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Gamma Interferon-Dependent Clearance of Cytomegalovirus Infection in Salivary Glands

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Cytomegalovirus (CMV), similar to other members of the *Herpesviridae* family, can establish both persistent and latent infections. Each of the CMVs that are found in many animal species replicates in the salivary gland, and oral secretion represents a source of horizontal transmission. Locally restricted replication characterizes the immunocompetent individual, whereas in the immunocompromised host, protean disease manifestations occur due to virus dissemination. The virus is cleared by immune surveillance, and $CD8^+$ T lymphocytes play a major role. Remarkably, certain cell types of salivary gland tissues are exempt from $CD8^+$ T-lymphocyte control of murine CMV infection and require the activity of $CD4^+$ T lymphocytes. The results presented here suggest that this activity is a function of Th1 cells. Neutralization of endogenous gamma interferon abrogated the antiviral activity of Th1 cells but not that of $CD8^+$ T lymphocytes in other tissues. Neutralization of endogenous gamma interferon did not interfere with the induction of the cellular and humoral immune response but acted during the effector phase. Recombinant gamma interferon could not replace the function of Th1 cells in vivo and had limited direct antiviral activity in vitro. The results therefore suggest that gamma interferon represents one, but not the only, essential factor involved in salivary gland clearance, establishment of CMV latency, and, eventually, the control of horizontal transmission.

Cytomegalovirus (CMV), a member of the herpesvirus family, establishes acute and chronic infections. After primary infection, CMV remains in the host in a latent state from which reactivation can occur. Infection of immunocompetent hosts is usually asymptomatic, whereas infection or virus reactivation in the immunocompromised host can cause severe and fatal disease. CMV is a serious problem for patients with organ transplants and represents the most frequent viral cause of death in patients with AIDS (6, 18).

Studies on infection of mice with murine CMV (MCMV), a model for CMV disease, have shown that although natural killer (NK) cell functions are associated with resistance to infection with high doses of virus, survival requires the presence of T lymphocytes (33). In our own studies concerned with the adoptive immunotherapy of the immunocompromised infected host, we were able to identify $CD8^+$ T lymphocytes as a major protective principle (reviewed in reference 16). With the mouse model, the definition of major antigens (24), the identification of antigenic peptides presented by major histocompatibility complex (MHC) class I molecules (26), the principles of variable antigen presentation during viral replication (3), and the construction of experimental recombinant vaccines (4, 5, 10, 32) have been carried out.

Still, analysis of the protective immune mechanisms operative during acute infection failed to explain a long-established notion, namely, the production of CMV in salivary gland tissues and virus excretion even by immunocompetent hosts. Ultrastructural examinations have located the acinar glandular epithelial cells as the site of chronic infection (9). Recently, we reported that, unlike in all other tissues tested, $CD8^+$ T lymphocytes alone cannot clear (11) and $CD4^+$ T lymphocytes are essential for the clearance of salivary gland tissues, a function that does not even require the contribution of $CD8^+$ T lymphocytes (12).

In this study, we have characterized the CD4 T-lymphocyte subset involved. We identified the protective CD4 subset as Th1 cells by the correlation between function and endogenous gamma interferon (IFN-γ) activity. We report that neutralization of IFN-γ abolishes the antiviral activity of $CD4^+$ but not of $CD8^+$ T lymphocytes. Because recombinant IFN-γ (rIFN-γ) cannot replace Th1 cells and has limited direct antiviral activity, this cytokine probably represents only one of the essential factors required for the establishment of viral latency in the infected host.

MATERIALS AND METHODS

Mice. Female BALB/c ($H\text{-}2^d$) and CBA/J ($H\text{-}2^k$) mice, 7 to 9 weeks old, were obtained from our own breeding colony. Thymectomized mice were prepared by the standard procedure for adult thymectomy (12).

Virus, virus titrations, and infection conditions. Sucrose gradient-purified mouse embryo fibroblast culture-propagated MCMV (Smith strain; code VR-194; American Type Culture Collection, Rockville, Md.) was used. MCMV in tissues was quantified by a plaque assay (27). The detection limit was 100 PFU of MCMV per organ homogenate. Virus titers (X versus Y) were regarded as significantly different for P (X 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).

A plaque reduction assay was used to test the in vitro antiviral activity of rIFN-γ. Second-passage mouse embryo fibroblasts, grown in Dulbecco's modified Eagle's medium supplemented with 3% fetal calf serum (GIBCO), were seeded in 24-well tissue culture plates (10^5 cells per well), and various concentrations of rIFN-γ were added. After 24 h, the monolayers were infected with MCMV (200 PFU/well). After adsorption for 30 min at 37°C, the plates were

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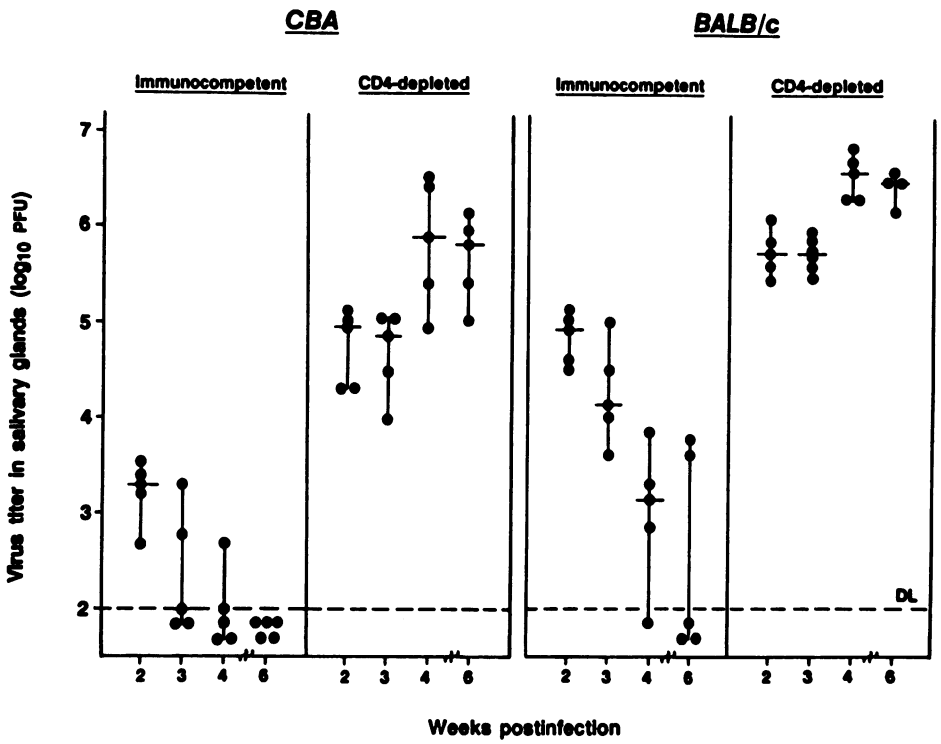

FIG. 1. Effect of CD4 subset depletion on the course of MCMV infection in the salivary gland of resistant and susceptible mouse strains. The effect of long-term CD4 subset depletion was studied in CBA and BALB/c mice. Individually scored titers are shown by solid circles (●), and median values are marked by horizontal bars (—). The dashed line indicates the detection limit (DL). Virus titers in groups of CD4-depleted mice differed significantly ($P = 0.005$) from virus titers in immunocompetent mice of either strain.

centrifuged at 800 × *g* for 30 min. The cultures were then overlaid with medium containing methyl cellulose and incubated for a further 96 h in the presence of rIFN-γ. For intraplantar infection, a dose of 2×10^5 PFU was used throughout.

Reagents and antibodies. Recombinant murine IFN-γ was generously provided by G. R. Adolf, Bender Vienna, Vienna, Austria. The specific activity of rIFN-γ (lot no. M3-RD48) was 10^7 U/mg, as determined by the L cell-encephalomyocarditis virus bioassay. Diluent vehicle for in vivo and in vitro studies was sterile Dulbecco's Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS; GIBCO Laboratories). Monoclonal antibodies (MAbs) included 191.1 (rat immunoglobulin G2b [IgG2b]) (2), which recognizes the murine CD4 antigen; 169.4 (rat IgG2b) (2) and 19.178 (mouse IgG2a) (8), both specific for the murine CD8 antigen; and R4.6A2 (rat IgG1), which neutralizes murine IFN-γ (30). Antibodies were partially purified from ascitic fluid or tissue culture supernatant by 50% ammonium sulfate precipitation, followed by dialysis in PBS (pH 7.6), and stored at −30°C until use. The concentration of rat IgG in the MAb stocks used in vivo was determined by a radial immunodiffusion assay with rat and mouse IgG standards (Serotec, Oxford, England). The IFN-γ-neutralizing capacity of MAb R4.6A2 stocks was determined in a cell protection assay on an L929 monolayer grown in 96-well flat-bottomed plates after infection with vesicular stomatitis virus, using a standard technique described elsewhere (18). The neutralizing capacity of the anti-IFN-γ MAb stock was ca. 5×10^4 U/mg.

In vivo treatment of mice. In vivo depletion of $CD4^+$ and $CD8^+$ T-lymphocyte subsets was carried out as described previously (11, 12). In thymectomized mice, long-term depletion of the CD4 and CD8 subsets was accomplished by intravenous treatment with anti-CD4 or anti-CD8 MAb, respectively, at a dose of ca. 1 mg for 3 successive days. A single antibody injection was repeated after 7 days. For short-term depletion of T-cell subsets, immunocompetent mice as well as adoptive lymphocyte transfer recipients were injected intraperitoneally with 1 mg of anti-CD4 or anti-CD8 MAb every fourth day, starting 1 h after infection and cell transfer. For in vivo neutralization of IFN-γ, mice were injected with 1 mg of R4.6A2 MAb in 0.5 ml of PBS for 2 successive days starting from the day of infection. Every third day, another single injection of 1 mg of anti-IFN-γ antibody was carried out. Experiments were terminated after 2 weeks.

The in vivo activity of rIFN-γ was tested by injecting mice with a single dose of 2×10^4 U of rIFN-γ intraperitoneally every other day for the test period of 2 weeks. Control mice received PBS.

The prophylactic adoptive cell transfer was performed as described previously (27). In brief, recipient mice were immunodepleted by total-body gamma irradiation, with 6 Gy delivered as a single dose. Spleen lymphocytes from donor mice were infused into the tail vein 2 h after irradiation and infection. Virus titers in tissues of recipients were determined 2 weeks later.

Cytofluorometric analysis. For cytofluorometric analysis (FACScan; Becton Dickinson & Co., Mountain View, Calif.) of the distribution of the CD4 and CD8 subsets, lymphocytes were stained simultaneously by phycoerythrin-conjugated anti-L3T4 MAb and fluorescein isothiocyanate-conjugated

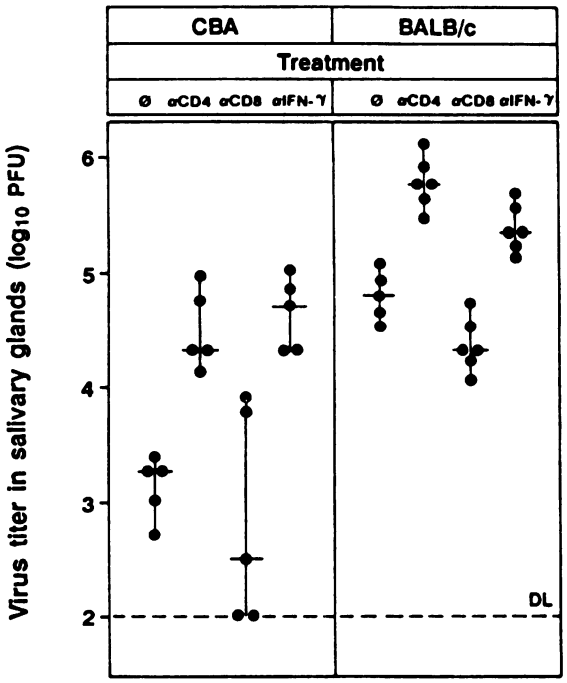

FIG. 2. Effect of IFN-γ neutralization on the course of MCMV infection in salivary glands. CBA and BALB/c mice were compared with respect to virus production in the salivary glands 2 weeks postinfection. Groups of mice were treated with antibodies to IFN-γ, CD4, or CD8. Control groups of mice received rat IgG (Ø). Titers in individual mice (●) and median values (—) are shown. DL, detection limit. There is a significant difference in virus titers between mice treated with anti-IFNγ and control serum for CBA mice ($P = 0.005$) and BALB/c mice ($P < 0.01$).

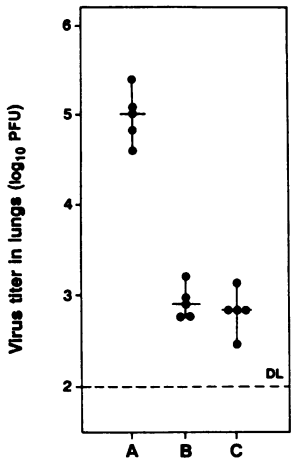

FIG. 3. Generation of antiviral effector cells in anti-IFN-γ-treated mice. Two weeks after infection, spleen cells (10^6) from donor mice were transferred into immunodepleted recipient mice to determine their antiviral activity. Depicted are titers in lungs for individual recipients, determined 2 weeks after cell transfer and infection (●), as well as median values (—). A, nonprimed donors; B, MCMV-primed donors treated with control rat IgG; C, MCMV-primed donors treated with anti-IFN-γ MAb. DL, detection limit.

anti-Lyt-2 MAb (Becton Dickinson). The expression of MHC class I antigens on thymocytes was analyzed by indirect immunofluorescence. The cells were incubated with hybridoma supernatant (28-14-8, anti-L^d) (21) as a first reagent, and after washing, the binding of the first antibody was visualized by the fluoresceinated secondary antibody (fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin [Becton Dickinson]). The fluorescence signal was triggered on particles larger than platelets, and propidium iodide (1 μg/ml)-stained dead cells were excluded by electronic gating. Relative fluorescence intensities were expressed in a log scale, with 10^4 cells analyzed.

Detection of specific antibodies. The enzyme-linked immunosorbent assay (ELISA) for quantitating MCMV-specific antibodies and the plaque reduction assay for determination of titers of virus-neutralizing antibodies have been described previously (10). The antibody response of mice to rat IgG, after treatment with rat MAb in vivo, was monitored by ELISA with purified rat IgG as the antigen.

RESULTS

CD4 subset required for virus clearance from salivary gland tissues. The importance of the CD4 T-lymphocyte subset in clearance of CMV infection and establishment of latency was shown previously for the BALB/c ($H\text{-}2^d$) strain of mice (11). This mouse strain is highly susceptible to MCMV disease, whereas other strains, such as CBA ($H\text{-}2^k$), are significantly more resistant (23). We therefore asked whether the requirement for CD4 lymphocytes for virus clearance characterizes only the susceptible BALB/c strain or represents a general mechanism. Immunocompetent and CD4 subset-depleted mice of both strains were infected with 2×10^5 PFU of MCMV, and the kinetics of virus clearance in the salivary gland, the organ which is cleared last during CMV infection, was studied. As shown in Fig. 1, the more resistant CBA mice had lower virus titers and cleared virus faster from the salivary glands than did BALB/c mice. Three to 4 weeks after infection, most of the CBA mice had already terminated the productive virus infection. CD4 subset depletion, however, led to augmented virus replication restricted to the salivary gland tissues, resulting in persistent MCMV infection comparable to the situation seen in CD4 subset-depleted BALB/c mice. In accordance with previously published data for the BALB/c strain (12), CD8 depletion did not prevent virus clearance in CBA mice (data not shown). Thus, the requirement for $CD4^+$ T lymphocytes apparently represents a general mechanism.

In vivo neutralization of endogenous IFN-γ prevents virus clearance. It was of interest to understand by what mechanism $CD4^+$ T lymphocytes contribute to virus clearance. By the type of cytokines they secrete, murine $CD4^+$ T-helper lymphocytes can be divided at the clonal level into at least two subsets (19), T-helper 1 cells (Th1) and T-helper 2 cells (Th2) (19). Th1 cells are characterized by the production of interleukin-2 (IL-2), IFN-γ, and lymphotoxin (20). We had observed that the protective effect of $CD4^+$ T lymphocytes requires the cooperation of the CD4 subset with other cells, which to us appeared indicative of cytokine involvement (12). To test the contribution of endogenously produced IFN-γ, mice were treated with anti-IFN-γ antibody R4.6A2 to neutralize the endogenous activity of IFN-γ. The effect of anti-IFN-γ treatment was compared with the known effect of anti-CD4 and anti-CD8 antibody treatment (Fig. 2). Neutralization of IFN-γ abolished virus clearance from the salivary gland to a degree similar to that resulting from CD4 subset depletion, whereas elimination of the CD8 subset had no

TABLE 1. In vivo neutralization of IFN-γ does not alter the virus-specific antibody response

Group	Treatment in vivo	No. of seropositive mice	Antibody titer	
			ELISA[a]	Virus neutralization[b]
Noninfected		0/5	<8	<8
Infected	Rat IgG	5/5	512	32
	Anti-CD4	0/5	<8	<8
	Anti-IFN-γ	5/5	256–512	64

[a] Serum was taken 3 weeks after MCMV infection, and virus-specific antibodies were determined by an ELISA. Data represent the reciprocals of serum dilutions (pooled from five mice) that gave a positive reaction (3 standard deviations above the control).

[b] The in vitro neutralizing activity of individual sera was determined as described in Materials and Methods. Data represent the reciprocals of the serum dilutions that gave 50% neutralization.

effect. These results suggest that Th1 cells releasing IFN-γ have a central role in T-lymphocyte-mediated MCMV clearance from salivary gland tissues.

IFN-γ neutralization does not prevent induction of the antiviral immune response. Cytokines have complex effects, and neutralization of IFN-γ could either block the generation of the specific immune response to MCMV or inhibit the effector phase. We first studied the effect of IFN-γ neutralization on generation of the protective T-lymphocyte response. Spleen cells from MCMV-infected and anti-IFN-γ-treated BALB/c mice and spleen cells from control mice receiving isotype-matched irrelevant antibody were tested for their capacity to limit virus spread after adoptive transfer into MCMV-infected and gamma-irradiated syngeneic recipients. The irradiation protocol of 6 Gy is equivalent to the lethal dose of 50% of the mice within 30 days and abolishes the capacity of recipient mice to mount a specific immune response during the test period (15, 27). Two weeks later, recipients were killed, and virus titers in their organs were determined (Fig. 3). Unlike cells from nonprimed cell donors (Fig. 3A), the same number of cells (10^6) from MCMV-primed mice showed a strong antiviral effect (Fig. 3B and C), which is the function of $CD8^+$ T lymphocytes. The degree of virus titer reduction was comparable between recipients of cells from MCMV-primed donors treated with anti-IFN-γ and recipients of cells from donors receiving only control antibody. Therefore, neutralization of IFN-γ did not apparently interfere with the maturation of antiviral effector cells.

To study the potential effect of IFN-γ on the production of antiviral antibodies, sera derived from anti-IFN-γ-treated mice and from control animals were analyzed for virus-specific antibodies. Titers were determined by an ELISA and by virus neutralization. No significant difference between these two groups of mice was seen, which indicated that IFN-γ neutralization is without effect on the magnitude and biological activity of the MCMV-specific antibody response (Table 1).

IFN-γ required for $CD4^+$ but not for $CD8^+$ T-lymphocyte effector functions. In the absence of a major effect of IFN-γ neutralization during the induction phase of the specific immune response, we studied whether IFN-γ neutralization did interfere with antiviral T-lymphocyte effector functions in vivo. This function of T lymphocytes was again tested in an adoptive cell transfer system. Unlike in the experiment described above, not only donors but also cell recipients were treated. MCMV-primed cell donors were either fully

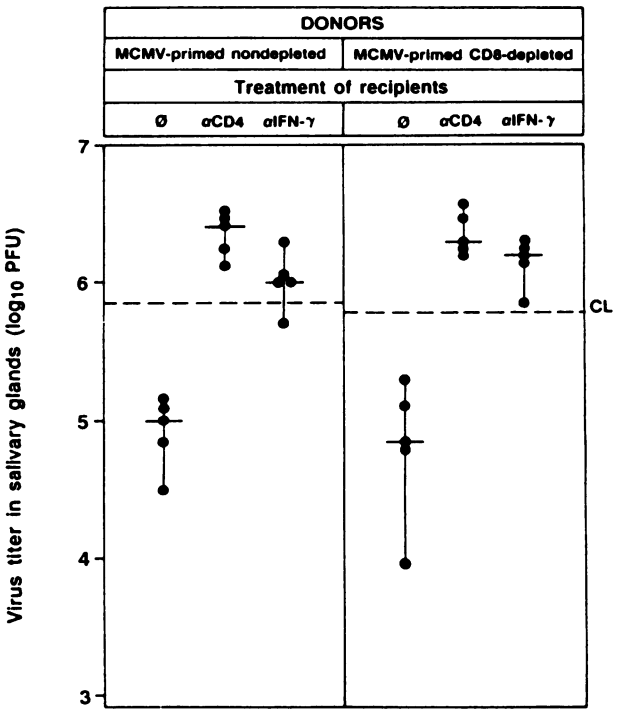

FIG. 4. Neutralization of endogenous IFN-γ prevents the antiviral activity of MCMV-primed $CD4^+$ lymphocytes in salivary gland tissues. Spleen cells (10^6) from immunocompetent or CD8 subset-depleted MCMV-primed donors were transferred into irradiated and MCMV-infected syngeneic recipient mice for in vivo assessment of antiviral activity. Recipient mice were divided into three subgroups, receiving either purified rat IgG (Ø), rat MAb to CD4, or rat MAb to IFN-γ. Virus titers in individual recipient organs, measured 2 weeks after transfer and infection (●), and median values (—) are shown. Control level (CL) represents the effect on cells from nonprimed control donors.

immunocompetent or CD8 subset depleted. The irradiated and MCMV-infected cell recipients received, on the day of cell transfer (2×10^5 cells), either control immunoglobulins or antibodies to CD4 or IFN-γ. Comparable effects on virus titers in salivary gland tissues were seen with donor cells from immunocompetent and from CD8-depleted mice (Fig. 4), demonstrating again that it is mainly the CD4 T-lymphocyte subset which contributes to virus clearance in this organ. In line with this fact was that treatment with anti-CD4 abolished the protective activity of both donor cell populations. Treatment of recipients with anti-IFN-γ had an effect comparable to that of anti-CD4 injection. Although not significant in this experiment, nevertheless, it was an invariable observation in several similar experiments that, after anti-CD4 treatment, the resulting virus titer was even higher than after anti-IFN-γ administration, indicating that anti-IFN-γ significantly but perhaps not completely eliminated the antiviral activity of the CD4 subset. Together, these data show that IFN-γ is required mainly during the effector phase and that it is crucial for the clearance of MCMV infection by the primed Th1 subset of $CD4^+$ T lymphocytes.

The situation was different when virus titers were studied in those tissues in which mainly $CD8^+$ T lymphocytes limit MCMV replication (Fig. 5). In the lungs and the spleen, elimination of the CD4 subset, as expected from the results of previous studies (11, 12), did not abolish the control of

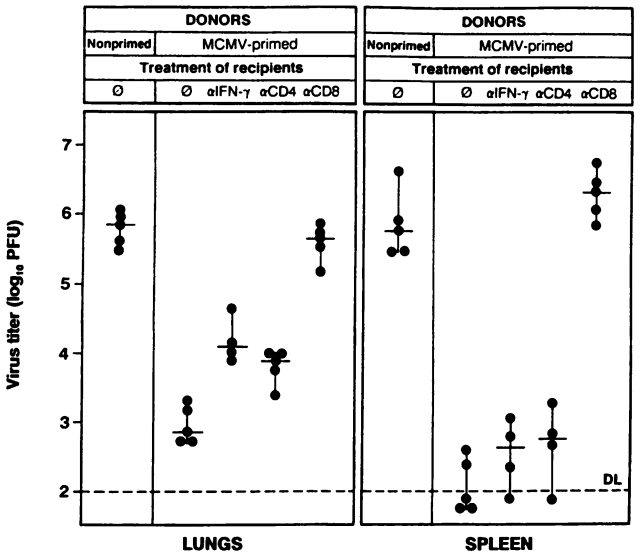

FIG. 5. Effect of IFN-γ neutralization on virus clearance from lungs and spleen after transfer of MCMV-primed lymphocytes. Spleen cells (2×10^5) derived from immunocompetent mice, either MCMV primed or nonprimed, were transferred into MCMV-infected recipient mice that were immunodepleted by sublethal gamma-irradiation. Recipients of cells received either purified rat IgG (Ø), rat anti-CD4, rat anti-CD8, or rat anti-IFN-γ. Shown are titers for individual recipients, determined 2 weeks after transfer. DL, detection limit.

virus spread. Although there was some effect of CD4 depletion on virus titers in the lungs, only elimination of the CD8 subset abolished the protective activity. Treatment of recipients with antibodies to IFN-γ had an effect similar to the removal of the CD4 T-lymphocyte subset. Therefore, unlike the effector function of the CD4 subset in the salivary gland, the activity of the CD8 subset operative in other tissues is not dependent upon endogenous IFN-γ activity.

rIFN-γ cannot replace the antiviral CD4 subset functions in vivo. CD4 T-lymphocyte-depleted mice develop a chronic productive infection of the salivary gland, whereas CD8 T-lymphocyte-depleted animals clear this tissue (12). Since anti-IFN-γ administration abolished the function of the CD4 subset in the salivary glands, we asked whether IFN-γ is the main effector molecule and whether it can replace the CD4 T-lymphocyte function in the salivary glands. Three experimental protocols were carried out (Table 2). First, infection and administration of rIFN-γ were initiated simultaneously in irradiated mice. Second, mice were depleted only for the CD4 subset, infected, and injected with rIFN-γ. Third, mice were depleted for the CD4 subset and infected, but rIFN-γ injection was delayed until 2 weeks after the infection. In all three groups, rIFN-γ treatment was continued for 2 weeks, and the effect on virus titers in the salivary gland was tested. In none of the experiments was a therapeutic effect of rIFN-γ administration detectable. This was concluded from the observation that in irradiated mice, the virus titers of saline- and rIFN-γ-injected mice were comparable (group A). rIFN-γ could not diminish or prevent the effect of CD4 subset removal (groups B and C). rIFN-γ injection, however, was not without any biological activity in vivo, since the lymphocytes reconstituting the thymus after irradiation that were obtained from mice treated according to the first protocol showed enhanced MHC class I antigen expression

TABLE 2. Injection of rIFN-γ does not affect MCMV replication

Protocol[a]	Treatment	Range of virus titers in salivary glands[b] ($\log_{10}$ PFU)
A	PBS	6.3–6.8
	rIFN-γ	6.1–6.3
B	Rat IgG	3.8–4.1
	Anti-CD4	5.1–5.7
	Anti-CD4 + rIFN-γ	4.9–5.6
C	Rat IgG	<2–3.8
	Anti-CD4	5.5–6.0
	Anti-CD4 + rIFN-γ	5.3–6.3

[a] A, Irradiated (6 Gy) and MCMV-infected BALB/c mice were injected with 2×10^4 U of rIFN-γ every other day for 2 weeks; B, thymectomized and MCMV-infected BALB/c mice were treated with anti-CD4 or control rat IgG and MCMV infected. rIFN-γ treatment (2×10^4 U) was initiated at the same time and continued every other day for 2 weeks; C, thymectomized BALB/c mice were depleted of $CD4^+$ T lymphocytes and MCMV infected. rIFN-γ (2×10^4 U) treatment was initiated after 2 weeks of infection and repeated every other day for another 2 weeks.

[b] Six mice were used per group. The data represent the range of titers found in individual mice.

(Fig. 6). Considering that a similar dose of rIFN-γ proved to be efficacious during bacterial (14, 29), parasitic (28), and viral (15) infection, we concluded that IFN-γ alone cannot substitute for the function of the CD4 subset.

Weak antiviral effect of rIFN-γ in vitro. The results described above predicted that rIFN-γ should not have a strong direct antiviral effect. This question was addressed by a plaque reduction assay (Fig. 7). Permissive murine embryo fibroblasts were incubated with different concentrations of rIFN-γ prior to infection with MCMV, and rIFN-γ was also maintained in the medium throughout the test period. Plaques were counted 96 h following infection. There was only a minimal effect of rIFN-γ on plaque numbers at physiological concentrations of the cytokine, indicating that the capacity of IFN-γ to induce an antiviral state in fibroblasts is low. Only at a concentration of 100 U of IFN-γ and higher was the number of MCMV plaques decreased significantly.

DISCUSSION

Limitation of acute infection and establishment of viral latency are the hallmarks of an efficient host immune surveillance during a herpesvirus infection. NK cells are active during the first days of infection (33), and the control of acute MCMV disease is an essential function of $CD8^+$ T lymphocytes (27), whereas final clearance of productive infection and establishment of latency in the salivary gland require $CD4^+$ T lymphocytes. In the absence of clearance, a persistent state of viral infection is established (11). The spread to other tissues is under control of the CD8 subset, and antibodies probably also contribute. Thus, $CD4^+$ T lymphocytes have a pivotal role in establishment of MCMV latency and the prevention of horizontal transmission. Consequently, it is necessary to evaluate their physiological role in vivo. Here we report three major findings. First, the antiviral activity of the $CD4^+$ T lymphocytes is a function of the Th1 subset; second, the protective effector function of $CD4^+$ but not of $CD8^+$ T lymphocytes is associated with the release of the cytokine IFN-γ; and third, the role of IFN-γ in the

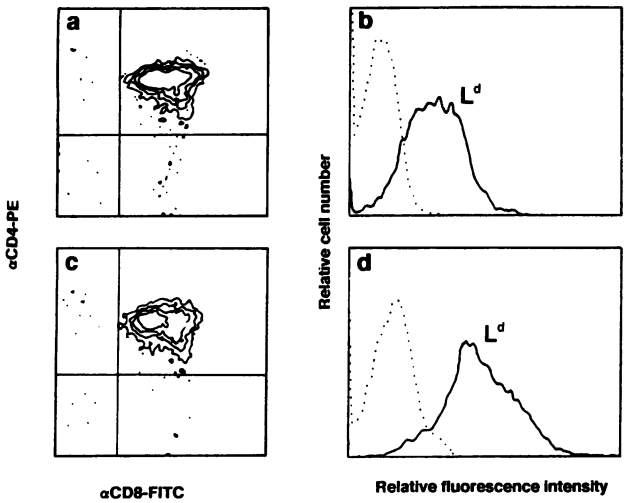

FIG. 6. Enhancement of MHC class I antigen expression after treatment with rIFN-γ in vivo. Cytofluorometric analysis of $CD4^+$ and $CD8^+$ cells and expression of MHC class I antigens (L^d) in thymocytes derived from sublethally irradiated and MCMV-infected mice 2 weeks after adoptive transfer of 10^5 nonprimed spleen cells. (a and b) Fluorescence profiles of mice treated with PBS. (c and d) Fluorescence profiles of mice injected with 2×10^4 U of rIFN-γ every other day for 2 weeks, starting on the day of infection. PE, phycoerythrin; FITC, fluorescein isothiocyanate.

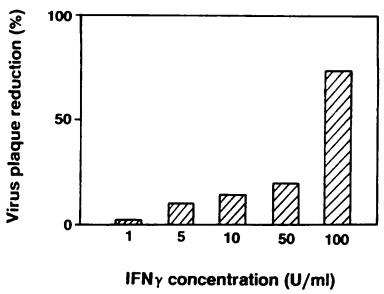

FIG. 7. Effect of rIFN-γ on MCMV replication in vitro. Murine embryonic fibroblasts were incubated for 24 h with the indicated concentrations of rIFN-γ prior to infection with 200 PFU of MCMV. rIFN-γ was maintained throughout the incubation period of 4 days, after which plaques were counted.

enhancement of host resistance to MCMV infection is probably not due to the direct antiviral activity of this cytokine.

Although intermediate patterns do exist, $CD4^+$ murine T-lymphocyte clones usually exhibit distinct patterns of cytokine production (20). The observation that Th1 cells have a beneficial effect during the immune response to parasite infections (22, 28) stimulated us to test Th1 cell function. Th1 cells but not Th2 cells produce IL-2, IFN-γ, and lymphotoxin, whereas Th2 but not Th1 cells release IL-4, IL-5, IL-6, and IL-10 (20). The significance of IFN-γ for virus clearance is established for members of the arenavirus (15, 17), the poxvirus (13), and the myxovirus (31) families. We therefore reasoned that, to identify a beneficial contribution of Th1 cells to the control of MCMV infection, neutralization of one of the essential cytokines of Th1 cells, IFN-γ, should provide a clue. Given the dichotomy of helper T cells, our observation that anti-IFN-γ antibody largely abolished CD4 T-lymphocyte-dependent virus clearance in salivary gland tissues suggests that the protective function is a property of the Th1 subset. This is in agreement with our observation that in vivo administration of anti-IL-4 antibody had no measurable effect on MCMV virus titers (10a).

$CD8^+$ T lymphocytes exhibit a cytokine secretion pattern which is indistinguishable from that of Th1 cells. Because MCMV-specific $CD8^+$ T lymphocytes have a powerful role in MCMV clearance, it was essential to define their contribution. The effect of $CD8^+$ T lymphocytes was excluded by the following results. First, virus clearance in the salivary gland is a function associated with CD4 cells, and mice lacking the CD4 subset do not clear this organ (11, 12). Second, as shown here, despite the presence of both subsets in the transferred effector cell population, the treatment of immune-cell recipients with either anti-CD4 or anti-IFNγ antibody resulted in the loss of protective function. Third, the antiviral effect of $CD8^+$ T lymphocytes was not abolished by IFN-γ neutralization. Therefore, the IFN-γ released under these conditions probably originates from the CD4 subset.

Neutralization of endogenous IFN-γ indicated a physiological role of this cytokine in the enhancement of host resistance to a herpesvirus. Due to the pleiotropic effect of IFN-γ, it was necessary to identify at which step cytokine neutralization interferes with the physiological immune response. IFN-γ could act during the induction phase, either by modulating the activation of lymphocytes, macrophages, or NK cells, by regulating MHC class I and class II expression, or by affecting the regulation of the isotype-specific antibody response, or during the effector phase of the immune response. This situation is apparently different for the different viruses that have been tested. In a retrovirus model, neutralization of endogenous IFN-γ inhibited the activation of specific cytotoxic T lymphocytes (7), whereas picornavirus and rhabdovirus responses were not affected (15). Our finding that the same conditions that prevented the T-cell-mediated clearance of salivary gland tissue allowed the generation of an effector cell response that was indistinguishable from that seen in the absence of IFN-γ neutralization led us to conclude that the major effect of IFN-γ neutralization seen in this study does not affect the induction of the cellular antiviral immune response.

IFN-γ neutralization in vivo did not cause detectable differences in the titer of neutralizing antibodies. There was, however, a clear effect on the relative representation of the different immunoglobulin isotypes (17a). The hypothesis that different antibody isotypes to MCMV play a major role was considered unlikely, since clearance of the salivary gland is not correlated with antibody titers even in the fully immunocompetent animal (25). Altogether, the experimental evidence suggested that the neutralization of endogenous IFN-γ should mainly affect the effector phase. Proof included the observation that the function of adoptively transferred $CD4^+$ T lymphocytes, but not of $CD8^+$ lymphocytes, was blocked by anti-IFN-γ antibody.

NK cells are also producers of IFN-γ. In experiments to show an antiviral function of enriched NK cells in the adoptive cell transfer model, however, about 100-fold more cells were required to see an effect on virus titers in the spleen, the organ most susceptible to the influence of transferred cells (1). Therefore, we considered the essential contribution of IFN-γ by NK cells unlikely.

How to explain the function of IFN-γ in the effector phase of Th1 cells? Our finding that IFN-γ could not replace the function of the CD4 subset in vivo and the limited activity of IFN-γ at physiological concentrations argue against a direct

role of this cytokine. It should be recollected that the basis of the persistent infection in salivary gland tissue is the immune escape of one cell type, glandular epithelial cells, from CD8 subset control (11). This is the only cell type we have identified so far that requires the function of the CD4 subset, and, as shown here, the additional presence of IFN-γ. Other cells in the salivary gland are still subject to CD8 subset control. Two mechanisms can be considered. The cytokine could be required for MHC class II upregulation and the subsequent recognition of MCMV peptides by $CD4^+$ T lymphocytes with cytolytic function. The other possibility, which is favored by us, is that IFN-γ must synergize with other CD4 subset-derived cytokines, or cytokines induced by IFN-γ in other cells, to result in virus clearance of this particular cell type.

In conclusion, our studies in a model system for CMV disease have revealed that, in contrast to other tissues, the acinar cells of the salivary gland are exempt from CD8 subset control. These cells represent a source of chronic productive virus infection and thus a source of horizontal CMV transmission. Infection of this cell type is under CD4 subset control, i.e., the control of Th1 cells. Virus clearance by Th1 cells requires the function of IFN-γ. Because rIFN-γ cannot replace Th1 cells and has little direct antiviral activity, IFN-γ probably acts in concert with other effector mechanisms which are presently under study.

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