To the Graduate Council:

I am submitting herewith a thesis written by Debra Prymowicz entitled "Development of a Limiting Dilution Assay to Enumerate the Frequency of Helper T Lymphocyte Precursor Cells Specific for Herpes Simplex Virus Type 1." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Barry T. Rouse, Major Professor

We have read this thesis
and recommend its acceptance:

Richard J Courtney

Robert N Moore

Accepted for the Council:

The Graduate School

DEVELOPMENT OF A LIMITING DILUTION ASSAY TO ENUMERATE THE FREQUENCY OF HELPER T LYMPHOCYTE PRECURSOR CELLS SPECIFIC FOR HERPES SIMPLEX VIRUS TYPE 1

A Thesis

Presented for the

Master of Science

Degree

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Debra Prymowicz

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To my family

and to Tim,

for their love and support.

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ABSTRACT

A limiting dilution assay system was developed to quantitate the frequency of Helper T Lymphocyte-precursors (HTL-P) specific for Herpes Simplex Virus Type 1 (HSV-1). Limiting dilution assay conditions for the estimation of optimal helper cell frequencies were determined. The HSV-specific HTL-P was demonstrated to be virus-specific for *in vivo* induction, *in vitro* clonal expansion and *in vitro* restimulation to promote interleukin 2 (IL 2) production. The cell type of the HTL-P was shown to be a Thy 1.2^+, Lyt 1.1^+ cell and the estimated frequency of the HTL-P could be increased 3-fold by Lyt 2.1 depletion of the responder population.

The utility of this limiting dilution approach in the evaluation of various immunization protocols was demonstrated. Live HSV-1 *in vivo* priming was shown to be required to stimulate virus-specific HTL-P as both heat- and UV-inactivated virus preparations failed to stimulate detectable helper cell frequencies. Soluble infected-cell extracts containing HSV-1 glycoproteins also failed to induce detectable virus-specific HTL-P.

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I. LITERATURE REVIEW

The Cell Mediated Immune Response

Cellular immunology is largely concerned with the study of interactions between several cell types in response to a foreign antigen. In the cell mediated immune (CMI) response, various populations of thymus-derived T lymphocytes play an essential role. The discovery of T cell markers has facilitated the study of the T cell response. Reif and Allen described an antigen which is expressed on all T lymphocytes (83). This is now known as the Thy 1 antigen and is found as two alleles (Thy 1.1 and Thy 1.2). The discovery of the Thy antigen allowed for the isolation or enrichment of T cells to more effectively study the cellular response.

Another set of T cell markers was described by Cantor and Boyse (11) which identified various functional subpopulations of T cells. By using alloantisera to define antigens selectively expressed on a T cell subclass, they asked the question: does the generation of functional T cell diversity precede the encounter with antigen? By using alloantisera directed against the Lyt 1 antigen expressed on mature helper T lymphocytes or against the Lyt 2,3 antigen expressed on mature cytotoxic or suppressor T lymphocytes, they demonstrated the presence of these subclasses of T cells from normal, unimmunized animals. This indicated that T cells are committed to express a given activity, be it helper, suppressor or cytotoxic, through a differentiative process that takes place prior to exposure to antigen and that effector cells are derived from different T cell maturational lines. They found that of

peripheral Thy^+ cells: 50% are Lyt $1^+,2,3^+$ precursors, 33% are Lyt 1^+ helper T cells and 6-8% are Lyt $2,3^+$ cytotoxic and suppressor T cells (11).

Another T cell marker was more recently described by Dialynus et al. (24), the L3T4a antigen. Using a monoclonal antibody specific for this antigen, they demonstrated that the expression of L3T4a by murine T cell clones correlated with class II MHC antigen reactivity. The monoclonal antibody could block class II antigen-specific functions by murine class II antigen-reactive T cell clones and they concluded that L3T4a is very similar to the human Leu 3/T4 antigen of human derived helper T lymphocytes. It has been suggested that the L3T4a antigen is, in fact, a more appropriate phenotypic marker for helper T lymphocytes since the Lyt 1 antigen described by Cantor and Boyse (11) is actually found on all T cells, although on helper T cells to a greater extent (24).

Cell mediated immunity is known to play an important role in the response to various foreign antigens, including viral antigens (7, 27, 44, 104). The activity of the cytotoxic T lymphocytes (CTL) may be an important effector mechanism in viral immunity. Through a series of multicellular interactions, the antigen-specific CTL become activated. When the circulating activated CTL comes in contact with an infected cell expressing a foreign antigen in combination with the appropriate self major histocompatibility complex (MHC) antigen, the infected cell is selectively destroyed.

The intricacies of multicellular interactions leading to the induction of CTL have only in the past two decades been established. A

number of cell types and/or their soluble products play a role, including: the antigen presenting cell (APC), the helper T lymphocyte (HTL) and its precursor (HTL-P), the cytotoxic T lymphocyte (CTL) and its precursor (CTL-P) and finally the suppressor T lymphocyte (STL) circuit.

The first among this series of interactions necessary to stimulate a virus-specific CTL response is a genetically-restricted interaction between the APC and the HTL-P (31, 76, 84). This interaction requires genetic compatibility of a class II MHC antigen. The APC, usually the macrophage or dendritic cell, is a cell which possesses the class II (MHC) I-region encoded antigen, Ia (50, 98). Swierkosz et al. studied the MHC genes that control the interactions between macrophages and helper cells and demonstrated that Ia is the relevant subregion involved in the recognition of complexed antigen by the HTL-P (100). The APC appears to have a dual function: the processing and presentation of antigen in the context of Ia to the HTL-P (40, 98, 108, 109) and the production of the soluble product interleukin 1 (IL 1) (29, 30, 69, 82, 96).

The APC-dependent nature of the HTL can be illustrated with continuous T helper cell cultures which require adherent cells plus antigen for their growth and maintenance (56).

The need for APC to stimulate antiviral immunity can be demonstrated with various viral systems. In the reovirus system, depletion of Ia bearing adherent cells such as occurs following UV treatment of splenocytes resulted in a failure to generate CTL (54). In the herpes simplex virus system, Ia positive APC are also

necessary for HSV-specific CTL induction (91, 92, 93). HSV immunity will be described in greater detail in the following section of this review.

Generally, the viral antigen to be presented to the HTL-P requires processing in some fashion by the APC. For example, if macrophages are inactivated such as by UV-light, and then cultured with antigen, they are unable to appropriately present antigen. If, however, the APC are inactivated after several hours of culture with antigen, the HTL-P is appropriately activated (109).

Once activated by antigen, the second role of the APC is the production of the soluble product interleukin 1 (IL 1). Interleukin 1 is biochemically defined as a protein of 12,000 to 18,000 daltons, with isoelectric points of 4.5-5.5 in the mouse, pH 2 insensitive and containing no Ia molecules. It is functionally defined as having an MHC-unrestricted, species-unrestricted activity which does not promote or maintain in vitro long term T cell cultures (1, 30). It has been suggested that the complexing of processed antigen with Ia enhances receptivity of the HTL-P to IL 1 (69) or that IL 1 may enhance the ability of the HTL-P to more tightly bind specific antigen so that preincubation of T cells with IL 1 simultaneously increases the antigen affinity and the lipid viscosity of the HTL-P. This results in the increased accessibility of membrane proteins (79) such as, perhaps, the antigen receptor.

Two T cell soluble products which effect APC function are colony-stimulating factor (CSF) and gamma interferon (IFN-γ). Colony-stimulating factor affects the APC by inducing proliferation and

stimulating the production of IL 1 and prostaglandins. Prostaglandin synthesis decreases the ability of the macrophage to present antigen, perhaps by inhibiting Ia expression (8). Gamma interferon, alternatively, induces Ia expression (3) so it appears that the balance of CSF and IFN-γ activities regulate APC activity.

The next interaction necessary for the formation of virus-specific CTL is between the HTL-P and the CTL-P via soluble products of the HTL. The combination of antigen in the context of Ia and IL 1 induce the antigen-specific HTL-P to clonally expand and to produce soluble factors such as interleukin 2 (IL 2). Studies using the human T cell leukemia line Jurkat demonstrated that both antigen and IL 1 were necessary for IL 2 specific mRNA transcription and that another signal was needed for IL 2 synthesis (110).

Interleukin 2 was first discovered by Gordon and Maclean and Kasakura and Lowenstein in 1965, as a soluble mitogenic factor found in human T cell supernatants after plant lectin stimulation (95). In 1976, Morgan et al. reported that medium from lectin-stimulated mononuclear cells would support the continuous proliferation of mitogen-activated human T cells (64). The use of this biological property allowed for the development of a sensitive microassay for IL 2 based on the ability to trigger T cell proliferation. A long term cytotoxic T cell line, the CTLL, when grown overnight in IL 2, would incorporate a tritiated-thymidine pulse in a dose-dependent manner (33).

Interleukin 2 is a lymphokine which induces the proliferation of antigen or mitogen-stimulated T cells. Its biochemical characteristics

include: a molecular weight of 30,000-35,000 in the mouse, 15,000 molecular weight in humans, an isoelectric point of 3.5-5.5, 2-mercaptoethanol insensitivity and pH 2 insensitivity, lack of Ia molecules, production requiring the presence of macrophages and T cells, MHC unrestricted activity and promotion or maintenance of *in vitro* long term T cell cultures. Historically IL 2 has been referred to as Thymocyte Mitogen Factor (TMF), T Cell Growth Factor (TCGF), Co-Stimulator, Killer Cell Helper Factor (KHF) and Secondary Cytotoxic T Cell Inducing Factor (SCIF) (1).

Interleukin 2 has characteristics similar to polypeptide hormones in that interaction with the target cell is by means of specific receptors. Radiolabeled IL 2 binding assays were developed using highly purified, internally labeled IL 2 to demonstrate that only IL 2 responsive T cells, such as CTL, express high affinity IL 2 specific binding sites (85). Thus, the IL 2-T cell interaction occurs via binding sites on membrane molecules which satisfy the criteria for hormone receptors in their high affinity, saturability, ligand specificity and target cell specificity (112). It has, in fact, been suggested that IL 2 receptor levels may determine the extent of T cell clonal expansion and the "tempo and magnitude of the T cell immune response" (13).

One target cell of the HTL product, IL 2, is the CTL-P. Whereas the HTL-P must be presented antigen in the context of the class II Ia antigen, the CTL-P is presented antigen in the context of a class I MHC antigen encoded in the K and D regions. Only CTL-P, which have been appropriately activated by a foreign antigen-class I self antigen

complex, possess IL 2 receptors (6). These are known as "poised" or "activated" CTL-P. The interaction of IL 2 with the IL 2 receptor causes a proliferative response of the CTL-P. The HTL may also produce a second soluble helper cell product, T Cell Differentiation Factor (TCDF) (105) which is thought to be a CTL-P maturational factor. Preliminary work with TCDF illustrated that when IL 2 activity was absorbed out of mitogen stimulated splenocyte supernatant fluids, remaining activity allowed for the development of a CTL activity without proliferation by CTL-P (105). Thus, it appears that helper cells produce factors necessary for the expansion of CTL-P and also for their differentiation into mature virus-specific CTL.

Various systems have been used to demonstrate the role of the HTL and its products to stimulate CTL activity. Finberg et al. used a hapten-modified self system to study the need for helper cells. Mice primed with TNP hapten-modified syngeneic splenocytes could serve as a population of radio-resistant, antigen-specific HTL which could augment the in vitro CTL response of normal splenocytes to TNP-modified syngeneic cells (32).

Pilarski (75) described an in vitro system in which antigen specific HTL are a requirement for CTL generation. In this system, CTL-P were not stimulated to yield mature CTL unless irradiated HTL were added to cultures.

The need for virus-specific HTL was shown in Influenza A and Sendai virus systems as in vivo primed Lyt 1^+ splenocytes were essential as a source of HTL for an in vitro primary CTL response against both viruses (4, 27).

In the HSV system, a mandatory role for HSV-specific HTL was also demonstrated (91, 93). This will be described in greater detail in the following section of this review.

Plate (77) was the first to demonstrate that the CTL response of HTL-depleted splenocytes could be restored by the addition of mixed lymphocyte culture supernatant fluid. In double chamber experiments with HTL and CTL-P separated by a nucleopore membrane, the actual helper effect was shown to be mediated by non-specific helper factors from Lyt 1^+ T cells (71, 107).

In essence then, the CTL response may be described as having three phases: phase one is the interaction of the APC and the HTL-P; phase two is the proliferation and maturation of the HTL and production of its soluble factors; phase three is the interaction of the CTL-P with antigen and the subsequent proliferation and maturation of the CTL via the helper cell soluble products. These interactions demonstrate the positive regulatory effect of the HTL and its product IL 2. In addition the CTL response is regulated negatively by a circuit of interacting suppressor T lymphocytes (STL).

The STL circuit consists of 3 cell types which interact by means of their soluble products. The first cell, T Suppressor-1 (TS-1), is an Lyt 1^+, I-J^+ T cell which expresses idiotype and produces a soluble factor T Suppressor Factor-1 (TSF-1). This factor activates the second cell, T Suppressor-2 (TS-2), an Lyt $1^-,2,3^+$, I-J^+ T cell which is anti-idiotypic and produces its soluble factor, T Suppressor Factor-2 (TSF-2). This stimulates the actual suppressor

effector cell which is an Lyt $2,3^+$, I-J^- T cell and produces the actual suppressive molecule, T Suppressor Factor-3 (TSF-3) (37).

In some systems the suppressor effector exerts its effects by inhibiting the CTL effector function (61, 86). In other systems, TS-3 negatively regulates an immune response by inhibiting helper functions (45, 86) or by passively absorbing IL 2 as it is produced (69a).

A summary of the cellular interactions leading to antiviral CTL induction and important regulatory features of this response are shown in Figure 1.

To study the immune response to a particular virus, the relevant considerations seem to lie in evaluating the regulation of the interactions which must occur to stimulate antiviral CTL activity. Since the helper cell and its products play a vital role in the regulation of the CTL response, one may better understand antiviral immunity by understanding the role of the helper T lymphocyte.

The Cellular Response to Herpes Simplex Virus

A major form of anti-HSV immunity appears to be cellular in nature (67, 80, 87). HSV-specific neutralizing antibody plus complement, while destroying virus-infected cells, does not prevent the spread of HSV (67). Conversely, cell-mediated cytotoxic responses seem to prevent viral spreading which suggests that CTL responses may occur early in viral maturation before infectious viral particles are produced (112).

While the suggestion was made that *in vivo* spread of HSV infections might be controlled by virus-specific CTL, early researchers failed to detect CTL in splenocytes of HSV-infected mice (44, 52, 72,

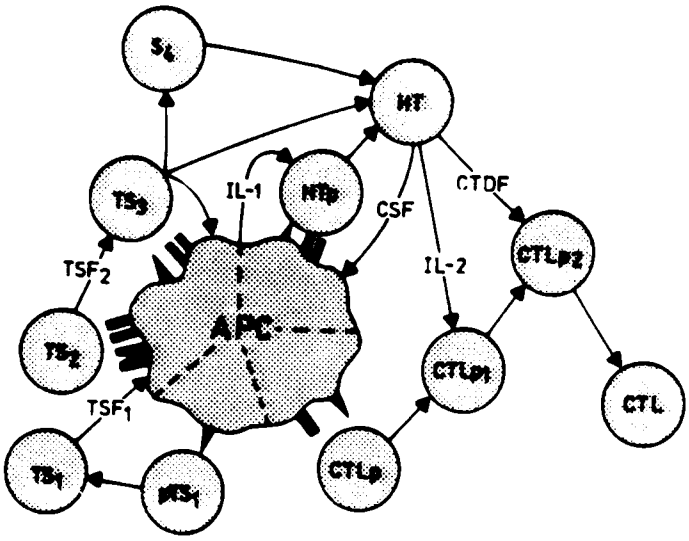

Figure 1. Cellular Interactions Leading to CTL Induction.

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94). Pfizenmaier et al. (72, 73) used a different approach to demonstrate that T cells of mice undergoing a local HSV infection, when transferred in vitro, would differentiate into CTL highly cytotoxic for HSV-infected targets. This has lead the way for many investigators to study the secondary CTL response to HSV (52, 89).

Various groups addressed the question of which viral specific antigens were targets for lysis by immune CTL. Lawman et al. (51) studied the role of the HSV envelope glycoproteins in CMI lysis of HSV-infected targets. They utilized a temperature-sensitive mutant defective in glycoprotein synthesis at the nonpermissive temperature and demonstrated that targets infected with the mutant virus were lysed less efficiently. They also treated infected targets with 2-deoxy-D-glucose and tunicamycin (glycoprotein synthesis inhibitors) and found that treatments significantly reduced target cell susceptibility to lysis. This work suggested that HSV-specific glycoproteins were the primary target for cell-mediated lysis of virus-infected cells. Similar studies by Carter et al. (14) also inferred that a primary target for anti-HSV CTL activity might be one or more HSV glycoproteins.

With the hypothesis that antiviral CTL might be an important immune response to HSV, the role of various immune cells and their products in stimulating anti-HSV CTL was evaluated.

Studies were done to assess the role of the antigen presenting cell in the in vitro induction of HSV-specific CTL (52, 89, 91, 92). It was demonstrated that removal of Ia-positive adherent splenocytes prevented a CTL response which could be restored by the addition of

peritoneal exudate cells (91, 92). Glutaraldehyde-fixed Ia-positive peritoneal cells or an Ia positive tissue culture line, $P388D_1$, required IL 1 to stimulate antiviral CTL. These results indicated that the antigen presenting cell was making two contributions to CTL development: antigen presentation in the context of Ia antigen and secretion of IL 1 (92).

It was also shown that Ia-positive, HSV-1 infectable B lymphocytes failed to serve as accessory cells, even in the presence of IL 1. This indicated the requirement for an additional accessory cell function, such as antigen processing, which B cells were incapable of providing (91).

Other experiments demonstrated that free antigen added to accessory cell-depleted primed splenocytes could stimulate CTL if IL 2 was added. This inferred that either antigen presentation on accessory cells is not a requirement for CTL-P or that the accessory cell type required for antigen presentation to the CTL-P is different than for the HTL-P (52, 89, 91).

Since the HTL is the vital regulator of cellular responses, it became clear that the role and induction requirements of the HTL in regulating anti-HSV CTL responses needed to be evaluated. It was found that splenocytes from mice primed in vivo with infectious HSV-1 could be restimulated in vitro with live or partially UV-inactivated HSV-1 to generate high levels of virus-specific CTL (52, 89, 93). In contrast, naive splenocytes or those taken from mice primed with heat or UV-inactivated HSV-1 (93) or purified HSV-1 glycoproteins incorporated into liposomes (67) failed to generate CTL after in vitro stimulation.

When it was found that near maximal levels of CTL activity could be induced if inactivated virus-primed cultures were supplemented with helper factors (52, 89, 91, 92), it was suggested that the inability of inactivated virus to induce CTL was associated with inadequate stimulation of the HTL population.

One could hypothesize that rather than failing to stimulate help, the quantity of inactivated virus preparations was insufficient to be antigenic (93). This was ruled out on the grounds that these virus preparations could induce helper cell-dependent antibody responses (67).

One could also consider the possibility of induction of an active suppressive mechanism by these particular forms of virus. Cell mixing experiments and supernatant fluids from cultures stimulated with inactivated HSV-1 failed to demonstrate suppression (93), although at this time the possible influence of active suppression cannot be ruled out entirely.

Limiting Dilution Analysis

As the field of cellular immunology developed, experiments in bulk culture containing several populations of immune cells, were the standard assay system for studying cellular interactions. As more became known in the field, it was obvious that to understand the role of each particular cell type, bulk culture conditions were insufficient. The microculture technique was developed to study cellular interactions on a smaller scale. Since more than one cell type is involved in an immune response, even in microcultures it was

difficult to assess the roles of individual cell types and a more complex system, termed limiting dilution analysis was established.

In vitro limiting dilution assays were developed by Lefkovits in 1972 and Quintans and Lefkovits in 1973 to estimate the frequency of B cell clones stimulated in response to various antigens. In the development of their limiting dilution assay systems, they performed a series of manipulations such that only one type of cell was limiting and all other cells required for the immune response would be saturating in each microculture (53). The limiting dilution approach to estimate immune cell precursor frequencies was extended to the field of cellular immunology with the development of a number of assay systems to quantitate CTL-P frequencies in response to allo- (10, 15, 17, 19, 23, 57, 101), hapten-modified-self (102) or viral (66, 68, 88, 90) antigens.

As the importance of the HTL in immunoregulation was clarified, various groups began to develop assays to quantitate the number of helper cell-precursors stimulated in response to various antigens. Miller and Stutman (62) developed a limiting dilution assay to quantitate the frequency of HTL-P stimulated in response to alloantigens. In their assay system, T cell depleted peritoneal stimulator cells were cocultured with varying numbers of responder cells in 24 well replicates. After a 5 day incubation period, plates were centrifuged and supernatant fluids were transferred to plates containing 10^4 CTLL cells, an IL 2 dependent cell line. After 18 hours of incubation, tritiated thymidine was added to each well and samples were harvested 6 hours later. Radioactivity was measured and

each well was scored as positive or negative for tritiated thymidine incorporation above or below the mean incorporation induced by coculturing with supernatant fluids from wells containing stimulators alone, plus three standard deviations of the mean. Each group of replicates in which some, but not all, microcultures gave positive responses was considered in the calculation of the HTL-P frequencies (78) by Poisson analysis. If a semilogarithmic plot of the responder number versus the fraction of negative wells per responder group gave a straight line in which the y intercept was close to 1 (53), this would indicate that limiting conditions were met. According to Poisson statistics, the responder number at which 37% microcultures were negative corresponded to the frequency of the responder cell, in this case the HTL-P. This group utilized their limiting dilution assay system to study the age-associated changes in the immune response, especially in terms of the effects on the HTL-P frequencies and IL 2 production (40, 60) and also to study helper cell heterogeneity (63).

A limiting dilution analysis protocol developed by Kronke et al. (48) determined the presence of an HTL-P in a given microculture by its ability to drive CTL-P to maturation. In this assay system, microcultures were set up with varying numbers of responders and a constant number of Lyt 2 positive syngeneic splenocytes as a source of CTL-P. Each culture was stimulated with allogeneic T cell depleted splenocytes and after a 7 day incubation period each culture was assayed for cytotoxicity against ^{51}chromium-labeled P815 tumor cell targets. In another procedure, Kronke et al. (48) used an assay system similar to that of Miller and Stutman to test the IL 2 activity of secondary mixed leukocyte culture supernatants with an IL 2 dependent

cell line after coculturing responders with allogeneic stimulators. Both limiting dilution protocols demonstrated the necessity requirements for Lyt 1 positive HTL-P for the induction of alloantigen-specific primary CTL responses and for IL 2 production (48).

Various other groups have utilized the limiting dilution approach to quantitate alloreactive HTL-P (21, 57, 99) or bacterial antigen-reactive HTL-P (28).

The question of T cell tolerance was also addressed using a similar limiting dilution assay (35). Neonatal Balb/c or C57Bl/6 mice were injected with (Balb/c x C57Bl/6)F_1 splenocytes to induce tolerance. Workers found that the frequency of HTL-P as well as CTL-P were significantly reduced in tolerized animals.

Hefeneider et al. (38) utilized the limiting dilution approach to quantitate the frequency of T cells producing a number of soluble products. Using the MRC-lpr autoimmune mouse model they demonstrated that the number of IL 2 and CSF producing cells present in these autoimmune animals was similar to the number found in normal mice. They concluded that faulty IL 2 production was not involved in the etiology of autoimmune disease (38). In other experiments, MacDonald et al. (42, 43, 46) and Krammer et al. (46) used limiting dilution analysis to quantitate the precursor frequencies of cells producing MAF, IFN and CSF. Using a similar approach, MacDonald also studied the frequency and specificity of IL 2 producing precursors in congenitally athymic mice (59).

It thus appears that limiting dilution analysis may be a useful approach to study cellular interactions and to assess in a quantitative

fashion, the ability of an antigen to induce a particular immune response.

Rationale for the Present Study

The field of cellular immunology has proceeded at an astounding pace since the early observations of cellular interactions (11, 12). Nonetheless, there is a great deal yet to be elucidated in the study of the cellular immune response, especially in the regulation of immune interactions. Since it is agreed that the helper T lymphocyte is a major modulator of antiviral CTL activity, one can hope to understand how to enhance virus-specific CTL by more appropriately stimulating the helper cell.

Previous work in our laboratory has suggested the important immunoregulatory role of the HTL in HSV immunity and has also suggested that the failure to stimulate antiviral CTL activity by some forms of virus might be due to a failure to stimulate the helper cell adequately or appropriately (52, 89, 91, 92, 93).

The limiting dilution approach to enumerating precursor frequencies is a valuable tool in that it enables one to quantitatively assess the magnitude of an immune response specific for a given antigen. Although various groups have seen the need to develop limiting dilution assays to enumerate helper cell frequencies in response to a number of antigens, an assay to quantitate virus-specific helper cell frequencies is, as yet, unprecedented.

The relevant contribution of this research lies in its ability to aid in the understanding of how to best stimulate a major regulator of

the cellular response, the helper T lymphocyte. This limiting dilution analysis could be adapted to other viral systems to evaluate the potential of other viral preparations to stimulate helper functions. In the development of future vaccines, for HSV and for other viruses, an important feature to be considered may be an immunogen's ability to stimulate the helper T lymphocyte, a vitally important immunoregulatory cell in the antiviral T cell response.

II. SPECIFIC AIMS

1. To determine the optimal assay conditions for a virus-specific limiting dilution approach to enumerating herpes simplex virus-specific helper T lymphocyte-precursors.
2. To report the frequency of HTL-P in the draining lymph node specific for HSV-1.
3. To evaluate the *in vivo* and *in vitro* virus requirements to stimulate HSV-specific HTL-P.
4. To define the cell type of the HSV-specific HTL-P.
5. To show the utility of this assay approach in the evaluation of various immunization protocols to stimulate a cell-mediated immune response.

III. MATERIALS AND METHODS

Mice

All C3H/HeJ (H-2^k) mice were obtained from the University of Tennessee Medical Research Center, Knoxville, TN and Balb/c mice from Cumberland View Farms, Clinton, TN. Mice were housed under conventional conditions and were used at 2-4 months for responder cells or up to 8 months for stimulator cells.

Medium

The medium used was RPMI medium 1640 (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO), penicillin (100 U/ml), streptomycin (100 ug/ml), gentamycin (5 ug/ml), 5×10^{-5} M 2-mercaptoethanol and buffered to pH 7.2 with 25 mM Hepes. Initial cell suspensions were prepared in Hepes-buffered Hank's balanced salt solution (HBSS). All antiserum and complement dilutions were made in RPMI 1640 plus 0.3% bovine serum albumin (BSA) and is referred to as RPMI-BSA.

Viruses

Herpes simplex virus type 1 strain KOS and HSV-2 strain 186 were grown in HEp-2 cells by infecting cells at a low multiplicity of infection and harvesting cell associated antigen as described previously (9). Heat inactivated HSV-1 was prepared by heating virus for 60 min. at 56°C. This procedure reduced the infectivity from 5×10^7 plaque forming units (pfu)/ml to <10 pfu/ml. Ultraviolet (UV) inactivated HSV-1 was prepared by exposing 5×10^7 pfu of virus at a distance of

5 cm to UV light from 2 GE5T8 germicidal bulbs (General Electric Co.) for 5 min. The remaining infectivity was 10 pfu/ml or less. Partially inactivated HSV-1 was prepared by exposure to UV light for 2 minutes to reduce cytopathic effects during in vitro culture. The infectivity titer of such preparations was 100-1000 pfu/ml. HSV-1 glycoprotein extracts were obtained from Dr. R. J. Courtney of this department and were prepared by solubilizing infected cells in 1% sodium deoxycholate or Tween 40 detergent solutions. Samples were vortexed, incubated for 60 min at 37°C and insoluble material was removed by centrifugation at 100,000 x g for 60 min (26). Influenza stain PR8 (obtained from Dr. J. Bennick, Wistar Institute, Philadelphia) was propagated in chicken eggs.

Preparation of IL-2-containing Con A supernatant fluid

Con A supernatants were prepared as described (34). Briefly, splenocytes were isolated from normal Lewis rats (Charles River, Wilmington, MA), washed in HBSS twice and resuspended in RPMI complete medium. Cells were seeded at 5 x 10^6 cells/ml and 20 mls were added to 35 mm culture dishes. Cells were stimulated with 5 ug/ml Concanavalin A (Pharmacia Fine Chemicals, Piscataway, NJ) at 37°C for 48 h under 5% CO_2. Supernatant fluids were pooled and cells and cellular debris were removed by centrifugation at 2000 x g for 15 min. Alpha-methyl mannoside (50 mM/ml) was added to neutralize Con A for 1 h and supernatant fluids were filter sterilized using a 0.45 micrometer vacuum-operated membrane filter unit (Gelman Sciences, Inc.), aliquoted and frozen at -20°C.

CTLL response to IL-2

CTLL cells clone 15G were kindly donated by Dr. Kendall Smith (Dartmouth College, NH). Cells were grown in RPMI complete medium with 5-10% IL-2 containing Con A supernatant fluid.

The CTLL response to IL 2 was titrated as described (34). CTLL cells were washed in RPMI complete medium and incubated at 37°C for 2 h to remove residual IL-2. Two-fold dilutions of Con A supernatant fluids in RPMI complete medium were added to 96 well flat bottom plates (Costar, Cambridge, MA) in 0.1 ml volumes. After incubation, CTLL cells were resuspended in RPMI complete medium at 5×10^4 cells/ml and 0.1 ml volumes were added to each well containing dilutions of the laboratory IL-2 source and to control wells containing RPMI complete medium. Plates were incubated for 18 h at 37°C in 5% CO_2. Cultures were pulsed with 0.5 µCi tritiated thymidine (ICN, Inc., Irvine, CA), harvested 6 h later using an automatic cell harvester (Flow Laboratories, Rockville, MD) and processed for Beta counts.

Preparation of splenocyte stimulator cells

To prepare stimulators, normal C3H/HeJ or Balb/c mice were sacrificed by cervical dislocation, spleens were aseptically removed and single-cell suspensions were prepared as described previously (52). Briefly, cells were minced and passed through 80 mesh wire screens into HBSS and erythrocytes were lysed by treating cell pellets with Tris-buffered 0.83% NH_4Cl at 37°C. Cells were washed twice in HBSS, once in RPMI complete medium and viable cells were enumerated. Splenocytes were T-cell depleted by treating with ascites anti-Thy 1.2 monoclonal

antibody (hybridoma H013.4, supplied by Dr. John Sprent, Wistar Institute, Philadelphia) and Low Tox Rabbit Complement (Accurate Chemical, Westbury, NY). Splenocytes were treated with anti-Thy 1.2 (1:400 dilution in RPMI-BSA) at 1 x 10^8 cells per ml antiserum at 4°C for 45 min. Cells were X-irradiated (2000 R) washed with RPMI-BSA and treated with complement (1:15 dilution in RPMI-BSA) at 1 x 10^7 cells/ml for 45 min at 37°C. Cells were washed, resuspended in RPMI complete medium and viable cells counted. After pelleting, known numbers of cells were then reacted for 1-2 hr at 37°C with 2 min UV inactivated HSV-1, HSV-2, or influenza virus at a multiplicity of infection of 1 (prior to inactivation) for viral stimulators of Balb/c splenocytes were untreated with virus, for the alloantigen limiting dilution assay. Stimulators were then adjusted to 5 x 10^5 cells per ml and added at 0.1 ml volumes to 96 well round bottomed culture dishes (Costar).

Preparation of responder cells

To prepare responder cells, C3H/HeJ mice were immunized via the footpads with 10^6 pfu of infectious HSV-1, inactivated HSV-1 (10^7 pfu prior to inactivation) or HSV-1 glycoproteins (100 μg). Five days later, popliteal lymph nodes (LN) were aseptically removed and single cell suspensions were prepared as described above. Normal LN cells were used as responder cells for the alloantigen specific HTL-P assay or immunized LN cells were used as responders for the virus-specific HTL-P assay.

To deplete responder cells of Lyt 2^+ lymphocytes, LN were treated with 0.5 ml monoclonal anti-Lyt 2.1 antiserum (Accurate

Chemical) (1:20 dilution in RPMI-BSA) per 10^7 cells for 45 min at 4°C. Cells were washed in RPMI-BSA and then treated with 1 ml complement (1:15 dilution) per 10^7 cells at 37°C for 45 min. Cells were washed in RPMI complete medium and enumerated.

Assay to determine the effect of HSV-1 on CTLL proliferation

CTLL cells were washed free of IL 2 and incubated in RPMI complete medium for 2 hours at 37°C in 5% CO_2. Cells were resuspended in RPMI complete medium at 5×10^4 cells per ml.

Dilutions of IL 2-containing Con A supernate were added to wells of a 96 well flat bottom plate (Costar) in 12 replicates of each of the following dilutions: 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64. Control wells were set up as 12 wells containing 0.1 ml of medium. Varying dilutions of HSV-1 were made up in 25 µl volume including the following concentrations of virus: 1×10^8, 5×10^7, 1×10^7, 5×10^6, 1×10^6, 5×10^5, 1×10^5, 5×10^4, 1×10^4, 5×10^3 and 1×10^3 pfu/ml HSV-1. For each set of IL 2 dilutions and corresponding medium control, a single dilution of virus was added.

CTLL cells were added to each well in 0.1 ml volumes containing 5000 CTLL cells. Cultures were incubated at 37°C in 5% CO_2 for 18 hours, pulsed with 0.5 µCi ^{3}H-TdR (ICN, Inc.) and harvested 6 hours later using an automatic cell harvester (Flow Laboratories).

Assay to determine the effect of HSV-1 containing supernatant fluid on CTLL proliferation

Normal C3H/HeJ splenocytes were T cell depleted and X-irradiated as described previously. Splenocytes were stimulated for 1 h at 37°C

with partially inactivated HSV-1 at a multiplicity of infection (MOI) equal to 1 or 5. Splenocytes at 5 x 10^5 cells in 0.15 ml which were infected with virus at an MOI of 1 or 5 or those which were uninfected, were added to round bottom wells in 24 well replicates. Plates were incubated for 18 h at 37°C in 5% CO_2. Plates were then centrifuged at 300 x g for 5 min and 0.1 ml of each supernatant was transferred to flat bottom wells (Costar) containing 5000 CTLL cells suspended in 0.1 ml of a known dilution of IL-2. Plates were incubated for 18 h, cultures were pulsed with 0.5 µCi ^{3}H-TdR (ICN, Inc.), harvested 6 h later using an automatic cell harvester (Flow Laboratories).

Limiting dilution protocol for alloantigen-specific HTL-P

C3H/HeJ (H-2^d) responder cells were added in 24 well replicates to 96 well round-bottom plates (Costar) with wells containing usually 1000, 500, 250, 125, 62.5, 31.25 and 15.125 cells per well in 0.1 ml RPMI complete medium. Balb/c (H-2^d) stimulators were added at 5 x 10^5 cells per well to wells containing responders and to 24 wells containing 0.1 ml RPMI complete medium. Plates were sealed and incubated for 7 days at 37°C in 5% CO_2 to allow for clonal expansion of putative HTL-P. After 7 days, cultures were centrifuged at 300 x g for 5 min, washed and restimulated with 5 x 10^5 fresh stimulators per well in 0.15 ml RPMI complete medium. Plates were reincubated for 24 h and then centrifuged at 300 x g for 5 min. Supernatant fluids were transferred to 96 well flat bottom plates and assayed for IL-2 activity using CTLL cells as described above.

Determination of the appropriate stimulator population

To determine the appropriate stimulator population to enumerate HSV-1 specific HTL-P, varying numbers of 5 day immune live HSV-1 primed lymph node responder cells were added in 24 well replicates to round bottom wells of a 96 well plate. For each set of responder cell titrations, one of the following stimulator populations was used: T-cell depleted and X-irradiated syngeneic splenocytes, T-cell depleted and HSV-1 stimulated syngeneic splenocytes, T-cell depleted, X-irradiated and HSV-1 stimulated syngeneic splenocytes or HSV-1 stimulated syngeneic splenocytes. All T-depletions and X-irradiation followed procedures described above.

Splenocytes were in vitro stimulated with HSV-1 by suspending cell pellets in 5 ml RPMI 1640 and adding partially inactivated virus at an MOI of 1 (prior to inactivation). Suspensions were incubated at 37°C for 1-2 hours. Cells were then set at 5×10^6 cells per well in RPMI complete medium and 0.1 ml was added to each well containing responder cells and to 24 wells containing medium. Plates were incubated at 37°C in 5% CO_2 for 9-10 d, centrifuged at 300 x g for 5 min, washed in RPMI complete medium and then restimulated with 5×10^5 T-cell depleted, X-irradiated, HSV-1 stimulated syngeneic splenocytes per well in 0.15 ml volumes. Plates were incubated for 24 h, centrifuged at 300 x g for 5 min and 0.1 ml of each supernatant fluid was transferred to wells of 96 well flat-bottom plates (Costar) and assayed for IL-2 activity as described above.

Determination of the appropriate clonal expansion period

To determine the optimum clonal expansion period for HSV-specific HTL-P *in vitro*, 5 x 10^5 T-cell depleted, X-irradiated, HSV-1 stimulated splenocytes, prepared as previously described, were cultured with varying numbers of 5 day immune LN responder cells. Three sets of responders were stimulated for 5, 9 or 15 days. Plates were centrifuged, cultures were washed and then restimulated with HSV-1 stimulators for 24 h. Plates were centrifuged at 300 x g, supernatant fluids were transferred to flat bottom wells of 96 well plates (Costar) and assayed for IL-2 activity as described above.

Determination of the appropriate in vitro restimulation period

To determine the optimal *in vitro* restimulation period to generate IL 2 production by expanded HTL-P clones, 5 day immune LN responder cells were set up in bulk culture with 10^6 cells per well in 1 ml, cultured in 24 well plates (Costar). Each culture was stimulated with 10^6 T-cell depleted, X-irradiated, HSV-1 stimulated splenocytes in 1 ml and plates were incubated for 9 days. Cultures were then restimulated with HSV-1 stimulators for varying lengths of time, including 6, 12, 18, 24 and 48 hours. At the appropriate time 0.1 ml supernatant fluids from 4 replicate wells were transferred to flat bottom wells of 96 well plates (Costar) and assayed for IL 2 activity as described above.

Limiting dilution protocol for HSV specific HTL-P

Responder cells were added in 0.1 ml 96 well round-bottom plates (Costar) as 24 well replicate series containing usually, 20,000,

10,000, 5,000, 2,500, 1,250, 625 and 312.5 responders per well. Stimulators were added (5 x 10^5 cells per well) to all wells containing responders and to 24 control wells containing 0.1 ml RPMI complete medium. Plates were sealed and incubated for 9 days at 37°C in 5% CO_2 to allow for clonal expansion of putative HTL-P. After 9 days, cultures were centrifuged at 300 x g for 5 min, washed twice and restimulated with 5 x 10^5 fresh stimulators (prepared exactly as described for initial stimulators) per well in 0.15 ml RPMI complete medium. Plates were re-incubated for 24 h and then centrifuged at 300 x g for 5 min. Supernatant fluids (0.1 ml) were removed with a multichannel pipette and added to 96 well flat bottom plates containing 5000 CTLL cells for the detection of IL-2 as described earlier.

Statistics

A baseline level of ^{3}H-TdR incorporation was arbitrarily chosen as the mean counts per minute of 24 wells containing stimulators but no responders plus 3 standard deviations of the mean. Each well in a given assay was scored as positive or negative for ^{3}H-TdR incorporation when compared to this baseline level. A semilogarithmic plot was made in which the percent negative wells within a group where both positive and negative wells were found, was plotted against the responder number.

A line of best fit was drawn using least squares analysis, and r^2 values and y intercepts were calculated. According to Poisson Analysis (53), if a line was drawn in which the y intercept was close to 1, this indicated that only 1 cell type was limiting in the

cultures; in this case the responder number, namely the HTL-P. For experiments presented in this report, all r^2 values were greater than 0.95 and y intercepts were between 0.92 and 1.2. From interpolation of the linear regression line at which 37% responders were negative, the frequency of the HTL-P was deduced.

IV. RESULTS

Use of CTLL cells to measure IL-2 production

Initial experiments were carried out to study the ability of an IL-2 dependent cell line to measure IL-2 activity. The CTLL cell line was cultured with dilutions of a laboratory Con A supernatant fluid containing IL-2 activity for 18 h and ^{3}H-TdR incorporation was measured as seen in Figure 2. A dose dependent proliferative response of CTLL cells to IL-2 was observed. High concentrations of supernatant fluid were inhibitory, proliferation reached a maximum value at a dilution of 1:32 and the proliferative response was titrated out at further dilutions of IL-2. Since even very low levels of IL-2 activity may be detected by CTLL cells, it is clear that the CTLL cell line may be useful for detecting the presence of the helper cell product, IL-2 and thereby for detecting the presence of the helper cell precursor (HTL-P) and its clonal progeny.

In order to determine the units of IL-2 in the laboratory Con A supernatant fluid, a titration curve such as that shown in Figure 2 was used. Units of IL-2 activity were estimated by taking the inverse of the dilution of IL-2 containing supernatant fluid which gave 50% of the maximal ^{3}H-TdR incorporation (95). In other words, from Figure 2, the maximal cpm were 25,525 and therefore 50% of this value was 12,762. Since this level of ^{3}H-TdR incorporation was found at a dilution of 1:70, 70 units of IL-2 per volume of supernatant tested was estimated as being present. Since supernatant fluids were added to microwells in

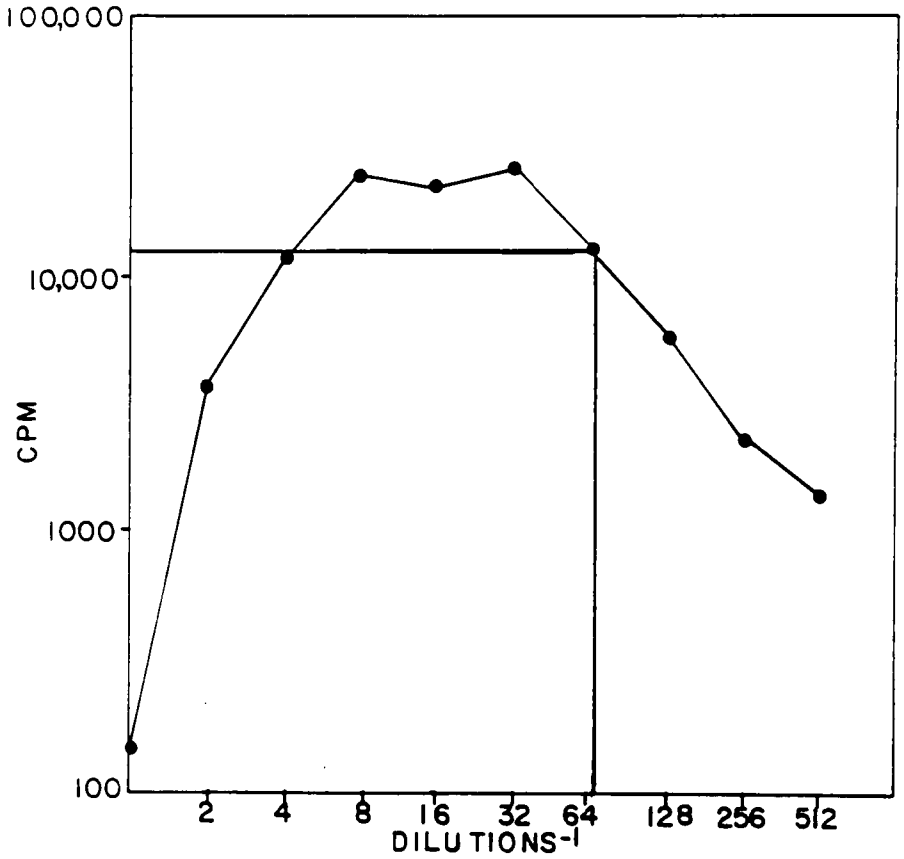

Figure 2. CTLL Proliferative Response to IL 2.

CTLL cells were washed free of IL 2 and 5000 CTLL cells were added to wells containing varying dilutions of IL 2. Cultures were incubated for 18 hours, pulsed with ^{3}H-TdR and harvested 6 hours later.

0.1 ml volumes, this indicates a value of 700 units of IL-2 activity/ml of laboratory Con A supernatant fluid.

Use of limiting dilution analysis to quantitate alloreactive HTL-P

The ability of IL-2 dependent cell lines to measure small quantities of IL-2 in microculture supernatant fluids was utilized in the development of limiting dilution assays to quantitate HTL-P frequencies. Considering our eventual goals of designing a limiting dilution approach to estimating HSV-specific HTL-P, we did preliminary experiments to measure the frequencies of alloreactive HTL-P following the protocol of Kronke et al. (48).

In this assay, Balb/c ($H\text{-}2^d$) T-cell depleted, X-irradiated splenocytes were used as stimulator cells and C3H/HeJ ($H\text{-}2^k$) LN cells were used as responders. Cells were cocultured for 7 days to clonally expand HTL-P, washed to remove any IL-2 produced up until this time and cultures were then restimulated with fresh stimulator cells for 24 h during which time maximal levels of IL-2 were produced (48). Supernatant fluids were removed at this time and transferred to 96 well flat bottom plates containing CTLL cells. After 18-24 h cultures were pulsed with ^3H-TdR and harvested 6 h later. Data was statistically analyzed as described.

Representative data points for an $H\text{-}2^k$ anti-$H\text{-}2^d$ HTL-P limiting dilution assay are shown in Figure 3. This demonstrates that as responder numbers increased, a higher percentage of positive wells were found. The fraction of negative microcultures for each responding group having both positive and negative cultures, was plotted versus

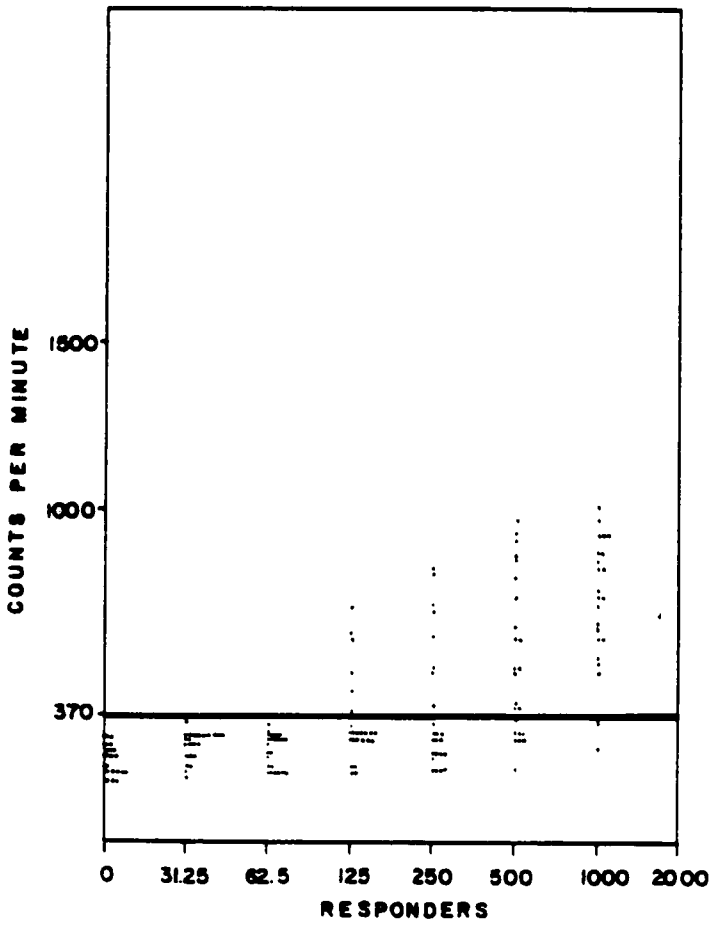

Figure 3. H-2^k-anti-H-2^d HTL-P Limiting Dilution Assay: CTLL Proliferation by Individual Microculture Supernatant Fluids.

Proliferation of CTLL cells induced by coculturing with supernatant fluids from cultures containing BALB/c stimulators plus varying numbers of C3H/HeJ responders. Each point represents an individual microculture. Line indicates 3 standard deviations above the mean of wells containing zero responder cells.

the responder number. This is shown in Figure 4. A semilogarithmic plot with little deviation from the origin indicated a good fit of the data and that conditions were met such that only one assay parameter was limiting, in this case the responder cell. According to Poisson statistics (53), the responder number at which 37% of the microcultures were negative indicated the frequency of the responder population, in this case the HTL-P. Therefore, for the experiment shown in Figure 4, of $H\text{-}2^k$ responder lymph node cells stimulated by $H\text{-}2^d$ alloantigen, an HTL-P frequency of 1:445 resulted. Data for 3 such alloantigen limiting dilution assays is shown in Table I.

HSV-1 inhibited CTLL proliferative response

In adapting an alloantigen specific limiting dilution protocol for a viral system, our initial concern was that HSV-1 used to stimulate putative HTL-P *in vitro* would inhibit the CTLL proliferative response to IL 2. As Table II shows, when varying dilutions of HSV-1 were added to CTLL cells cultured in dilutions of IL 2-containing supernatant fluids, an obvious inhibition of the CTLL proliferative response to IL 2 was evident.

Since the practical concern for the use of a limiting dilution approach was that remaining virus present in culture fluids after *in vitro* culture with HSV-stimulator cells might inhibit the CTLL proliferative response to IL-2 and thus make us unable to detect IL-2 producing helper cells in the responder population, the effect of HSV-1 containing supernatant fluids on CTLL proliferation was evaluated.

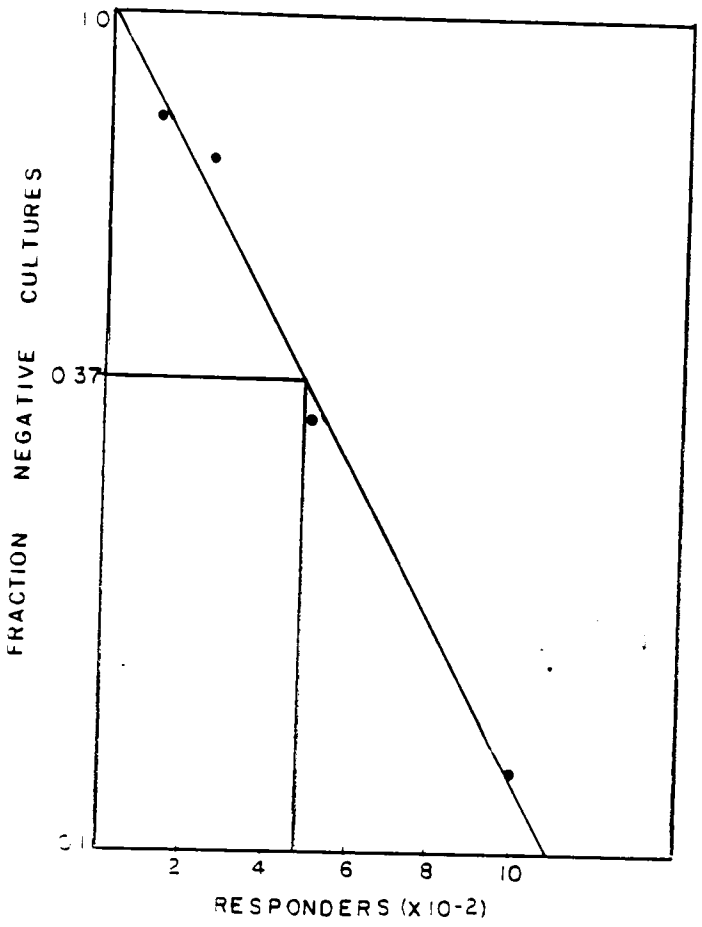

Figure 4. H-2^k-anti-H-2^d HTL-P Frequency.

Representative experiment illustrating an estimate of the frequency of H-2^k-anti-H-2^d IL-2 producing HTL-P in regional lymph node cells. Proportion of non-responding cultures for each responder group of 24 replicates was plotted logarithmically (y axis) against responder cell input (x axis) and the best fitting line was drawn by the least squares method and the precursor frequency wsa estimated by Poisson analysis (f = 1:481; r^2 = 0.99, y = 1.03).

Table I. $H\text{-}2^d$-anti-$H\text{-}2^k$ Alloantigen Limiting Dilution Assays.

Experiment	Frequency	R^2 value	Y intercept
1	1:481	0.99	1.03
2	1:553	0.98	.922
3	1:302	0.96	1.04

Data from 3 separate experiments to determine the frequency of $H\text{-}2^d$-specific HTL-P in the popliteal LN population of $H\text{-}2^k$ mice.

Table II. HSV-1 Effect on CTLL Proliferative Response to IL-2

	PFU of HSV-1											
	10^8	5 x 10^7	10^7	5 x 10^6	10^6	5 x 10^5	10^5	5 x 10^4	10^4	5 x 10^3	10^3	--
MC*	151	165	216	205	251	165	164	226	195	157	159	148
1:2	592	1126	1258	1557	1400	721	1719	1320	1365	1677	2114	1448
1:4	1484	2294	2923	3794	2603	2325	3137	3011	3165	3032	2685	3099
1:8	2807	2189	2743	3077	4164	4652	4233	4952	5826	4943	5619	4955
1:16	3404	6147	7126	3437	7751	5530	7717	5647	6836	6347	10531	10671
1:32	2187	7125	7373	8754	9429	8010	8873	8254	10812	9889	13452	13712
1:64	3102	4129	2960	3677	7088	7598	8340	8129	6360	8776	11644	12085

*Dilutions of IL 2 containing supernatant fluids.

Varying concentrations of HSV-1 were added to 10^4 CTLL cells cultured in varying dilutions of Con A stimulated IL 2 containing supernatant fluid cultures were incubated for 18 hours, pulsed with ^{3}H-TdR and harvested 6 hours later.

Normal splenocytes were T cell-depleted, X-irradiated and stimulated with partially inactivated HSV-1 at an MOI of 1 or 5. Then 5 x 10^5 stimulators were added to microwells and incubated for 24 hours. When supernatant fluids were transferred to wells containing CTLL cells cultured with known concentrations of a laboratory source of IL-2, ^{3}H-TdR incorporation of CTLL cells was significantly reduced, especially at low levels of IL-2. As Figure 5 illustrates, at a multiplicity of infection of 1, while stimulator supernatant fluids inhibited proliferation, they still gave demonstrable levels of ^{3}H-TdR incorporation above background for CTLL cells in medium containing no IL-2. At an MOI equal to 5, the proliferation of CTLL was inhibited such that ^{3}H-TdR incorporation was below background at lower levels of IL-2, thereby demonstrating that treatment of stimulator cells with a high MOI inhibited the CTLL proliferative response to IL-2. For this reason, in all limiting dilution experiments, stimulator populations were stimulated with HSV at an MOI equal to 1.

Determination of the appropriate stimulator population

To establish an HSV-specific limiting dilution analysis, the stimulator population able to optimally stimulate HTL-P frequencies was investigated. The responder cell chosen for this assay was the 5 day immune popliteal lymph node cell shown previously by our laboratory to be a good responder population for limiting dilution assays to enumerate CTL-P (90). Varying numbers of responders were cocultured with one of several stimulator populations as shown in Table III. Only in the case of stimulator cells which were T-cell depleted, X-irradiated

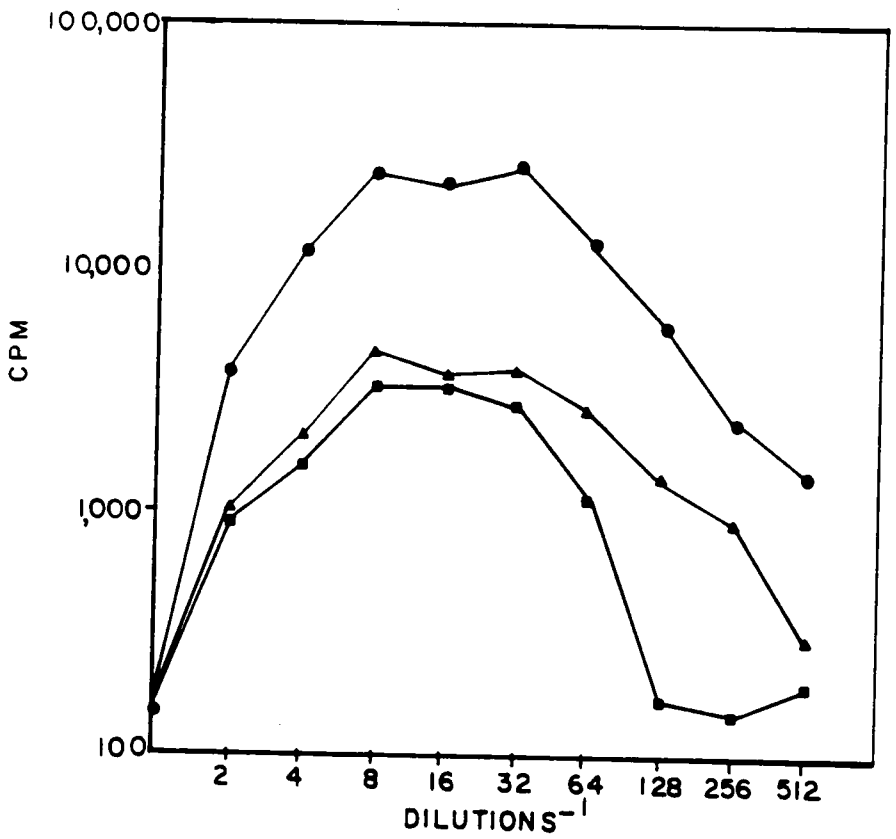

Figure 5. Effect of HSV-1 Containing Supernatant Fluids on CTLL Proliferation.

Normal splenocytes were T cell depleted, X-irradiated and HSV-1 stimulated with partially inactivated HSV-1 at a MOI of 1 (▲) or 5 (■). Then 5 x 10^5 stimulators were added to microwells and incubated for 24 hours, transferred to wells containing CTLL cells cultured with known concentrations of IL 2 and tritiated thymidine incorporation of CTLL cells was compared with a standard titration of IL 2.

Table III. Limiting Dilution Assays to Determine Appropriate Stimulator Population

Experiment	Stimulator	Frequency	R^2 value	Y intercept
1	T-depleted	*		
	X-irradiated			
	T-depleted, X-irradiated	1:2917	0.99	1.1
	HSV-1 stimulated			
	X-irradiated, HSV-1 stimulated	1:2227	0.98	0.86
	HSV-1 stimulated	*		
2	T-depleted, X-irradiated	*		
	T-depleted, X-irradiated, HSV-1 stimulated	1:7356	0.98	1.17
	X-irradiated, HSV-1 stimulated	1:3618	0.94	1.3
	HSV-1 stimulated	*		
3	T-depleted, X-irradiated	*		
	T-depleted, X-irradiated, HSV-1 stimulated	1:3102	0.96	1.05
	X-irradiated, HSV-1 stimulated	1:4816	0.92	1.2
	HSV-1 stimulated	*		

*Not determinable.

Various stimulator populations were tested for their ability to stimulate 5 day immune LN responder cells to generate detectable HTL-P frequencies. Stimulators and responders were cocultured for 9 days, washed and restimulated with identical stimulator populations and supernatant fluids were titrated.

and HSV-1 stimulated were acceptable r^2 values, y intercepts and reproducible frequencies obtained. Thus the appropriate stimulator population to optimally stimulate HTL-P was taken as T-cell depleted, X-irradiated, HSV-1 stimulated splenocytes.

Appropriate period of HTL-P clonal expansion

Another assay parameter analyzed in the design of an HSV-specific limiting dilution system was the optimal period of clonal expansion of HTL-P such that IL-2 was produced following restimulation. As shown in Table IV, only when cultures were incubated for a 9 day period prior to restimulation were data acceptable in terms of r^2 values, y intercepts and reproducible HTL-P frequencies. These results indicated that 5 days is too short a period to allow for detectable levels of IL-2 to be produced and that 2 weeks may be too long, presumably because factors such as medium depletion could interfere with the assay system. Consequently, 9 days appears to be an optimal time period for HTL-P clonal expansion.

Appropriate restimulation period of HTL-P clones to induce IL 2 production

A final parameter analyzed in the development of an HSV-specific limiting dilution assay was the proper period of *in vitro* stimulation of expanded HTL-P clones to produce optimal levels of IL 2, and therefore, a truer estimate of HTL-P frequencies. As Figure 6 shows, after a 9 day clonal expansion of immune LN cells maximal IL 2 levels were produced 18 h after restimulation of HTL-P clones. This time period

Table IV. Limiting Dilution Assays to Determine Appropriate Clonal Expansion Period of HTL-P

Expansion Period (Days)	R^2	Y intercept	Frequency
5	*		
9	0.98	1.17	1:7356
	0.99	1.1	1:2917
15	0.96	2.15	1:3615
	0.97	2.6	1:2846

*Not determinable.

HSV-1 stimulators and 5 day immune LN responder cells were cocultured for 5, 9 or 15 days to clonally expand HTL-P specific for HSV-1. Cultures were then washed, restimulated with HSV-1 stimulators and supernatant fluids were titrated for IL 2 activity as described. Data was analyzed by Poisson statistics to generate HSV-1 specific HTL-P frequencies.

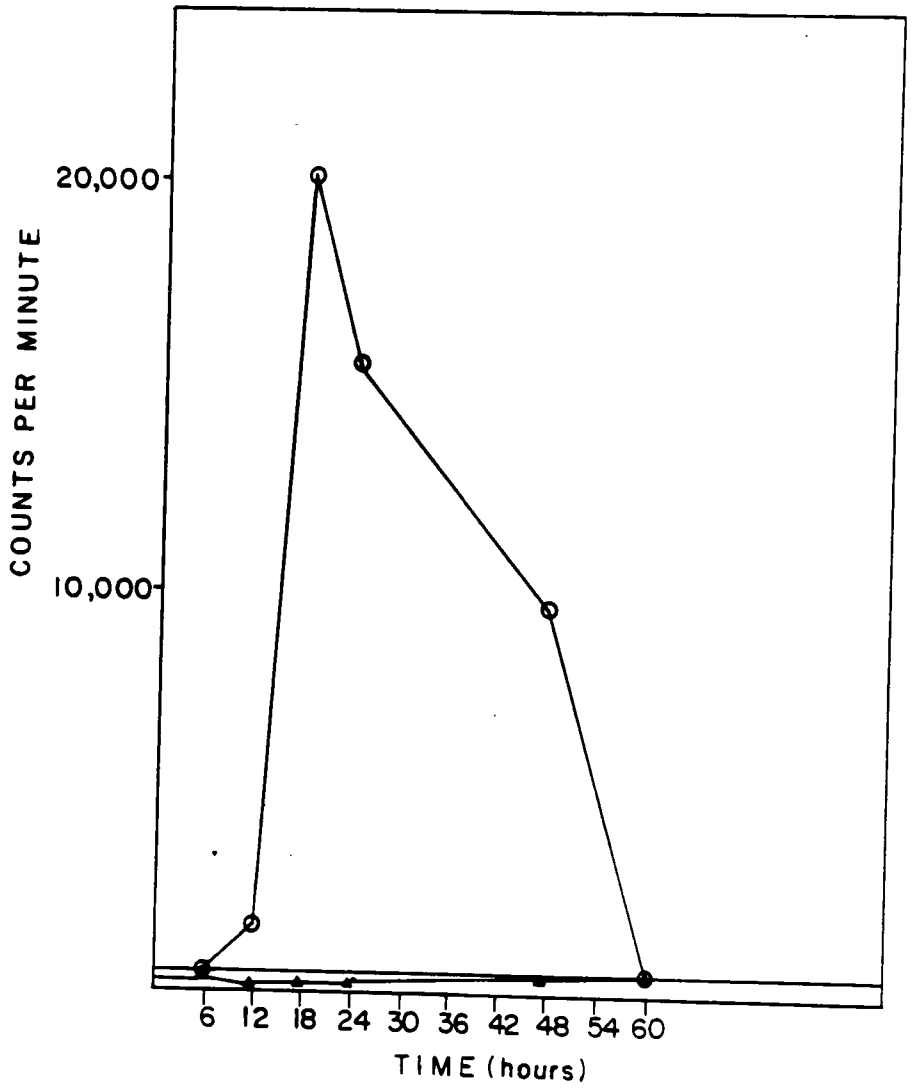

Figure 6. Time course of IL 2 Production After Restimulation of HTL-P Clones.

Five day immune LN responders were set up in culture of 1 x 10^6 cells and were stimulated with 1 x 10^6 T-depleted, X-irradiated, HSV-1 stimulated splenocytes (o) or 1 x 10^6 T-depleted, X-irradiated splenocytes (▲) for 9 days. Cultures were restimulated as before and 3 replicate supernatants were removed at various time points after restimulation and titrated for IL 2 activity with CTLL cells.

was, therefore, used in all limiting dilution experiments to restimulate HTL-P prior to testing supernatant fluids for IL 2 activity.

HSV specific limiting dilution assay

Once the optimal assay conditions for the virus-specific HTL-P limiting dilution assay were determined (Figure 7), this assay was used to estimate the frequency of HSV-1 specific HTL-P. Representative data points for a typical limiting dilution assay are shown in Figure 8. As was shown for the alloreactive HTL-P assay, as responder numbers increase, a higher percentage of wells positive for IL 2 activity could be detected. A semilogarithmic plot of the fraction of negative cultures for each responder group containing positive and negative wells is shown in Figure 9. This data demonstrated that limiting conditions of a single parameter were met. According to Poisson statistics, the frequency of HTL-P was estimated to be 1/4492. Table V gives frequencies for 9 such HSV-specific limiting dilution assays. Figure 9 also shows data demonstrating that if responder LN cells were depleted of Lyt 2 positive cells prior to *in vitro* culture, the HTL-P frequency was increased to 1/1319. Data for 3 typical limiting dilution assays is shown in Table VI.

Antigen requirements in vitro for HTL-P detection

To look at the requirements for HTL-P clonal expansion, LN responders were cultured with HSV-1, influenza or normal stimulator splenocytes for the 9 day expansion period. Cultures were then washed and restimulated with HSV-1-stimulators and the 24 hour supernatant fluids were tested for IL-2 activity. As Figure 10 shows, stimulation

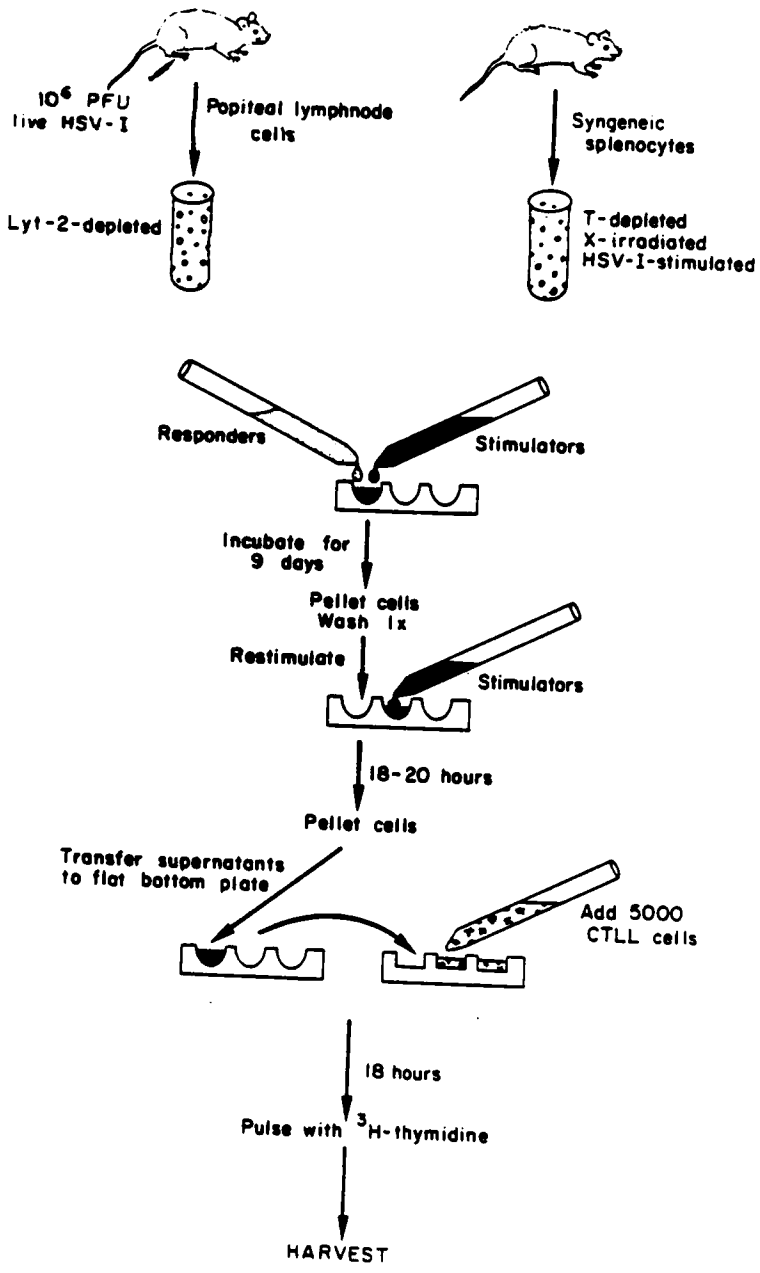

Figure 7. Limiting Dilution Assay Protocol for the Determination of HSV-1 Specific HTL-P.

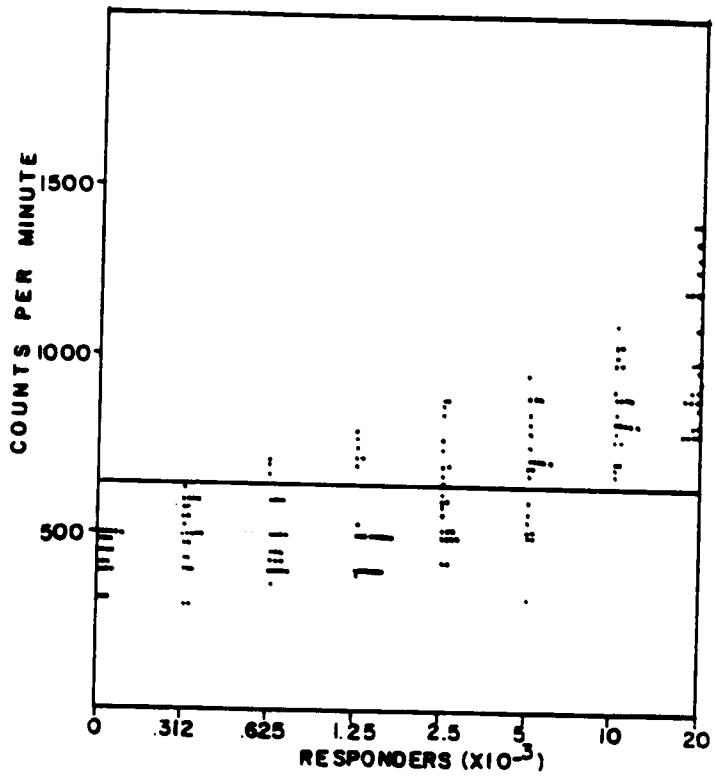

Figure 8. HSV-1 Specific HTL-P Limiting Dilution Assay: CTLL Proliferation by Individual Microculture Supernatant Fluids.

Proliferation of CTLL cells induced by coculturing with supernatant fluids from cultures containing HSV-stimulators plus varying numbers of responders. Each point represents an individual microculture. Line indicates 3 standard deviations above the mean of wells containing zero responder cells.

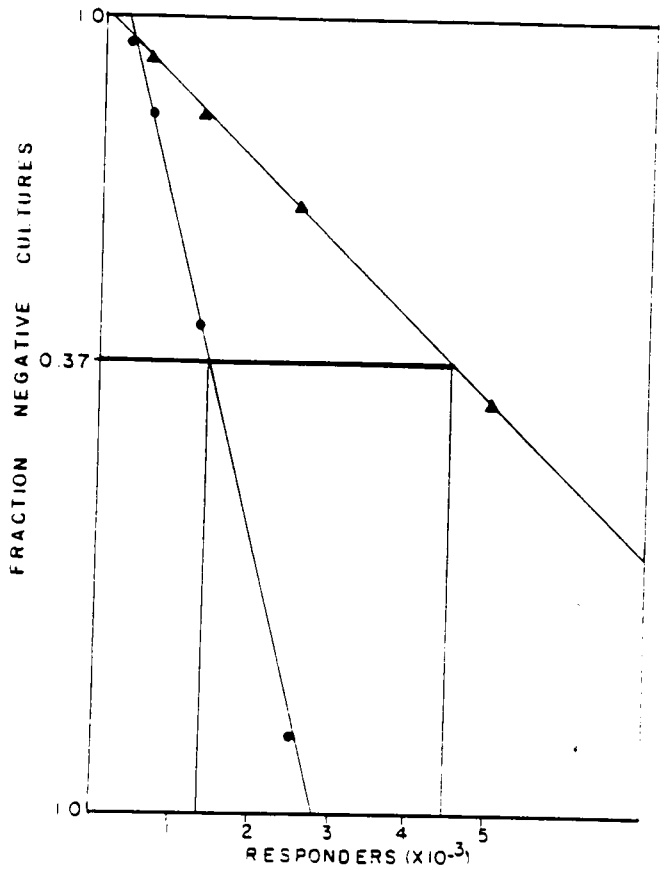

Figure 9. HSV-1 Specific HTL-P Frequencies for Untreated or Lyt 2-Depleted Lymph Node Cells.

Typical limiting dilution assay to estimate the frequency of HSV-specific IL-2-producing HTL-P in regional lymph node (LN) cells after 5 day priming with live HSV-1. Proportion of non-responding cultures for each responder group of 24 replicates was plotted logarithmically (y axis) against responder cell input (x axis) the best fitting line was drawn by the least squares method and the precursor frequency of untreated lymph node cells (▲) or Lyt 2 depleted cells (●) was estimated by Poisson analysis.

▲ : f = 1:4992; r^2 = 0.99; y intercept = 1.0

● : f = 1:1350; r^2 = 0.99; y intercept = 1.2

Table V. HSV-1 Specific Limiting Dilution Assays

Experiment	Frequency	R^2 value	Y intercept
1	1:3102	0.96	1.05
2	1:4400	0.97	1.1
3	1:5147	0.95	1.03
4	1:4492	0.99	1.0
5	1:7740	0.98	1.2

HSV-1 stimulators were cocultured with untreated 5 day immune responder cells for 9 days, cultures were washed and then restimulated. Supernatant fluids were titrated for IL 2 activity and HSV-1 specific HTL-P frequencies were determined using Poisson statistics.

Table VI. Lyt 2 Depletion of Responder Cells Leads to Enhanced HTL-P Frequencies

Experiment	Lyt 2 depletion	Frequency	R^2 value	Y intercept
1	-	1:4400	0.97	1.1
	+	1:1510	0.94	1.1
2	-	1:5147	0.95	1.03
	+	1:2473	0.98	1.05
3	-	1:4492	0.99	1.0
	+	1:1350	0.99	1.2

Immune LN responder cells were either untreated or Lyt 2 cell depleted prior to coculturing with HSV-1 stimulators. After 9 days cultures were washed, restimulated and supernatant fluids were titrated for IL 2 activity. Poisson statistics was used to determine HSV-1 specific HTL-P frequencies.

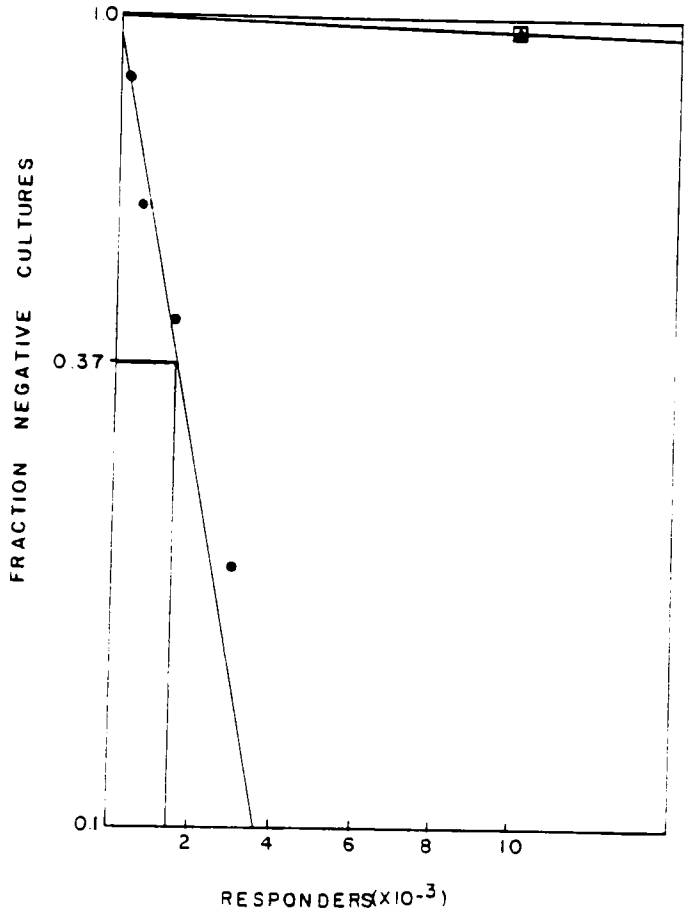

Figure 10. HSV is Required for In Vitro Clonal Expansion.

Live virus primed LN responders were clonally expanded with HSV-1 (●), influenza (▫) or normal (▲) stimulators for 9 days. Responders were restimulated with HSV-1 stimulators for 24 hours and HSV-1 specific HTL-P frequencies were estimated by Poisson analysis.

- ●: f = 1:1513, r^2 = 0.992; y = 0.92
- ▫: frequency not determinable
- ▲: frequency not determinable

with HSV-1 provided a frequency estimate of 1:1513. However, neither influenza- nor normal-stimulators generated sufficient positive cultures to permit an estimation of HTL-P frequencies. Data for 3 such experiments are shown in Table VII. Thus, in vivo primed helper cells show strict antigen requirements for clonal expansion of HTL-P in vitro. This contrasts with HSV-specific CTL precursors where two frequencies of cells were detectable in 5 day immune LN populations: an antigen dependent population with a frequency of approximately 1/2500 and an antigen independent population of approximate frequency 1/25,000.

Antigen requirements were also tested for during the second 24 hr stimulation after the initial 9 days expansion with HSV-1 stimulation. As shown in Figure 11, only cultures restimulated with HSV-1 stimulators gave detectable frequencies (1/3073). Those stimulated with normal stimulators or influenza stimulators produced zero or very few positive cultures precluding estimation of HTL-P frequency. Data for 3 experiments are listed in Table VIII. Thus the induction of IL-2 by HSV-1 expanded HTL is antigen specific.

Our data can provide no clues about the viral antigens of HSV-1 responsible for HTL-P stimulation but presumably they are shared with HSV-2 since HSV-1 primed LN cells stimulated with HSV-2 gave HTL-P frequency estimates of the same magnitude and those obtained following HSV-1 stimulation (Figure 12). Frequencies for 2 experiments are shown in Table IX. Thus HSV generated HTL-P are virus-specific but cross-reactive between HSV-1 and HSV-2.

Table VII. HSV-Specificity of HTL-P Clonal Expansion

Experiment	Stimulators	Frequency	R^2 value	Y intercept
1	HSV-	1:1513	0.9992	0.92
	Influenza-	*		
	Normal-	*		
2	HSV-	1:2056	0.95	1.1
	Influenza-	*		
	Normal-	*		
3	HSV-	1:3018	0.96	0.95
	Influenza-	*		
	Normal	*		

*Not determinable.

Immune LN responder cells were cocultured with HSV-1, influenza- or normal stimulators for 9 days to clonally expand HTL-P. Cultures were then washed, restimulated with HSV-1 stimulators and supernatant fluids were tested for IL 2 activity. Poisson statistics was used to determine HSV-1 specific HTL-P frequency.

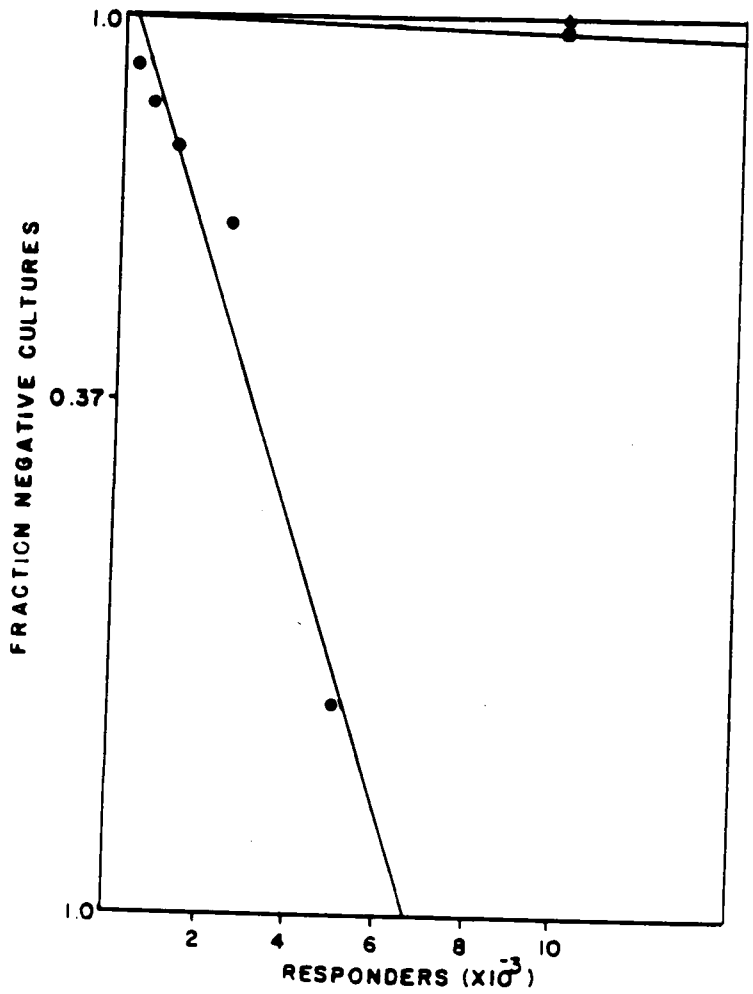

Figure 11. HSV-1 is Required for In Vivo IL 2 Production by Expanded Clones.

Live virus primed LN responders were clonally expanded with HSV-1 stimulators for 9 days and then restimulated with HSV-1 (●), influenza (■) or normal (▲) stimulators for 24 hours. HSV-1 specific HTL-P frequencies were estimated using Poisson analysis.

- ●: f = 1:3073, r^2 = 0.97, y = 1.06
- ■: frequency not determinable
- ▲: frequency not determinable

Table VIII. HSV-Specificity of HTL-P Clone IL-2 Production

Experiment	Stimulators	Frequency	R^2 value	Y intercept
1	HSV-	1:3073	0.97	1.06
	Influenza-	*		
	Normal-	*		
2	HSV-	1:4166	0.95	1.1
	Influenza-	*		
	Normal-	*		
3	HSV-	1:3802	0.99	1.18
	Influenza-	*		
	Normal	*		

*Not determinable.

Immune LN responder cells were cultured with HSV-1 stimulators for 9 days. Cultures were washed, stimulated with HSV-1-, influenza- or normal-stimulators. Supernatant fluids were titrated for IL 2 activity and Poisson statistics was used to determine HSV-1 specific HTL-P frequencies.

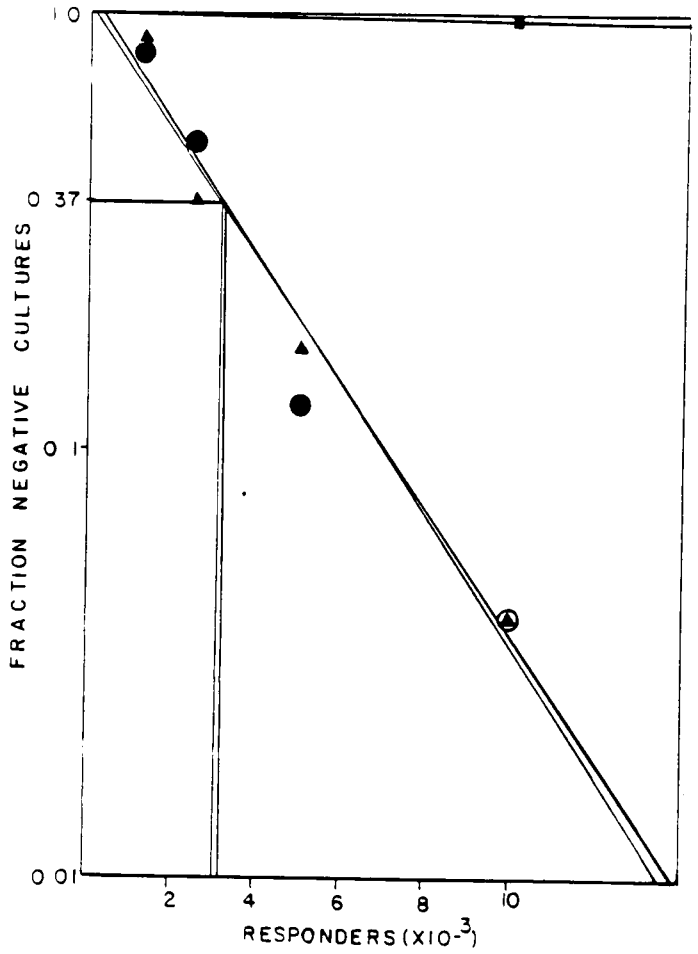

Figure 12. HSV Type Cross Reactivity of In Vivo HSV-1 Primed HTL-P.

HSV-1 primed LN responders were clonally expanded with HSV-1 (●), HSV-2 (▲) or normal (■) stimulators for 9 days and then restimulated with the same stimulator population as above for 24 hours. HTL-P frequencies were estimated using Poisson analysis.

● : f = 1:3102; r^2 = 0.98; y = 1.09
▲ : f = 1:3099; r^2 = 0.99; y = 1.05
■ : frequency not determinable

Table IX. HSV-1 vs. HSV-2 In Vitro Induction

Experiment	Stimulators	Frequency	R^2 value	Y intercept
1	HSV-1	1:3102	0.98	1.09
	HSV-2	1:3099	0.99	1.05
2	HSV-1	1:2473	0.98	0.94
	HSV-2	1:2341	0.99	1.2

Immune LN responder cells were cultured with HSV-1 or HSV-2-stimulators for 9 days. Cultures were washed, restimulated with the same stimulator and supernatant fluids were titrated for IL 2 activity. Poisson statistics was used to determine HTL-P frequencies.

Cellular nature of HSV-specific HTL-P

To identify the responsive cell type in our assay, microcultures were expanded for 9 days under conditions where approximately 30% of cultures were expected to be positive (and so according to Poisson statistics had a high likelihood of being the expanded progeny of a single HTL-P). Cultures were then split and treated with either complement alone or various antisera plus complement. After such treatment, cultures were incubated for 24 h with HSV stimulators and IL-2 was quantitated by CTLL proliferation. As shown in Figure 13, whereas treatment with anti-Thy 1.2 and anti-Lyt 1.1 reduced levels of CTLL proliferation to background levels, treatment with anti-Lyt 2.1 and anti-asialo GM1 had little effect. Thus the HSV specific HTL-P was assumed to be a Lyt 1+2- T lymphocyte.

Inactivated virus fails to prime HTL-P

Once our assay was developed and the *in vitro* requirements of the HTL-P established, we wished to demonstrate the utility of this approach to assess various forms of immunization. We first looked at the ability of inactivated virus to stimulate help. Responder cells were obtained from mice immunized with live or UV-inactivated HSV-1. Lymph node cells were cultured with HSV-1-stimulators for a 9 day expansion period, washed and restimulated. Then 24 hour supernatant fluids were tested for IL-2 activity. As Figure 14 shows, responder cells from mice primed with live virus gave an HTL-P frequency of 1:3400 while cells from mice primed with inactivated virus gave insuf

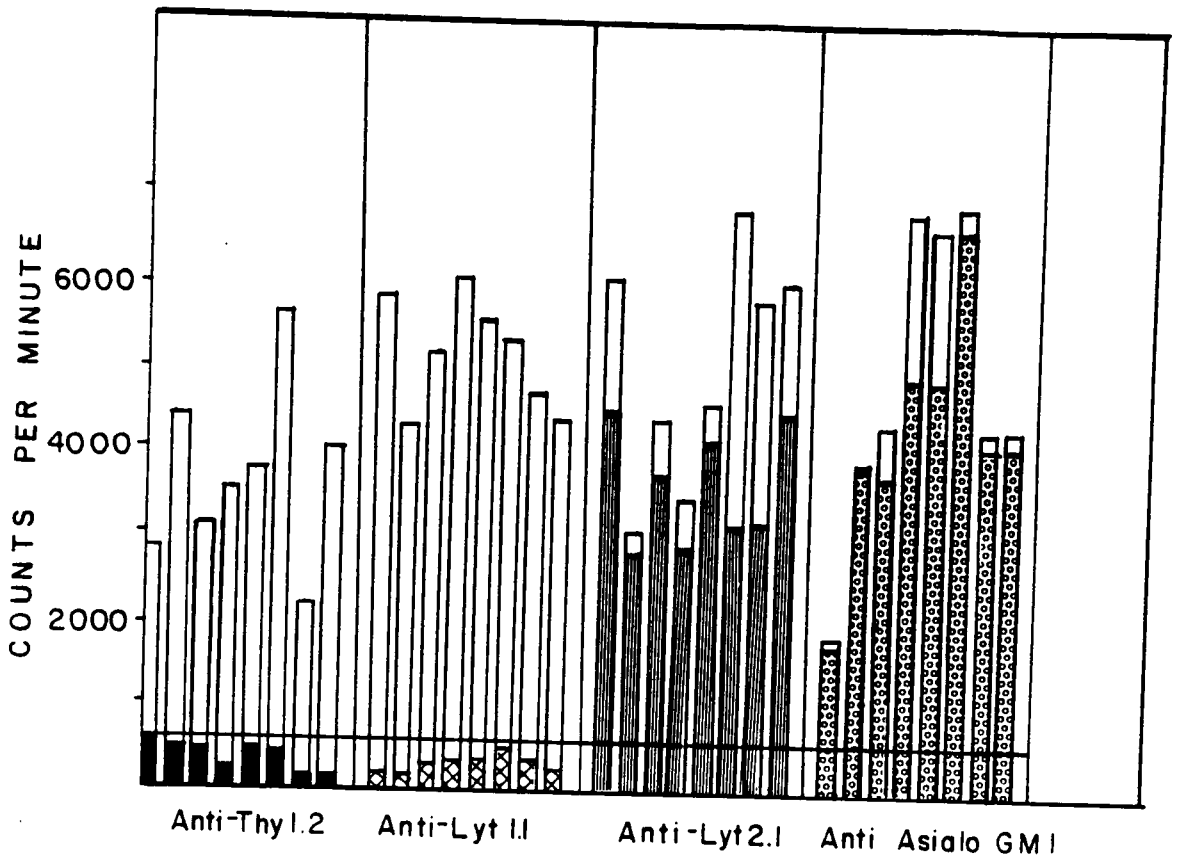

Figure 13. Cellular Nature of HSV-Specific HTL-P.

Characterization of the cell type of the HSV-specific HTL-P. Live virus primed responder LN cells (40,000 per well) were clonally expanded with HSV-1 stimulators for 9 days. Cultures were washed, split and corresponding wells were treated with antiserum plus complement (shaded bars) or complement alone. Cultures were then restimulated with HSV-1 stimulators for 24 hours and supernatants were titrated for IL-2 activity using the CTLL cell line. Line represents mean proliferation of 24 wells containing no responders.

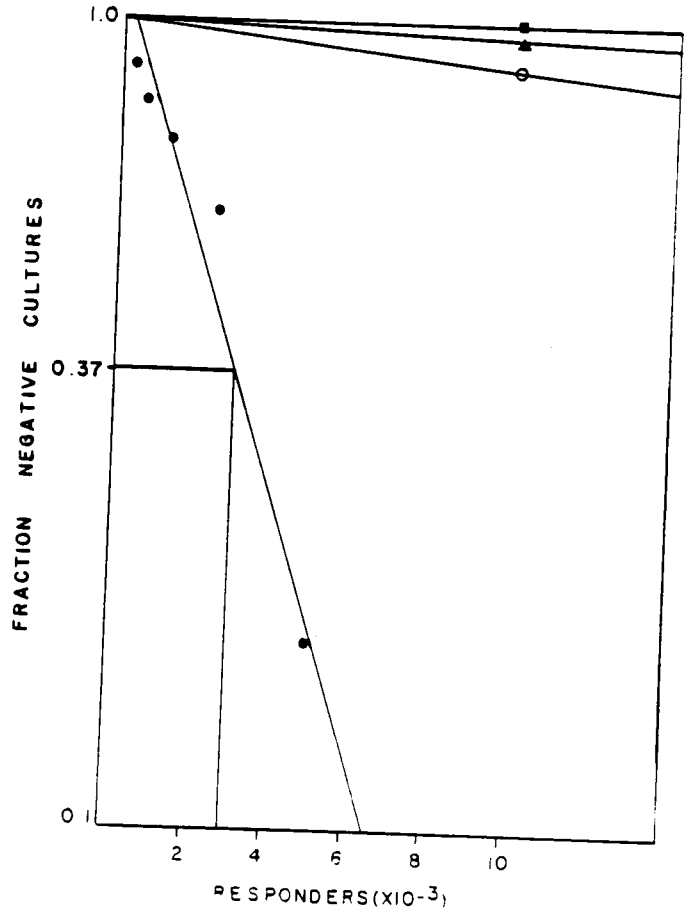

Figure 14. HSV-1 In Vivo Requirements.

LN cells from mice immunized with 10^6 PFU live (●), UV inactivated (○), heat-inactivated (▲) HSV-1 or 100 ug cell free HSV-1 glycoprotein extract (■) were used as responders in a typical limiting dilution assay and HSV-1 specific HTL-P frequencies were estimated using Poisson analysis.

- ● : f = 1:3073, r^2 = 0.97, y = 1.06
- ○ : frequency not determinable
- ▲ : frequency not determinable
- ■ : frequency not determinable

ficient HTL-P to permit estimation of their frequency. Data from 4 such experiments is found in Table X.

Soluble glycoproteins also fail to stimulate help

To further analyze the usefulness of our approach to study vaccine preparations, we assessed the ability of soluble HSV-1 glycoprotein extracts to stimulate help. Responder cells were obtained from mice primed with either live HSV-1 or a mixture of glycoproteins kindly donated by R. J. Courtney. Lymph node cells were cultured with HSV-1-stimulators for 9 days, restimulated and 24 hour supernatants were tested for IL-2 activity. As Figure 14 and Table X also illustrate, mice primed with soluble viral glycoproteins failed to generate detectable helper cells.

Table X. Live Virus Requirement for *In Vivo* Induction

Experiment	HSV-1 preparation	Frequency	R^2 Value	Y Intercept
1	Live	1:5147	0.97	1.05
	UV	*		
	HI	*		
2	Live	1:3400	0.99	1.2
	UV	*		
	HI	*		
3	Live	1:5892	0.98	1.16
	UV	*		
	HI	*		
4	Live	1:3915	0.98	0.92
	UV	*		
	HI	*		

*Not determinable.

LN responder cells from mice immunized with live-, UV-inactivated or heat-inactivated HSV-1 or a cell free extract containing HSV-1 glycoproteins were cultured with HSV-1 stimulators for 9 days. Cultures were washed, restimulated and supernatant fluids were titrated for IL 2 activity. Poisson statistics was used to determine HTL-P frequencies.

V. DISCUSSION

This report presents the first investigation to use limiting dilution analysis to estimate the frequency of virus-specific IL 2 producing helper T lymphocyte-precursors. The optimal assay parameters necessary to quantitate HSV-specific HTL-P were established and then used to assess the *in vitro* and *in vivo* induction requirements and the cellular characteristics of the HSV-1 specific HTL-P. The utility of this approach in the evaluation of various viral preparations to stimulate a helper cell response was also demonstrated.

Preliminary experiments were done in order to modify the alloreactive HTL-P limiting dilution assay developed by Kronke *et al.* (48). Viral stimulators were used in the form of T cell depleted, X-irradiated, HSV-1 stimulated syngeneic splenocytes. Responder cells containing the putative HTL-P were not detectable in normal mice, as are alloreactive HTL-P, but were readily detectable in the regional lymph nodes of mice primed 5 days earlier with live HSV-1. The optimal clonal expansion period of HTL-P clones was longer than that of most alloantigen systems, however when detecting alloreactive HTL-P in athymic mice, a 14 day clonal expansion period was needed (59). In this instance IL-2 was added to cultures which presumably aided cell survival. Perhaps a longer clonal expansion period, along with concommitant *in vitro* administration of IL-2, would lead to an enhanced HSV-specific HTL-P frequency, or the detection of HTL-P that do not require a secondary *in vitro* antigen stimulation (discussed subsequently).

The HSV-1 specific HTL-P frequency was determined to be 1:2470 to 1:5800. If responder cells were treated with anti Lyt 2 antiserum plus complement, according to the limiting dilution protocol for allo-antigen-specific HTL-P developed by MacDonald and Lees (59), a 2-3 fold increase in the HTL-P frequency resulted. This could be explained in two ways. One possibility is that by removing Lyt 2 positive cells from the responder pool, an enrichment of IL-2-producing Lyt 1 positive cells would lead to enhanced HTL-P frequencies. Since the proportion of Lyt 2 positive cells in the regional lymph node is only on the order of 10-20%, this possibility could only explain a slight increase in the frequencies detected. A second possibility is that during Lyt 2 cell depletion, a suppressor cell is removed from the responder population. The action of this suppressor cell could be to passively absorb IL-2 as it is produced thereby decreasing the levels of detectable IL-2 by our assay system. Alternatively an active form of suppression might occur in which case questions warranting attention are whether the suppressor mechanism exerts its effects at the level of the antigen presenting cell--HTL-P interaction or by inhibiting HTL-P clonal expansion or perhaps by suppressing IL-2 production by HTL-P clones.

The in vitro induction of HSV-1 specific HTL-P was addressed using this approach and it was found that HSV was required in vitro both during HTL-P clonal expansion and again during restimulation of clones to produce detectable frequencies. These results suggest a stricter requirement for in vitro HTL-P induction than for CTL-P induction, as a population of low frequency CTL-P can be detected under assay conditions where clonal expansion occurs in the absence of antigen, if

growth factors are provided (88). Other experiments demonstrated that responder cells from HSV-1 primed mice could be *in vitro* expanded and restimulated with HSV-2 as well as HSV-1, which suggested that shared epitopes of types 1 and 2 might be presented to HTL-P by antigen presenting cells in a similar fashion. Which of the HSV epitopes actually stimulate the virus-specific HTL-P is an interesting question needing evaluation as the interest in subunit vaccines grows.

The cellular nature of the HSV-specific HTL-P was studied. The helper cell-precursor was found to be a Thy 1 positive, Lyt 1 positive T cell. This insured that we were not detecting the presence of an IL-2 producing Lyt 2 positive cell, as has been described for some alloantigen systems (42) or an IL-2 producing natural killer cell which has recently been described by Kasahara *et al*. (41). With the suggestions by various groups for the ability of cells other than helper cells to produce IL-2, further studies to definitively prove that this IL-2-producing cell is indeed a helper cell may need to be done. Limiting dilution assays which were developed for alloreactive HTL-P to provide help to both antibody producing cells and CTL would be appropriate to determine the functional ability of the HTL-P described here to provide help in these systems.

The usefulness of this limiting dilution approach to evaluate the ability of virus preparations to stimulate a helper cell response was demonstrated in experiments to assess the ability of live or inactivated virus prepartions to prime for HSV-1 specific HTL-P. It was demonstrated that only when mice were primed with live HSV-1 were detectable HTL-P frequencies obtained. Mice primed with heat-inactivated or UV-

inactivated virus failed to stimulate help. These results confirm earlier work performed in this laboratory which demonstrated the inability of inactivated virus to prime for anti-HSV CTL activity (93).

One explanation for the failure of inactivated virus to stimulate helper cell induction may be the obvious suggestion that nonreplicating forms of virus fail to provide sufficient antigenic mass to appropriately stimulate antigen presenting cells (APC). Another possibility is that such forms of virus are unable to interact with class II antigens on APC but selectively interact with class I antigens, leading to in vitro induction of CTL-P but not HTL-P, as has been previously shown (93).

Cell free HSV-1 glycoprotein extracts were also shown to be unable to prime for detectable HTL-P. This also confirms earlier work from this laboratory which demonstrated that soluble glycoproteins failed to stimulate a CMI response, even when placed on the surface of liposomes (52).

In the efforts of many to enhance antiviral immunity, methods of selectively stimulating various components of the cellular response need to be determined. Since the helper cell plays a critical role in regulating this response, means of priming for HTL are essential in the development of anti-HSV vaccines. In light of the fact that acceptable vaccines will most likely be subunit in nature, methods of stimulating detectable HTL-P frequencies after glycoprotein immunizations need to be found. The use of acceptable adjuvants or glycoproteins in conjunc-

tion with more immunogenic liposomes, might lead to better stimulation of the HTL.

The limiting dilution approach is a useful tool to assess the ability of a potential vaccine to stimulate the helper cell component of cellular immunity. If the failure of potential vaccines is shown to be a failure to stimulate a helper cell response, administration of IL-2 in conjunction with such immunizations might lead to enhanced HSV responses, as has been recently suggested by our laboratory (Rouse, in press).

VI. CONCLUSIONS

1. The frequency of helper T lymphocyte-precursor specific for herpes simplex virus type 1 in the regional lymph node is 1:3102 to 1:7740.
2. The frequency of HTL-P is increased 3-fold by depletion of responder cell populations of Lyt 2^+ cells.
3. HSV is required *in vitro* for both precursor clonal expansion and then restimulation to detect HSV-specific HTL-P.
4. HTL-P stimulated by *in vivo* priming with HSV-1 may be *in vitro* stimulated with HSV-1 or HSV-2 to give similar frequencies of HSV-specific HTL-P.
5. Live virus is necessary to *in vivo* stimulate HSV-specific HTL-P; inactivated virus or HSV glycoprotein extracts fail to stimulate HTL-P.

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VITA

Debra Prymowicz was born in Summit, N.J. She attended elementary schools in Garwood and Westfield, N.J. and graduated from Westfield High School in June, 1978.

The following September she entered Lafayette College, Easton, PA and in May, 1982 she graduated with honors with a Bachelor of Arts Degree in Biology.

In the fall of 1982, she began work towards a Master's degree in the Microbiology Department of The University of Tennessee, Knoxville. This degree was awarded in December, 1984.