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Adoptive Immunotherapy of Murine Cytomegalovirus Adrenalitis in the Immunocompromised Host: CD4-Helper-Independent Antiviral Function of CD8-Positive Memory T Lymphocytes Derived from Latently Infected Donors

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The ability of memory T lymphocytes derived from latently infected mice to control murine cytomegalovirus disease in the immunocompromised host was studied by adoptive transfer experiments. At a stage of pathogenesis when virus had already colonized target tissues, a therapeutic antiviral function could be ascribed to the CD8$^+$ subset. This in vivo function was not restricted to sites in which intravenously infused lymphocytes usually are trapped or home in, such as the lungs or the spleen, respectively, but was also evident in the adrenal glands, a site to which antiviral effector cells have to specifically migrate. Specific infiltration of adrenal gland cortical tissue by donor-derived CD8$^+$ memory T lymphocytes was demonstrated. CD4$^+$ memory T lymphocytes had no antiviral effect by themselves and also were not required for the function of the CD8$^+$ effector cells in this short-term immunotherapy model. These findings should help settle the debate about which subset of T lymphocytes comprises the effector cells that can directly control cytomegalovirus infection in the murine model system.

Cytomegalovirus (CMV) latency is controlled by antiviral immunity. Only in the immunocompromised host does primary CMV infection or CMV recurrence result in severe disease. In bone marrow transplant recipients with opportunistic human CMV infections, interstitial pneumonia is considered the immediate cause of death (8), but other tissues are also affected, and serious CMV adrenalitis has been observed in a murine model system (15, 16). For patients with acquired immunodeficiency syndrome, CMV disease is one of the leading causes of mortality (7, 9), and adrenal necrosis with presumed herpesvirus etiology is a life-threatening symptom (17).

As a model for human CMV disease in iatrogenically immunocompromised patients, we studied murine CMV (MCMV) disease after immunodepletion by total-body γ irradiation of mice of the BALB/c strain. Previous work (12, 14) has demonstrated that T lymphocytes, obtained from draining popliteal lymph nodes of immunocompetent mice during an acute intraplantar infection with MCMV, can limit virus multiplication in tissues of immunodeficient cell transfer recipients. Such T lymphocytes were protective not only when given prophylactically before infection, but also when applied therapeutically after MCMV had colonized host tissues. So far, it has been shown for the lungs (14) and for the spleen (12) that the antiviral effect of T lymphocytes is a function of the CD4$^-$ CD8$^+$ subset and that low numbers of CD4$^-$ CD8$^+$ cytolytic effector cells specific for immediate-early membrane antigens of MCMV (5, 6, 10, 11) prevent lung tissue destruction and mediate survival (13).

On the basis of these findings, it was assumed that also in other tissues CD8$^+$ but not CD4$^+$ T lymphocytes comprise the antiviral effector cells. This assumption contradicts recent results of Shanley (15), who used the T-lymphocyte-deficient athymic *nu/nu* mutant of BALB/c mice as cell transfer recipient and concluded that in the adrenal glands, MCMV infection is controlled only by the CD4$^+$ CD8$^-$ subset of T lymphocytes.

Two hypotheses can be proposed to explain this discrepancy: either the effector mechanisms of protective immunity are different in the two model systems, or different T-lymphocyte subsets are responsible for controlling CMV infection at different sites. An experimental verification of either of these explanations would be of obvious interest for the understanding of CMV pathogenesis. These considerations prompted us to identify in our model system the T-lymphocyte subset that controls adrenal gland infection.

To ascertain that MCMV pathogenesis in irradiated wild-type BALB/c (12–14) and nude mutant (15, 16) mice is not disparate, we studied the histopathology of the adrenal glands (Fig. 1a and b). At day 14 after irradiation and intraplantar infection, at a time when mortality was already pronounced (13, 14), focal necroses were found rarely in the medulla but frequently throughout the cortex. Figure 1a shows an extended necrosis in the reticular zone adjacent to the medulla. By electron microscopy (Fig. 1b), stages of MCMV virion morphogenesis, including the formation of multicapsid virions, (18) were identified in a parenchymal cell of the reticular zone. In essence, virus multiplication and adrenalitis was also demonstrated in the irradiated host.

A therapeutic antiviral function during an established infection of tissue demands the presence of effector cells at the infected sites. It is known that lymphocytes administered in the caudal vein are trapped in the lungs (for a review, see reference 4), and it has been documented for other adoptive transfer models that donor-type T lymphocytes do home in lymphoid tissues, where they persist for long terms as memory cells (1). While an antiviral effect in the lungs and spleen of the recipient may therefore not require site-

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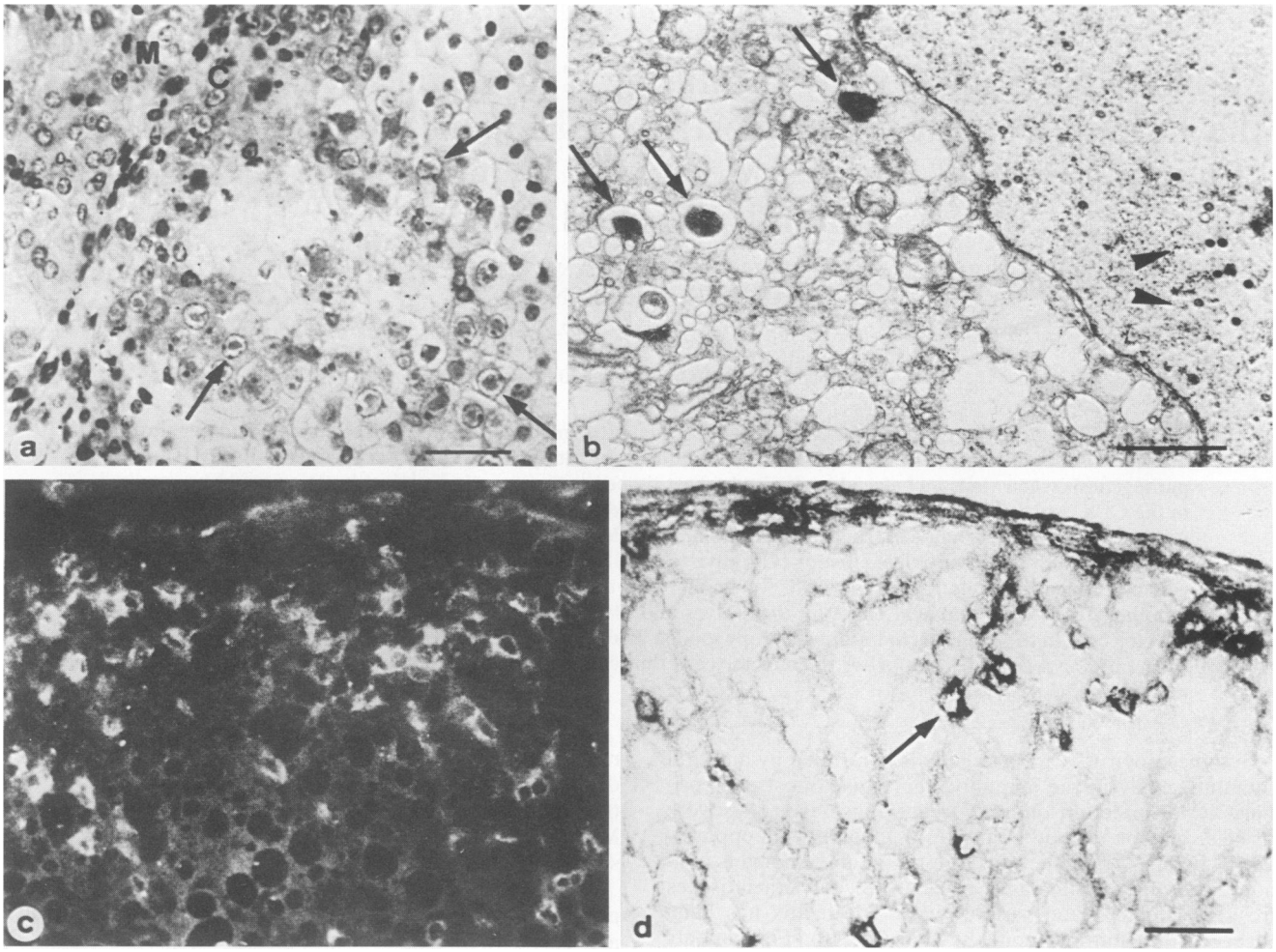

FIG. 1. Demonstration of adrenalitis and infiltration of the adrenal gland cortex by $CD8^+$ T lymphocytes. Adrenal gland tissue was isolated at day 14 p.i. (a and b) or at day 12 p.i. (c and d) from BALB/c mice infected in the footpad with 10^5 PFU of MCMV (strain Smith, ATCC VR-194) after immunodepletion by total-body γ irradiation with a single radiation dose of 6 Gy (14). (a) Paraffin section (5 μm) stained with hematoxylin and eosin and examined by light microscopy (bar, 30 μm), showing a pronounced focal necrosis in the zona reticularis of the cortex. M, Medulla; C, cortex. Infected parenchymal cells with characteristic inclusion bodies (arrows) are found in the periphery. (b) Electron micrograph (14, 18) showing stages of MCMV virion morphogenesis in an infected parenchymal cell (bar, 1 μm). The assembly of nucleocapsids in the nucleus (arrowheads) and the formation of multicapsid virions by budding of cytoplasmic nucleocapsid clusters into cytoplasmic vacuoles (arrows) are shown. (c and d) $CD8^+$ memory T lymphocytes (2×10^7) infused i.v. at day 6 p.i. Cells infiltrating the adrenal gland cortex were visualized immunohistologically in cryostat sections (8 μm) of frozen tissue by direct immunofluorescence with fluorescein isothiocyanate-labeled rat Mab anti-Lyt-2 (no. 1353; Becton Dickinson and Co.) (c) and by indirect immunoenzymatic staining with mouse Mab anti-Lyt-2.2 (hybridoma 19/178 [13, 14]) and alkaline phosphatase-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG (no. 115-055-072; Jackson Immuno-Research) with naphthol-As-MX-phosphate as substrate and fast blue BB as coupling agent (Fluka AG, Buchs, Switzerland) (d). The arrow in panel d points to membrane-stained cells. Panels c and d are shown at the same magnification (bar, 30 μm).

directed migration of transferred T lymphocytes, specific infiltration of infected tissue is needed for an antiviral function of effector T lymphocytes in the adrenal glands.

To test whether the reported failure of $CD8^+$ T lymphocytes to cope with adrenal gland infection (15) is caused by an inability to infiltrate that tissue, we transferred at day 6 postinfection (p.i.), at a time when virus had colonized adrenal gland tissue (unpublished observation), $CD8^+$ T lymphocytes derived either from spleens of latently infected donors (for explanation, see below) or, for control, from spleens of noninfected donors. In neither case was infiltration demonstrated before day 4 after transfer (day 10 p.i.). At day 6 after the transfer of nonimmune cells, only a few $CD8^+$ T lymphocytes became detectable by screening a series of sections (not depicted), whereas after the transfer of memory cells, infiltrating $CD8^+$ T lymphocytes were readily seen by different techniques in the multiformic and fascicular zones of the adrenal gland cortex (Fig. 1c and d). Single cells were also found scattered in the reticular zone. The delayed appearance of donor-derived cells and the ready detection of cells derived from latently infected donors strongly suggest a directed infiltration by antigen-specific T lymphocytes. Thus, this part of our study did not reveal any reason that $CD8^+$ T lymphocytes should not be operative in adrenal gland tissue also.

By measuring the virus titers in tissues, we confirmed our previous finding that $CD8^+$ T lymphocytes comprise antiviral effector cells operative in the lungs and in the spleen (12–14) and proved that in this respect the adrenal glands are not an exception (Fig. 2).

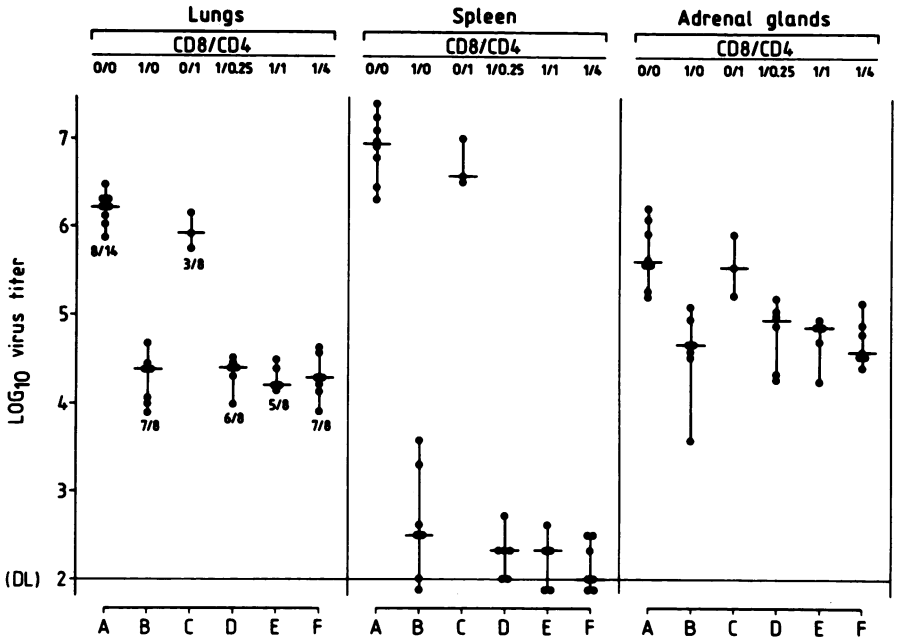

FIG. 2. Identification of the memory T-lymphocyte subset operating in a short-term antiviral immunotherapy model. Memory cells of either T-lymphocyte subset or defined mixtures of both were transferred i.v. into irradiated (6 Gy; γ irradiation) and infected (10^5 PFU of MCMV) recipients at day 6 p.i., i.e., according to the therapeutic schedule (12, 14). ●, Individual titers in tissues determined at day 14 p.i.; —, median values. The detection level (DL) was 100 PFU per organ. The proportion of recipients that had survived until the day of assay is given in the left panel. Columns: A, no transfer of lymphocytes (code 0/0); B, transfer of 10^6 T lymphocytes depleted of the $CD4^+$ subset (code 1/0); C, transfer of 10^6 T lymphocytes depleted of the $CD8^+$ subset (code 0/1); D to F, transfer of mixtures of both subsets composed as indicated.

We applied the therapeutic lymphocyte transfer protocol essentially as previously described (14), except that memory T lymphocytes were used instead of acutely sensitized lymphocytes. Spleens of 10-month-old latently infected BALB/c mice, infected the day after birth intraperitoneally with 100 PFU of MCMV (10), were taken as sources of memory T lymphocytes. Latent infection of the donors was ascertained by detecting recurrent virus in salivary glands after radiation-induced (6 Gy) reactivation (incidence of recurrence, 37%; data not shown). It is notable that the generation of cytolytic effector cells in memory T-lymphocyte populations requires specific restimulation (10), and thus it is expected that transferred memory cells must encounter antigen in the recipient to function. We have chosen this modified protocol because it allows us to evaluate the ability of memory T lymphocytes to cope with virus reactivation from latency.

The procedure was carried out as follows. Memory T lymphocytes were purified by nylon-wool depletion of B lymphocytes and accessory cells and depleted of the $CD4^+$ and $CD8^+$ subsets by treatment with monoclonal antibody (Mab) anti-L3T4a (GK 1.5, rat immunoglobulin G2b (IgG2b) and anti-Lyt-2.2 (hybridoma 19/178, mouse IgG2a), respectively (14). The purities of the resulting $CD8^+$ and $CD4^+$ populations were controlled by flow cytometric analysis (not shown) as described previously (13). In all three tissues tested, the transfer of $CD8^+$ T lymphocytes at day 6 p.i. significantly limited virus multiplication, as assessed by determining the virus titers in individual recipients at day 14 p.i. (Fig. 2, compare columns A and B [$P < 0.001$ by the one-sided Wilcoxon-Mann-Whitney exact rank sum test]). The transfer of the same number of $CD4^+$ T lymphocytes had no antiviral effect (Fig. 2, compare columns A and C [$P > 0.05$]). In agreement with previous results (12), the antiviral efficacy of therapeutically transferred cells was lower in the adrenal glands than in the lungs and spleen (Fig. 2, compare the respective panels), while prophylactic transfer was most effective in protecting against adrenal gland infection (12). An explanation may be that prophylactic transfer prevents the colonization of tissue by virus, whereas during established tissue infection, the delayed infiltration of the adrenal glands by effector T lymphocytes limits the time available for their operation in a short-term immunotherapy model.

We asked further whether memory $CD4^+$ T lymphocytes enhance the antiviral efficacy of memory $CD8^+$ T lymphocytes by providing help (Fig. 2, columns D to F). There was no indication of a helper effect when graded numbers of $CD4^+$ T lymphocytes were transferred with a constant number of $CD8^+$ T lymphocytes (Fig. 2, compare columns B and D to F [$P > 0.05$]). It is notable that $CD4^+$ memory T lymphocytes were operative when transferred with memory B lymphocytes, as monitored by helper-dependent seroconversion in the transfer recipient (data not shown). These data already strongly suggested that, at least for a short-term function, $CD8^+$ memory T lymphocytes do not require help from $CD4^+$ T lymphocytes.

It could be argued, however, that help is delivered by putative radiation-resistant helper cells of recipient type or by helper cells generated by autoreconstitution during the 6 days between irradiation and therapeutic lymphocyte transfer. To exclude this possibility, the irradiated and infected recipients received intravenous (i.v.) infusions of anti-CD4 Mab (GK 1.5, anti-L3T4a [3]) for an in vivo depletion of $CD4^+$ T lymphocytes (Table 1), a strategy established by Cobbold and colleagues (2). The antibody was administered 6 h before cell transfer, and to prolong the helper-depleted state, a second dose was given 2 days later. This regimen caused a rapid, complete, and lasting depletion of $CD4^+$ T

TABLE 1. CD4-helper-independent therapeutic antiviral function of $CD8^+$ memory T lymphocytes

Group	Experimental regimen[a]			Median value (n = 4) of virus titers (log_{10} PFU) in:		
	Irradiation and infection	Transfer	αCD4 in vivo[b]	Lungs	Spleen	Adrenal glands
A	+	−	−	5.8	5.9	5.4
B	+	+	−	4.3	3.0	2.5
C	+	+	+	4.6	3.2	3.6

[a] Purified $CD8^+$ memory T lymphocytes (2×10^6) were infused i.v. at day 6 p.i. into irradiated (6 Gy; γ irradiation) and infected (10^5 PFU of MCMV) recipients. Virus titers in tissues were measured at day 14 p.i.

[b] For in vivo depletion of $CD4^+$ T lymphocytes, 0.8 mg Mab GK 1.5 (anti-L3T4, rat IgG_{2b} [3]) contained in 2 mg of protein from partially purified ascites was administered i.v. 6 h before cell transfer. Dose 2 was given 2 days thereafter.

lymphocytes, as judged by cytofluorometric analysis of spleen and lymph node cells (data not shown).

The transfer of $CD8^+$ T lymphocytes also significantly limited virus multiplication in recipients depleted of putative residual $CD4^+$ helper cells (Table 1, compare groups A and C [$P = 0.025$ in all tissues tested]), and the antiviral efficacy of lymphocyte transfer was not significantly lowered (Table 1, compare groups B and C [$P > 0.1$ in lungs and spleen and $P = 0.1$ in adrenal glands]). We arrived at the same conclusion when we transferred nonseparated memory T lymphocytes and eliminated either the $CD4^+$ or the $CD8^+$ subset in vivo by infusing the respective antibodies (data not shown). These experiments thus demonstrated that $CD8^+$ memory T lymphocytes can operate in the transfer recipient also in the absence of helper cells of the $CD4^+$ subset.

In conclusion, our study has shown that memory T lymphocytes of the $CD8^+$ subset can infiltrate adrenal gland tissue and can control infection also at that site, whereas $CD4^+$ T lymphocytes do not exert a direct antiviral effect, nor are they essential as helper cells.

These findings are contrary to the conclusion drawn by Shanley from his experiments with the nude mouse model for CMV disease, in which sorted T-lymphocyte subsets were used for adoptive transfer (15). While we have now disproven the hypothesis that at different sites different T-lymphocyte subsets control MCMV infection, the possibility that the effector mechanisms of antiviral control differ in the two experimental models can still account for the obvious discrepancy. The main difference between the models is the presence of B lymphocytes and accessory cells, as well as natural killer cells, in nude mice. One interpretation could be that the antiviral effect of donor $CD4^+$ T lymphocytes observed by Shanley (15) has been mediated by one of these recipient cell types. However, before a final conclusion is reached, we think that a critical review of the experimental details of cell sorting may be useful. It is a disadvantage of positive selection that binding of antibodies causes the depletion of stained cells in vivo by opsonization (S. P. Cobbold and H. Waldmann, circulatory letter with recommendations for the use of rat IgG2b Mab to mouse T-lymphocyte subsets). In addition, the use of irreversible metabolic inhibitors to prevent antigen modulation (15) must be expected to negatively affect the ability of sorted cells to operate in vivo thereafter.

With respect to our previous hypothesis that cytolytic T lymphocytes of the $CD8^+$ subset are involved in preventing CMV recurrence in latently infected hosts (11), our present study gave the important new information that $CD8^+$ memory T lymphocytes derived from latently infected mice can indeed control MCMV multiplication as efficiently as or even better than acutely sensitized lymphocytes do (12, 14).

The finding that this function can be performed also in the absence of $CD4^+$ T lymphocytes may be of interest for the prevention of opportunistic CMV infection in CD4-subset-deficient patients with acquired immunodeficiency syndrome.

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