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Murine Cytomegalovirus Stimulates Natural Killer Cell Function But Kills Genetically Resistant Mice Treated with Radioactive Strontium

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Treatment of C3H/St mice with 100 μCi of ^{89}Sr weakened their genetic resistance to murine cytomegalovirus (MCMV) infection. The criteria utilized to detect increased susceptibility were: (i) survival of mice; (ii) numbers of MCMV-infected cells in the spleens and liver; and (iii) serum glutamic pyruvic transaminase levels. The natural killer (NK) cell activity of spleen cells from mice treated with ^{89}Sr is very low. However, the NK activities of spleen cells of both normal and ^{89}Sr-treated mice were greatly augmented 3 days after infection with MCMV. These NK cells lysed a variety of tumor cells and shared several features with conventional NK cells, but were not lysed by anti-Nk-1.2 serum (specific for NK cells) plus complement. Splenic adherent cells did not lyse tumor cells themselves but were necessary for the stimulation of NK cells by MCMV. The paradox of high NK cell function and poor survival in ^{89}Sr-treated mice infected with MCMV was a surprise. We conclude that these augmented NK cells, of themselves, cannot account for the genetic resistance of C3H/St mice to infection with MCMV.

Mice injected with the bone-seeking isotope, ^{89}Sr, lose their genetic resistance to grafts of normal allogeneic or parental-strain marrow cells (2) and to grafts of parental-strain lymphoma cells (19). Natural killer (NK) cell activity against certain, but not all, tumor cell targets is consistently low in such mice; this low activity cannot be boosted by interferon or interferon inducers (10). C57BL/6 mice treated with ^{89}Sr lose their genetic resistance to the leukemogenic (*Fv-2^r*) and the immunosuppressive (*Fv-3^r*) Friend virus complex (11, 13, 14). (C57BL/6 × DBA/2)F_1 mice treated with ^{89}Sr lose their genetic resistance to infection with the facultative intracellular bacterium, *Listeria monocytogenes*, but not to the extracellular pathogen, *Yersinia pestis* EV76 strain (3), and lose their genetic resistance to the encephalomyelitis induced by herpes simplex virus type 1 (18). We have tentatively concluded that a separate class of lymphoid cells, operationally called marrow-dependent cells, are critically involved in these host defense systems (4, 12). The concept of marrow-dependent cells, i.e., marrow as a central lymphoid tissue, is supported by the findings of low NK cell function and the inability to reject incompatible marrow cell grafts in mice with osteopetrosis induced by estradiol (26, 27).

We report here observations with murine cytomegalovirus (MCMV) which further support the hypothesis that marrow dependent cells depleted by ^{89}Sr treatment contribute to host defenses against infection. Human cytomegalovirus infections are becoming more frequent with the use of powerful immunosuppressive regimens (6, 24). MCMV may be a good model to study to understand the immunobiology of human cytomegalovirus infections, e.g., MCMV infections can be reactivated by deliberate immunosuppression of mice (20). During the acute phase of infection with MCMV, the spleen and liver appear to be important target organs. Although specific cell-mediated and humoral immunity to MCMV are very important in host resistance to MCMV (8, 9, 29), naturally occurring defense mechanisms may also be important. For example, augmentation of non-H-2-restricted cytotoxic activity, presumably NK activity, occurs shortly after virus infections (33), and MCMV is no exception (1, 23). The protective effect of such NK cells has not been determined, although there was a correlation between augmentation of NK activity by spleen cells and genetic resistance to MCMV (1). In contrast, we observed an augmentation of NK activity in ^{89}Sr-treated mice destined to die of MCMV infection a few days later.

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MATERIALS AND METHODS

Mice. (C57BL/6 × DBA/2) F_1 (B6D2F_1) and C57BL/6 mice were purchased from The Jackson

Laboratory, Bar Harbor, Maine, and A/St and C3H/St mice were purchased from West Seneca Laboratories, West Seneca, N.Y. SIM.R mice were bred in our mouse colony.

^{89}Sr treatment. B6D2F$_1$ or C3H/St mice 8 to 10 weeks old were injected intraperitoneally with 100 μCi of ^{89}Sr (Oak Ridge National Laboratory, Oak Ridge, Tenn.). Ten days later, each mouse was infused with 10^7 syngeneic marrow cells to provide marrow-derived but not marrow-dependent hemopoietic cells (2). At 3 to 5 weeks after marrow infusion, the mice were entered into experiments.

MCMV. The Smith strain of MCMV was obtained from the American Type Culture Collection, Rockville, Md. The virus was passaged in SIM.R mice, and salivary gland tissue was the source of several virus passages. The virus was titrated on monolayers of mouse embryo fibroblasts (second passage). Plaques were counted after 5 days of incubation, and the titers are expressed as plaque-forming units (PFU) per ml (28). Virus titers in the spleens and livers utilized dispersed cell suspensions and thus measured infectious centers.

Histopathology. Organs were fixed in 10% formaldehyde, and sections were stained with hematoxylin and eosin.

Survival. ^{89}Sr-treated and age control C3H/St or B6D2/F$_1$ mice were infected with various doses of MCMV (groups of five or eight mice) and were housed in a laminar-flow tent to minimize airborne infections. The mice were examined daily.

Enzyme levels. Mice were bled 3 days after infection, and the serum samples were number coded and sent to the hospital chemistry laboratory for the measurement of serum glutamic pyruvic transaminase.

Statistics. Fisher's exact probability test was used to analyze survival data, and Student's *t* test was used to compare virus titers.

Pretreatments of effector cells. Spleen cells (2×10^7) were treated with distilled water briefly to lyse erythrocytes, washed, and then incubated with a 1:80 dilution of hyperimmune CE anti-CBA serum containing a high titer of anti-Nk-1.2 antibodies (5a), a gift from Robert C. Burton, Transplantation Unit, Massachusetts General Hospital, Boston, Mass. After a 30-min incubation at 37°C with the antiserum, the cells were washed and incubated for 30 more min with a 1:8 dilution of preselected rabbit serum as a source of complement. The viability of spleen cells after this procedure is approximately 95%. This antiserum specifically lyses NK cells (5, 5a). A monoclonal anti-Thy-1.2 serum (New England Nuclear Corp., Boston, Mass.) was diluted 1:300 and incubated with 2×10^7 spleen cells for 30 min at 37°C. The cells were washed and later incubated with a 1:10 dilution of guinea pig serum (previously adsorbed with mouse spleen cells) as a source of complement for 30 min. This antiserum lyses most T cells. Filtration of spleen cells over columns of nylon wool was performed exactly as previously described (14). Depletion of spleen cells with Fc receptors was accomplished by rosetting spleen cells with sheep erythrocytes coated with polyclonal rabbit immunoglobulin G anti-sheep erythrocyte antiserum (Cordis Laboratories, Miami, Fla.) diluted 1:500. Thus, sensitized cells were incubated with spleen cells for 30 min at 37°C, the mixture was washed, and the cells were centrifuged in a density gradient (Lympholyte M; Cedarlane Laboratories, Ltd., Toronto, Ontario, Canada). The cells with Fc receptors were pelleted, and the cells not pelleted were assayed for cytotoxic cell function. Plastic adherent cells were obtained by incubating 3×10^7 spleen cells in 60-mm-diameter petri dishes in 3 ml of medium for 2 h. Nonadherent cells were gently rinsed off, and adherent cells were removed by a rubber policeman.

Pretreatment of mice. Cyclophosphamide (Mead Johnson Laboratories, Evansville, Ind.) was injected intraperitoneally 1 day before inoculation of MCMV or 2 days after infection and 1 day before testing for cytotoxic activity. The dose was 300 mg/kg of body weight. Carrageenan (Seakem-9; Marine Colloids, Inc., Rockland, Maine) was dissolved in saline by heating. Mice were inoculated intraperitoneally with 1 mg on 2 successive days, followed 1 day later by infection with MCMV.

Assay for cytotoxic cells. The procedure for the cytotoxic cell assay has recently been described in detail (10). Briefly, 2×10^4 ^{51}Cr-labeled tumor cells and various numbers of lymphoid cells to give effector/target ratios of 50:1 to 1.56:1 were incubated at 37°C in a 5% CO_2 in air humidified atmosphere for 4 to 24 h (depending upon the particular target cell) in volumes of 0.2 ml. The microtest plate was centrifuged and 0.1 ml of supernatant fluid was removed from each well for the determination of ^{51}Cr radioactivity. The formula for percent specific cytotoxicity in counts per minute (cpm) is: [experimental cpm − spontaneous cpm (target cells only)]/[maximal cpm (saponin lysis) − spontaneous cpm] × 100. The results presented were mean values from two or three experiments, each performed in triplicate. The standard errors of the mean were always less than 10% of mean values and are not presented.

Target cells. The YAC-1 lymphoma is from the A strain, the EL-4 lymphoma is from the C57BL strain, the L1210 lymphoma is of DBA/2 origin, FLD-3 is a Friend virus induced erythroleukemia cell line from BALB/c mice, C1-18 is a myeloma of C3H origin, and WEHI-164.1 is a fibrosarcoma from the BALB/c strain. The optimal times of incubations to detect lysis of these cells in NK assays were used and are: 4 h for YAC-1; 18 h for EL-4, L1210, C1-18, and WEHI 164.1; and 24 h for FLD-3.

RESULTS

Survival. Genetically susceptible B6D2F$_1$ mice treated with ^{89}Sr were about 10 times more susceptible to MCMV than were age control mice (Fig. 1). At a dose of 10^6 PFU, the mean survival times were 3.7 days for ^{89}Sr-treated mice and 7.5 days for the age control mice. However, by day 15, the percent survival was equivalent between the two groups. Genetically resistant age control C3H/St mice survived a dose of 10^6 PFU (Fig. 2). However, C3H/St mice treated with ^{89}Sr were very susceptible to MCMV, i.e., their genetic resistance was lost. All of the mice died after doses of 10^6 PFU, and some died even after doses of 10^4 PFU.

Viral titers and serum glutamic pyruvic

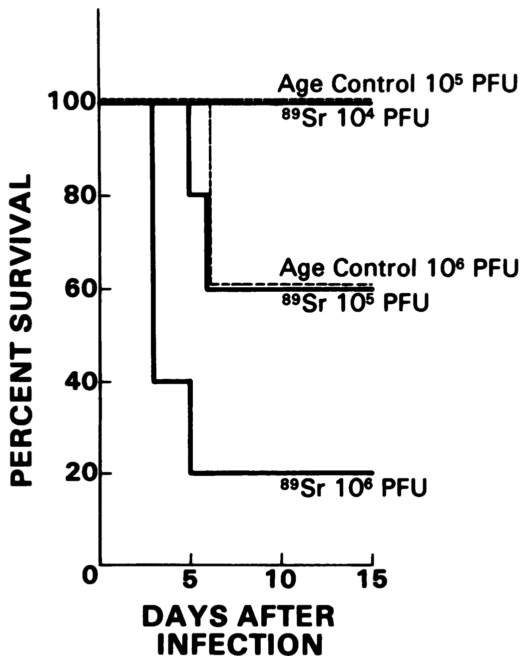

FIG. 1. *Survival of ^{89}Sr-treated B6D2F$_1$ mice infected with various doses of MCMV (groups of five mice).*

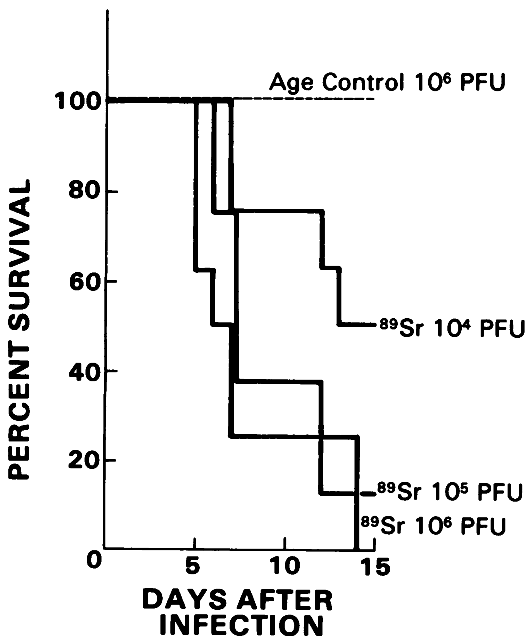

FIG. 2. *Survival of ^{89}Sr-treated C3H/St mice infected with various doses of MCMV (groups of eight mice).*

transaminase levels. The values for MCMV infectious centers in spleens and livers of age control and ^{89}Sr-treated B6D2F$_1$ and C3H/St mice 3 days after infection are presented in Table 1. Splenic viral titers were significantly higher in ^{89}Sr-treated mice of both types. The titers of MCMV in livers of age control B6D2F$_1$ mice were higher than that in livers of age control C3H/St mice. Treatment of C3H/St mice resulted in significantly higher liver titers, whereas age control and ^{89}Sr-treated B6D2F$_1$ mice had similar liver titers of MCMV. The serum glutamic pyruvic transaminase levels appeared to reflect the growth of MCMV in the livers and were high in age control and ^{89}Sr-treated B6D2F$_1$ mice and in ^{89}Sr-treated C3H/St mice, but were low in age control C3H/St mice (Table 1).

Histopathology. The spleens of age control C3H/St mice showed prominent signs of necrosis with depletion of cells both in the red pulp and white pulp (Fig. 3A and B). The spleens of mock-infected ^{89}Sr-treated C3H/St mice had increased hemopoiesis in the red pulp, with many megakaryocytes, as expected (Fig. 3C). After infection with MCMV, there was less evidence of necrosis in the spleens of mice treated with ^{89}Sr, although there was a diminution of lymphocytes in the white pulp and of hemopoiesis in the red pulp (Fig. 3D). Note the relative lack of megakaryocytes. The livers of mock-infected age control and ^{89}Sr-treated mice were within normal limits, histologically (data not shown). The livers of infected age control C3H/St mice had heavy leukocytic infiltration, but only isolated liver cells with giant inclusions. The liver cells were swollen and vacuolated (Fig. 4A). The frequency of liver cells with inclusions was far greater in C3H/St mice previously treated with ^{89}Sr, and there was a paucity of leukocytic infiltration (Fig. 4B). There were more infiltrating leukocytes in the liver of age control B5D2F$_1$ mice than of control C3H/St mice 3 days after infection, confirming a previous observation (28). However, there was less leukocytic infiltration in livers of ^{89}Sr-treated mice of both strains, particularly at later times after infection.

Cytotoxic activity in ^{89}Sr-treated and infant mice. MCMV stimulated the NK activity of spleen cells of B6D2F$_1$ mice against YAC-1 targets 3 days after infection. Ultraviolet light exposure of the virus prevented the NK boosting effect of MCMV (Table 2, experiment 1). The NK(YAC-1) activity of spleen cells of C3H/St or B6D2F$_1$ mice treated with ^{89}Sr was low, as expected. However, 3 days after infection with 10^5 PFU of MCMV, NK activity was very high in such mice (Table 2, experiments 2 and 3).

TABLE 1. *MCMV in spleens and livers and serum glutamic pyruvic transaminase (SGPT) levels of mice treated with ^{89}Sr*

Mice		Log PFU MCMV/10^6 cells[a]		SGPT[b]	
Strain	Pretreatment	Spleen	Liver	Mock	MCMV
B6D2F$_1$	Age controls	1.39 ± 1.53	4.90 ± 0.98	32	>350
B6D2F$_1$	^{89}Sr	4.67 ± 0.12[c]	5.28 ± 0.58	68	>350
C3H/St	Age controls	1.75 ± 0.44	3.55 ± 0.42	43	34
C3H/St	^{89}Sr	4.48 ± 0.49[c]	4.86 ± 0.70[c]	61	>350

[a] Infectious center assay performed 3 days after infection with 10^5 PFU of MCMV.
[b] Values expressed as international units per liter.
[c] Mean value significantly different from control, $P < 0.05$, by Student's t test.

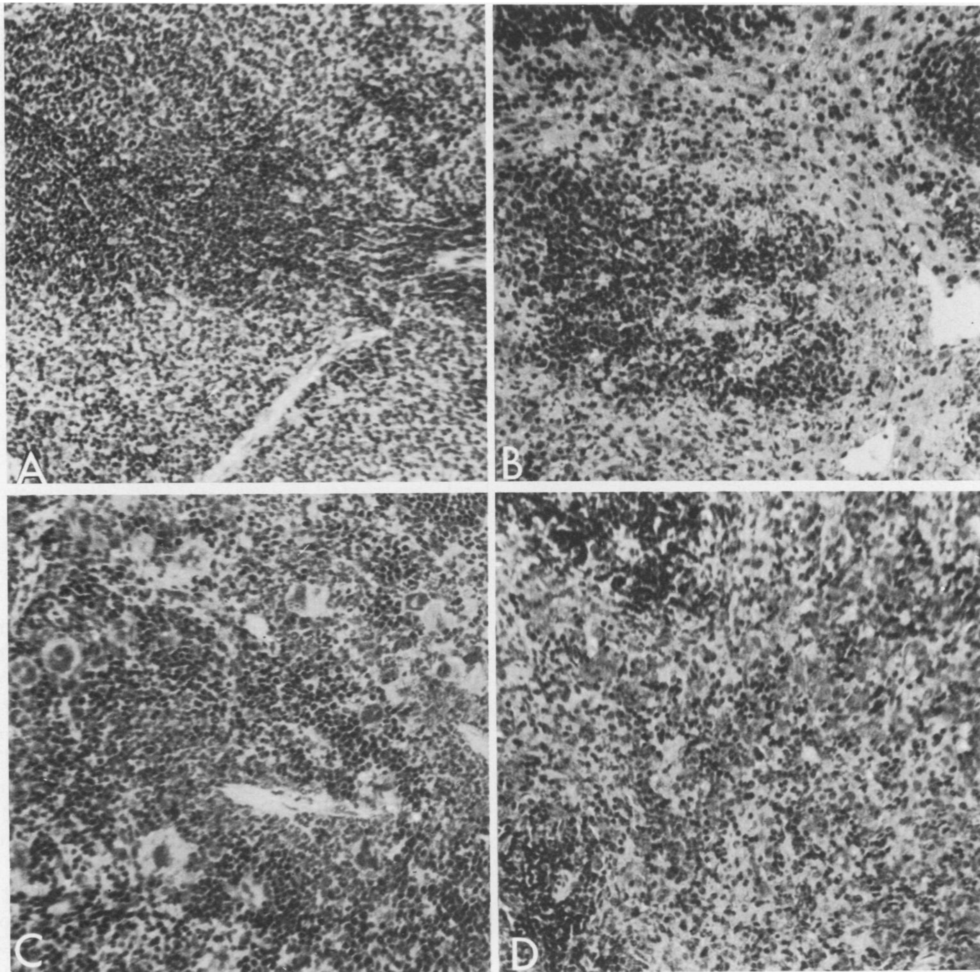

FIG. 3. *A, Spleen of age control mock-infected C3H/St mouse. B, Spleen of age control C3H/St mouse infected 3 days earlier with 10^5 PFU of MCMV; note the marked depletion of cells in the red and white pulp and the areas of necrosis. C, Spleen of mock-infected C3H/St mouse treated with ^{89}Sr; note the increased extramedullary hemopoiesis with numerous megakaryocytes. D, Spleen of infected C3H/St mouse previously treated with ^{89}Sr; there are fewer megakaryocytes, but granulopoietic cells remain in the red pulp and there is lesser evidence of necrosis. (Hematoxylin and eosin, ×100.)*

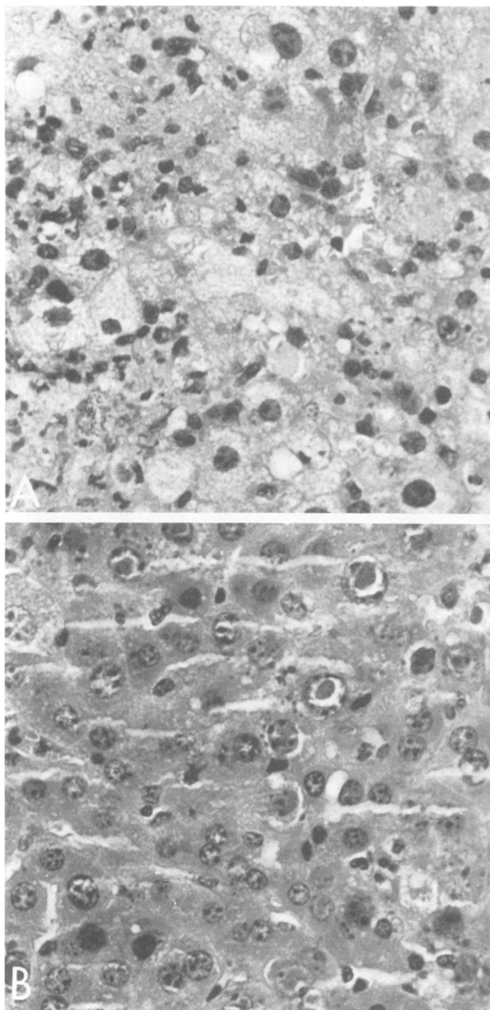

FIG. 4. *A, Liver of age control C3H/St mouse infected 6 days earlier with MCMV. The liver cells are swollen and vacuolated and there is intense leukocytic infiltration. However, inclusion bodies are seen only in rare cells. B, Liver of ^{89}Sr-treated mouse infected with MCMV. Note the lack of leukocytic infiltration associated with many inclusion bodies within liver cells. (Hematoxylin and eosin, ×250.)*

Two-week-old mice also had low NK(YAC-1) activity in their spleens. However, 3 days after infection with MCMV, their spleen cells were quite capable of lysing YAC-1 target cells (Table 3). This was true for mice of the A/St, C57BL/6, and C3H/St strains.

Characterization of cytotoxic cells. The NK activity in various organs of B6D2F$_1$ mice infected 3 days earlier with MCMV was measured by using YAC-1 target cells. Augmented activity was detected in the peritoneum (site of virus injection), spleen, and bone marrow, but not in lymph nodes or in the thymus (Table 4). The NK cells induced by MCMV in spleens of B6D2F$_1$ mice were not sensitive to anti-Nk-1.2 plus complement or anti-Thy-1.2 plus component, although NK cells of uninfected mice were sensitive to anti-Nk-1.2 plus complement (Fig. 5). The frequency of NK cells did not decrease after filtration over nylon wool columns. Furthermore, the augmented NK cells did not express Fc receptors detectable by rosette formation (Fig. 5). Cells that adhered to plastic and were recovered were not very active as cytotoxic cells for YAC-1 targets. Incubation of uninfected or infected spleen cells for 4 h at 37°C resulted in a large loss of activity against YAC-1 tumor cells (Fig. 5). Pretreatment of mice with cyclophosphamide, either before or after infection with MCMV, resulted in less NK activity. This drug also reduced cytotoxicity by spleen cells of uninfected mice (Fig. 5).

Stimulation of cytotoxic cells by virulent MCMV. During the course of these experiments, we observed that the late-passaged virus preparations were quite virulent, such that 10^5 PFU would kill a small fraction of C3H/St mice. Such virus preparations also seemed less able to augment NK cell activity 3 days after infection. We therefore passaged a virulent preparation, SG7, in tissue culture, utilizing mouse embryo fibroblasts and infecting at a 1.5 multiplicity of infection. Five days after infection of the monolayer, when 75% of the cells showed a cytopathic effect, the supernatant fluid was harvested. Age control and ^{89}Sr-treated B6D2F$_1$ and C3H/St mice were infected with 10^5 PFU of SG7 or TC1 or were mock infected with salivary gland tissue homogenate. The TC1 preparation of MCMV was much better able than the SG7 preparation to stimulate NK cells active against YAC-1 lymphoma cells 3 days after infection of age control or ^{89}Sr-treated B6D2F$_1$ mice (Table 5). Age control C3H/St mice responded equally well to the two virus preparations, but C3H/St mice previously treated with ^{89}Sr responded better to TC1. Roughly parallel findings were obtained by simply determining the numbers of nucleated cells per spleen (Table 5). SG7 decreased splenic cellularity, particularly in B6D2F$_1$ mice.

Spectrum of tumor cells lysed by cytotoxic cells. B6D2F$_1$ mice were used as donors of spleen cells to test for the range of tumor cells against which the augmented cytotoxic cells were active. The cytotoxicity against all of the tumor targets tested was augmented, although that against FLD-3 was least stimulated (Table 6). This has been a consistent finding. Even L1210 cells, which are resistant to lysis by NK cells of uninfected mice, were susceptible to lysis by these augmented cytotoxic cells.

TABLE 2. *Lysis of YAC-1 lymphoma cells by spleen cells of ^{89}Sr-treated mice infected with MCMV*

Expt	Mice		Virus inoculation[a]	Mean ± specific cytotoxicity[b]		
	Strain	Pretreatment		50:1	25:1	12.5:1
1	B6D2F$_1$	None	Mock	48.4	36.2	18.1
			MCMV	77.4	66.9	48.2[c]
			MCMV-UVL	47.3	34.7	20.2
2	B6D2F$_1$	Age controls	Mock	26.3	15.9	7.6
			MCMV	74.1	63.7	45.7[c]
		^{89}Sr	Mock	2.7	1.2	0.5
			MCMV	75.2	65.2	46.7[c]
3	C3H/St	Age controls	Mock	24.2	12.9	6.3
			MCMV	66.5	49.2	31.2[c]
		^{89}Sr	Mock	7.9	4.8	2.8
			MCMV	83.5	69.5	50.1[c]

[a] Salivary gland homogenate from uninfected (mock) or infected SIM.R donors diluted to give 10^5 PFU of MCMV per inoculum intraperitoneally 3 days before the assay. Exposure of the virus preparation to ultraviolet light (UVL) inhibited subsequent viral replication.
[b] Incubation time was 4 h. Numerical ratios indicate effector/target cell ratios.
[c] See footnote *c* of Table 1.

TABLE 3. *Lysis of YAC-1 lymphoma cells by spleen cells of 2-week-old mice infected with MCMV*

Mouse strain	Virus inoculation[a]	Mean % specific cytotoxicity[b]		
		50:1	25:1	12.5:1
A/St	Mock	1.4	0.8	0.1
	MCMV	47.0	31.6	19.2[c]
C57BL/6	Mock	15.1	12.4	6.3
	MCMV	54.2	38.5	34.6[c]
C3H/St	Mock	7.9	5.7	2.5
	MCMV	25.0	20.5	11.2[c]

[a, b, c] See footnotes *a, b,* and *c*, respectively, of Table 2.

TABLE 4. *Lysis of YAC-1 lymphoma cells by cells from various tissues of B6D2F$_1$ mice infected with MCMV*

Tissue	Mean % specific cytotoxicity[a]	
	Mock	MCMV[b]
Spleen	27.6	70.4[c]
Bone marrow	7.1	18.8[c]
Peritoneal cavity	1.5	32.8[c]
Lymph nodes	5.4	6.0
Thymus	0.4	0.3

[a, b, c] See footnotes *b, a,* and *c*, respectively, of Table 2. The effector/target cell ratio was 50:1; similar results obtained with ratios of 25:1 and 12.5:1.

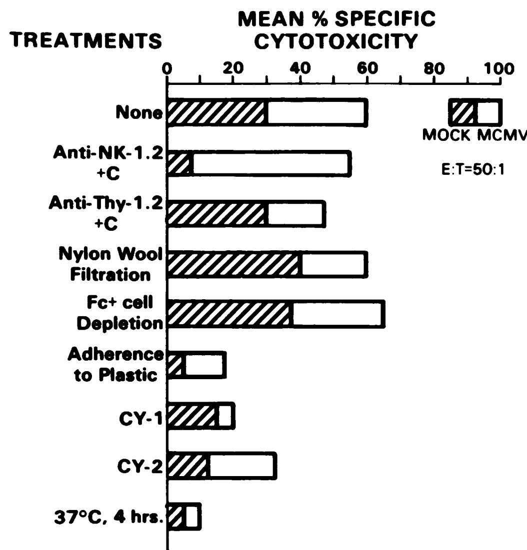

FIG. 5. *Characterization of spleen cells capable of lysis of YAC-1 lymphoma cells in spleens of B6D2F$_1$ mice infected with MCMV 3 days earlier. See text for details. CY-1, cyclophosphamide injected 1 day before infection and 4 days before assay; CY-2, cyclophosphamide injected 2 days after infection and 1 day before assay. The mean values were significantly less than control values ($P < 0.05$ by Student's t test) in the following groups: plastic adherent, CY-1, CY-2, 37°C, 4 h for both mock-infected and MCMV-infected mice and anti-Nk-1.2 for mock-infected mice only.*

Stimulation of cytotoxic cells in mice treated with carrageenan. To study the influence of macrophages on the boosting of cytotoxic cell function by MCMV, B6D2F$_1$ mice were injected on successive days with carrageenan before infection. On the day of infection, groups of mice received intravenous inocula of 6 × 10^6 plastic-adherent spleen cells (one spleen equiv-

TABLE 5. *Lysis of YAC-1 lymphoma cells by spleen cells of ^{89}Sr-treated mice infected with virulent (SG7) and tissue culture-passaged (TC1) MCMV preparations*

Mice		Virus inoculation[a]	Mean % specific cytotoxicity[b]			No. of spleen cells (10^6)
Strain	Pretreatment		50:1	25:1	12.5:1	
$B6D2F_1$	Age controls	Mock	25.4	14.9	8.8	116
		TC1-MCMV	86.6	75.3	69.4[c]	72
		SG7-MCMV	33.3	22.4	10.6[c]	40
$B6D2F_1$	^{89}Sr	Mock	1.2	0.3	0.4	66
		TC1-MCMV	66.8	56.8	34.9[c]	123
		SG7-MCMV	5.1	4.3	3.4[c]	14
C3H/St	Age controls	Mock	12.5	7.7	4.3	121
		TC1-MCMV	43.4	30.4	19.0[c]	129
		SG7-MCMV	44.1	28.5	17.8[c]	102
C3H/St	^{89}Sr	Mock	0.5	0.5	0.2	88
		TC1-MCMV	35.2	25.1	14.5[c]	82
		SG7-MCMV	20.1	13.3	8.2[c]	45

[a, b, c] See footnotes *a*, *b*, and *c* of Table 2.

TABLE 6. *Lysis of various tumor cell types by spleen cells of $B6D2F_1$ mice infected with MCMV*

Target cell	Assay time (h)	Virus inoculation[a]	Mean % specific cytotoxicity				
			50:1	25:1	12.5:1	6.25:1	1.56:1
YAC-1	4	Mock	26.3	15.9	7.6		
		MCMV	74.1	63.7	45.7[b]		
EL-4	18	Mock	10.3	4.2	3.2		
		MCMV	53.0	39.0	22.3[b]		
L1210	18	Mock	3.1	3.9	3.5		
		MCMV	25.3	15.0	10.7[b]		
C1-18	18	Mock		17.4		11.7	5.1
		MCMV		52.2		42.1	32.0[b]
WEHI 164.1	18	Mock		16.7		16.2	12.6
		MCMV		44.3		34.2	14.6[b]
FLD-3	24	Mock		25.9		17.4	9.6
		MCMV		32.6		30.8	20.4[b]

[a, b] See footnotes *a* and *c*, respectively, of Table 2.

alent) or 10 × 10^6 nonadherent spleen cells. Carrageenan prevented the boosting of NK activity by MCMV (Fig. 6). The infusion of adherent, but not nonadherent, spleen cells from syngeneic donor mice restored the boosting by MCMV.

DISCUSSION

The abrogation of genetic resistance to lethal infection by MCMV in C3H/St mice by prior treatment with ^{89}Sr (Fig. 2, Table 1) confirms and extends previous observations with other infectious agents (3, 13, 18). Genetically susceptible $B6D2F_1$ mice were somewhat more sensitive to MCMV after ^{89}Sr treatment (Fig. 1). The majority of experiments reported here were aimed at testing the hypothesis that depletion of marrow-dependent cells by ^{89}Sr abrogated genetic resistance to MCMV by affecting NK cells which are activated or boosted early during virus infections (33). This hypothesis seemed reasonable for two reasons: (i) NK activity against YAC-1 lymphoma cells was low and could not be boosted by interferon inducers or by interferon preparations themselves in mice treated with ^{89}Sr (10, 19) and (ii) there was a positive correlation between the degree of boosting of NK activity of spleen cells by MCMV and genetic resistance to MCMV (1).

However, there was an unexpected augmen-

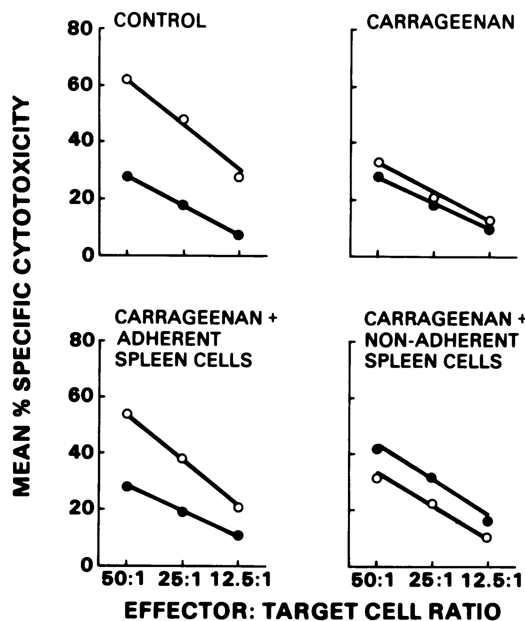

FIG. 6. *Prevention of MCMV-induced boosting of cytotoxicity by spleen cells for YAC-1 lymphoma target cells by carrageenan. Effects of infusions of splenic adherent and nonadherent cells. See text for details. Carrageenan was injected on 2 successive days before intraperitoneal infection with MCMV. Inocula of 6 × 10^6 adherent or 10 × 10^6 nonadherent spleen cells were infused at the time of infection. Symbols: ●, mock infection; ○, MCMV infection. The mean values were significantly less than control values ($P < 0.05$ by Student's t test) in the following groups: carrageenan, carrageenan + nonadherent spleen cells for MCMV-infected mice only.*

tation of NK activity against YAC-1 cells in genetically susceptible $B6D2F_1$ or resistant C3H/St mice previously treated with ^{89}Sr (Table 2). Similar observations were made in genetically susceptible and resistant infant mice, all of which have low NK(YAC-1) activity at that age and which are susceptible to the lethal effects of MCMV (28). These surprising observations certainly demand an explanation, and some of the experiments performed did provide some insight.

First of all, characterization of the cytotoxic cells (Table 4, Fig. 5) indicates that they belong to the broad category of natural killer cells of lymphocyte origin and are probably not macrophages. For example, plastic-adherent cells from spleens of MCMV infected mice were not active as effector cells, and the effectors did not adhere to nylon wool (Fig. 5). Also, activated macrophages (25) and promonocytes (17) require more than 4 h to kill YAC-1 target cells. NK cells reactive against YAC-1 targets are susceptible to suppression by cyclophosphamide and lose activity after incubation at 37°C for 4 or more h; the augmented cytotoxic cells were similarly affected. The resistance of the effectors to anti-Thy-1.2 plus complement suggests that they are not T cells, but the resistance of the effectors to anti-Nk-1.2 plus complement suggests that they are also not conventional splenic NK(YAC-1) cells (Fig. 5). Certain alloantisera plus complement did partially inactivate these NK cells at concentrations which lysed most of the spleen cells (data not shown), suggesting that the NK cells had not become totally resistant to complement-mediated lysis.

NK cells appear to be heterogeneous (5, 10, 15, 19, 19a) and we tested the cytotoxicity of MCMV-induced effectors against a panel of tumor cells which are used to detect the different NK cell types (Table 6). For example, in uninfected mice, the NK cells which lyse YAC-1 or C1-18 cells express the Nk-1.2 antigen and are depleted in spleens of ^{89}Sr-treated mice, whereas the opposite is true of NK cells which lyse WEHI 164.1 or FLD-3 cells (19a). MCMV stimulated effectors capable of enhanced lysis of all of these targets. The effectors could even lyse L1210 cells, which resist lysis by NK cells of normal mice (Table 6). These effectors do not easily fit into the Nk-1.2 antigen-negative, marrow-independent category of NK cells, because such NK cells are not susceptible to inhibition by incubation at 37°C for 4 h, and NK(WEHI 164.1) cells are not suppressed by cyclophosphamide treatment.

Based upon the data presented, how did MCMV augment NK activity in ^{89}Sr-treated mice? (i) MCMV may have stimulated a unique NK cell type which is marrow independent and Nk-1.2 antigen negative but which is sensitive to cyclophosphamide and to short-term incubation at 37°C. It follows that this cell does not function in uninfected mice. This augmentation was certainly unexpected, because we have observed that the near-normal levels of marrow-independent NK cell function against EL-4 lymphoma cells in ^{89}Sr-treated mice is actively suppressed after challenge with interferon inducers or El-4 cells themselves (19). A similar suppression of NK(FLD-3) activity occurs in ^{89}Sr-treated mice infected with Friend virus (unpublished observation by J. A. Lust, V. Kumar, and M. Bennett). (ii) MCMV could have stimulated many types of NK cells, both marrow dependent and marrow independent. The failure to detect Nk-1.2 antigens on the augmented NK cell could have been due to a diminution of antigen expression or resistance to complement-mediated lysis. MCMV may have bypassed the requirement for an intact marrow microenvironment by stimulating mononuclear phagocytic cells to function as an ectopic source of the stimulus needed to

cause differentiation of NK(YAC-1) cells in ^{89}Sr-treated mice. The studies with carrageenan and adherent spleen cells (Fig. 6) do suggest that macrophages are required for the stimulation of NK cells by MCMV. A similar observation was made in studies of activation of NK cells by BCG (31). There is a precedent for this concept in the erythropoietin field. Liver and spleen macrophages are the main source of erythropoietin in anephric animals (7, 21, 22).

We did observe that a virulent preparation of MCMV was capable of distinguishing genetically susceptible mice from genetically resistant mice and age control mice from ^{89}Sr-treated mice (Table 5) with respect to NK boosting. The study relating augmentation of the NK cell function with genetic resistance to MCMV also employed a virulent preparation of MCMV (1). Therefore, such cytotoxic cells may indeed have important functions in host defense against MCMV, particularly virulent forms of the virus.

In summary, the NK activity observed after MCMV infection appears to be a marrow-independent function. As in beige mice infected with lymphocytic choriomeningitis virus (32), the cytotoxic cells generated apparently do not play a significant role in curtailing viral synthesis (Table 1). Depending upon the virulence of the virus preparation, these NK cells do not appear to protect mice against lethal effects of MCMV. It would have been desirable to analyze NK cells reactive against MCMV-infected target cells. However, we have not been able to develop a reliable assay for such cells.

Since lack of NK cell function, by itself, cannot easily explain the loss of genetic resistance of C3H mice to MCMV after ^{89}Sr treatment, abnormalities in other host defense functions may have been involved. Two possibilities we consider are (i) increased suppressor cell function and (ii) defective production of, or response to, lymphokines. ^{89}Sr-treated mice infected with low doses of MCMV are later immune to otherwise lethal doses (unpublished observations), indicating that they can mount specific immunity to the virus if they survive the acute infection. However, macrophages infected with MCMV have immunosuppressive effects upon T lymphocytes (16). Since marrow-dependent cells seem to regulate suppressor cells in the Friend leukemia virus system (11, 14), it is conceivable that marrow-dependent cells regulate the immunosuppression by macrophages. The suppressive effect upon T lymphocytes might inhibit production of lymphokines, generation of cytotoxic T cells, or production of antibodies.

The histological features of ^{89}Sr-treated mice infected with MCMV suggest that there is a lesser degree of leukocytic infiltration in the liver and of necrosis in the spleen (Fig. 3 and 4). These defects could be due to a lack of production of lymphokines, e.g., chemotactic factors. In the herpesvirus model, ^{89}Sr-treated mice died of encephalomyelitis, but histology of the brain and spinal cord showed minimal infiltration of leukocytes (18). It is also conceivable that ^{89}Sr-treated mice do not secrete normal amounts of interferon in response to MCMV infection. Indeed, spleen cells of ^{89}Sr-treated mice do secrete low amounts of macrophage activating factor (A. Masuda and M. Bennett, Eur. J. Immunol., in press), and preliminary experiments indicate that interferon responses to MCMV in vivo and to concanavalin A in vitro are defective. Another explanation for the lack of leukocytic infiltration in livers of infected ^{89}Sr-treated mice is a relative lack of hemopoiesis in these animals which is further diminished after virus infection. There was no hemopoiesis in the bones of ^{89}Sr-treated mice, and MCMV inhibited splenic hemopoiesis (Fig. 3). In age control mice splenic hemopoiesis was also inhibited (Fig. 3), but the bone marrow cavities showed active hemopoiesis histologically (data not shown). Although we have certainly not determined the mechanisms by which ^{89}Sr weakens genetic resistance to MCMV, further analysis of this model may elucidate host defense functions against this interesting virus.

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LITERATURE CITED

1. **Bancroft, G. J., G. R. Shellam, and J. E. Chalmer.** 1981. Genetic influences on the augmentation of natural killer (NK) cells during murine cytomegalovirus infection: correlation with patterns of resistance. J. Immunol. **126**:988–994.

2. **Bennett, M.** 1973. Prevention of marrow allograft rejection with radioactive strontium: evidence for marrow-dependent effector cells. J. Immunol. **110**:510–516.

3. **Bennett, M., and E. E. Baker.** 1977. Marrow dependent (M) cell function in early stages of infection with *Listeria monocytogenes*. Cell. Immunol. **33**:203–210.

4. **Bennett, M., E. E. Baker, J. W. Eastcott, V. Kumar, and D. Yonkosky.** 1979. Selective eliminations of marrow precursors with the bone-seeking isotope ^{89}Sr: implications for hemopoiesis, lymphopoiesis, viral leukemogenesis and infection. Res J. Reticuloendothel. Soc. **20**:71–87.

5. **Burton, R. C.** 1980. Alloantisera selectivity reactive with NK cells: characterization and use in defining NK cell classes, p. 19–23. *In* R. B. Herberman (ed.), Natural cell mediated immunity against tumors. Academic Press, Inc., New York.

5a. **Burton, R. C, and H. J. Winn.** 1981. Studies on natural killer cells. I. NK cell-specific antibodies in CE anti-

CBA serum. J. Immunol. **126**:1985-1989.

6. **Fiala, M., J. E. Payne, T. V. Berne, T. C. Moore, W. Henle, J. Z. Montgomerie, S. N. Chatterjee, and L. B. Guze.** 1975. Epidemiology of cytomegalovirus infection after transplantation and immunosuppression. J. Infect. Dis. **132**:421-433.
7. **Gruber, D. F., J. R. Zucali, J. Wleklinski, V. LaRussa, and E. A. Mirand.** 1977. Temporal transmission in the site of rat erythropoietin production. Exp. Hematol. **5**: 399-407.
8. **Ho, M.** 1980. Role of specific cytotoxic lymphocytes in cellular immunity against murine cytomegalovirus. Infect. Immun. **27**:767-776.
9. **Howard, R. J., and J. S. Najarian.** 1974. Cytomegalovirus-induced immune suppression. I. Humoral immunity. Clin. Exp. Immunol. **18**:109-118.
10. **Kumar, V., J. Ben-Ezra, M. Bennett, and G. Sonnenfeld.** 1979. Natural killer cells in mice treated with ^{89}strontium: normal target binding cell numbers but inability to kill even after interferon administration. J. Immunol. **123**:1832-1838.
11. **Kumar, V., and M. Bennett.** 1976. Mechanisms of genetic resistance to Friend virus leukemia in mice. II. Resistance of mitogenresponsive lymphocytes mediated by marrow-dependent cells. J. Exp. Med. **143**:713-727.
12. **Kumar, V., and M. Bennett.** 1981. The biology of marrow dependent cells in mice, p. 145-160. *In* W. Waters (ed.), The handbook of cancer immunology, vol. 6. Garland STPM Press, New York.
13. **Kumar, V., M. Bennett, and R. J. Eckner.** 1974. Mechanisms of genetic resistance to Friend virus leukemia in mice. I. Role of ^{89}Sr-sensitive effector cells responsible for rejection of bone marrow allografts. J. Exp. Med. **139**:1093-1109.
14. **Kumar, V., T. Caruso, and M. Bennett.** 1976. Mechanims of genetic resistance to Friend virus leukemia. III. Susceptibility of mitogen responsive lymphocytes mediated by T cells. J. Exp. Med. **143**:728-740.
15. **Kumar, V., E. Leuvano, and M. Bennett.** 1979. Hybrid resistance to EL-4 lymphoma cells. I. Characterization of natural killer cells which lyse EL-4 cells and their distinction from marrow-dependent natural killer cells. J. Exp. Med. **150**:531-547.
16. **Loh, L., and J. B. Hudson.** 1980. Immunosuppressive effect of murine cytomegalovirus. Infect. Immun. **27**: 54-60.
17. **Lohmann-Mattes, M.-L., W. Domzing, and J. Roder.** 1979. Promonocytes have the functional characteristics of natural killer cells. J. Immunol. **123**:1883-1886.
18. **Lopez, C., R. Ryshke, and M. Bennett.** 1980. Marrow-dependent cells depleted by ^{89}Sr mediate genetic resistance to herpes simplex virus type 1 infection in mice. Infect. Immun. **28**:1028-1032.
19. **Luevano, E., V. Kumar, and M. Bennett.** 1981. Hybrid resistance to EL-4 lymphoma cells. II. Association between loss of hybrid resistance and detection of suppressor cells after treatment of mice with ^{89}Sr. Scand. J. Immunol. **13**:563-571.

19a. **Lust, J. A., V. Kumar, R. C. Burton, S. P. Bartlett, and M. Bennett.** 1981. Heterogeneity of natural killer cells in the mouse. J. Exp. Med. **154**:306-317.

20. **Myo, D. R., J. A. Armstrong, and M. Ho.** 1977. Reactivation of murine cytomegalovirus by cyclophosphamide. Nature (London) **267**:721-723.
21. **Peschle, C., G. Marone, A. Genovese, C. Magli, and M. Cordorelli.** 1976. Hepatic erythropoietin: enhanced production in anephric rats with hyperplasia of Kupffer cells. Brit. J. Haematol. **298**:951-956.
22. **Peschle, C., G. Marone, A. Genovese, I. A. Rappaport, and M. Cordorelli.** 1976. Increased erythropoietin production in anephric rats with hyperplasia of the reticuloendothelial system induced by colloidal carbon or zymosan. Blood **47**:325-336.
23. **Quinnan, G. V., and J. E. Manischewitz.** 1979. The role of natural killer cells and antibody-dependent cell-mediated cytotoxicity during murine cytomegalovirus infection. J. Exp. Med. **150**:1549-1554.
24. **Rand, K. H., R. B. Pollard, and T. C. Merigan.** 1978. Increased pulmonary superinfections in cardiac transplant patients undergoing primary cytomegalovirus infection. N. Engl. J. Med. **298**:951-956.
25. **Roder, J. E., M. L. Lohmann-Mattes, W. Domzig, R. Kiessling, and O. Haller.** 1979. A functional comparison of tumor cell killing by activated macrophages and natural killer cells. Eur. J. Immunol. **9**:283-288.
26. **Seaman, W. E., T. D. Gindhart, J. S. Greenspan, M. A. Blackman, and N. Talal.** 1979. Natural killer cells, bone, and bone marrow: studies in estrogen-treated mice and in congenitally osteopetrotic (*mi/mi*) mice. J. Immunol. **122**:2541-2547.
27. **Seaman, W. E., T. C. Merigan, and N. Talal.** 1979. Natural killing in estrogen-treated mice responds poorly to poly I.C. despite normal stimulation of circulating interferon. J. Immunol. **123**:2903-2905.
28. **Selgrade, M. K., and J. E. Osborn.** 1974. Role of macrophages in resistance to murine cytomegalovirus. Infect. Immun. **10**:1383-1390.
29. **Starr, S. E., and A. C. Allison.** 1977. Role of T-lymphocytes in recovery from murine cytomegalovirus infection. Infect. Immun. **17**:458-462.
30. **Stutman, O., C. Paige, and E. F. Figarella.** 1979. Natural cytotoxic cells against solid tumors in mice. I. Strain and age distribution and target cell susceptibility. J. Immunol. **121**:1819-1826.
31. **Tracey, D. E.** 1979. The requirement for macrophages in the augmentation of natural killer cell activity by BCG. J. Immunol. **123**:840-845.
32. **Welsh, R. M., Jr., and R. W. Kiessling.** 1980. Natural killer cell response to lymphocytic choriomeningitis virus in beige mice. Scand. J. Immunol. **11**:363-367.
33. **Welsh, R. M., and R. M. Zinkernagel.** 1977. Heterospecific cytotoxic cell activity induced during the first three days of acute lymphocytic choriomeningitis virus infection in mice. Nature (London) **268**:646-648.