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CD8-Positive T Lymphocytes Specific for Murine Cytomegalovirus Immediate-Early Antigens Mediate Protective Immunity

MATTHIAS J. REDDEHASE,[1] WOLFGANG MUTTER,[1] KONRAD MÜNCH,[1] HANS-JÖRG BÜHRING,[2] AND ULRICH H. KOSZINOWSKI[1]*

Federal Research Centre for Virus Diseases of Animals[1] and Medical University Clinic,[2] D-7400 Tübingen, Federal Republic of Germany

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We have shown in a murine model system for acute, lethal cytomegalovirus (CMV) disease in the immunocompromised natural host that control of virus multiplication in tissues, protection from virus-caused tissue destruction, and survival are mediated by virus-specific $CD8^+$ $CD4^-$ T lymphocytes. Protection from a lethal course of disease did not result in a rapid establishment of virus latency, but led to a long-lasting, persistent state of infection. The $CD8^-$ $CD4^+$ subset of T lymphocytes was not effective by itself in controlling murine CMV (MCMV) multiplication in tissue or essential for the protective function of the $CD8^+$ $CD4^-$ effector cells. The antiviral efficacy of the purified $CD8^+$ $CD4^-$ subset was not impaired by preincubation with fibroblasts that presented viral structural antigens, but was significantly reduced after depletion of effector cells specific for the nonstructural immediate-early antigens of MCMV, which are specified by the first among a multitude of viral genes expressed during MCMV replication in permissive cells. Thus, MCMV disease provides the first example of a role for nonstructural herpesvirus immediate-early antigens in protective immunity.

Cytomegalovirus (CMV) disease is one of the leading reasons for mortality in immunocompromised patients. Opportunistic primary or recurrent CMV infection is involved in the acquired immunodeficiency syndrome (22, 23) and also causes interstitial pneumonia in bone marrow transplant recipients after iatrogenic immunosuppression (21, 25). In the immunocompetent host, CMV infection is effectively controlled, and virus persists in a latent state. CD8 (T8)-positive cytolytic T lymphocytes (CTL) have been implicated in the recovery from CMV infection (25). Viral gene expression in lymphocytes and monocytes of seropositive patients was found to be arrested after the expression of immediate-early (IE) genes (33), the first set of herpesvirus genes transcribed under permissive conditions (8), and CTL have been reported to recognize infected cells at an early time during the viral replication cycle (1).

We have studied protective immunity to CMV in a murine model system with particular respect to the virus-specified antigens involved. The present state of knowledge can be briefly reviewed as follows.

(i) During acute, intraplantar infection of immunocompetent BALB/c mice, replication of murine CMV (MCMV) is restricted to glandular epithelial cells in the salivary glands, while after immunodepletion by total-body gamma irradiation, virus can colonize several other tissues, including lung tissue, in which it causes interstitial pneumonia (32). (ii) Protective immunity can be conferred to immunodepleted recipients by transfer of specifically sensitized T lymphocytes that contain the CD8 (Lyt-2)-positive subset (32). The antiviral efficacy of such cells can be enhanced by application of interleukin 2 (IL-2) in vivo (31). (iii) A high proportion of the sensitized CTL precursors contained in donor T lymphocyte populations are specific for cell membrane antigen(s) specified by IE genes. IE antigens are nonstructural viral antigens (29, 30). (iv) A particular IE membrane antigen defined by the L^d-restricted CTL clone IE1 (26) is expressed in L($H\text{-}2^k$) fibroblasts cotransfected with the major histocompatibility complex (MHC) gene L^d and IE genes of MCMV (16, 17). In infected cells, this antigen can be detected after enhanced, selective expression of IE genes and also during the late phase of MCMV replication (27). (v) The major IE protein of MCMV, pp89, has a regulatory function in viral gene expression and is located in the nucleus of the infected cell (10, 11, 13, 15, 16). Gene *ie*I, encoding pp89, lacks nucleotide sequences that would predict membrane-spanning domains (12).

Based on the finding that T lymphocytes of the CD8 subset mediate protective immunity (31, 32), it has been a tacit assumption in our working hypothesis that IE-specific CTL perform a protective function. In this communication we provide the first evidence that CD8-positive T lymphocytes specific for IE antigens can protect from a lethal course of infection.

MATERIALS AND METHODS

Short-term T-lymphocyte lines. In vivo-sensitized T lymphocytes were propagated in vitro as IL-2-dependent short-term lines to deplete for other cell types before probing the antiviral efficacy in immunodepleted cell transfer recipients. The lymphocytes were derived from draining popliteal lymph nodes of immunocompetent BALB/c ($H\text{-}2^d$ haplotype) donors 8 days after intraplantar infection with 10^5 PFU of MCMV (strain Smith, VR-194; American Type Culture Collection, Rockville, Md.) and expanded in vitro for 8 days in round-bottomed 0.2-ml microcultures at a cell density of 5×10^4 cells per well in the absence of viral antigen and with no feeder cells (28). The culture medium (minimal essential medium alpha without nucleosides; GIBCO Laboratories; for supplements see reference 26) contained 100 U (0.88 pmol) of recombinant human IL-2 (rhIL-2) (lot 89050/84802) with a specific activity of 7.3×10^6 U/mg of protein (generously supplied by the Sandoz Forschungsinstitut, Vienna, Austria). Lymphokine-activated killer (LAK) cells (34) were generated by propagating popliteal lymph node-derived lym-

* Corresponding author.

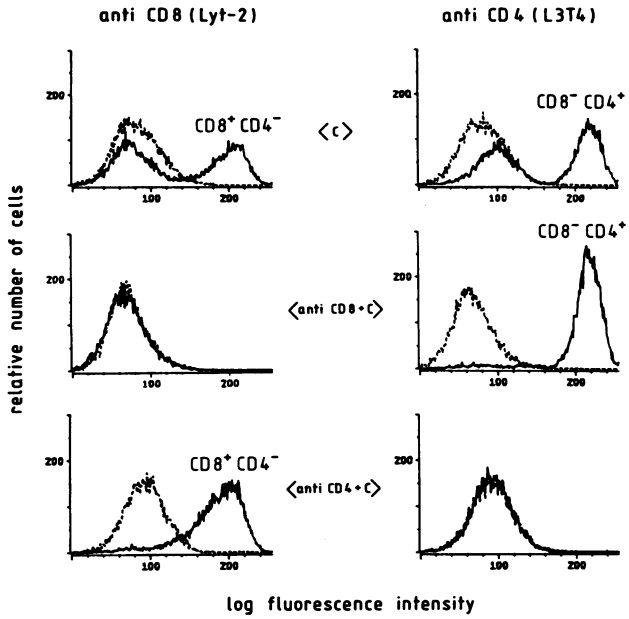

FIG. 1. Cytofluorometric analysis of purified T-lymphocyte subsets. Lymphocytes sensitized in vivo by intraplantar infection were propagated with IL-2 in vitro and then either treated with complement (C) alone (first row) or depleted of the CD8- and CD4-positive subsets by treatment with anti-CD8 (second row) or anti-CD4 (third row), respectively, and C. The residual cells were stained specifically with anti-CD8 MAb (left column) or with anti-CD4 MAb (right column). Results are expressed on a log fluorescence scale, with 10^4 cells analyzed. The control profiles obtained by staining with fluorescein isothiocyanate-conjugated second antibody alone are shown superimposed with dashed contours.

phocytes of noninfected BALB/c mice under the same culture conditions.

Depletion of the CD8- and CD4-positive subsets was achieved by treatment with monoclonal antibodies (MAbs) anti-Lyt-2.2 (immunoglobulin G2a [IgG2a]; hybridoma 19/178 [7]) and anti-L3T4 (GK 1.5, IgG2b [3]), respectively, and complement (32).

Cell surface phenotyping. For single-parameter flow cytometric analysis (FACS IV; Becton Dickinson FACS Systems, Sunnyvale, Calif.) of the CD phenotype, lymphocytes were stained indirectly with MAbs anti-Lyt-2.2 (anti-CD8) and GK with 1.5 (anti-CD4) and fluorescein-conjugated affinity-purified $F(ab')_2$ fragments of goat anti-mouse IgG and goat anti-rat IgG (Jackson Immuno Research, Avondale, Pa.), respectively (26). The fluorescence signal was triggered on particles larger than platelets, and propidium iodide (2 μg/ml)-stained dead cells were excluded by electronic gating. Relative fluorescence intensities were expressed on a log scale, with 10^4 cells analyzed.

Adoptive lymphocyte transfer. Lymphocyte transfer was performed essentially as described previously (32). BALB/c recipients immunodepleted by total-body gamma irradiation were infected in one hind footpad with 10^5 PFU of MCMV at 2 h after intravenous infusion of lymphocytes. The radiation dose was adapted to cause 50% mortality in noninfected recipients within a period of 30 days. In the experiments shown, this condition was fulfilled at a dose of 6 Gy.

Tissue virus titers. Infectious MCMV in lungs, spleen, and salivary glands (pooled parotid, greater sublingual, and mandibular glands) was quantitated 14 or 60 days postinfection by a plaque assay as described previously (32). The detection limit was 50 PFU of MCMV per organ homogenate. Sets of virus titers (x and y) are regarded as significantly different for P (x versus y) $< \alpha = 0.05$ (one-sided), where P denotes the observed probability value and α denotes the selected significance level (Wilcoxon-Mann-Whitney exact rank sum test).

Antigen-specific effector cell depletion and cytolytic assay. BALB/c mouse embryo fibroblasts (MEF) and simian virus 40-transformed B10.D2 (14) or C57BL/6 fibroblasts (lines KD2SV and B6/WT-3, respectively) selectively synthesizing IE proteins (IE-MEF, IE-KD2SV, IE-B6/WT-3) were prepared by infection with 20 PFU of MCMV per cell in the presence of cycloheximide (50 μg/ml), which was replaced after 3 h by actinomycin D (5 μg/ml) to prevent transcription of early- and late-phase genes. Selective presentation of viral structural antigens (S-MEF, S-KD2SV) was achieved by infection with high doses of MCMV (200 PFU/cell) in the presence of actinomycin D to prevent de novo antigen synthesis (26, 29).

For antigen-specific depletion, lymphocyte populations depleted of CD4-positive lymphocytes were incubated three times at a ratio of 1:1 for 30 min at 37°C on monolayers of either noninfected or MCMV antigen-expressing MEF. The cytolytic activity of the population recovered after the third incubation was measured in a standard 3-h ^{51}Cr release assay with 1,000 labeled IE-KD2SV or S-KD2SV target cells per well.

In situ hybridization of lung tissue sections with cloned MCMV DNA fragments. The technique for preparing tissue sections and the hybridization procedures have been described previously (32). MCMV DNA *Hin*dIII fragments A, B, D, and C (4), tritiated by nick translation to a specific activity of 3×10^7 dpm/μg, were used for detecting viral genome in tissue. Autoradiographs were exposed for 4 days and examined by light microscopy. Formation of silver grains proved to reflect mainly DNA-DNA hybridization, as treatment of tissue sections with RNase (200 μg/ml, 30 min at 37°C) before hybridization did not significantly reduce grain frequency, while DNase digest (100 μg/ml, 30 min at 37°C) resulted in a more than 95% reduction in the number of silver grains. The sensitivity of the in situ hybridization was determined by relating the number of silver grains to the number of viral genome equivalents measured in a Southern blot by using defined numbers of MCMV genomes as standards. At the exposure time of 4 days, one silver grain was found to signify the presence of ca. 5 genome equivalents.

RESULTS

Antiviral efficacy of purified T-lymphocyte subsets. Previous studies (31, 32) have demonstrated that depletion of CD8-positive lymphocytes abrogates the ability of a sensitized lymph node cell population to control MCMV multiplication in tissues of immunodepleted cell transfer recipients. Cooperation with CD4-positive lymphocytes proved to be dispensable, while a requirement for donor-derived CD4-negative accessory cells has not been formally excluded. For relating antigen specificity to antiviral in vivo function of effector cells, we have now studied the properties of purified T-lymphocyte subsets after in vitro IL-2-mediated clonal expansion in the absence of antigen.

The composition of in vitro IL-2-propagated lymphocyte populations, derived from draining lymph nodes of infected donors, was determined by cytofluorometric analysis (Fig. 1). The population comprised two distinct subsets: $CD8^+$ $CD4^-$ lymphocytes (left column in the array of fluorescence

histograms) and $CD8^-$ $CD4^+$ lymphocytes (right column). There was no indication of the existence of a non-CD4 non-CD8 population, demonstrating the depletion of B lymphocytes and accessory cell types during clonal expansion of T lymphocytes. Treatment with anti-CD8 MAb plus complement (second row in the array of fluorescence histograms) yielded a pure $CD8^-$ $CD4^+$ population, while treatment with anti-CD4 MAb plus complement (third row) yielded a pure $CD8^+$ $CD4^-$ population. The CD8 population contained cells with a low CD8 antigen density, a phenomenon that can be observed also with cloned long-term CTL lines (26).

The in vivo antiviral activity of these purified, accessory cell-depleted T-lymphocyte subsets was probed by prophylactic adoptive transfer into immunodepleted, lethally infected recipient mice (32), employing the lungs as a sensitive indicator site of MCMV replication (Fig. 2). LAK cells (34) generated by IL-2-mediated in vitro activation of lymphocytes derived from popliteal lymph nodes of noninfected donors did not affect virus multiplication in the lungs of transfer recipients (compare the virus titers in columns A and B; A versus B1, $P > 0.1$; A versus B2, $P > 0.1$). This control demonstrated that under the conditions of the experiment and within the dose range tested, an antiviral function could not be ascribed to LAK cells. Compared with it, nonseparated specifically sensitized lymphocyte populations (composed as depicted in Fig. 1, first row) markedly limited MCMV multiplication in lung tissue (compare columns A and C in Fig. 2). Note that transfer of 20,000 specific T lymphocytes proved to be significantly more effective than transfer of 10-fold this amount of LAK cells (B2 and C1, $P = 0.01$). A pure $CD8^-$ $CD4^+$ population was not able to exert

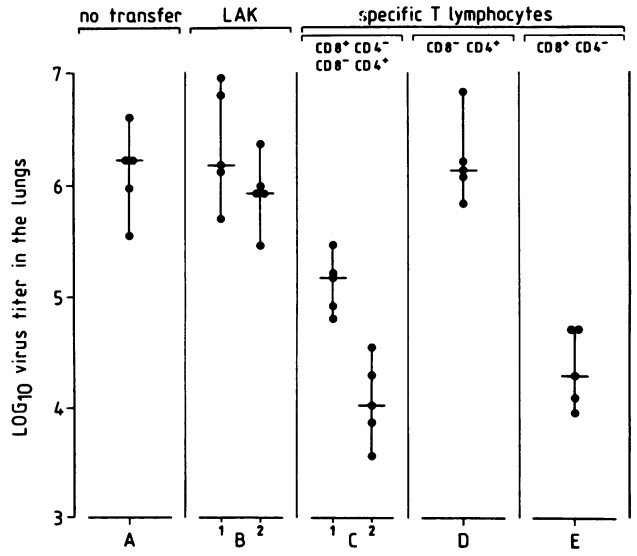

FIG. 2. Identification of the in vivo antiviral T-lymphocyte subset. The in vivo antiviral efficacy of lymphocytes was tested in lethally infected recipients by means of adoptive transfer. Virus titers in the lungs were determined at day 14 postinfection in five randomly collected surviving recipients per group. Individual titers are signified by closed circles, and median values are marked by horizontal bars. (A) No transfer of lymphocytes. (B) Transfer of 2×10^4 (lane 1) and 2×10^5 (lane 2) lymphokine (IL-2)-activated nonspecific killer cells. (C) Transfer of 2×10^4 (lane 1) and 2×10^5 (lane 2) specifically sensitized, IL-2-propagated T lymphocytes. (D) Transfer of 2×10^5 group C lymphocytes depleted of the CD8-positive subset. (E) Transfer of 2×10^5 group C lymphocytes depleted of the CD4-positive subset.

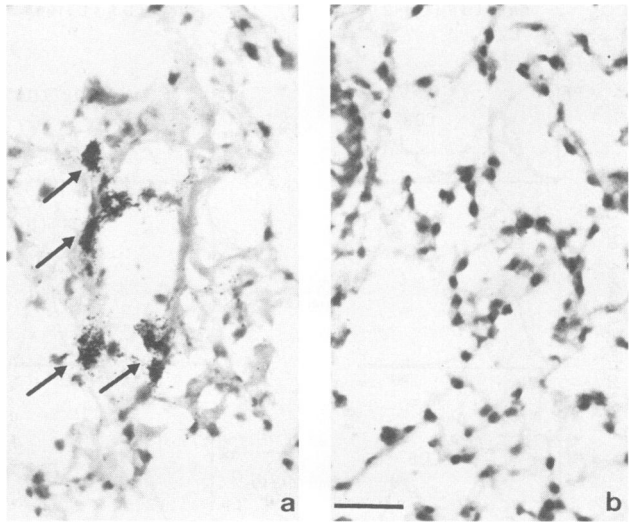

FIG. 3. In situ demonstration of the antiviral effect of CD8-positive T lymphocytes. Infected cells in thin sections (5 to 10 μm) of lung tissue were visualized at day 14 postinfection by in situ hybridization of viral nucleic acid with a mixture of plasmid-cloned MCMV DNA fragments as specific probe. (a) Transfer of 2×10^5 CD4-positive T lymphocytes; for virus titer see Fig. 2, column D. (b) Transfer of 2×10^5 CD8-positive T lymphocytes; for virus titer see Fig. 2, column E. Arrows in panel a point to clusters of infected cells. Both sections are shown at the same magnification. Bar, 30 μm.

an antiviral effect (D versus A, $P > 0.1$), while a pure $CD8^+$ $CD4^-$ population was as effective as the unseparated population (C2 and E, $P > 0.1$).

Evidence for prevention of virus-mediated histopathology by transfer of $CD8^+$ $CD4^-$ T lymphocytes was documented by in situ hybridization of lung tissue sections with cloned genomic fragments of MCMV (Fig. 3). While after transfer of T lymphocytes depleted of the CD8 subset several clusters of infected cells could be detected with ease in each lung tissue section at day 14 postinfection (Fig. 3a), MCMV DNA was only rarely detectable in lung tissue by screening a series of sections (Fig. 3b) after transfer of $CD8^+$ $CD4^-$ T lymphocytes. The more dispersed appearance of the tissue in Fig. 3a reflects the widening of the alveolar septa during interstitial MCMV pneumonia, as has been demonstrated in a previous report (32), whereas the tissue in Fig. 3b was phenotypically normal. This finding verified that the antiviral activity of CD8 effector cells was not accompanied by cell-mediated histopathology, but rather protected from virus-mediated tissue destruction.

In conclusion, the antiviral effector function in vivo can be assigned to the CD8-positive subset of specifically sensitized T lymphocytes. The CD4-positive subset was neither effective by itself nor essential for the function of the CD8 effector cells.

CD8-positive T lymphocytes specific for IE antigens control MCMV replication in vivo. In Fig. 4, the in vitro cytolytic activity of a purified CD8-positive effector cell population is related to its in vivo antiviral function. In that experiment, the frequency of IL-2-receptive, i.e., sensitized, CTL precursors (IL-CTLp [28, 30]) specific for antigens displayed by fibroblasts that selectively expressed IE genes of MCMV was estimated by limiting-dilution analysis (maximum-likelihood method [5]) to be 1 of 7,680 (95% confidence range, 1 of 5,550 to 1 of 10,640; $P = 0.78$), which was in good

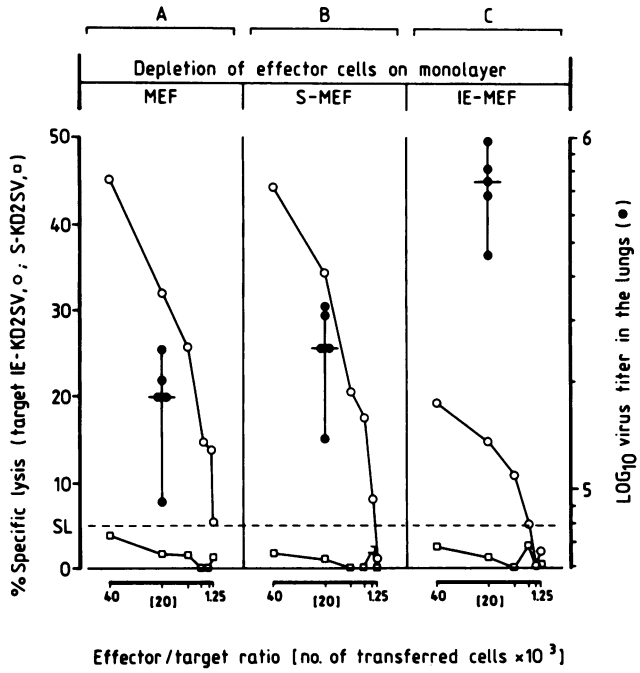

FIG. 4. In vitro effect and in vivo function of CD8-positive T lymphocytes specific for IE antigen(s) of MCMV. A purified $CD8^+$ $CD4^-$ effector cell population (Fig. 1, third row) was incubated for antigen-specific depletion on monolayers of noninfected fibroblasts (A; MEF) or of fibroblasts that either presented viral structural (S) antigens (B; S-MEF) or expressed viral IE antigens (C; IE-MEF). CTL activity and specificity in vitro (scale on the left, open symbols) were determined by lysis of the target cells S-KD2SV (open squares) and IE-KD2SV (open circles) at the effector-to-target cell ratios indicated. The dashed line represents the significance level for specific lysis, defined by the upper 95% confidence limit of spontaneous lysis. The in vivo antiviral function was tested by adoptive transfer of 20,000 cells (equal to the number of effector cells at the in vitro effector-to-target cell ratio of 20). The virus titers in the lungs of five transfer recipients per group were determined individually at day 14 postinfection (scale on the right, closed circles). The median values are marked by horizontal bars.

accordance with previous estimates (30), while the frequency of IL-CTLp specific for viral structural (S) antigens was <1 of 80,000.

The polyclonal lymphocyte population obtained after IL-2-mediated expansion was first depleted of the CD4 subset (Fig. 1, third row) and then incubated for antigen-specific depletion on fibroblast monolayers. CD8-positive lymphocytes recovered from noninfected monolayers (Fig. 4A) lysed syngeneic IE target cells (IE-KD2SV, $H\text{-}2^d$) with high efficacy. The recognition of IE antigens was MHC restricted, as MHC-mismatched IE target cells (IE-B6/WT-3, $H\text{-}2^b$) were not lysed (not depicted). Also not lysed were syngeneic fibroblasts that displayed viral S antigens (S-KD2SV). Note that S-KD2SV was an appropriate target for lysis by CTL clones specific for S determinants of MCMV (26). This result thus documented the absence of S-specific CTL in that effector cell population and reconfirmed that IE antigens and S antigens are separate entities recognized by different CTL (26, 29). Accordingly, preincubation on monolayers carrying S antigens did not affect the anti-IE-directed lytic efficacy of the population (Fig. 4B), whereas contact with monolayers that expressed IE genes profoundly did so (Fig. 4C).

The in vivo antiviral efficacies of the three selected populations were compared by testing the ability of 20,000 cells to limit MCMV multiplication in the lungs of immunodepleted recipients (Fig. 4). While preincubation on S monolayers had no significant effect (A and B, $P = 0.1$), depletion of IE-specific effector cells on IE antigen-expressing monolayers (panel C) resulted in a significant increase in virus titers (A and C, $P = 0.005$; B and C, $P = 0.005$; and A and B versus C, $P < 0.001$) to almost the control level (Fig. 2, column A).

Altogether, these data established that CD8-positive lymphocytes specific for IE antigens of MCMV are able to limit virus multiplication in vivo.

Protection from a lethal course of disease and establishment of persistent infection. Two important questions remained to be answered: (i) was the numerically and histologically significant antiviral effect also significant quo ad vitam, and (ii), if so, did infection in surviving cell transfer recipients then attain a latent or a persistent state (9)?

For the correct evaluation of the results shown in Fig. 5, it is important to recollect some basic facts of the experimental model detailed in a previous report (32). Immunodepletion as a consequence of total-body gamma irradiation with a radiation dose of 6 Gy (equal to a lethal dose referred to as LD50/30 in reference 37) causes, on average, 50% of adult (8- to 10-week-old) female BALB/c mice to die within an observation period of 30 days. Without exception, immunodepleted mice die of acute CMV disease after infection, while immunocompetent mice all survive.

CD8-positive lymphocytes selected for the recognition of viral nonstructural antigens (Fig. 4B) were transfused into lethally infected recipients (Fig. 5). Without lymphocyte transfer, all mice died within a demarcated period of time

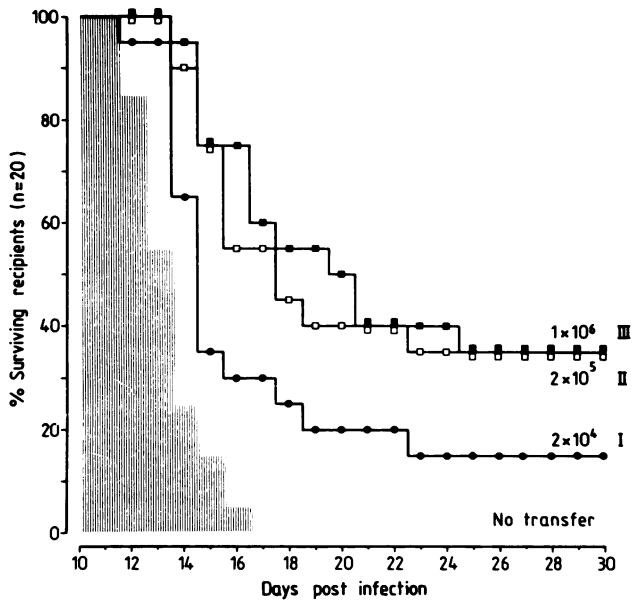

FIG. 5. Protection from a lethal course of MCMV disease by CD8-positive T lymphocytes. The diagram illustrates the mortality in a set of 20 immunodepleted, infected mice (shaded area) and the protection mediated by prophylactic adoptive transfer of 2×10^4 (group I, closed circles), 2×10^5 (group II, open squares), and 1×10^6 (group III, closed squares) $CD8^+$ $CD4^-$ T lymphocytes, which were selected for the recognition of viral nonstructural antigens (as shown in Fig. 4B). In one of the recipients (in group II) that had survived until the end of the 30-day monitoring period, necrosis at the plantar site of infection was not cured. This individual died at day 42 postinfection (not depicted).

postinfection (Fig. 5, shaded area). The terminal stage of CMV disease was signified by a severe wasting syndrome, extensive hemorrhages spreading from the plantar site of infection, and necrosis in several tissues, including the lungs, the liver, and the adrenal glands. Transfer of only 20,000 specific lymphocytes significantly retarded death from CMV disease, and 3 of 20 mice survived (Fig. 5, graph I). Note that hemorrhages in the footpad developed in all mice until day 10 postinfection and were completely healed in the survivors by day 30. Mortality could be further reduced by increasing the number of lymphocytes transferred, but reached a plateau level (compare graphs II and III). This result is well comprehensible, because antiviral T lymphocytes cannot be expected to remedy radiation sickness, but at best can annul the consequences of opportunistic viral infection.

A month later, at day 60 postinfection, titers of MCMV in tissue were determined for the salivary glands, the spleen, and the lungs of survivors from all three lymphocyte transfer groups (Fig. 6). With two exceptions (group I, recipient 3, and group II, recipient 6), the presence of infectious virus in the salivary glands indicated that viral latency was not established. With one exception (group III, recipient 4), viral replication had ceased in the spleen, while infectious virus was detected in the lungs in two of three (group I), three of six (group II), and two of seven (group III) recipients. The individual scoring revealed only a few mismatches in the rank of virus titers observed in salivary glands and lungs (group II, recipients 2, 3, and 4). In these recipients, MCMV was present in the salivary glands but not in the lungs. Consistent with the known fact that salivary glands represent the most permissive site for MCMV replication (20, 32), the reverse case was not observed, and the virus titers in the salivary glands were significantly higher than those in the lungs of the same individuals.

Seroconversion was observed in all individuals, indicating autoreconstitution of humoral immunity, but there was no correlation between MCMV-specific serum antibody titers and virus titers in any of the organs tested (data not shown).

In conclusion, CD8-positive T lymphocytes specific for IE antigens of MCMV protected from a lethal course of disease but did not prevent persistent infection.

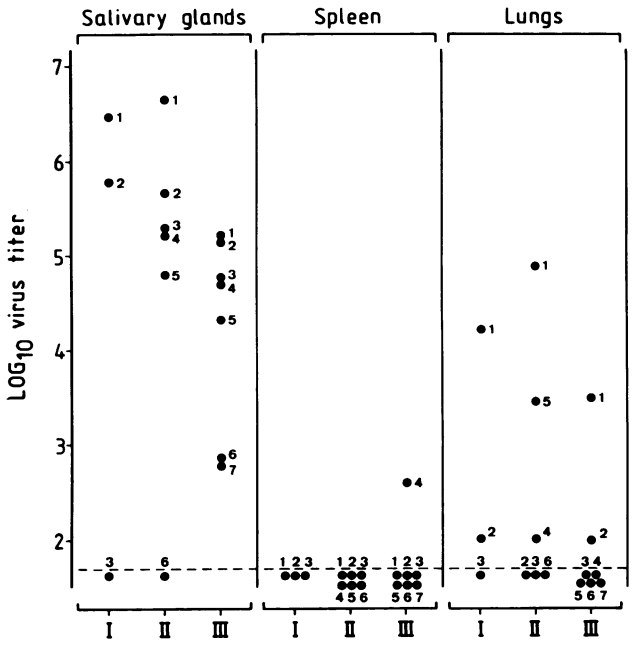

FIG. 6. Establishment of persistent MCMV infection. The survivors of the protection experiment shown in Fig. 5 (groups I, II, and III) were screened at day 60 postinfection for the presence of infectious MCMV in the salivary glands, the spleen, and the lungs. The individuals in each group are signified by numbers according to the rank of the virus titer in the salivary glands. The dashed line represents the detection limit.

DISCUSSION

Recent findings in viral immunology have established that virion envelope glycoproteins are not the only viral gene products that can be recognized by CTL in conjunction with class I major histocompatibility glycoproteins. Antigenic determinants can also be derived by antigen processing, i.e., by degradation or biochemical derivation, from nonglycosylated virion internal proteins (38–41) or from regulatory viral proteins which are not constituents of the virus particle but are present in transformed cells (2, 6).

Whether or not a significant role in eliciting protective immunity can be ascribed to processing-dependent antigens is a topic of current interest with possible relevance to future design of vaccines. MCMV infection offers the advantage of studying lethal virus disease, protection against it, and the conditions for establishing persistent and latent infection in the natural host.

There is a current debate about the T-lymphocyte CD subset operative in vivo in the control of MCMV disease. In the BALB/c mutant *nu/nu*, CD4 lymphocytes have been reported to be the antiviral effector cells in the adrenal glands (35). We have chosen the irradiated BALB/c mouse for a model because interstitial pneumonia caused by opportunistic primary or recurrent human CMV infection is a clinical problem in leukemia patients after irradiation (21, 25). Our present data concur with our previous studies (31, 32) in the conclusion that control of virus multiplication in the lungs is a function of the CD8 subset of specifically sensitized T lymphocytes and prove in addition that purified $CD8^+$ $CD4^-$ effector cells do not require the help provided by CD4 lymphocytes to protect against tissue destruction and mediate survival.

The mechanism by which CD8 lymphocytes exert their antiviral effect in vivo is still open to question, and a solution of this problem is beyond the scope of this contribution. Two aspects, however, deserve consideration. First, remarkably few cells were required for conferring protection (Fig. 3 and 5), and second, only specifically sensitized lymphocytes inhibited virus multiplication (Fig. 2). A seemingly low probability for antiviral effector cells to encounter their targets in vivo has been adduced as an argument in favor of a lymphokine effect (18). Studies by Lukacher et al. (19) and by Zinkernagel et al. (42) have provided evidence for direct in vivo cytolysis by CTL. From recent work by Oldstone et al. (24), it can be concluded that these mechanisms do not necessarily exclude each other. In MCMV disease, control of virus multiplication in the lungs by CD8 effector cells was not accompanied by cell-mediated tissue destruction. This finding, however, cannot be taken as evidence against direct cytolysis. MCMV infection in tissue is focal, spreading from single cells initially colonized by virus. The occurrence of infected cells in distinct clusters (Fig. 3a) illustrates this fact. Elimination of only a few infected cells at an early time during infection, although histopathologically asymptomatic, can result in a significant reduction in virus load,

measurable at a later stage. Thus, the finding that low numbers of antiviral effector cells suffice for protection can be explained by early eradication of a few infected centers.

Two months after protection against a lethal infection by adoptive transfer of $CD8^+$ $CD4^-$ lymphocytes (Fig. 5), persistent virus replication was detected in the salivary glands of most and in the lungs of some survivors (Fig. 6), although these individuals appeared healthy, had cured the hemorrhages at the plantar site of infection, and had reconstituted their immune system, as manifested by a humoral immune response to MCMV. There was no indication of a role for antiviral antibody in the control of MCMV replication, as antibody titers in serum did not correlate with virus titers in any of the organs tested. Persistent infection and tissue distribution of MCMV during reconstitution of the immune system after irradiation resemble the infection in newborn mice, while in adult immunocompetent mice the lungs are not affected and MCMV replication is confined to the salivary glands, where it ceases after about 6 weeks (32; G. M. Keil et al., unpublished observations).

The occurrence of substantial virus replication in the lungs of phenotypically healthy survivors (Fig. 6) indicates that infection at that site is not inevitably lethal. Adrenal cortical dysfunction (31, 36) or failure in hematopoiesis (W. Mutter, M. J. Reddehase, F. Busch, H.-J. Bühring, and U. H. Koszinowski, manuscript in preparation) might turn out to be a more decisive factor.

The most important aspect in our results concerns the antigen specificity of protective effector cells. An in vivo antiviral function could be attributed to $CD8^+$ $CD4^-$ T lymphocytes that displayed an in vitro CTL activity against target cells selectively expressing nonstructural IE gene products, but not against target cells presenting viral structural antigens (Fig. 4A). Recognition by CTL of nonstructural antigens encoded during the early and late phases of MCMV replication could not be assessed, because selective expression of early- and late-gene products in the absence of IE gene products is technically not feasible as yet. Note that pp89, the major IE protein (13), once synthesized during the IE phase, remains detectable in infected cells throughout the viral replication cycle and is synthesized again during the late phase (27). For this reason, antigen-specific depletion of lymphocytes and testing of the residual population was the more conclusive approach to the identification of antiviral effector cells. Significant reduction in both in vitro IE-specific lytic activity and in vivo antiviral function by preincubating sensitized $CD8^+$ $CD4^-$ T lymphocytes on monolayers that selectively expressed IE antigens positively identified IE-specific T lymphocytes of the CD8 subset as in vivo-operating antiviral effector cells.

It is still open to question why, in the immunocompetent host, CTL specific for structural antigens of MCMV occur less frequently than IE-specific CTL (30) and are often not detectable at all. It has been discussed previously (30) that in the immunocompetent host, infection is not productive in tissues, except for the salivary glands, and hence effective priming of CTL precursors specific for virion structural antigens may not occur. Based on the present findings it could be speculated that, by eradicating infected cells at an early stage, the high in vivo antiviral efficacy of IE-specific effector cells is directly responsible for the failure to generate a structural antigen-specific CTL response.

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LITERATURE CITED

1. **Borysiewics, L. K., S. Morris, J. D. Page, and J. E. P. Sissons.** 1983. Human cytomegalovirus-specific cytotoxic T lymphocytes: requirements for in vitro generation and specificity. Eur. J. Immunol. **13**:804–809.
2. **Campbell, A. E., F. L. Foley, and S. S. Tevethia.** 1983. Demonstration of multiple antigenic sites of the SV40 transplantation rejection antigen by using cytotoxic T lymphocyte clones. J. Immunol. **130**:490–492.
3. **Dialynas, D. P., D. B. Wilde, P. Marrack, A. Pierres, K. A. Wall, W. Havran, G. Otten, M. R. Loken, M. Pierres, J. Kappler, and F. W. Fitch.** 1983. Characterization of the murine antigenic determinant, designated L3T4a, recognized by monoclonal antibody GK 1.5: expression of L3T4a by functional T cell clones appears to correlate primarily with class II MHC antigen-reactivity. Immunol. Rev. **74**:29–56.
4. **Ebeling, A., G. M. Keil, E. Knust, and U. H. Koszinowski.** 1983. Molecular cloning and physical mapping of murine cytomegalovirus DNA. J. Virol. **47**:421–433.
5. **Fazekas de St. Groth, S.** 1982. The evaluation of limiting dilution assays. J. Immunol. Methods **49**:R11–R23.
6. **Gooding, L. R., and K. A. O'Connell.** 1983. Recognition by cytotoxic T lymphocytes of cells expressing fragments of the SV40 tumor antigen. J. Immunol. **131**:2580–2586.
7. **Hämmerling, G. J., U. Hämmerling, and L. Flaherty.** 1979. Qat-4 and Qat-5, new murine T-cell antigens governed by the Tla region and identified by monoclonal antibodies. J. Exp. Med. **150**:108–116.
8. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of at least three groups of viral proteins. J. Virol. **14**:8–19.
9. **Jordan, M. C.** 1978. Murine models of cytomegalovirus latency, p. 805–810. *In* J. G. Stevens, G. J. Todaro, and C. F. Fox (ed.), Persistent viruses. Academic Press, Inc., New York.
10. **Keil, G. M., A. Ebeling-Keil, and U. H. Koszinowski.** 1984. Temporal regulation of murine cytomegalovirus transcription and mapping of viral RNA synthesized at immediate-early times after infection. J. Virol. **50**:784–795.
11. **Keil, G. M., A. Ebeling-Keil, and U. H. Koszinowski.** 1987. Immediate-early genes of murine cytomegalovirus: location, transcripts, and translation products. J. Virol. **61**:526–533.
12. **Keil, G. M., A. Ebeling-Keil, and U. H. Koszinowski.** 1987. Sequence and structural organization of murine cytomegalovirus immediate-early gene 1. J. Virol. **61**:1901–1908.
13. **Keil, G. M., M. R. Fibi, and U. H. Koszinowski.** 1985. Characterization of the major immediate-early polypeptides encoded by murine cytomegalovirus. J. Virol. **54**:422–428.
14. **Knowles, B. B., M. Koncar, K. Pfizenmaier, D. Solter, D. P. Aden, and G. Trinchieri.** 1979. Genetic control of the cytotoxic T cell response to SV40 tumor-associated specific antigen. J. Immunol. **122**:1798–1806.
15. **Koszinowski, U. H., G. M. Keil, H. Volkmer, M. R. Fibi, A. Ebeling-Keil, and K. Münch.** 1986. The 89,000-M_r murine cytomegalovirus immediate-early protein activates gene transcription. J. Virol. **58**:59–66.
16. **Koszinowski, U. H., G. M. Keil, H. Schwarz, J. Schickedanz, and M. J. Reddehase.** 1987. A nonstructural polypeptide encoded by immediate-early transcription unit 1 of murine cytomegalovirus is recognized by cytolytic T lymphocytes. J. Exp. Med. **166**: 289–294.
17. **Koszinowski, U. H., M. J. Reddehase, G. M. Keil, and J. Schickedanz.** 1987. Host immune response to cytomegalovirus: products of transfected viral immediate-early genes are recognized by cloned cytolytic T lymphocytes. J. Virol. **61**:2054–2058.
18. **Lehmann-Grube, F., U. Assmann, G. Löliger, D. Moskophidis, and J. Löhler.** 1985. Mechanism of recovery from acute virus infection. I. Role of T lymphocytes in the clearance of

lymphocytic choriomeningitis virus from spleens of mice. J. Immunol. **134**:608–615.
19. **Lukacher, A. E., V. L. Braciale, and T. J. Braciale.** 1984. In vivo effector function of influenza virus-specific cytotoxic T lymphocyte clones is highly specific. J. Exp. Med. **160**:814–826.
20. **Mayo, D. R., J. A. Armstrong, and M. Ho.** 1977. Reactivation of murine cytomegalovirus by cyclophosphamide. Nature (London) **267**:721–723.
21. **Meyers, J. D., N. Flournoy, and E. D. Thomas.** 1980. Cytomegalovirus infection and specific cell-mediated immunity after marrow transplant. J. Infect. Dis. **142**:816–824.
22. **Moskowitz, L., G. T. Hensley, J. C. Chan, and K. Adams.** 1985. Immediate causes of death in acquired immunodeficiency syndrome. Arch. Pathol. Lab. Med. **109**:735–738.
23. **Niedt, G. W., A. Roger, and A. Schinella.** 1985. Acquired immunodeficiency syndrome. Clinicopathologic study of 56 autopsies. Arch. Pathol. Lab. Med. **109**:727–734.
24. **Oldstone, M. B. A., P. Blount, P. J. Southern, and P. W. Lampert.** 1986. Cytoimmunotherapy for persistent virus infection reveals a unique clearance pattern from the central nervous system. Nature (London) **321**:239–243.
25. **Quinnan, G. V., Jr., N. Kirmani, A. H. Rook, J. F. Manischewitz, L. Jackson, G. Moreschi, G. W. Santos, R. Saral, and W. H. Burns.** 1982. Cytotoxic T cells in cytomegalovirus infection: HLA-restricted T lymphocyte and non-T lymphocyte cytotoxic responses correlate with recovery from cytomegalovirus infection in bone marrow transplant recipients. N. Engl. J. Med. **307**:7–13.
26. **Reddehase, M. J., H.-J. Bühring, and U. H. Koszinowski.** 1986. Cloned long-term cytolytic T-lymphocyte line with specificity for an immediate-early membrane antigen of murine cytomegalovirus. J. Virol. **57**:408–412.
27. **Reddehase, M. J., M. R. Fibi, G. M. Keil, and U. H. Koszinowski.** 1986. Late-phase expression of a murine cytomegalovirus immediate-early antigen recognized by cytolytic T lymphocytes. J. Virol. **60**:1125–1129.
28. **Reddehase, M. J., G. M. Keil, and U. H. Koszinowski.** 1984. 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. J. Immunol. **132**:482–489.
29. **Reddehase, M. J., G. M. Keil, and U. H. Koszinowski.** 1984. The cytolytic T lymphocyte response to the murine cytomegalovirus. II. Detection of virus replication stage-specific antigens by separate populations of in vivo active cytolytic T lymphocyte precursors. Eur. J. Immunol. **14**:56–61.
30. **Reddehase, M. J., and U. H. Koszinowski.** 1984. Significance of herpesvirus immediate early gene expression in cellular immunity to cytomegalovirus infection. Nature (London) **312**:369–371.
31. **Reddehase, M. J., W. Mutter, and U. H. Koszinowski.** 1987. In vivo application of recombinant interleukin 2 in the immunotherapy of established cytomegalovirus infection. J. Exp. Med. **165**:650–656.
32. **Reddehase, M. J., F. Weiland, K. Münch, S. Jonjic, A. Lüske, and U. H. Koszinowski.** 1985. Interstitial murine cytomegalovirus pneumonia after irradiation: characterization of cells that limit viral replication during established infection of the lungs. J. Virol. **55**:264–273.
33. **Rice, G. P. A., R. D. Schrier, and M. B. A. Oldstone.** 1984. Cytomegalovirus infects human lymphocytes and monocytes: virus expression is restricted to immediate-early gene products. Proc. Natl. Acad. Sci. USA **81**:6134–6138.
34. **Rosenberg, S. A., and M. T. Lotze.** 1986. Cancer immunotherapy using interleukin-2 and interleukin-2-activated lymphocytes. Annu. Rev. Immunol. **4**:681–709.
35. **Shanley, J. D.** 1987. Modification of acute murine cytomegalovirus adrenal gland infection by adoptive spleen cell transfer. J. Virol. **61**:23–28.
36. **Shanley, J. D., and E. L. Pesanti.** 1986. Murine cytomegalovirus adrenalitis in athymic nude mice. Arch. Virol. **88**:27–35.
37. **Storer, J. B.** 1966. Acute responses to ionizing radiation, p. 427–446. *In* E. L. Green (ed.), Biology of the laboratory mouse. McGraw-Hill Book Co., New York.
38. **Townsend, A. R. M., F. M. Gotch, and J. Davey.** 1985. Cytotoxic T cells recognize fragments of the influenza nucleoprotein. Cell **42**:457–467.
39. **Townsend, A. R. M., J. Rothbard, F. M. Gotch, G. Bahadur, D. Wraith, and A. J. McMichael.** 1986. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell **44**:959–968.
40. **Yewdell, J. W., J. R. Bennink, M. Mackett, L. Lefrancois, D. S. Lyles, and B. Moss.** 1986. Recognition of cloned vesicular stomatitis virus internal and external gene products by cytotoxic T lymphocytes. J. Exp. Med. **163**:1529–1538.
41. **Yewdell, J. W., J. R. Bennink, G. L. Smith, and B. Moss.** 1985. Influenza A virus nucleoprotein is a major target antigen of cross reactive anti-influenza A virus cytotoxic T lymphocytes. Proc. Natl. Acad. Sci. USA **82**:1785–1789.
42. **Zinkernagel, R. M., E. Haenseler, T. Leist, A. Cerny, H. Hengartner, and A. Althage.** 1986. T cell-mediated hepatitis in mice infected with lymphocytic choriomeningitis virus. Liver cell destruction by H-2 class I-restricted virus-specific cytotoxic T cells as a physiological correlate of the ^{51}Cr-release assay? J. Exp. Med. **164**:1075–1092.