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ANALYSIS OF CYTOTOXIC T LYMPHOCYTE PRECURSOR FREQUENCIES AGAINST HERPES SIMPLEX VIRUS

A Thesis

Presented for the

Master of Science

Degree

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Julia Ann Heath

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For my parents, who have made innumerable sacrifices, in order that my brother and I could have the education that they themselves desired and deserved, but could never have.

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ABSTRACT

A limiting dilution microculture system is described in which the frequencies of cytotoxic T lymphocyte precursors reactive against herpes simplex virus type 1 were determined. Analysis by Poisson statistics demonstrated that the estimated frequency of HSV-1 reactive cells in the spleens of normal mice was less than 1/250,000. In contrast, spleen cells from HSV-1-immune mice stimulated in vitro with HSV-1-infected irradiated splenocytes resulted in CTL-p frequencies between 1/6519 and 1/15632. The same responder population of splenocytes when heterologously stimulated with HSV-2-infected stimulators yielded much lower frequencies of anti-HSV-1 CTL-p, 1/22045-1/49196.

To analyze the cross-reactivity between HSV-1 and HSV-2, mice were infected in vivo with HSV-2, and the resulting immune splenocytes were then heterologously or homologously stimulated in vitro. When the effectors were assayed against HSV-1-infected target cells, CTL-p frequencies of 1/13664-1/18476 and 1/12784-1/16644 were obtained for homologously and heterologously stimulated splenocytes, respectively.

Split culture analysis comparing cytotoxicity against syngeneic and allogeneic virus-infected targets provided evidence for specificity, H-2 restriction and the T cell nature of the CTL-p. It was determined that the putative CTL-p derived cytolytic effector progeny were eliminated by treatment with anti-Lyt 2.1 plus complement, thus indicating that the effector progeny were Lyt $1^{-}2^{+}$ cells.

These experiments demonstrate the feasibility of using the limiting dilution analysis as a sensitive and quantitative means to measure the cell mediated immune response to HSV-1 antigens.

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ABBREVIATIONS

A31 = strain BALB/c clone A31

DCS = donor calf serum

FCS = fetal calf serum

m.o.i. = multiplicity of infection

p.f.u. = plaque forming units

HSV-1 = herpes simplex virus type 1

HSV-2 = herpes simplex virus type 2

Con A = concanavalin A

CTL-p = cytotoxic T lymphocyte precursor

HTL-p = helper T lymphocyte precursor

PFC = plaque forming cells

LDA = limiting dilution analysis

PEC = peritoneal exudate cells

MLC = mixed lymphocyte culture

CSF = colony stimulating factors

TCGF = T cell growth factor

IL-1, IL-2, IL-3 = interleukin type 1 and type 2 and type 3

CHAPTER I

LITERATURE REVIEW

Introduction

Because more than one cell type is involved in the immune response to an antigenic stimulus, it is necessary that all components, i.e., factors and cell types, needed for such a response be present in the culture. There are, however, problems inherent in working with such complex, "saturated" systems, i.e., it is difficult to obtain selective information on a single component or variable contained in such a system. An alternative approach is to interfere with this saturation by titrating or limiting one necessary component, while all other components remain saturating. This idea of limiting one component serves as the basis for the limiting dilution analysis.

The principle upon which the limiting dilution analysis is based is not a new one, e.g., extinction processes in physics are based on this idea; but only recently has it been applied in immunology to estimate the frequency of relatively rare cells, e.g., influenza-specific cytotoxic T lymphocyte precursors (CTL-P) (8). Initially, the limiting dilution technique was used in in vivo cell transfer experiments, but this proved to be too costly and time consuming requiring large numbers of animals. In 1972, Ivan Lefkovits developed the in vitro limiting dilution analysis which was more cost efficient and less time consuming (15). The in vitro limiting dilution analysis requires that the titrated cell type be present in a wide range of concentrations, whereas all other cells are present in constant, saturating amounts.

The Poisson distribution is used to analyze the data and estimate the frequency of the titrated cell type. One must first score the microculture wells as either responding or nonresponding. By plotting the fraction of nonresponding cultures versus the cell input on a semilog plot and using the method of least squares, one can find the best fitting straight line which passes through the origin indicating a single-hit event. By interpolating at the level of 37% nonresponding cultures, the size of the sample containing only one precursor cell can be estimated. (See Methods and Materials).

Limiting dilution analysis is a relatively new technique in the field of cellular immunology. The earliest immunological work using this in vitro limiting dilution technique involved the titration of B-precursor cells and T-helper cells (14, 34). Prior to 1976, most of the information on cytotoxic T lymphocytes (CTL's) was derived from bulk assays, but these could give no estimate of the frequencies of CTL's. In 1976, Skinner and Marbrook found that this in vitro limiting dilution analysis could be used to study the cytotoxic T-cell system (30). Most of this work has involved CTL-P cells reactive against alloantigens. More recently, in vitro limiting dilution analysis has been used in studies of viral-specific CTL-P, as well as, suppressor T-cells and lymphokine-secreting T-cells.

Frequency of Helper T Lymphocyte Precursors (HTL-p)

The in vitro limiting dilution analysis technique has been used by a number of immunological researchers in a myriad of different

systems. Some of the earliest work utilizing the LDA involved the estimation of the frequency of helper T lymphocyte precursor (HTL-p).

Waldmann et al. used the LDA to determine the frequency of T cells mediating specific and nonspecific help (34). SRBC-educated thymus cells were titrated in LD microcultures with B cells and SRBC, in order to determine the frequency of Tsrbc that were capable of facilitating an anti-SRBC response by B cells. The frequency of HTL-p was found to be 8×10^{-5}. Irradiated keyhole limpet hemocyanin (KLH)-primed spleen cells could facilitate an anti-SRBC response by B cells in a nonspecific manner at an estimated frequency of 4×10^{-5} or 1 in 25,000.

Because SRBC is a particulate antigen, any anti-SRBC response would be facilitated by both "specific" and "nonspecific" help. Therefore, this system could not be used to ascertain whether all SRBC specific HTL were the same as those mediating nonspecific facilitation. However, the use of hapten coupled to a soluble protein carrier (TNP-KLH) as an antigen allowed the authors to determine if the T cells mediating specific help were the same as those mediating nonspecific facilitation, because the anti-hapten response to TNP-KLH cannot be helped by the nonspecific mechanism. The frequency of KLH-primed cells which were capable of helping a specific fashion was 11×10^{-6} to 9×10^{-6}. The data from these experiments suggest that the frequency of specific helper cells was lower than that of

nonspecific helper cells, thus implying the possibility of two different T cell subpopulations which mediate specific and nonspecific help.

Melchers et al. used a somewhat different LDA approach to determine the frequencies of SRBC-reactive as well as HRBC- and CRBC-reactive HTL-p cells within the normal T cell pool by using xenogeneic RBC as T-dependent antigens and measuring "help" as the response of a B cell population in terms of direct plaque-forming cells (PFC) (40). Nylon-wool-purified C57BL/6 splenic T cells were activated in bulk culture with Con A and then cultured in LD conditions with irradiated peritoneal exudate cells (PEC) and Con A supernate (supernate of Con A-induced rat spleen cells). T cells from LD microcultures were then cultured with B cells and SRBC (CRBC or HRBC) for 4 days, and the responses were assayed by counting the number of direct PFC/well.

Interestingly, the plots of the fraction of negative cultures versus cell input showed decreases and increases in the fraction of negative cultures in relation to the T cell concentration, i.e., showed definite reproducible peaks of response at 40-200 T cells/well, 600-3000 T cells/well, and 20,000-40,000 T cells/well. To exclude possible artifacts, the authors tested whether the help observed in this system was indeed T cell mediated:

1. Helper activity was not due to Con A supernate as the addition of Con A supernate directly into cultures with B cells and RBC did not produce a response.
2. By preparing T cell-enriched and T cell-depleted populations, it could be shown that help from the titrated cells was due to T cells.

The data did not fit a single-hit Poisson distribution, but instead suggest a multiphase limiting-dilution curve with each peak or responsiveness reflecting a different pre-Th population which becomes suppressed at increasing cell concentrations. Unfortunately, the final interpretation of the curves will depend upon a mathematical model that permits the analysis of multihit results, i.e., a system that allows for estimating accurate frequencies from multihit LD. The polyclonal activation by Con A may allow for the determination of not only the effector precursor under study (in this case HTL-p) but also those cells that can modify the generation and function of the effector; thus these complex LD curves may reflect important cellular interactions which serve to regulate effector functions.

Several groups have estimated the frequencies of alloreactive CTL-p by adding limiting numbers of responders to microcultures which contained alloantigen and TCGF. Lutz et al. described a similar approach to estimate the precursor frequency of alloreactive helper T lymphocytes (HTL) which cooperate with CTL to generate cytolytic activity (39). In order to estimate the precursor frequency of HTL which react to a single haplotype, limiting numbers of responding C57BL/6 spleen cells were cultured in LD conditions with cloned L3 CTL cells (which require either HTL or TCGF for growth and thus serve as cytolytic indicator cells for HTL activity) and either semiallogeneic BDF_1 or allogeneic DBA/2 stimulating cells. Approximately 1 out of 1000 C57BL/6 spleen cells developed helper activity in response to BDF_1 alloantigen, while C57BL/6 helper activity in

response to BDF_1 alloantigen, while C57BL/6 helper cells reacted to DBA/2 alloantigen at a frequency of approximately 1 in 830.

Because different alloantigens appear to stimulate distinct T lymphocyte subsets in MLC, LDA was used to measure which alloantigens would stimulate the highest frequency of HTL-p. Approximately 28-fold more B10.A (2R) HTL-p responded to DBA/2 (H-2K-IE^d, D^d; Mls^a) than to B10.A ($H\text{-}2D^d$) stimulating cells. In order to determine which H-2 alloantigens best stimulate HTL-p the frequency of HTL-p which responded to H-2-congenic stimulating cells was estimated by LDA. Alloantigens encoded by genes of the H-2 IA-IE region stimulated approximately 6-fold more HTL-p than alloantigens encoded by the H-2K or D regions. Non-H-2 alloantigens (including Mls) stimulated approximately 20-fold more HTL-p than H-2K or D gene products, and 2.5 fold more HTL-p responded to non-H-2 than to an entire H-2 haplotype. The Mls^b alloantigens stimulated very few HTL-p when compared to Mls^a. These results obtained by LDA tend to support the hypothesis that alloantigens which are encoded by the H-2 I and non-H-2 Mls regions preferentially stimulate HTL.

Frequency of Factor Producing T Cells

T cells are known to produce a variety of soluble factors which effect a number of different functions. Among these T cell derived factors are IL-2 (TCGF), immune interferon (IFN-γ), colony-stimulating factors (CSF), B cell growth factor (BCGF), and T cell replacing factor (TRF). Recently, limiting dilution analysis has been

used to determine the frequency of T cells producing CSF, IL-2, IL-3 and IFN-γ (42).

Colony-stimulating factors (CSF) are glycoproteins necessary for the formation of colonies of mature hematopoietic cells from single immature progenitor cells known as colony-forming cells (CFC). A variety of cells can produce CSF, including T cells, and the number of colonies which develop in semisolid agar can be used to determine the CSF concentration contained in biological fluids. Staber et al. used LDA to determine if normal T cells produce CSF in vitro and whether neutrophil-granulocyte-macrophage CSF (GM-CSF), megakaryocyte CSF (Meg-CSF), eosinophilic granulocyte CSF (Eo-CSF) and erythroid cell CSF (E-CSF) are produced by different T cell subsets, as well as determine the frequency of CSF producing T cells (41).

C57BL/6 (B6) spleen cells were initially stimulated in bulk culture with Con A. Light density blasts were separated and subsequently grown in LD microcultures with allogeneic irradiated PEC and TCGF. Following a 10 day growth period in which cells were fed with rat spleen cell conditioned medium (RSCM), cells were washed to remove any CSF contained in the RSCM and resuspended in medium with Con A. After overnight incubation, the supernatants of all wells were assayed for CSF in semisolid agar containing CBA bone marrow cells. Seven days later, the bone marrow cultures were scored for growth and the cellular composition of the colonies determined.

The growth frequency of Con A-activated T cells initially plated in LD was 1 out of 2-3 cells, while the frequency of CSF producing

cells was approximately 1/6. It was found that the supernatant of a single T cell clone could contain different types of CSF, however, the production of Meg-CSF, E-CSF, and Eo-CSF occurred only concomitantly with GM-CSF production. Thus, through the use of LDA, it could be demonstrated that a large proportion of normal T cells can produce CSF upon mitogen induction.

Another very important immuno-modulator is interferon (IFN). Interferon has a wide variety of activities: induction of natural killer cells (NK) (43), the inhibition of viral replication and growth of certain tumors. There are three types of interferon, leukocyte (IFN-α), fibroblast (IFN-β), and immune (IFN-γ). Using long-term in vitro clones of normal mouse T cells stimulated with mitogen in the presence of TCGF but in the absence of adherent cells, Krammer et al. succeeded in inducing high quantities of IFN-γ, thus establishing that T cells alone could produce IFN-γ (44). However, it remained to be shown if IFN-γ was produced by a particular T cell subset. In a later paper, Krammer et al. used LDA to study the frequency of IFN-γ-producing cells and whether CTL could produce IFN-γ (42).

Normal C57BL/6 spleen cells were activated by Con A and subsequently grown in microcultures under LD conditions. The clonal progeny of cells plated in LD were divided and tested for (1) lectin-dependent cytolytic activity on EL-4 tumor targets in order to determine the frequency of CTL regardless of specificity and (2) the quantity of IFN-γ released by Con A stimulation. The frequency of growing Con A-activated B6 spleen cells was 1/5.9 cells. The

frequency of CTL-p was 1/26 and IFN-γ producing cells 1/8, i.e., approximately 1/3 and 1/2 Con A-activated splenic T cells were CTL or released IFN-γ, respectively. To determine the relationship of IFN-γ producing cells and cytotoxic cells, individual microcultures were divided and then tested for both activities. Some cultures were found to lack both activities. IFN-γ could be released by noncytotoxic as well as a large portion of CTL. Cytotoxic activity was not necessarily associated with IFN-γ release as some cultures had CTL activity but did not produce IFN-γ, however, high cytolytic activity was associated with high IFN-γ titers.

These results provide the first frequency estimate of IFN-γ producing cells and also demonstrate that LDA can be used to culture and expand T cells from normal animals to a clone size suitable for functional tests.

IL-2 (TCGF) is a soluble immunoenhancing factor which is produced by activated T helper cells and which acts in a nonspecific, i.e., genetically unrestricted, manner. IL-2 is produced by T cells following stimulation with either T cell mitogen or specific antigen. The LDA has been used to determine the frequency of alloantigen-responsive precursors of IL-2-secreting mouse T helper cells in spleen cell populations (45).

BALB/c spleen cells were stimulated by allogeneic $CB6F_1$ T cell depleted resident peritoneal cells (RPC) in LD microcultures. The IL-2 content of the supernatants of the microcultures was assayed by the ability of the supernate to sustain the growth of the IL-2

dependent CTLL-A2 long-term cytotoxic T cell line as determined by ^{3}H-thymidine uptake. The frequency of pHTL in unfractionated spleen cell populations was 3.4 per 10^{3} splenocytes or 1/292 cells.

For other strain combinations in which only MIs incompatibility was present, the frequencies of pHTL were 1/62-1/120 for unfractionated spleen cells and 1/29 for splenic T cells. In combinations in which both MIs and MHC antigens were involved, the results were comparable to those involving MIs incompatibility alone with the estimates for splenocytes being between 1/85 and 1/147, and for T cells between 1/40 and 1/158. In MHC-disparate combinations, the frequencies are somewhat lower, 1/292-1/1004 splenocytes and 1/226-1/240 T cells.

Thus, depending on the particular stimulus, 1 T cell in every 30-300 can generate detectable levels of IL-2. MIs reactive T cells seem to be about 5- to 8-fold more frequent than those responsive to MHC antigen. These results are consistent with those of Lutz et al. (39) who found that 2.5 fold more HTL-p responded to non-H-2 than to an entire H-2 haplotype and 20-fold more HTL-p responded to non-H-2 alloantigens than to H-2K or D gene products.

IL-3 is a lymphokine, produced by mitogen- and alloantigen-stimulated T cells (47) and also by the myelomonocytic leukemia WEHI-3, which promotes the proliferation of clonal cultures of certain bone marrow-derived mouse cell lines. The same LDA technique as above was later used to determine the frequency of T helper cells secreting IL-3 and to study the correlation between IL-2 and IL-3 production by HTL (46).

CBA/H-T6J responder splenocytes were cultured in LD with anti-Thy-1 + C treated (C57BL/6J x CBA/H-T6J)F_1 stimulators. The frequency of the IL-3 helper precursor cell varied between 1 responder cell in 220 and 1 cell in 1353. The frequency of IL-3 producing pHTL appear to be somewhat lower than those found previously for IL-2-producing pHTL. The authors, however, suspect that the true frequency of IL-3 producing pHTL is approximately 2-fold greater and has been underestimated because of the relative insensitivity of the IL-3 assay to the lowest levels of IL-3 produced in LD conditions. By assaying the supernatants of these short-term cultures for both IL-2 and IL-3, it could be shown that essentially all wells that produce IL-3 also produce IL-2. The amount of IL-2 produced in any individual well correlates strongly with the IL-3 production in the same well. These data suggest that both lymphokines are produced by a single class of T cell.

Determination of the Frequency of Proliferating T Cell Precursors (PTL-p) and CTL Precursors (CTL-p)

Before the LDA was used to estimate the frequency of CTL-p, the frequency of antigen-specific responding cells had been difficult to assess. The methods used to estimate the frequencies of the CTL-p's included the incorporation of ^{3}H-TdR into DNA (36), removal of cells from the circulating pool (37) or the ability of cells to form virus plaques (38). In 1976, Skinner and Marbrook introduced the use of LDA to determine th frequency of CTL-p. Using a rather crude system of dimples in the base of an acrylamide vessel to segregate the

effector cell clones, they determined the frequency of precursor cells in a CBA spleen able to generate a cytotoxic response against DBA mastocytoma cells to be 1 per 1700 spleen cells (30).

A value for the frequency in a cell population of CTL-p specific for a given determinant would be more significant if it could be compared with a value for the total number of CTL-p present. Wilson et al. described a LDA of all CTL-p, i.e., an LDA system that assays all members of the functional CTL-p subclass regardless of antigenic specificity (48). In order to bypass specific antigen requirements at the CTL-p triggering step, Con A was used to polyclonally activate the CBA spleen or thymus cells which served as the responders. PHA was then used in conjunction with the tumor target P815, so as to bypass the specificity of CTL-target binding. In the spleen, approximately 1 spleen cell in 8.3 was found to be a CTL-p, and in the thymus, 1 cell in 29. These results demonstrate that it is possible through the use of LDA to estimate the total number of functional T cells of the cytotoxic class in developing T cell populations.

The proliferative response of T cell populations to the mitogens phytohemagglutinin (PHA) and concanavalin A (Con A) in bulk culture has been widely used as a general index of T cell function. However, in bulk culture assays, the results depend on the balance and interaction of helper, suppressor and accessory cells, as well as the number of T cells initially stimulated to divide. The LDA offers an alternative to bulk assays and allows one to determine the frequency of the T cell precursor of the proliferative response, i.e., the clonal

response is determined only by the presence or absence of the PTL-p. The PTL-p should include samples of T cells of the helper, suppressor and cytotoxic lineages (49).

To enumerate the T cells capable of responding to Con A stimulation by proliferation, mouse spleen or thymus cells were cultured in LD with Con A, supernate from Con A stimulated rat spleen cells and irradiated mouse spleen cells as fillers. ^{3}H-TdR uptake by the microcultures was used as an indication of proliferation, and cultures were defined as positive when ^{3}H-TdR incorporation exceeded the mean control uptake by 3 standard deviations. Seventy-to-one hundred percent of the individual T cells cultured in the presence of Con A could give rise to clones of progeny cells. The clonal progeny were found to contain Lyt $1^{+}2^{-}3^{-}$ and Lyt $1^{+}2^{+}3^{+}$ T cells. The PTL-p population also contained CTL as determined by the ^{111}In-release method, and every clone found positive by the CTL assay had also been positive for proliferation.

There are several possible applications for the high cloning efficiency (70-100%) of this LDA system: enumerate the number of T cells capable of expansion (as above), functional analysis of a selection of expanded T cells, and use the cloned microcultures as a starting point for permanent T cell lines.

Medullary and cortical thymocytes can be separated based on their ability to bind peanut agglutinin (PNA), i.e., medullary are PNA^{-} and cortical are PNA^{+}. There has been much controversy about the immunocompetence of PNA^{+} thymocytes based on the findings of bulk

culture assays. To reinvestigate the immunocompetence of cortical PNA^+ thymocytes, Wei-Feng et al. used a LDA system similar to those mentioned previously to enumerate all CTL-p and all PTL-p in medullary and cortical thymocytes (50). The results of the LDA indicated that the vast majority of both CTL-p and PTL-p were present in the PNA^- or medullary-type thymocytes. A very low frequency of functional precursors was found in the PNA^+ or cortical-type fraction, and this could be attributed to PNA^- contaminants. Thus it appears that all the functional activity of the thymus is concentrated in the PNA^- thymocytes and that the PNA^+ thymocytes are inactive.

Frequency of CTL-p Directed Against Alloantigens

When allogeneic lymphocytes are cultured together, CTL are generated which can be detected by their ability to specifically lyse target cells bearing the sensitizing alloantigens. Several authors have applied LDA to allogeneic MLC and calculated the frequency of alloreactive CTL-p in lymphoid cell suspensions (30). More recently, the in vitro LDA has been used for the determination of the frequencies of CTL-p that are reactive against modified alloantigens and mutant H-2 antigens as well as alloantigens.

Most previous determinations of the frequencies of CTL-p responding to alloantigens involved combinations of responders and stimulators which differed not only at the H-2 locus but also at multiple minor histocompatibility (minor H) loci. Recently, LDA has been used to investigate the role of MHC antigens alone in primary

allogeneic responses in vitro (51). To determine the frequencies of CTL-p responding to H-2 antigens alone, splenocytes from H-2 congenic strains of mice were used as both responder and stimulator cells. (H-2 congenic strains should not differ at any minor-H loci.) These frequencies were then compared to frequencies obtained using strain combinations differing at the minor-H loci in addition to H-2. The results obtained from several combinations of responder and stimulator cells showed no significant differences in the CTL-p frequencies between H-2 congenic and H-2 allogeneic combinations. Thus multiple minor-H differences between responder and stimulator cells do not increase the frequency of CTL-p, and consequently minor-H differences do not appear to contribute significantly to allogeneic responses.

CTL can recognize a wide variety of antigens which cross-react with the antigen originally used to sensitize the CTL. Investigators interested in the cellular basis of cross-reactivity have turned to the examination of CTL clones generated under LD conditions in MLC systems (52). In primary allogeneic MLC populations assayed against specific and third-party targets, three phenotypically distinct sets of CTL clones could be observed, i.e., specific with a mean frequency of 81%, heteroclitic (reactive against third-party but not specific targets) with a frequency of 11% and cross-reactive with a frequency of 8%. To determine if the specificity of all CTL in each clone remained homogeneous throughout the clone's expansion, 22 clones were subcloned and later tested separately for CTL activity against specific and third-party targets. Twenty of the subclones retained their specificity

phenotypes and two changed their specificity phenotypes. The CTL activities of the two clones which changed were very low and near the detection limits of the assay, therefore, it could be concluded that the specificity phenotype of the clones remained stable for at least a short time. Perhaps, more importantly, these results demonstrated that not only was it possible to generate CTL clones in LDA, but it was also possible to subclone the microcultures and later perform functional testing on the subclones.

As shown previously, LDA can be used to study cross-reactivity (52), Teh used the LDA technique to estimate the frequency of CTL-p specific for trinitrophenyl (TNP)-modified alloantigens and also to estimate the degree of cross-reactivity between allodeterminants and TNP-modified self determinants (54). C57BL/6 (B6) H-2^b-nu/+ lymph node cells were stimulated with either unmodified or TNP-modified DBA/2 (D2) H-2^d allogeneic spleen cells. The microcultures were then tested for cytotoxicity against unmodified or TNP-modified targets. The frequency of CTL-p specific for TNP-modified alloantigens was approximately 13% of the CTL-p frequency to unmodified alloantigens, thereby demonstrating that CTL-p to TNP-modified alloantigens do exist in normal mice. To study the cross-reactivity between allo-determinants and TNP-modified self determinants, B6 anti-D2 CTL were generated in microcultures and then tested against either D2 or B6-TNP Con A blasts, 16% of the cytotoxic clones activated by allo-antigens (2786 per 10^6 responders) cross-reacted with TNP-modified self determinants (455 per 10^6 responders).

Functional studies have indicated that T cells with different immunologic activities can be distinguished on the basis of the Lyt phenotype, i.e., Lyt-1, Lyt-2, Lyt-3. It was originally proposed that alloreactive precursor cells that respond in MLC could be divided into 2 different functional subsets: Lyt $1^+2^-3^-$ which includes helper cells for the generation of CTL and Lyt $1^-2^+3^+$ cells that differentiate into CTL (56). Recent studies on the Lyt phenotype of CTL and CTL-p have produced conflicting results. In an attempt to further clarify the controversy by quantitatively investigating the Lyt phenotype of CTL-p, Cerottini and MacDonald described the LDA of alloantigen-reactive T-lymphocytes and the subsequent determination of the Lyt phenotype of the CTL-p reactive against normal and mutant H-2 alloantigens (55).

Nylon-wool purified splenic T cells from B6-Thy 2.1 mice were treated with either anti-Lyt 1.2 or anti-Lyt 2.1 serum plus C, and the remaining viable cells were placed in LD cultures with irradiated DBA/2 stimulators and 2°MLC SN. The lytic activity of each microculture was assayed against P815 targets. The number of CTL-p remaining after treatment was reduced 15- and 100-fold by treatment with anti-Lyt 1.2 and anti-Lyt 2.1 sera, respectively. The effect of treatment with anti-Lyt 1.2 or anti-Lyt 2.1 serum plus C on the frequency of B6 Lyt 2.1 CTL-p directed against mutant K^{bml} alloantigens was also assessed. The reduction in the frequency and absolute numbers of antimutant CTL-p by either antibody treatment was of the same degree as that observed with anti-DBA/2 CTL. In both cases,

treatment of the responders with antiserum directed against Lyt-1 or Lyt-2 alloantigens reduced the absolute number of CTL-p by 85-94% and 98-99%, respectively. These results indicate that the majority of C57BL/6 CTL-p responsive against normal or mutant K^{bml} alloantigens exhibit the Lyt 1^+2^+ phenotype.

Even after the removal of Lyt 2^+ cells in the anti-DBA/2 system, many PTL-p could still be observed. These PTL-p yielded Lyt 1^+2^- progeny which were unable to directly lyse either P85 or DBA/2 blasts, but could mediate PHA-dependent lysis of P815 targets. It appears then, that alloreactive T cells can be subdivided into at least 2 subsets: Lyt 1^+2^+ precursors of specific CTL and Lyt 1^+2^- precursors of effectors which can mediate lectin-dependent lysis.

Using LDA, Goronzy et al. compared the CTL-p frequencies determined in polyclonally (Con A) activated T cells with those determined in alloantigen-activated T cells, and found the CTL-p frequencies to be similar (53). However, whereas the alloantigen-activated T cells contained only a single type of CTL-p, the polyclonally activated T cells showed an additional CTL-p of lower frequency. At higher concentrations in the polyclonally activated T cells, the frequent CTL-p were suppressed, while even at very high cell concentrations, inhibition of the rare CTL-p could not be demonstrated. Analysis of the CTL-p frequencies based on Lyt phenotypes revealed in the alloantigen-activated T cells a single CTL-p whose frequency depended on the Lyt phenotype of the T cell population placed in the LD. Lyt-1 T cells appeared to be completely devoid of CTL-p whereas

Lyt-23 T cells were only slightly depleted of CTL-p. In the polyclonally activated T cells, the Lyt-1 cells were also depleted of CTL-p cells. In the Lyt-23 cells, two populations were found whose frequencies were similar to those of unselected T cells. The results tend to suggest that the majority of the CTL-p are of the Lyt-123 phenotype and appear within the frequent set of CTL-p, whereas the minority are of the Lyt-23 phenotype and are within the infrequent set of CTL-p.

Effects of Priming, Irradiation, BUdR Treatment on Frequency of CTL-p

Standard proliferation assays have been used in the past to analyze the response of lymphocytes sensitized in vitro, however, the proliferative response can vary widely for the same individual. The LDA technique can be used to estimate the frequency of antigen-reactive cells (ARC) sensitized in vitro and provides more reproducible results than the proliferation assays. Gebel et al. used LDA to estimate the frequency of human peripheral blood T lymphocytes (PBL) that proliferate in response to in vitro immunization with keyhole limpet hemocyanin (KLH) (57). The ARC frequency of lymphocytes after primary immunization with KLH ranged from 1:23,800 to 1:52,631. In some experiments, in vitro KLH-primed lymphocytes were rechallenged in secondary LDA culture with KLH. The ARC frequency increased from 7- to 20-fold from primary to secondary culture, i.e., the ARC frequency in secondary culture increased to 1:123 to 1:7247.

The effects of immunization upon the frequencies of CTL-p have been assessed in another type of LDA. The precursor frequencies and

the regulation of 2,4,6-trinitrophenyl (TNP) specific CTL-p in unimmunized and immunized T cell populations were studied using LDA (63). Normal lymph node or spleen cells or draining LN cells from mice sensitized on the abdominal skin with 2,4,6-trinitrochlorobenzene (TNCB) or injected under the abdominal skin with TNP-coupled cells and spleen cells primed with TNP-coupled cells in vitro, were stimulated with Con A and then plated in LD culture. The microcultures were then tested for cytotoxic activity on normal and TNP-coupled syngeneic targets. Normal LN cells contained three CTL-p sets of graded frequencies, the more frequent sets of CTL-p were subject to a suppressive mechanism, whereas the rare set wasn't suppressed. Normal spleen cells, however, contained only a single, rare CTL-p set. Using two different in vivo immunization protocols and primary in vitro stimulation, three independently regulated sets of CTL-p with frequencies similar or identical to the CTL-p frequencies in normal LN cells could be found early (1-2 days) after antigen priming in LN as well as the spleen. Later after priming (3 days), a change to a single non-suppressed precursor set equal in frequency to the most frequent set in normal LN could be demonstrated in both spleen and LN. In contrast to normal LN, the frequent CTL-p set was not suppressed in immune LN. In normal spleen cells, only one rare set of CTL-p could be observed. The appearance of more frequent sets of CTL-p was also observed in the spleens of mice pretreated with cyclophosphamide. Release from suppression can, therefore, be achieved by in vivo and in vitro priming with antigen and by cyclophosphamide pretreatment.

These data would suggest the possibility that the main feature of cytotoxicity generation may be the release of CTL-p from suppression, rather than from clonal expansion of CTL-p.

Frequency of Viral CTL-p

The in vitro limiting dilution analysis technique has been used mainly in the studies of only four viral systems, i.e., influenza, MSV-MLV (murine sarcoma-leukemia virus complex), rabies and herpes simplex. This technique has been used to estimate the frequencies of viral specific CTL-p, to determine the antigenic specificities of the CTL-p's and their clonal progeny and in some cases to further characterize the phenotype of the CTL's.

The priming of mice with MSV-MLV complex results in the production of tumors which will regress spontaneously in vivo, and the lymphocytes from such animals can be restimulated in vitro by syngeneic MLV (Moloney leukemia virus)-induced tumor cells (23, 27). Brunner, MacDonald, and Cerottini used a limiting dilution mixed leukocyte-tumor cell microculture to estimate the frequencies of CTL-p in BALB/c and C57BL/6 regressor spleens reactive against syngeneic Moloney leukemia virus-induced lymphoma cells (4). Various numbers of responding C57BL/6 or BALB/c regressor spleen cells were cultured for 7 days with supernate from secondary mixed leukocyte culture (2°MLC SN) irradiated syngeneic spleen cells, and irradiated lymphoma cells which served as a source of antigen. The CTL activity of these microcultures was then assayed against syngeneic tumor cells. The

mean CTL-p frequency in C57BL/6 regressor spleens was 1/600 and 1/1300 for BALB/c mice. This represents a 10- to 20-fold increase in CTL-p frequencies over normal spleen CTL-p frequencies. These frequencies were even comparable to those of CTL-p reactive against alloantigens (16). The authors also tested the specificity of the lytic activity of the clonal progeny of the CTL-p reactive against the syngeneic tumor cells. The majority of the clonal microcultures--73% of the C57BL/6 and 87% of BALB/c--were found to lyse syngeneic but no allogeneic tumor cells, thus indicating H-2 restriction. However, a small minority of the positive C57BL/6 microcultures were "double-reactors," i.e., lysed both syngeneic and allogeneic targets equally well. Although there were BALB/c cultures which lysed both targets, none of these clearly appeared to be double-reactors, instead the cultures were found to preferentially lyse the syngeneic targets. CTL-p with specificity for the allogeneic targets only were either absent entirely or present at low frequencies, as in only one experiment, which involved C57BL/6 cultures, were allogeneic targets preferentially lysed. These results tend to indicate some heterogeneity at the clonal level.

In a later paper by these same authors, limiting dilution analysis was used to compare the frequency of CTL-p, at the onset of tumor regression, in the cells infiltrating the MSV-MLV induced tumors, peripheral blood lymphocytes (PBL), and spleen cells C57BL/6 mice previously injected with MSV-MLV complex (5). The authors found a 5- to 10-fold enrichment in the frequency of CTL-p present in the

lymphoid cells recovered from the tumor as compared to the blood or spleen cells. Next, the proportion of cells in the spleen, PBL, and tumor expressing Thy-1 and Lyt-2 surface markers was determined. In order to quantitate the T cell subsets, monoclonal antibodies directed against Thy-1 or Lyt-2 alloantigens followed by fluoresceinated anti-immunoglobulin were used to stain lymphoid cells from each tissue. Samples were then analyzed by flow microfluorimetry. The CTL-p frequencies previously obtained for the spleen, blood and tumor-derived cells were then recalculated based on the relative T cell content of each tissue. As compared with the spleen cells or PBL's, the CTL-p in the tumor tissue were 5- to 8-fold enriched in Thy-1^+ cells and 4- to 6-fold enriched in Lyt-2^+ cells.

CTL populations derived from the tumors and obtained by limiting dilution techniques were expanded by stimulation with irradiated syngeneic tumor cells in cultures containing irradiated syngeneic spleen cells and 2°MLC SN. These expanded populations were then tested for cytolytic activity and specificity against four different tumor target types. The five tumor-derived clones showed specificity for the syngeneic MLV-derived tumor target, i.e., showed strong cytolytic activity, and lacked cytolytic activity against MLV-induced allogeneic targets or against syngeneic and allogeneic unrelated, i.e., non-MLV-derived, tumor targets. Thus the H-2 restricted cytolytic activity previously seen in MLV-specific CTL's derived from C57BL/6 regressor spleens is also found in tumor-derived C57BL/6 CTL's.

In a 1978 paper by Komatsu et al., the <u>in vitro</u> limiting dilution analysis was used to determine the frequency of CTL-p generated in a

primary response against influenza A-infected cells (12). The responder cells were spleen cells taken from CBA/J mice. The stimulator cells were taken from the spleens of cyclophosphamide-treated mice and then infected with type A/Jap virus. The frequency of CTL-p reactive against influenza A-infected syngeneic targets was 1.9×10^{-5}. To further characterize the cells involved in the antiviral response, CBA spleen cells and infected stimulators were set up in bulk cultures. After 2 days in culture, the cells from the cultures were treated with anti-Thy 1 and complement. The cytotoxicity against virally infected targets was almost completely abrogated.

Using limiting dilution analysis, Owen et al. examined the frequency and specificity of memory CTL's in the spleens of BALB/c mice primed with PR8 influenza A virus (19). The authors studied the possible requirement for helper T cells in the differentiation of the CTL-p by adding either Con A supernate or purified IL-2 (interleukin 2 or T cell growth factor). The results were variable, i.e., in some cases the presence of Con A SN had little effect on the frequency of CTL-p and in others the addition of Con A SN resulted in a significant increase. Therefore, to avoid variability due possibly to the different proportions of CTL-p and helper T cells in the responding spleens and to insure that the CTL-p was the limiting cell, either Con A SN or IL-2 was added to all other cultures. The frequency of memory CTL in mice primed with PR8 and restimulated <u>in vitro</u> with the homologous virus ranged from 1 in 1600 to 1 in 4800. The frequency of CTL's capable of lysing syngeneic targets infected with the heterologous HK

virus were from 1 in 1700 to 1 in 4700. Therefore, at least 80% of the PR8 specific CTL's have lytic activity against both PR8- and HK-infected syngeneic targets. It was possible to further analyze the specificity of these CTL's by expanding the positive cultures and then testing these clones against syngeneic targets infected with different A strain viruses. (To expand the cultures, cells from positive cultures were transferred to conical centrifuge tubes and cultured for 7 days in the presence of Con A supernate stimulators, and α-methyl mannoside. The cultures were then split and the aliquots tested against the various targets.) In most instances, the targets infected with the heterologous viruses were lysed to a similar extent as the homologously infected targets. These results indicate that the majority of PR8-primed specific CTL's are cross-reactive.

The addition of anti-Thy 1.2 and complement to the cultures immediately prior to the assay resulted in an abrogation of all virus-specific lysis, and the majority of the cultures were found to preferentially lyse virally infected syngeneic targets rather than infected allogeneic targets. These results argue against ADCC and NK (natural killer cell) killing and instead, indicate that the effector is a T cell and that the response is H-2 restricted.

Askonas et al. investigated whether the main priming effect of influenza virus infection by the intranasal or intraperitoneal route is to induce memory CTL-p or to enhance T-helper activity (2). Using the in vitro limiting dilution assay, the frequency of CTL-p in the spleens of normal mice was compared to that of mice primed with influenza

virus. Spleen cells from primed or unprimed BALB/c mice were cultured in the presence of stimulators (spleen cells infected with influenza virus and irradiated), irradiated T-helper cells from primed donors, and irradiated normal spleen cells used as fillers. Infection with influenza virus increased at least 10-fold the level of splenic memory CTL-p in comparison with normal mice. Both routes of infection resulted in frequencies of $1\text{-}2 \times 10^{-4}$ CTL-p in the spleen. However, without the addition of irradiated T-helper cells from primed mice or the supernate of Con A-simulated rat spleen cells, maximal expression of CTL-p was not achieved. The frequency plot of responder wells does not go through the origin without the addition of additional T-helper cells or Con A supernate, thus T-helper cells are limiting after infection.

The specificity of the T-helper cells primed by influenza infection was investigated to determine if the T-helper cells would be cross-reactive for influenza A viruses as were the CTL-p. Irradiated spleen cells from an A/x-31 primed donor were tested for the ability to enhance the induction of cross-reactive cytotoxic T cells by stimulating with either A/x-31 or A/USSR infected stimulators and testing for cytotoxicity against A/Jap infected targets. When A/x-31 primed responder spleen cells were stimulated with A/x-31 infected stimulators, almost all of the cultures gave a positive cytotoxic response in the presence of additional irradiated A/x-31 primed T-helper cells. The same T-helper population with the same number of CTL-p stimulated A/USSR resulted in 75% of the cultures having cytotoxic activity

against the A/Jap infected targets. Thus the T-helper cells from A/x-31 primed donors do help the induction of cytotoxic T cells by A/USSR virus and so at least a part of the influenza-primed T-helper cells are cross-reactive for the different types of influenza A virus.

Ashman investigated the long-term persistence of influenza A-reactive cytotoxic T-memory cells and of the T-helper cells that are necessary for the generation of virus-specific CTL responses (1). Ashman found that CTL's from two-year-old influenza A/Jap primed mice showed a weaker response against influenza-infected targets when compared to 6-8 week old controls. To better define this defect in aged mice, helper cells from young and old mice were assayed for their ability to promote the generation of influenza-specific CTL in unprimed spleen cells stimulated with infected syngeneic cells. The T-helper cells in aged mice were not defective in their ability to present help. The frequency of memory CTL-p was then determined by in vitro limiting dilution analysis. In aged mice, the CTL-p frequency was 1/15,000 spleen cells and in young mice was 1/8,000. Thus both T-helpers and CTL's are present and active in aged influenza-primed mice, but the frequency of memory CTL-p declines with ages resulting in a weakened immune response.

Reddehase et al. have used three different in vitro limiting dilution protocols to estimate the frequency and determine the activation of CTL and CTL-p present in vivo after rabies infection and the response to allogeneic cells was used as the control (24). Lymph node cells of unprimed C57BL/6 mice were cultured in the presence of

antigen-presenting stimulator cells, i.e., allogeneic DBA/2 spleen cells or rabies-infected fibrosarcoma cells, and IL-2. The frequency of alloantigen reactive CTL-p was 1/100-1/1000 cells, while the frequency of rabies-specific CTL-p was 0.5-3 precursors per popliteal lymph node (1/27,700; 1-6 per 10^6).

In the second protocol, mice were primed in vivo by footpad injection of either mitomycin-C-treated allogeneic DBA/2 cells or rabies virus. Lymph node cells from primed mice were cultured in vitro with antigen-presenting stimulators and IL-2. This protocol allows for secondary exposure in vitro of CTL-p to antigen and to further interleukin-mediated expansion. In the allogeneic control, the relative frequency remains essentially unchanged after priming 1/197. However, in the rabies system there is a 100-fold increase in the frequency 1/16,270 (52-71 per 10^6).

The third protocol is based on the idea that exposure of the CTL-p to antigen results in the differentiation of the IL-2 insensitive CTL-p to the IL-2 sensitive IL-CTL-p, i.e., the IL-CTL-p expresses a receptor for IL-2 and can be further expanded in the presence of IL-2 without antigen. In vivo primed antigen-reactive cells were cultured in vitro in the presence of IL-2 alone, so that the frequencies of IL-CTL-p could be determined by the in vitro limiting dilution analysis. In the allogeneic system, the frequency of IL-CTL-p is not significantly different from the frequency obtained after secondary exposure in vitro to antigen, 1/290. However, in the rabies system, the IL-CTL-p frequency was 1/60,990 (14-19 per 10^6) which indicated

that most in vivo sensitized CTL-p fail to mature to IL-CTL-p in vitro to produce active effectors.

Rouse et al. established a limiting dilution assay to estimate the frequency of CTL-p reactive against HSV-1 (26). CBA/CA mice were primed by intraperitoneal (i.p.) injection of HSV-1 infectious virus or of heat inactivated virus. Spleen or lymph node cells were used as responders 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 was found to be less than 1/250,000. The frequency of positive microcultures in spleen cells from HSV-1 primed mice was 1/3,500-1/15,670. It was found to be necessary to add TCGF to the cultures, in order to obtain data giving a straight line and thus indicating a single limiting cell; however, the frequencies of CTL-p were not significantly increased when T-helper cells (irradiated splenocytes from primed animals) were added to the cultures. This is in contrast to Askonas et al. who found maximal expression of CTL-p only in the presence of additional T-helper cells, but Askonas et al. also reported that Con A supernate could be used instead of additional T-helpers (2). To test the specificity of the cultures, the microcultures were split and aliquots were assayed against infected syngeneic targets, uninfected syngeneic targets, infected allogeneic targets, or vaccinia-infected syngeneic targets. The frequencies in the controls

were 10- to 50-fold less than against the syngeneic infected targets. In assays against infected and uninfected targets, some double positives were found, similar to the findings of Brunner et al. (4). The majority of cultures were found to lyse only syngeneic infected targets as opposed to allogeneic infected targets. Cytotoxicity was greatly reduced by treatment of the cultures with anti-Lyt 2 sera. These results suggest that the effector cell is a T lymphocyte. To characterize the phenotype of the HSV-1 reactive CTL-p, responder cells were enriched for T cells by passage over nylon wool and then treated with either anti-Thy 1.2, anti-Lyt 1.1 or anti-Lyt 2.1 plus complement. Treatment of the precursors with any of these antibodies plus complement almost completely eliminated the response, thus indicating that the phenotype of the CTL-p is Lyt $1^{+}2^{+}$. Immunization with inactivated virus resulted in frequencies substantially lower than those obtained with live virus.

Conclusion

The utility of the in vitro limiting dilution analysis has been shown to go beyond the determination of the frequencies of rare cells:

1. LDA provides a highly efficient method of cloning.
2. LDA makes it feasible to screen large numbers of clones for specificity and function, particularly the semiautomated LDA.
3. Clones can be screened and selected for further expansion. These subclones can then be split and assayed for a variety of functions simultaneously.

4. Cloned microcultures could possibly be used as a starting point for permanent cell lines.

In the future, researchers will find more and different applications for the in vitro limiting dilution analysis.

CHAPTER II

METHODS AND MATERIALS

Cells and Viruses Used

Strain Ls cells ($H\text{-}2^k$) were obtained from Johannes-Gutenburg Unversitat, Mainz, W. Germany. Strain BALB/c 3T3 clone A_{31} ($H\text{-}2^d$) was obtained from Dr. Ray Tennant, Biology Division, Oak Ridge National Laboratory. Strain EL-4 thymoma cells ($H\text{-}2^b$) were obtained from Dr. John Farrar, National Institute of Dental Research. Strain HEp-2 cells were obtained from Flow Laboratories.

Strain Ls cells and strain BALB/c 3T3 clone A_{31} (abbreviated A_{31}) were grown in McCoy's SA medium supplemented with 10% donor calf serum (DCS). Strain EL-4 cells were grown in RPMI 1640 containing 10% fetal calf serum (FCS). Cell strain HEp-2 was cultured in McCoy's SA medium containing 10% donor calf serum. HSV-1 strain KOS was grown in HEp-2 cells by infecting cells at low multiplicity of infection (m.o.i.) and harvesting cell-associated virus as described previously (13). HSV-2 strain 186 was also grown in HEp-2 cells as described for HSV-1 strain KOS. The viral stocks used had infectivity titers ranging from 10^8 to 3×10^8 plaque forming units (p.f.u.) per milliliter. To UV-inactivate the virus, 10^8 p.f.u. of virus was exposed to UV light provided by two G15T8 germicidal bulbs (General Electric Co., Waterford, N.Y.) at a distance of 5 cm for 2 minutes.

Mouse Immunization and Preparation of Spleen Cells

C3H/HEJ mice ($H\text{-}2^k$) were obtained from the University of Tennessee Medical Research Center or from Cumberland View Farms, Clinton, Tennessee. Some groups of animals were infected intraperitoneally with 0.1 ml inoculum containing 10^7 p.f.u. of infectious HSV-1 KOS. Other animals were first infected intraperitoneally with 0.1 ml inoculum containing 10^3 p.f.u. UV-inactivated HSV-2 186 and three weeks subsequent to the initial injection were given 10^3 p.f.u. of infectious virus followed four weeks later by 10^4 p.f.u. of infectious virus. At least four weeks following the final infection, mice were sacrificed by cervical dislocation and their spleens aseptically removed.

Single cell suspensions were prepared by teasing spleen cells through 80-mesh sieves into Hank's balanced salt solution (HBSS). After one wash, erythrocytes were lysed by brief exposure at 37°C to 0.85% NH_4Cl in .17 M Tris buffer (pH 7.2). Cells were then washed in HBSS twice more, the cell aggregates were removed, and the viable cells were counted.

Responder spleen cells were suspended in RPMI 1640 containing 10% FCS (Gibco, Grand Island Biological Co., Grand Island, N.Y.), 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 g/ml), and 5×10^{-5} M 2-mercaptoethanol.

For in vitro culture under limiting dilution conditions, varying responder cell numbers (4×10^4 to 2.5×10^3 cell/well) were added in 0.1 ml of medium to U-bottomed wells of 96 well microtiter plates (Linbro). Twenty-four replicates were used for each responder cell

number. To the responder cells were added 2×10^5 stimulator cells in 0.1 ml of culture medium containing rat T cell growth factor and α-methyl mannoside. After cell addition, the microplates were sealed with plastic tape, and the cultures were incubated in a humidified CO_2 (5%) atmosphere for 7 days. At the end of the 7-day incubation period, the microcultures were tested for cytotoxicity.

To prepare the stimulator cell populations, normal spleen cells were infected for 2 hours with UV-irradiated HSV-1 KOS or HSV-2 (approximately 1 p.f.u./cell before UV irradiation) in 50 ml centrifuge tubes. After infection, cells were irradiated (2000R), washed and resuspended in culture medium containing 10% (final concentration T cell growth factor (TCGF) and 50 mM (final concentration) α-methyl mannoside. In some experiments, these virus-infected normal spleen cells were used as a source of antigen; and in other experiments, uninfected normal spleen cells (irradiated) were used as stimulators.

Preparation of T Cell Growth Factor (TCGF) Containing Supernatant Fluid

Spleen cells were isolated from Lewis rats (Charles River, Wilmington, MA) and suspended at a concentration of 1.25×10^6/ml in RPMI 1640 and 10% FCS. Aliquots of 20 ml suspension were placed in plastic 100 mm petri dishes and stimulated with 3.0 g/ml of concanavalin A (Con A) (Pharmacia, Uppsala, Sweden). Cells were cultured for 25 hours at 37°C in a humidified CO_2 (5%) incubator, after which the supernatant fluids were harvested and freed of cells and cellular debris by centrifugation at 200 xg for 30 minutes. Con A was

neutralized by adding 50 mM of α-methyl mannoside or removed by absorption to a Sephadex G-25 (10). Supernatant fluids were then sterilized by filtration through a 0.2 micrometer membrane filter (Nalgene), aliquoted and stored at -70°C.

Antiserum and Complement Treatment

In some experiments, at the end of the 7 day incubation period, the microcultures were split into three subcultures with a multichannel micropipette (Flow Laboratories, McLean, V.A.). After centrifugation of the plates at 1000 rpm for 5 minutes, the medium was flicked off and the subcultures washed twice with HBSS. To deplete the culture of Lyt 2^+ or Lyt 1^+ cells, 2 subcultures were incubated with a 1:30 dilution of either anti-Lyt 2.1 or anti-Lyt 1.1 obtained from Cedarlane Laboratories, Wesbury, N.Y. for 45 minutes at 0°C followed by 30 minutes at 37°C with a 1:10 dilution of rabbit low mouse toxicity complement (Cedarlane Labs). The third subculture was treated with complement alone. All subcultures were then tested for cytotoxicity.

Cytotoxicity Assay

At the end of the incubation period, cultures were split into two subcultures using a multichannel micropipette. The microplates were centrifuged at 1000 rpm for 5 minutes to pellet the cells. The supernatant fluids were flicked off and replaced with 1000 target cells suspended in 150 µl of RPMI 1640 plus 5% DCS. Medium control wells received target cells only. The total releasable ^{51}Cr was obtained by exposing target cells to 1% Triton X-100. To prepare the target cells,

Ls cells were infected with HSV-1 KOS or HSV-2 186 at a high multiplicity (5-10 p.f.u./cell) for 150 minutes at 37°C. In a few experiments, target cells were infected overnight with HSV-2. After the first 30 minutes, 200 µci/ml of $Na_2\ ^{51}CrO_4$ were added (New England Nuclear Corp., Boston, M.A.). Two hours later, the cells were washed twice with DCS and viable cells counted by the Trypan blue exclusion test. Each microwell received 1000 target cells, plates were centrifuged at 1000 rpm for 5 minutes, and then the cultures were incubated at 37°C for 6 hours. In some assays involving the HSV-2 target system, cultures were incubated for 4, 5, 6, 7 or 8 hours. At the end of the 6 hour incubation, 100 µl of supernatant were removed for the measurement of radioactivity. Cultures in which the radioactivity exceeded by 3 standard deviations (SD) that of the medium control or that in cultures with stimulators but without responders were considered as positive.

Statistical Analysis

The Poisson distribution was used to evaluate the data and to estimate the frequency of CTL-precursors. If a cell type being titrated in limiting dilution microcultures is randomly and independently distributed among a number of wells, then the number of precursors per well should follow the Poisson distribution:

$$Fi = \frac{e^{-u}}{i!} \cdot u^{i} \qquad (1)$$

where Fi is the expected fraction of wells with I precursors when there are a mean of u precursors per well. If all the wells with one or more precursors are scored as positive, then Fo which is the expected fraction of negative wells equals e^{-u},

$$Fo = e^{-u} \quad (2)$$

The mean number of precursors per well (u) is equal to the number of added responder cells (N) times the precursor frequency, f. Therefore, the number of added responders (N) should be proportional to -1n (Fo). When there is a mean of one precursor per well (u = 1), the $Fo = e^{-1} = 0.368$. At this point, the precursor frequency (f) should be 1/N (35).

The number of negative microcultures as a fraction of all cultures was plotted on a logarithmic ordinate against the responding cell number on the linear X-axis. The method of least squares was used to determine a regression line of best fit (32). Frequencies were calculated from the slope of the regression line. The r^2 values and Y-intercepts were determined.

CHAPTER III

RESULTS

Demonstration of Anti-HSV-1 Cytotoxic T Lymphocytes (CTL) in HSV-1-Immune Splenocytes Homologously and Heterologously Stimulated In Vitro

When spleen cells from normal mice were cultured under limiting dilution conditions with either HSV-1- or HSV-2-infected stimulator cells and TCGF, the frequency of positive cultures was too low to compute statistically. This was true even when 4×10^4 lymphocytes were used as responders (maximum cell number compatible with culture system). Thus, the frequency of anti-HSV-1 CTL-p in normal spleen cell populations was estimated to be lower than 1/250,000 (the limit of sensitivity of the test system).

Following stimulation of spleen cells from HSV-1-immune mice (4 weeks or more postinfection) with homologously infected, i.e., HSV-1-infected, stimulators, positive microcultures resulted. Although there were variation between experiments, the frequency of anti-HSV-1 CTL-p in nucleated spleen cells was between 1/6519 and 1/15632 (Table I) (Figures I and II). In Figure II, the results of a representative experiment are shown demonstrating that the levels of specific cytotoxicity in cultures recorded as positive varied markedly but were as high as 75% specific lysis. In order to better define the cross-reactivity of HSV-CTL-p, the same responder population of splenocytes from HSV-1-immune mice was heterologously stimulated in vitro, i.e., exposure to strain HSV-1 in vivo and stimulation

Table I. Frequency of anti-HSV-1 CTL-p. HSV-1 and HSV-2 immune splenocyte populations were placed in limiting dilution microcultures with either HSV-1 or HSV-2 infected viral stimulators and TCGF. After 7 days of stimulation, the microcultures were assayed against 1000 ^{51}Cr labelled HSV-1 infected syngeneic Ls cells. Poisson distribution was used to evaluate the data and estimate the frequency of anti-HSV-1 CTL-p present in the responder populations.

Experiment	Effector : Stimulator	Frequency	y-intercept	r^2
1	HSV-1 : HSV-1	1/6519	1.01	.99
	HSV-1 : HSV-2	1/23088	1.15	.96
	HSV-2 : HSV-1	1/14403	1.05	.96
	HSV-2 : HSV-2	1/17172	1.05	.98
2	HSV-1 : HSV-1	1/11762	.98	.98
	HSV-1 : HSV-2	1/22045	1.05	.92
	HSV-2 : HSV-1	1/12784	1.01	.97
	HSV-2 : HSV-2	1/13664	1.06	.98
3	HSV-1 : HSV-1	1/7788	1.02	.97
	HSV-1 : HSV-2	1/27674	1.10	.97
	HSV-2 : HSV-1	1/15203	1.07	.96
	HSV-2 : HSV-2	1/14904	1.08	.98
4	HSV-1 : HSV-1	1/15524	1.05	.98
	HSV-1 : HSV-2	1/28596	.98	.96
	HSV-2 : HSV-1	1/16644	1.05	.97
	HSV-2 : HSV-2	1/18476	1.00	.97
5	HSV-1 : HSV-1	1/15632	1.02	.95
	HSV-1 : HSV-2	1/49196	1.04	.99
	HSV-2 : HSV-1	1/15493	1.08	.96
	HSV-2 : HSV-2	1/17834	1.08	.99

Figure 1. Comparison of the frequencies of CTL-p specific for syngeneic HSV-1 infected Ls cells in nucleated splenocytes from C3H/HEJ mice previously infected with either HSV-1 or HSV-2. The method of least squares was used to determine a regression line of best fit, and frequencies were calculated from the slope of the regression line.

HSV-1-immune splenocytes were stimulated by normal syngeneic irradiated spleen cells and factor (x-x) or HSV-1-infected syngeneic irradiated splenocytes and factor (——). Also shown are the responses of the same population of splenocytes stimulated by HSV-2-infected splenocytes and factor (· —— ·).

In the same experiment, HSV-2-immune splenocytes were stimulated by normal irradiated splenocytes and factor (x —— x), HSV-1-infected spleen cells and factor (--), or HSV-2-infected splenocytes also with factor (·——·).

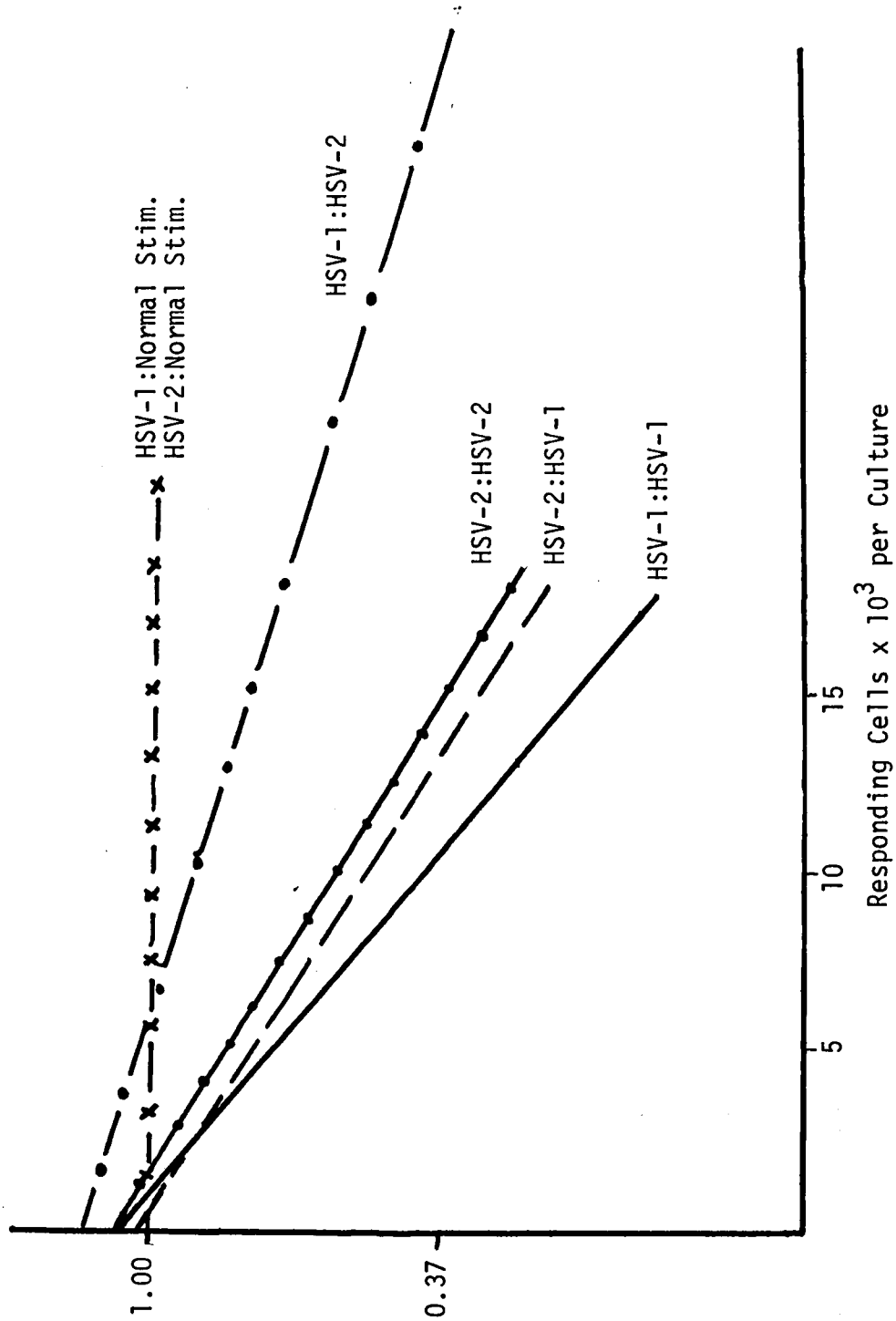
HSV-1:Normal Stim.
HSV-2:Normal Stim.
HSV-1:HSV-2
HSV-2:HSV-2
HSV-2:HSV-1
HSV-1:HSV-1
1.00
0.37
5
10
15
Responding Cells x 10^3 per Culture

Figure 1.

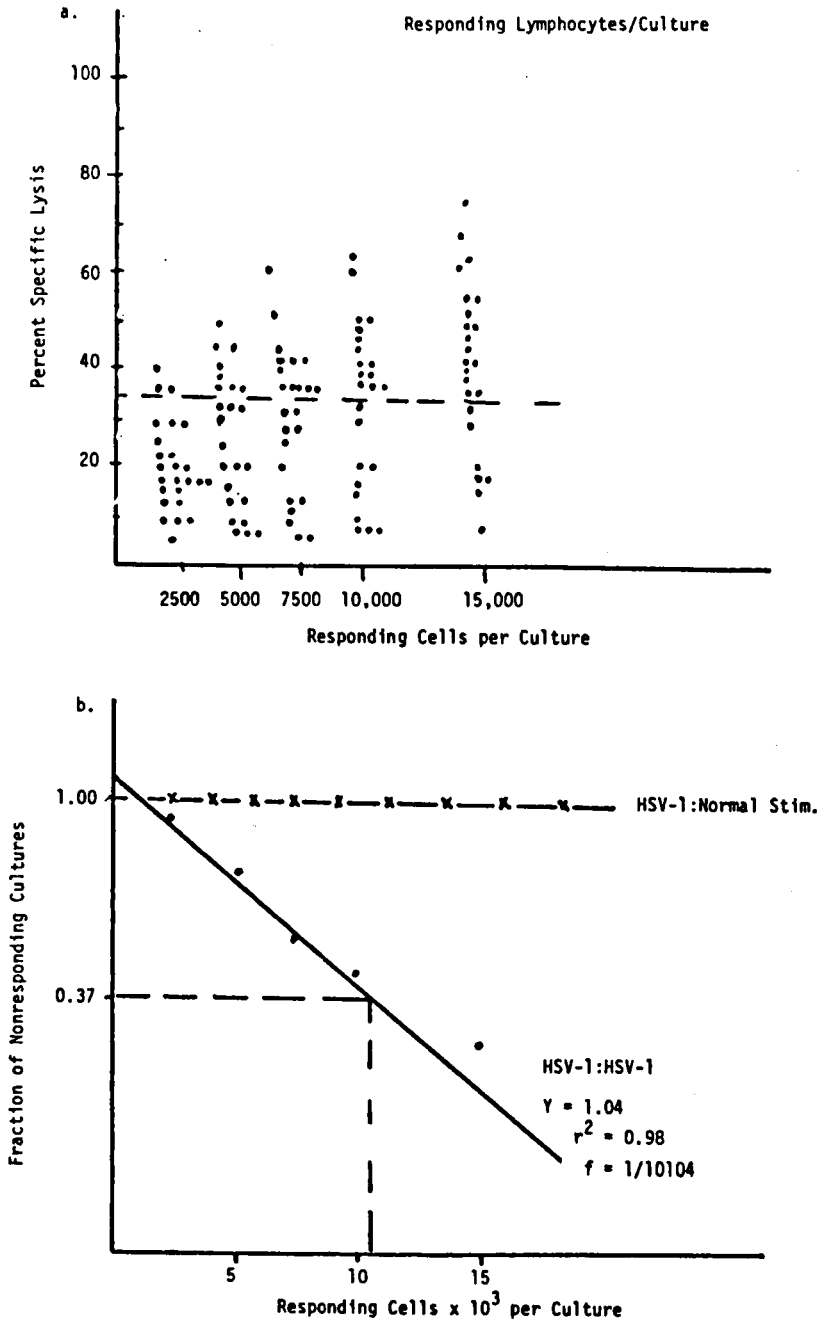

Figure 11. Percent-specific ^{51}Cr release values for each microculture (a). The dashed line represents three standard deviations above the mean of control cultures that contained no responder cells. Minimum estimate of the frequency of CTL-p specific for HSV-1 in HSV-1-immune splenocytes (b). Responding cells were stimulated with either normal syngeneic irradiated spleen cells (x —— x) or HSV-1-infected splenocytes and factor (——).

in vitro with HSV-2, and then tested for cytotoxicity against HSV-1-infected target cells. The frequency of positive cultures was much lower than that of the homologously stimulated cultures ranging from 1/22045-1/49196 (Table I) (Figures I and III). To obtain positive cultures, it was necessary in both the strain combinations to stimulate responder cells with viral antigen, as the frequency was too low to compute in the microcultures containing normal, i.e., uninfected, stimulators and HSV-1-immune splenocytes. Thus the frequency computed either in nonimmune spleen cell populations or in the presence of normal, uninfected stimulators was markedly below that seen in both of the strain combinations of viral stimulated cultures.

Demonstration of Cross-Reactive Anti-HSV-1 CTL in HSV-2-Immune Mice

To analyze the cross-reactivity between HSV-1 and HSV-2, mice were infected in vivo with HSV-2, and the resulting primed spleen cells were then stimulated in vitro with either the homologous or heterologous serotype. The effectors were assayed against HSV-1-infected target cells. The frequency of positive cultures in those responder populations stimulated in vitro with HSV-2 viral antigens was between 1/13664-1/18476 (Table I) (Figures I and IV). Splenocytes from HSV-2-primed mice cultured with HSV-1-infected stimulators gave similar results with frequencies of 1/12784-1/16644 (Table I) (Figures I and V). As before, responders plus normal stimulators and factor did not yield positive cultures thus indicating a need for restimulation in vitro with viral antigen. These results demonstrate

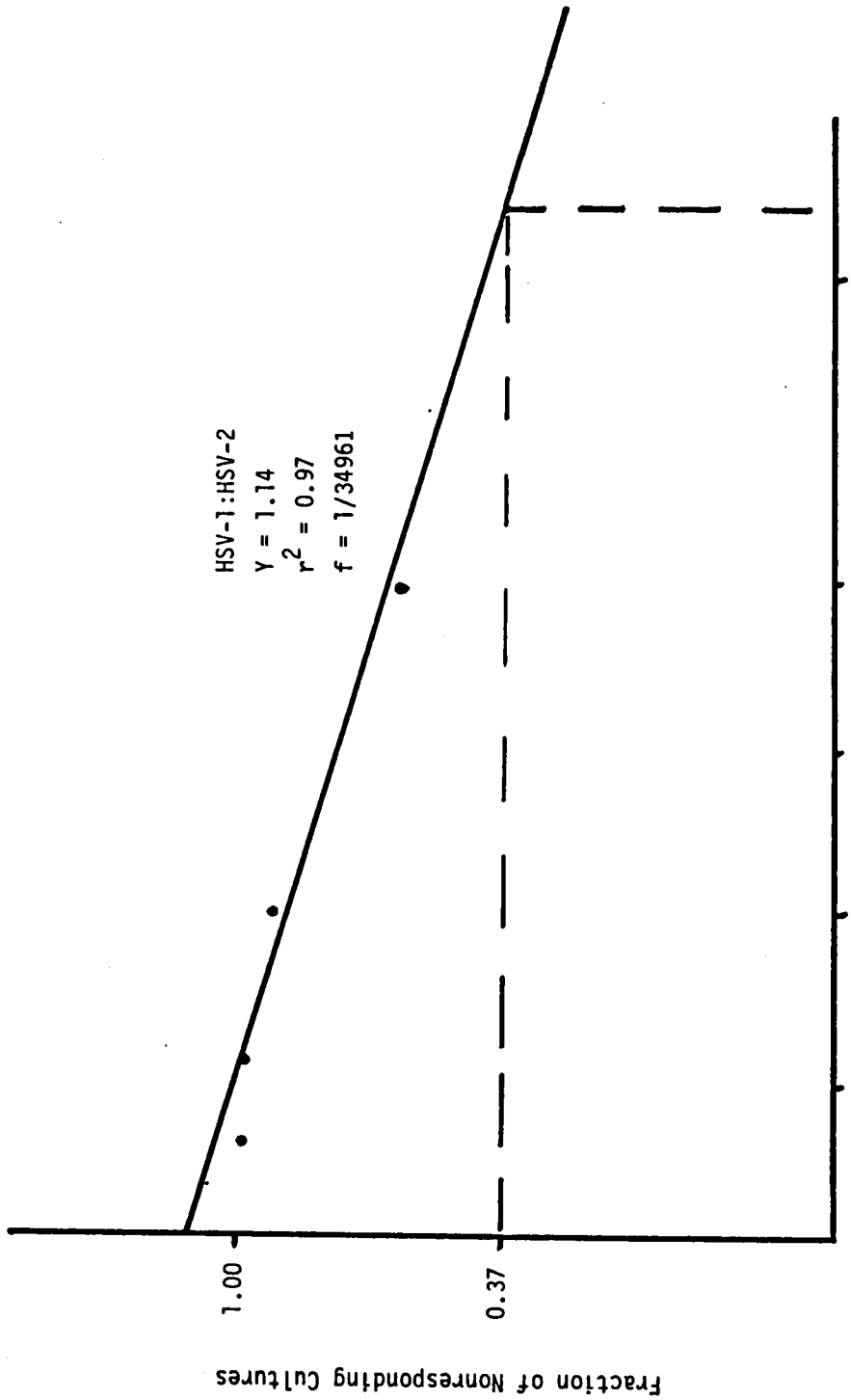

Figure III. Responding HSV-1-immune splenocytes were stimulated with HSV-2-infected syngeneic irradiated spleen cells in the presence of TCGF. The minimum estimate of the frequency of CTL-p specific for syngeneic HSV-1-infected Ls cells was determined by Poisson distribution.

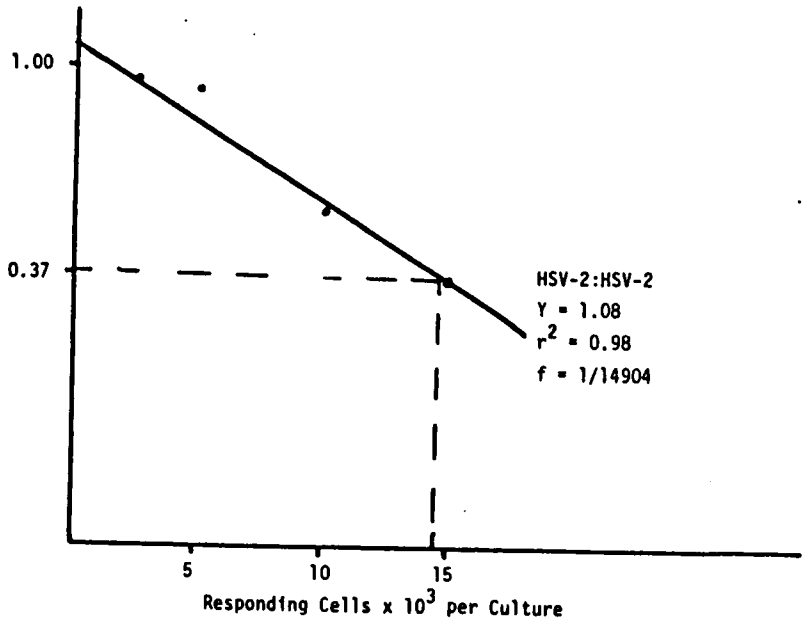

Figure IV. HSV-2-immune splenocytes were stimulated in the presence of TCGF with HSV-2-infected irradiated spleen cells and then tested for cytotoxicity against HSV-1-infected Ls cells. The frequency of CTL-p reactive against HSV-1 was estimated using Poisson statistics.

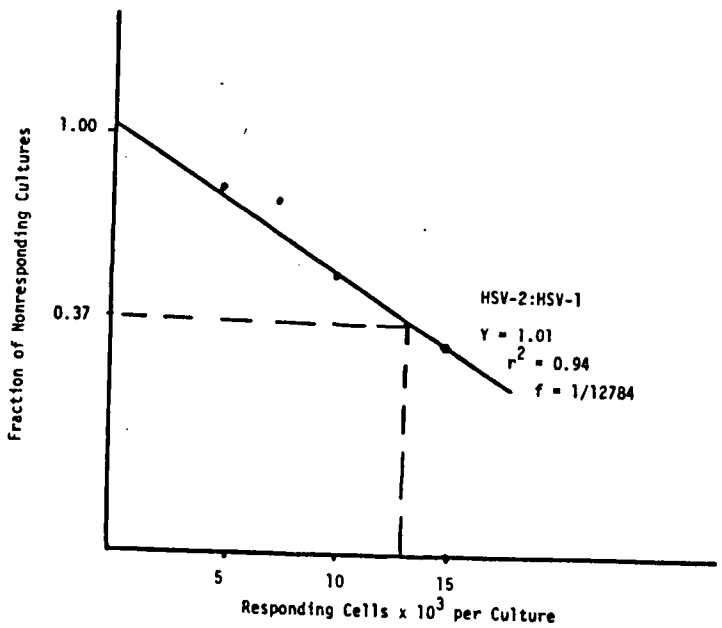

Figure V. HSV-2-immune splenocytes were stimulated in the presence of TCGF with HSV-1-infected splenocytes and tested for cytotoxicity against HSV-1-infected Ls cells.

the presence of cross-reactive CTL-p in both the homologous and heterologous strain combinations is only slightly lower than that of the HSV-1 type-specific CTL-p obtained in the homologous HSV-1 effector:HSV-1 stimulator combination and is higher than the heterologous combination.

Evidence That Detected Cells Were HSV-1 Specific T Cells

For several viral systems, activation of NK cells has been reported to precede the virus specific CTL response, and a number of the methods used to generate CTL's will also generate NK activity (64). It has been demonstrated that NK cells will grow and divide in culture when they are provided TCGF (64). This may result in confusion as to the level of killing that is actually mediated by CTL. However, NK cells are by definition distinct from self-restricted T lymphocytes by their lack of self-restriction. Thus, the test for self-restriction may be used to discriminate between NK cells and T cells.

To assess viral specificity and determine self-restriction, 48 replicates initially plated at 4×10^4 responders per well were cultured for 7 days, the microcultures were then split and alliquots tested against syngeneic infected target cells or uninfected syngeneic targets, or virus infected allogeneic cells. Positive microcultures were observed with the syngeneic infected targets only (Table II). All other targets resulted in levels of lysis well below that defined as positive. These results demonstrate anti-HSV specificity and H-2 or

Table II. Demonstration of self-restriction. Forty-eight microcultures containing HSV-1 or HSV-2 immune responding splenocytes, homologously or heterologously infected stimulators and TCGF were plated at 4×10^4 responders per well. At the end of the 7 day incubation period, microcultures were split and aliquots assayed against infected or uninfected syngeneic Ls target cells or against infected allogeneic target cells.

	Target Cell			
Effector : Stimulator	Ls-HSV-1	Ls	A_{31}-HSV-1	EL-4-HSV-1
HSV-1 : HSV-1	$\frac{48}{48}$	$\frac{0}{48}$	$\frac{0}{48}$	$\frac{0}{48}$
HSV-1 :HSV-2	$\frac{23}{48}$	$\frac{0}{48}$	$\frac{0}{48}$	$\frac{0}{48}$
HSV-2 : HSV-2	$\frac{37}{48}$	$\frac{0}{48}$	$\frac{0}{48}$	$\frac{0}{48}$
HSV-2 : HSV-1	$\frac{40}{48}$	$\frac{0}{48}$	$\frac{0}{48}$	$\frac{0}{48}$

self-restriction, and thus argue against NK killing and instead indicate that the cytotoxic cell is a T cell.

Phenotype Determination of the Effector CTL

T lymphocyte differentiation (Lyt) markers provide an additional means to discriminate between NK and self-restricted T cells. At the end of the seven day culture period and immediately prior to testing for cytotoxicity, microcultures containing the putative CTL-p derived cytolytic effector progeny were split, and aliquots were treated with either anti-Lyt 1.1 or Lyt 2.1 sera plus complement or complement alone. The addition of anti-Lyt 2.1 plus C to the cultures immediately prior to the assay resulted in an abrogation of all virus-specific lysis. However, treatment of the responders with either anti-Lyt 1.1 and C or complement alone did not eliminate cytotoxic activity. Of 48 positive HSV-1 effector:HSV-1 stimulator cultures examined, elimination of cytotoxicity occurred in all 48 microcultures following treatment with anti-Lyt 2.1 sera and C (Table III). Similar results were obtained for other strain combinations: 23 of 23, 41 of 41 and 25 of 25 for HSV-1:HSV-2, HSV-2:HSV-2 and HSV-2:HSV-1, respectively. These results indicate that the effector clonal progeny of the CTL-p were Lyt $1^{-}2^{+}$ cells.

Determination of the Frequency of HSV-2-Specific CTL-p in HSV-2-Immune Spleen Cells and the Frequency of Cross-Reactive Anti-HSV-2 CTL-p in HSV-1-Immune Splenocytes

It was hoped that in the present work it would be possible to demonstrate the presence of and determine the frequency of CTL-p

Table III. Phenotype determination of effector CTL. At the end of the seven day culture period and prior to testing for cytotoxicity, 48 microcultures initially plated at 4×10^4 responders per well were split into three aliquots, which were then treated with a 1:30 dilution of either anti-Lyt 2.1 or anti-Lyt 1.1 for 45 minutes at 0°C followed by 30 minutes at 37°C with a 1:10 dilution of rabbit complement or complement alone. Treated responders were assayed for cytocoxicity against HSV-1 infected ^{51}Cr labelled Ls Cells.

Effector : Stimulator	α-Lyt 2.1 + C	α-Lyt 1.1 + C	Complement alone
HSV-1 : HSV-1	$\frac{0}{48}$	$\frac{48}{48}$	$\frac{48}{48}$
HSV-1 : HSV-2	$\frac{0}{23}$	$\frac{23}{23}$	$\frac{23}{23}$
HSV-2 : HSV-1	$\frac{0}{25}$	$\frac{25}{25}$	$\frac{25}{25}$
HSV-2 : HSV-2	$\frac{0}{41}$	$\frac{41}{41}$	$\frac{41}{41}$

that are type-specific and cross-reactive for the HSV-1 and HSV-2 serotypes. Although anti-HSV-1 CTL-p could be demonstrated in both HSV-1 and HSV-2-immune spleen cells and the frequency of the anti-HSV-1 CTL-p determined, it was not possible, however, to demonstrate the presence of anti-HSV-2 CTL-p in either HSV-1- or HSV-2-immune cells nor to determine the frequency of cross-reactive and HSV-2 specific CTL by limiting dilution analysis with any certainty or reproducibility.

In the great majority of assays performed, significant levels of lysis of HSV-2-infected targets could not be demonstrated resulting in a large number of negative microcultures and consequently extremely low frequencies, often lower than 1/250,000. In a very few instances, it was possible to determine the frequency of anti-HSV-2 CTL-p, however, the results were inconsistent and unreliable. Representative results are shown in Table IV.

In an attempt to better define the HSV-2 system and hopefully obtain more positive results, a series of kinetic experiments were performed including overnight infection of the target cells with HSV-2. Because the HSV-1 LDA assay system worked with consistency and reproducibility, these experiments were concerned with HSV-2 target system only. Overnight infection of the targets with HSV-2 resulted in low number of positive wells even at high numbers of responders (Table V).

All previous experiments had been conducted using a 6 hour incubation of targets and effectors, therefore, in some cytotoxicity

Table IV. Frequency of anti-HSV-2 CTL-p. Limiting dilution microcultures containing TCGF and either homologously or heterologously stimulated HSV-2 and HSV-1 immune splenocytes were assayed, after 7 days of stimulation, against 1000 ^{51}Cr labelled HSV-2 infected Ls target cells. The frequency of anti-HSV-2 CTL-p was estimated by Poisson distribution.

Experiment	Effector : Stimulator	Frequency	y-intercept	r^2
1	HSV-1 : HSV-1	1/188629	1.01	.99
	HSV-1 : HSV-2	1/250000	1.00	.97
	HSV-1 : norm	1/250000	1.00	.98
	HSV-2 : HSV-1	1/250000	1.02	.96
	HSV-2 : HSV-2	1/109292	1.02	.98
	HSV-2 : norm	1/250000	1.01	.94
2	HSV-1 : HSV-1	1/80470	1.04	.99
	HSV-1 : HSV-2	1/70956	.99	.96
	HSV-1 : norm	1/250000	1.01	.97
	HSV-2 : HSV-1	1/63194	1.02	.98
	HSV-2 : HSV-2	1/44104	1.01	.93
	HSV-2 : norm	1/250000	1.00	.99
3	HSV-1 : HSV-1	1/28939	1.15	.98
	HSV-1 : HSV-2	1/23350	1.13	.99
	HSV-1 : norm	1/250000	1.01	.97
	HSV-2 : HSV-1	1/22302	1.06	.93
	HSV-2 : HSV-2	1/13776	1.14	.92
	HSV-2 : norm	1/250000	1.03	.98

Table V. Overnight infection of Ls cells with HSV-2. Forty-eight replicated microcultures containing homologous and heterologous effector-stimulator combinations were initially plated at 4×10^4 responders per well. At the end of the 7 day culture period, the microcultures were assayed for cytotoxicity against Ls cells which had been infected overnight with HSV-2.

Effector : Stimulator	Experiment #1	Experiment #2	Experiment #3
HSV-1 : HSV-1	$\frac{0}{48}$ (25%)[a]	$\frac{4}{48}$ (30%)[a]	$\frac{7}{48}$ (27%)[a]
HSV-1 : HSV-2	$\frac{7}{48}$	$\frac{5}{48}$	$\frac{6}{48}$
HSV-1 : norm	$\frac{0}{48}$	$\frac{2}{48}$	$\frac{0}{48}$
HSV-2 : HSV-2	$\frac{6}{48}$	$\frac{8}{48}$	$\frac{12}{48}$
HSV-2 : HSV-1	$\frac{4}{48}$	$\frac{3}{48}$	$\frac{5}{48}$
HSV-2 : norm	$\frac{0}{48}$	$\frac{1}{48}$	$\frac{0}{48}$

[a]Percent spontaneous background release of ^{51}Cr.

assays, the target and effector cells were incubated at 37°C for varying lengths of time after which the cultures were centrifuged to pellet cells and the supernatant removed for measurement of radioactivity. As can be seen, the maximum number of positive wells with a reasonable background release was obtained with 6 hour incubation (Table VI). Incubation for shorter periods of time resulted in lower numbers of positive wells, whereas incubation for longer time intervals resulted in a much higher background release and consequently, a reduced number of positive microcultures. These findings are consistent with those of Carter et al. (7) in which HSV-2-infected targets were less susceptible to lysis by either HSV-1 or HSV-2 CTL and varying the m.o.i. or the time of infection did not increase the lytic susceptibility.

Table VI. Effects of varying length of effector-HSV-2-target incubation on the number of positive microcultures. Forty-eight microcultures were plated at 4×10^4 responders per well. Responding populations were HSV-1 and HSV-2 immune splenocytes stimulated with normal infected irradiated splenocytes, HSV-1 infected spleen cells or HSV-2 infected spleen cells in the presence of TCGF. At the end of 7 day culture period, the responding populations were tested for cytotoxicity against HSV-2 infected ^{51}Cr labelled Ls cells. Targets and effectors were incubated at 37°C for varying lengths of time, i.e., 4, 5, 6, 7 or 8 hours and 100 µl of supernate were removed for measurement of radioactivity.

Effector : Stimulator	Experiment #1	Experiment #2	Experiment #3
4 hours			
HSV-1 : HSV-1	1/48 (12%)[a]	3/48 (14%)[a]	5/48 (10%)[a]
HSV-1 : HSV-2	1/48	1/48	3/48
HSV-1 : norm	0/48	0/48	0/48
HSV-2 : HSV-1	2/48	4/48	7/48
HSV-2 : HSV-2	5/48	6/48	6/48
HSV-2 : norm	0/48	0/48	1/48
5 hours			
HSV-1 : HSV-1	4/48 (14%)	7/48 (20%)	5/48 (12%)
HSV-1 : HSV-2	5/48	8/48	4/48
HSV-1 : norm	0/48	0/48	0/48
HSV-2 : HSV-1	10/48	9/48	7/48
HSV-2 : HSV-2	8/48	12/48	13/48
HSV-2 : norm	1/48	0/48	0/48
6 hours			
HSV-1 : HSV-1	14/48 (15%)	15/48 (13%)	8/48 (12%)
HSV-1 : HSV-2	11/48	13/48	13/48
HSV-1 : norm	0/48	0/48	0/48
HSV-2 : HSV-1	13/48	16/48	14/48
HSV-2 : HSV-2	21/48	17/48	19/48
HSV-2 : norm	0/48	0/48	0/48
7 hours			
HSV-1 : HSV-1	12/48 (37%)	13/84 (28%)	9/48 (25%)
HSV-1 : HSV-2	8/48	7/48	3/48
HSV-1 : norm	0/48	0/48	0/48
HSV-2 : HSV-1	16/48	8/48	10/48
HSV-2 : HSV-2	15/48	12/48	11/48
HSV-2 : norm	0/48	1/48	0/48
8 hours			
HSV-1 : HSV-1	8/48 (35%)	6/48 (32%)	3/48 (36%)
HSV-1 : HSV-2	5/48	8/48	2/48
HSV-1 : norm	0/48	0/48	2/48
HSV-2 : HSV-1	9/48	11/48	10/48
HSV-2 : HSV-2	13/48	13/48	7/48
HSV-2 : norm	0/48	1/48	1/48

[a]Percent spontaneous background release of ^{51}Cr.

CHAPTER IV

DISCUSSION

The purpose of the present investigation was to better define the cross-reactivity between HSV-1 and HSV-2 by determining the frequency of anti-HSV-1 and anti-HSV-2 CTL-p present in HSV-1 and HSV-2 immune spleen cell populations homologously or heterologously stimulated in vitro and to demonstrate the utility of the limiting dilution analysis technique in determining the frequencies of these specific and cross-reactive CTL-p.

Unlike allogeneic responsiveness, it was not possible to demonstrate anti-HSV-1 CTL-p in normal mice stimulated in vitro with either HSV-1 or HSV-2. Consequently, the frequency of anti-HSV-1 CTL-p in in normal mice was estimated to be less than 1/250,000. Similar results have been reported for the rabies virus system (24) with the frequency of anti-rabies CTL-p in normal mice also estimated at less than 1/250,000 (1.6 per 10^6 cells). In contrast to normal mice, anti-HSV-1 CTL-p could be detected in both homologously and heterologously stimulated HSV-1 immune splenocytes. Estimated frequencies for the homologous strain combination, i.e., HSV-1 effector:HSV-1 stimulator, varied from 1/6519 (153 per 10^6 nucleated splenocytes) to 1/15632 (63 per 10^6). However, the frequency of anti-HSV-1 CTL-p in heterologously stimulated HSV-1 primed splenocytes was much lower ranging from 1/22045 (45 per 10^6 cells) to 1/49196 (20 per 10^6 cells).

CTL-p which cross-reacted with HSV-1 were detectable in spleen cells from HSV-2 immune mice. Upon homologous antigenic stimulation of HSV-2 primed spleen cells, cross-reactive anti-HSV-1 CTL-p were present at frequencies of 1/13664 (73 per 10^6 cells) to 1/18476 (54 per 10^6 cells). Similar results were obtained with the heterologous, i.e., HSV-2 effector:HSV-1 stimulator, combination with frequencies ranging from 1/12784-1/16644 (78 per 10^6 and 60 per 10^6 cells, respectively). To obtain positive microcultures, it was necessary in all serotype combinations to stimulate responders with viral antigens, the frequency was too low to compute in the microcultures containing normal, uninfected stimulators.

It was important to establish that the frequency being estimated was that of CTL-precursors and not that of some other type of cytotoxic cell reactive against HSV-1 infected targets, e.g., NK cells. Because NK cells grow and divide in the presence of TCGF and because CTL's have been shown to produce interferon which is an NK cell activator (42), it was especially important to exclude the possibility of NK killing. The results of assays against infected and uninfected syngeneic or infected allogeneic targets show that the cytotoxicity was virus specific and H-2 restricted, making it unlikely that NK cells are involved as NK killing is not self-restricted. Furthermore, the cytolytic effector progeny expressed the Lyt 2 alloantigen, an antigen not express on NK cells (64).

Recently, Rouse et al. reported that the precursors of anti-HSV CTL were Lyt 1^+2^+ cells (26). This finding taken with the one

presented here that the effector progeny are of the Lyt 1^-2^+ phenotype would indicate a shift in phenotype during in vitro culture. Reddehase has made similar observations with another herpes virus, murine cytomegalovirus (MCMV). The majority of CTL-p were found to be of the Lyt 1^+2^+ phenotype with a minority of the Lyt 1^-2^+ phenotype. During in vitro culture, however, the Lyt 1^+2^+ cells changed to the Lyt 1^-2^+ phenotype. What possible role the loss of the Lyt 1 antigen could play in the maturation and differentiation of CTL-p will require further investigation.

Cross-reactive CTL have been observed in a number of systems including allogeneic (54, 52) as well as herpes simplex (6) and influenza viral systems (19). The results of the present investigation demonstrate the presence of cross-reactive CTL-p in homologously or heterologously stimulated HSV-2-immune spleen cells, and the frequencies of the cross-reactive CTL-p are very similar, 1/13664-1/18476 and 1/12784-1/16644 for HSV-2 effector:HSV-2 stimulator and HSV-2 effector:HSV-1 stimulator, respectively. Thus, it appears that HSV-1 and HSV-2 viral antigens are nearly equal in their ability to stimulate cross-reactive CTL-p. These frequencies are only somewhat lower than those obtained with homologously stimulated HSV-1 immune splenocytes tested against HSV-1-infected targets, 1/6519 to 1/15632. These results tend to support Tevethia's finding that, in bulk culture, highly reactive CTL were generated by HSV-2, and these HSV-2 CTL were as cytotoxic as HSV-1 CTL against HSV-1-infected target cells (6). Perhaps this high degree of cytotoxicity was due to the similar frequencies of responding HSV-1 and HSV-2 CTL.

HSV-2 viral antigens were less effective in stimulating anti-HSV-1 CTL-p in HSV-1 immune populations resulting in a lower frequency of anti-HSV-1 CTL-p, 1/22045 to 1/49196. This finding may in part explain the reportedly lowered susceptibility of HSV-2-infected targets to lysis by HSV-1 CTL. Perhaps the reduced lytic ability of HSV-1 CTL against HSV-2 targets stems from a relative inability of HSV-2 antigens to stimulate HSV-1 immune cells resulting in a reduced frequency of cross-reactive anti-HSV-2 CTL-p. The findings of Glorioso et al. would suggest this, homologously stimulated HSV-1 immune lymph node cells tested against both HSV-1 and HSV-2-infected targets yielded frequencies of 1/16554 for HSV-1 targets and 1/59116 for HSV-2 targets (66).

In the present work, the frequency of anti-HSV-1 CTL in both HSV-1 and HSV-2 immune spleen cell populations could be determined, however, determining the frequency of cross-reactive and HSV-2 specific cytotoxic T lymphocytes by limiting dilution analysis was not so readily possible. Results obtained from limiting dilution assays using the HSV-2 target system were not consistent and reliable. These mostly negative results are likely due to a reduced susceptibility of the HSV-2-infected target to lysis by either HSV-1 or HSV-2 CTL. There is, however, some disagreement among researchers as to the lytic susceptibility of the HSV-2 target. The results of this investigation are consistent with the findings of Carter et al. (6) who report a reduced susceptibility of the HSV-2 target to lysis.

Using bulk culture assays, Carter and colleagues have described problems inherent in the use of HSV-2 infected target cells due to an

apparent reduced susceptibility of these HSV-2 targets to lysis by both HSV-1 and HSV-2 generated CTL (6). Cytotoxic T lymphocytes (CTL) were generated by immunizing C57BL/6 mice in the hind foot pads with 10^5 p.f.u. of HSV-1 or HSV-2 per foot pad. The draining lymph nodes were removed 5 days postimmunization and lymphocyte suspensions were prepared. After three days of incubation, the CTL generated were tested for cytotoxicity against ^{51}Cr-labeled syngeneic HSV-1 and HSV-2 infected target cells.

The CTL generated in response to four HSV-1 strains (KOS, 17, HFEM, mP) were tested against targets infected with KOS, 17 HFEM, or mP. The HSV-1 targets were all equally susceptible to lysis by the HSV-1 induced CTL yielding high levels of percent specific ^{51}Cr release (at 40:1, 28-49%). Similar experiments were conducted with HSV-2 strains 186, G and GP6. Then the HSV-2 induced CTL were reacted against HSV-2 infected targets, significant but low values of percent specific ^{51}Cr release were obtained (at 40:1, 3.6-24.6%). However, when the HSV-2 CTL were tested against HSV-1-infected targets, 2-4 fold more ^{51}Cr release was obtained, the levels of release being comparable to those observed with HSV-1 CTL reacted against HSV-1 infected targets (at 40:1, 32-40%). The HSV-1 induced CTL were also tested for cytotoxicity against HSV-2-infected targets. The results were comparable to those of HSV-2 CTL tested against HSV-2 targets, i.e., significant but low levels of percent specific ^{51}Cr release. Thus, HSV-1 and HSV-2 will induce cross-reactive populations of CTL, HSV-2-induced CTL are as cytotoxic as HSV-1 CTL

against HSV-1-infected targets, but HSV-2-infected targets are less susceptible to lysis by either HSV-1 CTL or HSV-2 CTL.

Carter et al. investigated several possible reasons for this variance in the lytic susceptibility of HSV-1-infected and HSV-2-infected targets. Infectious center assays were performed to determine if similar numbers of cells were infected with HSV-1 and HSV-2. No difference was found between the number of cells infected by either HSV-1 KOS or HSV-2 186 that were capable of forming an infectious center. The results of growth assays of HSV-1 strains KOS, 17 and HFEM and HSV-2 strains 186, G and GP6 in mouse b1/6 WT-3 cells and also in the human cell line HEp-2 indicate that 186, G, and GP6 replicate with approximately the same efficiency as HSV-1 17 with KOS and HFEM showing more efficient replication in WT-3 cells. Thus, it would appear that the lowered susceptibility of HSV-2-infected cells to lysis is not due to an inefficient infection of the cells.

To determine the effect of increased m.o.i. on the lytic susceptibility of HSV-2-infected targets, C57BL/6 WT-3 cells were infected with either HSV-1 KOS or HSV-2 186 at varying m.o.i. from 1 to 20 and then used as targets for CTL induced by either HSV-1 KOS or HSV-2 186. Increasing the m.o.i. of either HSV-1 or HSV-2-infected targets did not increase the value of the percent specific ^{51}Cr release.

Because the replicative cycles of HSV-1 and HSV-2 have been shown to differ, Carter et al. examined the time course of susceptibility between 2 and 16 hours p.i. of HSV-1- and HSV-2-infected cells to lysis by HSV-1 and HSV-2 CTL in order to determine if there was an

optimal time for the expression of antigens which react with the CTL (9). HSV-1-infected targets were susceptible to lysis by HSV-1 CTL and HSV-2 CTL between 2 and 16 hours, and the level of susceptibility increased between 2 and 12 hours. HSV-2-infected targets, however, were not highly susceptible to lysis by either HSV-1 or HSV-2 CTL. A slight increase in lysis is observed between 2 and 16 hours postinfection.

Because the lysis of HSV-infected cells by antibody and complement has been shown to involve the HSV-specific glycoproteins, infected targets expressing these glycoproteins should be susceptible to lysis by HSV-immune serum and complement (17, 7). The results of studies by antibody-dependent complement mediated lysis demonstrated that the lowered lytic susceptibility of HSV-2 targets was not due to a lack of expression of the viral glycoproteins on the target cell surface, as the HSV-2-infected targets were as susceptible to lysis by antibody and complement as the HSV-1-infected targets.

The cold cell competition assay was used to determine if recognition of the HSV-2-infected target by the CTL occurs. Varying number of HSV-1- and HSV-2-infected, unlabeled, i.e., cold, cells were mixed with HSV-1-infected ^{51}Cr-labeled target cells and then HSV-1 and HSV-2 CTL were added to the target cells. The ability of the "cold" cells to compete with the ^{51}Cr-labeled target cells was measured as a reduction in the percent specific ^{51}Cr release. HSV-2-infected cold cells were found to be as efficient as the HSV-1-infected cold cells in reducing the lysis of ^{51}Cr-labeled, HSV-1-infected cells by either

HSV-1 or HSV-2 CTL; thus both HSV-1 and HSV-2 targets must be equally recognized by the CTL.

In contrast to the findings of Carter et al. (6), Eberle et al. (65) demonstrated significant lysis of HSV-2-infected targets by both HSV-1- and HSV-2-immune cells. To examine the specificity of anti-HSV CTL, splenocytes from mice primed with either HSV-1 or HSV-2 were restimulated in vitro in bulk culture with either the homologous or heterologous HSV serotype. Whereas Carter et al. reported that HSV-2 CTL were more cytotoxic for HSV-1-infected targets than for HSV-2-infected target cells, Eberle et al. demonstrated that homologously stimulated HSV-2 CTL when tested against HSV-1 targets resulted in much lower levels of lysis than when reacted against HSV-2 targets, 72% versus 50%. However, heterologously stimulated HSV-2-primed cells gave comparable levels of lysis against both target types, 39% and 40% for HSV-1 and HSV-2 targets, respectively. Carter et al. also reported that HSV-2-induced CTL were as cytotoxic as HSV-1-induced CTL against HSV-1-infected targets. Eberle et al. found this to be true only in the case of heterologously stimulated HSV-1 CTL. The HSV-1 effector:HSV-1 stimulator combination resulted in 85% specific ^{51}Cr when tested against HSV-1-infected targets, whereas heterologously stimulated HSV-1 CTL and both the homologously and heterologously stimulated HSV-2 tested against HSV-1 targets resulted in significantly lower levels of lysis. The results of Carter et al. indicated that HSV-2-infected cells were 2-3 fold less susceptible to lysis using either HSV-1 or HSV-2 induced CTL. Eberle demonstrated

that HSV-1-CTL either homologously or heterologously stimulated, resulted in significant but lower levels of lysis than the HSV-2:HSV-2 combination tested against HSV-2 targets but were comparable to the HSV-2:HSV-1 combination. Thus, cells restimulated with homologous virus exhibited greater killing of target cells infected with the homologous virus than of targets infected with the heterologous HSV serotype. In contrast, in vitro stimulation of either HSV-1 or HSV-2 immune spleen cells with the heterologous virus resulted in lowered but nearly equivalent killing of HSV-1- or HSV-2-infected targets.

The results presented here clearly demonstrate the effectiveness of the limiting dilution analysis technique as a quantitative means to measure the cell mediated immune response to antigen and to determine the specificity of that response. It is obvious, however, that further investigation of the HSV-2 target system is needed in order to settle any discrepancies concerning the lytic susceptibility of the HSV-2 target. Is the reduced frequency of anti-HSV-2 CTL reported by Glorioso due to a reduced susceptibility of the HSV-2 target to lysis? Or is the seemingly reduced lytic susceptibility the result of an inability of HSV-2 antigens to stimulate anti-HSV-2 CTL and thus causing a lowered frequency of anti-HSV-2 CTL? Research beyond the scope of this work is needed to answer these questions.

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VITA

Julia Ann Heath was born on May 25, 1957, in Harriman, Tennessee. She attended elementary schools in Harriman and graduated from Harriman High School in June 1975. The following September she entered Roane State Community College. While at Roane State, she served as a senator on the SGA. In 1977, she transferred to The University of Tennessee, Knoxville, and in August 1979, she received a Bachelor of Arts degree in Biology, graduating with high honors.

She entered the Graduate School of The University of Tennessee, Knoxville, in June 1980. She was awarded the Master of Science degree in the field of Microbiology in June 1985. Currently, the author is attending medical school at the University of Tennessee Center for the Health Sciences in Memphis and will be graduating in June, 1988.

The author is a member of Phi Beta Kappa, Phi Kappa Phi, and Gamma Beta Phi honor fraternities.