingly, several studies have reported a chronic stress state in patients infected with the Human Immunodeficiency Virus (HIV). These patients display an increase in basal plasma cortisol levels (41-43), however, with parallel decreases in plasma ACTH levels, suggesting that cortisol secretion may not be stimulated by pituitary ACTH (41). The mechanism for the cortisol secretion in HIV-infected patients is unknown. Since resident macrophages have been identified in human adrenals (30) and macrophages of HIV-infected patients serve as important reservoirs of the virus (44), infected adrenal macrophages may be stimulated to secrete local ACTH that causes cortisol secretion.

2. Immunosuppressive effects mediated by the local immune-adrenal axis

The release of glucocorticoids is normally under the negative feedback inhibition of high circulating glucocorticoids acting at both the hypothalamus and anterior pituitary to downregulate CRF and ACTH production, respectively (22, 23). In contrast, ACTH production by activated adrenal macrophages does not appear to be subjected to

glucocorticoid-mediated negative feedback inhibition since POMC transcripts in GVHD adrenals remain highly expressed despite elevated levels of circulating glucocorticoids. The loss of the negative feedback inhibition results in chronic elevated levels of glucocorticoids which can be detrimental to the host. Glucocorticoids are well known to have multiple immunosuppressive effects. High levels of endogenous glucocorticoids inhibit T cell functions by downregulating the production of IL-2 (45) and the levels of p56lck and p59fyn, two src family tyrosine kinases involved in T cell activation (46), inhibit macrophage phagocytic functions, as well as antigen processing and presentation (47), block the synthesis of inflammatory cytokines including IL-1, IL-6 and TNF-α (48), decrease NK cell activity (49), and induce programmed cell death in immature T and B precursors and in mature T and B lymphocytes (50, 51). We propose that gut-derived LPS and/or activation of endogenous viruses provide the stimulus for adrenal macrophages to produce ACTH. Thus chronic infection with bacteria or viruses can continuously activate adrenal macrophages to produce local ACTH which maintains persistent elevated levels of glucocorticoids. The sustained adrenal hyperactivity therefore renders the host severely immunosuppressed, leading to the appearance of opportunistic superinfections. The induction of the local immune-adrenal axis may provide an explanation for the severe imunosuppression often observed in certain viral and bacterial infections (4, 7, 35). In addition, this mechanism

may also perpetuate the immunodeficiency observed in patients infected with HIV.

C. A MODEL OF LATE GRAFT FAILURE

BMT is the treatment of choice for many hematological malignancies, immunodeficiency diseases, aplastic anemia and radiation injury (52). Even in fully HLA-matched bone marrow transplant recipients, GVHD remains a major complication including morbidity and mortality (52, 53). The incidence of GVHD is even greater in HLA-mismatched BMT (52, 53). Over the years, several strategies were used to control or ameliorate GVHD. Drugs such as methotrexate, cyclophosphamide, cyclosporin A and corticosteroids are routinely employed to control GVHD by interfering with donor T cell activation and proliferation (52-55). However, doses at which these drugs control GVHD also cause unwanted side-effects including interstitial lung disease, mucositis, and nephrotoxicity (56-58). Despite the use of these drugs, at least 15% of BMT patients die of complications related to GVHD indicating the need for a more effective way of preventing or controlling the disease (59-61).

Experimental studies in rodents show that mature T cells present in the

bone marrow are responsible for inducing GVHD (62, 63) and GVHD does not occur when the graft is devoid of mature T cells (64-66). Further studies in other animal models also demonstrate that prevention of GVHD can be achieved by removing T cells from the bone marrow (67-69). Based on these animal models, numerous centres carry out transplantation of T cell depleted bone marrow (TCDBM) in human patients. As predicted, the incidence and severity of GVHD are markedly reduced (70-75). Unfortunately, the rate of graft failure or rejection of recipients transplanted with HLA-matched allogenic TCDBM increases to 10%, and 50% with HLA-mismatched allogenic TCDBM, in contrast to 1-5% of patients receiving unmodified bone marrow (71-73, 76, 77). Most of the cases of graft failure occurs 7 days and up to 200 days after an initial period of engraftment implying that graft failure is a late phenomenon. The exact mechanism of graft failure is unknown. It is postulated that the different techniques used to deplete T cells from the bone marrow cause stem cell damage and hence promote graft failure. This is not the case since graft failure does not occur in patients transplanted with autologous bone marrow devoid of T cells by the same depleting techniques utilized for allogenic bone marrow (78). Instead, residual host resistance is believed to be the principal mechanism responsible for graft failure or rejection.

In chapter 3 we describe a late graft rejection mechanism mediated by the

induction of a $CD8^+$ effector cell of host origin that is sensitive to high levels of glucocorticoids. The effector cell in our model appears to target donor non-T cells, most likely including hematopoietic stem cells present in the splenic graft. This suggests that the effector cell may also target hematopoietic stem cells present in the bone marrow and may play a role in the late rejection of TCDBM. Interestingly, many groups of investigators observed that there is an increase of $CD8^+$ cells of host origin among the peripheral blood mononuclear cells emerging at the time of late graft failure after TCDBM transplantation in human patients (79-85). The lack of GVHD occurrence in clinical TCDBM transplantation circumvents the use of immunosuppressive drugs such as corticosteroids. Two studies in which prednisolone was used resulted in the disappearance of the activated host derived $CD8^+$ cells and the reversal of graft failure (83, 84, 86). The host-anti-donor $CD8^+$ cells appear to be resistant to the pretransplant conditioning regiment consisting of total or partial irradiation and cyclophosphamide (83, 84, 86). The clinical findings are supportive of our studies, showing that treatment of the recipient mice with high dose of exogenous glucocorticoids eliminates the host-anti-donor $CD8^+$ effector cell and promotes engraftment. Thus it seems that a glucocorticoid sensitive $CD8^+$ effector cell of host origin is activated after TCDBM transplantation and mediates late graft failure. As mentioned above, corticosteroids are rarely utilized in TCDBM transplantation because of the low incidence of

GVHD. In light of these studies, corticosteroids should be used more readily for patients transplanted with TCDBM in order to eliminate the glucocorticoid-sensitive host-anti-donor $CD8^+$ effector cells and thus promote engraftment without the risk of GVHD occurrence.

Graft failure is rarely observed in patients with unmodified BMT, although GVHD is a major complication. This may be explained on the basis of the elimination of the host-anti-donor $CD8^+$ cells with high doses of corticosteroids used to control GVHD. Furthermore, in patients receiving unmodified BMT without being treated with exogenous glucocorticoids, GVHD can trigger the local immune-adrenal axis to secrete high amounts of glucocorticoids which then eliminates the host-anti-donor effector $CD8^+$ cells and abrogates graft failure.

E. A MODEL OF AUTOIMMUNITY: synthesis of our results

Despite the wealth of knowledge of the immune system , the pathogenesis of autoimmune diseases still remains obscure. Autoimmune diseases are pathological conditions in which the immune system is activated to react

to the host tissues and inflicts injuries. No single theory or mechanism can explain the development of autoimmune diseases. At least four factors have been postulated; genetic, viral, endocrine and psychoneuroimmunological. In the present discussion, a speculative hypothesis will be put forth on the role of the endogenous glucocorticoids in the pathogenesis of autoimmune diseases.

In the Parental → F1 model, the parental lymphoid cells react vigorously against the F1 hybrid tissues. Since the parental lymphoid cells share the same MHC antigens as the F1 hybrid, they are recognized as self and are not rejected by the F1 hybrid. Hence, the immune reaction of the parental lymphoid cells against the F1 tissues is perceived by the F1 hybrid as an autoreaction. In fact, GVHD can result in the development of autoimmunity and autoimmune diseases (87-92). Results from chapters 3 and 4 show that a glucocorticoid sensitive F1 effector cell is induced to eliminate the parental graft, suggesting the F1-anti-parental mechanism may be activated to eliminate autoreactive cells (ie. parental lymphoid cells). It is of interest to note that the F1-anti-parental effector cell is $CD8^+$, and $CD8^+$ suppressor/cytotoxic cells are shown to suppress or lyse autoreactive cells (93, 94). We therefore speculate that the physiological role of the F1-anti-parental $CD8^+$ effector cell may be to protect against autoimmunity and the development of autoimmune diseases. Hence,

elimination or suppression of the effector cell by glucocorticoids can result in the development of autoimmune diseases. Interestingly, autoimmune diseases tend to develop with age, and the basal levels of glucocorticoids are also increased with age due to a defect in the glucocorticoid-mediated negative feedback inhibition (95-99). The increase in basal levels of glucocorticoids may suppress or eliminate the $CD8^+$ effector cells that protect against autoimmunity, thereby, contributing to the development of autoimmune diseases with aging. This hypothesis is in accord with findings that $CD4^+$ cells stay at a relatively constant level in adult and senescent stages, while $CD8^+$ cells gradually decline with aging (100). Autoimmune diseases are also more often diagnosed in persons exposed to chronic stress (101-107). Based on our hypothesis, the chronic stress triggers the persistent release of glucocorticoids in the circulation to suppress or eliminate the $CD8^+$ effector cells that protect against autoimmunity, thus leading to the development of autoimmune diseases.

The results obtained in the studies of endogenous glucocorticoids on GVHD have revealed an alternate pathway of glucocorticoid secretion that may be important in the understanding of the pathogenesis of certain viral and bacterial infections. In addition, a speculative model has been constructed to associate the role of endogenous glucocorticoid with the development of autoimmune diseases. These findings give rise to new questions about the

interaction of the endocrine and immune systems and in particular, how the endocrine system may regulate autoimmunity.

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