

Efficacious Control of Cytomegalovirus Infection after Long-Term Depletion of CD8⁺ T Lymphocytes

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Although the relative contribution of different immune effector functions to clearing tissues of cytomegalovirus is controversial, the contribution of CD8⁺ T lymphocytes has generally been accepted as essential. In this report, we show that under certain conditions the CD8⁺ T-lymphocyte subset can be dispensable for clearance of cytomegalovirus. Mice depleted of the CD8⁺ T-lymphocyte subset eliminated infectious virus with a clearance kinetics similar to that of normal mice. Adoptive transfer studies revealed that the limitation of virus spread required the cooperation between the CD4⁺ subset and other cells. Comparison between protective functions generated in fully immunocompetent and in CD8⁻ mice demonstrated that elimination of the CD8⁺ subset before infection altered the quality of the antiviral immune response. The compensatory protective activity gained by CD4⁺ cells in CD8⁻ mice was absent in normal mice recovering from virus infection.

Human cytomegalovirus (HCMV), a member of the herpesvirus family, is endemic in all human populations. Similar to other herpesvirus infections, acute HCMV infection is followed by chronic infection or viral latency from which reactivation can occur. Congenital HCMV infection of the immunologically immature fetus represents the most common viral intrauterine infection, and HCMV is also the major cause of viral morbidity and mortality in immunocompromised patients. The status of the immune system dictates whether infection is asymptomatic or leads to clinical or even fatal disease. Since reactivation can also occur in seropositive subjects, cellular immune mechanisms appear to have a major role in HCMV control. Accordingly, the activity of cytolytic T lymphocytes was found to correlate with recovery of transplant patients from primary HCMV infection (26). The role of CD4⁺ T lymphocytes and the protective effects of antibodies are not clear. Prophylactic administration of anti-HCMV immunoglobulin can reduce the severity of HCMV disease and has an effect on the incidence of HCMV pneumonitis, whereas therapeutic application has been mainly without protective effects (41).

To study the immune mechanisms in the infected host, infection of mice with murine cytomegalovirus (MCMV) has been used as a suitable animal model (13). Evidence has been provided that primed CD8⁺ T lymphocytes can protect against subsequent lethal MCMV disease (24, 28, 31, 32, 34). This protective function of the antecedent CD8⁺ T-lymphocyte activity was documented by cell transfer experiments and by the selective activation of antiviral CD8⁺ T lymphocytes (8, 14). The experiments invariably showed that whenever CD8⁺ T lymphocytes were activated, they played a dominant role in protection. The significance of antibody is not clear, since prophylactic transfer of antiserum to MCMV has protective activity, whereas immunoglobulin therapy of established infection does not prevent viral spread (38). Similarly, the contribution of sensitized CD4⁺ T lymphocytes is controversial (28, 36). These data show clearly that a preceding activation of both cellular and humoral immune

functions can contribute to protection against subsequent primary MCMV infection or reinfection.

The relative importance of the different immune effector mechanisms that are generated during primary infection is not revealed by these studies. Therefore, we have started to analyze the contribution of the CD4⁺ and the CD8⁺ T-lymphocyte subsets to MCMV clearance. The experimental approach has been the depletion of lymphocyte subsets *in vivo* before and during infection. We have reported recently that mice can control acute MCMV infection in the absence of the CD4⁺ subset (15). CD4⁻ mice clear most infected organs but develop a chronic infection confined to the salivary glands. In the study reported here, we tested the alternative situation. Mice were depleted of the CD8⁺ T-lymphocyte subset and then infected with MCMV. The results show that recovery from acute MCMV infection can proceed in the absence of the CD8⁺ T-cell subset. Although virus clearance was almost indistinguishable between normal and CD8⁻ mice, major differences were revealed by the analysis of immune effector functions.

MATERIALS AND METHODS

Elimination of T-lymphocyte subsets *in vivo*. Female BALB/c mice 4 to 5 weeks of age were thymectomized under chloral hydrate anesthesia according to standard surgical procedures. *In vivo* depletion of T-lymphocyte subsets was carried out as described by Cobbold et al. (6). Depletion of the CD8⁺ subset was accomplished by daily intravenous injections of thymectomized mice (for time schedule, see Fig. 1) with 1 mg of rat immunoglobulin G2b (IgG2b) anti-murine CD8 monoclonal antibody (MAb) YTS 169.4 (6) for 3 successive days. After that period, CD8⁺-depleted mice were injected weekly with 0.25 mg of mouse IgG2a anti-Lyt 2.2 (CD8) MAb 19/178 (10) throughout the experiment. For depletion of CD4⁺ T lymphocytes, mice were injected intravenously with 1 mg of rat IgG2b anti-mouse CD4 MAb YTS 191.1 (6) for 3 successive days. The antibodies used for *in vivo* treatment were enriched from ascitic fluid by precipitation with 50% ammonium sulfate and subsequent dialysis against phosphate-buffered saline. The concentration of specific antibody was determined by immunodiffusion, using rat and mouse IgG standards (15).

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Monitoring of T-cell subsets. Depletion efficacy of T-lymphocyte subsets in cell suspensions was tested by single-parameter cytofluorometric analysis for the presence of the CD4⁺ and CD8⁺ subsets as described previously (15). The depletion efficacy of T-lymphocyte subsets after treatment with anti-CD4 and anti-CD8 MAb in tissues was tested by indirect immunoperoxidase staining of frozen tissue sections as described by Hsu et al. (12).

Virus and infection conditions. The Smith strain of MCMV (VR-194; American Type Culture Collection, Rockville, Md.) was propagated in BALB/c mouse embryo fibroblasts as described previously (29). Mice were infected by injection of 2×10^5 PFU of MCMV into one hind footpad. T-cell subset-depleted mice were infected after the third injection of rat MAb, and recipients for the adoptive transfer were infected 2 h after irradiation and cell transfer.

Determination of virus titers in tissues. MCMV in tissues was quantified by plaque assay (34). The detection limit was 100 PFU of MCMV per organ homogenate. Virus titers (x and y) were regarded as significantly different for $P(x \text{ versus } y) < \alpha = 0.05$ (one sided), where P is the observed probability value and α is a selected significance level (Wilcoxon-Mann-Whitney exact rank sum test).

Adoptive lymphocyte transfer. The prophylactic adoptive cell transfer was performed as described previously (34). Recipient mice were subjected to total-body γ irradiation with 6 Gy 2 h before cell transfer. Donor spleen lymphocytes to be transferred were either used as nonseparated cell suspension or enriched to a purity of more than 95% T lymphocytes by passage through nylon-wool columns. If necessary, T-lymphocyte subsets were depleted by treatment with MAb to CD4 (9) or CD8 (10), respectively, and complement.

Generation of cytolytic effector cells and cytolytic assay. Splenocytes and mesenteric lymph node lymphocytes were obtained 4 weeks after infection with MCMV. Lymphocytes (2×10^6 cells per ml in 4-ml cultures) were restimulated in vitro with 10^5 PFU of MCMV per ml and 5×10^5 stimulator cells per ml. At day 3 of culture, 25 U of recombinant human interleukin-2 (Sandoz Forschungsinstitut, Vienna, Austria) was added per ml, and cultures were tested for cytolytic activity at day 6. KD2SV cells (simian virus 40-transformed kidney fibroblasts from B10.D2 [H-2^d] mice) were used as target cells after incubation with a synthetic peptide containing the antigenic epitope recognized by BALB/c cytolytic T lymphocytes (CTL) in pp89, an immediate-early-phase regulatory protein of MCMV (8, 16, 33). The cells were pulsed with 100 μ M of 19-mer peptide P(161-179) during the 90-min ⁵¹Cr-labeling period, and excess peptide was removed by washing of target cells before incubation with effector cells. A standard 3-h cytolytic assay was performed (30) with 10^3 target cells and graded numbers of effector cells in twofold-dilution steps. Data represent the mean percentage of specific lysis from three replicate cultures.

Detection of virus-specific antibodies. An enzyme-linked immunosorbent assay (ELISA) for quantifying MCMV-specific antibodies in sera by using infected mouse embryo fibroblasts as a source of antigens was used (14). Titers were read from the half-maximal optical density after subtraction of values from control lysates of noninfected fibroblasts.

Tumor transplantation. A/J (H-2^a)-derived sarcoma I (SaI) tumor was propagated in ascitic form in A/J mice. SaI expresses major histocompatibility complex (MHC) class I antigens, including K^k, D^d and L^d, but is devoid of class II molecules on the cell surface (7, 40). Ascites-passaged SaI tumor cells were harvested from A/J mice 5 to 7 days after

intraperitoneal inoculation of 5×10^6 tumor cells. Cells were washed in phosphate-buffered saline and injected (5×10^5 tumor cells) subcutaneously into the lateral thoracic skin in thymectomized BALB/c mice. Tumor diameter was measured with a caliper in two dimensions at a 90° angle, and tumor size is expressed as mean tumor diameter.

RESULTS

Experimental design. The experiments were designed to study the ability of mice to control MCMV infection after ablation of the CD8⁺ T-lymphocyte subset. Because even fully immunocompetent mice require at least 6 weeks for clearance of tissues from infectious virus and establishment of viral latency, a protocol for efficient long-term depletion of T-lymphocyte subsets was essential. The time schedule for the treatments and assays is outlined in Fig. 1. In a previous report, we described the course of MCMV infection in long-term CD4⁻ mice (15). In that study, the state of CD4⁺ subset deficiency was maintained by weekly injection of rat MAb to murine CD4. Because of the risk of an antiglobulin response to xenogeneic antibodies, this protocol could not be applied to long-term depletion of CD8⁺ T lymphocytes. Therefore, thymectomized mice were used to prevent the repopulation of peripheral lymphoid organs. Mice were first injected with MAb of rat origin on 3 consecutive days before infection with MCMV. The efficacy of T-cell subset depletion was controlled by cytofluorometry and immunohistology. Whereas a low number of CD8⁺ T lymphocytes was still detectable in lymphoid tissues 6 weeks after treatment of mice with rat MAb to murine CD8 alone, the number of these residual cells was reduced to the background level by additional weekly injection of murine MAb to CD8 (data not shown). Functional CD8⁺ T-lymphocyte inactivation was monitored by testing the capacity to generate MCMV-specific CTL and by testing the rejection of an allogeneic tumor graft differing in MHC class I molecules. Thymectomized mice were also depleted of the CD4⁺ T-lymphocyte subset to provide either CD4⁻ or CD8⁻ mice under conditions that allow comparison between previously published data and the results presented here. The state of MCMV infection in immunosuppressed and control mice was monitored by screening individual mice collected randomly from the respective samples. The antiviral effect of lymphoid cells was tested by adoptive transfer into MCMV-infected syngeneic recipient mice that were immunodepleted by 6 Gy of irradiation (34). This adoptive transfer regimen was developed previously as a model for cytoimmunoprophylaxis of CMV disease and can be used as an in vivo assay for estimating the strength of the T-lymphocyte-mediated antiviral immunity generated in the donor mice (24, 28, 31, 32, 34).

Functional depletion of the CD8⁺ subset in vivo. Immunosuppressed and control mice were infected with MCMV. None of the CD4⁻ mice but also none of the CD8⁻ mice showed signs of illness or succumbed to MCMV infection. Four weeks later, spleen cells were isolated and restimulated with MCMV in vitro, and the generation of a CTL response was tested (Fig. 2). CTL from both MCMV-primed immunocompetent mice and CD4⁻ mice were able to lyse KD2SV (H-2^d) fibroblast targets pulsed with synthetic peptide P(161-179). This peptide represents the epitope recognized by CTL in the immediate-early protein pp89, an immunodominant antigen of MCMV recognized by antiviral CD8⁺ CTL (8, 14, 16, 17, 30, 33, 42). In vivo depletion of the CD8⁺ T-lymphocyte subset abolished generation of the CTL response.

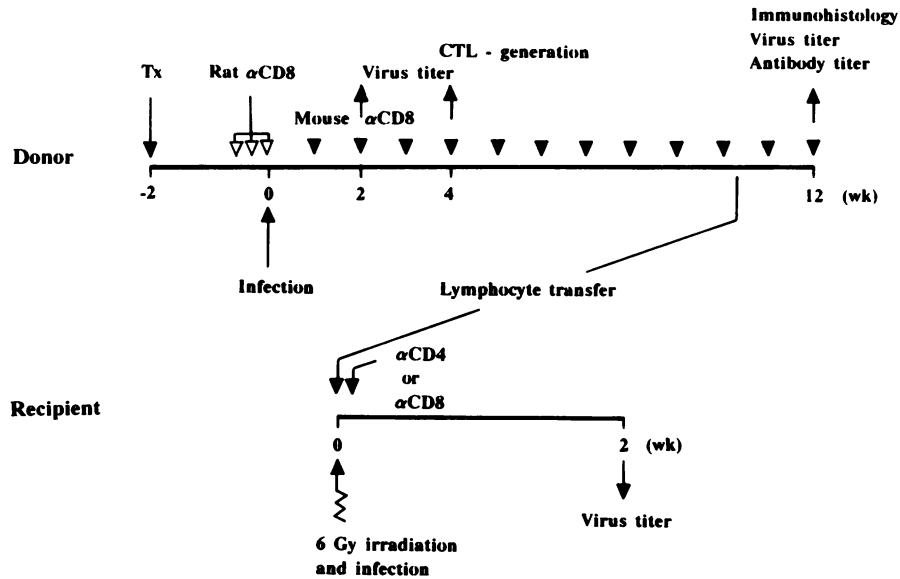


FIG. 1. Experimental regimen and time schedule. Thymectomy (Tx) was carried out 2 days before infection. CD8⁺ T lymphocytes were eliminated by injection with rat MAb to CD8 (αCD8; ∇), followed by maintenance therapy with mouse MAb to CD8 (▼). At various times, mice were sacrificed for determination of virus titers in organs, for testing of CTL activities, etc. (▲). Protective capacities of lymphocytes generated in donors were tested by adoptive lymphocyte transfer. In transferred cells, subpopulations were depleted by specific MAb plus complement. Cell transfer recipients received 6 Gy of irradiation before infection to ablate their capacity to mount a specific immune response during the test period.

The frequency of CTL precursors to MCMV is less than 1 in 10³ CD8⁺ T lymphocytes for the antigen tested (17, 30). Reduction of this number by 100-fold in CD8⁺-subset-depleted mice could make these cells difficult to detect in vitro,

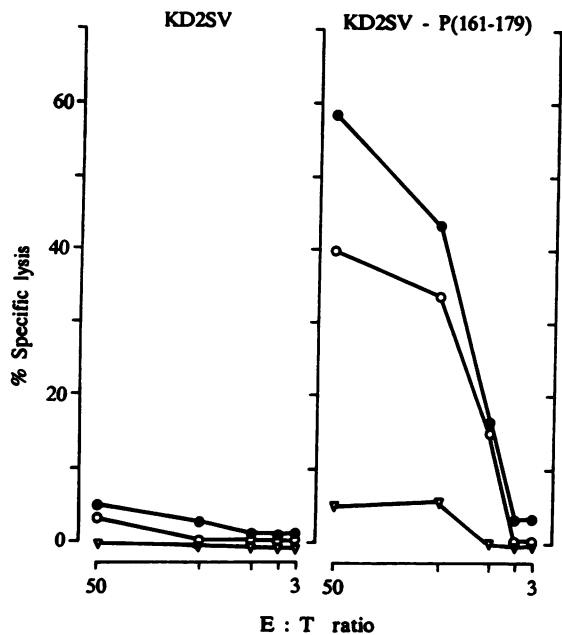


FIG. 2. Absence of virus-specific CTL in mice depleted of CD8⁺ T lymphocytes. The cytolytic activity to the antigenic peptide P(161-179) of the MCMV immediate-early protein pp89 is shown for CD4-depleted (○), CD8-depleted (∇), and nondepleted (●) mice. Effector cells were spleen lymphocytes obtained 4 weeks after MCMV infection and in vitro restimulation with MCMV for 6 days.

although they might still contribute to antiviral defense in vivo. The number of CD8⁺ T cells reactive to alloantigens is much higher than the number of cells reactive to nominal antigens. Thus, if the elimination of reactive CD8⁺ T cells was not complete and if such residual cells, albeit not detectable as CTL in vitro, did contribute to protection in vivo, this effect should be much more pronounced in response to alloantigen. The effect of CD8⁺ subset depletion on protective immunity in vivo to an MHC class I-different tumor allograft was determined (Fig. 3). Immunocompetent control mice rejected the tumor graft by 20 days after transplantation. The progressive tumor growth observed in the group of CD8⁻ mice and the death of these animals within 5 weeks indicated that the depletion protocol could be used also for long-term functional ablation of CD8⁺ T lymphocytes.

Clearance of infectious virus in tissues of CD8⁻ mice. The state of infection in T-lymphocyte-subset-depleted mice was studied by measuring infectious virus in organs at different times after infection. CD4⁻ mice exhibit a delayed clearance of the lungs and completely fail to clear the salivary glands, in which a persistent infection is established (15). As expected, the combination of thymectomy and CD4⁺ subset depletion led to the same result (Fig. 4). By contrast, there was remarkably little difference in virus clearance between normal and CD8⁻ mice. Although the lungs of some mice still contained a low titer of virus at 2 weeks postinfection (p.i.), the virus was effectively cleared at 4 weeks p.i. in all mice tested. No difference was detected in the clearance kinetics of the salivary glands between immunocompetent and CD8⁻ mice. Previous studies have shown that the salivary glands represent the organ that is most difficult to clear from MCMV by the immune system (15, 21, 31, 34). Therefore, despite the important protective effect of CD8⁺ T lymphocytes, MCMV clearance can be achieved in the absence of this subset.

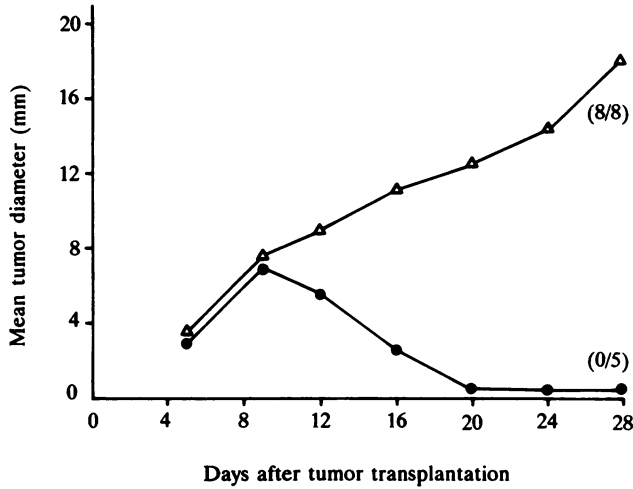


FIG. 3. Effect of CD8⁺ subset depletion on growth of an MHC class I-disparate allograft. BALB/c mice were thymectomized and treated with rat MAb to murine CD8 and then for 3 weeks with murine MAb to murine CD8. SaI tumor cells (5×10^5) were injected subcutaneously. For CD8⁺-subset-depleted mice (Δ), tumor diameter measurements represent the means from eight mice. For immunocompetent control mice (\bullet), tumor diameter measurements represent the means of five mice. The proportions of mice dying from or surviving tumor growth are shown in parentheses.

Cells from CD8⁻ mice require the CD4⁺ subset for virus clearance. Depletion of the CD4⁺ subset results in a marked reduction of the virus-specific antibody response (15). When the antibody response in thymectomized mice was tested 12 weeks p.i., again the CD4⁻ mice generated at best marginal antibody titers, whereas the immunocompetent and the CD8⁻ mice had developed titers in the range of 512 to 1,024 (data not shown). Spleen cell transfer into lethally infected recipients was used to assay and to compare the efficacy and the effector cell specificity of antiviral immune functions operative in normal and subset-depleted mice.

The results are documented for the spleen as an indicator organ of recipient mice at day 14 p.i. and adoptive transfer of 2×10^5 cells (Fig. 5), but essentially the same results were obtained for other organs tested, such as lungs and adrenal glands (not shown). The difference between the virus titers (about four log₁₀ steps) seen after transfer of spleen cells isolated from noninfected, immunocompetent donors and spleen cells from MCMV-primed, immunocompetent mice as donors (Fig. 5a) is a measure of the antiviral activity present in 2×10^5 immune spleen cells. It was found that cells from all three test groups (MCMV-primed immunocompetent mice, primed CD4⁻ mice, and primed CD8⁻ mice) limited virus spread. To investigate the contribution of the CD8⁺ and the CD4⁺ subsets to antiviral activity, the recipients of cells were injected with MAb to either CD4 or CD8 to ablate the in vivo function of the respective transferred cell subset and also to prevent a contribution of subsets

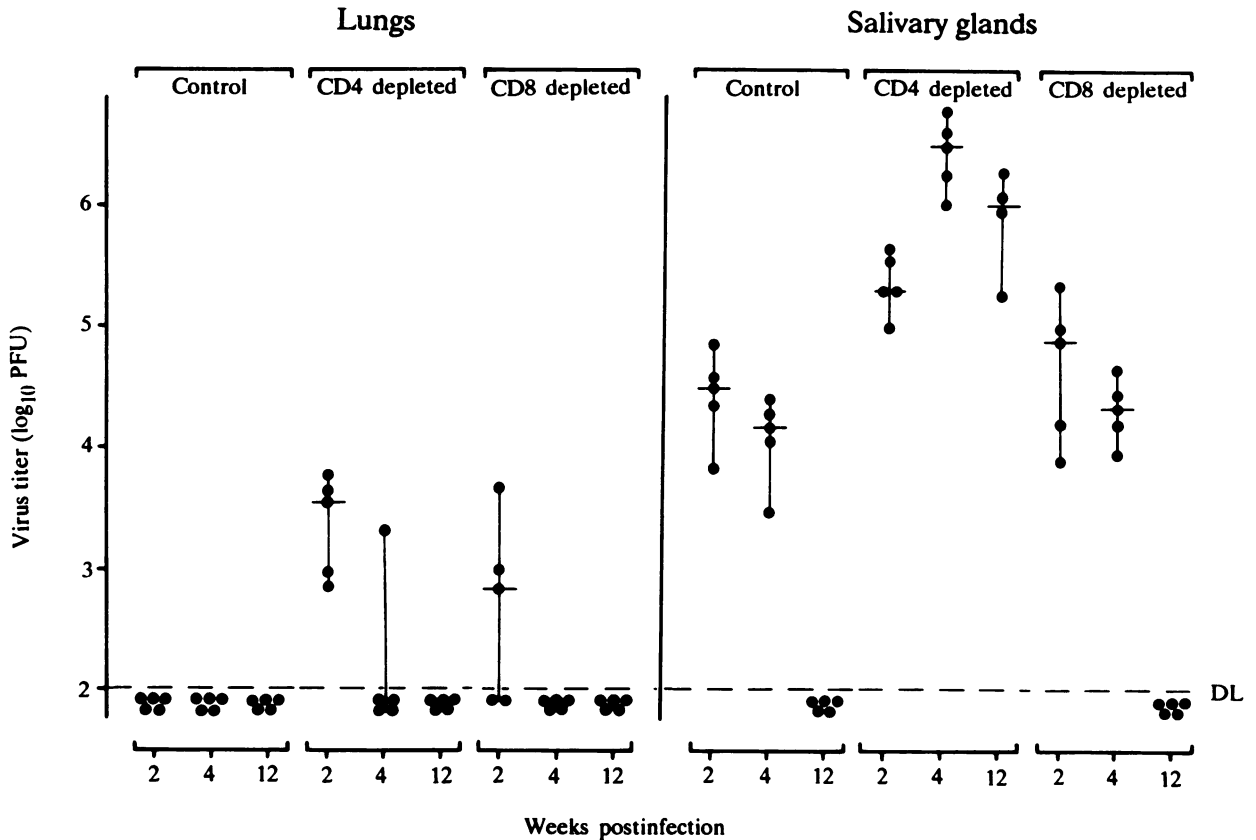


FIG. 4. Effect of CD8⁺ subset depletion on the course of MCMV infection. A comparison was made at 2, 4, and 12 weeks p.i. between virus production in the salivary glands and lungs of nondepleted control mice and of mice depleted of either the CD8⁺ or CD4⁺ subset of T lymphocytes. Individually scores titers (\bullet) and median values ($—$) are shown. DL, Detection limit.

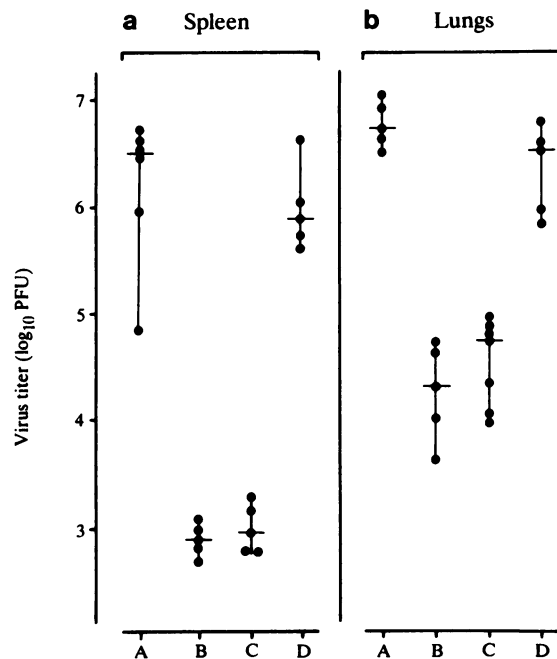


FIG. 6. Absence of direct antiviral activity in T lymphocytes from $CD8^-$ mice. Nylon-wool-enriched splenic T lymphocytes (2×10^5 cells) were transferred intravenously into immunodepleted recipient mice for in vivo assessment of antiviral activity. Shown are titers in individual recipients, measured 2 weeks after cell transfer and infection (\bullet), and median values (—). Columns: A, nonprimed donors; B, nondepleted MCMV-primed donors; C, $CD4^+$ -subset-depleted, MCMV-primed donors; D, $CD8^+$ -subset-depleted, MCMV-primed donors.

the absence of $CD4^+$ T-helper functions (15). The difference that we observed between normal and $CD4^-$ mice was that $CD4^-$ mice exhibited an altered virus clearance pattern and developed a chronic MCMV infection of the acinar glandular epithelial cells in salivary gland tissue. Because even a low number of $CD8^+$ T lymphocytes can have profound effects on virus clearance, the new finding, namely, that $CD8^+$ T lymphocytes can be negligible, was somewhat disconcerting, and it was important to rigorously exclude the putative contribution of residual $CD8^+$ T lymphocytes. Significant numerical reduction of $CD8^+$ T lymphocytes was achieved by a combination of treatment with rat and mouse MAb specific for the murine CD8 molecule, which led to the efficient long-term removal of antigen-positive cells. The functional absence of MCMV-specific $CD8^+$ T lymphocytes was shown in several independent assays. First, $CD8^-$ mice did not generate CTL specific for MCMV. Second, $CD8^-$ mice did not reject an allogeneic tumor graft. Third, whereas protective activity in normal and in $CD4^-$ mice was associated with a direct function of $CD8^+$ T lymphocytes, this did not apply to T lymphocytes from $CD8^-$ mice. Fourth, the protective function of cells from $CD8^-$ mice was destroyed by treatment of recipients with antibody to CD4 but not to CD8. Collectively, these data show that the $CD8^+$ subset did not contribute to the virus clearance in $CD8^-$ mice. Thus, the first conclusion that can be drawn is that the course of infection is not altered in mice deficient for the $CD8^+$ subset.

The availability of MAb to T-lymphocyte surface molecules has stimulated in vivo and in vitro analyses of antiviral functions of T-lymphocyte subsets (43). Studies on herpes

simplex virus (HSV) (25), influenza A virus (20), lymphocytic choriomeningitis virus (LCMV) (1, 18, 22), ectromelia virus (5), and MCMV (15, 28, 31, 32, 34) have consistently stressed the importance of the contribution of $CD8^+$ T lymphocytes to virus clearance. However, the relative role of each of the major T-lymphocyte subsets is apparently quite different for each of the virus infections studied. For instance, deletion of the $CD4^+$ T-lymphocyte subset does not prevent the elimination of LCMV (1, 22) or influenza A virus (20) but causes a delayed local clearance of HSV (25) and results in site-restricted persistent MCMV infection (15). Even more striking are the differences in mice deficient for the $CD8^+$ T-lymphocyte subset: this situation leads to the complete inability to clear LCMV (1, 22), whereas influenza A virus-infected mice still eliminate the virus (20), and HSV-infected mice clear the infection of the skin but develop a severe infection of the nervous system (25). As reported here, clearance of infectious MCMV in $CD8^-$ mice is similar to that of normal mice. Thus, there is no general assignment of a determinative role in vivo to either T-lymphocyte subset in recovery from viral infection. Instead, a detailed examination is required in every virus infection under study, and no prediction can be made even for related viruses such as the family of herpesviruses.

At first glance, it does not appear surprising that $CD8^-$ mice can eliminate MCMV with a clearance kinetics almost indistinguishable from that of fully immunocompetent mice. These mice were still able to generate antibodies to MCMV, and the situation seen in $CD8^-$ mice could merely reflect the residual capacity of the natural immune response after subtraction of the important, albeit perhaps not indispensable, part that $CD8^+$ T lymphocytes may play. This view is tenable only as long as the protective principles active in normal and in $CD8^-$ mice are not compared. Only the characterization of cells active after transfer reveals the difference. Fully immunocompetent mice do contain both T-lymphocyte subsets; nevertheless, the transfer of antiviral activity required the presence of the $CD8^+$ subset. Elimination of the $CD8^+$ subset did not reveal an important residual protective capacity of the remaining $CD4^+$ cells, although their cooperation was essential for antibody production in cell recipients. Thus, the result in $CD8^-$ mice, namely, that $CD4^+$ cells contribute to the control of virus spread, is not identical with the antiviral activity residing in the spleen of immunocompetent mice after MCMV infection and subsequent withdrawal of $CD8^+$ T lymphocytes. The $CD4^+$ subset apparently gains a major compensatory protective function only in mice that do not contain $CD8^+$ T lymphocytes at the time of infection. Therefore, the second conclusion to be drawn is that the MAb treatment has altered the quality of the antiviral immune response.

The compensatory protective function exerted by the $CD4^+$ subset in $CD8^-$ mice required the cooperation of other cells. These cells may include B cells or other cells that respond to factors released by the $CD4^+$ subset or both. Antibody responses in those mice were equivalent to the responses of immunocompetent controls whether examined by Western immunoblot (K. Münch, unpublished data), by ELISA, or by neutralization in the presence of complement (data not shown). Thus, to detect differences in the antibody response, further analyses of the isotype pattern and of the in vivo protective activities of antisera from such mice are required. If there are differences, we expect them to be in the quality of the antibody response because we could not detect a correlation between antiviral antibody titers and virus clearance (32). Another mode of cooperation between the

CD4⁺ subset and other cells could be the release of factors resulting in the activation of nonspecific host defense mechanisms, as shown for an HSV-specific CD4⁺ T-lymphocyte clone (19). This T-helper cell clone derived from immunocompetent mice could protect mice from fatal infection. This function was associated with the release of lymphokines, including gamma interferon, which in turn increased the viricidal activity of macrophages (35). Liberation of various lymphokines could also activate other nonspecific host defense mechanisms, such as natural killer cells, that are thought to be important in the early defense of the host against CMV (2, 4, 27, 37, 39).

Our new observation that the immune response following ablation of either T-lymphocyte subset can still protect against CMV infection may stimulate research into viral antigens essential for stimulation of the CD4⁺ subset that mediates protection, into the definition of the protective principle resulting from the cooperation between the CD4⁺ subset and other cells, and into the mechanisms that regulate MCMV control in the immunocompetent host preferentially via the CD8⁺ subset. Murine CD4⁺ T-helper lymphocytes can be divided into at least two types (23). At the clonal level, these T-helper cells exhibit distinct but overlapping functions with regard to lymphokine secretion and cell cooperation. Data indicate that the expansion of distinct CD4⁺ T-lymphocyte subsets that are correlates of the Th1 and Th2 cells defined at the clonal level *in vitro* can be associated *in vivo* with control or progression of disease caused by intracellular pathogens (11). A testable speculation is that the long-term depletion of CD8⁺ T lymphocytes alters the contribution of the two T-helper cell subsets to the control of MCMV infection.

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