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Frequency of Cytotoxic T Lymphocyte Precursors to Herpes Simplex Virus Type 1 as Determined by Limiting Dilution Analysis

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The conditions for establishing a limiting dilution assay to measure cytotoxic T lymphocyte precursors (CTL-P) against herpes simplex virus type 1 (HSV-1) were determined. Analysis by Poisson statistics demonstrated that the estimated frequency of HSV-1-reactive cells in the spleens of normal mice was less than 1/250,000. In contrast, mice immunized previously with infectious HSV-1 demonstrated a CTL-P frequency between 1/3,500 and 1/15,670. The generation of a maximum cytotoxic T lymphocyte response required that mice be primed in vivo with infectious virus. Immunization with inactivated virus either failed to elicit detectable CTL-P frequencies or gave frequencies markedly less than those induced with infectious virus. To obtain positive cultures, the responder cell population had to be exposed to stimulator splenocytes expressing viral antigens. Normal splenocytes without virus or normal splenocytes with T cell growth factor did not result in significant cytotoxicity. Split culture analysis comparing cytotoxicity against syngeneic and allogeneic virus-infected targets provided evidence for specificity, *H-2* restriction, and the T cell nature of the CTL-P. It was determined that precursors were eliminated by treatment with anti-Thy 1, Lyt 2.1, or Lyt 1.1, indicating the CTL-P were Lyt 1^+2^+ cells. Cytotoxicity was reduced after treatment of the responders with anti-Lyt 2 plus complement, which gave further evidence of the T cell nature of the cytotoxic T lymphocytes. These experiments demonstrated the feasibility of using the limiting dilution approach as a highly sensitive and quantitative means to measure the cell-mediated immune response to HSV-1 antigens.

Until recently, cellular immunology lacked single-cell assays, and T cell reactivity had to be measured by the effector functions induced in bulk cultures of highly heterogeneous cell mixtures. However, improvements in cell culture technology, particularly the knowledge that clones of T cells can sometimes be maintained indefinitely in media containing appropriate soluble factors, permitted the development of analytical approaches such as limiting dilution analysis (11, 22, 23, 26). Thus, by measuring the activity of the clonal progeny of single cells in sensitive culture systems, the frequency of cytotoxic T lymphocyte precursors (CTL-P) within various populations can be estimated, and the fine immunological specificity of CTL-P can be analyzed. Few reports have applied the limiting dilution approach to viral antigens (2, 7, 12, 16, 19), but wide application is to be expected since the technique should provide answers about the cross-reactivity of T cells to viral antigens and since it represents a more quantitative approach for measuring responses in various immunization protocols. In this report, we document the conditions for establishing a limiting dilution assay to measure CTL-P against herpes simplex virus type 1 (HSV-1). We show that whereas anti-HSV-1 CTL-P are not present in sufficient numbers to be detected in normal splenocyte populations, the estimated frequency of CTL-P in animals exposed to live virus is around 1/5,000. In contrast, the frequency of CTL-P in animals exposed to inactivated virus is markedly lower, providing a possible explanation for the failure of inactivated virus to elicit cytotoxic T lymphocytes (CTL) as detectable by the standard bulk culture techniques (9).

MATERIALS AND METHODS

Virus. HSV-1 strain KOS was propagated in HEp-2 cells as described by Bone and Courtney (3). The viral stocks used had infectivity titers of $10^{8.2}$ PFU/ml. To effect inactivation, virus was heated at 56°C for 60 min

or was UV irradiated for 2 min. These procedures reduced infectivity by 7 and 5 logs, respectively.

Mice. CBA/Ca ($H\text{-}2^k$) mice were obtained from Olac Ltd., Show's Farm, Blackthorn, United Kingdom. Mice were at least 8 weeks old at the time of immunization. To effect immunization, mice were infected intraperitoneally with 10^5 PFU of HSV-1, a dose which most survived. Animals were usually used 6 weeks or more after infection. Animals were also immunized with heat-inactivated (HI) virus with from one to three doses of 10^8 PFU administered intraperitoneally and subcutaneously. Spleens were taken from such mice 2 to 3 weeks after the final infection.

In vitro culture under limiting dilution conditions. Spleens or lymph nodes were removed, and suspensions of single nucleated cells were prepared as described elsewhere (9). Viable cells were counted and suspended in a medium containing a 50:50 mixture of RPMI 1640 and Click supplemented with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 2 mM glutamine, sodium bicarbonate, 5×10^{-5} M 2-mercaptoethanol, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.). Various numbers of cells (usually 4×10^4 to 1×10^3) were added in 0.1 ml of medium to the U-shaped wells of 96-well microplates. Either 24 or 32 replicates were used for each responder spleen cell number. To the responder cells were added 2×10^5 stimulator cells (in 0.1 ml) suspended in culture medium and containing rat T cell growth factor (TCGF) and 50 mM methyl mannoside. The preparation of rat TCGF has been described elsewhere (27). Basically, rat splenocytes (5×10^6 cells per ml) in medium were stimulated for 48 h with 5 μg of conconavalin A per ml. The supernatant fluids were collected, filter sterilized, and stored at −20°C until used. To ensure that enough TCGF was used, trial limiting dilution experiments were performed with various final concentrations of TCGF. For the batch used in the reported experiments, 7.5% was found sufficient to attain maximum numbers of responder cultures, and 12% was routinely used to ensure excess.

To attain viral antigen stimulation, spleen cells were infected for 2 h with UV-irradiated HSV-1 (approximately 1 PFU per spleen cell before viral inactivation) in siliconized glass tubes. After infection, cells were irradiated (1,800 to 2,000 R), washed, and suspended in medium. In some experiments, uninfected normal spleen cells (irradiated) were used. In other experiments, immune spleen cells were used as a source of help. Such cells were irradiated with 1,200 R and used at 10^5 cells per culture. To these cells, 10^5 virus-infected normal spleen cells irradiated at 2,000 R were added as a source of antigen. After these cells were added, the microculture lids were sealed with plastic tape, and the cultures were incubated for 6 to 7 days in a humidified atmosphere of 5% CO_2.

Assay of cytotoxicity. At the end of the incubation period, cultures were split into two subcultures. After centrifugation at 1,000 rpm for 5 min to pellet cells, the supernatant fluids were removed, and target cells were added in 150-μl volumes of RPMI 1640 containing 5% fetal calf serum. Control wells received target cells alone; some of these wells were used to ascertain the total releasable ^{51}Cr by the addition of a detergent. The target cells were prepared in various ways. Either LS cells ($H\text{-}2^k$) or 5 μg of LPS blasts ($H\text{-}2^k$ or $H\text{-}2^b$) stimulated for 48 h were used. To effect virus infection, cells were infected at high multiplicities (5 to 10 PFU per cell) with HSV-1 for 150 min. After the first 30 min, 200 μCi of $Na_2{}^{51}CrO_4$ per ml was added. After 2 h, the cells were washed twice and incubated a further 90 min at 37°C in 5% CO_2. After a further wash, the cells were counted, and their radioactivity was measured. This was usually in the order of 2 cpm per cell. Each microwell received 1,000 cells, and the cultures were incubated at 37°C for 6 h, after which they were centrifuged to pellet cells and 100 μl was removed with a multichannel pipette to measure radioactivity. Cultures in which the radioactivity exceeded by three standard deviations that of the medium control or (more usually) that of cultures with stimulator cells but without responders were considered as positive. For each culture, the percentage of specific release was determined as [(test release − control release)/(total release − control release)] × 100. The level of release in medium control varied, but during 6-h assays for LS and infected LS cells it was between 8 and 20%.

In some experiments, bulk cultures were used to measure the generation of HSV-1-specific CTL. These measurements were made as described previously (9).

Statistical analysis. The calculation of frequencies from limiting dilution data was based on the Poisson distribution. The number of negative microcultures as a fraction of all cultures was plotted on a logarithmic ordinate against the responding cell numbers on the *x* axis. By using the least squares method (25), a regression line of best fit was determined. Frequencies were then calculated from the slope of the regression lines by Poisson statistics. If not specifically reported, frequency data included were selected with r^2 values above 0.92, giving intercepts between 0.92 and 1.15. Additional analysis by the chi-square minimization method (24) revealed close convergence of frequency estimates.

Cell separation. In certain experiments, T cell-enriched fractions were obtained by the nylon wool filtration technique of Julius et al. (6). T cells were also fractionated into Lyt 1^+ and Lyt 2^+ fractions by negative selection by treating cell preparations with monoclonal anti-Lyt sera (New England Nuclear Corp., Boston, Mass.) and rabbit complement (C) (Accurate Chemical and Scientific Corp., Westbury, N.Y.) according to methods described by the reagent manufacturers. Basically, cells were treated with either anti-Lyt 1.1 or anti-Lyt 2.1 serum for 60 min at 0°C, washed, and then treated with C for 45 min at 37°C. Dead cells were removed by Ficoll-Hypaque flotation (density, 1.090) before cells were added to cultures. Anti-Thy 1.2 and C treatment (with monoclonal anti-Thy 1.2 supplied by Jon Sprent, Wistar Institute, Philadelphia, Pa.) was performed as described previously (9).

RESULTS

Demonstration of anti-HSV-1 CTL. After the culture of spleen or lymph node cells from normal mice under limiting dilution conditions with stimulator cells expressing viral antigen and factor, the frequency of positive cultures was too low to compute statistically. This was true

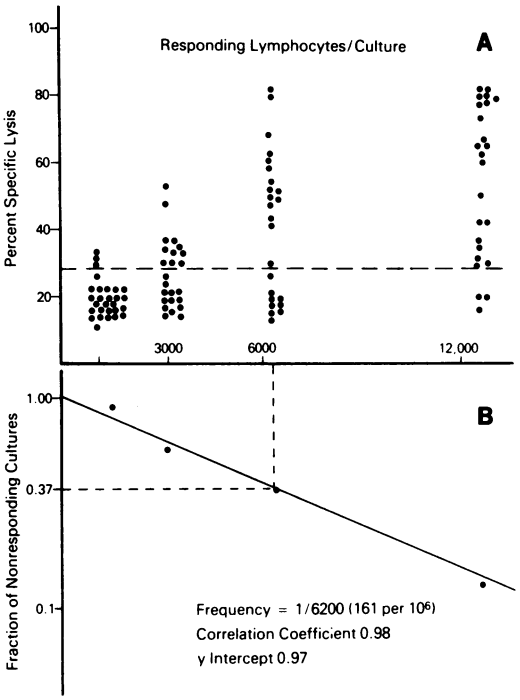

FIG. 1. Minimum estimate of the frequency of CTL-P specific for syngeneic HSV-1-infected LS cells in nucleated splenocytes from CBA mice 8 weeks after infection with HSV-1. (A) Percentage of specific ^{51}Cr release for each microculture. The dashed line represents three standard deviations above the mean of control cultures that contained no responder cells. (B) Proportion of nonresponding cultures plotted on a logarithmic scale (y axis) against cell input (x axis). The best-fitting straight line was fitted by the method of least squares, and the precursor frequency was estimated by Poisson statistics.

even when 5×10^4 lymphocytes were used as responders, a quantity that overcrowds microcultures. Thus, the frequency of anti-HSV CTL-P in normal spleen and lymph node populations was judged to be lower than 1/250,000. However, upon antigen stimulation of spleen cells from HSV-1-immune mice (6 weeks or more after HSV-1 infection), positive microcultures resulted. Although variations occurred between experiments, the frequency of positive cultures in nucleated spleen cells was between 1/3,500 and 1/15,670. Figure 1 shows the results of a representative experiment, demonstrating that levels of specific cytotoxicity in cultures recorded as positive vary markedly but are as high as 82.5% specific lysis. To obtain positive cultures, it was necessary to stimulate responder cells with viral antigen. Thus, although the frequency computed either in the presence of normal splenocytes or in the presence of splenocytes and rat TCGF was usually above what was detectable in non-immune spleen cell populations (but in some cases too low to compute), the frequency was markedly below that seen in virus-stimulated cultures (Fig. 2).

To obtain data to which a straight line could be fitted after plotting, it was necessary to include a source of TCGF in the culture fluids. Fluids from splenocytes stimulated with concanavalin A, which contained TCGF, were routinely used, but similar results were also obtained with partially purified TCGF from the fluids of mouse splenocytes stimulated with concanavalin A (data not shown). In the absence of TCGF, positive microcultures often occur at incidence levels and degrees of cytotoxicity similar to those in cultures which contain TCGF as a source of help. However, in the absence of TCGF, computation of CTL-P frequencies was not possible since the data did not indicate that a single cell was limited. In some experiments, it appeared that two or more cells were responsible for positive cultures (Fig. 2).

It is to be noted that CTL-P were readily detectable in the HSV system provided that TCGF was included in the system as a source of

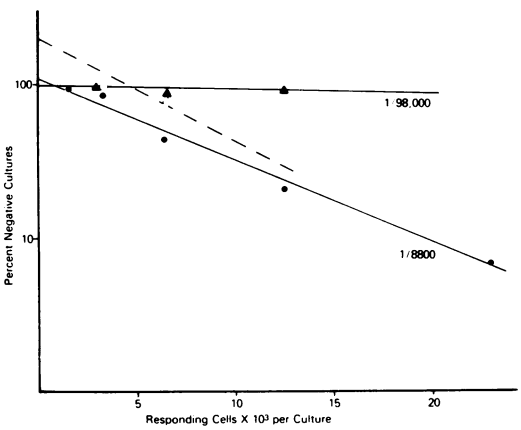

FIG. 2. Minimum estimate of frequency of CTL-P specific for syngeneic HSV-1-infected LS cells in nucleated splenocytes from CBA mice 3 months after infection with HSV-1. The best-fitting straight lines by the least-squares method and the frequencies estimated by Poisson statistics are shown for cultures of immune splenocytes stimulated by virus-infected syngeneic irradiated spleen cells and TCGF (●) and by normal syngeneic irradiated spleen cells and TCGF (▲). Also shown are the responses of the same population of immune splenocytes stimulated by virus-infected splenocytes but without TCGF (○). In the same experiment, normal splenocytes stimulated by virus-infected spleen cells and TCGF and immune splenocytes stimulated by normal spleen cells but without TCGF gave responses too low for frequencies to be computed.

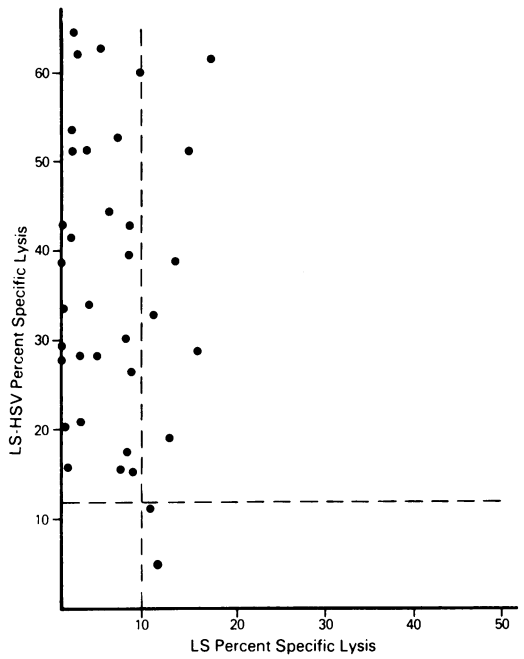

FIG. 3. Cytolytic activity of individual cultures with responder immune spleen cells from HSV-immune mice stimulated in the presence of TCGF with irradiated virus-infected normal spleen cells. After 7 days of stimulation, each was split and assayed against 1,000 ^{51}Cr-labeled virus-infected syngeneic LS cells and uninfected cells in a 6-h assay. The dotted lines represent the mean spontaneous release plus three standard deviations determined in groups of cultures containing no responder cells but with irradiated virus-infected spleen cells and TCGF. This defines the lower limit of positive cytolytic activity.

help. Others have observed that to obtain maximum frequencies and to obtain regression lines that intercept on the ordinate close to the origin of a semilogarithmic plot when plotting responder number versus percentage of negative cultures, helper cells must be added to the system (2). This was not our experience; the frequencies of CTL-P were not significantly increased when irradiated splenocytes were added from animals immunized with live or inactivated virus. Indeed, in one of three experiments, the addition of irradiated splenocytes from animals given HI virus lowered the CTL-P frequency by half. Whether this can be attributed to suppression is under investigation.

Evidence that detected cells were HSV-1-specific T cells. Several approaches were used to provide evidence that virus-specific CTL-P were being counted and that these cells were T lymphocytes. To assess viral specificity, cultures were split, and samples were assayed against syngeneic virus-infected target cells, uninfected targets, virus-infected allogeneic cells, or syngeneic cells infected with vaccinia virus. Against all targets, positive microcultures were detectable, but only from cultures of antigen-stimulated immune splenocytes. However, the frequencies of CTL-P were much lower (10- to 50-fold) with various control targets than with syngeneic virus-infected targets, and the levels of specific cytotoxicity in controls were also less. Data on split-culture analysis of microcultures assayed against virus targets and against uninfected cells are shown in Fig. 3. Some double positives occurred, but cytotoxicity levels against uninfected cells were lower, and such reacting cells were less frequent than cells which reacted with infected cells alone. It is possible that the double positives represent natural killer (NK) cells or a combination of NK cells and CTL-P.

The results on split-culture analysis comparing cytotoxicity against syngeneic and allogeneic virus-infected targets provide further evidence

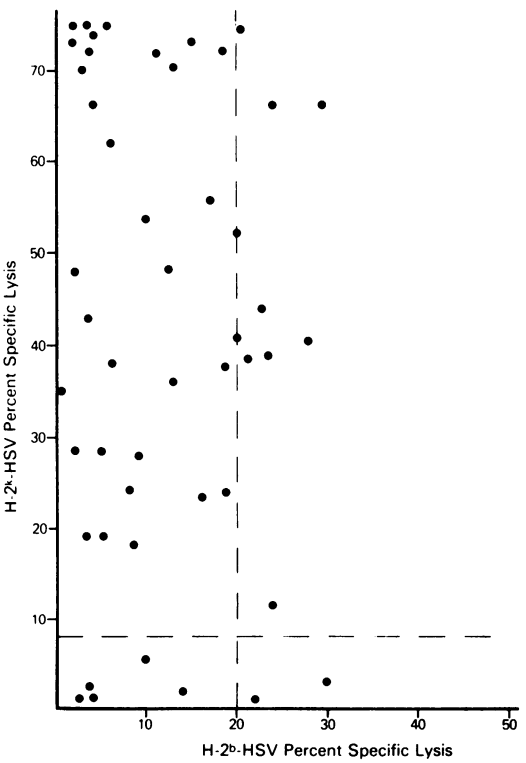

FIG. 4. Cytolytic activity of individual cultures with responder immune spleen cells from HSV-1-immune mice stimulated in the presence of TCGF with irradiated virus-infected normal spleen cells. After 7 days of culture, wells were split and assayed against 1,000 ^{51}Cr-labeled syngeneic virus-infected LS cells or virus-infected LPS blast cells from C57BL/6 mice (*H-2$_b$*). The dotted lines represent the mean spontaneous release plus three standard deviations determined in groups of cultures containing no responder cells to define the lower limit of positive culture activity.

TABLE 1. Estimated frequency of anti-HSV CTL-P after fractionation and pretreatment with alloantisera and C[a]

Responder population	Estimated frequency of CTL-P	
	Expt 1	Expt 2
Intact spleen	1/7,650	1/5,360
Nylon nonadherent	1/3,050	1/1,385
C-pretreated nylon nonadherent	1/4,200	ND[b]
Anti-Ly 2.1 + C	>1/200,000	1/63,000
Anti-Ly 1.1 + C	>1/200,000	1/87,000
Anti-Thy 1.2 + C	>1/200,000	ND

[a] Pooled immune spleens from CBA mice 3 months after infection with HSV-1. Cells were stimulated with normal irradiated splenocytes infected with UV-irradiated HSV-1 for 7 days in the presence of TCGF. The responder cells were either untreated or were passed over nylon wool to enrich for T cells. After this step, the cell populations were treated with antisera at 0°C for 60 min, washed, and treated with rabbit C for 45 min at 37°C. The frequencies were estimated as described in the text.

[b] ND, Not determined.

for the specificity and the T cell nature of the CTL-P (Fig. 4). Again, the great majority of cells lysed only the syngeneic target, and when significant lysis of the allogenic target was observed, it was usually lower than against syngeneic cells. Occasionally, examples of lysis of only the allogeneic target were observed, such cells perhaps exhibiting alloreactivity. This possibility needs further examination. Specificity analysis experiments against vaccinia virus-infected syngeneic targets were also performed. The frequency against such targets was by far lower, and the levels of cytotoxicity were much less than against syngeneic HSV-infected targets (data not shown). Taken together, these approaches demonstrate anti-HSV specificity and strongly imply that cytotoxic cells are T cell in nature.

Further evidence for the latter concept came from experiments in which cultures were split, and one-half was treated with anti-Lyt antisera and C and the other with C alone. After treatment, the two aliquots were assayed for their cytotoxicity against syngeneic virus-infected targets. By this approach, the levels of cytotoxicity were markedly reduced with anti-Lyt 2 serum (mean 63.2% specific release in positive cultures versus a mean of 7.3%; 9.1% was three standard deviations above the release value of control cultures). Of 46 positive cultures examined, reduction or elimination of cytotoxicity occurred in 44, indicating the T cell nature of at least the majority of the cytotoxic cultures. Whether cultures without reduced activity after anti-Lyt 2 treatment are NK cells requires further investigation.

The precursors of anti-HSV CTL are Lyt 1^+2^+. To establish the cellular nature of the anti-HSV CTL-P, experiments were performed in which responder cells were enriched for T cells by passage over nylon wool, after which they were treated with C and with anti-Thy 1.2, anti-Lyt 1.1, or anti-Lyt 2.1. After dead cell removal by Ficoll-Hypaque flotation, cells were subjected to limiting dilution analysis. The CTL-P were assumed to be T cell in nature since their frequency in nylon column passage cells was enriched compared with unfractionated nucleated splenocytes (Table 1). Furthermore, the precursors were virtually eliminated after treatment with anti-Thy-1, Lyt 2.1, or Lyt 1.1, indicating that the CTL-P were Lyt 1^+2^+ cells.

Induction of anti-HSV-1 CTL by immunization with inactivated viral preparations. It is frequently the case when immunizing with inactivated viral preparations that negligible or nonexistent cell-mediated responses are elicited in vivo, particularly as determined by the measurement of CTL responses by bulk culture techniques (1, 4, 9). Thus, when immunizing against HSV-1 with inactivated virus, CTL are not elicited, and animals are usually not sensitized for a secondary CTL response inducible in vitro (9). To analyze this situation by the more sensitive limiting dilution approach, animals were immunized with HI virus on one or more occasions, after which spleen cells were removed for anti-HSV CTL assays by limiting dilution as well as by bulk culture. The results from such experiments were variable, but in bulk culture only splenocytes from animals given live virus yielded positive virus-specific cytotoxicity (data not shown). By limiting dilution, the frequency of CTL-P in splenocytes from mice that received HI virus was invariably lower than in mice given live virus; in mice immunized once, the frequency of CTL-P was always greater than 10-fold (Table 2). Even in mice immunized three times with HI virus, the CTL-P frequencies were still lower in those infected on one occasion with live virus. We have previously shown that antibody titers of such repeatedly immunized mice are similar to those immunized with live virus (15). Our results indicate that the apparent failure to generate a CTL response after in vivo immunization with inactivated virus may be associated with insufficient induction and expansion of the CTL-P population. Perhaps when CTL-P are induced, the failure to detect them in bulk culture is attributable to insufficient help or to active suppression. Experiments to investigate the situation are clearly required.

DISCUSSION

Our results demonstrate that the limiting dilution approach can be applied to estimating the

TABLE 2. Comparison of estimated frequencies of CTL-P in mice immunized with HI HSV-1 and mice infected with infectious HSV-1

Animal	Exposure[a]	Frequency of CTL-P[b]	r^2	y Intercept	Control frequency	Difference[c]
1	1	1/238,000	0.9	0.99	1/7,246	32.8
2	1	>1/250,000			1/7,246	>32
3	1	1/215,000	0.92	0.94	1/13,300	16.2
4	1	1/130,000	0.9	0.94	1/10,200	12.7
5	2	1/85,000	0.88	1.1	1/12,800	6.6
6	2	1/31,000	0.94	1.1	1/3,500	8.9
7	3	1/200,000	0.97	0.91	1/6,200	32.3
8	3	1/16,000	0.86	0.96	1/5,500	2.9
9	3	1/12,500	0.88	0.96	1/3,500	3.6

[a] Activity measured 2 to 3 weeks after immunization. Repeated immunizations were at intervals of 3 to 4 weeks.

[b] Estimated frequency of CTL-P in splenocytes from mice infected 6 weeks or more previously with infectious HSV-1. Regression coefficients were better than 0.92, and y intercepts were between 0.94 and 1.1

[c] Calculated by dividing frequency in mice receiving inactivated virus by the control frequency in mice given infectious virus.

minimal frequency of CTL-P against HSV-1 viral antigens. In marked contrast to allogeneic responsiveness, anti-HSV-1 CTL-P could not be detected in normal mice, and consequently the frequency of CTL-P was estimated to be less than 1/250,000, the limit of sensitivity of the test system. This value must be compared with the CTL-P frequency of 1/100 to 1/1,000 in unprimed alloantigen systems (12, 29). Since the frequency of anti-HSV-1 CTL-P in naive cell populations is so low, it is perhaps not surprising that so far it has proven impossible to generate primary CTL responses in vitro against HSV-1. A low or undetectable frequency of CTL-P in naive mice seems to occur for all viral antigens studied to date. Thus, the frequency estimates of anti-rabies virus CTL-P in normal lymph nodes is less than 1/250,000, values of 0.5 to 3 per 10^6 cells being recorded (19). In influenza, the most extensively investigated system (16), some could not detect CTL-P in normal splenocytes, but others could, although not in specific-pathogen-free mice (2). To detect anti-influenza CTL-P in normal mice, irradiated cells from primed mice have to be added as a source of help (2). However, we failed to reveal detectable HSV-1-specific CTL-P in T cell populations of normal mice by adding T helper cells or factors thereof to the culture system.

In marked contrast to the failure to detect anti-HSV-1 CTL-P in normal mice, it was easy to detect such cells in primed animals. Estimated frequencies varied, ranging from 1/3,500 (286 per 10^6 nucleated splenocytes) to 1/15,670 (64 per 10^6 nucleated splenocytes). To detect the CTL-P and obtain data that could be analyzed by Poisson statistics, it was necessary to include in the system concanavalin A-stimulated spleen cell supernatant fluids rich in TCGF. In the absence of TCGF, positive microcultures were demonstrable, but the data were irregular, and straight-line plots passing close to the origin of the y axis were never obtained. It is well known that to obtain analyzable data by limiting dilution, the limitation should be a single cell (10). Work on bulk systems has clearly shown that CTL generation requires the interaction of numerous cell types, including accessory cells and at least two subsets of T cell, namely CTL precursors and helper cell precursors (30). The latter type of cell serves to produce factors such as TCGF and T cell differentiation factor (28), required for the full development of antigen-triggered CTL-P (31). Consequently, to demonstrate CTL-P, it is necessary to provide an excess of helper cells or their soluble products required for CTL-P differentiation.

It was important to establish that in our culture system we were estimating the frequency of CTL precursors and not of other types of cytotoxic cells such as NK cells or antibody-dependent cells, both of which are potent against HSV-1-infected targets (5, 20). It was especially important to exclude NK cells as the explanation of the cytotoxicity since the factor used in the system could contain the NK cell activator interferon, and indeed it has recently been shown that CTL themselves can produce interferon on antigen stimulation (8, 14). We consider it unlikely, however, that NK cells were being detected since the cytotoxicity was virus specific and clearly *H-2* restricted. Furthermore, the CTL expressed the Lyt 2 alloantigen, which is not expressed on NK cells (18). Finally, the CTL precursors were shown to express Thy 1, Lyt 1, and Lyt 2 alloantigenic markers, which have been shown by others to identify cells of the T cell lineage (13).

Perhaps the most interesting aspect of our study was the observation that the generation of a maximum CTL-P response requires that mice be primed in vivo with infectious virus. This priming was necessary on only one occasion, and although the topic has not yet been carefully investigated, there seemed no obvious difference in the estimated frequencies of CTL-P in splenocytes taken 6 weeks or 6 months after a single priming with infectious HSV-1. In contrast, immunization with inactivated virus either failed to elicit detectable CTL-P frequencies or gave frequencies which were usually markedly less than those induced with infectious virus. Consequently, the failure to detect CTL responses by the less sensitive in vitro bulk culture after in vivo immunization with inactivated virus could be explained by insufficient expansion in vivo of CTL-P. However, other explanations must also be considered, such as insufficient help or the presence of suppression. Regarding the former, we have shown elsewhere that inactivated virus may fail to stimulate helper cells in vivo or to prime for the restimulation of such cells in vitro (21; D. S. Schmid, B. T. Rouse, and H. Wagner, manuscript in preparation). Also arguing in favor of insufficient help is the observation that bulk cultures from animals given inactivated virus in vivo may sometimes respond if stimulated by virus plus TCGF but not if stimulated with virus alone (Rouse, unpublished observation). Pfizenmaier et al. (17) have clearly demonstrated that suppression may operate to prevent HSV-specific CTL responses. They demonstrated that a CTL response by lymph node cells from recently infected mice does not occur after direct culture but requires an additional 3 to 4 days of in vitro culture to abrogate suppression.

Since there is an interest in developing vaccines composed of subunits against many viruses, including herpesviruses, it is important to establish that such preparations are capable of eliciting components of immunity, such as cell-mediated immunity, that are involved in antiviral defense. The limiting dilution approach provides a highly sensitive and quantitated way to assess immunization protocols for their efficacy at eliciting CTL precursors that may play a role in protection. We are performing experiments along these lines.

ACKNOWLEDGMENT

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