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THE ROLES OF THE CD4+ (T HELPER) LYMPHOCYTE IN REGULATING THE MURINE CYTOTOXIC T LYMPHOCYTE RESPONSE TO HERPES SIMPLEX VIRUS TYPE 1

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Carmen Maria Mercadal

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This is dedicated to my family :

my mother who made many sacrifices so that I could have the education that she always desired but never had,

my father who inspired me to pursue that which I wanted,

and to my brother Mark and my sisters Melanie and Frances,

who made growing up fun and who continue to show me immense love and support

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ABSTRACT

This communication presents an approach for identifying the requirement of CD4+ T lymphocytes in the induction and expansion of HSV-1 specific CTL *in vivo*. Rat monoclonal antibodies were used to selectively deplete mice of CD8+ and CD4+ T cells *in vivo* in order to assess the requirements of CD4+ T lymphocytes in the induction and expansion of HSV-1 specific CTL. These antibodies produced greater than 95% depletion of their respective T cell subsets as determined by flow cytometry. Functional depletion of CD4+ cells was evident by supressed antibody response towards Rabbit IgG and suppressed neutralizing antibody response to HSV-1,both T-dependent antigens. Additional functional evidence was shown by a reduction of HSV-1 specific delayed-type hypersensitivity. CD4+ deficient mice could generate a memory HSV-1-specific CTL response, yet acutely infected mice could not generate HSV-specific CTLs. This response was strain specific.

In addition to the aforementioned studies, a limiting dilution microculture system was utilized to calculate the frequencies of CTL-p reactive against HSV-1 in CD4+ depleted and non-depleted mice. Tazwell analysis (160) demonstrated that the estimated frequency of HSV-1 reactive cells in the lymph nodes of non-depleted immune mice was 1/6063, while in immune, CD4+ depleted mice the frequency was 1/9462. In addition to this, lymph node cells from these mice, when restimulated *in vitro* with HSV-1 infected irradiated splenocytes, demonstrated CTL-p frequencies of 1/2654 and 1/1177 respectively. These observations clearly demonstrate that CD8+ CTL-ps can be

induced in a CD4+-deficient environment. However, there may be different requirements in both systems as to the expansion and differentiation of these cells. The results suggest that the expansion of CTL-p to their aggressor status in the primary HSV-1-specific CTL response in CD4+ deficient animals is dependent upon soluble growth factors, these factors probably being secreted by a Th cell. The implications of these observations respective to the induction and subsequent expansion of HSV-1-specific CD8+ CTLs are discussed.

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IFN	=	interferon
NK	=	natural killer
Ab	=	antibody
Ag	=	antigen
CTL	=	cytotoxic T lymphocyte
HSV-1	=	Herpes Simplex Type 1
CTL-p	=	cytotoxic T lymphocyte precursor
Th	=	T helper lymphocyte
Th-p	=	T helper lymphocyte precursor
LCMV	=	lymphocytic choriomeningitis virus
VSV	=	vesicular stomatitis virus
MCMV	=	murine cytomegalovirus
TMEV	=	Theiler's murine encephalomyelitis virus
IL	=	interleukin
MHC	=	major histocompatiblity complex
DTH	=	delayed type hypersensitivity
CMI	=	cell mediated immunity
TCR	=	T cell receptor
^{3}H-Tdr	=	tritiated thymidine
$TCID_{50}$	=	T cell infectious dose-50 (the dose of virus responsible for 50% of cytopathic effect seen)

CHAPTER I

INTRODUCTION

The interaction between the CD4+ T cell and the CD8+ T cell subsets in the immune response to viral infection *in vivo* still remains to be fully elucidated. It is known that the functions of both cell types are governed by their respective Major Histocompatibility (MHC) makeup, CD4+ T cells being restricted to Class II MHC and CD8+ T cells being restricted to class I MHC (1,2,13). In viral infections, it has been shown that the CD8+ T cell is responsible for the irradication of the virus and the lysis of virus-infected cells(36). The generation of a primary alloreactive CTL response *in vitro* requires the interaction between CD4+ T helper cells, antigen presenting cells and CTL-p (1,3,37,65,66). CD4+ T cells produce IL-2 and other lymphokines to drive the proliferation , expansion, and terminal differentiation of CD8+ CTL. In the past two years, great strides have been made to glean information about the interactions of these lymphocyte population in influenza virus(152), coxsackie virus(150), TMEV(146,147), LCMV(148,149), VSV(148), and HSV(153). In the latter reference, Nash *et al.* have shown the physical and functional depletion of CD4+ T cells *in vivo* had no effect on the ability of CBA mice to generate a CTL response to HSV-1. Nash, along with others, have thus proposed a CD4+ independent pathway of CTL induction and expansion *in vivo*.

In this paper, the antibodies described by Fitch *et al.* (145,156) were used to deplete selectively CD4+ and CD8+ T cell subsets *in vivo* in order to study the role played by these T cells in the CTL response against HSV-1. In

particular, the role of the CD4+ T lymphocyte was studied in the induction of an HSV-1-specific CTL response. In addition, limiting dilution cultures were designed in order to estimate the frequency of HSV-1-specific CTL-p in CD4+ depleted mice and compare that frequency with the frequency in intact (non-depleted) mice. It has been reported previously by this lab and others (135,157), that the frequency of HSV-1 specific CTL-p that can be induced in a normal system ranges from from 1/100 - 1/11,000.

We addressed the following questions pertaining to the interaction of the CD4+ T cell with the CD8+T cell in the induction of an HSV-1-specific CTL response. Can lytically active CTL cells be induced in the absence of CD4+ T cells *in vivo*? Is this induction equal in an acute HSV-1-specific CTL response as compared to a memory HSV-1-specific CTL response? Is the frequency of CTL-p in CD4+ depleted mice comparable to the frequency found in intact mice?

The results indicate that virus-specific CD8+ T cells can be induced in CD4+ depleted mice, but questions arise as to their subsequent expansion and differentiation, since the primary HSV-1-specific CTL response and the memory CTL response differed. Time and soluble growth factor considerations had to be taken in account in order to differentiate between the requirements of CTL-p expansion and differentiation in the two different assay systems. The requirement for soluble growth factors was addressed and the results show that these factors are sufficient in order to restore the HSV-1-specific primary CTL response. The implications of this study and the requirement for CD4+ T lymphocytes is discussed.

CHAPTER II

SURVEY OF THE LITERATURE

The Host Immune Response to a Viral Infection

The host immune response to a viral infection is greatly varied and finely regulated. Non-specific mechanisms of immunity provide a first line of defense in response to a viral infection. Specific immune responses involving complex interactions between immune effector cells, regulatory cells, and soluble mediators, represent the more long-term antiviral response. (1,2,3) Nonspecific immune mechanisms pertinent to the control of virus infection include the complement system, tissue and blood-borne phagocytic cells, and the induction of virus-inhibitory interferons. Non-specific antiviral mechanisms may act alone or synergistically with components of the specific immune response. For instance, interferons can affect immunocompetent and natural killer cells (NK) cells. The specific immune response is highly dependent on the regulatory activities of T lymphocytes and their products to promote specific antibody (Ab) production by B lymphocytes, stimulate macrophage microbicidal activity and induce cytotoxic T lymphocytes (CTL) capable of destroying virus infected cells (4,5). A major role has been described for the CTL as the predominant immune component in many viral infections (6-12), a few representatives being lymphocytic choriomeningitis virus (LCMV), murine cytomegalovirus (MCMV), influenza virus, and herpes simplex virus (HSV) types 1 and 2.

The CTL represents a subset of T-lymphocytes specialized for contact-lysis of cells bearing antigen (Ag) for which they have been previously sensitized. CTL's are derived from bone marrow precursors which mature in the thymus where the greater portion of their differentiation occurs. Once the CTL matures and emerges from the thymus, it is tolerant to self and restricted to gene products of the Major Histocompatibility Complex (MHC)[reviewed by Zinkernagel and Doherty](13). It also bears surface Ag's Thy1+Lyt1+2+L3T4- (1). The cytotoxic process has been well investigated *in vitro* (13-18), but its role *in vivo* still remains to be clarified.

The Involvement of T Lymphocytes in Anti-Viral Immunity

Demonstration of cell-mediated immunity to virus antigens has been of interest since Jenner first inoculated cowpox fluids into a milk maid who previously had been exposed to cow pox and observed a very marked display of inflammation at the site of injection. This represented what today is known as Delayed Type Hypersensitivity (DTH). Then, in 1967, Allison (14) was able to take peritoneal cells from guinea pigs who had been immunized with vaccinia virus and transfer the DTH reaction to non-immune recipients. He could not achieve this with serum antibody. This represented formal proof that the reaction was cell-mediated. The importance of immune cellular infiltration was further supported by Rager-Zisman and Allison. They found that mice infected with HSV and treated with cyclophosphamide (an immunosuppressive agent) died. These mice showed no cellular infiltrates, whereas protected mice showed near normal cellular infiltrates(15). Passive protection of the

immunosuppressed mice could be achieved by injecting either immune spleen cells or antibody together with normal spleen cells. Other observations in immunosuppression show that procedures which suppress cell-mediated immunity to a greater extent than humoral immunity increase the severity of infections with poxviruses, herpesviruses and measles (16-18).

Thus, cell-mediated immunity can play a number of roles in protecting hosts against viral infections. First, cell-mediated immune responses can recruit monocytes into sites of virus multiplication through the release of chemotactic factors and activation factors such as gamma-interferon, macrophage chemotactic factor, and leukocyte chemotactic factors. (19). Secondly, lymphocytes responding to antigens or mitogens liberate interferon, and the local release of interferon from leukocytes infiltrating lesions may protect host cells, including macrophages, from viral infection. Third, antigen sensitized lymphocytes may recognize virus-specific antigens on the surfaces of infected cells and destroy them, thus preventing further viral replication (15).

The kinetics of viral growth and induced immune response was summarized first by Fenner in 1949 with poxvirus(20). When poxvirus is injected in the footpad of a mouse, it localizes and replicates in the regional lymph nodes. The virus then passes via lymph to the blood, and subsequently seeds into the liver, spleen or skin. The recovery from viral infection begins between 4-6 days after infection and seems to depend mainly upon the presence of activated T cells in the circulation which recognize viral antigen in the lesions, and then provokes an inflammatory cell infiltration of the necrotic areas. The developing immune response can be measured by DTH, cellular cytotoxicity, or transferable cellular immunity. It can usually be detected about

the time virus titers start to decrease and reaches highest levels soon after the virus is cleared from the body. However, antibody responses or antibody-dependent complement mediated cytotoxicity (ADCMC) become measurable when virus is no longer detectable and remains at substantial levels long after virus is undetectable.

Immunopathology

The immune response to viral infection may frequently contribute to the pathology of the disease itself. The prototype is the disease that results following intracerebral inoculation of adult mice with LCMV (49). These studies suggested that cellular components may be important in clearing the virus from the lungs and contributing to the observed pathology. The fatal meningitis can be completely prevented by immunosuppression with x-irradiation, anti-lymphocyte serum, cytotoxic drugs, or neonatal thymectomy. The disease is entirely due to the mouses's own vigorous cell-mediated immunity (CMI) reaction. In the model murine influenza system, the DTH response to influenza contributes to the pathology of infection in the murine lung. Intravenous transfer of Lyt1+ (CD4+) T cells with DTH activity greatly increased the amount of cellular infiltration and edema in the lung and accelerated the death of the animals (50,51).

Immunopathology of herpesvirus infections has been reviewed by Rouse (52). The immunopathology is usually associated with persistent infections due to the virus. Usually the immunopathology, when T cell mediated, is in the form of DTH. These reactions may be severe and extremely prolonged, and there is

considerable tissue damage due to the infiltration of mononuclear cells in the infected area (53). Other immunopathological effects include direct target destruction by CTL and inflammatory mediators released from damaged tissue cells.

There are situations where HSV antigens persist for long periods without replication and cell destruction. The best example is with herpesvirus latency and the second example is in stromal keratitis. In the murine model of herpetic stromal keratitis, accumulating evidence indicates an immunopathologic reaction mediated by T lymphocytes. This has been shown by Metcalf *et al.* (54) that athymic mice, injected with the RE strain of HSV developed a small degree of epithelial keratitis which eventually cleared up, while normal mice infected under the same conditions developed an epithelial keratitis followed by a deep stromal reaction and permanent scarring. Histological studies show that the corneas of mice are heavily infiltrated with both mononuclear and polymorphonuclear leukocytes and as a result, severe necrotizing lesions develop. Adoptive transfer of the stromal response was abrogated upon removal of the T lymphocytes with specific antisera and complement. Further evidence that the stromal reaction was immunopathological was shown by adoptive transfer experiments. Spleen cells from HSV-immune mice restimulated in vitro with HSV were transferred to athymic mice. This cell population consisted predominantly of T cells which expressed high levels of HSV-specific H-2 restricted cytotoxicity, contained helper cell activity and could mediate a DTH response (55-57). If indeed T cells were involved in the immunopathological reaction, then the spleen cells should elicit a prompt response in the athymic mice. Indeed, the athymic mice, when given these cells adoptively, developed a

necrotizing stromal reaction and scarring similar to those observed in euthymic mice. This further implicated that the stromal response was due to T lymphocytes. Presently, there is a study by Newell *et al.* that delineates the actual mechanism and T cell subset involovement of this stromal reaction *in vivo* (58).

The Role of CTL as an Anti-Viral Effector Cell

A role for CTL in the host defense to viral infection has been postulated based on their capacity to specifically lyse virus-infected syngeneic cells in culture. Among the most definitive demonstrations of CTL activity *in vivo* are the models of host defense to primary virus infection in mice with LCMV, poxvirus, Sendai virus, and Influenza virus (21-24).

In the murine influenza system, Yap *et al.* (25,26) and Wells *et al.* (27) have shown that adoptive transfer of immune cells possessing cytotoxic activity to influenza virus-infected mice promotes their recovery and reduces pulmonary virus titers. The cells responsible for this *in vivo* effector function bore the Thy 1 and CD8 surface marker, required Class I homology between donor and recipient for *in vivo* activity, and exhibited significant influenza-virus specific cytotoxic activity *in vitro* (26). This finding was extended by Lin and Askonas (28) who showed that a clone line of killer T cells transferred to mice 24 hours after infection had a similar effect. However, these clones secreted gamma-interferon. Consequently, in 1982, several groups of researchers (29-31) documented the secretion of lymphokines by CTL populations. This finding seemed to support the hypothesis of Zinkernagel and

Althage (32) that, in addition to direct cytolysis, virus-specific CTL could exert their anti-viral effect *in vivo* by release of lymphokines, most notably gamma-interferon, with direct or indirect anti-viral activity. Based on these criteria, it was deduced that CTL represented the T cell subclass responsible for conferring protection in the murine influenza system. Lukacher *et al.* provided more insight to the role of CTL *in vivo* by developing specific CTL clones against Influenza A virus (33). In this study, the activity of the clones was examined after transfer into syngeneic mice that had been infected with a lethal dose of influenza A virus. One clone recognized only subtype H2N2 and promoted recovery from the lethal infection in a sub-type specific fashion. However, another clone, which exhibited cross-reactivity to two subtypes, likewise protected the mice against each of the different subtypes. Yet, when mice were simultaneously infected with a lethal dose of two different subtypes, the subtype-specific CTL clone could not promote recovery and reduced the pulmonary virus titer of the strain representative of the subtype recognized. The results of their study implied that immunity to the virus was specific and this supported the concept that CTL mediated their effect via direct cytolysis of virus-infected cells. Speel *et al.* (34) and Lundstedt (35) were the first to described that virus-immune lymphocytes destroyed target-cells of virus infected cells *in vitro*, as assessed in a microcytotoxicity assay. This cytotoxic activity can be monitored by ^{51}Cr (in the form of sodium chromate) release *in vitro*. Cells that express the appropriate target antigens for CTL are incubated in the presence of this high energy gamma emitter for a sufficient period of time to permit the uptake of the chromium into the cells. The target cells are then washed free of any extraneous chromium, and coincubated with specifically-

activated CTL at several effector-target cell ratios. These are allowed to incubate for a short time after which supernatant fluid is drawn from each test well and chromium release is measured. Any level of chromium release above background is used as a relative measure of CTL-mediated target cell damage. Since these early studies, it has become evident that the T cell immune system, particularly CTL, plays a crucial role in clearing virus infected cells. Consequently, in most viral diseases, the generation of a CTL response is of the utmost importance .

The Host Immune Response to HSV

The specific host response to infection with HSV is very complex (36,37,48) including both cellular and humoral components. Natural resistance mechanisms, mediated by macrophages, NK cells and interferon represent an important early barrier to the virus, although the establishment of long-lasting anti-viral defense is due to the specific immune system.

NK cells represent a population of non-T, non-B lymphoid cells that have the functional capability to lyse a variety of tumor, virus-infected, and hematologic cell types (38-40). They are thought of as representing a functionally specialized lymphoid class derived from T cell progenitors and may exert a non-specific cell-mediated cytotoxicity against virus-infected targets. Lysis by NK cells does not require prior exposure to antigen or the presence of target sensitizing antibody. Contrary to the histocompatibility restriction found with CTLs, NK cells can lyse cells of different strains and species (39,40). They become activated during the early stages of virus infections and interferon

enhances their activation. At later stages of the infection however, the interferon levels decrease, along with NK cell activation and blastogenesis (41,42).

Whereas both humoral and cell-mediated responses are readily induced by herpesviruses, it is clear that the action of T cells is central for recovery from the primary infection and in the control of recrudescent lesions. This evidence has been shown by studies with a variety of inbred and congenic mouse strains in order to study the MHC restriction of T-cell responses, and the availability of monoclonal antibodies to specific T cell subsets has enabled a detailed analysis of the antiviral T-cell response.

Distinct T-cell subsets recognize Ag in association with different MHC products. This provides a means of identifying the subsets which operate in protective immunity against viral infections. Several groups have identified the products of the murine K, D, and L (Class I) as the restricting elements for anti-viral CTL directed to a variety of different viruses (43-45).

Howes *et al.* (46) found that immune protection against HSV-1 could be conferred by immune spleen cells possessing either Class II or Class I MHC compatibility and that long-lasting protection is conferred only by cells with I-region compatibility. In other words, their findings show a role for the Th and CTL in antiviral immunity, but complete protection required the operation of both cell types. Subsequently, Nash analyzed the MHC restriction of DTH to HSV. He observed that, in mice, the H-2K, D and H-2 I-A regions are important for the accelerated clearance of infectious HSV. These results were comparable with the results of Howes *et al.* Yet, compatibility with H-2K, D structures alone was not sufficient to reduce virus titers, and I-region compatibility alone is

sufficient for DTH transfer but not for the full antiviral effect. This suggests that interactions between the cell types governed by these regions is necessary *e.g.*, the Th and CTL (47). Therefore, it is evident that the action of T cells, particularly CTL, is central for recovery from primary infection and in the control of recrudescent lesions caused by the latent virus. Both heterogeneous and cloned populations of anti viral CTL were shown, upon adoptive *in vivo* transfer into infected recipients, to inhibit replication of HSV and to alter the outcome of lethal infection (1,2).

The Induction of HSV- Specific CTL Cells

The mechanisms of induction, expansion, and terminal differentiation of virus-specific CTL *in vivo* are highly complex. *In vitro* mechanisms have been well documented (59-64). Schmid *et al.* (65,66), along with other investigators have studied in detail the induction of HSV specific CTL cells (67). In mice HSV- specific CTL can be detected in draining lymph nodes 4 days after injection with live HSV-1 or HSV-2 (67), killer activity rises until day 6 and declines by day14 when it becomes undetectable.

The induction of HSV-specific CTL requires interaction between a number of cell types and can be divided into two stages (37). In the first stage, lytically active CTL precursors (CTL-p) recognize specific antigen that is presented by an antigen presenting cell (APC) in association with Class I MHC molecules. The CTL-p is thereby stimulated via its antigen specific receptor (TCR). In this instance, IL-2 and other cytokine receptors are induced on the surface of the cell. The APC's also physically present processed antigen (Ag)

in the context of the appropriate Class II molecule to an antigen-specific helper T lymphocyte precursors (Th-p)(69,70). The APC can probably be a macrophage or dendritic cell (59,63,69,71,72). Treatment with monoclonal antibodies (mAb) to Ia diminishes the ability of CTL cells to mount an anti-viral response (65,73,74). Once the macrophage is activated by the presence of Ag, it secretes interleukin-1 (IL-1), which is a soluble protein factor that is a primary requirement for the generation of lytically active CTL (75-77). IL-1 enhances the ability of the Th lymphocyte to bind specific Ag in context with Class II MHC (Ia), and it primes the Th for the release of lymphokines that further augment the generation of the CTL. This role in lymphokine release defines the central position of the Th cell in the antiviral immune response(78,79). Under appropriate stimulus, the Th releases a number of products, the most prominent of which are interleukin-2 (IL-2), interleukin-4 (IL-4), and gamma-interferon (g-IFN)(78,80).

In the second stage of CTL induction, the various growth factors, lymphokines and cytokines bind to their respective receptor on the membrane of activated CTL-p. This results in proliferation of the CTL-p and then differentiation into lytically active CTL. Subsequent to maturation, the mechanisms by which CTL lyse virus infected target cells can be separated into several phases. In the initial phase, cell-cell contact between the CTL and its target cell is established. Next, the "lethal hit" is delivered to the target cell. Once the lethal hit has been delivered, the CTL is no longer required for completion of lysis of the target cell. The target cell is "programmed for lysis", *i.e.*, a cascade of events occurs within the target cell which eventually leads to the lysis of that cell (81).

The mechanism of triggering for target lysis by the CTL involves the specific binding of antigen with the TCR (T cell receptor). The TCR is a cell surface heterodimer composed of an acidic alpha chain and a more basic beta chain. It is noncovalently associated with at least five nonpolymorphic CD3 subunits. These subunits have been designated γ, ∂, ε, ζ, and η (82-86). In mouse T-lymphocytes, the TCR/CD3 complex has been shown to transduce signals from the cell surface to the interior of the cell(84,87), causing the hydrolysis of phosphitidyl inositol-bis-phosphate and generating second messengers which ultimately activate protein kinase C and cause a rise of cytosolic calcium. In the CTL, this promotes the production of serine esterases contained in the CTL granules (88-90), and secretion of lymphokines (*e.g.* gamma-interferon). These granules which are released via exocytosis during the lytic event also contain pore-forming molecules such as cytolysin, perforin, and cytotoxin, and are responsible for target cell damage and lysis(91-92).

Other molecules also play a role in CTL interactions with Ag expressed on the target cell. These include Lyt-2/CD8 and LFA molecules (93-96). Anti-LFA monoclonal antibodies can block the formation of CTL-target conjugates (97), and removal of CD8 from the CTL surface by trypsin under conditions which preserves MHC and other surface molecules, induced the loss of specific killing and conjugate forming ability (98).

Limiting Dilution Analysis

As the field of cellular immunology developed, it became necessary to develop more sophisticated techniques in order to understand the role of particular immune cell types. Previously, the standard assay system consisted of bulk cultures containing several population of immune cells. The microculture technique was developed to study cellular interactions on a smaller scale. Even so, it was still difficult to assess the roles of individual cell types and a more complex system, termed limiting dilution analysis (LDA) was established.

In vitro limiting dilution assays were developed by Lefkovits in 1972 to estimate the frequency of B cell clones stimulated in response to various antigens. In the development of their assay system, they performed a series of manipulations such that only one type of cell was limiting, in titrated amounts, and all other cells required for the immune response would be present in constant, saturated amounts in each microculture (113). The limiting dilution approach to estimate immune cell precursor frequencies was extended to the field of cellular immunology with the development of a number of assay system to quantitate CTL-p frequencies in response to allo-antigens (114-118), hapten-modified-self antigens (119), or viral antigens (68,120-122).

Before the LDA was used to estimate the frequency of CTL-ps, the frequency of antigen-specific responding cells had been difficult to assess. The methods used to estimate the frequencies included the incorporation of ^{3}H-Tdr into DNA (123), removal of cells from the circulating pool (124), or the ability of cells to form virus plaques (125). Skinner and Marbrook, in 1976, found the *in vitro* LDA to study the CTL system (126). Most of his work

involved the frequency of CTL-p reactive against allo-antigen. Using CBA mice spleen cells as effector CTL, they determined the frequency of precursor cells to generate a cytotoxic response against DBA mastocytoma cells to be 1/1700 spleen cells. By studying the distribution of positive and negative cultures as a function of the number of responder cells cultured, it is possible to deduce something about the conditions required for CTL-p activation and about the frequency of CTL-p responding to various allo-antigens.

In order to perform a LDA to measure CTL-p frequencies, one must establish the following conditions:

1) CTL-p must be the least frequent required cell in the suspension being diluted *i.e.*, it is the limiting cell.

2) The culture conditions must be such that a single CTL-p can survive and produce viable progeny after activation.

3) The assay for CTL must be sufficiently sensitive to detect the progeny of a single CTL-p *i.e.*, the assay must clearly distinguish cultures containing a single clone of CTL from non-responding cultures (127)

The *in vitro* LDA technique has been used mainly in the studies of four viral systems: influenza (128-132), murine sarcoma-leukemia virus complex (MSV-MLV)[133,134], rabies (135) and HSV (68,122,136). This technique has been used to estimate the frequencies of viral specific CTL-p, to determine the antigenic specificities of the CTL-ps and their clonal progeny and in some cases, to further characterize the phenotype of the CTL's.

Rouse *et al.* established a limiting dilution assay to estimate the frequency of CTL-p reactive against HSV-1 (68). CBA/CA mice were primed by intraperitoneal (ip) injection of HSV-1 infectious virus or heat-inactivated

virus. Spleen or lymph node cells were used as responder cells and were cultured under limiting dilution conditions in the presence of IL-2 containing medium and either infected or uninfected irradiated spleen cells which serve as stimulators for antigen presentation. The microcultures were then tested for cytotoxicity against infected targets. The frequency of HSV-1 specific CTL-p in normal spleen or lymph node cells of immune mice was found to be less than 1/250,000. In contrast, lymph node cells from these mice, when restimulated in vitro with HSV-1 infected, irradiated splenocytes demonstrated CTL-p frequencies of 1/3,500- 1/15,670. The majority of cultures were found to lyse only syngeneic infected targets as opposed to allogeneic infected targets.

The CD4+ T Helper/Inducer Cell

CD4 is a cell surface glycoprotein expressed on most thymocytes and on the predominant subset of peripheral T lymphocytes. The CD4 is expressed primarily on helper/inducer T cells and the expression of this surface protein has been found to correlate best with Class II restriction (99). Murine T cells that expressed CD4 were at first found to include cytotoxic cells whose specificity was associated with Class II MHC Ags. Therefore, Swain *et al.* (100) and Reinherz *et al.* (101) suggested that CD4 may bind to class II MHC in order to control the activation of CD4+ T cells. However, it seems that its primary function on T cells is to increase the avidity of the interaction between T cells and antigen presenters or target cells. Current evidence suggests a role as an adhesion molecule or as a signalling molecule or a combination of both (102).

With the advent of mAbs that could deplete *in vitro*, the role of the CD4

molecule to CD4+T cell function could be elucidated. Anti-CD4 mAbs do modulate CD4+ T cell responses. They inhibit the activation of CD4+ T cells (103), namely that the production of IL-2 and IL-3 is concomitantly reduced(104,105), the response of T cell hybridomas to Ag presented by syngeneic APC, and to allogeneic cells is suppressed (106-110), and the activation and proliferation of CD4+ T cells in situation independent of MHC Class II is inhibited. These findings all suggest that anti-CD4 mAbs simply inhibit the recognition of Ag-MHC Class II complexes by T cells because CD4 is an integral component of the antigen recognition and binding structures. The cDNA and gene encoding CD4 have been isolated and sequenced. Analysis of the amino acid sequence predicted by the nucleotide sequence indicates that CD4 is a member of the immunoglobulin gene superfamily (111-112).

The Role of the CD4+ T Cell in the Regulation of the Immune Response to Virus

The induction of CTL responses depends on the interaction of several cell types and the mechanism of induction involves a Th cell dependent pathway. The availability of mAbs to specific T cell subsets has enabled a detailed analysis of the anti-viral T cell response (142), especially the role of the CD4+ T cell in regulating the immune response to virus. Attempts to determine the function the the CD4+ T cell antigen are based largely upon the use of anti-CD antibodies to prevent normal CD4+ T cell function. The mAbs developed to the CD4+ cells have been used to clarify the role of CD4+ T cells in allograft rejection across transplantation barriers (137,142), to confirm that CD4+ cells are necessary in primary and secondary humoral responses (138), to determine the role of

CD4+ T cells in various autoimmune disorders (139-141), to determine the role of CD4+ cells in T-cell immunity involving CTL cells, and to produce a long-term state of antigen-specific tolerance (142).

Cobbold *et al.* (1984) were the first to use mAB to specific T cell subsets *in vivo* and thereby assess the immunoregulation of these cells with respect to immune functions such as Ab production, and delayed type hypersensitivity. They used rat monoclonal IgG2b antibodies against CD4+ and CD8+ T cells, since they found that rat IgG2b mAbs gave substantial and long term depletion of their target T cells. When CD4+ cells were depleted in CBA mice (95% shown by flow cytometry), IgG Ab production against sheep red blood cells, a Th dependent Ag, was totally eliminated, but IgM Ab was still present. These mice did not mount an antibody response to injected rat Ig (immunoglobulin) either. The mice were primed with HSV in the ear pinna and 24 hours later measured for delayed-type hypersensitivity (DTH), an Ia restricted T cell reaction (47). This response was also suppressed. They also found that mice depleted of either CD8+ or CD4+ cells could not produce CTL's upon allogeneic stimulation (143). From this they concluded that the generation of CTL depends on the presence of both CD4 (helper/inducer), and cytotoxic precursors. When they mixed cells from CD4+ and CD8+ depleted animals, they could reconstitute the allogeneic CTL response.

In contrast to the aforementioned studies, Buller *et al.* (144), using rat mAb GK1.5 (98,145) to deplete CD4+ T cells *in vivo* found that unlike Cobbold, who still could show that IgM Ab titers to SRBC were still present after *in vivo* depletion of CD4+ T cells, the IgM response was markedly reduced as well as the IgG response. Secondly, neutralizing Ab to ectromelia virus, another

Th cell dependent response, was assessed. Buller found that neutralizing Ab titers were nonexistent in CD4+ depleted mice. In this study, the direct effect of CD4+ cell depletion on the induction of an ectromelia virus-specific CTL response was also assessed. Buller showed that in control mice that were not depleted with anti-CD4 mAB and in CD4+ depleted mice, the CTL activity generated by spleen cells were equivalent.

Other researchers have also studied the effects of *in vivo* CD4+ and CD8+ depletions in other viral infections such as Theiler's murine encephalomyelitis virus (TMEV)(146-147), LCMV (148-149), Coxsackie virus (150), MCMV (151), Vesicular stomatitis virus (VSV)(148), Influenza virus(152), and HSV (153).

Leist *et al.* (148) along with Ahmed *et al.* (149), analyzed the roles of T lymphocyte subsets in the antiviral host defense to LCMV. Treatment with anti-CD4 decreased the ability of T cells to provide help for LCMV- specific antibody production. Also, all mice treated with anti-CD4 mAB could generate an LCMV specific CTL response that was of sufficient magnitude to control the viral infection. Depletion of the CD4 cells had no effect on the ability of immune cells to eliminate virus from carrier mice. In mice treated with anti-CD8 mAB, induction of CTL was reduced by greater than 99%, and primary swelling of the footpad, a CD8 mediated response in LCMV, could not be detected. Immune cells treated with anti-CD8 were unable to clear virus from persistently infected mice.

Leist's results concurred with Ahmed, except for the fact that while also examining the role of CD4+T cells in the generation of LCMV-specific CTL response, he also found that anti-CD4 treated mice mounted a readily detectable

CTL response, although the number of lytic units per spleen was approximately 10-fold lower than control mice.

These results, taken together, suggest that LCMV-specific CD8+ T cells are essential for effective immune therapy of persistently infected mice. Also, a deficiency of CD4+ helper cells is likely to have a more severe effect on humoral responses than on the induction of CTL responses.

A very important implication of these results is that CD4+ T helper cells may not be required for the generation of LCMV-specific CTL and that the necessary lymphokines, such as IL-2, are produced by the CTL themselves or by other CD8+ T cells. The existence of CD8 "helper" T cells and the ability of CD8+ CTL clones to produce IL-2 has been shown in other systems (29). It is tempting to speculate that the CD8+ cells provide their own source of "helper" factors, or else, receive induction signals from other cells. However, the residual population of CD4+ T cells cannot be ruled out as being sufficient to provide help for the CTL response.

These results suggest several possibilities for the induction of virus-specific CTL. First, the induction of CTL to virus does not require help provided by CD4+ cells and is dependent solely on the response of CD8+ cells to Class I plus Ag. Second, there could exist two distinct populations of CD8+ CTL-p, one dependent on CD4+ T cells. Third, the CTL response to Ag may progress from an early stage of Th-dependence to a later stage of Th-independence (144).

Nash *et al.* (153) studied the different roles of CD4+ and CD8+ cells in acute HSV-1 infections. He found, as others have, that CD4-deficient animals failed to produce either primary delayed type hypersensitivity or specific antibodies to the virus, but cytotoxic T cell responses were induced and even

augmented in comparison with infected, normal (non-depleted) animals. In mice deficient of CD8+ cells, CTL activity is completely abrogated. He concludes that this clearly demonstrates that antiviral CD8+ CTL can be induced in a CD4+ deficient environment, which is contradictory to the dogma that the induction of CD8+ CTL responses *in vitro* requires CD4+ Th cells. These Th cells are required to supply the necessary signals for activating CTL-p to lytically active CTL cells.

Whatever the mechanism involved in CD8+ T cell activation, it is clear that a deficiency in CD4+ cells actually leads to an augmented CTL response to HSV. Nash concluded that immune T cells exert different anti-HSV activity in different target tissues (skin and nervous system). In the skin, CD4+ T cells act as the principal antiviral T cell, whereas in the nervous system, CD8+ T cells act as the principal antiviral cell. Still, both populations of T cells are active in the elimination of acute HSV infections, and the elimination of HSV is achieved only when recipient and donor were compatible both at the H-2D^{K} (Class I), and at the H-2 IA (ClassII) regions.

Rationale for the Present Study

Numerous studies have shown that the induction of CTL responses *in vivo* do not require the presence of Th cell. Consequently, it becomes important to to further evaluate and understand the activation requirements of the CTL-p and the other cells involved in its induction and regulation.

The work presented in this thesis was designed to specifically determine the role of the Th cell in the induction of HSV-1 specific CTL cells. In order to

accomplish this, we had available two rat mAbs directed to the CD4+ T cell (GK1.5) and the CD8+ T cell (2.43) that could deplete *in vivo*. Subsequently, we could study the characteristics, role, and induction requirements of the CTL and the Th cell involvement in this process. The ultimate umportance of this work will perhaps lie in its potential to clarify the roles of the Th cell and the CTL in the induction of an immune response to HSV-1. Furthermore, the technique of in vivo T cell depletion for studying the immunology of infectious diseases is important. This technique should be relevant to studying chronic virus infections, where immune mechanisms are implicated in the persistence of viruses and the roles of these cells in the immunopathology involved in many viral infections can also be clarified.

CHAPTER III

SPECIFIC AIMS

A) Deplete mice with mAB to CD4+ (GK1.5) *in vivo*

1) Test for physical depletin of CD4+ Th cell subset

FACS analysis (flow cytometry)

2) Test for functional depletion of CD4+ Th cell subset

Induction of HSV-1-specific DTH response

Assay for neutralizing Ab to HSV-1

Assay for specific Ab to Rabbit IgG

Assay for specific Ab to a T independent Ag (LPS- for control)

3) Assess the ability of the CD4+-depleted mice t generate an HSV-1-specific CTL response as compared to intact mice.

a) Using 3 day bulk culture techniques, assess the primary response

b) Using 5 day bulk culture techniques, assess the memory response

4) Assess the frequency of HSV-1-specific CTL precursors in CD4+-depleted mice and compared to intact mice.

Limiting Dilution Analysis

B) Deplete mice with mAb with CD8+ (2.43) *in vivo*

1) Test for physical and functinal depletion and mentioned with GK1.5 depletion.

2) Compare the HSV-1-specific response in the depleted mice as compared to intact mice and compare to the GK1.5 treated mice.

C) Investigate the role of soluble growth factors and growth factor secreting cells in the HSV-1-specific CTL response in CD4+-depleted mice

a) Using 3-day bulk culture techniques, add soluble growth factors to CD4+-depleted cultures *in vitro*

b) Using 3-day CTL blastogenesis techniques, add Ag-stimulated, x-irradiated splenocytes to CD4+-depleted cultures *in vitro*

CHAPTER IV

MATERIALS AND METHODS

Virus

HSV-1 strain KOS was grown on Vero cells (ATCC) supplemented with McCoy's 5A medium with 10% heat inactivated donor calf serum (GIBCO Laboratories, Grand Island, NY), 100 IU/ml penicillin, 100ug/ml streptomycin, and 10mM L-glutamine. Virus was titered on Vero cells and TCID50 per milliliter was determined 5 days after infection. Virus was aliquoted in .5ml amounts and stored at -80°C. Vaccinia virus and vaccinia virus bearing recombinants were grown in HeLa cells supplemented with S-MEM(GIBCO) with 100lu/ml penicillin, 100ug/ml streptomycin, 10mM L-glutamine, and supplemented with 10% heat inactivated horse serum (GIBCO). The media was buffered to pH 7.o with 25mM Hepes and 7.5% Na2HCO3. Virus was titered on CV1 cells (ATCC) and PFU per milliliter was determined 3 days after infection. Virus was aliquoted and stored at -80°C.

Virus was UV irradiated by exposing 2 $x10^8$ $TCID_{50}$ to a germicidal lamp (Sylvania Electric Products, Danvers, Mass.) at a distance of 3 cm. for 2 minutes. This resulted in a reduction oth the viral titer to approximately 10^2 $TCID_{50}$/ml. This residual infectious virus is necessary for optimum CTL generation as reported previously (154).

Mice

Male and female BALB/c Byj mice (H-2d), female C3H/Hen (H-2k), and female C57BL/6 (H-2b) (Harlan Sprague Dawley, Indianapolis, IN) mice 6-10 weeks of age were used throughout the experiments. Animals were anesthesized prior to any experimental procedure using methoxyflurane (Pitman-Moore, Washington Crossing, NJ).

Monoclonal Antibodies and T-cell subset depletion

The GK1.5 (anti-CD4+) hybridoma cell line was obtained from American Type Culture Collection, Rockville, MD, and has been described previously (98,145). The 2.43 (anti-CD8+) hybridoma cell line was a generous gift from Dr. Frank Fitch (University of Chicago, IL) and has also been described previously (155). The hybridoma cell line HB151 (ATCC) secretes a rat monoclonal antibody (IgG2b) that is non-reactive with any antigen. Mice treated with anti-CD4, anti-CD8 or nonspecific antibody were inoculated with .5mg of antibody (as determined by ELISA) intravenously on days -2, +2, and +3 with day 0 representing the day of HSV-1 inoculation. These mice were then killed on day 5 for primary CTL assays. Mice used for memory (in vitro restimulation with antigen) were reinoculated with the same volume of antibody on days +7, +14, +21 and +28 in order to maintain depletion. The extent of depletion was measured by flow cytometry.

Flow Cytometry

Cells from lymph nodes of individual mice (depleted and non-depleted) were obtained at various times during the course of experiments to determine

the rate of CD4+ of CD8+ antigen expression following treatment with anti-CD4 or anti-CD8. An aliquot of cells was incubated on ice for 1 hour with lug of anti-CD4 or anti-CD8 antibody in PBS media containing 1% bovine serum albumin and 0.2% sodium azide (Fischer Scientific Company, Fair Laron, NY). This was followed with two washes in PBS and counterstaining with fluorescein-conjugated goat anti-rat antibody (Southern Biotechnology Associates, Birmingham, AL). Cells were then washed two times and single color fluorescence analysis was performed using a FACstar PLUS fluorescence activated cell sorter (Becton-Dickinson), generously provided by Dr. Ronald Worthington.

Mouse Immunizations and Preparation of Lymphocyte Cultures

To prepare stimulator cells normal BALB/c mice were killed by cervical dislocation, their spleens were aseptically removed, and single cell suspensions were prepared. The spleens were minced and passed through 80-mesh wire screens into RPMI 1640 (GIBCO) supplemented with 10% HI FCS, 7mM L-glutamine, 5x10-5M 2ME, (100IU/ml) penicillin, (100ug/ml) streptomycin, was buffered to pH 7.2 with 25mM HEPES and 7.5% Na2HCO3 (RPMI complete medium). Erythrocytes were removed from the spleen cell populations by treating the cell pellets with Tris-buffered 0.83% NH_4Cl. After being pelleted by centrifugation the cells were x-irradiated (2400 rads), washed twice and specific number of cells were infected for 2 hours at 37°C with UV-inactivated virus at a multiplicity of infection (MOI) 1. The cells were thein suspended in RPMI-complete medium and were used as stimulator cells for limiting dilution analysis.

HSV immune splenocytes were obtained from mice which had been injected intraperitoneally at 4 and 8 weeks before use with 0.1 ml of inoculum containing 5×10^6 $TCID_{50}$ of HSV-1. Vaccinia-immune splenocytes were obtained from mice which were injected intraperitoneally 2 weeks before use with 5×10^5 PFU of vaccinia virus.

Immune lymph node cells were obtained from mice which had been injected in the footpad and in each pinna with a 0.02 ml inoculum containing 10^6 $TCID_{50}$ of HSV-1.

Delayed Type Hypersensitivity Assay

DTH testing was performed using a modification of (157). Five mice from each group were tested for their ability to mount an HSV1 specific DTH response. Mice were primed with 5×10^6 $TCID_{50}$ HSV-1 KOS subcutaneously. Six days later, they were challenged by injecting 50 ul of UV-inactivated HSV-1 containing 1×10^6 $TCID_{50}$ into the right rear footpad. The left footpad received 50ul of Vero cell lysates and served as a non-specific swelling control. Footpad swelling was measured 24 and 48 hours after challenge with spring loaded calipers (Oditet, West Germany).

Neutralizing Antibody Determination

Serum from immune, non-depleted mice and immune GK1.5 and 2.43 depleted mice was collected on days 7, 14, 21 and 28 (day 0 represents HSV-1 priming). Heat-inactivated serum was diluted in threefold dilutions and for each dilution, 50ul were aliquoted into four replicate wells of a flat-bottom microtiter plate (Costar, Cambridge, Mass). To each well an equal volume of

media containing 150 $TCID_{50}$ HSV-1 KOS was added, the plates were sealed, and incubated at 37°C overnight. Subsequently, 50ul of media containing Low Tox rabbit complement diluted 1/4 were added to each well and the plate was resealed and incubated at 37°C for another hour. Finally, 5×10^3 Vero cells were added to each well (50ul), the plates sealed, and incubated at 37°C. After five days, the plates were examined and the serum titer determined as the dilution that neutralized 50% of the virus.

Enzyme-Linked Immunosorbent Assay (ELISA)

Rabbit IgG- and LPS-specific antibody in the serum of non-depleted, CD4 depleted, and CD8 depleted mice was assayed in a solid phase indirect ELISA. Immunlon 1 flat bottom microtiter plates (Dynatech, Chantilly, VA.) were coated with either Rabbit IgG or E. coli LPS 055, sealed, and incubated overnight at 4°C. Subsequently, after washing, 0.1 ml of serum (diluted 1:50) was added to the first well, two-fold dilutions of the serum were made, and allowed to incubate at 37°C for an hour. After washing and blocking, 0.05 ml of horse radish peroxidase labelled goat anti-mouse immunoglobulins were added (Southern Biotechnology, Birmingham, AL), and incubated for an hour. After extensive washing, development with 2,2'- Azinobis (3-ethylbenzthiazolene sulfonic acid)[Sigma Chemical Co., St. Louis, MO] was allowed at room temperature for 10-20 minutes, then stopped with 1.5% Potassium Fluoride. Samples were read at wavelenth 405nM on a Biotech EIA plate reader (Biotech Instruments, Inc. Burlington, VT).

Cytotoxic T Lymphocyte Assay

CTL cultures were generated according to the method of Pfizenmaier *et. al.* (158). Five day after inoculation of 10^6 $TCID_{50}$ HSV-1 in the foot pad and subcutaneously in both pinna, the draining popliteal lymph nodes and cervical lymph nodes were removed and single cell suspensions were prepared. Viable cells were adjusted to 4×10^6 per milliliter in RPMI-complete medium and plated as 5 ml aliquots in 6 well plates (Costar) in the absence of exogenously administered HSV-1 antigen. After 3 days, the cells were washed and viable cell concentrations were adjusted to the desired final effector/target cell ratio. 0.1 ml of these effector cells were added to replicate 96-well V-bottom plates (Dynatech, Chantilly VA). Target cells were ^{51}Cr labeled in RPMI-complete medium and 0.1 ml was added to each V-bottom well. The target cells were either the standard mock-infected or virus infected syngeneic or allogeneic continous fibroblast lines. After the addition of the target cells, the assay plates were centrifuged (200 x g, 2 min) and incubated at 37°C for 4 hours. The ^{51}Cr released from the lysed cells into the supernatant fluid was measured, and the percent specific lysis was determined by the following equation:

$$\frac{\text{experimental release - spontaneous release}}{\text{total release - spontaneous release}}$$

Memory bulk CTL cultures were generated according to the method of Lawman *et al.* (154). Briefly, 8 - 10 weeks after inoculation of 5×10^6 $TCID_{50}$ of HSV-1 KOS intraperitoneally, the spleens were removed and single cell suspensions prepared. Viable cells were adjusted to 2×10^6 in RPMI-complete medium and cultured at 37°C as 5ml aliquots in 6 well plates. Each

aliquot was stimulated with UV-inactivated HSV-1 KOS at an MOI of 1. After 5 days, the cells were washed and viable cells were used as CTL effectors in a standard 4 hour chromium release assay as mentioned above.

CTL-p Limiting Dilution Culture

CTL-p limiting dilution cultures has been previously described (156). Briefly, 5 days after the inoculation of $5x10^6$ TCID50 of HSV-1 KOS into the rear footpads and ear pinna of intact and CD4+ depleted mice, the draining popliteal and cervical lymph nodes were removed and single cell suspensions prepared. Various dilutions of viable cells (which served as responder cells) were made in 96-well round bottom plates (Corning) in RPMI 1640 complete-media supplemented with 50% NCTC 109 (GIBCO). One half of the responder cells were then stimulated with 5×10^5 x-irradiated (2400 rads) normal, non-immune splenocytes and the other half of the responder cells were stimulated with splenocytes that were restimulated with UV-inactivated HSV-1 at an MOI of 1 (as mentioned above). The total well volume was 0.2 ml of RPMI-complete medium containing 6% concanavalin A-derived rat T-cell growth factor (with 2-mM alpha methyl-mannoside) prepared as described previously (158). After 7 days incubation at 37°C, the cells were washed and used as CTL effectors in a split well culture analysis comparing cytotoxicity against syngeneic and allogeneic virus-infected fibroblastic targets.

Cytotoxic T Lymphocyte Blastogenesis

Lymphocyte preparations were cultured in RPMI-complete medium as described previously by Horohov *et al.* (159) with minor modifications. HSV-1

primed draining popliteal lymph nodes and cervical lymph nodes were removed as described previously in this text. Viable cells were suspended at a concentration of 4×10^6 per ml and 0.1 ml of this suspension was aliquoted in 96-well round bottom plates (Corning). Stimulator cells were prepared as mentioned previously. 1×10^5 or 5×10^5 x-irradiated cells were added to the test wells in a volume of 0.1 ml. These stimulator cells were infected with HSV-1 at an MOI of 1, mock-infected, or infected with Vaccinia virus at an MOI of 1, the plates were sealed, and incubated at 37°C for 3 days or 5 days. Control wells received no stimulator cells. Subsequently, on the 3rd or 5th day, the cells were washed and used as CTL effectors in a split well culture analysis comparing cytotoxicity against syngeneic and allogeneic virus-infected fibroblastic targets. To one of the split cultures, 1uCi of [^{3}H]thymidine (Tdr: ICN Pharmaceuticals, Inc., Irvine CA) was added to each well, and the plates were incubated for an additional 18 hours. The incorporation of [^{3}H]-Tdr into cellular DNA after this time was used as a measure of lymphocyte proliferation.

Statistical Analysis

For limiting dilution analysis, a baseline level of ^{51}Cr release was arbitrarily chosen as the mean counts per minute of 30 wells containing non-immune or virally stimulated stimulators but no responders plus 3 standard deviations of the mean. Each well in a given assay was scored as positive or negative for ^{51}Cr release when compared to this baseline level. A semilogarithmic plot or Scatchard analysis was made in which the percent negative wells within a group where both positive and negative wells were found, was plotted against the responder number.

The statistical analysis was performed according to Taswell *et al.* (160), and a line of best fit was drawn using least squares analysis, confidence intervals (95% fit), and frequencies were calculated from this line by linear regression analysis.

Statistical relevance was calculated by Student's t test in all other assays.

CHAPTER V

RESULTS

Quantitation of T-Cell Subsets After Treatment With Anti-CD4 and Anti-CD8 mAbs.

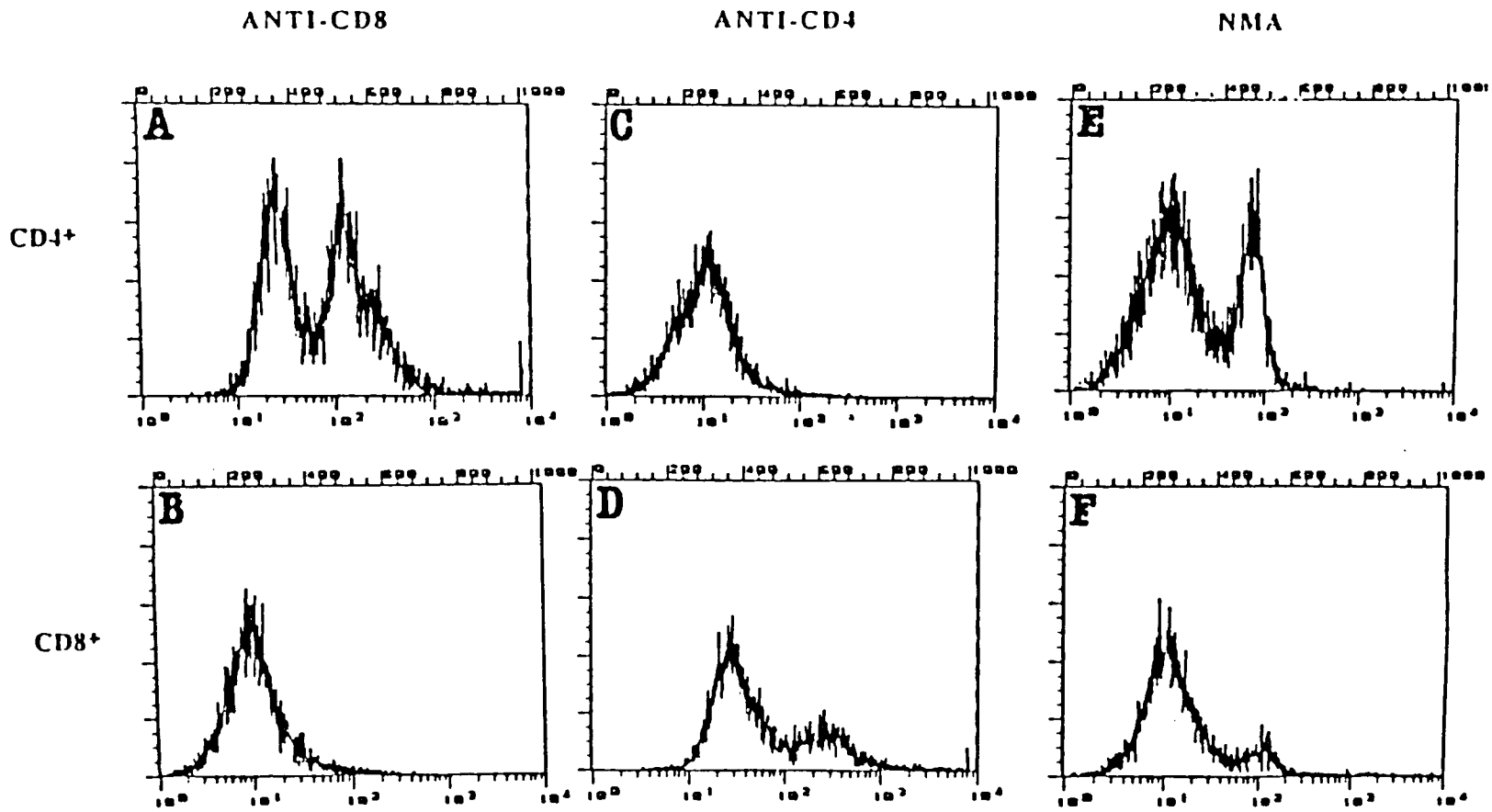
To determine the effectiveness of antisera treatment on depletion of T lymphocyte subsets, lymph nodes from test and control mice were analyzed for CD4+ and CD8+ T cells by flow cytometry. Three injections of GK1.5 (anti-CD4+- 500ug each on days -2 , +2, and +3) and 3 injections of 2.43 (anti-CD8+- 700ug on the same days) caused >95% depletion of CD4+ T cells and >90% depletion of CD8+ T cells respectively. Mice injected three times with HB151 (normal mouse ascites) showed no decrease in the population of CD4+ or CD8+ T cells. The data in Figure 1 are representative and depict profiles of lymph node cells at 7 days post HSV-1 immunization (day 0). Profiles of test animals taken at the termination of experiments were similar to those shown in Figure 1 (data not shown). These results concur with the results obtained by others with these mAbs (99,138) and demonstrate that these regimens of Ab depletion give near-total depletion of the T-cell subsets in question, at least within the limits of detection by flow cytometry.

Effect of T Cell Depletion on the Induction of DTH

Several studies were performed to determine if there was any functional evidence for the persistence of CD4+ cells in mice treated with mAb GK1.5. First, normal (intact) and mAB GK1.5 treated mice were immunized with HSV

Fig. 1. Phenotypic Determination of Lymph Node Cells After Treatment with Monoclonal Antibodies

Lymphocytes of individual mice which received antiserum treatment on days -2, +1, and +3 (with day 0 representing the day of HSV-1 priming) were obtained on day 7 from the lymph nodes. Cells were then stained and analyzed for the presence of CD4+ and CD8+ T lymphocytes by flow cytometry. Panels A,C, and E represent the CD4+ population of T lymphocytes from mice treated with anti-CD8, anti-CD4, and normal mouse ascites (NMA) respectively. Panels B, D, and F represent the population of CD8+ T lymphocytes from mice treated with anti-CD8, anti-CD4, and NMA respectively.


ANTI-CD8
ANTI-CD4
NMA
CD4+
CD8+
A
B
C
D
E
F

and tested to see if they could mount an HSV-1-specific DTH response. DTH is a response known to be dependent upon T-cell help (48). Normal, non-immune BALB/c mice were injected with either anti-CD4, anti-CD8, or normal mouse ascites (NMA)on days -2, +1 and +3. At day 0, mice from each group were primed with HSV-1 i.p. and challenged with UV-inactivated HSV-1 via foot pad 6 days later. Footpad swelling was measured 24 and 48 hours later. As shown in Figure 2, depletion of CD4+ T cells significantly reduced the amount of virus specific DTH (64%), whereas depletion of CD8+ T cells had no effect upon the extent of DTH. These results verify the fact that DTH, in the HSV system, is a CD4+ T lymphocyte (Class II restricted) response as characterized by Nash *et al* (47). The response is not abrogated completely by the depletion of CD4+ T cells and this is probably due to the residual CD4+ T cells that remain after the depletion regimen.

Antibody Response to HSV-1, Rabbit IgG, and *E. coli* LPS in Depleted Mice.

To further assess the functional depletion of CD4+ T cells in mAb-treated mice, the ability of CD4 -depleted BALB/c mice to generate an antibody response to T-helper dependent Ags as well as a T-independent Ag was investigated. Three groups of mice were injected with anti-CD4 or anti-CD8 on days -2, +1 and +3, and were kept depleted with weekly injections of antibody . On day 0, the mice were injected with HSV-1, E. coli LPS 055, or rabbit IgG i.p., and at different time intervals were bled to determine specific levels of antibody. Neutralizing antibody response to HSV-1 was measured as well as the antibody response to rabbit IgG and LPS (determined by ELISA). As shown in Table 1, normal mice immunized with HSV-1 developed high titers of

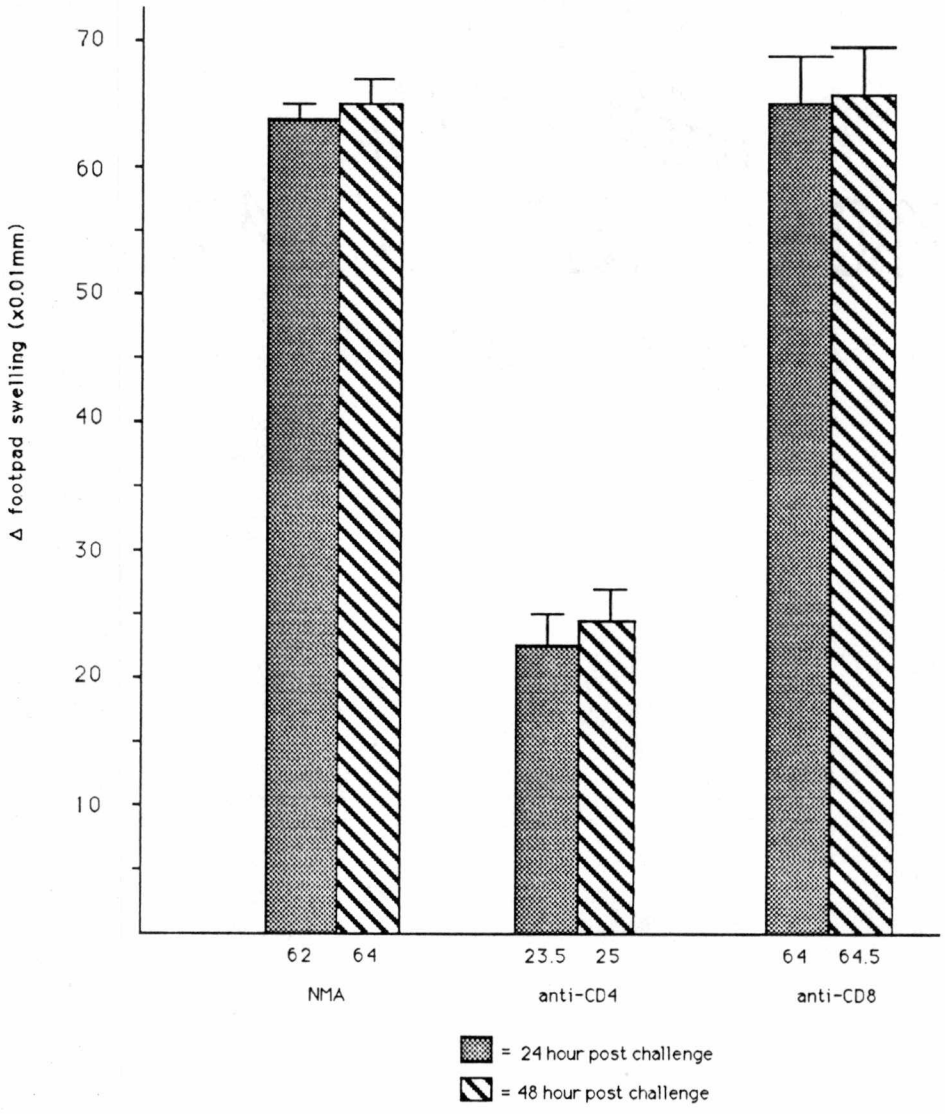


Figure 2. Effect of in vivo T cell depletion on the induction of an HSV-1-specific DTH response.

BALB/c mice were depleted with GK1.5 or 2.43 as mentioned in Materials and Methods, with day 0 representing priming with 5 x106 Tcid50 of HSV-1 for DTH induction. At day 6 following priming with HSV-1, mice from each treatment group were challenged with UV-inactivated HSV-1 (106 TCID50) in the right footpad in order to elicit a DTH response. Vero cell extracts were injected in the left footpad and served as a non-specific swelling control. Footpad were measured at 24 and 48 hours post challenge. Results are depicted as the mean footpad swelling with numbers below each bar representing the numerical value.

Table 1. Specific Antibody Response in Intact and T Cell Subset-Depleted Mice

Treatment	Antigen		Antibody Titre			
			day			
			7	14	21	28
none	Rabbit IgG [a]		<50	100	ND	400
anti- CD4	Rabbit IgG		<50	<50	ND	<50
none	E. coli LPS 0155 [b]		320	640	ND	2560
anti- CD4	E. coli LPS 0155		320	1280	ND	5120
none	HSV-1 (neutralizing) [c]	+C'	840	1360	1778	2548
		-C'	<7	14	56	89
anti- CD4	HSV-1	+C'	<7	<7	<7	68
		-C'	<7	<7	<7	<7

[a,b]titer calculated by taking the mean plus three standard deviations of the normal serum absorbance values. The titer was determined as the first value giving a positive result above the normal value.

[c]Titer calculated by using a modification of Spearman-Rankin LD_{50}. The titer was expressed as that dilution in which 50% of the virus was neutralized.

ND= not done

Table 1- continuation.

BALB/c mice were injected with GK1.5 (anti-CD4+) or HB151 (NMA) on days -2, +1,+3 and were kept depleted with weekly injections of GK1.5. Day 0 represented priming with each Ag i.p. into each group of mice. Mice were bled on various days and specific Ab titer was determined in all cases by ELISA except HSV-1, which was determined by neutralization assay. Titers are expressed as means plus or minus the standard deviation of 5 mice per group. Five preimmune mice served as a negative control, and their Ab titers were below the detection limits of the assays.

neutralizing Abs which rose until day 28 (completion of the assay). Mice immunized with Rabbit IgG showed similar results. By comparison, depletion of CD4+ T cells abrogated the antibody response to HSV-1 and the neutralizing antibody response to rabbit IgG (both T-dependent Ags).

In the case of LPS, which is a T-independent Ag, depletion of CD4+ T cells did not abrogate the LPS-specific Ab response. LPS-specific Ab titers in CD4+depleted mice rose until day 28 and was 1:2560,while in normal mice, the Ab titer was 1:5120 (day 28). Both values were not statistically different ($P<0.005$). These results confirm the ability of mAb GK1.5 depletion to abrogate Th-dependent Ab responses.

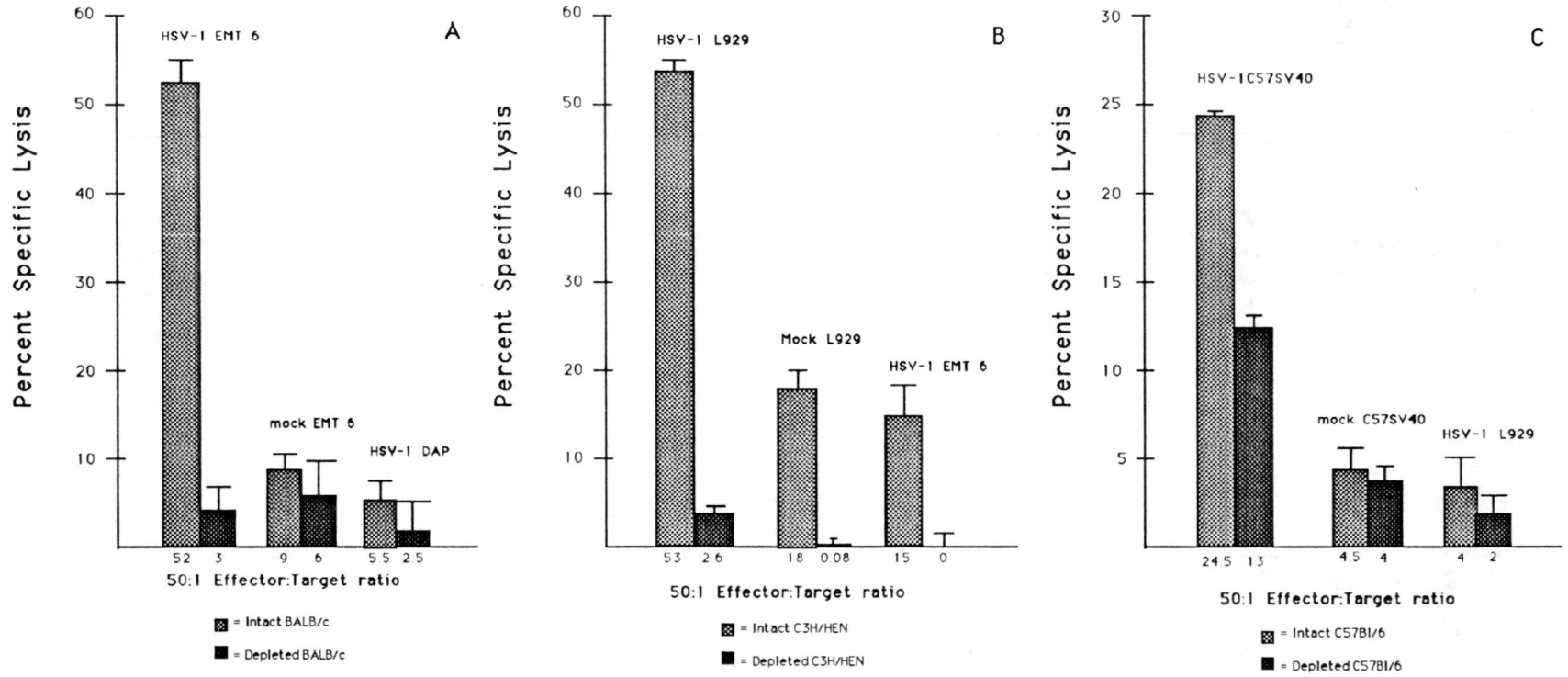
Induction of Cytotoxic T Cells in Subset-Depleted Mice

To assess the direct effect of T cell subset depletion on the induction of a CTL response, draining lymph node cells were obtained 5 days after injection of 10^6 $TCID_{50}$ in the footpad and ear pinna of intact and T cell subset depleted BALB/c, C_3H/HEN and $C_{57}Bl/6$ mice. The popliteal and cervical lymph nodes were then used in a 3-day bulk CTL assay and then mixed at various effector to target ratios with ^{51}Cr-labelled virus-infected or mock infected autologous cells or allogeneic cells. According to previous studies in the HSV system (153), *in vivo* depletion of CD4+ T cells prior to the inoculation of HSV-1 did not inhibit the subsequent development of HSV-1 specific CTL.

However, according to results shown in Figure 3 and 4, anti-CD4+ treated mice could not mount an HSV-1 specific CTL response. The reduction in percent specific lysis was 94%, 95%, and 46% for BALB/c, C_3H, and $C_{57}Bl/6$ respectively. However, the response in C_3H mice was variable. In most

Figure 3. HSV-1 Specific CTL Activity in Intact and CD4+ Depleted Mice.

Lymphocytes which were either mock (NMA) or CD4+ depleted were obtained from the popliteal and cervical lymph nodes of acutely infected BALB/c, C_3H/HEN, or $C_{57}Bl/6$ and were cultured in a 3 day bulk culture assay. The cells were used as a source of CTL effectors in a 4-hr chromium release assay. Target cells were infected with the respective viruses shown (multiplicity of 5) and labelled with $Na_2{}^{51}CrO_4$ (250μCi) for 4 hr prior to the assay. Target cells were autologous EMT 6 ($H-2^d$), L 929 ($H-2^k$) or C57SV40 ($H-2^b$) and allogeneic DAP ($H-2^k$), EMT 6, or L929 for BALB/c, C_3H and $C_{57}Bl/6$ respectively. Results are shown as the mean value of 12 replicate cultures at a 50:1 effector to target ratio plus or minus one standard deviation.


A
HSV-1 EMT 6
mock EMT 6
HSV-1 DAP
Percent Specific Lysis
60
50
40
30
20
10
52 3
9 6
5.5 2.5
50:1 Effector:Target ratio
= Intact BALB/c
= Depleted BALB/c
B
HSV-1 L929
Mock L929
HSV-1 EMT 6
Percent Specific Lysis
60
50
40
30
20
10
53 2.6
18 0.08
15 0
50:1 Effector:Target ratio
= Intact C3H/HEN
= Depleted C3H/HEN
C
HSV-1C57SV40
mock C57SV40
HSV-1 L929
Percent Specific Lysis
30
25
20
15
10
5
24.5 13
4.5 4
4 2
50:1 Effector:Target ratio
= Intact C57Bl/6
= Depleted C57Bl/6

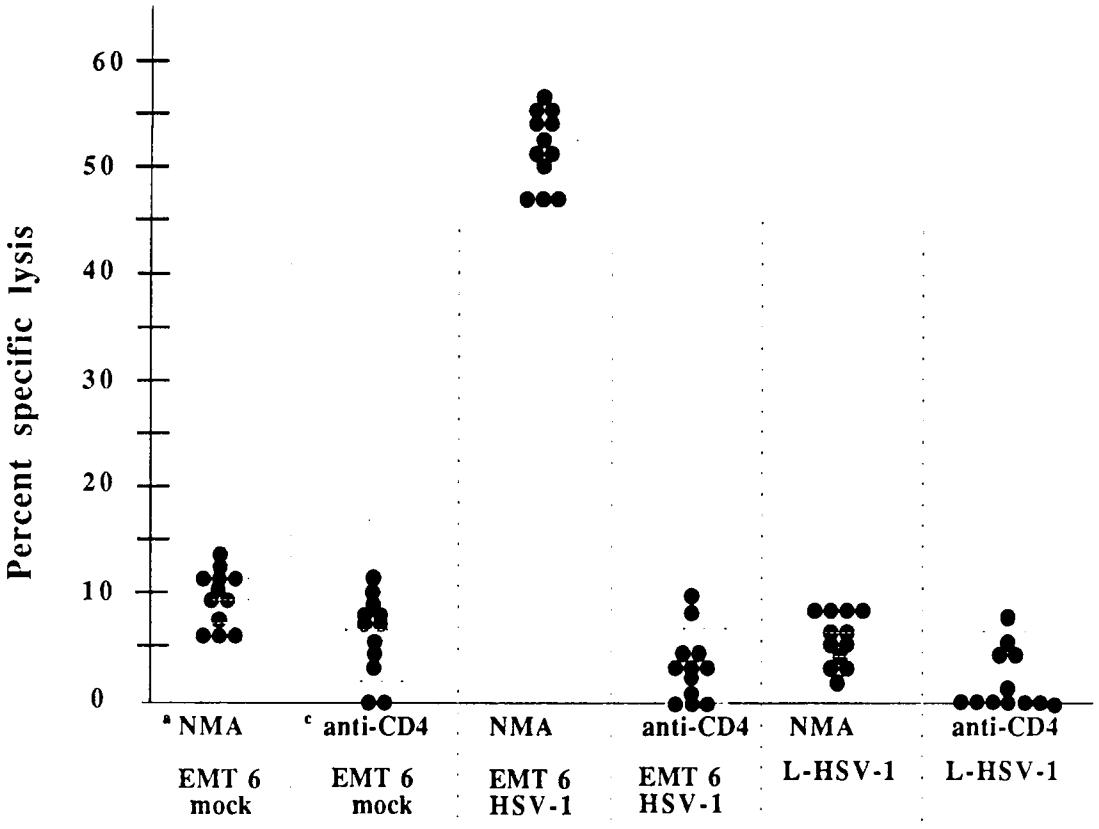


Figure 4. HSV-1-Specific CTL Activity in Intact and CD4+ Depleted BALB/c Mice.

Lymphocytes which were either mock (NMA) or CD4+ depleted were obtained from the popliteal and cervical lymph nodes of acutely infected BALB/C mice and were cultured as described in the legend to Figure 3. The cells were used as a source of CTL effectors in a 4-hr chromium release assay. Target cells were infected with the respective viruses shown (multiplicity of 5) and labeled with $Na_2{}^{51}CrO_4$ (250μCi) for 4 hr prior to the assay. Target cells were autologous EMT 6 ($H\text{-}2^d$) or allogeneic DAP ($H\text{-}2^k$). Percent specific lysis was determined as described in Materials in Methods. Results are shown as individual values of 12 replicate cultures, with the broken lines lines representing the mean plus or minus one standard deviation at an effector to target ratio of 50:1.

instances CD4+ depleted C3H mice could mount an HSV-1 specific CTL response comparable to normal mice. These results are seen in Figure 5. Nevertheless, the depletion of CD8+ T cells resulted in a markedly diminished HSV-1 specific CTL response in BALB/c (23% specific lysis in intact mice versus 3% specific lysis in CD8+ depleted mice- an 87% reduction). These results are shown in Figure 6.

The killing observed in these experiments was MHC-restricted, *i.e.*, no killing of H-2^k fibroblast cell line (SJ.1,DAP, and L929 for BALB/c) was observed and effector ratio dependent (Figure 7). The killing was also virus-specific, in that the CTL's could not lyse Vaccinia infected target EMT 6 (H-2^d). These results were consistent in all three strains of mice.

The mice were also examined for their ability to generate an HSV-1-specific memory CTL response. According to Nash, mice that are CD4+ depleted can mount an HSV-1-specific memory CTL response. Normal mice are immunized with HSV-1 i.p., and boosted 4 weeks later with Ag. After 8 weeks, the spleens from these mice are excised, restimulated *in vitro* with Ag, and set up in a bulk culture assay. After 5 days, these cells are used as effector CTL cells at varying effector to target ratios. Chromium labelled cells are added and percent specific lysis is calculated after a brief incubation period. Anti-CD4+ depleted mice demonstrated levels of HSV-1-specific CTL activity comparable to the levels of lysis seen in non-depleted animals. These results are seen in Figure 8. These results concur with Nash *et al.* (153), and suggest that the induction of HSV-1-specific memory CTL cells is helper-cell independent.

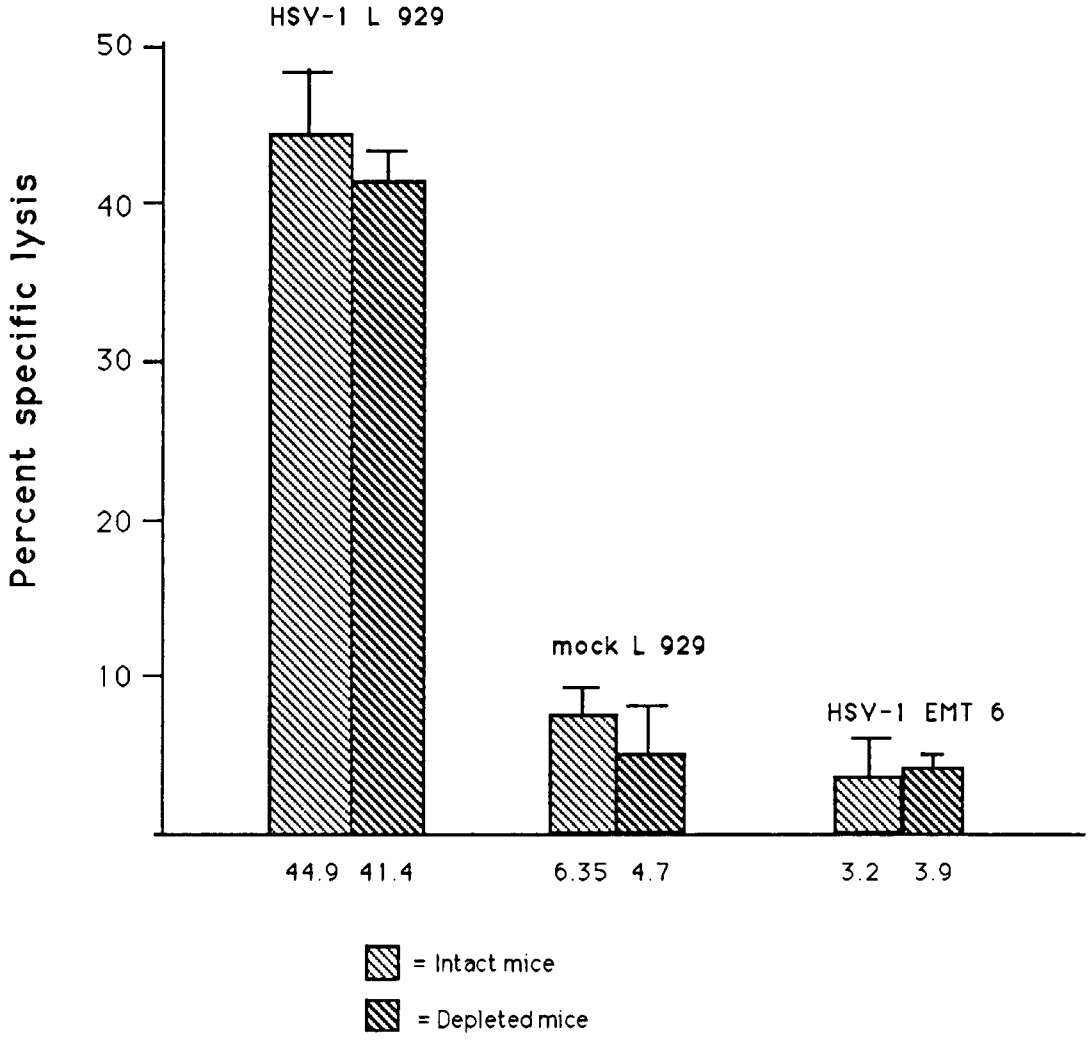


Figure 5. HSV-1 Specific CTL Activity in Intact and CD4+ Depleted C3H/HEN Mice.

Lymphocytes which were either mock (NMA) or CD4+ depleted were obtained from the popliteal and cervical lymph nodes of acutely infected C_3H/HEN mice and were cultured as described in the legend to Figure 3. The cells were used as a source of CTL effectors in a 4-hr chromium release assay and target cells for C_3H mice are the same as mentioned in the legend to Figure 3. Results are shown as the mean values of 12 replicate cultures plus or minus one standard deviation at an effector to target ratio of 50:1. The number below each bar represents the average lysis for the respective target.

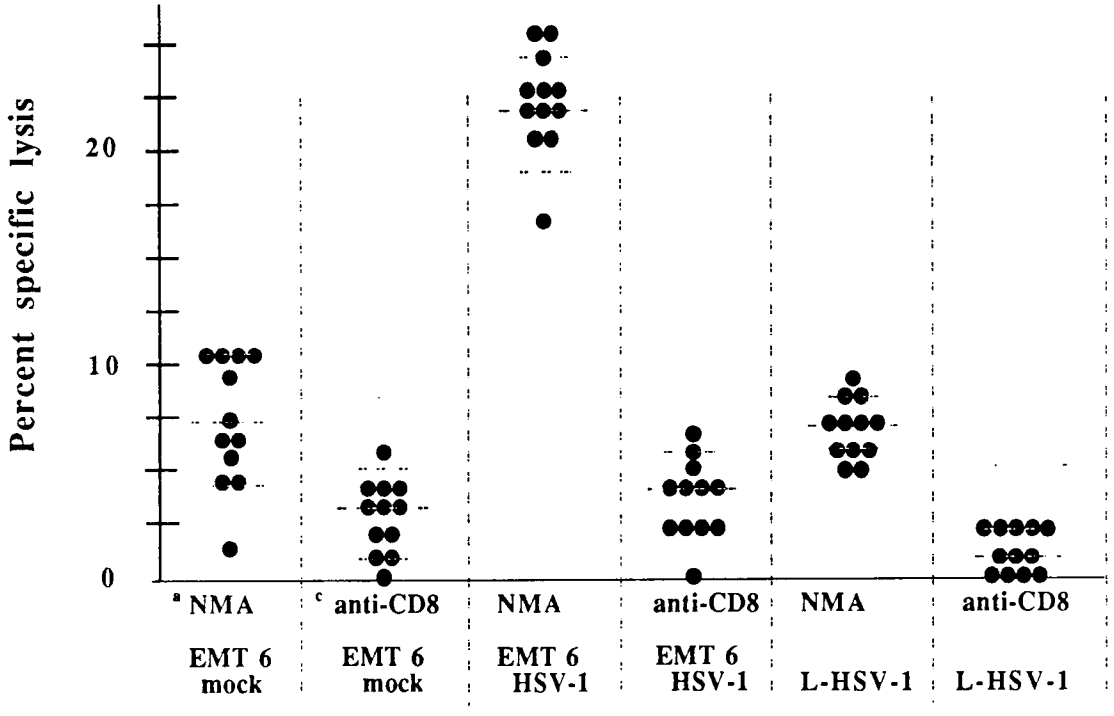


Figure 6. Loss of HSV-1-Specific CTL Response in Intact and CD8+ Depleted BALB/c Mice.

Lymphocytes which were either mock (NMA) or CD8+ depleted were obtained from the popliteal and cervical lymph nodes of acutely infected BALB/c mice and were cultured as a source of CTL effectors in a 4-hr chromium release assay as described in the legend to Figure 3. Target cells were autologous EMT 6 cells (H-2^d) or allogeneic L 929 (H-2^k). Results are shown as individual values of 12 replicate cultures at a 50:1 effector to target ratio, with the broken lines representing the mean plus or minus one standard deviation.

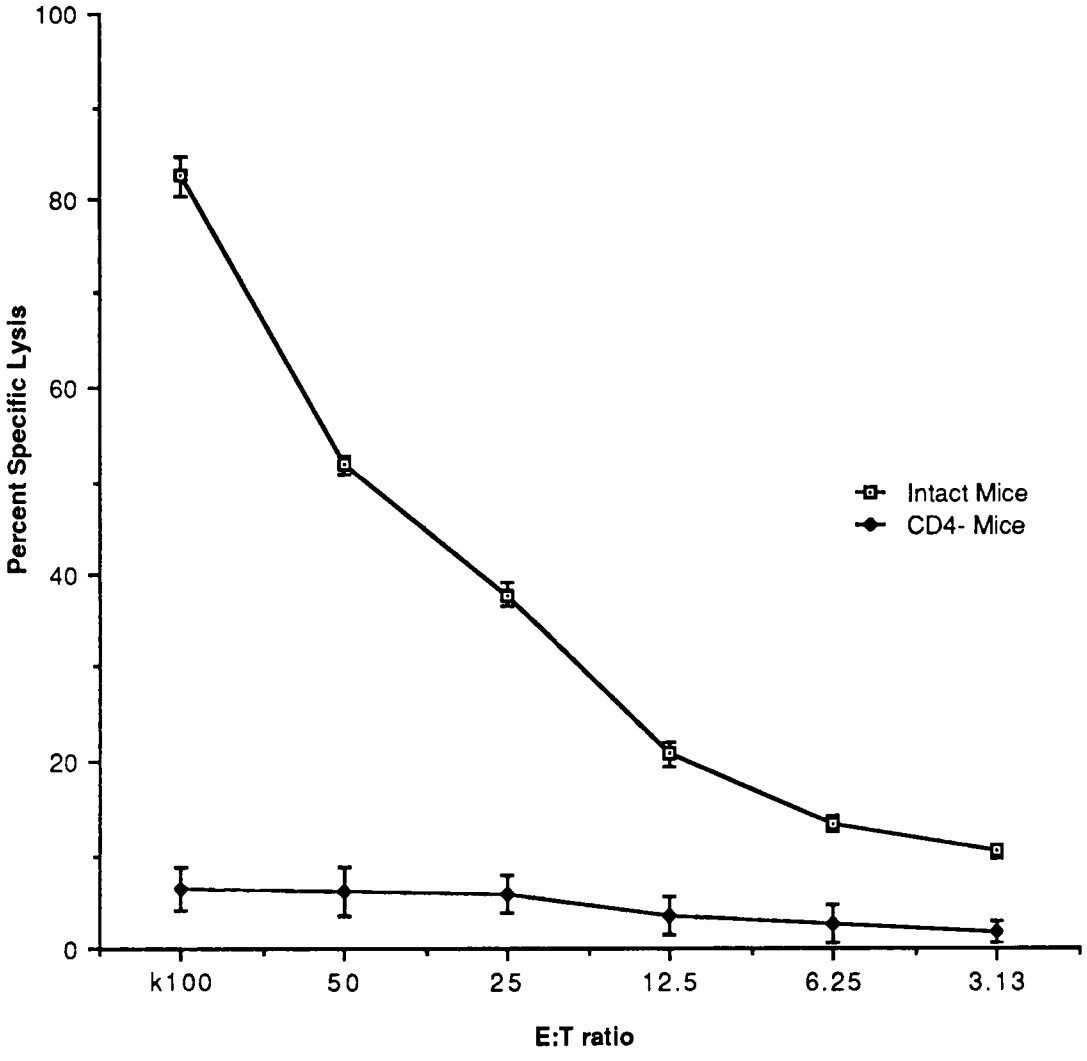


Figure 7. HSV-1-Specific CTL Activity in Intact and CD4+ Depleted BALB/c Mice at Varying Effector to Target Ratios.

Lymphocytes which were either mock (NMA) of CD4+ depleted were obtained from the popliteal and cervical lymph nodes of acutely infected BALB/C mice and were cultured as described in the legend to Figure 3. The cells were used as a source of CTL effectors in a 4-hr chromium release assay and target cells are the same as mentioned in the legend to Figure 4. Results are shown as the average mean values of 12 replicates at each effector to target ratio used plus or minus one standard error of the mean.

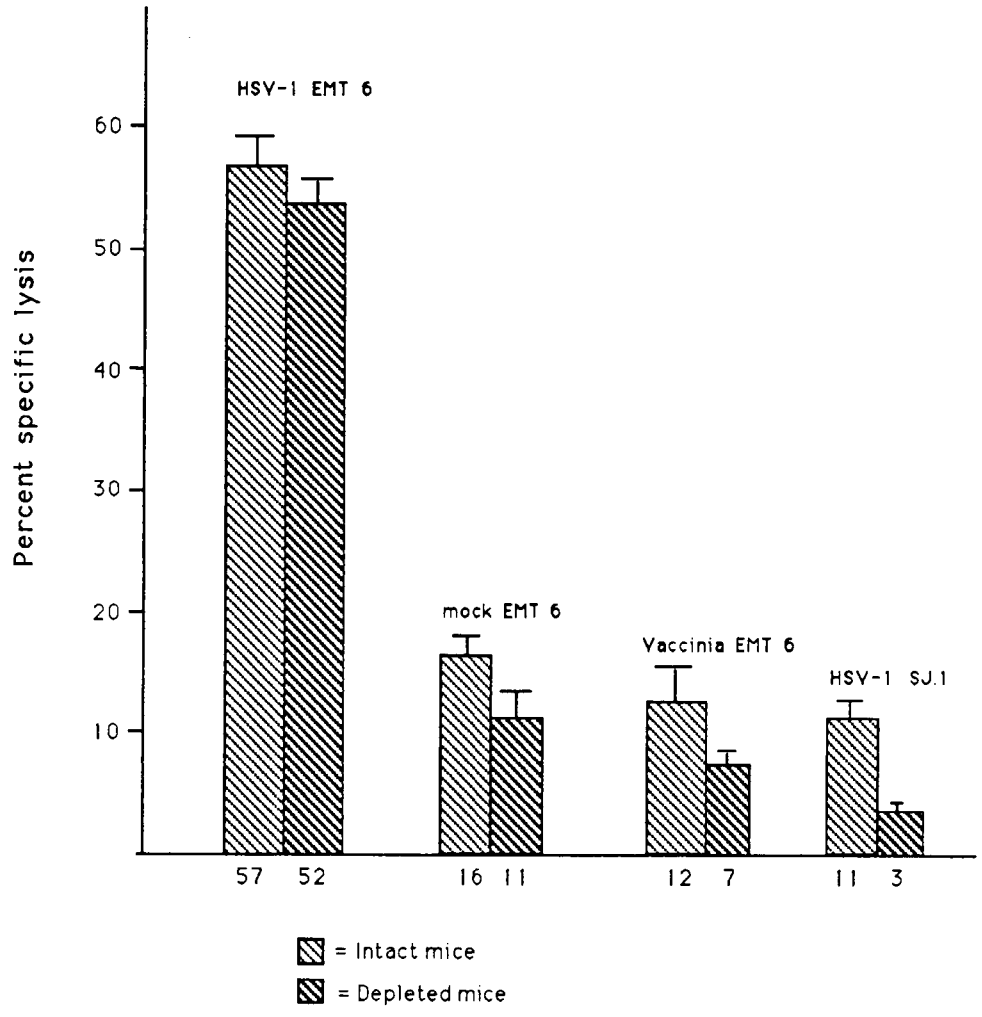


Figure 8. Memory HSV-1-Specific CTL Response in Intact and CD4+ Depleted BALB/c Mice.

Lymphocytes which were either mock (NMA) or CD4+ depleted were obtained from the spleens of BALB/c mice which had been injected with 5×10^6 $TCID_{50}$ of HSV-1 at day 0 and 4 weeks later. The splenocytes were cultured with UV-inactivated HSV-1 in a 5 day bulk culture assay. The cells were used as a source of CTL effectors in a 4-hr chromium release assay as described in the legend to Figure 3. Target cells were autologous EMT 6 cells ($H\text{-}2^d$) or allogeneic SJ.1 cells ($H\text{-}2^k$). Results are shown as the mean value of 12 replicate cultures plus or minus one standard error of the mean. The E:T ratio for above figure is 25:1. The values below each bar represents the mean CTL lysis at that ratio for the respective target.

Frequency of CTL-p in CD4+ Deficient Mice Using Limiting Dilution Analysis

The results seen in the previous sections may be explained as follows: The induction of HSV-1 specific CTL in CD4+ deficient BALB/c may require the presence of growth factors provided by a helper T cell or alternatively, the problem may be a lack of CTL-p that are able to respond to HSV. To address this, a limiting dilution analysis was performed in order to quantitate the CTL-p in CD4+ depleted mice versus non-depleted mice. The assay was set up so that the CTL-p number was the limiting factor, while all other factors required for the expansion of these cells were present in saturating amounts. The results are seen in Figure 9 and Figure 10 with frequencies from two separate experiments listed in Table 2. The HSV-1 specific CTL-p frequency in CD4+ depleted mice (1/2857) was comparable to the frequency found in non-depleted mice (1/6398) [using Taswell analysis]. Furthermore, the use of HSV-1 infected stimulators augmented the frequency of CTL-p in both depleted (1/1054) and non-depleted mice (1/5319). These results demonstrate that CTL-p are present in CD4+ depleted mice at levels comparable to those seem in non-depleted mice.

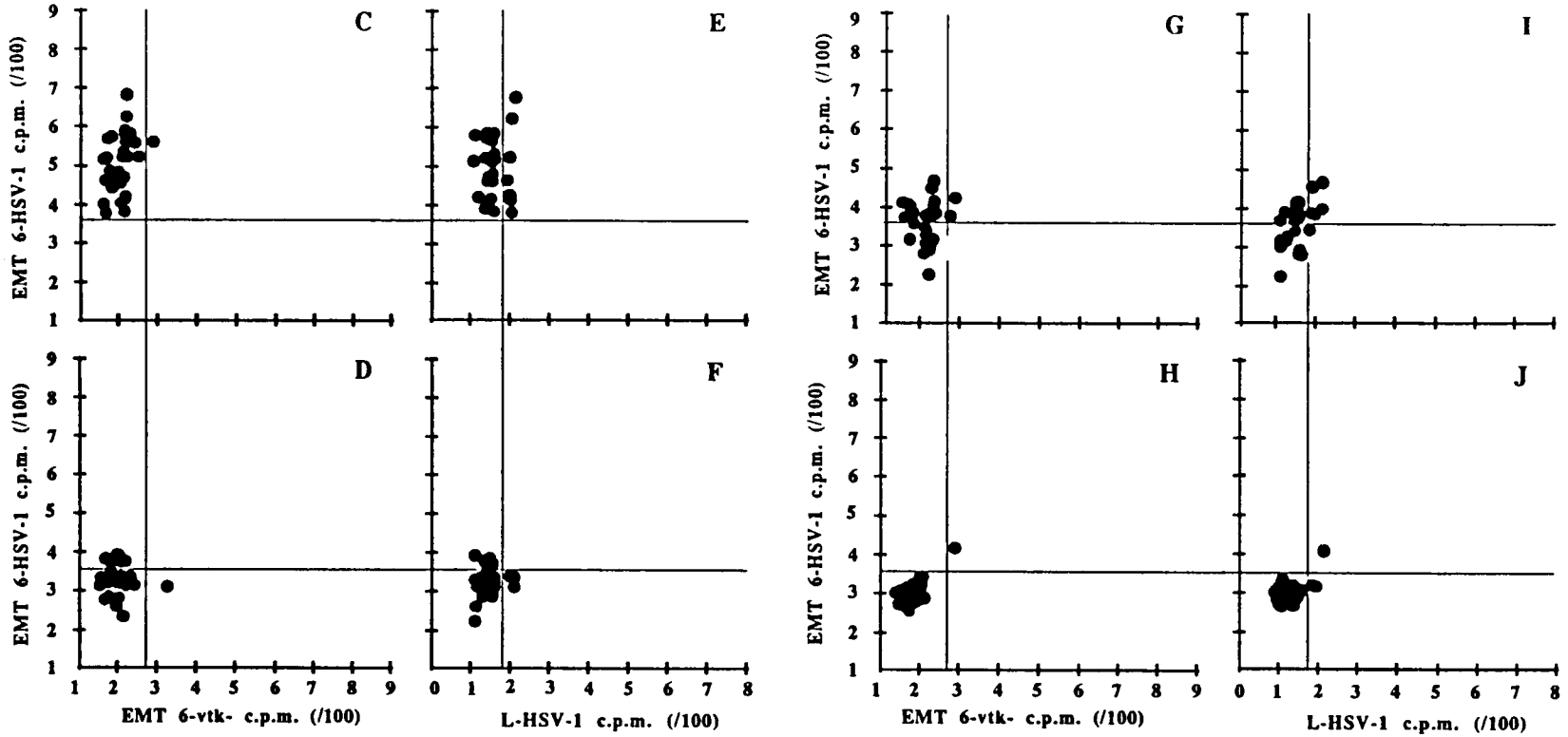
Effect of Helper Factors on the Induction of HSV-1 Specific CTL Responses in CD4+ Subset Depleted Mice.

The results obtained thus far in the limiting dilution indicate that the induction of HSV-1-specific CTL cells does occur in CD4+-subset depleted mice. The CTL-p frequency is comparable in intact and subset depleted mice, however, the mice cannot generate a primary HSV-1-specific CTL response. Since the limiting dilution analysis requires that all other factors required for the

Figure 9. Limiting Dilution Split Well Analysis of CD4+-Depleted and Intact BALB/c Mice

Panels C and E represent individual well values at 20,000 effector cells/well comparing HSV-1 EMT 6 against EMT 6-vtk- and L-HSV-1 respectively (intact mice)
Panels D and F represent individual well values at 3,000 effector cells/well comparing HSV-1 EMT 6 against EMT 6-vtk- and L-HSV-1 respectively (intact mice)
Panels G and I represent individual well values as described for Panels C and E (CD4+-depleted mice)
Panels H and J represent individual well values as described for Panels D and F (CD4+-depleted mice)

Lymphocytes which were either mock or CD4+ depleted were obtained from the popliteal and cervical lymph nodes of acutely infected BALB/c mice. The cells were then used in a 7 day limitng dilution analysis where the number of effector cells was limiting and exogenous growth factors were added in saturating amounts. The cells were used as a source of CTL effectors in a 4-hr chromium release split well analysis after 7 days. Target cells were the same as described in the legend to figure 8. Results are shown as individual values of 30 replicate cultures using Normal Stimulator cells. The lines represent the cut-off point (mean + 3 standard deviations) calculated from microcultures containing stimulator cells only.


C
E
G
I
D
F
H
J
EMT 6-HSV-1 c.p.m. (/100)
EMT 6-vtk- c.p.m. (/100)
L-HSV-1 c.p.m. (/100)

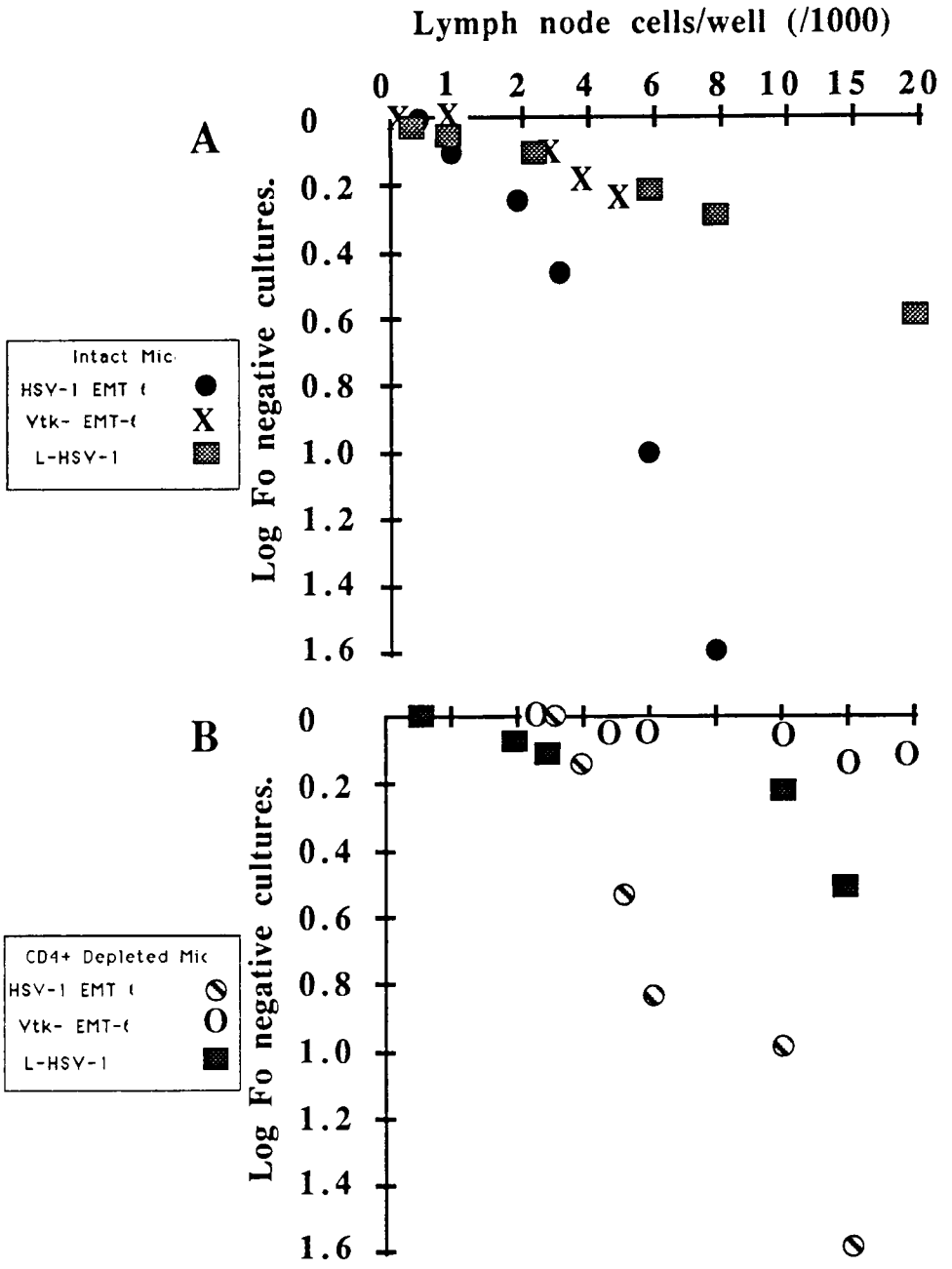


Figure 10. Single Hit Kinetics of HSV-1-Specific CTL-p Frequencies in Intact and CD4+ Depleted BALB/c Mice.

Panel A represents the lines for each target in intact BALB/c mice
Panel B represents the line for each target in CD4+-depleted BALB/c mice

Lines were calculated by Taswell analysis

Table 2. HSV-1-Specific CTL-p Frequencies in Intact and CD4+ Depleted BALB/c Mice

Target	Stimulator	Treatment	CTL-p frequency (95% Confidence Limits)
EMT 6-HSV-1	NS	None	1/6063 (4803-8218) 1/6398 (5239-8218)
	VS		1/2654 (2262-3209) 1/5319 (4378-6777)
	NS	anti-CD4+	1/9462 (7821-11974) 1/2857 (2353-3636)
	VS		1/1177 (930-1603) 1/1054 (847-1397)
EMT 6-Vik-EMT 6-mock	NS	None	1/243,588 (200619-309982) 1/296,655 (145653-808062)
	VS		1/155,404 (129519-194218) 1/115,607 (74755-254916)
	NS	anti-CD4+	1/103,447 (65670-243549) 1/239,776 (190147-324461)
	VS		1/343,304 (225654-717265) 1/87,498 (71602-112466)
L-HSV-1	NS	None	1/250,070 (214901-943544) 1/102,967 (59807-369917)
	VS		1/186,353 (163228-213614) 1/129,367 (49289-207101)
	NS	anti-CD4+	1/62,160 (32796-594176) 1/74,607 (33786-358346)
	VS		1/69,243 (62474-418477)

Frequencies were calculated according to the statistical method of Taswell. The frequencies are listed for each target in CD4+ depleted and intact mice, with the number in parenthesis representing the 95% confidence limits of the frequency.

subsequent expansion and differentiation of these cells be present in saturating amounts, it can be speculated that in order for mice to generate a primary HSV-1 CTL response, the presence of growth factors that are supplied by a "helper cell", or the presence of Th cells is actually required. Therefore, these results suggest that while the induction of HSV-1-specific CTLs is helper cell independent (as has been shown by other investigators), the subsequent expansion and terminal differentiation of these CTL-p into lytically active CTL cells is Th cell dependent (or helper factor dependent). These results also suggest that antigenic restimulation with HSV-1 is required. In order to test this hypothesis, it was necessary to perform a primary CTL assay where helper factors such as IL-2, TCGF and/or Ag (HSV-1) were added to the responding cultures. For this, a CTL blastogenesis assay was set as follows: BALB/c mice were depleted with anti-CD4+ on days -2, +1, and +3 with day 0 representing HSV-1 priming. The popliteal and cervical lymph nodes were excised on day 5 and the cells were cultured with the various factors and held for three days, after which ^{51}Cr-labelled autologous virus-infected or mock infected EMT 6 cells or SJ.1 cells were added to each microculture. The results are shown in Figures 11 and 12. The response was virus-specific and MHC-restricted (data not shown).

The addition of varying concentrations of con A supernatants (a source of IL-2 and other growth factors) was able to restore the HSV-1-specific CTL response in CD4+ depleted BALB/c mice. In fact, the lysis observed reached a peak of 18% with 3% con A sups (this includes IL-2 and Ag), compared to control levels which was 3%. This represents an 83% increase over 0 control

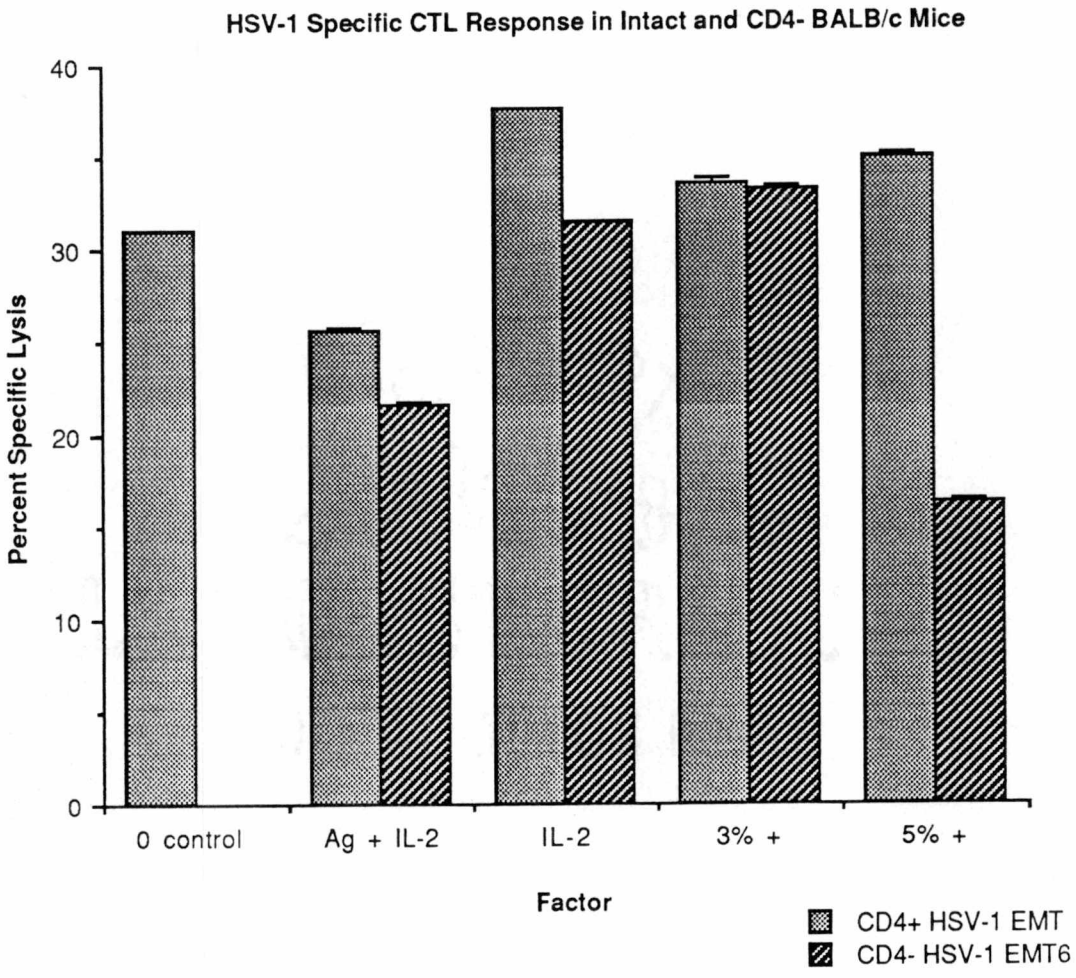


Figure 11. The Addition of Added Soluble Growth Factors Restore the Primary CTL Response in CD4+ Deficient BALB/c Mice.

Lymphocytes which were either mock (NMA) or CD4+ depleted were obtained from the popliteal and cervical lymph nodes of acutely infected BALB/C mice and 4×10^5 cells were cultured in a 96-well rb microtitre plate. The cultures were stimulated *in vitro* with Ag alone, or varying concentrations of con-A-stimulated splenocyte cultures (RF) plus Ag and recombinant IL-2 (rIL-2). The cells were used as a source of CTL effectors in a split well 4-hr chromium release assay after 3 days. Target cells were set up as described in the legend to figure 4 with the allogeneic target being SJ.1 ($H\text{-}2^k$). Percent specific lysis was determined as described in Materials in Methods. Results are shown as the mean values of 12 replicate cultures, plus or minus one standard deviation.

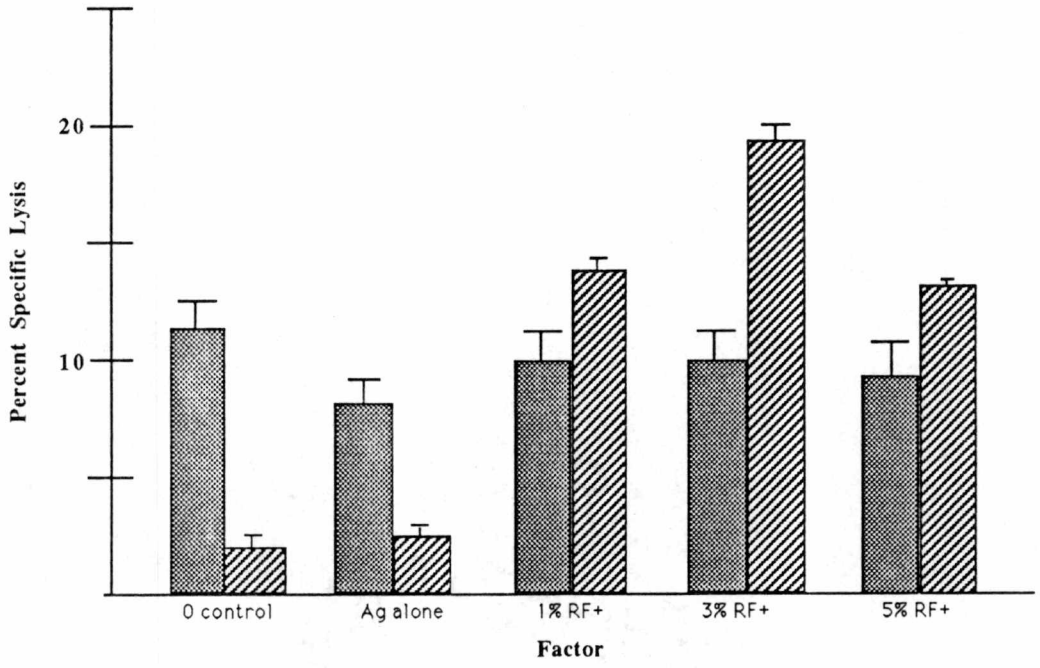


Figure 12. The Addition of rIL-2 Alone is Sufficient to Restore the HSV-1-Primary CTL Response in CD4+ Depleted BALB/ c Mice.

Lymphocytes which were either mock (NMA) or CD4+ depleted were obtained from the popliteal and cervical lymph nodes of acutely infected BALB/C mice and were set up as described in the legend to Figure 10. The cultures were stimulated *in vitro* with varying concentrations of con-A-stimulated splenocyte cultures (RF) plus Ag and recombinant IL-2, Ag plus rIL-2 or recombinant IL-2 alone. The cells were used as a source of CTL effectors in a split well 4-hr chromium release assay after 3 days. Target cells were set up as described in the legend to figure 10. Percent specific lysis was determined as described in Materials in Methods. Results are shown as the mean values of 12 replicate cultures, plus or minus one standard deviation.

values. Once the concentration of con A sups reaches 5%, the lysis starts to decrease. This could be due to toxic effects of the TCGF on the CTL cells when present in high amounts. Recombinant IL-2 alone is sufficient to restore CTL activity in CD4+ depleted mice (as seen in Figure 11), however, Ag alone has no effect on the levels of lysis.

Effect of Immune and Non-Immune Splenocytes on the HSV-1 Specific CTL Response in CD4+ Depleted Mice

The results given in the previous section suggest that the expansion of the CTL-p cells into lytically active CTL cells in CD4+ depleted mice is helper-factor dependent, the absence of CD4+ T cells being "replaced" by added soluble factors. The question now arises as to whether these factors are secreted by the T helper cell or by the CD8+ T cells that are in the culture. It can probably be ruled out that these factors are secreted by the CD8+ T cells in culture, since no lysis is seen in these depleted cell cultures. With factors present, one would see HSV-1-specific CTL lysis. Therefore, these factors are probably secreted by the T helper cell that is absent.

In order to assess whether this observation is correct, another CTL Blastogenesis assay was set up using whole splenocytes isolated from HSV-1 immune, non-immune, and Vaccinia immune mice. These splenocytes were stimulated *in vitro* with HSV-1 or Vaccinia and were added to cells obtained from the lymph nodes of acutely infected mice. After three days, these cells were used as CTL effectors in a split well analysis. Chromium labelled target cells were added to the effectors and ^{3}H-Tdr was added to the remaining effector cells in order to assess whether or not they were proliferating.

These results are seen in Table 3. Splenocytes added to CD4+ depleted cultures are not able to restore CTL activity, nor is proliferation seen in depleted cultures irrespective of added splenocytes. Interestingly, the addition of splenocytes abrogated the CTL response in intact mice, yet the responser cells all exhibit high levels of proliferation.

Table 3. HSV-1-Specific CTL Activity and Proliferation in Intact and CD4+ Depleted BALB/c Mice After Addititon of Ag-Stimulated Splenocytes.

Stimulator	Quantity	Treatment	Percent Specific Lysis	^{3}H-Tdr uptake (cpm)
None	10^5	None	11 +/- 1.25%	13833 +/- 2544
normal non-immune			1.61 +/- 1.82%	12551 +/- 1357
normal viral-stimulated			1.99 +/- 1.38%	11911 +/- 2568
immune			3.0 +/- 0.95%	13353 +/- 2726
immune CD4+depleted			3.0 +/- 1.5%	16447 +/- 4439
Vaccinia			3.4 +/- 1.3%	22233 +/- 2227
None	10^5	anti-CD4+	1.25 +/- 1.65%	824 +/- 256
normal non-immune			-2.39 +/- 1.24%	891 +/- 183
normal viral-stimulated			-1.86 +/- 1.08%	2332 +/- 679
immune			-0.05 +/- 1.47%	3758 +/-930
immune CD4+depleted			0.74 +/- 1.15%	2381 +/- 347
Vaccinia			-0.46 +/- 1.85%	8177 +/- 1705

Lymphocytes which were either mock (NMA) or CD4+ depleted were obtained from the popliteal and cervical lymph nodes of acutely infected BALB/C mice and were set up as described in the legend to Figure 10. The cultures were stimulated *in vitro* with 10^5 HSV-1 or Vaccinia stimulated x-irradiated splenocyte cultures. The cells were used as a source of CTL effectors in a 4-hr chromium release split well analysis after 3 days. Target cells were set up as described in the legend to figure 4. 1 µCi of ^{3}H-Tdr was added to the remaining cells in the split culture and ^{3}H-Tdr uptake was measured. Percent specific lysis was determined as described in Materials in Methods. Results are shown as the mean values of 12 replicate cultures, plus or minus one standard deviation.

CHAPTER VI

DISCUSSION

This study documents the use of monoclonal antibodies to manipulate the immune response. In the response to HSV-1, there are differential requirements for CD4+ T helper lymphocytes in the induction of antiviral antibody and CTL responses. Monoclonal Abs specific for the CD4+ (GK1.5) and CD8+ (2.43) Ags on T lymphocytes have been used in this study to probe the immune response to HSV-1 in the murine system. The results demonstrate, in keeping with previously published expectations, that mice treated with anti-CD4 exhibited suppression of T helper dependent responses, *e.g.*, DTH and Ab responses to HSV (figure 2 and Table 2). Contrary to this, a T-independent response (Ab response to E. coli LPS 055) was not suppressed (Table 2).

The primary focus of this study was the role of the Th lymphocyte in the generation of HSV-1-specific CTL cells. Nash *et al.* (153) have shown that CTL responses to HSV-1 are induced in CD4+ depleted mice and are actually augmented above those observed in untreated mice. This has been observed in other viral systems as well including LCMV, Coxsackie virus, Ectromelia virus and Influenza virus (152,144,148-150). This suggests common pathways for Ag-presentation, activation and clonal expansion in the responses to these viruses.

CTL's play an important part in controlling viral infections and the induction of CTL responses in a Th cell-depleted mouse is quite unexpected since all *in vitro* studies clearly indicate that CD4+ T helper cells are needed to

provide those factors which induce CTLs into lytically their active status (37). In order to activate CTLs, two distinct Ag activated stages are necessary. The first stage is the Ag activation of Th cells. This is an independent stage and begins with the presentation of Ag on the surface of an APC, which is Ia+, and also secretes IL-1. IL-1 drives the Th cell into proliferation and induces the subsequent secretion of various soluble growth factors that are needed by the CTL to expand and differentiate. CTL differentiation and expansion encompasses the second stage of the CTL response. This sequence is dependent on the Th cell. HSV-1 is presented to the CTL-p in the context of Class I Ags (K or D) and subsequently, Ag and Th factors cause the expansion and differentiation of these precursor cells into lytically active CTLs.

It seems that these events which have been well characterized *in vitro* represent those events underlying the *in vivo* CTL response. Yet, the results shown in this study contradict the results seen by Nash and other researchers. When lymph nodes are removed from CD4+ depleted, acutely-infected mice (5 days after HSV-1 injection) and assessed for their efficacy in generating a primary CTL response, the results in three strains of mice tested indicate that no reponse can be generated (Figure 3). Leist *et al.* (148) reported similar findings whereby LCMV-infected C_{57}Bl/6 mice, depleted with anti-CD4+ mAb failed to generate an effective LCMV-specific CTL response. CD4+ depletion reduced the lytic units per spleen by a factor o f 10 when compared to the response generated by thymectomized but otherwise untreated mice. This finding supports the notion that T help is involved in the generation of a primary CTL response *in vivo* (161).

The results reported herein show that in all strains of mice tested, the CTL response in CD4+-depleted mice could be suppressed by 50-95%. However, in C_3H/HEN mice, this was,for the most part, not the case. Instead, these mice generated a CTL response in CD4+ depleted animals comparable to the levels seen in non-depleted mice (Figure 4). FACS analysis demonstrated >95% depletion in all cases leaving incomplete depletion as an unlikely explanation for this. This suggests that genetic mechanisms may be involved in controlling the generation of a primary CTL response in H-2^k mice (both C3H and Nash's CBA mice are H-2^k). This has not been investigated at this time.

The remainder of the study concentrated on utilizing the BALB/c mouse since it consistently showed total abrogation of the primary CTL response (Figure 3, 5 and 7). When comparing the primary HSV-1-specific CTL response in these CD4+ depleted mice against the memory CTL reponse, it was found that a memory CTL response could be generated at levels comparable to those seen in intact mice (Figure 8). In order to show this, spleen cells isolated from mice 8 weeks after HSV-1 infection were restimulated *in vitro* with virus and assessed for CTL activity. Nash *et al.* (153) demonstrated responsiveness in depleted mice following restimulation *in vitro* and likewise in the present study, CD4+ depleted mice demonstrated a capacity for CTL response following restimulation. The mice were maintained in a depleted state by weekly injections of mAb, yet a long-term memory response was demonstrable. This suggests that the CD4+ T cell population is not needed for the generation or maintenance of this CTL population, nor for its expansion upon secondary restimulation *in vitro*. Prior to the *in vitro* culture, spleen cells from both intact and Ab treated mice could be depleted of CD4+ T cells *in vitro* by using

anti-CD4 Ab and complement with no effect on CTL activity noted (data not shown).

The difference noted between primary CTL responses and this memory response could be explained in a number of ways. First, the time period involved in assessing for the memory response and primary response must be taken into consideration. The memory reponse is measured 8 weeks after infection with HSV-1, while the primary response is measured in acutely infected mice (only 5 days). The primary response then could be dependent upon rapid Th cell expansion. Second, the memory response is measured after the mice are boosted *in vivo* with HSV-1 and the lymphocytes are restimulated *in vitro* with Ag, not so with the primary response. The reexposure of the virus could cause continual expression of viral Ags on cells that could stimulate the CTL directly in the absence of help. Also, one cannot rule out the possible existence of two separate populations of CTL-p cells that are capable of responding to Ag as proposed by Buller *et al.* (144). One population would be helper cell dependent, *i.e.*, requiring Th cells and their factors, and the other population would be T-helper cell independent, *i.e.*, they could circumvent the requirement for help and thereby expand and differentiate into lytically active CTLs. Klarnet *et al.* (162) examined the role of a certain subset of CD8+ T cells termed HITc which are capable of producing IL-2 and proliferate in response to Ag without the cooperation of CD4+ T cells. These cells expressed both Th and CTL functions. These cells also expressed IL-1 receptors and required IL-1 for the subsequent secretion of IL-2. Their data demonstrated that two subpopulations of CD8+ CTLs existed. One subset does express IL-1 receptors

and does not secrete IL-2 after Ag stimulation. In contrast, the second subset does express IL-1 receptors and will secrete IL-2 subsequent to Ag stimulation.

In order to investigate these responses at the level of the CTL-p, limiting dilution analyses was performed in order to quantitate the HSV-1-specific CTL-p in intact and CD4+ depleted mice. In this assay, only the number of responder cells (from acutely infected lymph nodes) was limiting while Ag and other growth factors were present in saturating amounts. The results demonstrate that CTL-p are present at comparable frequencies in CD4+ depleted and intact mice (Figure 10 and Table 2). CTL-p are clearly induced *in vivo* in CD4+ depleted mice and the CD4+ T cell subset is not needed for the generation and maintenance of the CTL-p. Therefore, the lack of demonstrable CTL in primary cultures may be due to the absence of helper factors necessary to expand the activated CTL-p. In the LDA, the helper factors are supplied exogenously. It is possible that this requirement for growth factors could be met by added soluble factors or by competent factor-secreting cells.

To investigate the requirement for Th factors to expand CTL-p into lytically active CTL cells, a CTL blastogenesis culture system was set up to measure a primary response. In this assay system, lymph nodes are excised from acutely infected intact and CD4+ depleted mice and set up in a microculture plate. Growth factors that are supplied by Th cells (i.e., IL-2, and IL-4, were added back into the culture in varying amounts; these factors were all derived in the Con-A-stimulated rat splenocyte cultures). UV-inactivated HSV-1 was also added to see whether this was an additional or sole requirement for CTL maturation. CD4+ depleted mice could indeed mount a primary CTL response when the growth factors were added back (Figure 11 and

12). Cytotoxic levels were comparable and even enhanced over the levels of lysis seen in intact mice. These results suggest that the expansion of CTL-p and terminal differentiation is indeed factor dependent. Interestingly, the requirement for factor-dependent expansion could apparently be supplied by recombinant IL-2 (rIL-2) alone. This observation has been reported by other researchers who found that in limiting dilution cultures IL-2 alone can provide the necessary help for polyclonally activated primary CTL-p ().

The question now arises as to whether these factors are secreted by a Th lymphocyte or by the CD8+ T lymphocyte itself. To address this, primary bulk cultures were supplemented with HSV-1-stimulated or Vaccinia-stimulated x-irradiated splenocytes. The results reported here show that with the addition of these Ag-stimulated splenocytes, no restoration of the CTL response could be seen in CD4+ depleted cultures (Table 3). Interestingly, no CTL response was seen in the non-depleted cultures that had received stimulator splenocytes. The cells in the non-depleted cultures did proliferate as compared to the CD4+ depleted cultures (shown by ^{3}H-Tdr uptake). This suppressive effect could be due to the release of prostaglandins or other inhibitory agents (163).

There are several possible explanations for the complexities noted for CTL induction in the studies outlined here. First, as shown by primary CTL response, the induction of CTL-p *in vivo* is T-helper independent (supported by the LDA), but the expansion and terminal differentation of these CTL-p into their aggressor status is Th cell dependent. This dependence on CD4+ helper cells appears to lie primarily in a requirement for secreted growth factors. This has been supported by Schmitt *et al.* (164) studying the *in vitro* reactivity of CD8+ T cells toward hapten 2,4,6-trinitorphenol (TNP)-modified

syngeneic stimulator cells. The use of stimulators enriched for DC and supplementation of the culture medium with IL-1 and colony-stimulating factor (CSF), and IL-3 are critical for the proliferation and IL-2 production of CD8+ T cells. These factors increased the reactiveness of CD8+ T cells and were necessary to obtain optimal T cell responses. Second, there remains the possibility that CD4+ T cells control the secretion of the added exogenous cytokines. Consequently, the activation of CD8+, IL-2 producing, H-2 restricted T cells would be indirectly dependent on CD4+ T cells. Interestingly, only a minority (<10%) of clonally developing TNP-specific CD8+ T cells appear to be bifunctional (possessing both the properties of IL-2 production and CTL activity).

In a study by Horohov *et al.* (165) where they studied the induction of human influenza virus-specific memory T lymphocytes, they found that the differentiation of CTL-p which subsequently develop into effector cells is dependent upon the presence of exogenous helper factors. They demonstrated that human CTL-p require a differentiation signal that is distinct from IL-2 and IFN. Addition of IL-2 alone was sufficient for inducing proliferation, however maximal maturation and cytolytic activity occurred when conditioned medium containing growth factors was added after the IL-2. It may not be possible to extrapolate from the human to the mouse, yet this offers additional evidence for the requirement of growth factors in order to generate a primary HSV-1-specific CTL response.

In generation of an HSV-1-specific memory response, Ag restimulation *in vitro* is a requirement for the subsequent expansion of the CTL-p. This does not seem to be the case in acutely infected mice. Inaba *et al.* (104) reported

that dendritic cells are capable of stimulating CD8+ T cells directly during responses to transplantation antigens. In fact, these Ags when presented on lymphoid dendritic cells, activate CD8+ T cells to become lymphoblasts with modest cytolytic activity. This response does not occur when the Ags are presented on macrophages or foreign lymphocytes. This mechanism may operate in long term memory responses. Also, another report by Boog *et al.* (166) reports that stimulation with dendritic cells (DC) obviates the CD4+ T helper requirement in CTL responses to the male antigen H-Y, both *in vitro* and *in vivo*. Besides being highly efficient at class II-restricted presentation of Ag to Th, DC were also superior in the presentation of Ag in the context of class I molecules to CTL cells. They conclude from their studies that because DC have high class I molecule expression, and they do have a unique ability to induce cluster formation (making them better presenters of Ag), they are more efficient in triggering CD8+ cells to secrete IL-2, thereby bypassing the requirement for CD4+ T cells. However, these dendritic cells may not be able to stimulate CTL-p directly in acutely infected mice (due to the time constraints between this assay system and a memory CTL assay system) leading to a greater level of Th dependency in these more short-term initial responses.

The possibility of two separate existing CD8+ populations was mentioned earlier, the first population being Th-dependent and the second being Th-independent for their maturation. This observation has been supported by Schmitt *et al.* (164) and Heeg *et al.* (167). In the latter reference, Heeg *et al.* analyzed *in vivo* the CD4+ T cell subset-independent reactivity of CD8+ T cells towards transplantation Ags. Their CD4+ depleted mice showed no physical and functional properties attributed to CD4+ T cells, however, the mice still

reacted to class I MHC alloantigens *in vivo* and developed Ag-specific cytolytic activity. Moreover, they found that IL-2 producing T lymphocyte and cytolytic CD8+ T lymphocyte-precursor frequencies increased up to 20-fold. Yet, the CD8+ T cells in CD4+ depleted mice could function independently of CD4+ T cells provided that they were stimulated *in vitro* with class I MHC-bearing stimulator cells. This *in vitro* result strongly suggests T-T cell interactions within the CD8+ T-cell subset. The results given here do not support this contention, nor do they necessarily refute it. Given the apparent factor-dependence of the primary CTL response to HSV, it would seem that the key to CTL responsiveness rests in a requirement for activation and expansion of factor-secreting cells, not in responding CTL-p subsets.

It will be important to assess if the CTL response obtained with the BALB/c, $C_57Bl/6$, and the C_3H/HEN mice is strain-dependent. It could be proposed that the balance between Th-dependent and Th-independent CTL response to a specific Ag is genetically determined. If this is true, then it could be proposed that mouse strains such as C_3H and CBA mice may follow a Th-independent pathway for CTL induction and subsequent maturation, while other mouse strains such as BALB/c and $C_57Bl/6$ may be biased towards a pathway requiring Th-cells. Support for this is found in work by Sprent *et al.* (28-buller), where they found that, for a single mouse strain, there is a hierarchy of responses by CD8+ T lymphocytes to different Ags that cannot be explained by variations in precursor frequencies.

A final explanation for the complexity noted between the acute and memory HSV-1-specific CTL response could be due to the residual population of CD4+ cells. It cannot be ruled out that a residual population of CD4+ cells may

have been sufficient enough to provide help for the CTL response, especially in longer term cultures such as the memory response.

It is possible that CTL responses to some viruses may be totally Th-cell dependent. Studies addressing this issue will better define the role of CD4+ T cells in antiviral host defense mechanisms and may provide a clearer understanding of the pathogenesis of acquired immunodeficiency syndrome, a disease characterized by a deficiency of CD4+ T cells and susceptibility to opportunistic infections (149).

In conclusion, this study demonstrated that CD8+ T cells can be induced *in vivo* in a CD4+ deficient environment. All physical and functional depletion of CD4+ T cells is supported by FACS analysis, DTH response, and Ab response. CD8+ precursor cells can expand and differentiate into lytically active CTLs only during a memory HSV-1-specific CTL response, yet the acute primary CTL response is dependent on the addition of exogenous growth factors. This suggests that CD4+ T lymphocytes are required for the generation of a primary HSV-1-specific CTL response, but not the memory response. This observation has recently been reported by Jennings *et al.* (168) who investigated the requirement for CD4+ T lymphocytes in the generation of HSV-1-specific CTL responses in $C_{57}Bl/_{6}$ mice. They found that these mice could not mount a primary CTL response but this did not affect the levels of HSV-specific CTL precursors expanded *in vitro* in the presence of exogenous Ag and IL-2. He suggested, as this study has, that the CD4+ T lymphocyte are not required for the initial expansion of HSV-specific CTLp following priming, but were not essential for the priming itself. Their analysis of the secondary (memory) response indicated that the CD4+ T subset was not required for either the

generation or maintenance of the memory CTL population, nor for its expansion upon secondary restimulation *in vitro*. This also concurs with the results in this study. They confirmed this by restimulating the secondary cultures with paraformaldehyde-fixed stimulator cells. These cells lack the expression of class II glycoproteins and are unable to release viral antigen for subsequent processing by class II-expressing APCs. However, this did not affect the generation of memory HSV-specific CTL. In fact, the levels of CTL lysis in this system were comparable to the levels of lysis in the system stimulated by unfixed stimulator cells. Interestingly, they also suggest that IL-2 is a critical factor in the CD4-independent expansion of HSV-specific memory CTL.

Most importantly, the observations reported here indicate the importance of T cell depletion *in vivo* ("immunosurgery" as coined by Nash *et al.*) as a technique for studying the immunobiology of infectious diseases. There are at least two ways in which aspects of immunoregulation can be investigated along these lines. Firstly, subset-depleted mice can be used to elucidate the T cells involved in different aspects of the response to viral infections. Secondly, T-cell subsets can be manipulated at any time during a response to foreign Ag or in autoimmune diseases, in order to probe the events which regulate the immune system as a whole. This technique would be particularly relevant to studying chronic virus infections and the involvement of T lymphocytes in the pathology of virus diseases, which is still poorly understood.

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VITA

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