CYTOTOXIC CELLS INDUCED DURING LYMPHOCYTIC CHORIOMENINGITIS VIRUS INFECTION OF MICE

I. Characterization of Natural Killer Cell Induction*

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While acute viral infections commonly induce the proliferation of specific clones of bone marrow-derived lymphocytes (B cells) and thymus-derived lymphocytes (T cells), profound nonspecific immunostimulatory (as well as depressive) effects may also occur in the infected animal (1). As examples, lactic dehydrogenase virus infection enhances antibody response to nonviral antigens (2), and Epstein-Barr virus infection elicits polyclonal proliferation of human B cells (3). A viral infection may activate and recruit into areas of inflammation phagocytic cells, such as macrophages and polymorphonuclear (PML) leukocytes (4.5).[1] These presumably function to phagocytize cellular debris in areas of necrotized tissue but may provide immunoregulatory functions as well (6). How a virus infection affects so many cells of the immune system is not clearly understood, but the induction of interferon by virus-infected cells (7) and the release of other cell products (lysosomal enzymes, histamine, serotonin, etc.) by dying cells may contribute to a generalized immune stimulatory or inflammatory reaction (8).

We have used the murine-lymphocytic choriomeningitis virus (LCMV) system as a model to study the host response to a viral infection. Adult mice infected intraperitoneally with LCMV develop an antibody response, measured in immune complexes as early as 4 days postinfection (9, 10), and a virus-specific T-cell response as early as 5-6 days postinfection (11-13). The T-cell response directly or indirectly controls the virus infection, as mice genetically or experimentally depleted of T cells fail to eradicate the virus (14), and administration of immune T cells into infected mice lowers the titer of virus (15). T cells have also been implicated in the immunopathology and death of mice infected intracranially with LCMV (11, 14), since transfer of T cells to virus-infected immunosuppressed mice results in a fatal immunopathological lesion. Also associated with the acute LCMV infection of adult mice is the

[1] Abbreviations used in this paper: A, antibody: C, guinea pig complement; ECC, early cytotoxic cell; LCMV, lymphocytic choriomeningitis virus; MEM, Eagle's minimal essential medium; NDV, Newcastle disease virus; NK, natural killer (cell); PFU, plaque-forming unit; PML, polymorphonuclear leukocytes; UV, ultraviolet; VSV, vesicular stomatitis virus.

activation of macrophages as early as 4 days postinfection (5). Their specific role in the immunopathology of the LCMV infection is unknown, but macrophages are found in the inflammatory lesions in the meninges of intracranially infected mice (M. B. A. Oldstone, personal communication).

Recently, we reported that the infection of mice with LCMV elicits cytotoxic cells in the spleen and peritoneum before the advent of the T-cell response (16). These cells resembled natural killer (NK) cells, which have been reported and described in other laboratories (17–22). During the past year several other laboratories have reported similar types of cytotoxic cell activity associated with virus infections in mice (23–25), suggesting a role in virus infections for lymphoid cells that are neither T cells, B cells, macrophages, or PML. Here we report the characterization and induction properties of LCMV-induced NK cells.

Materials and Methods

Viruses. The viruses used in these studies were the Armstrong (26) and WE (26) strains of LCMV, propagated in L-929 cells. Also used were vesicular stomatitis virus (VSV)-Indiana strain (American Type Culture Collection), Newcastle disease virus (NDV)-strain Herts (27), and Pichinde virus (28).

Cells. L-929 (L) cells were propagated in Eagle's minimal essential medium (MEM) supplemented with antibiotics (streptomycin, penicillin, and fungizone), glutamine, and 10% heat inactivated (56°C, 30 min) fetal bovine serum (Flow Laboratories, Inc., Rockville, Md.). L cells persistently infected with LCMV (L [LCMV]) were cultivated the same as uninfected cells. L (LCMV) cells expressed viral antigens on the cell surface and were used as target cells for virus-specific T-cell killing. Properties of these cells have been previously reported (29). They release predominantly defective-interfering LCMV and little or no detectable standard virus. F9 teratoma cells derived from H-2b mice but lacking H-2 antigenic expression were cultivated in Dulbecco's medium on gelatin-coated monolayers as described (30).

Infection of Mice. The following strains of mice were used: AKR, BALB/c, B10.BR, B10.D2, C57BL/6, CBA/N × DBA/2 F_1, C3H/HeJ, C3H/St, C3H/St × DBA/2 F_1, NZB × NZW F_1, SWR/J, BALB/c, athymic homozygous nude mice off a BALB/c background, and heterozygous nude/ +litermates. These mice were bred in the vivarium at the Scripps Clinic and Research Foundation or were from Strong Laboratories, Del Mar, Calif. The CBA/N × DBA/2 F_1 mice were a kind gift from Dr. A. Sher, National Institutes of Health, Bethesda, Md. Unless otherwise stated, experiments were done with 1- to 4-mo old male mice.

Mice were injected intraperitoneally with 0.1 ml of virus preparation in MEM. At various times after injection the mice were sacrificed, and spleen and/or peritoneal cells were harvested and pooled, usually from three to five mice. Peritoneal cells were removed by washing the peritoneal cavity with 6 ml of MEM lacking serum but containing 20 U/ml heparin (Lipo-Hepin, Riker Laboratories, Inc., Northbridge, Calif.). Spleens were removed and placed in complete MEM. Spleen cells were prepared by gentle squeezing of spleens with forceps, and by filtration through a nylon mesh to remove debris. Spleen and peritoneal cells were sedimented at 1,000 rpm in an International PR-2 centrifuge for 5 min and resuspended in 0.83% NH_4Cl (in water) for 5 min to remove erythrocytes. They were then pelleted, centrifuge-washed with complete MEM, and resuspended finally in complete MEM containing 0.01 M Hepes (Calbiochem, San Diego, Calif.) buffer (Hepes-MEM) for use in cytotoxicity assays. In some instances, erythrocytes were not removed from spleen cell preparations, but this did not materially affect the results.

Cells will be designated by the time after infection they were harvested, i.e., D0 control = uninfected mice, D3 = 3 days after infection with LCMV, D8 = 8 days after infection with LCMV (T cells). For the purpose of discussion, early cytotoxic cells (ECC) will be those cytotoxic cells induced before the virus specific T-cell response.

Lymphocytotoxicity Assays. Target cells were labeled with sodium chromate (^{51}Cr) (New England Nuclear, Boston, Mass.) at a concentration of 20 μCi/pellet of 5 × 10^5 cells/40 min at 37°C. After labeling, the cells were centrifuge-washed three to five times with 10 ml MEM and resuspended in Hepes-MEM at 1 × 10^5 cells/ml. The cells were then dispensed in 100-μl portions

into wells of microtiter plates (Bioquest, BBL & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md. 3040). Effector spleen or peritoneal cells at various effector to target multiplicities (200:1, 100:1, 50:1, 25:1, 12.5:1) were added in 100-μl quantities to the labeled targets. Each test sample was plated in quintuplicate. For a spontaneous lysis control, 100 μl Hepes-MEM was added in place of effector cells. For a 100% lysis control, 100 μl of 1% Nonidet P-40 was added to cells 15 min before harvest. Microtiter plates were incubated for 14–16 h (unless otherwise stated) on a rocker in a humidified 37°C CO_2 incubator. At the time of harvest, the cells were pelleted by centrifuging the plates at 1,000 rpm for 5 min in the 279 rotor of the International PR-2 centrifuge. From each well a 100-μl sample was carefully pipetted and transferred to a test tube for radioactivity counting. Samples were counted for released ^{51}Cr in a Searle model 1185 automatic gamma counter. Data were processed with a Hewlett-Packard 9825A computer which calculated standard deviations and standard errors for each set of data. Data are expressed as:

$$\text{percent specific } ^{51}Cr \text{ release} = \frac{(\text{cpm test sample} - \text{cpm MEM control})}{(\text{cpm NP-40 control} - \text{cpm MEM control})} \times 100.$$

All data presented in this paper were from replica samples with standard deviations of < 10% of the mean. Background ^{51}Cr release from L cells in 16-h assays was usually about 25–30%.

Sera and Reagents. Antibody to theta antigen was a gift from Dr. Robert Blandon, Canberra, Australia. Rabbit antibody to mouse immunoglobulin (Ig) was a gift from Dr. Michael Oldstone, Scripps Clinic and Research Foundation. An Fab_2 preparation directed against mouse immunoglobulin was a gift from Dr. Steven Russell from IgG of a goat immunized with mouse Ig (IgG and IgM). Guinea pig anti-LCMV serum and guinea pig Fab directed against LCMV were prepared as described previously (27, 31). Guinea pig serum was harvested and frozen at −70°C to be used as a complement source. It was absorbed against spleen leukocytes before use to remove antibodies directed against mouse cells.

Glass and Nylon Wool Columns. Lymphocytes were separated on glass wool and nylon wool (32) columns as described.

X-Irradiation of Mice. C3H/St mice were subjected to irradiation with 1,000 R cesium-137, using a Gamma Cell 40, Atomic Energy of Canada, Ltd. Mice were infected with LCMV immediately after radiation and fed water containing antibiotics (oxytetracycline hydrochloride [Pura-Mycin], Ralston Purina Co., St. Louis, Mo.).

Strontium-89 Treatment of Mice. 6 wk old C3H/St mice were injected intraperitoneally with 0.1 ml of strontium-89 (Oak Ridge National Labs, Oak Ridge, Tenn.) containing 100 μCi. After 4 wk the mice received a similar dose of Sr^{89}. 1–8 wk later mice were infected with LCMV and tested for cytotoxic cell activity. Mice were given water containing antibiotics after Sr^{89} administration.

Interferon Production and Assay. Mouse interferon was prepared by exposing monolayers of L cells to NDV inactivated with ultraviolet light (UV). The culture fluid was harvested 36 h postinfection, adjusted to pH 2 for 1 h at room temperature, and then readjusted to pH 7. Interferon was assayed by the 50% plaque reduction assay of Wagner (33), using L cells as the targets and 30–60 plaque-forming units of VSV as the challenge virus. Briefly, L-cell monolayers were exposed to dilutions of culture fluid of 20% mouse organ suspensions in MEM for 16–24 h. The plates were then washed with MEM and challenged with VSV. Plaques were counted 2 days later, and the interferon titer in units was calculated as the reciprocal of the dilution of interferon resulting in a 50% plaque inhibition.

Results

Kinetics of ECC Production and Activity. After intraperitoneal injection of the Armstrong strain of LCMV into 6- to 10-wk old C3H/St male mice, cells cytotoxic to cultured fibroblasts appeared in the spleen and peritoneum as early as 1 day postinfection (Table I). These we refer to as ECC. ECC activity was comparable in spleen and peritoneal cell populations on a cell to cell basis. Uninfected L cells and L (LCMV) cells were killed with equal efficiency during the first 4 days after infection, with peak ECC activity occurring 3 days postinfection. On days 6–8 postinfection the L(LCMV) cells were killed at much

TABLE I

Time of Appearance of Cytotoxic Cells in Spleen and Peritoneum

Effector cell	Day postinfection	^{51}Cr Release from target cells		
		L	L (LCMV)	F-9
			%	
Spleen cells	0 (Control)	2	−1	7
100:1*	1	7	3	12
	2	20	19	33
	3	34	28	56
	4	19	22	42
	5	21	31	35
	6	12	47	41
	7	14	81	ND
	8	7	83	ND
Peritoneal cells	0 (Control)	0	−3	−1
50:1*	1	2	5	21
	2	19	17	36
	3	18	17	40
	4	13	10	49
	5	16	15	56

Spleen cells and peritoneal cells from LCMV-infected C3H/St mice were tested for cytotoxic activity against various targets at different days postinfection of the mice.

* Effector: target ratio.

greater efficiency than the uninfected cells (Table I), correlating with the advent of the well described virus-specific cytotoxic T-cell response. F9 cells, derived from an H-2b mouse and expressing no H-2 antigens (30) were killed in a pattern resembling the uninfected L cells. ECC were also generated after intravenous injection of C3H/St mice with 10⁵-10⁶ plaque-forming units (PFU) of the WE strain of LCMV. Under these conditions the peak in ECC activity occurred on the 4th-day postinfection. No ECC were produced by injection of mice with the culture fluid from L cells (used to grow the virus) or with preparations of L-cell-propagated LCMV that had been inactivated by ultraviolet irradiation. This suggested that LCMV replication was required for the ECC response under conditions of infection with 2 × 10⁴ PFU.

Dose-response curves of the day 3 ECC revealed a linear relationship between the concentration of effector cells and resulting cytotoxicity. This indicated that cells were killed in a single hit kinetic pattern. The medium from ECC cultured for 2-24 h was not cytotoxic to L cells. Cytotoxicity was detected as early as 1 h after mixing populations of effector and target cells (Fig. 1). On L-cell targets most killing occurred within the first 4 h after admixture.

Characterization of ECC

ADHERENCE AND PHAGOCYTIC PROPERTIES. We reported earlier that when peritoneal ECC were exposed to plastic surfaces for 2-4 h at 37°C, most ECC activity was associated with cells removed from the monolayer by gentle
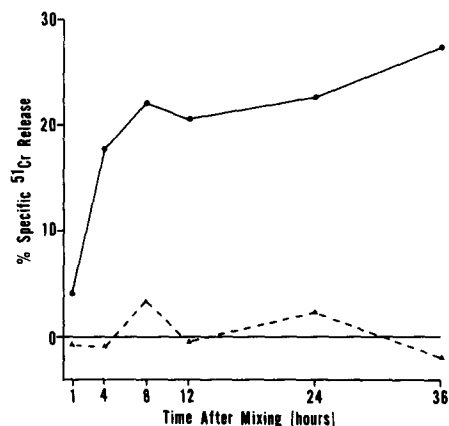
FIG. 1. Time-course of D3 cell cytolytic activity. Spleen leukocytes from C3H/St mice infected 3 days previously with LCMV were tested for their ability to kill L cells at different times after admixture. Leukocytes from uninfected mice served as controls. D3 effector cells: ●—●—● Control effector cells: ▲----▲----▲

washing (16). Here we report that spleen ECC also are not adherent to plastic with greater than 80% of the cytotoxic activity associated with the nonadherent fractions of spleen cells exposed to plastic surfaces for 2 h at 37°C. Addition of the culture fluid from the nonadherent cells to the adherent cells did not enhance the cytotoxic activity of the adherent cells. Passage of day 3 spleen cells through glass wool columns reduced the polymorphonuclear leukocyte and macrophage contamination to less than 3% as detected histologically, and it reduced the phagocytic cell population to less than 2%, as detected by zymosan uptake. Under those conditions the cytotoxic cell activity was completely recovered from the glass wool-depleted cells (Table II).

Spleen cells were also depleted of phagocytic cells by iron filings and a magnet. This technique reduced the phagocytic cell level to less than 2% of the cell population. No cytotoxic cell activity was removed by this treatment (Table II).

Spleen cell preparations were sedimented in Ficoll-Hypaque gradients to pellet PML from the cell preparation. Cells taken from the nonsedimented fraction possessed more cytotoxic activity on a cell to cell basis than untreated cells. We conclude from these experiments that the ECC is nonadherent and nonphagocytic. It is neither a macrophage nor a PML and therefore probably belongs to the lymphocyte series.

SURFACE ANTIGENS. T cells were depleted from spleen cell populations by antibody to theta antigen and guinea pig complement. During these tests this treatment reduced cytotoxic T-cell activity by 78–95% but did not significantly reduce radioactive thymidine uptake of treated lymphocyte preparations stimulated with the specific B-cell mitogens lipopolysaccharide and lipid A protein (34). In several experiments the ECC activity on a cell to cell basis was not significantly affected by this treatment (Table II), suggesting that the ECC is not a T cell.

B cells were depleted from spleen cell populations by antibody to mouse immunoglobulin and complement. This treatment eliminated at least 85% of

TABLE II

Characterization of ECC

Treatment	Efficacy	Specific ^{51}Cr release from L cells	
		Treated	Untreated control
		%	
Glass wool (100:1)	<2% Phagocytes <3% Macrophages and PML	16	16 (Untreated)
Iron filings + magnet (100:1)	<2% Phagocytes	34	36 (Sham depleted)
Antibody to θ + C′ (100:1)	>80% Reduction in T-cell activity No effect on B cells	26	21 (C′ Treated)
Antibody to Ig + C′ (50:1)	No effect on T cells <7% B Cells	8.2	9.3 (C′ Treated)
Nylon wool (50:1)	<2% Macrophages and PML <15% B Cells <11% Fc Receptor + cells	19	27 (Incubated at 37°C for duration of column)
Incubation at 37°C, 16 h (100:1)	No reduction of T-cell activity	−2.4	16 (Tested before incubation)
Trypsin (0.5%, 40 M, 37°C) (200:1)	55% Reduction of T-cell activity	37	36 (Sham treated)
Trypsin (2.5%, 40 M, 37°C) (200:1)	>85% Reduction of T-cell activity	5.5	36 (Sham treated)
EDTA (6 mM in assay well) (100:1)	>85% Reduction of T-cell activity	−0.9	34 (PBS Treated)
Antibody to LCMV + C′ (100:1)	Eliminates >90% lymphocyte infective centers	20	22 (C′ Treated)

Spleen leukocytes taken from C3H/St mice infected with LCMV 3 days earlier were treated as stated and tested for cytotoxic activity on L-cell targets. The efficacy column describes pertinent results for each treatment. Phagocytic cells were quantitated by zymosan uptake. Macrophages and PML were identified by histological staining. T-cell activity was measured by examining effects of treatment on LCMV-specific cytotoxic T cells taken from mice 8 days postinfection. B cells were identified by immunofluorescent staining with antibody to mouse Ig. Infective centers were measured on LCMV plaque assay plates. A brief description of the controls for each treatment is listed in the untreated control column.

* Effector to target ratio.

the B cells as judged by failure to respond to B-cell mitogens, but removed neither the LCMV-specific cytotoxic T-cell activity nor the response of the cultures to concanavalin A, a T-cell mitogen (35). This treatment did not remove the ECC activity (Table II). Passage of spleen cells through nylon wool columns removed 85% of the surface Ig-positive cells (monitored by immunofluorescence) and 90% of the Fc receptor-bearing cells (as shown by rosetting technique). In four experiments this procedure only reduced the ECC activity by an average of 37% (Table II). These data suggested that the ECC was not a B cell.

Physical and Chemical Properties

HEAT SENSITIVITY. The day 3 ECC were highly sensitive to heat inactivation at 37°C. Fig. 1 demonstrates that most cytotoxic activity occurred within 4
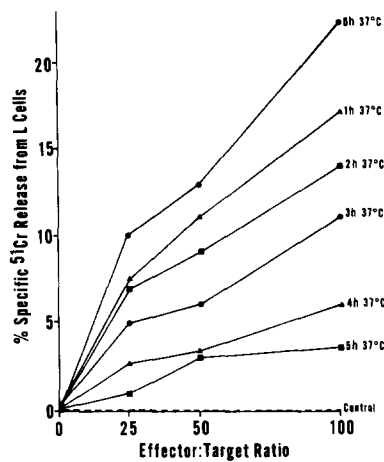
FIG. 2. Heat (37°C) sensitivity of D3 cells. Spleen leukocytes from C3H/St mice 3 days after infection with LCMV were suspended at 2×10^7 cells/ml in MEM and incubated either on ice or at 37°C in a humidified CO_2 incubator. All samples were incubated 5 h with different ratios of incubation at 37°C vs. 4°C. At the end of the incubation the cells were tested for cytotoxicity against L cells.

h after mixing effector and target cells. This was probably a function of ECC lability at 37°C, as greater then 80% of the ECC activity was removed by incubation at 37°C for 5 h before exposure to target cells (Fig. 2), and the activity was totally depleted after incubation overnight (Table II). In contrast, T-cell activity was not reduced at all even after incubation at 37°C for 16 h.

TRYPSIN SENSITIVITY. ECC activity was resistant to treatment with 0.5% trypsin at 37°C for 40 min; in contrast, this treatment lowered the cytolytic T-cell activity (Table II) by 55%. Trypsin at a concentration of 2.5% did inactivate ECC (Table II).

EDTA SENSITIVITY. Incorporation of 6 mM EDTA into the microtiter wells reduced the lytic activity of both the ECC and the T cells by greater than 85% (Table II).

Virological Properties. Treatment of ECC with antibody to LCMV plus complement did not affect the cytotoxic activity, indicating that the ECC were not actively producing LCMV antigens on the cell surface (Table II). This treatment removed more than 90% of LCMV-infected lymphocytes scoring as plaques in a virus infectivity assay (M. Doyle, personal communication). Incorporation of Fab directed against LCMV antigens in the assay wells also did not inhibit cytotoxic activity (not shown).

Failure to Arm Control Lymphocytes. The ECC killed a variety of cell targets (syngeneic, allogeneic, and xenogeneic) in the absence of added antibody to those cells and in the absence of mouse serum (16). ECC also killed cells when fetal bovine serum was not included in the cytotoxicity assay wells. Further, ECC activity was not inhibited by depletion of B cells from the population with anti-Ig + C' (Table II). These experiments suggested that antibody was not required for cell killing and that the killing was probably not due to K cells, cytolytic antibody-dependent lymphocytes described mostly in human systems (36). Yet the possibility existed that K cells in the population

could be armed with antibody in low enough quantities to escape immune cytolysis by antibody to Ig plus complement. Such antibody could be produced by B cells polyclonally stimulated by the LCMV infection. To test this hypothesis we first determined whether serum isolated from day 3 mice could arm spleen cells from control mice to kill L cells. At twofold serum dilutions of 1:4 (25% in assay MEM) to 1:128 there was no effect on the killing of L cells by day 3 ECC or on the lack of cell killing by control spleen cells. In a second type of experiment, day 2 and day 3 spleen cells were isolated from infected mice and then incubated overnight at 37°C. This treatment eliminated ECC activity but should not have eliminated B-cell activity. This unwashed preparation, containing active B cells and their secreted antibody, was exposed to L cells in the presence or absence of fresh control spleen cells, which should have intact K cells. No lysis of L-cell targets was observed. Finally, day 2 and day 3 cells were heat inactivated at 37°C overnight and then incubated with L cells for 24 h. Control spleen cells were added to the treated L-cell preparations and again no killing was observed. The results of these experiments are inconsistent with the hypothesis that B cells have armed K cells with antibody to the cell targets. We have also failed to block ECC-mediated lysis of L cells with Fab_2 fragments directed against mouse Ig in preliminary experiments, but it is not certain that these Fab_2 fragments block cytotoxicity induced by all the different antibody classes.

Effect of Irradiation on ECC Induction

X-IRRADIATION. Mice subjected to 1,000 rads of X-irradiation were infected with LCMV immediately thereafter and later tested for ECC and cytolytic T-cell activity. Table III shows that, whereas these mice produced no detectable cytolytic T-cell activity, they did elicit a reduced yet significant ECC response. In contrast, cells 7 days postinfection did not kill LCMV-infected targets, indicating a complete abrogation of virus-specific T-cell activity.

^{89}SR IRRADIATION. It has been reported that NK cell activity in mice is sensitive to treatment in vivo with ^{89}Sr, a calcium analogue which deposits in bone and kills bone marrow cells (37). In our system C3H/St mice showed a depressed ECC response to LCMV infection 6–8 wk after the second injection with 100 μCi ^{89}Sr (Table IV). Treatment of mice with ^{89}Sr did not however, significantly impair the development of the virus-specific cytotoxic T-cell response.

Induction of ECC Activity in Various Strains of Mice. The induction of cells cytotoxic to uninfected L cells occurred in all strains of mice tested after infection with LCMV (Table V). No correlations could be made between induction of cell cytotoxicity and H-2 type. T-cell-deficient nude mice produced levels of ECC comparable to or greater than virus-infected heterozygous T-cell-sufficient controls. CBA/N × DBA/2 male mice, which have a sex linked deficiency in the B-lymphocyte response to thymus-independent antigens (38), produced levels of ECC comparable to or higher than their relatively normal female litter mates. C3H/HeJ mice, known to be poor responders to certain B-cell mitogens (39), were among the higher ECC producers of the strains tested.

Role of Age and Sex. Both male and female mice produced comparable levels of ECC after LCMV challenge. The highest levels of ECC were induced in

TABLE III

Effect of X-Irradiation on ECC and T-Cell Induction

	Specific ⁵¹Cr release	
	Exp. 1 (L cells)	Exp. 2 (L cells)
	%	
Control	-1.8	-2.1
D3	25*	45*
X-Ray control	-1.2	ND
X-Ray D3	10*	11*
	Exp. 1 (L (LCMV) cells)	Exp. 2 (L (LCMV) cells)
Control	6.5	-1.6
D7	81*	81*
X-Ray D7	0.03	-1.5

C3H/St mice were irradiated with 1000 rads as described in Materials and Methods and immediately thereafter infected with LCMV. In all determinations SE < 5% of mean.

* Statistically significant elevation in cytotoxic activity.

TABLE IV

Depletion of ECC but not T-Cell Activity by Treatment of Mice with ⁸⁹Sr

	No.	Specific ⁵¹Cr release from matched pairs	
		⁸⁹Sr-Treated	Untreated
		%	
D3 Cells on L-cell targets	1	20	45
	2	-0.9	23
	3	4.9	25
	4	4.7	21
	5	32	37
	6	9.4	19
	7	16	27
		Average = 12	28 $P < 0.01$
D7 or 8 cells on L (LCMV)-cell targets	1	70	56
	2	40	56
	3	74	81
		Average = 61	64 P = NS

C3H/St mice were injected twice with 100 μCi ⁸⁹Sr as described in Materials and Methods. 4–10 wk after the final inoculation treated mice and age-matched, antibiotic-fed controls were injected with LCMV and tested for ECC 3 days postinfection and T cells 7–8 days postinfection. Numbers represent data from individual mice. Effector: target ratios were 100 for D3 cells and 50 for D7–8 cells.

5- to 10-wk old mice (Table VI). Mice greater than 1 yr of age gave a lesser response. Cytotoxic cell activity was induced in C3H/St mice infected less than 24 h after birth. The levels of ECC 3 days after infecting newborns were low but significant. The C3H/St mice infected at birth did not develop a measurable virus-specific cytotoxic T-cell response, but characteristically carried virus in their tissues for the remainder of their lives.

Induction of ECC in Immune and Carrier Mice. T and B lymphocytes

TABLE V
Induction of ECC in Different Strains of Mice

Strain	Mouse H2	Specific ^{51}Cr release from L cells		
		Unchallenged	Challenged	(Day)*
			%	
AKR	k	0.1	12	(2)
BALB/c	d	-0.7	15	(3)
BALB/c Nude	d	-2.0	46	(3)
B10.BR	k	4.0	13	(4)
B10.D2	d	4.0	26	(2)
C57BL/6	b	0.0	36	(3)
CBA/N × DBA2F₁ ♂	k × d	2.7	24.1	(3)
CBA/N × DBA2F₁ ♀	k × d	2.5	15.4	(3)
C3H/HeJ	k	1.6	48	(3)
C3H/St	k	5.3	52	(2)
SWR/J	q	-4.1	12	(3)
NZB × NZWF₁	d × z	5.1	53	(3)

Different strains of mice were examined for cytotoxic cell activity 2–4 days after infection with LCMV and compared to unchallenged controls. L cells were used as targets at an effector to target ratio of 100:1. Unless otherwise designated, male mice 1–4 mo old were tested. The assays were done at several different occasions, so the relative cytotoxic activities from different strains of mice cannot be directly compared. All data in the challenged column represent statistically significant increases above the unchallenged mice.

* Day postinfection.

characteristically proliferate in an anamnestic memory response after secondary exposure to a given antigen. The following experiments were done to determine whether the ECC behaved similarly when exposed to a second antigenic stimulus. 2 mo after intraperitoneal immunization with infectious LCMV or Pichinde virus, unstimulated mice possessed no ECC or cytotoxic T-cell activity. After intraperitoneal challenge of LCMV immune mice with LCMV, a secondary cytotoxic T-cell response developed, as has been described (40). In contrast, secondary challenge of immune mice did not elicit an ECC response (Table VII). These mice were judged capable of making such a response, as infection of LCMV-immune mice with Pichinde virus as well as infection of Pichinde virus-immune mice with LCMV both elicited ECC.

Four C3H/St male mice infected at birth with LCMV and chronically shedding LCMV did not demonstrate cytotoxic spleen cell activity. None of three persistently infected SWR/J mice had cytotoxic spleen cell activity, nor two persistently infected BALB/c mice. However, uncharacterized cytotoxic cell activity was found in the peritoneum of those same BALB/c LCMV-carrier mice.

Role of Interferon in ECC Induction. We questioned by which mechanism LCMV infection induced ECC activity. While ECC activity was detected 1 day

TABLE VI

Influence of Age and Sex on ECC Activity

Age at inocula-tion	Sex	% Specific ⁵¹Cr release from L cells	
		Unchallenged	Challenged
<24 h	Pooled	−2.6	13.3
2 wk	Pooled	−1.1	14.7
5 wk	♂	2.9	45
5 wk	♀	2.9	56
10 wk	♂	2.7	53
10 wk	♀	3.9	34
10 mo	♂	−1.5	22
10 mo	♀	−1.7	22
14 mo	♂	−1.2	13
14 mo	♀	−0.4	17

Spleen leukocytes from C3H/St mice of different ages and sexes were tested for cytotoxic activity on L cells. Challenged mice received an injection of LCMV 3 days before harvest. All data in the challenged column represent statistically significant increases above the unchallenged mice.

TABLE VII

Induction of ECC Cells in Immune Mice

Challenge	Specific ⁵¹Cr release from L cells Mouse type		
	Normal	LCMV Im-mune	Pichinde virus immune
	%		
None	2.3	0.6	2.0
LCMV	36*	0.5	30.0*
Pichinde virus	20*	14.1*	3.2

C3H/St mice were immunized by intraperitoneal inoculation of 2 × 10⁴ PFU LCMV or 10⁵ PFU Pichinde virus. 2 mo later the immune mice and normal mice were challenged with similar doses of LCMV or Pichinde virus. 3 days later spleen leukocytes were tested for cytotoxic activity against L cells at effector: target ratios of 100:1.

* Statistically significant elevation of cytotoxic activity.

postinfection, no virus-related B cell, T cell, or activated macrophage response has been detected before 4 days postinfection (5, 9–13). We and others (41–43) have recently reported that LCMV induces interferon production in mice, and Fig. 3 demonstrates that it is synthesized as early as 1 day postinfection. In C3H/St mice, high levels of interferon were detected the first 3 days postinfection, correlating with maximum ECC activity (Fig. 3). Interferon levels dropped significantly on the 4th-day postinfection, and at this time ECC activity also began its decline. Both interferon and ECC levels were relatively low after the 5th-day postinfection.

Culture fluids rich in interferon were prepared by infecting L cells with UV-inactivated NDV. Intraperitoneal injection of nu/nu or C3H/St mice on 4 successive days with 512 U of interferon in 0.5 ml induced high levels of
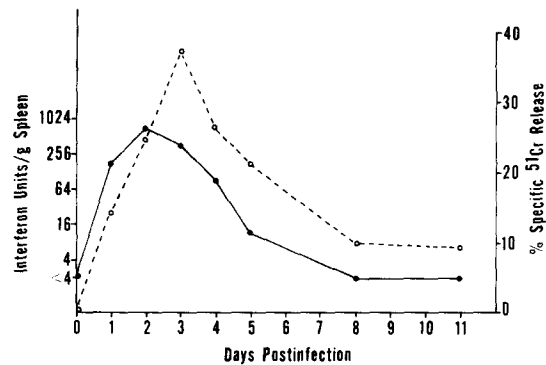
FIG. 3.   Spleen interferon and ECC in C3H/St mice during LCMV infection. C3H/St mice were infected with LCMV and spleens were harvested at different times postinfection. Spleen leukocytes were isolated and tested for cytotoxicity against L cells (data listed are at effector:target ratios of 100). The leukocytes were pooled with the remainder of the spleen, and 20% suspensions were prepared in MEM. After clearing debris by sedimenting at 1,000 g for 5 min, the supernates were tested for interferon activity. Data are from two experiments using two and three mice, respectively, for each time point. Cytotoxicity O----O----O Interferon/g spleen ●—●—●
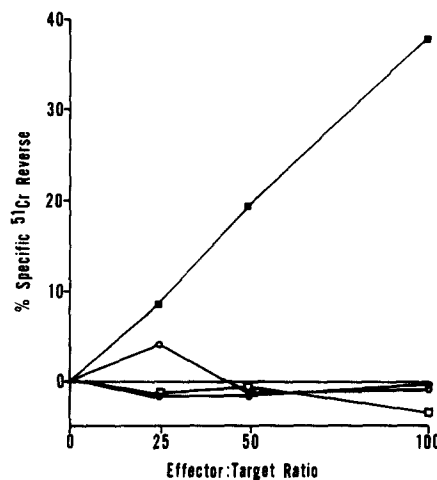

FIG. 4.   Induction of cytotoxic cells by interferon-containing culture fluids. Athymic nude (BALB/c) mice were injected intraperitoneally with pH 2-treated and neutralized L-cell culture fluids containing or lacking interferon (1,012 U/ml). Mice received 0.5-ml injections on 4 successive days. Peritoneal and spleen cells were harvested 3 h after the final injection and tested for cytotoxic activity on L cells. O—O—O, Control spleen cells; □—□—□, control peritoneal cells; ●—●—●, interferon spleen cells; ■—■—■, interferon peritoneal cells.

cytotoxic peritoneal but not spleen cell activity. Fig. 4 shows data derived from nude mice; similar levels of cytotoxic cells were also induced in C3H/St mice. Injections of both the interferon preparation and the culture fluid from uninfected L cells caused a two to fourfold increase in the number of peritoneal cells, but the latter treatment elicited no cytotoxic cell activity (Fig. 4). Plastic

TABLE VIII

Correlation of ECC Activity, Interferon, and Virus Synthesis in Different Strains of Mice

Days postinfec-tion	Strain of mouse			
	C3H/St	C57BL/6	BALB/c nu/+	BALB/c nu/nu
Lysis*	55	36	34	31
3 Interferon‡	160	1,280	640	640
PFU§	3.6	4.6	4.4	4.1
Lysis	23	53	0.5	44
7 Interferon	40	<20	40	1,280
PFU	1.8	<2	1.5	5.0
Lysis	9.5	45	8.7	74
11 Interferon	<20	<20	<20	80
PFU	1.0	<1	1.5	5.3

Mice were infected and spleens treated as described in Fig. 4. In this experiment, two spleens were used per sample, but other experiments revealed similar data. Uninfected control mice of each strain had no detectable ECC, interferon, or PFU.

* Percent ^{51}Cr release from L cells at effector: target ratio of 100.

‡ Interferon units per spleen.

§ Log_{10} PFU per spleen.

adherence studies indicated that virtually all cytotoxic cell activity was associated with the nonadherent fractions.

Several strains of mice were examined to correlate levels of virus, interferon, and ECC in the spleen at different days postinfection (Table VIII). In C3H/St and BALB/c nu/+ mice all three parameters correlated positively with high levels on D3 postinfection and lower levels on D7 and 11. nu/nu mice continued to produce high levels of virus and NK cells, but had reduced interferon activity 11 days postinfection. C57BL/6 mice continued to produce ECC 11 days postinfection even after major reductions in both virus titers and interferon had occurred.

Discussion

This report demonstrates that during a virus infection the host may generate cytotoxic lymphoid cells which are neither T cells, B cells, PML, nor macrophages. These rapidly generated cytotoxic cells (ECC) kill both virus infected and uninfected targets, but the fact that they respond locally to an interferon stimulus suggests a possible mechanism for localizing or regulating the spread of the virus infection. The LCMV-induced ECC did not adhere to plastic or glass wool and were unaffected when phagocytic cells were removed by iron depletion. Therefore, cytotoxic cell activity, demonstrated in the relative absence of PML or macrophages, was likely due to lymphocytes. The ECC are not T cells, as they were induced in thymus-deficient nude mice, and their activity resisted treatment with antibody to theta antigen and complement. ECC are not B cells, as (a) the activity was resistent to treatment with antibody to Ig and complement, (b) most activity passed through nylon wool columns, and (c) activity was induced in mice with hyporesponsive B cells (Table V). We could not

demonstrate a role for antibody, as normal spleen cells could not be armed to kill L-cell targets with serum from infected mice, and ECC activity was not depleted by treatment with anti-Ig plus complement to kill cells bearing cytophilic Ig. The resistance of ECC to trypsin also argues against cell bound antibody. In contrast to T or B cells the ECC did not demonstrate virus specificity and did not anamnestically respond to a secondary LCMV challenge. Relative to the virus-specific T-cell activity, the ECC were significantly more heat (37°C) labile in culture, insensitive to X-irradiation of mice, but sensitive to ^{89}Sr irradiation of mice.

The ECC reported here appear to satisfy the criteria for NK cells as described in other laboratories. The mouse NK cells are nonadherent, nonphagocytic lymphocytes lacking T-cell (theta antigen) and B-cell (surface immunoglobulin) markers (17–22). They are cytotoxic to a number of tumor cell lines, particularly T-cell lymphomas, without apparent antibody mediation. A useful property for identification of NK cells is that their cytolytic activity is destroyed by heating in culture for 5 h at 37°C (19). NK cell cytotoxicity is generally resistant to treatment of mice with X-irradiation (22) at doses which completely abrogate T- and B-cell proliferation. However, treatment of mice with ^{89}Sr reduces NK cell activity, as NK cells are believed to be derived from the bone marrow (37). Thus, with the exception of having additional target specificities, the LCMV-induced ECC have the properties of NK cells.

Rodda and White (44) earlier reported nonspecific cytotoxic cell activity in the peritoneum of mice infected 2–3 days previously with Semliki forest virus. They did not identify the cytotoxic cells as NK cells at that time, but this work inspired us to look for early cytotoxic cells in the LCMV system (16). Recently, MacFarland et al. published an extension of the earlier work, concluding that both Semliki forest virus and Kunjin virus induced cytotoxic cells resembling NK cells (25). The NK cells induced by LCMV have nearly identical properties to the NK cells reported by MacFarland et al. (25), including resistance to 0.5% trypsin, which is a still controversial property of NK cells. Wolfe et al. reported the induction of NK-like cells with the living bacterium, Bacillus Calmette-Guérin (45). With the exception of trypsin sensitivity, their NK cell shares many of the properties of the LCMV-induced NK cells, and we have found trypsin studies to be potentially controversial due to difficulties in controlling autodigestion of the enzyme. Herberman et al. (23) recently reported the induction of cytotoxic cells in nude and normal mice by a number of viral and bacterial agents, as well as tumor cells. Although the cytotoxic cells were not characterized for each inducing agent, they, too, are probably NK cells. Of interest is a paper by Wong et al. (24) suggesting that similar types of cells may be induced in female but not male mice infected with Coxsackie virus.

While much needs to be learned about the induction of NK cells, information has been gathered from this work. This is the first report that X-irradiation does not prevent the induction of NK cells. Since irradiation at 1,000 rads is a high dose and totally blocks T-cell induction (Table III), this experiment suggests that NK cells can be directly activated or recruited without a requirement for cell division. A possible candidate for an activator of NK cells is interferon as L-cell culture fluids rich in interferon induced NK cell activity

(Fig. 4). We and others reported earlier that LCMV induces interferon in mice (41–43) and in this report we correlate the induction of interferon with the induction of the NK cell response. Gidlund et al., have shown that purified interferon as well as interferon inducers stimulate NK cell activity in mice.[2] Our work is compatible with and confirms their findings and supports the concept that the recently described viral, bacterial, and cellular inductions of NK cells may act by a common interferon mechanism. Whether interferon induces NK cell activity directly or does so by causing cells such as macrophages to release chemotactic or lymphostimulatory lymphokines is not clear at this time.

It also is not known if other factors are required for the maintenance of NK cell activity. Clearly in our studies the induction of spleen NK cell activity correlates with induction of interferon as well as PFU synthesis (Table VIII, Fig. 4). However, the continued expression of NK cell activity does not correlate with interferon levels. NK levels in nude mice increased after interferon levels had declined, and C57BL/6 mice continued to produce NK cells for several days after both virus and interferon had fallen to undetectable levels. While the continued expression of NK cells in nude mice may be directly or indirectly related to a deficient T-cell response, it is difficult to postulate an explanation for the C57BL/6 mice at this time.

The induction of NK cells after infection of newborn mice with LCMV is the earliest evidence of a cellular response to this virus infection during the chronic disease. These mice become persistently infected with LCMV for life. They do not develop a detectable cytotoxic T-cell or activated macrophage response, and antibody can usually only be detected weeks later in the form of immune complexes (9). LCMV carrier mice did not maintain high levels of NK cells in their spleens. This correlates with what is known about interferon in these mice, as interferon is produced shortly after newborns are infected (43) but not in long-term carriers (46). Although not investigated in our system, the failure to generate an NK cell response during secondary immunization of adult mice with LCMV may have been due to a poor inteferon response, demonstrated often in other secondary virus infections (47).

Endogenous (unstimulated) NK cells when assayed against sensitive target cells peak in activity between 1 and 2 mo of age of the mouse, but very little activity is seen in cells from newborn animals (19, 48). The LCMV-induced NK cell activity, as tested against a normally insensitive target, also peaked at 5–10 wk of age, but significant activity was induced in newborns. This indicates that inactive NK cells or NK cell precursors do exist in significant quantities in newborns.

The significance of NK cell induction to the progression of a viral infection is unclear at this time, but the activation or recruitment of cytolytic lymphocytes by interferon-producing cells seems a possible mechanism for limiting virus spread. There is strong evidence, however, that NK cells may be important for the immunosurveillance of tumors (22, 49) and related to the cells responsible for rapid rejection in host anti-graft reactions (22). It is obvious that an understanding of the mechanism regulating the induction and suppression of

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[2] Gidlund et al. Manuscript submitted for publication.

such cytotoxic cell activity may shed light on the control of immunosurveillance and contribute to the understanding of acute virus disease.

Summary

Lymphocytic choriomeningitis virus (LCMV) infection of C3H/St, nude (BALB/c background), and other mice induced high levels of natural killer (NK) cell activity in the spleen and peritoneum. L-929 cells were used as targets and were not lysed by spleen or peritoneal cells from uninfected mice. The cytotoxic cells were characterized as NK cells because they were nonadherent, nonphagocytic lymphocytes lacking θ and immunoglobulin antigens on their plasma membranes. Their activity was sensitive to 6 mM EDTA and to heating for 5 h at 37°C, but resisted treatment with 0.5% trypsin. No role for antibody could be demonstrated in these assays. Relative to cytotoxic T-cell activity, the induction of NK cell activity was resistant to X-irradiation of mice with 1,000 rads but was sensitive if mice were first treated with Strontium-89, a bone-seeking isotope.

NK cells were induced by LCMV in all tested strains of mice. In C3H/St mice NK cell activity was detected as early as 1 day and peaked at 3 days postinfection. Maximum activity in C3H/St mice was observed in mice 5–10 wk of age, but significant NK activity was also induced in newborns, which subsequently carried virus in their tissues for the duration of their lives. Older LCMV-carriers did not have detectable spleen NK cell activity.No memory or anamnestic response could be demonstrated for NK cell induction. NK cell activity was not induced by LCMV challenge of LCMV-immune mice, but was induced in those mice by infection with Pichinde virus, a closely related virus.

The advent of NK cell activity correlated with the synthesis of interferon in LCMV-infected mice. Culture fluids lacking virus infectivity but containing interferon induced cytotoxic cell activity in nude and C3H/St mice. These experiments suggest that LCMV induced NK cells via an interferon-dependent mechanism. When studied in several strains of mice, the continued expression of NK cell activity did not seem to directly correlate with spleen interferon levels, suggesting that additional factors may play a role as well in maintaining the activity of the NK cell in vivo.

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