0022-1767/84/1321-0482$02.00/0
THE JOURNAL OF IMMUNOLOGY
Vol. 132, No. 1, January 1984
Printed in U.S.A.

THE CYTOLYTIC T LYMPHOCYTE RESPONSE TO THE MURINE CYTOMEGALOVIRUS

I. Distinct Maturation Stages of Cytolytic T Lymphocytes Constitute the Cellular Immune Response during Acute Infection of Mice with the Murine Cytomegalovirus[1]

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Limiting dilution (LD) analysis with two modifications, the expansion and the restimulation LD assay, led to the detection and quantification of two distinct *in vivo* maturation stages within the lineage of virus-specific self-restricted CTL after infection of mice with the murine cytomegalovirus (MCMV). A low frequency set, representing an average of 15% of the specifically activated CTL-P in a draining lymph node, generated virus-specific lytic activity in the absence of antigen, solely under expansion conditions provided by growth and differentiation interleukins. These cells were considered to be active and were denoted antigen-independent or interleukin-receptive CTL-P (IL-CTL-P). A high frequency set required additional antigen *in vitro* to generate functionally active clones, and therefore the cells were termed antigen-dependent. Both sets are present *in vivo* simultaneously at the peak of the acute immune response and represent antigen-activated cells because their existence strictly depends on a preceding priming event. IL-CTL-P disappear quickly after acute infection and are absent during the memory state. It is proposed that the isolation of IL-CTL-P could serve to detect viral antigen expression during persistent and/or recurrent herpes virus infections.

Usually, infections with cytomegaloviruses, a subfamily of herpesviruses termed Betaherpesvirinae according to their biological properties (1), do not cause serious illness unless the host is immunologically immature or deficient. The major threats for host and environment are the sequelae of reactivation from latent or persistent infection. It has been suggested that T cells rather than B cells play a decisive role in the control of murine cytomegalovirus (MCMV)[3] infection (2, 3). The contribution of cellular immune responses to a balanced virus-host interaction cannot be understood in detail without a more precise analysis of the cellular dynamics induced by and involved in the complex process of infection. With regard to a potential role of cellular immunity during latent or persistent infection, it is of special importance to discriminate between the activated and the resting fractions of *in vivo*-sensitized antigen-specific T lymphocytes.

Our previous limiting dilution study on rabies virus infection, designed as a model system for a lethal situation, led to the distinction of two sets of sensitized T lymphocytes present in the draining lymph node (LN) during the acute immune response (4). One set of cells required antigen to generate a functionally active progeny whereas the other did not. In that study it was not shown whether the latter fraction belongs to the lineage of H-2-restricted CTL and it also remained unclear whether the appearance of sensitized cells of different maturation stages is generally seen after viral infection or is a typical property of the lethal rabies infection. The present study demonstrates that during acute infection with MCMV, despite the completely different pathogenesis, both sets of cells appear in relative proportions similar to that observed in the rabies system. In addition, it is now proved that the antigen-independent interleukin-receptive cytolytic T lymphocyte precursors (IL-CTL-P) indeed belong to the lineage of H-2-restricted CTL.

The low frequency IL-CTL-P are considered representatives of an active immune response *in vivo* because they appear only locally and transiently in LN draining the site of infection, and activation by antigen *in vitro* is not required for the generation of a functionally active progeny.

Received for publication August 2, 1983.
Accepted for publication October 4, 1983.
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[1] This work was supported by Grant Ko 571/8 from the Deutsche Forschungsgemeinschaft.
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[3] Abbreviations used in this paper: CTL, cytolytic T lymphocyte; CTL-P, cytolytic T lymphocyte precursor; IL-CTL-P, interleukin-receptive CTL-P; LD, limiting dilution; LN, lymph node; MC, minimum Chi-square estimation; MCMV, murine cytomegalovirus; MEF, mouse embryo fibroblast; ML, maximum likelihood estimation; pfu, plaque-forming unit; pMC, probability observed in MC; f, frequency.

MATERIALS AND METHODS

Animals. Original breeding pairs of BALB/c (H-2^d haplotype) and C57BL/6 (H-2^b haplotype) mice were obtained from the Institut für Versuchstierforschung, Hannover, Germany, and were thereafter maintained in our colony and routinely controlled for absence of murine hepatitis virus. Both strains express the number 2 allele of the lymphocyte cell surface markers Thy-1, Lyt-1, and Lyt-2. Six- to 8-wk-old female mice were used in the reported experiments. Female Sprague Dawley rats were purchased from Fa. Ivanovas (Kisslegg, Germany) at the age of 8 wk and were used for the preparation of xenogeneic lymphocyte growth and maturation factors (interleukins).

Viruses. MCMV (strain Smith, ATCC VR-194, mouse salivary

gland virus) was obtained from the American-type culture collection (Rockville, MD) and was propagated on BALB/c mouse embryo fibroblasts (MEF). The cloned ERA strain of rabies virus was originally obtained from T. J. Wiktor, Wistar Institute Philadelphia, and was propagated on the syrian hamster fibroblast cell line BHK-21 and purified as described (5).

Culture media. RPMI 1640 (Serva, Heidelberg, Germany) was routinely supplemented with 2 mM L-glutamine, 1 mM sodium-pyruvate, 5×10^{-5} M 2-mercaptoethanol, and, if not indicated otherwise, with 10 mM HEPES buffer (pH 7.2), 100 U/ml penicillin, and 0.1 g/liter streptomycin. Minimum essential medium (MEM-α) without nucleosides (GIBCO Europe, Karlsruhe, Germany) was supplemented as described, but with no additional sodium-pyruvate. MEM with Earle's salts (Serva) was supplemented as described, except for sodium-pyruvate and 2-mercaptoethanol. All media contained mycoplasma-screened heat-inactivated fetal calf serum (FCS) (Seromed, München, Germany) at doses indicated. PBS-A used during virus purification is phosphate-buffered saline devoid of Ca^{2+} and Mg^{2+}.

Preparation of MEF. MEF were prepared from 15 to 17 day-old BALB/c or C57BL/6 mouse embryos by trypsinization (0.25% trypsin-0.125% EDTA) and subsequent selection of fibroblasts during two 4- to 6-day culture passages (MEM + 5% FCS, 5% CO_2, 37°C, humidified atmosphere) in plastic tissue culture flasks (2×10^7 cells per 75 cm^2, no. 5375; Lux-Miles, Naperville, IL).

Preparation of partially purified MCMV stock virus. Second passage MEF were infected at a multiplicity of 0.01 plaque-forming units (pfu)/cell and the supernatant medium (titer about 10^6 pfu/ml) was harvested after 4 to 6 days of culture. Semipurification was performed by a three-step centrifugation procedure: cell debris was removed at 7000 × G in a Beckman JA 10 rotor and the virus was sedimented at 17,000 × G for 3 hr in a Beckman JA 14 rotor. After resuspension, the virus was layered on top of a 15% sucrose-PBS-A cushion and then resedimented at 68,000 × G for 60 min in a Beckman SW 41 rotor. The final sediment was resuspended in PBS-A and stored frozen at −70°C. This material was screened for mycoplasma contamination (6, 7) and was used as a stock virus (2×10^8 pfu/ml) for priming and for target cell preparation.

Virus infection of MEF for stimulator and target cell formation. Second passage MEF were infected as monolayers before reaching confluence with a dose of 0.05 pfu MCMV/cell under the influence of an 800 × G-30 min centrifugal field. This centrifugal infection results in a 20- to 80-fold increase in infectivity, termed "centrifugal enhancement" (8). The cells were used 16 to 20 hr post infection; i.e., after the onset of viral DNA and structural protein synthesis. Rabies virus (strain ERA) infection of MEF was performed with a multiplicity of 10 pfu/cell for 20 to 24 hr in MEM + 5% FCS containing 5 mg/ml DEAE-dextran (Serva) to improve virus adsorption. The efficiency of infection was 100% as assessed by direct immunofluorescence.

Priming for the induction of in vivo immune responses. Mice were infected by injection of 10^2 to 10^6 pfu MCMV in 0.1 ml 0.15 M NaCl into the left hind footpad. The animals were killed 2 days to 3 wk after virus application and LN served as a source for immune (ipsilateral popliteal) or control (contralateral popliteal, inguinal, axillary, cervical) responder cells.

Limiting dilution (LD) microcultures. Graded numbers of LN lymphocytes were plated into 96-well round-bottomed microtiter plates (No. 650101; Greiner, Nürtingen, Germany), with all tested concentrations present in 12 replicates on each individual plate. In addition, all plates contained 12 cultures set up with all components except for responding lymphocytes to serve as low controls. Information was then obtained by combining the results of several (at least two) identically designed but independent plates. This proceeding served to control for divergent development during the culture period due to variable quality of individual microplates. Two principal protocols were applied. Expansion protocol: the cultures (0.25 ml final vol) were adjusted to 20% v/v IL. The supernatant from Con A-activated spleen cells, which was depleted from low m.w. suppressor factors (9) and was tested for support of growth and maturation (10), was termed IL; restimulation protocol: the cultures received 10^4 MCMV-infected MEF as stimulator cells in addition to IL. Only when the two protocols were directly compared was the expansion protocol modified by adding 10^4 noninfected MEF as filler cells. In our experiments, the addition of MEF or spleen filler cells had no effect on the precursor frequency estimates. Incubation was performed for 5 to 7 days under standard conditions.

Separation of lymphocytes by discontinuous density gradient centrifugation. Lymphoblasts and dense cells were separated by flotation-sedimentation (11). Sediments of 6×10^7 LN cells were suspended in 6 ml isoosmotic Percoll (Pharmacia, Uppsala, Sweden) of the intended separation density, layered on top of a high density cushion (2 ml; $\rho = 1.09$ g/ml) in a 10 ml polystyrene tube (No. 163 160, Greiner) and covered with low density Percoll (2 ml; $\rho = 1.03$ g/ml). After centrifugation (1250 × G, 45 min) the fractions were analyzed for activity in expansion limiting dilution (LD) microcultures.

Selective depletion of immune lymphocytes before in vitro expansion. The monoclonal antibodies and the C3PO hybridoma (12) were generously provided by Catherine Mark and Z. A. Nagy, Max-Planck-Institute for Biology, Tübingen. C3PO produces anti-Lyt-1.2 monoclonal IgM antibody and the supernatant was used diluted 1/10 (cytotoxic titer, 1/3200). The anti-Lyt-2.2 19-178 hybridoma supernatant (originally obtained from U. Hämmerling) was applied undiluted (cytotoxic titer, 1/380). Supernatant from anti-Thy-1.2 IgM producing hybridoma HO-13-4 (13) was used in a dilution of 1/10 (cytotoxic titer 1/5500). The cytotoxic titer was determined in a BALB/c thymocyte reference system from the 50% maximal activity observed. The immune lymphocytes were subjected to two cycles of treatment with antibody and complement (C).

Phenotype determination of effector CTL. The microcultures, now containing grown CTL-clones, were split fivefold, and the identical 0.04 ml fractions were transferred to corresponding recipient wells and two cycles of antibody plus C treatment were performed. Finally, 10^3 ml target cells were added for the cell-mediated lympholysis (CML) assay.

Target cells and cytolytic assay. The 3 h CML-assay was performed with technical modifications based on established methods (14–16). The label efficiency of target cells ranged between 1.6 to 3.2 cpm/cell, and spontaneous release (LC) ranged from 10 to 20% of the total incorporated radioactivity (HC). If not otherwise indicated, only the specific lysis detected on infected syngeneic BALB/c MCMV-MEF target cells is given in figures and tables.

Calculations for the precursor frequency estimation. According to the theory of LD (for review see Reference 17), a culture is considered to have received at least one precursor cell if significant lytic activity can be detected. In the present study, the 99% tolerance limit (one sided) of the low control normal distribution was used to discriminate positive and negative cultures. Calculated as mean LC + $t(n\text{-}1, a/2 = 0.01) \times SD \times (1 + 1/n)^{0.5}$ (where n denotes the number of LC replicates, a the selected significance level, SD the standard deviation and t the Student *t*-distribution value for n-1 degrees of freedom), this limit is directly related to the number of low control replicates. The precursor frequencies were calculated by two established independent methods: the maximum likelihood (ML) estimation (18) and the minimum Chi-square (MC) estimation (19). According to the proposition of Taswell (19), an estimate was accepted when the probability value was $p > 0.05$ and the ML/MC divergence < 10%.

RESULTS

Kinetics and priming dose dependence of IL-CTL-P generation. Female BALB/c mice were primed by injection of various doses of infectious MCMV into the left hind footpad and cells from the ipsilateral popliteal LN were tested at the indicated time intervals for IL-CTL-P activity in the expansion protocol (Table I). This was defined by *in vitro* culture of preactivated lymphocytes under LD conditions in the presence of growth and maturation factors (IL), avoiding selective restimulatory influences of antigen (4). To reduce the number of *in vitro* parameters, cultures were usually set up (for exception see Fig. 1) without any addition of filler cells, following the general line to define the genuine *in vivo* expressed repertoire.

Peak frequencies of IL-CTL-P were observed between day 4 and day 8, depending on whether relative or absolute frequencies are concerned. At day 14 the number of IL-CTL-P already decreased and was less than $f = 1/10^5$ cells after 3 to 6 wk (not shown). There was no substantial activation in the contralateral LN. Despite of the strong cellular reactions in draining regional LN, the virus finally appeared in the salivary gland, which is the privileged target organ for MCMV replication during the persistent phase of infection (3.5×10^3 to 2.2×10^4 pfu/individual salivary gland 3 wk post infection with 10^4 pfu footpad). The activation was a function of the infec-

TABLE I

Kinetics of the relative and absolute IL-CTL-P frequency in regional LN draining the site of infection[a]

Site of Detection Popliteal LN	Time (day post infection)	Relative Frequency (95% confidence limits)	Probability (MC evaluation)	Average Number Cells/LN ($\times 10^{-6}$)	Absolute Frequency per Popliteal Lymphnode
Ipsilateral					
	0	$< 10/10^{-6}$	—	1.2	<10
	2	1/78300 13 (7-17)/10^6	0.473	4.2	50 (30-70)
	4	1/13500 74 (54-94)/10^6	0.264	9.0	670 (490-850)
	8	1/15000 67 (46-87)/10^6	0.521	17.5	1170 (800-1520)
	14	1/37900 26 (20-35)/10^6	0.527	7.9	200 (160-280)
Contralateral					
	8	1/59000 17 (12-24)/10^6	0.232	0.8	< 20
	14	1/97000 10 (7-15)/10^6	0.389	1.1	< 20

[a] LD microcultures (N = 24) were set up under expansion conditions with lymphocytes from BALB/c mice (five groups of 10 age-matched animals each) infected earlier with 10^4 pfu MCMV at the time intervals indicated. The absolute IL-CTL-P frequency was estimated by correcting the relative frequency (determined by MC and ML estimation, MC demonstrated only) for the average number of cells per LN, which was measured for each group from the pooled popliteal LN. In the case that far more than 1/e (36.8%) of the cultures were scored negative at the highest test dose (10^5 responder cells) the frequency could not be estimated with certainty and was indicated as $< 10/10^6$ cells.

tion dose. The increment of IL-CTL-P frequency was remarkably low (f = 1/25300 after 10^2 pfu to f = 1/4900 after 10^6 pfu) and thus only fivefold after increasing the viral dose by 10^4. In conclusion, IL-CTL-P are detectable only locally and transiently in draining LN, and their frequency depends upon the antigen dose.

Demonstration of two sets of activated CTL-P. Cultivation of primed LN lymphocytes in the absence or presence of antigen revealed two different precursor frequencies (Fig. 1). The low frequency obtained in the expansion protocol refers to cells in an antigen-independent stage (IL-CTL-P), whereas the increase in frequency obtained in the restimulation protocol is due to a majority of cells that grow to cytolytic clones only when cultured in the presence of IL and additional antigen in immunogenic form. In Figure 1, two examples are selected from a series to demonstrate the variance in the frequency estimates obtained in separate experiments. Despite the numerical differences, the basic observation was confirmed and even a similar ratio between antigen-dependent and -independent cells was found.

The frequency of specific CTL-P in nonimmune mice was too low to be estimated with certainty (f < 1/160 000) when popliteal LN cells or cells pooled from axillary, inguinal, and cervical LN were tested. This is in line with observations in other self-restricted systems including rabies virus (4), herpes simplex virus (20), H-Y antigen (21), and minor-H antigen (22). Both sets of cells found during primary infection, therefore, represent specifically activated cells.

Under LD conditions residual viral antigen is not responsible for expansion of activated CTL-P. Restimulation by residual viral antigen could account for the activity observed in the expansion protocol, instead of the real antigen-independent expansion of preactivated cells. Indeed, co-cultivation experiments led to the detection of about one infectious center per 10^6 cells. Consequently, the protocol for bulk cultures without antigen (23) is not applicable to the MCMV system because few infectious centers could disseminate virus and thereby provide restimulation conditions. In LD protocols, the infected cells within the tested population become limiting before the various types of precursors under test and hence do not interfere; the observation of single hit conditions (Fig. 1) supports this view.

The presentation of noninfectious viral fragments or processed antigen, however, was not yet excluded. For this purpose, the capacity of 10^5 day 8 immune Lyt-2.2-depleted LN lymphocytes to serve as stimulator cells for day 21 memory cells, which were already free from IL-CTL-P, was analyzed. By cultivation for 7 days in the presence of IL the tested stimulator cells could not elicit a secondary response (f < $1/10^5$), whereas the conditions provided by IL and a dose of 0.1 pfu infectious virus per responder cell were effective for *in vitro* recall of memory in the same experiment (f = 1/15228; 66(45-86)/10^6 pMC

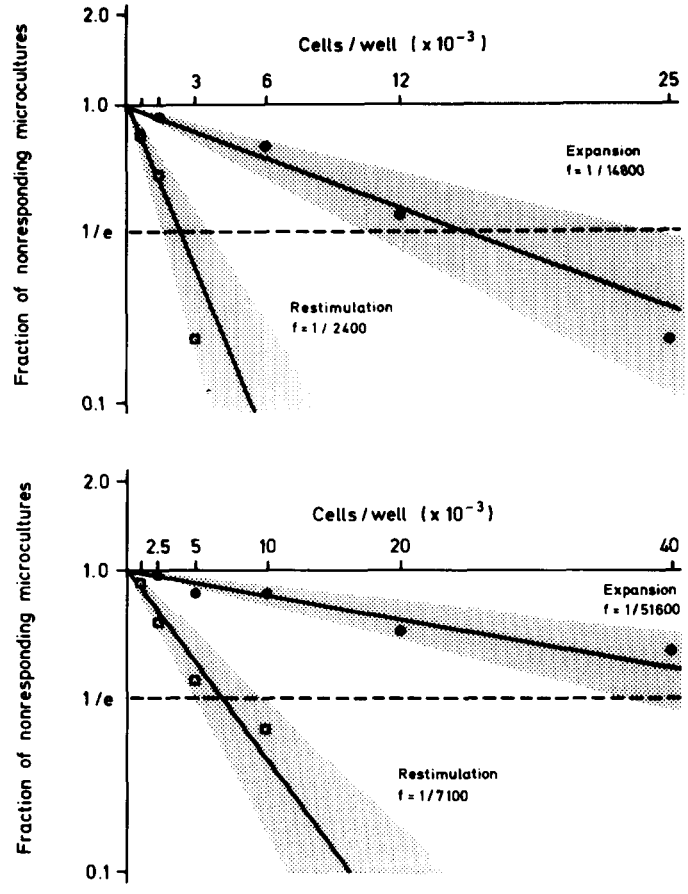

Figure 1. At the peak of a primary immune response activated lymphocytes of the cytolytic lineage are present in two distinct maturation stages. After infection with 10^4 pfu MCMV, day 8 immune lymphocytes were analyzed for the presence of antigen-independent and antigen-dependent cells by expansion (10^4 noninfected MEF filler cells) and by restimulation (10^4 MCMV-infected MEF stimulator cells). Because a direct comparison was performed on replicate microplates set up with the same cell suspension to start the dilution series, the observed relation is not affected by the uncertainty in the determination of the cell number. The *shaded areas* represent the 95% confidence limits of the MC estimates.

= 0.245). Thus, the antigen independent state of IL-CTL-P is a property of these cells and is not simulated by residual viral antigen.

IL-CTL-P reside in the lymphoblast fraction of the primed lymph node. It has been previously shown that a few days after antigen priming blast cells do not reside in the cortical follicles but rather in the paracortical region of the LN (24). *In situ* blast formation is also observed during MCMV infection (histologic studies not shown) and clearly lymphoblasts are the candidates for activated T cells. For a functional analysis, day 8 immune lymphocytes were separated into a low density lymphoblast fraction and a high density fraction. Both were analyzed for IL-CTL-P activity in the expansion LD assay (Fig. 2). The control group, devoid of IL (panel *B*), provided evidence that the observed activity was due to IL-dependent cells. The density threshold in this experiment was selected to obtain a significant depletion in the dense fraction rather than to obtain optimal enrichment of activated cells in the blast fraction. Clearly, IL-CTL-P belong predominantly to the lymphoblast fraction (compare panels *C* and *D*).

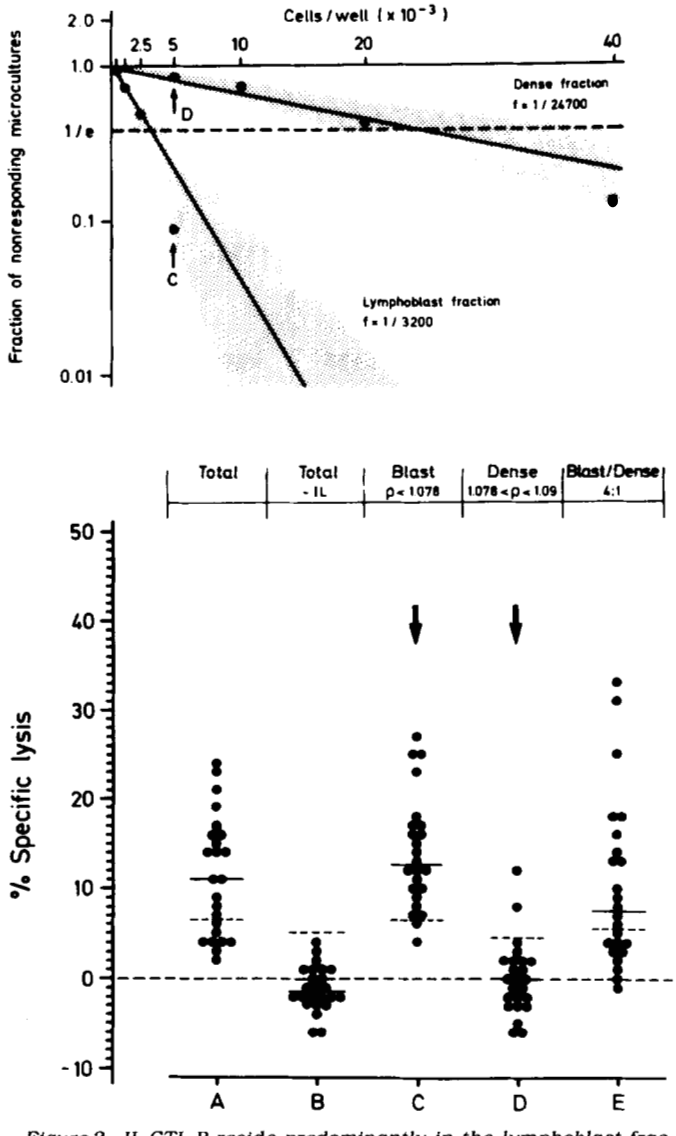

Figure 2. IL-CTL-P reside predominantly in the lymphoblast fraction of the primed LN. Physical separation on day 8 (10^5 pfu MCMV) sensitized lymphocytes in a density step gradient yielded 78.3% lymphoblasts ($\rho < 1.078$ g/cm^3) and 21.7% dense cells ($1.078 < \rho < 1.09$) with regard to recovered cells. The few interphase cells ($\rho = 1.078$) were discarded. The unseparated population with and without IL (*panels A* and *B*), the separated fractions (*panels C* and *D*), and the reconstituted population (*panel E*) were analyzed in the expansion LD assay (data of individual cultures demonstrated for 5×10^3 cells). The frequency in unseparated cells was 1/3667: 273(192–353)/10^6 pMC 0.943. The *dashed horizontal bars* represent the 99% tolerance limits of the low controls (n = 24). The solid horizontal bars indicate the positions of the median values (N = 24). The mean values are not shown because LD data are not normally (Gaussian) distributed. The *shaded areas* in the *upper figure* show the MC 95% confidence limits.

Interleukin sensitivity of IL-CTL-P and specificity of the cytolytic progeny. By definition, IL-CTL-P grow in the presence of IL, and thus the final functional activity generated can be expected to depend on the dose of IL, which determines the degree of clonal expansion. After a 6-day period of growth in the absence of antigen, individual oligoclonal cultures (25), initially set up with 10^5 day 6 immune LN cells, were split into five identical fractions and were tested for specificity on the target cells (Fig. 3*A*) and for the correlation control twice on MCMV-infected syngeneic MEF (Fig. 3*B*). The response was specific at all tested doses of help in the sense that there was no significant lysis of the noninfected syngeneic target MEF as well as of BALB 3T3 cells. High doses of IL, however, favored the generation of lytic activity against cells infected with an unrelated virus (rabies virus, strain ERA). Nonspecific activities due to increasing doses of IL 2 have also been reported recently in an analysis of CTL specific for minor H antigens (22). Consequently, either CTL could not discriminate between the different targets or various independent clones were expanded under such conditions, possibly including natural killer (NK) cells (26).

This alternative was tested by Spearman Rank Correlation analysis (27; Table II). The experiment was constructed as described above, with the modification that two out of four identical fractions of individual cultures were tested on MCMV-infected MEF and the other two on ERA-infected MEF. The activities against the two targets under test were independently distributed, indicating that there was no antigenic linkage on the population level. Thus, at higher concentrations of IL additional clones of cytolytic cells start to grow *in vitro*. In cultures set up with high numbers of activated lymphocytes, some cytolytic activity was generated even in the absence of external help (Fig. 3*B*). Presumably, an IL-producing helper cell is present at low frequencies in primed responder populations. This cell type is diluted out at lower cell numbers (compare Fig. 2, panel *B*).

Self restriction and Lyt-phenotype distinguish IL-CTL-P from activated NK cells. Thy-1$^+$ NK cells are capable of utilizing IL (26, 29), and for many viral systems including MCMV (30) their activation has been reported to precede the virus-specific CTL response. Because NK cells are not self restricted (29, 31), the test for self restriction (Fig. 4) was decisive for defining the nature of IL-CTL-P.

The test was performed in vice versa fashion to investigate whether each responder strain lyses the infected syngeneic target cell with preference over the infected allogeneic target cell; this preference could be demonstrated. It was consistently observed that the immune response in C57BL/6 (H-2^b) towards MCMV was low compared with BALB/c (H-2^d), and some lytic activity on infected as well as on noninfected allogeneic target cells

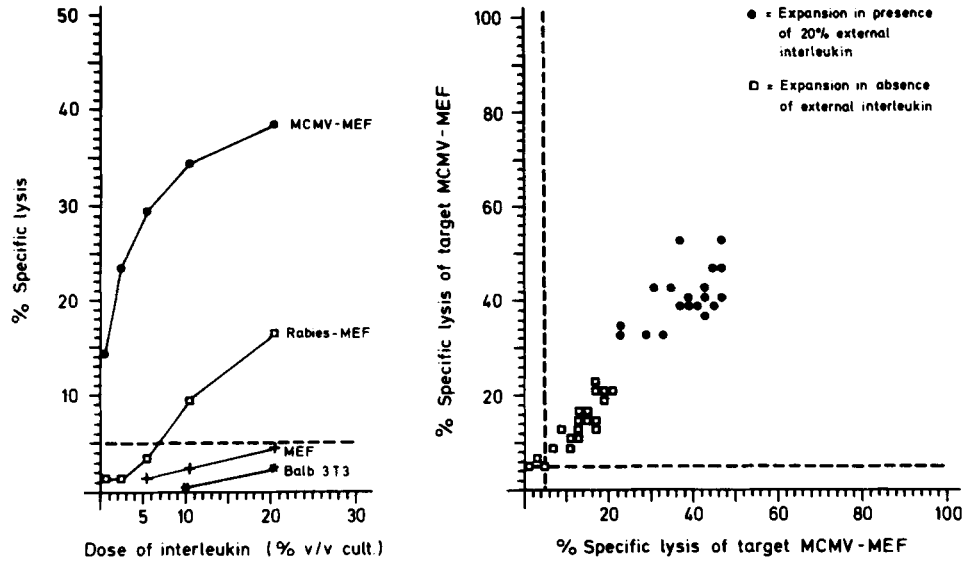

Figure 3. IL-CTL-P specific for the priming antigen utilize IL more efficiently than do precursors with deviating recognition capacity. Day 6 immune LN cells (10^5 pfu MCMV) were expanded for 6 days under oligoclonal conditions (10^5 cells/well) with various doses of IL. The cultures (N = 20) were split fivefold and were tested twice on the relevant infected syngeneic target BALB/c MCMV-MEF, and for control on noninfected BALB/c MEF or BALB 3T3 as well as on BALB/c MEF infected with the unrelated rhabdovirus rabies, strain ERA. The *left part* of the figure shows the median values as a function of the IL dose. The *dashed lines* represent the minimum positive detection level that is defined by the 99% tolerance limit of the MCMV-MEF low control.

TABLE II

Cells infected with MCMV or with the unrelated rabies virus (strain ERA) are recognized by separate CTL populations[a]

Target Comparison	R_s	p	Correlation Hypothesis
MCMV 1 – MCMV 2	0.382	0.012	accepted
ERA 1 – ERA 2	0.448	0.004	accepted
MCMV 1 – ERA 1	0.227	0.090	rejected
MCMV 2 – ERA 2	0.166	0.164	rejected
MCMV 1 – ERA 2	0.178	0.147	rejected
MCMV 2 – ERA 1	0.199	0.119	rejected

[a] After a 6-day *in vitro* expansion period, oligoclonal cultures, initially set up in 36 replicates (N = 36) with 10^5 day 6 *in vivo* (10^5 pfu) immune LN cells, were split fourfold (0.05 ml) and were tested for lytic activity twice on MCMV-MEF (MCMV1 and MCMV2) and twice on ERA-MEF (ERA1 and ERA2). The individual cultures were ranked according to their lytic activity and the Spearman rank correlation coefficient (R_s) was calculated (27). For $R_s \times (N-1)^{0.5}$ the attached probability values p (27) were looked up in tables of the standard normal probability function (28). The correlation hypothesis was accepted for positive R_s and $p < 0.05$ (one-tailed test).

was seen. The number of cultures responding to infected syngeneic cells, however, was with 19 out of 24 cultures, significantly higher ($p = 0.0016$, Fisher-Yates exact probability test) than those responding to infected allogeneic cells (eight out of 24 cultures).

T lymphocyte differentiation (Lyt) markers (32) provide an additional and independent means to discriminate between NK and self-restricted T cells. Figure 5 demonstrates that IL-CTL-P-derived cytolytic BALB/c effector cells are of the Thy-1^+, Lyt-1^-,2^+ phenotype. Also, after treatment of C57BL/6 effector cells with anti-Lyt-2.2 antibody plus C the preference for infected syngeneic target cells was abolished (data not shown).

In addition, the phenotype of the activated precursors was tested (Fig. 6). IL-CTL-P were selectively depleted by two cycles of bulk treatment with the indicated monoclonal antibodies and C before IL-mediated antigen-free expansion. In several experiments, the majority of IL-CTL-P were found to be of the Thy-1^+, Lyt-1^+,2^+ phenotype, distinct from their *in vitro* progeny. The Lyt-1-depleted population could not be reconstituted to function by adding the same amount of Lyt-2-depleted cells (compare panels *C* to *E*). This indicates that the impaired responsiveness was due to depletion of Lyt-1^+,2^+ IL-CTL-P and was not caused by depletion of Lyt-1^+,2^- helper T cells (32), which might have been necessary in addition to IL. In conclusion, in BALB/c mice IL-CTL-P and their progeny definitely belong to the lineage of H-2-restricted virus-specific CTL.

DISCUSSION

The present report demonstrates that MCMV-specific CTL-P present in the draining LN during acute infection can be isolated, quantitated, and tested for differential functional activity by using a sensitive LD technique. After cultivation of sensitized cell populations in the presence or absence of stimulating viral antigen, the fraction of CTL-P was determined that had reached an antigen-independent state *in vivo* and could be selected by the ability to utilize IL (IL-CTL-P). This set represents only a minority (15%) within the total number of specifically sensitized CTL-P. The majority of cells still require antigen *in vitro* to generate a functionally active progeny.

Thus, even during acute infection the majority of sensitized cells can only be analyzed under conditions that do not discriminate between memory cells, resting or activated CTL-P, and CTL. Only a minority of cells, the IL-CTL-P, behave like mature CTL with regard to growth and function. The kinetic study indicates that during the course of an infection the majority of sensitized cells do not reach the IL-CTL-P stage. Frequency estimates in presence of antigen, therefore, probably overestimate the number of cells that actively take part in the effector cell response at a given stage of infection. These data confirm and extend similar observations made by studying another viral system, the lethal rabies infection of mice (4). In addition, it is shown that the *in vivo*-activated IL-CTL-P belong to the lineage of self-restricted CTL, as defined by functional and serologic criteria. They reside predominantly in the lymphoblast fraction of the draining LN, with little tendency to migrate as active cells to other LN and occurring only transiently during acute infection. These observations, together with the fact that priming is necessary, indicate that the *in vivo* active T cell fraction is measured and exclude nonspecific activation of cells by IL *in vitro* (33).

The finding that the majority of the IL-CTL-P express the Lyt-1^+, 2^+ phenotype justifies their designation as "precursors" in the sense that they are not terminally differentiated (34). The denotation "interleukin-receptive" requires further comments: resting T cells cannot

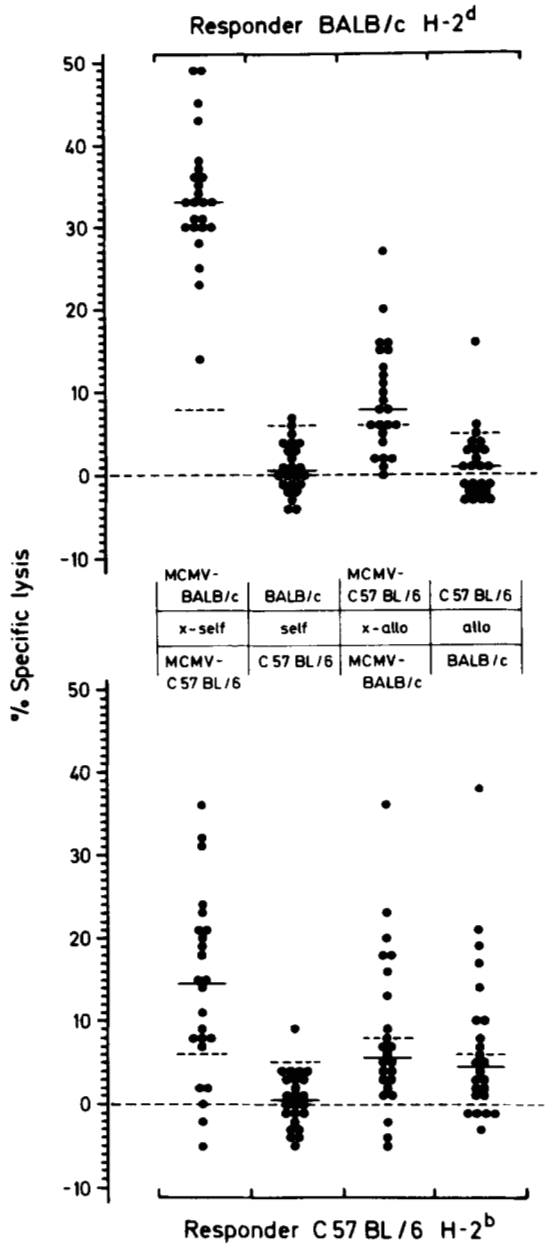

Figure 4. The progeny of IL-CTL-P are self-restricted CTL. BALB/c and C57BL/6 mice were infected with 10^5 pfu MCMV and at day 6 oligoclonal cultures (10^5 cells/well) were set up under expansion conditions. After 6 days *in vitro* the cultures (N = 24) were split fourfold (0.05 ml) and were tested on the indicated four types of target cells in a vice versa fashion. The *dashed horizontal bars* represent the 99% tolerance limits of the particular low controls (n = 24) and the *solid horizontal bars* indicate the positions of the median values.

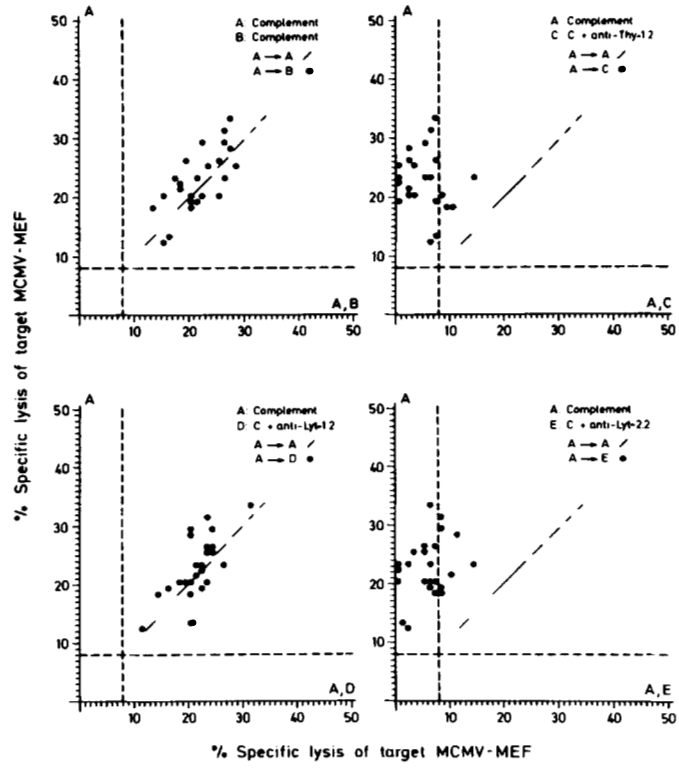

Figure 5. IL-CTL-P derived effector CTL express the Lyt-1$^-$, 2$^+$ phenotype. Oligoclonal expansion cultures (10^5 pfu MCMV, day 6 *in vivo*, 10^5 cells/well) were split fivefold (0.04 ml) and the effector cells were treated with antibody plus C (group C: HO-13-4 anti-Thy-1.2, group D: C3PO anti-Lyt-1.2, group E: 19-178 anti-Lyt-2.2). The *dashed lines* represent the 99% tolerance limit of the low control. The comparison A → A/indicates the theoretical correlation line, whereas the comparison A → B● demonstrates the true variance observed in the correlation control.

utilize IL and activation is required to render them receptive (35). IL allow the proliferation and maintenance of cytolytic T cell lines and clones (36). It is uncertain, however, how many different types of IL are required and in which precise sequence they act to drive a cell after sensitization by antigen through proliferation and differentiation to effector cell function. In contrast to former two-signal models (37), more recent results suggest a distinction between growth IL (IL 2) mediating cycling of activated T cells and other factors involved in maturation (10, 38, 39). Because in all protocols described here IL-CTL-P were detected not by proliferation but by the lytic activity of their clonal progeny, all IL-CTL-P must have received a differentiation signal. Thus, not the *in vivo*-acquired sensitivity to the growth promoting IL 2 but rather the sensitivity to differentiation factors could be the rate limiting property.

The expansion of sensitized cells in the presence of antigen is a widely applied protocol that reactivates self-restricted CTL (25, 40). The maturation state of the precursors during early stages of infection is, however, a subject for discussion. One interpretation could be that antigen-dependent CTL-P precede IL-CTL-P in the maturation pathway and represent an immature state. Phase-shifted maturation may result for various reasons, such as differential affinity of the precursors, delayed activation of cells due to asynchronous antigen encounter, or modulating influences of *in vivo* help and suppression. Another explanation is given by the "balance of growth" model in its more complex multicompartment version (41). This model proposes that memory cells and activated cells are in balance at all stages of differentiation. The increase in frequency after restimulation with antigen could then reflect the activation of early memory cells.

We propose that the separate sets observed in our experiments reflect distinct maturation stages in the sequence from the precursor to the terminally differentiated CTL. If this view is correct, only the fraction of IL-CTL-P can take part in the actual antiviral CTL response at a given moment of infection, whereas antigen-dependent CTL-P should not contribute directly to effector activity *in vivo*. The determination of IL-CTL-P frequency and specificity then provides an approach to detect *in vivo* active T cells that can be applied even in the presence of

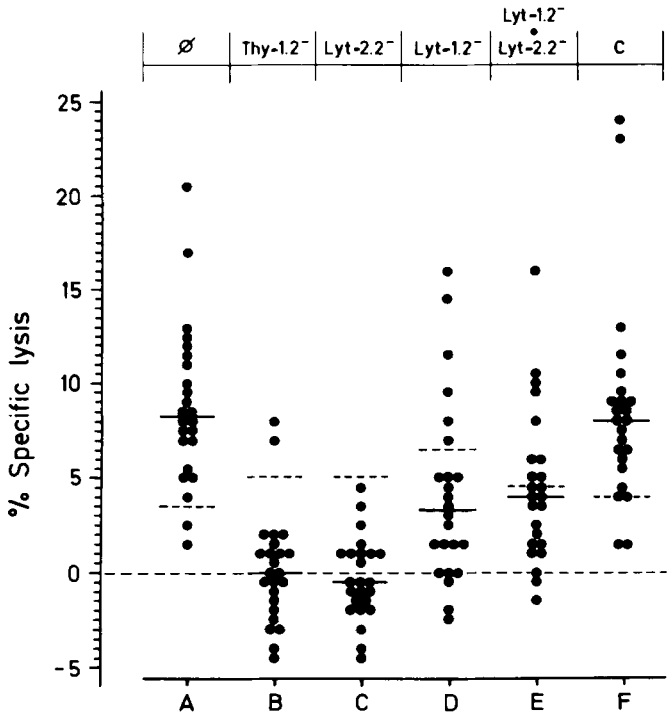

Figure 6. The majority of IL-CTL-P express the Lyt-1⁺, 2⁺ phenotype. Day 6 *in vivo* sensitized LN lymphocytes (10^4 pfu MCMV) were treated under bulk conditions with antibody plus C before the *in vitro* IL-mediated expansion phase. The depleted populations were then analyzed in the expansion LD assay for the presence of IL-CTL-P. Data from individual cultures (N = 24) are shown for an input of 40×10^3 responder cells/well (*panel A*: nontreated; *panel B*: HO-13-4 anti-Thy-1.2 + C; *panel C*: 19-178 anti-Lyt-2.2 + C; *panel D*: C3PO anti-Lyt-1.2 + C *panel E*: mixture 40×10^3 group C + 40×10^3 group D; *panel F*: C control). The frequency observed in the C control was 1/36 800; 27(20–35)/10^6; pMC 0.463 and in the Lyt-1.2-depleted population 1/158706; 6(3–9)/10^6; pMC 0.472. The *dashed horizontal bars* indicate the 99% tolerance limits of the low controls and the *solid horizontal bars* sign the positions of the median values.

memory T cells. This discrimination between the activated and the resting fractions of specific CTL is required for studying the potential role of CTL during persistent/latent herpes virus infection, and was the main reason for this work. Because we have found IL-CTL-P in the absence of detectable amounts of infectious virus during the latent stage of MCMV infection (to be published), it appears that the identification of such cells can serve to detect viral genome activities even at nonproductive stages of viral infection.

Acknowledgments. We thank Mss. Anke Lüske, Gabriele Röhrs, and Waltraud Umrath for the assistance, Ms. Sabine Grau for typing the manuscript, and Mr. Michael Reddehase for designing the computer software.

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