JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1989, p. 2316–2323
0095-1137/89/102316-08$02.00/0

Vol. 27, No. 10

Enumeration of Viral Antigen-Reactive Helper T Lymphocytes in Human Peripheral Blood by Limiting Dilution for Analysis of Viral Antigen-Reactive T-Cell Pools in Virus-Seropositive and Virus-Seronegative Individuals†

KATHLEEN A. CLOUSE,[1] PATRICK W. ADAMS,[2] AND CHARLES G. OROSZ[2,3]*

Department of Microbiology, Georgetown University, Washington, D.C. 20007,[1] and Therapeutic Immunology Laboratories, Department of Surgery, Division of Transplantation,[2] and Comprehensive Cancer Care Center,[3] The Ohio State University, Columbus, Ohio 43210

Received 3 April 1989/Accepted 13 July 1989

A limiting-dilution analysis technique was developed which enumerates human T cells with the capacity to secrete T-cell growth factors such as interleukin 2 after contact with herpes simplex virus type 1 (HSV-1) or cytomegalovirus (CMV) antigens (operationally defined as virus-reactive helper T cells [HTL]). By using this limiting-dilution analysis technique, the peripheral blood of HSV-seropositive individuals was analyzed for the frequency of HSV antigen-reactive HTL and for the ability either to proliferate or to secrete detectable T-cell growth factors in conventional HSV antigen-stimulated lymphocyte cultures. We found that the magnitudes of the latter two responses did not correlate directly with the frequency estimates of HSV antigen-reactive HTL. The study was expanded to analyze both HSV and CMV reactivities within individuals. Those who were seropositive for HSV or CMV were found to have relatively high HTL frequencies for the viral antigens to which they were sensitized. However, those who were seronegative for one of the viruses often had HTL reactive with that virus in their peripheral blood. These latter HTL frequencies were highly variable and ranged from undetectable to quite prominent, even within the same individual at different times. In general, it was found that viral antigen-reactive serologic activity does not necessarily reflect the status of viral antigen-reactive cell-mediated immunity in humans and that viral antigen-induced T-cell responses may be unexpectedly complex, rather than absent, in individuals who are seronegative for a particular virus.

Herpes group viruses can cause severe, life-threatening complications in individuals whose viral immunity is genetically (44) or pharmacologically (22) compromised. Clinical assessment of immunity to herpes simplex virus (HSV) usually involves a serologic analysis of peripheral blood for the presence of HSV-reactive antibodies. In general, the presence of such antibodies (i.e., a seropositive response) is considered to be evidence of existing antiviral immunity. In contrast, a seronegative response is generally considered to indicate an individual who is susceptible to a viral infection due to an apparent lack of protective antiviral immunity. Although specific antibodies can protect against a subsequent viral challenge (1), humoral immunity (serologic status) does not reflect the ability to recover from established infections (11) or to protect against reactivation of latent herpesviruses (28). Furthermore, clinical observations have implicated cell-mediated immunity as an essential component for recovery from viral infections caused by herpes group viruses (14). The extent to which viral antigen-reactive cell-mediated immunity correlates with serologic status is even more uncertain (39, 46).

T cells appear to play a major role in the resolution of viral infection. In vitro studies have shown that (i) human peripheral blood mononuclear cells (PBMC) from sensitized individuals can be induced to proliferate after exposure to viral antigen (19, 34, 39, 46), (ii) viral antigen-specific cytolytic T lymphocytes can be generated from sensitized PBMC (32, 36) and have been cloned (45), and (iii) PBMC from sensitized individuals secrete lymphokines, including interleukin 2 (IL-2), after contact with viral antigens (8, 15). Thus, in vitro evidence suggests that the cellular immune responses to these viruses involve both helper T lymphocytes (HTL) and cytotoxic T lymphocytes (12, 17).

In most of these studies, assessment of T-lymphocyte reactivity has been nonquantitative, due primarily to the technical design of the available assays. For example, detection of proliferation, lymphokine secretion, or cytotoxic activity in mixed lymphocyte-virus cultures demonstrates the presence of viral antigen-reactive lymphocytes but provides little information regarding the size or efficiency of the responding T-cell population. Furthermore, active T lymphocytes are not detectable in such cultures if they did not constitute a relatively large proportion of the responding population (31), and their detection may be influenced by suppressive multicellular interactions (23). An assay of T-lymphocyte behavior which would circumvent these problems is limiting-dilution analysis (LDA). LDA can detect T lymphocytes with defined antigen specificity and functional capacity even when they are present in very small numbers. In addition, some (20, 23, 24) but not all (40) LDAs have been shown to be insensitive to regulatory mechanisms functioning in other culture systems. Furthermore, the LDA is a quantitative immunologic technique that monitors T-cell numbers and thus allows analysis of changes in the sizes of specific T-lymphocyte subpopulations.

Although LDAs of antigen-specific cytotoxic T lymphocytes (5, 30) and HTL (26, 29) have been reported (5, 30),

* Corresponding author.

† Paper no. 34 from the Therapeutic Immunology Program, Department of Surgery, Division of Transplantation, The Ohio State University, Columbus.

histocompatibility antigens, rather than viral antigens, have been the topic of these studies. Since HTL apparently orchestrate a variety of immune responses, including cytotoxic T-lymphocyte generation, we have developed an LDA technique which enumerates HSV type 1 (HSV-1) antigen-reactive or cytomegalovirus (CMV) antigen-reactive T lymphocytes that can secrete T-cell growth factors (TCGF) and are thus operationally defined as HTL. Using this technique, we have found that approximately 0.1 to 0.01% of PBMC from individuals who are seropositive for HSV-1 have the capacity to secrete TCGF upon contact with viral antigen. However, seronegativity did not necessarily preclude cellular HSV antigen reactivity in the LDA. Similar observations were made regarding reactivity to CMV antigen.

MATERIALS AND METHODS

Virus. Stock HSV-1 strain F, produced in human epidermoid carcinoma no. 2 cells, was kindly provided by Patricia Spear of the University of Chicago, Chicago, Ill. CMV strain Towne that was grown in human foreskin fibroblasts, sucrose gradient purified, and heat inactivated was kindly provided by Richard Gehrz, Virology Research Laboratory, St. Paul Children's Hospital, St. Paul, Minn.

Viral antigens. Purified HSV-1 was prepared by using the methods described by Gehrz et al. (13) and Spear and Roizman (38). Briefly, human foreskin fibroblast cells were infected with stock HSV-1 lysate (10^9 PFU/ml) at a multiplicity of infection of 2 PFU per cell and were incubated at 37°C until the cytopathic effect involved 90% of the monolayer. Supernatants from infected cells and the cytoplasmic extracts obtained after disruption of infected cells in a Dounce homogenizer were pooled and clarified by centrifugation at 7,500 × *g* for 20 min. The virus was sedimented from the clarified medium and concentrated by centrifugation at 131,000 × *g* for 1 h and then purified by rate zonal centrifugation in a 10 to 50% sucrose gradient at 131,000 × *g* for 1 h. The opalescent band containing the virions was aspirated and stored at −80°C until used. Control (mock) antigen was prepared in the same manner by using uninfected human foreskin fibroblast cultures. The purified HSV-1 antigen and mock antigen preparations contained 850 and 600 μg of protein per ml, respectively, as determined by the method of Lowry et al. (20). All viral antigen preparations (purified virus and cell lysates) were inactivated by exposure to UV irradiation from two G15T8 germicidal bulbs (General Electric Co., Waterford, N.Y.) for 4 min at a distance of 5 cm. No plaques were detected on a human foreskin fibroblast monolayer after inactivation. Viral antigens were then titrated to determine the optimal dilution for induction of lymphocyte proliferation.

Viral antibody determinations. Viral antibody titers to CMV and HSV-1 in normal donors were determined by the Clinical Immunology Laboratory at the Ohio State University by using a microdilution enzyme-linked immunosorbent assay. Serum antibodies determined by enzyme-linked immunosorbent assay were calculated from a standard curve, with values of >0.22 indicating sensitization.

Culture medium. The tissue culture medium (RPMI complete medium) used for these experiments was RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 5×10^{-5} M 2-mercaptoethanol, and 15% (vol/vol) human serum (heat-inactivated pooled human serum from nontransfused males) unless otherwise indicated.

Isolation of human PBMC. Mononuclear cells were isolated from the heparinized blood of normal individuals after Ficoll-Hypaque gradient centrifugation (4). The cells were washed three times with phosphate-buffered saline (PBS) and resuspended in RPMI complete medium.

Human monocytes. Human monocytes were isolated from PBMC after centrifugation on a discontinuous Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient by using a modification of the method of Ulmer and Flad (42). Briefly, PBMC (50×10^6 to 100×10^6) were suspended in 2.25 ml of Percoll solution (density, 1.080 g/ml) in a 17- by 100-mm sterile polypropylene tube (U600; Fisher Scientific Co., Pittsburgh, Pa.). The cell suspension was then overlaid with 1.5-ml portions of Percoll solutions of decreasing densities (1.070, 1.068, 1.066, 1.064, and 1.062 g/ml) followed by 0.75 ml of 1× Hanks balanced salt solution. Density gradient centrifugation was carried out at 390 × *g* for 30 min at room temperature in a swing-out rotor with an IEC table centrifuge (Fisher). The upper band located at the interface above the Percoll solution with a density of 1.062 g/ml contained the majority of the monocytes. These cells were carefully removed with a sterile Pasteur pipette, washed at 4°C with 1× Hanks buffered saline solution, suspended in RPMI complete medium containing 15% human serum, and kept on ice before experimental testing. This cell preparation contained >90% OKM1-positive accessory cells and <1.5% contaminating CD3-positive lymphocytes, with approximately 70% staining positive for nonspecific esterase.

Primary mixed leukocyte-virus culture. PBMC from HSV-seropositive or -seronegative donors were isolated as above. Responder cells (10^5) were cultured in complete medium alone or with various dilutions of UV-inactivated HSV-1 antigen in 200 μl of complete medium in U-bottomed microdilution wells. The cultures were incubated at 37°C in 5% CO_2 for 6 days, which was the optimum period as determined by preliminary kinetic studies. After an 8-h terminal pulse with [^{3}H]thymidine (1 μCi per well), the cells were harvested and counted, and the results from triplicate wells were expressed as mean counts per minute.

Assay for human TCGF production. TCGF production was detected in virus-stimulated cultures using TCGF-dependent murine CTLL-20 cells. This cell line is known to respond to human IL-2, but, like other lymphokine-dependent cell lines, its pattern of lymphokine reactivity is not completely understood. We presume that all lymphokines which are mitogenic for CTLL-20 cells are TCGF. Hence, we defined the lymphokine(s) detected in this study as TCGF without attempting to document the production of a particular growth factor, such as IL-2.

The CTLL-20 cells were maintained by weekly transfer of 10^4 viable cells to upright 25-cm^2 (Corning T4160) tissue culture flasks containing 10 ml of Dulbecco modified Eagle medium (GIBCO) supplemented with 5×10^{-5} M 2-mercaptoethanol, additional amino acids (35), 2% (vol/vol) fetal calf serum, and 5% supernatant from rat concanavalin A-induced splenocytes. Mixed leukocyte-virus cultures were set up in U-bottomed microdilution plates as indicated above, except in 100 μl of medium. After 24 h of incubation at 37°C in 5% CO_2, the plates were gamma irradiated (2,500 R) and 100 μl of Dulbecco modified Eagle medium containing 5% human serum and 10^3 CTLL-20 cells was added to each well of the irradiated plates, which were then reincubated for 24 h, including an 8-h terminal pulse with [^{3}H]thymidine (1 μCi per well). Control plates received no CTLL-20 cells. Thymidine incorporation was measured as indicated above.

Limiting-dilution microculture conditions. The limiting-

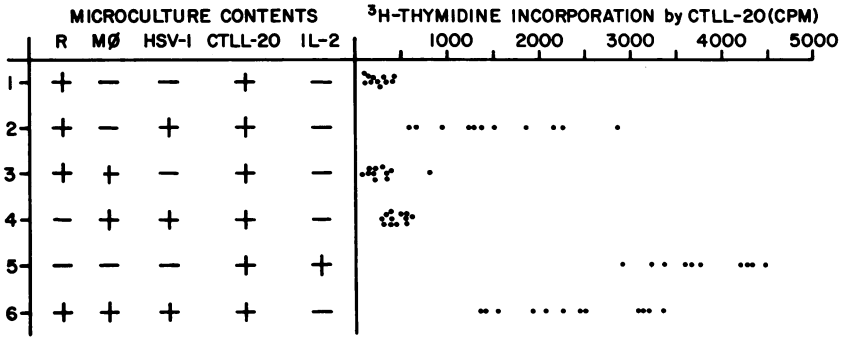

FIG. 1. Contribution of the various culture components to [^{3}H]thymidine incorporation in HSV antigen-reactive LDA microcultures. Various components of LDA microcultures were tested alone or in combination with other components for [^{3}H]thymidine incorporation. Timing of component additions (including [^{3}H]thymidine) and irradiation schedule were as described in Materials and Methods. The LDA components include responder cells (R; 20,000 PBMC per microwell), irradiated autologous monocytes (MØ; 500 Percoll-purified PBMC per microwell), HSV-1 antigen (0.8 μg of protein per ml), IL-2-dependent CTLL-20 cells (1,000 cells per microwell), and IL-2 (10% [vol/vol] human IL-2; Cell Products, Inc.). Shown is the level of [^{3}H]thymidine incorporation observed in each of the replicate microwells under each experimental condition.

dilution microculture conditions were adapted from a method of TCGF detection described previously (7, 8). Briefly, 25 μl of a dilution of responder PBMC in RPMI complete medium (usually in replicate sets of 24 microwells for each of eight twofold serial responder cell dilutions starting with 20×10^3 cells per well) were incubated with 5×10^2 autologous, Percoll-purified, gamma-irradiated (7,500 R) monocytes per well plus an appropriate dilution of viral antigen in a total volume of 75 μl. After 4 to 6 h, the microculture plates were gamma irradiated with 2,500 R and incubated overnight at 37°C in 5% CO_2. The following morning, 10^3 CTLL-20 cells in 25 μl of medium were added to each microdilution well. After 6 h of incubation, each microwell received 0.25 μCi of [^{3}H]thymidine. The microcultures were incubated for 8 h, and the contents of each well were aspirated onto glass fiber filters. The [^{3}H]thymidine incorporation was determined by scintillation spectrophotometry in a beta counter.

LDA. Minimal estimates of the HTL frequency were calculated by analysis of the Poisson distribution relationship between the number of responder cells added to the limiting-dilution microwells and the percent replicate microwells which failed to produce detectable TCGF (41). Limiting-dilution microcultures were considered positive for IL-2 production if [^{3}H]thymidine incorporation by CTLL-20 cells was greater than the [^{3}H]thymidine incorporation (mean plus 3 standard deviations) observed in 24 replicate microwells which contained LDA stimulator cells and CTLL-20 cells but no responder cells. Frequency calculations were made by using the zero-order term Poisson equation linearized to the form $\ln Y = -Fx + \ln A$, where x is the number of responder cells per well, Y is the percentage of negative microwells, F is the HTL frequency defined as the negative of the slope of the line, and A is the y-axis intercept. Experimental x and y values were fitted to the equation by chi-square minimization analysis (41). This analysis results in a frequency estimate ($1/f$), a frequency range corresponding to the 95% confidence limits of the frequency estimate (range), and the chi-square estimate of probability (P) for the frequency estimate. In these studies, a P value of >0.05 indicates that the data conform to the zero-order term Poisson equation and that the frequency estimate is statistically acceptable.

RESULTS

Description of the analytic system. We have described elsewhere (29) a limiting-dilution culture system that enables detection and quantitation of alloantigen-reactive HTL. We have adapted this protocol to allow detection of HTL (defined operationally by the capacity to secrete TCGF regardless of cell surface phenotype) that are reactive with specific viral antigens. The general design of the protocol was as follows. Dilutions of responder cells were cultured with viral antigens for a period sufficient to allow initial T-cell activation events to occur. The responder cells were then gamma irradiated to impair their ability to proliferate (9) while allowing them to continue lymphokine secretion (7, 43). To detect lymphokine secretion, CTLL-20 cells were added to the cultures and allowed to compete with responder cells for TCGF. When TCGF was present, the CTLL-20 cells proliferated, which could be detected by [^{3}H]thymidine incorporation. Since the responder cells were irradiated, they did not incorporate significant amounts of [^{3}H]thymidine. Hence, [^{3}H]thymidine incorporation in the LDA microcultures is indicative of TCGF production by the viral antigen-stimulated HTL, and thus is indicative of the presence of activated HTL.

In the allogeneic LDA, alloantigens are presented via foreign monocytes. Since the accessory cell requirement for alloantigen-driven responses can be met with either autologous or allogeneic monocytes (25), accessory cells are never limiting, even at high dilutions of the responder cell population. In contrast, T cells respond to viral antigen only in the presence of MHC-compatible accessory cells (18). Since accessory cells are usually present in lower numbers than T cells in human PBMC, accessory cells, rather than responder T cells, could theoretically become the limiting factor for T-cell function at high PBMC dilutions. To circumvent this problem, viral LDAs were supplemented with a concentration of autologous accessory cells that would not impair HTL function (6) but would facilitate TCGF production.

These theoretical considerations are supported by the data in Fig. 1, for which each component of the LDA microculture was tested alone or in combination with other components for the ability to contribute to [^{3}H]thymidine incorpo-

ration by CTLL-20 cells. In this series of experiments, PBMC from HSV-seropositive individuals were used as a source of responding lymphocytes. We observed no significant CTLL-20 cell proliferation in the presence of either responding PBMC alone (indicating lack of detectable spontaneous TCGF production) or isolated monocytes plus HSV antigen. Substantial [^{3}H]thymidine incorporation by CTLL-20 cells was observed in the presence of responding PBMC plus HSV antigen; responding PBMC plus gamma-irradiated, autologous monocytes and HSV antigen; or exogenous IL-2. At the relatively low responder cell dilution used in this experiment, autologous monocytes were not limiting, so HSV antigen-induced TCGF production occurred in the absence of additional monocytes. Nonetheless, the presence of additional monocytes did not interfere with TCGF production, but rather facilitated production and/or detection of TCGF in these microcultures. These data indicate that these culture conditions can be used to detect HSV antigen-induced TCGF production by human PBMC from HSV-seropositive individuals.

In the next series of experiments we used this protocol to establish limiting-dilution microcultures. Various dilutions of responding PBMC from HSV-seropositive individuals were cultured with a constant number of gamma-irradiated, autologous monocytes and a standard concentration of HSV antigen or mock viral antigen (see Materials and Methods) as a control. These microcultures were later gamma irradiated and overlaid with CTLL-20 cells, pulsed with [^{3}H]thymidine, and tested for [^{3}H]thymidine incorporation. The [^{3}H]thymidine incorporation in each microculture is shown in Fig. 2. In microcultures containing HSV antigen, the magnitude of [^{3}H]thymidine incorporation was directly related to the number of responding PBMC present. As the number of responding PBMC decreased, both the magnitude of [^{3}H]thymidine incorporation and the number of replicate microwells that developed significant [^{3}H]thymidine incorporation were diminished. The microwells were considered positive if [^{3}H]thymidine incorporation exceeded the mean plus 3 standard deviations of the incorporation in replicate microcultures containing all microculture components except responding PBMC. In contrast to HSV antigen-induced responses, few of the limiting-dilution microcultures containing mock viral antigen displayed positive [^{3}H]thymidine incorporation. Analysis of these data by the chi-square minimization method suggested by Taswell (41) yielded a frequency estimate (*f*), a 95% confidence interval for the estimate (range), and a chi-square evaluation of probability (*P*). The frequency of HSV-reactive PBMC in the individual under study was 1 cell per 12,390 PBMC, whereas the frequency of cells in the same PBMC population that could respond to the mock viral antigen was 1 cell per 536,407 PBMC (Fig. 3).

We next determined the relationship between the frequency of HSV antigen-reactive HTL in a PBMC population and the ability of that PBMC population to proliferate or secrete detectable TCGF in conventional lymphocyte cultures. For these experiments, PBMC from different HSV-seropositive individuals were concurrently evaluated for HSV-reactive HTL frequency by LDA and for HSV antigen-induced proliferation in conventional lymphocyte cultures. Some populations were also evaluated for HSV antigen-induced TCGF production in conventional lymphocyte cultures as detected by a procedure described elsewhere (8). Contact of PBMC with HSV antigen induced significant TCGF production and prominent proliferative responses in conventional lymphocyte cultures (Table 1). However, the

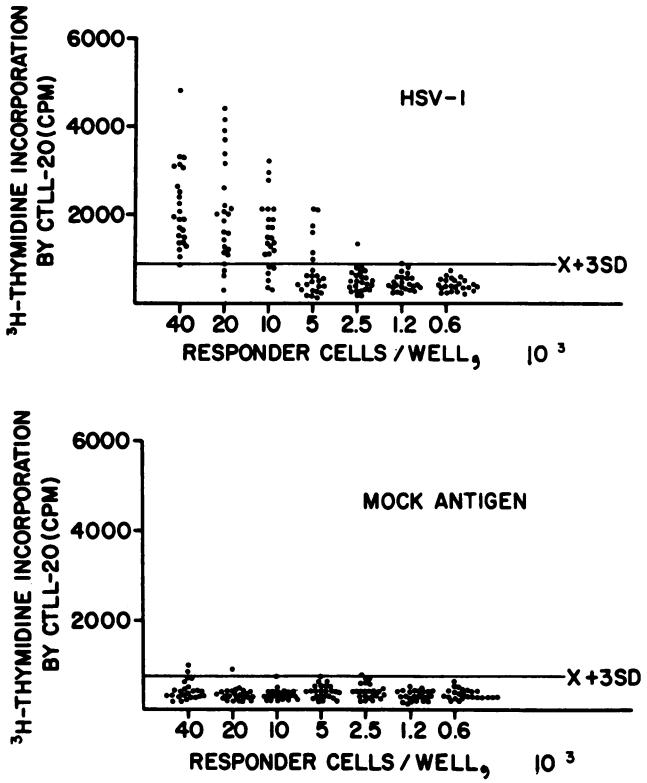

FIG. 2. [^{3}H]thymidine incorporation in HSV antigen-reactive limiting-dilution microcultures. Various dilutions of human PBMC from donor 5, ranging from 40,000 to 600 cells per well, were analyzed for HSV-1 antigen-induced IL-2 production according to the LDA protocol in Materials and Methods. Shown are the levels of [^{3}H]thymidine incorporation in each responder cell dilution in 24 replicate microwells when the LDA cultures were stimulated with HSV-1 antigen (0.8 μg of protein per ml) or mock antigen (0.8 μg of protein per ml) as indicated. Microwells were considered positive for [^{3}H]thymidine incorporation when levels exceeded the mean plus 3 standard deviations (X + 3SD) of [^{3}H]thymidine incorporation observed in 24 replicate microwells that contained all LDA microculture components except responder cells.

magnitude of these two responses did not directly correlate with the estimated frequency of HSV antigen-reactive HTL in the PBMC. This lack of correlation was not unexpected, given the quantitative nature of the LDA and the qualitative nature of the other two assays.

It has been reported that PBMC from seronegative individuals fail to proliferate when incubated in conventional lymphocyte cultures with appropriate viral antigens (3, 46). Our previous studies have shown that PBMC from HSV-seronegative individuals which fail to proliferate when stimulated in vitro with HSV antigen also fail to produce detectable levels of TCGF in high-density lymphocyte cultures (8). This suggests that HSV-seronegative individuals may lack HSV antigen-reactive HTL. If so, PBMC from individuals who are HSV seropositive should have LDA-detectable HTL, while PBMC from HSV-seronegative individuals should not. However, when the PBMC from two HSV-seronegative individuals were analyzed, the frequency of HSV-reactive HTL for one individual (no. 8) was significantly reduced relative to the number of HSV-reactive HTL detectable in the peripheral blood of HSV-seropositive individuals (Table 2). In contrast, a high HSV-reactive HTL

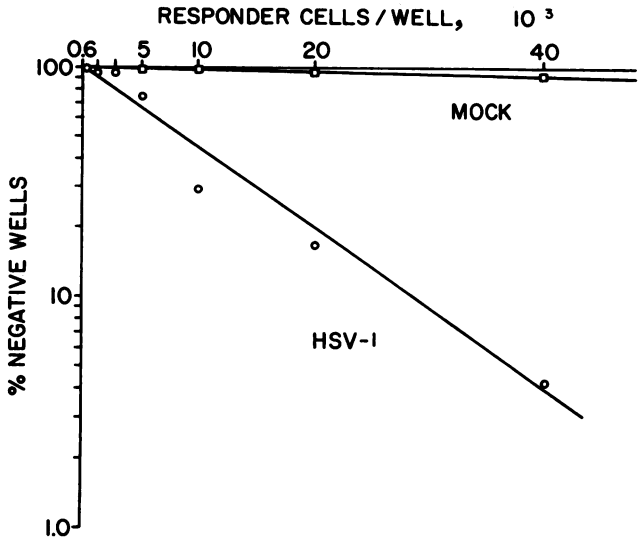

FIG. 3. Frequency analysis of [^{3}H]thymidine incorporation in limiting-dilution microwells. Data illustrated in Fig. 2 are redisplayed in a graph showing responder cells per microwell (linear scale) versus percent negative microwells (logarithmic scale). As determined by chi-square minimization analysis, these data result in the following frequency estimates. HSV-1 antigen: *f*, 1/12,390; range, 1/9,665 to 1/17,253; *P*, 0.2062. Mock antigen: *f*, 1/536/407; range, 1/260,695 to 1/9,312,016; *P*, 0.9879.

frequency was determined for the second seronegative individual (no. 9) in separate experiments and could not be attributed to variable LDA culture conditions. Repeated serum testing by enzyme-linked immunosorbent assay consistently failed to detect anti-HSV antibodies in the HSV-seronegative individuals (data not shown).

Analysis of the relationship between serologic status and HTL frequency. In order to examine the relationship between serologic status and HTL frequency more closely, we monitored the frequency of HTL with reactivity to CMV antigen along with the frequency of HSV antigen-reactive HTL. Since HSV and CMV are closely related herpes group viruses but are not cross-reactive with each other in conventional lymphocyte cultures (13, 46), individuals who are HSV seropositive and CMV seronegative should have LDA-detectable, HSV antigen-reactive HTL but not CMV antigen-reactive HTL. The reciprocal relationship should exist for individuals who are HSV seronegative and CMV seropositive. Experimental data confirming these relationships would also demonstrate the viral antigen specificity of the HTL that are detected by LDA.

To verify serologic status, sera from a number of healthy individuals were analyzed for HSV-reactive and CMV-reactive antibodies by using an enzyme-linked immunosorbent assay method. The PBMC from some of these individuals were then tested by LDA for frequency of HSV-reactive and CMV-reactive HTL. The optimal concentrations of CMV antigen (0.9 μg of protein per ml) and HSV antigen (0.8 μg of protein per ml) used in the limiting-dilution microcultures were determined in advance in separate experiments (data not shown). The PBMC were also tested for frequency of alloantigen-reactive HTL by using a protocol described in a separate communication (29).

The results of these experiments are shown in Table 3. In general, we observed that PBMC from individuals who were seropositive for either virus contained relatively high numbers of HTL that could react to appropriate viral antigens. However, in several experiments PBMC from individuals who were seronegative for either virus also had detectable numbers of HTL that could react to appropriate viral antigens. When these data were examined more closely, the

TABLE 1. Relationship between viral antigen-induced proliferative response, IL-2 production, and HTL frequency in human peripheral blood

Expt no.	Donor (patient no.)	Proliferation[a] (cpm, 10^3)	IL-2 production[b] (cpm, 10)	HTL frequency[c] (*f*)
1	1	25.8	12.0	1/432
2	2	27.4	13.5	1/4,481
3	3	16.4	6.1	1/2,090
4	4	8.3	ND[d]	1/1,442
5	5	7.7	ND	1/3,515
6	6	21.5	ND	1/3,485
7	7	32.8	ND	1/5,501

[a] [^{3}H]thymidine incorporation determined in primary mixed leukocyte-virus culture as described in Materials and Methods.
[b] [^{3}H]thymidine incorporation by CTLL-20 cells determined as described for assay of human IL-2 production in Materials and Methods and in reference 14.
[c] Determined as described for limiting-dilution microculture conditions and analysis in Materials and Methods.
[d] ND, Not determined.

TABLE 2. Detection of HSV-reactive T cells in peripheral blood of HSV-seronegative individuals

Expt no.	HSV donor (patient no.)	HSV-reactive serum status[a]	HTL frequency (f^{-1})
1	5	+	12,390
	8	−	319,740
2	6	+	2,727
	9	−	12,736
3	6	+	3,485
	9	−	5,519

[a] Determined as described for viral antibody determination in Materials and Methods.

TABLE 3. Relationship between serologic status and HTL frequency

Expt no.	Date (mo/day/yr)	Patient no.[a]	Serologic status for:		HTL frequency (f^{-1})		
			HSV	CMV	HSV[b]	CMV[c]	Allo[d]
1	11/5/87	6	+	−	5,758	25,538	6,805
		5	+	+	4,193	2,191	3,564
2	11/19/87	6	+	−	3,485	8,003	4,183
		7	+	+	5,501	3,519	2,168
3	12/8/87	6	+	−	2,498	6,366	ND[e]
4	12/15/87	6	+	−	2,727	34,954	7,203
5	12/3/87	4	+	−	1,442	<200,228	818
		5	+	+	3,515	2,558	ND
6	4/15/88	10	+	−	555	5,369	ND
		4	+	−	1,267	20,903	ND
7	4/20/88	11	+	+	2,042	568	ND
		12	−	−	40,191	34,994	ND
		13	−	−	78,957	13,154	ND

[a] Untreated PBMC donors.
[b] HSV-1 antigen; 0.8 μg of protein per ml plus 500 autologous monocytes.
[c] CMV antigen; 0.9 μg of protein per ml plus 500 autologous monocytes.
[d] A total of 5,000 irradiated (7,500 R) allogeneic PBMC-derived monocytes added to LDA microcultures in the absence of additional autologous monocytes or viral antigens (29).
[e] ND, Not determined.

following relationships became apparent. (i) The three individuals (no. 5, 7, and 11) who were seropositive for both HSV and CMV had relatively high HSV antigen-reactive and CMV antigen-reactive HTL frequencies. (ii) The two individuals (no. 12 and 13) who were seronegative for both HSV and CMV had moderate but detectable HSV antigen-reactive and CMV antigen-reactive HTL frequencies. (iii) The three individuals (no. 4, 6, and 10) who were seropositive for only HSV and seronegative for CMV had consistently higher frequencies of HTL reactive with HSV compared with those observed for CMV within the same experiment. Interestingly, these three latter individuals had highly variable but usually detectable frequencies of HTL reactive with CMV, for which they were seronegative. For example, one HSV-seropositive, CMV-seronegative individual (no. 6), who was tested four times in a 1-month period, had variable sequential CMV antigen-reactive HTL frequencies of 1/25,538, 1/8,003, 1/6,366, and 1/34,954 while displaying nearly constant simultaneous HSV antigen-reactive (1/5,758, 1/3,485, 1/2,498, and 1/2,727) and alloreactive (1/6,805, 1/4,183, and 1/7,203) HTL frequencies. This latter observation was unexpected and raises some interesting questions regarding the immunobiology of antiviral activity.

DISCUSSION

The participation of HTL in immune responses to viral infection has been difficult to evaluate or monitor, due primarily to a lack of suitable immunologic techniques. The immunologic techniques that have been available to date are all based on conventional lymphocyte culture techniques. Hence it is possible to detect lymphocyte proliferation, lymphokine secretion, and CTL generation in vitro when lymphocytes are cultured with viral antigens. However, detection of these responses demonstrates only the presence or absence of viral antigen-reactive T cells in the lymphocyte population but provides little information regarding the size of the viral antigen-reactive population. Without this information, it is not possible to understand the dynamics of viral immunity before, during, or after viral infection.

Unlike other assays of cellular immunity, LDA enumerates antigen-reactive lymphocytes and thus allows monitoring of dynamic changes in the sizes of various lymphocyte populations. Although one published report made use of a modified LDA to determine the frequency of human viral antigen-specific, proliferating T lymphocytes (16) that were assumed to be $CD4^+$ HTL (10), we are unaware of published reports describing LDA techniques for detection of human viral antigen-reactive, lymphokine-secreting T cells. Therefore, we developed an LDA that could enumerate T cells which secrete TCGF after contact with viral antigens. Virus-induced TCGF was detected via a bioassay involving murine CTLL-20 cells. These cells proliferate in response to human or murine IL-2 and murine IL-4, but not human IL-4 (27). Since the pattern of lymphokine reactivity of CTLL-20 cells appears to be complex and incompletely understood, we used CTLL-20 cell proliferation to indicate the presence of TCGF without defining the particular growth factor(s) in question. For the purposes of the studies discussed here, antigen-induced TCGF production is considered to be the operational definition of an HTL. Since recent LDA studies utilizing lectins and alloantigens suggest that there is no absolute correlation between the surface phenotype and the functional potential of human peripheral blood T lymphocytes (26), our operational definition of HTL is independent of considerations regarding cell surface phenotype or any collateral T-lymphocyte functions of the TCGF-secreting cells.

Using this LDA technique, we monitored HTL frequency from HSV-seropositive individuals and compared these frequencies with those obtained by using conventional assays of viral antigen-induced proliferation and lymphokine secretion (Table 1). Although we observed that there is little correlation between the quantitative estimate of HTL number and the qualitative assessments of T-cell responses based on proliferation or lymphokine secretion, this is not surprising since murine studies (24) have shown that IL-2 production under limiting-dilution conditions was much greater (on a per cell basis) than under conventional, high-density culture conditions. Furthermore, these conventional culture systems are particularly sensitive to multicellular interactions that could be suppressive (23), causing a net response that bears little relationship to the number of precursor cells present. However, it was of interest to find that PBMC from HSV-seronegative individuals contained detectable HSV-reactive HTL (Table 2). Others have reported that virus-reactive cell frequencies could only be obtained in PBMC from individuals who were seropositive for a particular virus (16). It should be noted that these authors monitored the proliferation of T cells rather than the production of TCGF as an index of HTL function in limiting-dilution microcultures.

When the relationship between virus-reactive serologic status and viral antigen-reactive HTL frequency was examined in greater detail by using CMV and HSV, we observed that individuals who are seropositive for either virus have relatively high (comparable with alloantigen-reactive HTL) HSV antigen-reactive or CMV antigen-reactive HTL frequencies (Table 3). This was not unexpected, since we had previously observed that PBMC from seropositive individuals were efficient at producing lymphokines when cultured with appropriate viral antigens (8). However, as found initially with HSV, CMV-reactive HTL could still be detected by LDA in PBMC populations from some CMV-seronegative individuals (Table 3). These latter frequencies appeared to be highly variable and ranged from undetectable to quite prominent, even within the same individual at different times.

Initially, HSV and CMV antigen reactivities were compared in these experiments to verify the viral antigen specificity of the HTL responses that were detected in the limiting-dilution microcultures. Because of the experimental results, further investigations are necessary to provide definitive data in this regard. Interestingly, murine studies utilizing LDA techniques (23) detected HTL that displayed patterns of cross-reactivity to influenza viral determinants that differed markedly from specificities determined by conventional culture systems (2). Furthermore, recent studies using Western (immuno-) blot analysis and immunoprecipitation techniques reported on antigenic cross-reaction between proteins of human HSV, CMV, and Epstein-Barr virus (33) and between HSV and varicella-zoster virus (37). This raises the possibility that cross-reactivity to herpesvirus proteins could be reflected by the HTL themselves, with the antigen specificity determined by antigen-specific regulatory cells that are more effective at high densities, as suggested by others (23). However, whether such cross-reactivities could result in the highly variable HTL frequencies observed for seronegative individuals is not known.

Theoretically, there are a number of immunologic explanations for the seronegativity of a given individual for a particular virus. Ranging from simplest to most complex,

these include (i) lack of prior exposure to viral antigens, (ii) lack of cellular ligands for viral binding and infection, (iii) lack of viral antigen-reactive B-cell clones, (iv) lack of viral antigen-reactive helper T-cell clones, and (v) presence of a virus-responsive regulatory mechanism which actively impairs the function of viral antigen-reactive B cells or HTL. Our data appear to eliminate the fourth possibility. The other possibilities require further investigation. Although the data concerning HTL in seropositive and seronegative individuals are not definitive, there is certainly enough information to warrant continued investigation in this area. Since these data could not have been obtained with conventional lymphocyte culture techniques, these experiments also illustrate the effectiveness and utility of LDA as a research tool for investigating some of the more subtle aspects of viral antigen-induced T-cell behavior.

ACKNOWLEDGMENTS

We thank Laurie Scovell for her technical assistance during these studies, Cindy Narcross for her secretarial assistance in the preparation of the manuscript, and Teri Bailey for her administrative efforts regarding our research program

This work was supported in part by a grant from the Kidney Foundation of Central Ohio, by a grant from the Department of Surgery Medical Research and Development Fund, and by Public Health Service grants AI20851 and AI34774 from the National Institutes of Health.

LITERATURE CITED

1. **Anderson, W., and E. Kilbourne.** 1961. Immunization of mice with inactivated herpes simplex virus. Proc. Soc. Exp. Biol. Med. **107**:518–520.
2. **Balachandran, N., D. E. Oba, and L. M. Hutt-Fletcher.** 1987. Antigenic cross-reactions among herpes simplex virus types 1 and 2, Epstein-Barr virus, and cytomegalovirus. J. Virol. **61**: 1125–1135.
3. **Berle, E. J., and E. Thorsby.** 1980. The proliferative T cell response to herpes simplex virus (HSV) antigen is restricted by self HLA-D. Clin. Exp. Immunol. **39**:668–675.
4. **Boyum, A.** 1968. Separation of leukocytes from blood and bone marrow. Scand. J. Clin. Lab. Invest. **21**:77–89.
5. **Bryan, D. J., C. E. Pinto, and T. C. Fuller.** 1983. Direct estimation of the frequency of human cytotoxic T lymphocytes and their precursors following *in vitro* allosensitization. Hum. Immunol. **6**:31–44.
6. **Chouaib, S., and D. Fradelizi.** 1982. The mechanism of inhibition of human IL-2 production. J. Immunol. **129**:2463–2468.
7. **Clouse, K., P. Adams, J. Sheridan, and C. Orosz.** 1987. Rapid method for purification of human T lymphocytes for further functional studies. J. Immunol. Methods **105**:253–262.
8. **Clouse, K. A., F. H. Bach, and C. G. Orosz.** 1984. Detection of HSV-1-induced lymphokine production in human cells by a direct indicator cell addition assay. J. Immunol. **132**:2961–2967.
9. **Conrad, R. A.** 1969. Quantitative study of radiation effects in phytohemagglutinin-stimulated leukocyte cultures. Int. J. Radiat. Biol. **16**:157–161.
10. **Engleman, E. G., C. J. Benike, C. Metzler, P. A. Gatenby, and R. L. Evans.** 1983. Blocking of human T lymphocyte function by anti-Leu 2 and anti-Leu 3 antibodies: differential inhibition of proliferation and suppression. J. Immunol. **130**:2623–2628.
11. **Evans, C., H. Slavin, and G. Berry.** 1946. Studies of herpetic infection in mice. IV. The effect of specific antibodies on the progress of virus within the central nervous system of young mice. J. Exp. Med. **84**:429–447.
12. **Finberg, R., and B. Benacerraf.** 1981. Induction, control and consequences of virus-specific cytotoxic T cells. Immunol. Rev. **58**:157–180.
13. **Gehrz, R. G., S. C. Marker, S. O. Knorr, J. M. Kalis, and H. H. Balfour, Jr.** 1977. Specific cell-mediated immune defect in active cytomegalovirus infection of young children and their mothers. Lancet **ii**:844–847.
14. **Glasgow, L.** 1970. Cellular immunity in host resistance to viral infection. Arch. Intern. Med. **126**:125–134.
15. **Haahr, S., L. Rasmussen, and T. Merigan.** 1976. Lymphocyte transformation and interferon production in human mononuclear cell microcultures for assay of cellular immunity to herpes simplex virus. Infect. Immun. **14**:47–54.
16. **Hayward, A. R., M. J. Herberger, J. Groothuis, and M. R. Levin.** 1984. Specific immunity after congenital or neonatal infection with cytomegalovirus or herpes virus. J. Immunol. **133**:2469–2473.
17. **Howes, E. L., W. Taylor, N. A. Mitchison, and E. Simpson.** 1979. MHC matching shows that at least two T-cell subsets determine resistance to HSV. Nature (London) **227**:67–68.
18. **Klein, J., and Z. A. Nagy.** 1983. MHC restriction and Ir genes. Adv. Cancer Res. **17**:233–317.
19. **Lopez, C., and R. O'Reilly.** 1977. Cell-mediated immune responses in recurrent herpesvirus infections. I. Lymphocyte proliferation assay. J. Immunol. **118**:895–902.
20. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
21. **Melchers, I., K. Fey, and K. Eichmann.** 1982. Quantitative studies on T cell diversity. III. Limiting dilution analysis of precursor cells for T helper cells reactive to xenogeneic erythrocytes. J. Exp. Med. **156**:1587–1603.
22. **Meyers, J., N. Flournoy, and E. Thomas.** 1980. Infection with herpes simplex virus and cell-mediated immunity after marrow transplant. J. Infect. Dis. **142**:338–346.
23. **Miller, R. A., and C. S. Reiss.** 1984. Limiting dilution cultures reveal latent influenza virus-specific helper T cells in virus-primed mice. J. Mol. Cell. Immunol. **1**:357–368.
24. **Miller, R. A., and O. Stutman.** 1982. Enumeration of IL 2-secreting helper T cells by limiting dilution analysis, and demonstration of unexpectedly high levels of IL 2 production per responding cell. J. Immunol. **128**:2258–2264.
25. **Mizel, S.** 1982. Interleukin 1 and T cell activation. Immunol. Rev. **63**:51–72.
26. **Moretta, A.** 1985. Frequency and surface phenotype of human T lymphocytes producing interleukin 2. Analysis by limiting dilution and cell cloning. Eur. J. Immunol. **15**:148–155.
27. **Mosmann, T., T. Yokota, R. Kastelein, S. Zurawski, N. Arai, and Y. Takabe.** 1987. Species specificity of T cell stimulating activities of IL-2 and BSF-1 (IL-4): comparison of normal and recombinant human IL-2 and BSF-1 (IL-4). J. Immunol. **138**: 1813.
28. **Nahmias, A., and B. Roizman.** 1973. Infection with herpes simplex viruses 1 and 2. N. Engl. J. Med. **289**:781–789.
29. **Orosz, C. G., P. W. Adams, and R. M. Ferguson.** 1987. Frequency of human alloantigen-reactive T lymphocytes. II. Method for limiting dilution analysis of alloantigen-reactive helper T cells in human peripheral blood. Transplantation **43**:718–724.
30. **Orosz, C. G., P. W. Adams, and R. M. Ferguson.** 1988. Frequency of human alloantigen-reactive T lymphocytes. III. Cyclosporine has an inhibitory effect on human CTL and CTL precursors independent of a CsA-mediated helper T cell dysfunction. Transplantation **46**(Suppl.):73S–79S.
31. **Orosz, C., N. Zinn, L. Sirinek, and R. Ferguson.** 1986. *In vivo* mechanisms of alloreactivity. I. Frequency of donor-reactive cytotoxic T lymphocytes in sponge matrix allografts. Transplantation **41**:75–83.
32. **Pfizenmaier, K., A. Starzinski-Powitz, M. Rollinghoff, D. Falke, and H. Wagner.** 1977. T cell-mediated cytotoxicity against herpes simplex virus-infected target cells. Nature (London) **265**:630–632.
33. **Reiss, C. S., and S. J. Burakoff.** 1981. Specificity of the helper T cell for the cytotoxic T lymphocyte response to influenza viruses. J. Exp. Med. **154**:541–546.
34. **Russell, A.** 1974. Cell-mediated immunity to herpes simplex virus in man. J. Infect. Dis. **129**:142–146.
35. **Ryser, J., J. C. Cerottini, and K. Brunner.** 1978. Generation of cytolytic T lymphocytes *in vitro*. IX. Induction of secondary

CTL response in primary long-term MLC by supernatants from secondary MLC. J. Immunol. **120**:370–377.
36. **Sethi, K., I. Stroehmann, and H. Brandis.** 1980. Human T-cell cultures from virus-sensitized donors can mediate virus-specific and HLA-restricted cell lysis. Nature (London) **286**:718–720.
37. **Shiraki, K., T. Okuno, K. Yamanishi, and M. Takahashi.** 1982. Polypeptides of varicella-zoster virus (VZV) and immunological relationship of VZV and herpes simplex virus (HSV). J. Gen. Virol. **61**:255–269.
38. **Spear, P. G., and B. Roizman.** 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpes virion. J. Virol. **9**:143–159.
39. **Starr, S., S. Karatela, S. Shore, A. Duffy, and A. Nahmias.** 1975. Stimulation of human lymphocytes by herpes simplex virus antigens. Infect. Immun. **11**:109–112.
40. **Stockinger, B.** 1984. Cytotoxic T cell precursors revealed in neonatally tolerant mice. Proc. Natl. Acad. Sci. USA **81**: 220–223.
41. **Taswell, C.** 1981. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. J. Immunol. **125**:1614–1619.
42. **Ulmer, A., and H. D. Flad.** 1979. Discontinuous density gradient separation of human mononuclear leukocytes using Percoll as a gradient medium. J. Immunol. Methods **30**:1–10.
43. **Vie, H., and R. A. Miller.** 1986. Estimation by limiting dilution analysis of human IL-2-secreting T cells: detection of IL-2 produced by single lymphokine-secreting T cells. J. Immunol. **136**:3292–3297.
44. **Woodruff, J. F., and J. J. Woodruff.** 1975. T lymphocyte interaction with viruses and virus-infected tissues. Prog. Med. Virol. **19**:120–160.
45. **Yasukawa, M., and J. Zarling.** 1984. Human cytotoxic T cell clones directed against herpes simplex virus-infected cells. I. Lysis restricted by HLA class II MB and DR antigens. J. Immunol. **133**:422–427.
46. **Zaia, J. A., P. L. Leary, and M. J. Levin.** 1978. Specificity of the blastogenic response of human mononuclear cells to herpesvirus antigens. Infect. Immun. **20**:646–651.