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Lysis of Uninfected and Virus-Infected Cells In Vivo: a Rejection Mechanism in Addition to That Mediated by Natural Killer Cells

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To examine the lysis of virus-infected cells in vivo, uninfected and lymphocytic choriomeningitis virus (LCMV)-infected L-929 cells were labeled in vitro with [^{125}I]-iododeoxyuridine and implanted intravenously into mice. Natural cytotoxicity against both uninfected and virus-infected cells was demonstrated in normal uninfected mice, but LCMV-infected cells were cleared from the lungs and whole bodies more rapidly than uninfected cells. Treatment of L-929 cells with defective interfering LCMV inhibited standard virus synthesis and protected the target cells from enhanced in vivo rejection. The in vivo rejection was apparently mediated by a cellular constituent of the host immune response and not simply a result of virus-induced cytopathic effects on the target cell, as hydrocortisone acetate and cyclophosphamide each reduced rejection of both target cell types and eliminated the enhanced rejection of LCMV-infected cells. The enhanced rejection of LCMV-infected cells was not restricted by histocompatibility antigens, indicating that classic T-cell recognition was not involved in the lysis, and since the enhanced rejection of LCMV-infected cells was mediated by mice treated with cobra venom factor, complement was also not involved in the lysis. Although moderate levels of interferon (102 U/ml) were present in the sera and although there was a modest activation of natural killer (NK) cells in the lungs of LCMV-infected cell recipients but not uninfected cell recipients, the enhanced rejection of virus-infected cells did not appear to be NK cell mediated. Normal mice and mice depleted of NK cell activity by in vivo treatment with antibody to asialo ganglio-*n*-tetraosylceramide (AGM1) rejected uninfected and LCMV-infected L-929 cells similarly. This antibody markedly inhibited the rejection of NK-sensitive YAC-1 cells. In addition to the natural cytotoxicity directed against virus-infected cells, a second nonspecific rejection mechanism appeared in response to treatment protocols which induced interferon. Polyinosinic-polycytidylic acid and infection with LCMV augmented in vivo rejection of both uninfected and LCMV-infected L-929 cells but eliminated the differential rejection of the virus-infected cells. Infection with LCMV also augmented the in vivo rejection of the NK-sensitive target cell, YAC-1. In vivo treatments with anti-AGM1 sera only moderately inhibited the elevated rejection of uninfected and LCMV-infected L-929 cells, indicating that the enhanced rejection of these target cells was predominantly mediated by a mechanism other than that mediated by NK cells. In contrast, rejection of YAC-1 cells in both uninfected and LCMV-infected mice was dramatically reduced by treatment with anti-AGM1 serum. These results demonstrate two in vivo rejection mechanisms (other than NK cells) which may contribute to the control of virus infection: (i) a selective natural cytotoxicity directed against virus-infected cells and (ii) a nonselective rejection mechanism activated during virus infection. These may be two manifestations of the same mechanism, but both appear to be distinct from those mediated by AGM1-positive NK cells.

Natural immunity may play an important role in the initial defense against virus infection. This may take the form of direct inactivation of viruses by serum components, inhibition of virus synthesis by interferon (IFN), or perhaps direct lysis of virus-infected cells. The latter point, i.e., direct or preferential lysis of virus-infected cells in vivo, has never been demonstrated. It has been hypothesized to occur because virus-infected cells are frequently more susceptible to lysis by nonimmune leukocytes in vitro. The cell type most commonly associated with such lysis is the natural killer (NK) cell, although other cell types could also be involved (for a review, see reference 34).

Virus infections induce IFN which in turn activates NK cells to become more cytolytic (28, 33). The sensitivity of virus-infected target cells to lysis mediated by NK cells has been examined in several different in vitro systems (3, 28, 36). Although many reports indicate that virus-infected target cells are more sensitive in vitro than uninfected cells, Welsh and Hallenbeck (36) have demonstrated that depending on the target cell and virus examined, sensitivity to lysis can be shifted in either positive or negative directions after virus infection of the target cell. It has been suggested that the sensitivity to NK cell-mediated lysis may be modulated by a variety of factors. Increased sensitivity to NK cells may result from the induction of IFN which activates NK cells during the assay (28), the increased expression of structures on the target cell that facilitate NK cell binding (36), and inhibition of target cell membrane repair systems (17). Other cells, including cytotoxic macrophages and a surface immunoglobulin-positive, asialo ganglio-*n*-tetraosylceramide (AGM1)-negative, lymphocyte-like cell (34a), are thought to mediate antiviral natural cytotoxicity but have received less study.

To assess the natural cytotoxicity against virus infections

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in vivo, we examined the rejection of [^{125}I]iododeoxyuridine (IUDR)-labeled uninfected and virus-infected cells from mice. The data presented in this paper demonstrate that (i) lymphocytic choriomeningitis virus (LCMV)-infected cells are cleared more rapidly than uninfected cells in vivo, even though they are not preferentially lysed by nonimmune leukocytes in vitro, (ii) the rejection is mediated by a host response sensitive to treatments with immunopharmacological agents but distinct from NK cells, and (iii) a nonspecific rejection mechanism, in addition to that mediated by NK cells, is activated during virus infection.

MATERIALS AND METHODS

Mice. C3H/St mice were purchased from West Seneca Laboratories, West Seneca, N.Y. They were maintained in our facilities for at least 3 weeks before use. C57BL/6 mice were originally purchased from Jackson Laboratories, Bar Harbor, Maine, and then bred in our own facilities. Unless stated otherwise, female mice at 10 to 16 weeks of age were used.

Virus. The Armstrong strain of LCMV was propagated in L-929 cells for use in these experiments. Standard (S) LCMV was harvested from acutely infected cells. Defective interfering (DI) LCMV was harvested from a persistently infected L-929 cell line and concentrated 100-fold for use in these experiments (37). The Indiana strain of vesicular stomatitis virus was used in the IFN assays.

Cells. The YAC-1 cell line is a Moloney leukemia virus-induced T-cell lymphoma of mouse strain A/Sn origin; it is extremely sensitive to lysis by endogenous (control) NK cells (16). The YAC-1 cell line was maintained in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with antibiotics (penicillin and streptomycin), glutamine, and 10% heat-inactivated fetal bovine serum (M. A. Bioproducts, Walkerville, Md.). L-929 is a continuous *H-2*^{k} fibroblast line relatively insensitive to endogenous NK cell-mediated lysis but is lysed by NK cells activated in vivo during virus infection (33). MC57G is a continuous *H-2*^{b} fibroblast cell line. L-929 and MC57G cells were grown in Eagle minimal essential medium (MEM; GIBCO) supplemented with antibiotics, glutamine, and 10% fetal bovine serum. Three days before in vivo rejection experiments, the fibroblast lines were seeded at 1.5×10^6 cells per 75-cm^2 flask and either left uninfected or infected with 0.1 PFU of S LCMV per cell (multiplicity of infection, 0.1). Uninfected YAC-1 target cells were seeded at 0.2×10^6 cells per ml 1 day before the experiment.

In vivo rejection assay. Target cells were labeled in vitro in a 5% CO_2 incubator at 37°C. Label, 20 μCi of IUDR ([^{125}I]IUDR; New England Nuclear Corp., Boston, Mass.), was added to 20 ml of 10% fetal bovine serum medium 18 h before target cell harvest. The labeled cells were greater than 90% viable, as shown by trypan blue dye exclusion. After extensive washing, the cells were resuspended at 10^6 cells per ml in either RPMI 1640 or MEM medium, and 0.5 ml (5×10^5 cells) was injected per mouse intravenously (i.v.) via the tail vein. Uninfected and LCMV-infected cells incorporated comparable levels of ^{125}I (≈200,000 cpm in 5×10^5 cells), and 80 to 90% of the label was precipitable in 5% trichloroacetic acid. Retention of radioactivity in whole bodies was measured at Scripps Clinic and Research Foundation (La Jolla, Calif.) in a Baird atomic spectrometer containing a well sufficiently large to insert a mouse. Counts retained in isolated organs were measured in a Beckman gamma 5500 counter (Beckman Instruments, Inc., Palo Alto, Calif.). The percentage of ^{125}I counts remaining at various times after injection was equal to [(the counts recovered in whole bodies or isolated organs)/(counts injected into the mouse at time zero)] × 100.

In vivo treatments. Mice were treated with hydrocortisone acetate in suspension (Hydrocortone; Merck Sharp & Dohme, West Point, Pa.) by intraperitoneal (i.p.) injection of 2.5 mg at 3 days and 3.75 mg at 1 day before use in in vivo rejection experiments. Cyclophosphamide (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 0.85% NaCl immediately before use, and mice were given (i.p.) 300 mg/kg of body weight 4 days before the experiments. Cobra venom factor (CVF; Cordis Laboratories, Miami, Fla.) was reconstituted in ice-cold water and stored at −70°C. CVF was diluted 1:2 in cold (4°C) phosphate-buffered saline immediately before use, and 15 U was administered i.p. on 3 and 1 days before the experiments (5). Mice were infected i.p. with 10^4 PFU of LCMV at various times before use. Polyinosinic-polycytidylic acid [poly(I):(C); Sigma] was made up at 0.4 mg/ml in 0.85% NaCl, and mice received (i.v.) a dose of 5 mg of poly(I):(C) per kg of body weight 1 day before use. Antiserum to anti-AGM1 (10) was diluted 1:5 in RPMI 1640 medium immediately before use, and the equivalent of 20 μl of undiluted serum was administered i.v. 2 days before the experiment.

In vitro cytotoxicity assays. Spleen effector cells were isolated as previously described (2, 33). Spleen cells were teased apart in RPMI 1640 medium and filtered through a nylon mesh. Cells were centrifuged at 400 × *g* for 5 min, resuspended in 0.83% NH_4Cl to lyse the erythrocytes, pelleted, and washed in RPMI 1640 medium. A modification of the protocol described by Puccetti et al. (24) was used to isolate lung effector cells with minimal contamination by peripheral blood cells. Mice were anesthesized, the interior vena cava was severed, and 10 ml of cold RPMI 1640 medium, containing 10 U of heparin per ml, was flushed through the lungs by injection into the right ventricle. The lungs were then removed, washed in RPMI 1640 medium, and minced into small pieces. The pieces were incubated at 4°C in medium for 1 h, flushed up and down in a syringe, filtered through fine gauze, and finally centrifuged at 400 × *g* for 5 min.

Microcytotoxicity assays were performed in vitro as previously described (2, 33). Briefly, target cells (10^6) were labeled with sodium chromate (^{51}Cr; New England Nuclear) for 1 h (37°C) at a concentration of 100 μCi per pellet of cells. After labeling, cells were centrifuged and washed, and 10^4 target cells were used per assay. Medium was added to cells used for spontaneous lysis determinations, and 1% Nonidet P-40 was added shortly before harvest to a set of target cell wells to determine 100% lysis. Microtiter plates were incubated from 4 to 18 h. Before harvest, plates were centrifuged at 200 × *g* for 5 min, and 0.1 ml was collected for radioactivity counting. Quadruplicate samples counted had standard deviations of less than 5% of the mean. Data are expressed as percent specific ^{51}Cr release (lysis) = 100 × [(cpm of test samples − cpm of medium controls)/(cpm of Nonidet P-40 controls − cpm of medium controls)], where cpm is the counts per minute. Spontaneous release never exceeded 10% in short (4 h) assays or 35% in long (18 h) assays.

IFN assays. Blood was collected from the retroorbital sinus of anaesthetized mice. After clotting, the serum was obtained by centrifugation. Lungs were harvested from individual animals, homogenized in 2.5 ml of 10% fetal bovine serum-MEM, and centrifuged at 400 × *g* to remove cellular debris. Samples for IFN assays were serially diluted

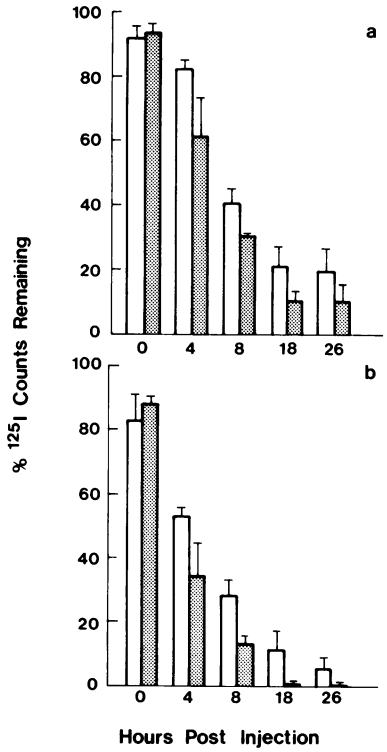

FIG. 1. Clearance of uninfected and LCMV-infected L-929 cells in vivo. Mice, C3H/St females at 12 weeks of age, were injected i.v. with 5×10^5 ^{125}I-labeled cells that were prepared as described in the text. At intervals between 0 and 26 h postinjection, groups of three animals were sacrificed, and the percentages of uninfected (□) and LCMV-infected (▩) cell counts remaining (± standard deviations) in whole bodies (a) and in isolated lungs (b) were determined.

twofold and used in a microtiter plate assay with L-929 cells as described elsewhere (4). After an 18- to 24-h incubation with the IFN test samples, L-929 cells were challenged with vesicular stomatitis virus and scored 2 and 3 days later for reduction in cytopathic effects.

Immunofluorescence assay. Cell surface antigens were stained directly with monospecific anti-LCMV guinea pig immunoglobulin G conjugated to fluorescein isothiocyanate as previously described (37). The percentage of cells expressing viral antigens was calculated after 200 cells were counted.

Assay for complement depletion. The effectiveness of complement depletion in vivo by CVF was assayed by examining the ability of serum from treated mice to provide C3 to sensitized sheep erythrocytes. The sheep erythrocytes were sensitized by treatment with a rabbit antiserum against sheep erythrocytes (a gift from Tulia Lindsten, Karolinska Institute, Stockholm, Sweden). The formation of erythrocyte-antibody complement (EAC) complex was assayed by rosette formation to human peripheral lymphocytes as described by Carlson and Terres (5).

Statistical analysis. Standard deviations were determined on the arithmetic means. Statistical significance was determined, and the *P* value was obtained by the two-tailed Student *t* test.

RESULTS

Differential rejection of virus-infected cells in vivo. Uninfected and LCMV-infected L-929 cells were labeled with [^{125}I]IUDR in vitro and injected into the tail vein of histocompatible C3H/St mice. Immediately after injection, greater than 90% of the counts of either uninfected or LCMV-infected L-929 cells were detected in the whole bodies of recipient mice (Fig. 1a). The cells were primarily localized in the lungs, from which over 80% of the counts were recovered (Fig. 1b). Although there was no difference in the initial implantation of uninfected and virus-infected cells, the LCMV-infected cells were cleared more rapidly than the uninfected cells. By 18 h postinjection, only 11% of the LCMV-infected cell counts remained in the whole bodies of injected mice, whereas 21% of the cell counts remained in those mice injected with uninfected cells.

Rejection of ^{125}I-labeled target cells from the whole body correlated directly with the clearance from the lungs; the difference between the percent counts remaining in whole bodies was comparable to the difference between the percent counts remaining in the lungs (Fig. 1 and Table 1). Use of the lung, rather than whole body, to measure counts eliminated the problem of residual whole body counts due to iodine localization in the thyroid or bladder. Livers and spleens never retained significant counts (Table 1). The implantation into and rejection from the lungs was therefore examined more closely.

Cell implantation and retention in the lungs was dependent on the viability of target cells. Dead cells were rapidly cleared from the lungs; by 4 h postinjection, when >50% of the counts contained in live L-929 cells were recovered in the lungs, <1% of the counts contained in the frozen and thawed cells were recovered (data not shown). The terminology percent counts recovered and percent cells recovered are used interchangeably in the remainder of this paper.

Defective interfering virus-induced resistance. DI LCMV specifically blocks S LCMV synthesis and inhibits production of LCMV antigens on the membranes of infected cells (35, 37). To examine the effect of DI virus on the in vivo rejection of S virus-infected cells, L-929 cells were exposed to DI LCMV before infection with S LCMV. In addition to inhibiting the expression of LCMV surface antigens, this treatment induced resistance to the in vivo rejection directed against virus-infected cells (Table 2). At a time when only 20% of the ^{125}I counts contained in S LCMV-infected cells were recovered in the lungs, 40% of the ^{125}I counts contained in either uninfected cells or cells infected first with DI virus and then challenged with S virus could be recovered. Differential rejection in vivo of LCMV-infected cells, therefore, required S LCMV synthesis and was not due to contamination of the S LCMV stock with another microorganism.

In vivo rejection after depletion of cellular constituents. The LCMV infection of L-929 cells is relatively noncytopathic in

TABLE 1. Organ distribution of radioactivity

Labeled target cell	% Injected counts remaining in:[a]			
	Total body	Lung	Liver	Spleen
Uninfected L-929	50.9 ± 9.2	19.8 ± 3.9	1.2 ± 0.24	0.54 ± 0.13
LCMV-infected L-929	39.3 ± 3.7	11.2 ± 3.0	1.2 ± 0.12	0.37 ± 0.12

[a] Target cells were prepared as described in the text. Six C3H/St female mice (12 weeks old) were used per group. Animals were sacrificed at 8 h postinjection. Data are expressed as the percent injected counts recovered ± the standard deviation.

TABLE 2. Defective interfering virus induces resistance to standard virus infection and in vivo rejection

Treatment of L-929 cells[a]		% of cells expressing surface LCMV antigens in expt 1[b]	% of cell counts remaining in vivo in:[c]	
First	Second		Expt 1	Expt 2
MEM	MEM	<0.5	40.2 ± 3.7	41.2 ± 1.4
DI LCMV	MEM	<0.5	ND[d]	45.0 ± 2.1
DI LCMV	S LCMV	<0.5	37.4 ± 7.0	40.5 ± 6.3
MEM	S LCMV	62	22.6 ± 2.6	18.8 ± 2.7

[a] DI LCMV was collected from culture fluids of L-929 cells persistently infected with LCMV and concentrated down 100-fold. L-929 cells (1.5×10^6 per 75-cm^2 flask) were treated with either MEM or 1 ml of concentrated DI LCMV for 1.5 h and washed. The cells were then challenged with MEM or S LCMV (multiplicity of infection, 0.1) for 1.5 h, washed again, and incubated in 25 ml of MEM for 3 days. Cells were labeled with 20 μCi of [^{125}I]IUDR for 18 h before harvest.

[b] Surface antigens were measured by immunofluorescence.

[c] Five C3H/St female mice were used per group. Lungs were harvested and counted at 4 h postinjection. Data are expressed as percent of injected counts recovered ± standard deviation.

[d] ND, Not done.

vitro, but it was nevertheless important to determine whether the differential rejection in vivo was due simply to a virus-induced cytopathology or to a host response function. Treatments with either hydrocortisone acetate or cyclophosphamide inhibit several cellular functions of the immune system, including NK cell-mediated lysis (14, 21, 25, 26). Treatments with hydrocortisone acetate that reduced spleen cell yields by 70% reduced in vivo rejection of both uninfected and LCMV-infected cells (Fig. 2a). In addition, the statistically significant differential rejection between uninfected and virus-infected cells was no longer apparent. Virtually identical results were obtained when animals were treated with cyclophosphamide under conditions that reduced spleen cell yields by greater than 90% (Fig. 2b). Since the recoveries in the drug-treated recipients were the same for both the uninfected and LCMV-infected cells, it can be concluded that the differential rejection of virus-infected cells was due to a drug-sensitive constituent of the host response and was not simply a result of virus-induced lysis of infected cells.

In contrast to the results described above, treatment protocols that depleted complement activity did not alter differential rejection of virus-infected cells. Treatments with CVF that reduced serum C3 levels by >90%, as measured by the ability to form erythrocyte-antibody complement complexes, slightly increased in vivo rejection of both uninfected and LCMV-infected L-929 cells but had no effect on the differential rejection (Fig. 3).

Histocompatibility requirements. To determine whether in vivo rejection was restricted to histocompatible target cells infected with LCMV, the rejection of *H-2*^{k} cells (L-929) and *H-2*^{b} cells (MC57G) was examined in an *H-2*^{k} mouse strain, C3H/St, and an *H-2*^{b} mouse strain, C57BL/6. Both the histoincompatible LCMV-infected cells and the histocompatible LCMV-infected cells were rejected more rapidly than their uninfected counterparts (Table 3), indicating that the enhanced rejection of LCMV-infected cells in vivo was not restricted by *H-2* antigens.

Augmentation of in vivo rejection by virus infection and poly(I):(C). NK cells are activated during LCMV infection of

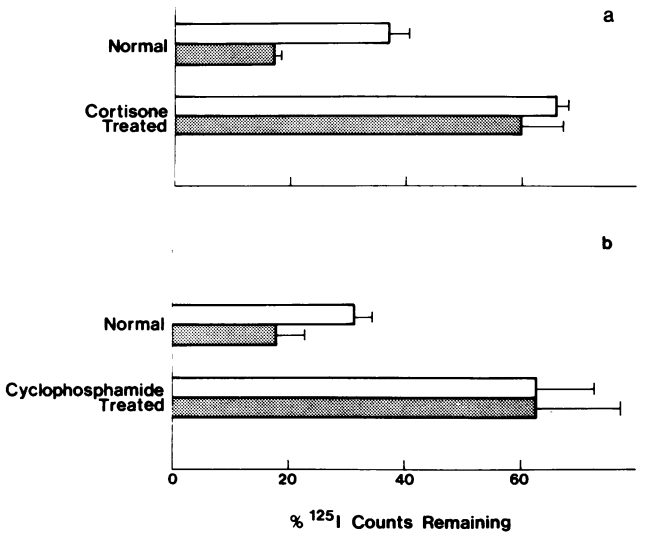

FIG. 2. Reduction of in vivo clearance and differential rejection. Uninfected and LCMV-infected L-929 cells were prepared and labeled with ^{125}I. Mice were injected i.v. with 5×10^5 labeled cells. (a) Mice were treated i.p. with hydrocortisone acetate (2.5 mg on day 3 and 3.75 mg on day 1 before the assay). Under these conditions, splenic NK cell activity was reduced from control levels of 18 to 6% as measured in a 4-h ^{51}Cr release assay against YAC-1 target cells (effector-to-target cell [E/T] ratio = 50/1). Lungs from mice receiving uninfected (□) and LCMV-infected (▨) cells were harvested and counted 8 h after injection of target cells. Levels of significance were $P < 0.001$ for normal mice and $P > 0.05$ for cortisone-treated mice. (b) Mice were injected i.p. with 300 mg of cyclophosphamide per kg of body weight 4 days before the assay. Under these conditions, splenic NK cell activity was reduced from 18 to 2% (E/T ratio = 50/1) and lung NK cell activity was reduced from 10 to 2% (E/T ratio = 10/1), as measured in a 16-h ^{51}Cr release assay against YAC-1 target cells. Lungs were harvested at 5 h post-injection with ^{125}I-labeled cells. Levels of significance were $P < 0.005$ for normal mice and $P > 0.5$ for cyclophosphamide-treated mice.

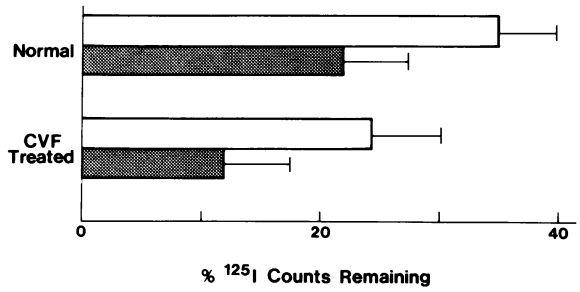

FIG. 3. Differential rejection of virus-infected cells after complement depletion. Mice were complement depleted by treatment with 15 U of CVF on 1 and 3 days before assay. Under these conditions, complement was reduced >90% as measured by the use of serum in an EAC rosette assay (see text). In contrast, splenic NK activity was unaltered; when measured in a 4-h ^{51}Cr release assay, control NK cells mediated 4% lysis of YAC-1 target cells, and cells isolated from CVF-treated animals mediated 6% lysis (E/T = 50/1). Mice were injected i.v. with ^{125}I-labeled L-929 cells. Lungs were harvested from mice receiving uninfected (□) and LCMV-infected (▨) cells and counted 5 h after injection. Levels of significance were $P < 0.001$ for normal mice and $P < 0.001$ for CVF-treated mice.

TABLE 3. Differential rejection of LCMV-infected cells in histoincompatible mice

Target H-2 type	Target cell[a]	% Injected counts remaining[b]	
		C57BL/6 Mice ($H\text{-}2^b$)	C3H/St Mice ($H\text{-}2^k$)
$H\text{-}2^b$	MC57G	30.6 ± 6.4	38.8 ± 8.5
	LCMV-MC57G	22.8 ± 6.9	9.3 ± 8.0
$H\text{-}2^k$	L-929	53.4 ± 8.0	60.9 ± 8.1
	LCMV-L-929	36.0 ± 4.2	40.3 ± 0.95

[a] LCMV-MC57G, LCMV-infected MC57G cells; LCMV-L-929, LCMV-infected L-929 cells.

[b] Target cells were prepared as described in the text. Recipient mice were 10- to 16-week-old females. Results with MC57G and LCMV-MC57G cells were compiled from four different experiments with a minimum of three animals per group. Results with L-929 and LCMV-L-929 cells came from a single experiment with three animals per group. Lungs were harvested at 4 to 5 h postinjection.

mice to lyse a broad range of target cells more efficiently; activated NK cells not only lyse YAC-1 cells more rapidly, but also lyse target cells resistant to endogenous NK cells such as uninfected and LCMV-infected L-929 cells (Table 4). The elevated NK cell activity peaks 2 to 4 days postinfection with LCMV and then subsides. Augmented NK cell-mediated lysis could be demonstrated with cells isolated from the lungs of mice at the time of peak spleen NK cell activity. In contrast to the NK cells isolated from the lungs of uninfected mice, which mediated only 5% lysis, NK cells prepared from the lungs of mice on day 3 postinfection with LCMV mediated 17% lysis of YAC-1 target cells in a ^{51}Cr release assay. Cytotoxic T cells specific for histocompatible cells expressing LCMV antigens develop later and peak at 7 to 10 days postinfection (Fig. 4a) (33, 39). The effect of these mechanisms on the rejection of uninfected and LCMV-infected cells in vivo was examined. Both uninfected and LCMV-infected mice had greater than 95% of the counts located in the lungs immediately after injection. The clearance of uninfected and LCMV-infected L-929 cells was, however, markedly augmented on day 3 postinfection, and the differential rejection of virus-infected cells was no longer apparent (Fig. 4b). The augmented rejection on day 3

TABLE 4. Sensitivity of [^{125}I]IUDR-labeled L cells to spleen NK cell-mediated lysis[a]

E/T ratio	% Specific ^{51}Cr release mediated by NK cells in:			
	Control[b]		Activated[c]	
	L	LCMV-L	L	LCMV-L
12	ND[d]	ND	5.9	7.3
25	−4.4	−2.5	8.7	8.9
50	−2.2	−4.1	11.5	13.8
100	−3.1	−0.5	13.3	16.3

[a] Uninfected L-929 (L) and LCMV-infected L-929 (LCMV-L) cells were prepared and labeled with [^{125}I]IUDR as described in the text.

[b] Control effector cells were isolated from spleens of 4-week-old mice. Cytotoxicity was determined in an 18-h assay. Spontaneous release was 28 and 33% for L cells and LCMV-L cells, respectively.

[c] Activated effector cells were isolated from the spleen of 6-week-old mice 2 days postinfection with LCMV (10^4 PFU i.p.). Cytotoxicity was determined in a 5-h assay, and spontaneous release was 16 and 19% for L cells and LCMV-L cells, respectively.

[d] ND, Not done.

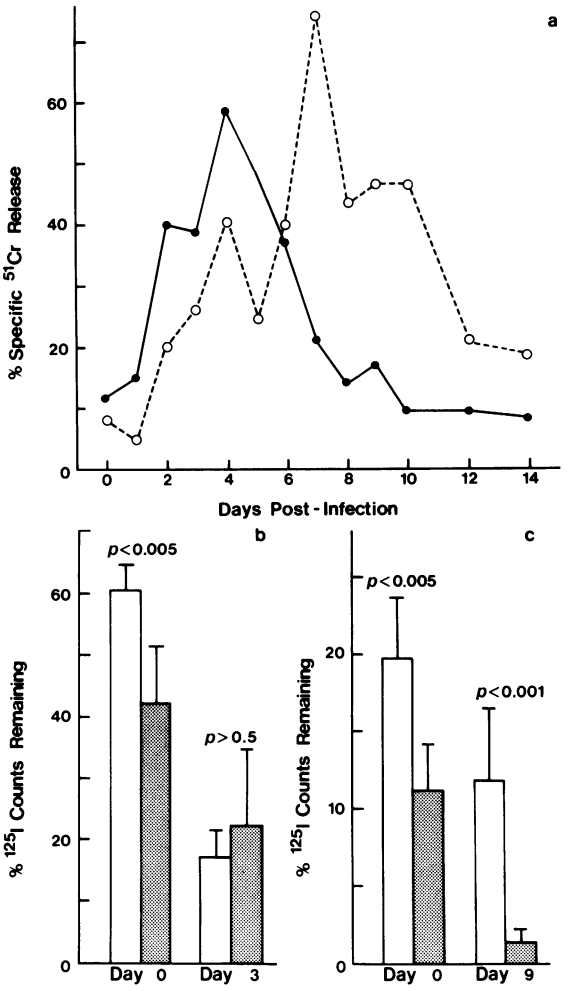

FIG. 4. In vivo rejection during viral infection. (a) In vitro cytotoxicity mediated by cells isolated from C3H/St mice at various days after infection with 10^4 PFU of LCMV. Spleen cells were prepared and used in a ^{51}Cr release assay for 4 h (E/T ratio = 50/1) against YAC-1 target cells (●) and for 18 h (E/T ratio = 100/1) against LCMV-infected L-929 cells (○). Uninfected (□) or LCMV-infected ( ) L-929 cells were labeled with ^{125}I for use in the in vivo rejection assay. Twelve-week-old female C3H/St mice were used in groups containing three to seven animals. (b) Lungs were harvested 4 h after i.v. injection of labeled target cells from control mice and mice on day 3 postinfection with LCMV. (c) Lungs were harvested 8 h after i.v. injection of labeled target cells from control mice and mice on day 9 postinfection with LCMV.

postinfection was apparently mediated by a cellular constituent of the host response, as treatments with cyclophosphamide before LCMV infection dramatically inhibited the rejection in infected mice (data not shown). On day 9 postinfection, a time when in vitro assays demonstrated that NK cell-mediated activity was declining but cytotoxic T-cell activity was high, the LCMV-infected cells were again rejected more rapidly than uninfected cells (Fig. 4c).

Since poly(I):(C) induces IFN and activates NK cells in vivo (6, 26), the effect of poly(I):(C) on the rejection of uninfected and LCMV-infected L-929 cells was also determined. Poly(I):(C) injections augmented spleen NK cell activity and caused an increase in the in vivo rejection of the uninfected L-929 cells such that both uninfected and LCMV-

infected cells were cleared to the same extent at 4 h postinjection (Fig. 5).

IFN levels and NK cell activity after rejection of LCMV-infected cells. Labeling cells with [^{125}I]IUDR did not alter LCMV synthesis; supernatants from control and [^{125}I]IUDR-labeled, LCMV-infected L-929 cells both contained 10^6 PFU of LCMV per ml (data not shown). In addition, the sensitivity of L-929 cells to NK cell-mediated lysis in vitro was not significantly enhanced after labeling with [^{125}I]IUDR. Uninfected L-929 cells and LCMV-infected L-929 cells labeled with ^{51}Cr are relatively resistant to endogenous NK cells isolated from control uninfected mice and are killed equally well by activated NK cells isolated on day 3 postinfection with LCMV (36). [^{125}I]IUDR-labeled uninfected and LCMV-infected L-929 cells also had similar sensitivities to NK cell-mediated lysis in vitro (Table 4).

Although LCMV does not readily induce IFN in culture (38), high levels of IFN are generated in vivo during LCMV infection of mice (33). The levels of IFN in the serum and lung in recipients of LCMV-infected cells was examined. Although no IFN could be detected in the supernatants of LCMV-infected L-929 cell cultures, within 4 h these cells induced an average of 102 U per ml of serum and 120 U per lung of recipient mice. IFN was not detected (<4 U) in mice that received uninfected L-929 cells.

NK cells were isolated from the lungs of mice that were injected i.v. with either uninfected or LCMV-infected L-929 cells. The isolated effector cells were tested in a ^{51}Cr release assay against YAC-1 target cells. The NK cell activity mediated by cells recovered from the lungs of LCMV-infected L-929 cell recipients was slightly elevated as compared with that mediated by NK cells recovered from the lungs of uninfected L-929 cell recipients, but neither population was very active (Fig. 6); in a 12-h assay, the maximum killing by the NK cells isolated from the lungs of 14-week-old mice was <10% against YAC-1 target cells. No cytotoxicity could be detected against L-929 cells with effector cells isolated from either uninfected or LCMV-infected cell recipients (data not shown).

Effect of NK cell depletion on in vivo rejection in normal mice. To examine the role of NK cells in the rejection of uninfected and LCMV-infected cells, mice were treated with anti-AGM1 serum in vivo under conditions which selectively eliminated endogenous and activated NK cells for several days (Table 5) (4, 10). Injection of 20 μl of anti-AGM1 at 2 days before the experiment completely eliminated spleen NK cell activity recovered from both uninfected and LCMV-infected cell recipients but did not inhibit IFN induction (Table 5). Anti-AGM1 treatments did not alter implantation of the NK-sensitive target cell, YAC-1, in the lung but significantly inhibited the in vivo rejection of this target cell. Immediately after injection, 91% of the YAC-1 cell counts were recovered in the lungs of normal animals and 88% were recovered in the lungs of anti-AGM1-treated animals. At 2 h postinjection, a time at which <2% of the injected YAC-1 cell counts were recovered in the lungs of normal animals, over 30% of the YAC-1 cell counts were recovered in the lungs of anti-AGM1-treated mice (Fig. 7). In contrast, treatments with anti-AGM1 had relatively little effect on the in vivo rejection of either uninfected or LCMV-infected L-929 cells, and the differential rejection of LCMV-infected cells was still observed (Fig. 7).

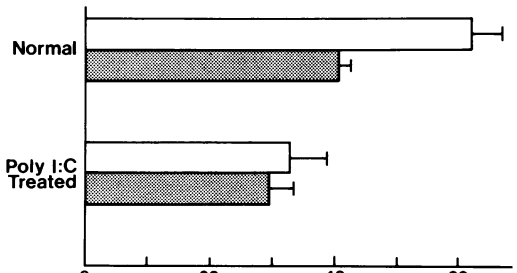

FIG. 5. Poly(I):(C)-induced augmentation of in vivo rejection and loss of differential rejection. Mice, C3H/St females at 15 weeks of age, were either untreated or treated with poly(I):(C) (5 mg/kg of body weight) 1 day before assay. Under these conditions, splenic NK cell activity was elevated from 7 to 13% cytotoxicity as measured in a 4-h ^{51}Cr release assay against YAC-1 target cells (E/T ratio = 50/1). Labeled (^{125}I) uninfected (□) and LCMV-infected (▨) L-929 cells were injected i.v., and lungs were harvested 4 h later. Levels of significance were $P < 0.001$ for normal mice and $P > 0.2$ for poly(I):(C)-treated mice.

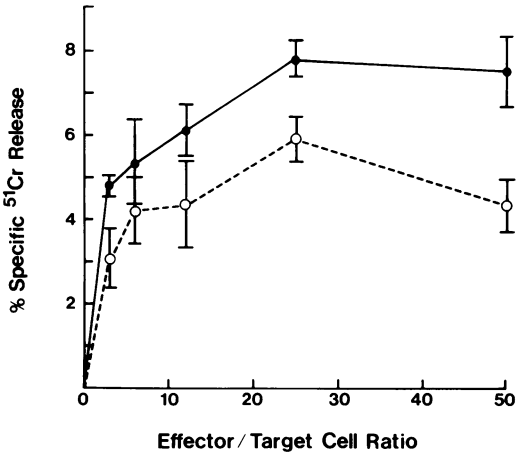

FIG. 6. NK cell activity isolated from the lungs after in vivo rejection of virus-infected cells. Groups of 10 mice, C3H/St females at 14 weeks of age, were injected i.v. with either uninfected (○) or LCMV-infected (●) L-929 cells. Four hours later effector cells were isolated from the lungs as described in the text. Cytotoxicity (± standard deviation between replicate samples) was determined in a 13-h ^{51}Cr release assay against YAC-1 target cells.

Effect of NK cell depletion on augmented rejection. We next examined the effect of anti-AGM1 treatment on the augmented rejection observed during virus infection, originally shown in Fig. 4b. Mice infected with LCMV 3 days before the experiment and then treated with anti-AGM1 had high levels of IFN but were drastically depleted of spleen NK cell activity (Table 5, experiment 2). The in vivo rejection of YAC-1, uninfected L-929, and LCMV-infected L-929 cells was augmented in virus-infected mice, but only the elevated rejection of YAC-1 target cells was dramatically reduced by treatment with anti-AGM1 (Fig. 8). Treatment of LCMV-infected mice with anti-AGM1 inhibited in vivo rejection such that the YAC-1 cell counts recovered in the lungs of activated, anti-AGM1-treated mice was almost threefold higher than those recovered in control uninfected mice (Fig. 8). Although it is clear that NK cells isolated from LCMV-infected mice lyse uninfected and LCMV-infected L-929 cells in vitro (Table 4) (36), only a small proportion of the

TABLE 5. Sensitivity of NK cells to treatment with anti-AGM1 in vivo[a]

Expt	Target cell in vivo rejection	Mice[b]	U/ml of serum IFN[c]	% Lysis of YAC-1 cells mediated by spleen cells[d]
1	L-929	Control	<1	9.3
		Control + anti-AGM1	1	−3.4
	LCMV-L-929[e]	Control	110	14.4
		Control + anti-AGM1	85	0.5
2	L-929	Control	4	2.2
		LCMV-infected	1,024	37.8
		LCMV-infected + anti-AGM1	768	0.60
	LCMV-L-929	Control	75	7.9
		LCMV-infected	18,000	32.5
		LCMV-infected + Anti-AGM1	41,000	1.7

[a] Target L-929 cells were prepared as described in the text. Sera and spleens were harvested from groups of four to six mice 4 h after injection of target cells.

[b] Mice, C3H/St females at 16 to 17 weeks of age, were used uninfected (control) or on day 3 postinfection with 10^4 PFU of LCMV (LCMV-infected). Mice, either control or LCMV-infected, were depleted of NK cell by injection i.v. with the equivalent of 20 μl of anti-AGM1 serum 2 days before harvest.

[c] IFN assays were run as described in the text.

[d] Spleen cells were processed and used in a ^{51}Cr release assay against YAC-1 target cells. The results reported for experiment 1 were obtained in an 18-h assay at an E/T ratio of 25:1. Those reported for experiment two were obtained in a 5-h assay at an E/T ratio of 100:1.

[e] LCMV-L-929, LCMV-infected L-929 cells.

augmented in vivo rejection against these target cells during virus infection was inhibited by treatment with anti-AGM1; the cell counts recovered with either uninfected or LCMV-infected cells were still significantly lower in LCMV-infected anti-AGM1-treated mice than the counts recovered in control uninfected mice (Fig. 8).

DISCUSSION

We believe this to be the first documentation that virus-infected target cells are preferentially and rapidly lysed within nonimmune mice. By examining the clearance of [^{125}I]IUDR-labeled cells in vivo, it was demonstrated that uninfected mice rejected LCMV-infected L-929 cells more rapidly than uninfected L-929 cells (Fig. 1). Pretreatments with DI LCMV reduced the synthesis of S LCMV (Table 2) and the rate of in vivo rejection, indicating that the rejection required LCMV synthesis and was not a consequence of a contaminant in the LCMV stock. The clearance of ^{125}I counts was mediated by the host and was not simply a result of virus-induced cytopathic effects in the cells; treatments with hydrocortisone acetate and cyclophosphamide, which inhibited cellular constituents of the immune system, ablated the differential rejection of LCMV-infected cells (Fig. 2). Although NK cells were among the cellular constituents inhibited by the drug regimens used (14, 25), NK cells did not appear to mediate the differential rejection of LCMV-infected cells; treatments with anti-AGM1 serum in vivo depleted NK cell activity and significantly depressed the in vivo rejection of the NK-sensitive target cell, YAC-1, but had little effect on the rejection of either uninfected or LCMV-infected L-929 cells (Fig. 7).

In addition to the natural cytotoxicity directed against virus-infected target cells, a nonspecific rejection mechanism activated on day 3 postinfection with LCMV could also be demonstrated (Fig. 4). NK cells activated at this time (Fig. 4) mediated rejection of YAC-1 target cells, but the enhanced rejection of both uninfected and LCMV-infected L-929 cells was probably mediated by mechanisms in addition to that mediated by NK cells; treatments with anti-AGM1 serum significantly depressed the augmented rejection of YAC-1 cells but did not dramatically alter the augmented rejection of either uninfected or LCMV-infected L-929 cells on day 3 postinfection (Fig. 8). It is not known

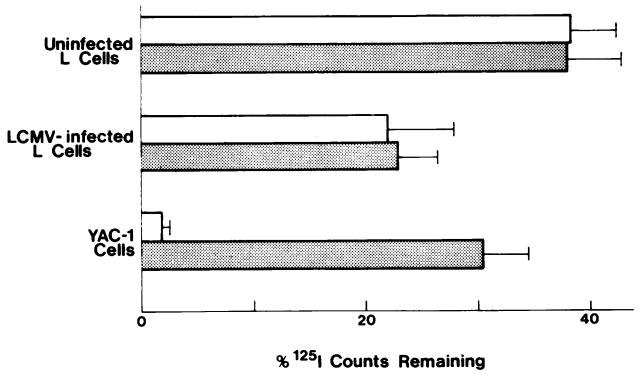

FIG. 7. Rejection in normal mice depleted of NK cell activity. C3H/St females, at 12 weeks of age, were either untreated (□) or NK cell depleted (▨) by i.v. injection of the equivalent of 20 μl of anti-AGM1 serum 2 days before the experiment. This treatment resulted in a greater than 10-fold reduction of splenic and lung NK cell activity when measured in vitro with a ^{51}Cr release assay against YAC-1 target cells. Labeled (^{125}I) target cells were prepared, and 5 × 10^5 cells were injected i.v. Lungs were harvested from uninfected and LCMV-infected L-929 cell recipients 4 h postinjection. Lungs from YAC-1 cell recipients were harvested 2 h postinjection. Levels of significance were $P > 0.5$ for uninfected L cells, $P > 0.5$ for LCMV-infected L cells, and $P < 0.001$ for YAC-1 cells.

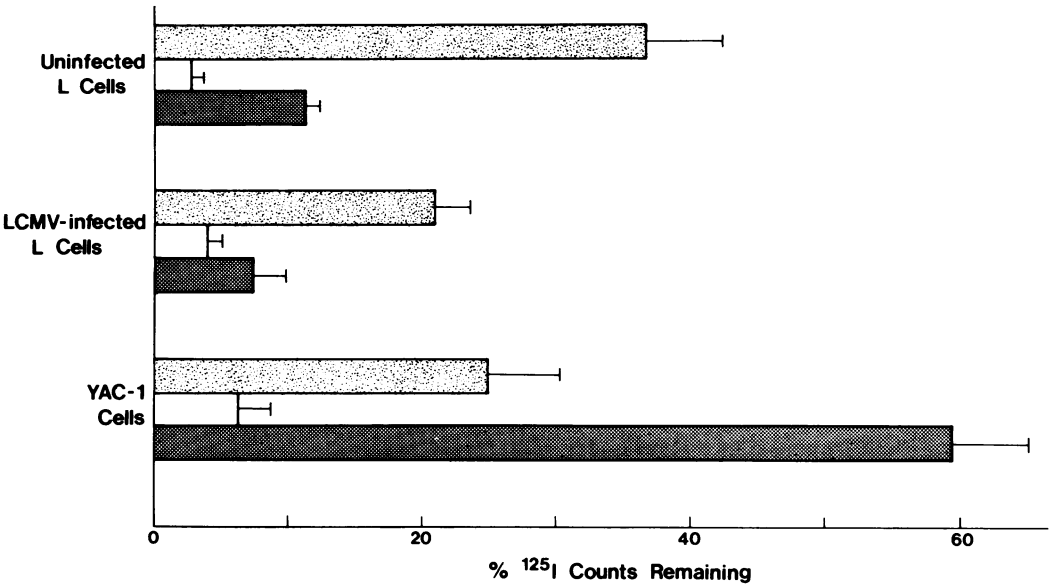

FIG. 8. Effect of anti-AGM1 treatments on the augmented rejection observed in vivo during viral infection. Uninfected L-929, LCMV-infected L-929, and YAC-1 target cells were labeled (^{125}I). Cells (5×10^5) were injected i.v. into recipient mice that were treated in the following manner: untreated control (▨); infected with 10^4 PFU of LCMV 3 days before the assay (□); and infected with 10^4 PFU of LCMV 3 days before and NK cell depleted 2 days before the assay by injection of the equivalent of 20 μl of anti-AGM1 serum (▩). Lungs were harvested from L-929 recipient mice at 4 h postinjection and from YAC-1 recipient mice at 1.5 h postinjection.

whether this nonspecific rejection mechanism observed on day 3 postinfection with LCMV is mediated by the same mechanism as the differential rejection of virus-infected cells in uninfected mice (Fig. 7). The differential rejection in normal mice and the augmented rejection in stimulated mice could be localized and generalized manifestations of the same phenomenon. Exposure to LCMV products on the surface of virus-infected cells in normal mice may locally activate AGM1-negative effector cells to lyse target cells. Experiments to determine whether LCMV-infected cells were activating a nonspecific rejection mechanism in uninfected mice have been inconclusive. When labeled L-929 cells were injected in the presence of unlabeled LCMV-infected L-929 cells at different ratios, the rejection of the uninfected cells was either not altered or only marginally augmented with questionable statistical significance (P always greater than 0.05; data not shown).

Evidence has been presented recently to support a role for NK cells in the regulation of tumor cell growth and the control of virus infection (4, 9–12, 15, 22, 23, 25, 27, 32). Minato et al. have shown that there is a good correlation between resistance to NK cell-mediated lysis in vitro and tumor cell growth in vivo in nude mice and that persistent infection with certain RNA viruses can render tumor cells more sensitive to both processes (22). Selective depletion of NK cells in vivo by treatment with anti-AGM1 serum has been shown to result in increased tumor cell growth (10, 15) and spreading during experimental metastasis (9). Specific in vivo reduction of NK cell activity in control uninfected mice also results in a reduction in the clearance of ^{125}I-labeled YAC-1 target cells extremely sensitive to in vitro lysis mediated by endogenous NK cells (9, 23). We extended the previous work by examining the in vivo rejection of ^{125}I-labeled YAC-1 cells and show that not only is the rejection in control uninfected mice mediated by NK cells (Fig. 7) but also that on day 3 postinfection with LCMV there is an elevated rejection of YAC-1 target cells mediated by NK cells (Fig. 8).

Implanted tumor cells are not always sensitive to an AGM1-dependent mechanism. When Kawase and co-workers (15) examined the effect of in vivo NK cell depletion on the tumorigenicity of cells that were either sensitive or resistant to NK cell-mediated lysis in vitro, they found that NK cells regulated NK-sensitive tumor cell growth in vivo but had little effect on NK-resistant tumor cells. The studies presented in this paper demonstrate that the in vivo rejection of L-929 cells is in direct contrast to the rejection of YAC-1 cells. L-929 cells are relatively resistant to in vitro lysis mediated by endogenous NK cells and are only moderately sensitive to lysis mediated by NK cells isolated from mice on day 3 postinfection with LCMV (Table 3) (33). These cells are, nevertheless, cleared in vivo by some mechanism (Fig. 2) other than that mediated by AGM1-positive NK cells (Fig. 7). In addition, a rejection mechanism predominantly independent of AGM1-positive cells is highly active on day 3 postinfection with LCMV (Fig. 8).

Complement has also been implicated in the defense against virus infection. Cells infected with LCMV can activate complement (37), and complement-depleted animals are more susceptible to Sindbis virus infection (13). Complement does not, however, appear to play an important role in the in vivo rejection of LCMV-infected L-929 cells since treatment with CVF depleted serum complement levels but did not eliminate the enhanced rejection of these target cells (Fig. 3).

These results apparently indicate that at least two rejection mechanisms are present and activated in the lung during LCMV infection. The first is NK cell mediated, presumably activated during LCMV infection by viral IFN and directed against YAC-1 cells (Fig. 7 and 8). The second rejection mechanism is directed against L-929 cells and is not sensitive to anti-AGM1 serum (Fig. 7 and 8). Since leukocyte prepara-

tions from lungs contain macrophages, NK cells, and a population of a natural cytotoxic (NC) cells clearly distinct from NK cells (24, 29; unpublished data), these populations must be considered. Both macrophages and NC cells differ from NK cells in their expression of AGM1 antigen, target cell range, and presence in older animals after NK cell activity has declined (18, 30, 31). Although both of these populations have been reported to be relatively resistant to the cyclophosphamide treatments used here, a cyclophosphamide-sensitive cell may be required to activate either macrophages or NC cells upon exposure to LCMV-infected target cells. Experiments to delineate the cell mediating the lysis are hampered, at this time, by the lack of suitable reagents to specifically eliminate either macrophages or NC cells in vivo. Recently, it has been demonstrated that NC cells could be activated in vitro by interleukins other than IFN (18, 19). Since such interleukins may be generated during LCMV infection, rejection mechanisms in addition to those mediated by NK cells could be activated during viral infections.

Several cellular effector mechanisms, in addition to that mediated by NK cells, may play a role in natural cytotoxicity against virus infections. An effector cell mediating preferential lysis in vitro of herpes simplex virus type 1 (HSV-1)-infected fibroblasts has recently been isolated from human peripheral blood (8, 20). This population of cells, NK (HSV-1) cells, is clearly different from the classic NK cell in physical properties and antigen expression (7). Welsh et al. have characterized a mouse virus killer cell, different from the NK cell, which is AGM1 negative and mediates lysis of mouse hepatitis virus-infected cells in vitro (34a). It has been reported that hamster peritoneal macrophages activated by vaccinia virus infection kill virus-infected target cells (S. K. Chapes and W. A. F. Tompkins, RES J. Reticuloendothel. Soc., p. 47, 1979). It seems, therefore, that the natural immunity to virus infection may be comprised of several effector mechanisms that function against particular viruses and target cells. Resistance may thus result from a composite response that is defined by the insult. We have previously demonstrated that L-929 cells infected with herpes simplex virus or Sindbis virus are all cleared more rapidly in vivo than uninfected cells (1). The rejection of virus-infected cells in vivo may thus represent a general defense mechanism against virus infection.

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LITERATURE CITED

1. **Biron, C. A., and R. M. Welsh.** 1982. Activation and role of natural killer cells in virus infections. Med. Microbiol. Immunol. **170**:155–172.
2. **Biron, C. A., and R. M. Welsh.** 1982. Blastogenesis of natural killer cells during viral infection *in vivo*. J. Immunol. **129**:2788–2795.
3. **Blazar, B., M. Pattaroyo, E. Klein, and G. Klein.** 1980. Increased sensitivity of human lymphoid lines to natural killer cells after induction of the Epstein-Barr viral cycle by superinfection or sodium butyrate. J. Exp. Med. **151**:614–627.
4. **Bukowski, J. F., B. A. Woda, S. Habu, K. Okumura, and R. M. Welsh.** 1983. Natural killer cell depletion enhances virus synthesis and virus-induced liver necrosis *in vivo*. J. Immunol. **131**:1531–1538.
5. **Carlson, G. A., and G. Terres.** 1976. Antibody induced killing *in vivo* of L1210/MTX-R cells quantitated by passively immunized mice with ^{31}I-iododeoxyuridine labeled cells and whole body measurement by retained radioactivity. J. Immunol. **117**:822–829.
6. **Djeu, J. Y., J. A. Heinbaugh, H. J. Holden, and R. B. Herberman.** 1979. Augmentation of mouse natural killer cell activity by interferon and interferon inducers. J. Immunol. **122**:175–181.
7. **Fitzgerald, P. A., R. Evans, D. Kirkpatrick, and C. Lopez.** 1983. Heterogeneity of human NK cells: comparison of effectors that lyse HSV-1-infected fibroblasts and K562 erythroleukemia targets. J. Immunol. **130**:1663–1667.
8. **Fitzgerald, P. A., P. Von Wusson, and C. Lopez.** 1982. Role of interferon in natural kill of HSV-1 infected fibroblasts. J. Immunol. **129**:819–823.
9. **Gorelik, E., R. H. Wiltrout, K. Okumura, S. Habu, and R. B. Herberman.** 1982. Role of NK cells in the control of metastatic spread and growth of tumor cells in mice. Int. J. Cancer **30**:107–112.
10. **Habu, S., H. Fukui, K. Shimura, M. Kasai, Y. Nagai, K. Okumura, and N. Tamoki.** 1981. *In vivo* effects of anti-asialo GM_1. Reduction of NK activity and enhancement of transplanted tumor growth in nude mice. J. Immunol. **127**:34–38.
11. **Hanna, N., and R. C. Burton.** 1981. Definitive evidence that natural killer (NK) cells inhibit experimental tumor metastasis *in vivo*. J. Immunol. **127**:1754–1758.
12. **Hanna, N., and I. Fidler.** 1980. Role of natural killer cells in the destruction of circulating tumor emboli. J. Natl. Cancer Inst. **65**:801–809.
13. **Hirsch, R. L., D. E. Griffin, and J. A. Winkelstein.** 1978. The effect of complement depletion on the course of Sindbis virus infection in mice. J. Immunol. **121**:1276–1278.
14. **Hochman, P., and G. Cudkowicz.** 1977. Different sensitivities to hydrocortisone of natural killer cell activity and hybrid resistance to parental marrow grafts. J. Immunol. **119**:2013–2015.
15. **Kawase, I., D. L. Urdal, C. G. Brooks, and C. S. Henney.** 1982. Selective depletion of NK cell activity *in vivo* and its effect on the growth of NK sensitive and NK resistant tumor cell variants. Int. J. Cancer **29**:567–574.
16. **Kiessling, R., E. Klein, and H. Wigzell.** 1975. Natural killer cells in the mouse. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. Eur. J. Immunol. **5**:112–117.
17. **Kunkel, L. A., and R. M. Welsh.** 1981. Metabolic inhibitors render "resistant" target cells sensitive to natural killer cell-mediated lysis. Int. J. Cancer. **27**:73–79.
18. **Lattime, E. C., S. T. Ishizaka, G. A. Pecoraro, G. Koo, and O. Stutman.** 1982. NC cells do not express NK-associated cell surface antigens and are not culture activated NK cells, p. 187–199. *In* R. B. Herberman (ed.), NK cells and other natural effector cells. Academic Press, Inc., New York.
19. **Lattime, E. C., G. A. Pecoraro, and O. Stutman.** 1983. The activity of natural cytotoxic cells is augmented by interleukin 2 and interleukin 3. J. Exp. Med. **157**:1070–1075.
20. **Lopez, C., D. Kirkpatrick, P. Fitzgerald, C. Y. Ching, R. W. Pahwa, R. A. Goop, and E. M. Smithwick.** 1982. Studies of the cell lineage of the effector cells that spontaneously lyse HSV-1 infected fibroblasts [NK (HSV-1)]. J. Immunol. **129**:824–828.
21. **Mantovani, A., W. Luini, G. Peri, A. Vecchi, and F. Spreafico.** 1978. Effect of chemotherapeutic agents on natural cell-mediated cytotoxicity in mice. J. Natl. Cancer Inst. **61**:1255–1261.
22. **Minato, N., B. R. Bloom, C. Jones, J. Holland, and L. Reed.** 1979. Mechanism of rejection of virus persistently infected tumor cells by athymic nude mice. J. Exp. Med. **149**:1117–1133.
23. **Pollack, S. B., and L. A. Hallenbeck.** 1982. *In vivo* reduction of NK activity with anti-NK 1 serum: direct evaluation of NK cells in tumor clearance. Int. J. Cancer **29**:203–207.
24. **Puccetti, P., A. Santoni, C. Riccardi, and R. B. Herberman.** 1980. Cytotoxic effector cells with the characteristics of natural killer cells in the lungs of mice. Int. J. Cancer **25**:153–158.
25. **Riccardi, C., T. Barlozzari, A. Santoni, R. Herberman, and C.**

Cesarini. 1981. Transfer to cyclophosphamide-treated mice of natural killer (NK) cells and *in vivo* natural reactivity against tumors. J. Immunol. **126**:1284–1289.
26. **Riccardi, C., P. Puccetti, A. Santoli, and R. B. Herberman.** 1979. Rapid *in vivo* assay of mouse natural killer cell activity. J. Natl. Cancer Inst. **63**:1041–1045.
27. **Riccardi, C., A. Santoli, T. Barlozzari, P. Puccetti, and R. B. Herberman.** 1980. *In vivo* natural reactivity of mice against tumor cells. Int. J. Cancer **25**:475–486.
28. **Santoli, D., G. Trinchieri, and H. Koprowski.** 1978. Cell mediated cytotoxicity against virus-infected target cells in humans. II. Interferon induction and activation of natural killer cells. J. Immunol. **121**:532–538.
29. **Stein-Streilein, J., M. Bennett, and V. Kumar.** 1983. Natural killer (NK) and natural cytotoxic (NC) cells in the lung. Fed. Proc. **42**:1379.
30. **Stutman, O.** 1980. Ontogeny and other age-related effects of natural cytotoxic (NC) cells in mice, p. 231–240. *In* R. B. Herberman (ed.), Natural cell-mediated immunity against tumors. Academic Press, Inc., New York.
31. **Stutman, O., E. F. Figarella, C. J. Paige, and E. C. Lattime.** 1980. Natural cytotoxic (NC) cells against solid tumors in mice: general characteristics and comparison to natural killer (NK) cells, p. 187–229. *In* R. B. Herberman (ed.), Natural cell-mediated immunity against tumors. Academic Press, Inc., New York.
32. **Talmadge, J. E., K. M. Meyers, D. J. Prieur, and J. R. Starkey.** 1980. Role of NK cells in tumor growth and metastasis in *beige* mice. Nature (London) **284**:622–624.
33. **Welsh, R. M.** 1978. Cytotoxic cells induced during lymphocytic choriomeningitis virus infection of mice. I. Characterization of natural killer cell induction. J. Exp. Med. **148**:163–181.
34. **Welsh, R. M.** 1981. Natural cell-mediated immunity during viral infection. Curr. Top. Microbiol. Immunol. **92**:83–106.
34a. **Welsh, R. M., C. A. Biron, J. Bukowski, S. Habu, M. V. Haspel, K. Holmes, K. Okumura, and D. Parkers.** 1983. Regulation and role of natural cell mediated immunity during virus infection, p. 21–41. *In* F. A. Ennis (ed.), Human immunity to viruses. Academic Press, Inc., New York.
35. **Welsh, R. M., and M. J. Buchmeier.** 1979. Protein analysis of defective interfering lymphocytic choriomeningitis virus and persistently infected cells. Virology **96**:503–515.
36. **Welsh, R. M., and L. A. Hallenbeck.** 1980. Effect of virus infections on target cell susceptibility to natural killer cell-mediated lysis. J. Immunol. **124**:2495–2497.
37. **Welsh, R. M., and M. B. A. Oldstone.** 1977. Inhibition of immunological injury of cultured cells infected with lymphocytic choriomeningitis virus: role of defective interfering virus in regulating viral antigenic expression. J. Exp. Med. **145**:1449–1468.
38. **Welsh, R. M., and C. J. Pfau.** 1972. Determinants of lymphocytic choriomeningitis interference. J. Gen. Virol. **14**:177–187.
39. **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.