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Cytotoxic Cells Induced During Lymphocytic Choriomeningitis Virus Infection of Mice: Natural Killer Cell Activity in Cultured Spleen Leukocytes Concomitant with T-Cell-Dependent Immune Interferon Production†

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The characteristics and specificities of spleen and peritoneal cytotoxic cells generated during lymphocytic choriomeningitis virus (LCMV) infection of C3H/St mice were examined. Activated natural killer (NK) cell activity was identified in fresh leukocyte populations from the 2nd to 8th days postinfection, whereas virus-specific cytotoxic T-cell activity was detected from the 6th to 14th days. When leukocytes were cultured overnight at 37°C before assay, T-cell activity was still observed, but nonspecific activated NK cell-like cytotoxicity was only detected on the 6th and to a lesser degree the 8th day postinfection. Overnight culture of leukocytes taken earlier in the infection eliminated their NK cell activity. Similar activities were seen with spleen cell, plastic-adherent peritoneal cell, and nonadherent peritoneal cell populations. The virus-specific cytotoxicity observed with adherent peritoneal cells was due to contamination with cytotoxic T cells, as shown by *H-2*-restricted cytotoxicity and sensitivity to anti-theta antibody and complement. The nonspecific cultured day 6 effector cell from either the spleen or peritoneum displayed killing specificities and other physical properties identical to those of activated NK cells, but had sensitivities to anti-theta antibody and complement intermediate between activated day 3 NK cells and cytotoxic T cells. Culture stable NK-like cells were not found in athymic nude mice, suggesting a T-cell-dependent mechanism. Whereas LCMV spleen homogenates contained 10-fold-higher levels of interferon at day 2 than at day 6 postinfection, substantially more (nearly 20-fold) interferon was made in cultures of day 6 cells than day 2 cells. Spleen interferon was predominantly type I, whereas the culture interferon was predominantly type II, as shown by acid lability studies. Significant levels of interferon were produced by nylon-wool-passed day 6 spleen cells, and virtually all interferon production was eliminated by treatment of either day 2 or day 6 cells with antibody to theta antigen and complement, suggesting that T cells produced the interferon in vitro. Furthermore, athymic nude mice had no culture-stable NK cells 6 days postinfection, and spleen cells from them failed to produce significant levels of interferon in vitro. Addition of interferon (type I, fibroblast) to cultured C3H spleen cells reactivated the NK cells in control and D2 spleen cell preparations, but did not affect the already elevated levels of cytotoxicity in day 6 cultures, suggesting that the NK cells in the day 6 culture were already activated. Our results suggest that T cells responding to LCMV infection secrete interferon type II which causes the continued activation of NK cells in culture. The resulting population of activated NK cells therefore appears to be relatively stable in culture and to express more theta antigen because of this T-cell dependence. Although one could mistakenly attribute the nonspecific cytotoxicity observed in these studies to be nonspecific or allospecific cytotoxic T cells or cytotoxic macrophages, more careful examination shows that they are most likely activated NK cells. It may also be inferred from these results that T cells locally responding to viral antigens in vivo may secrete interferon and locally activate NK cells, which may in turn be responsible for some of the T-cell-dependent pathology in LCMV infection.

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Lymphocytic choriomeningitis virus (LCMV) infection of mice results in the induction of natural killer (NK) cell activity early in the infection and the proliferation of virus-specific cytotoxic T cells later in the infection (3, 21, 24, 27). The NK cell has been characterized as a nonadherent, nonphagocytic lymphocyte bearing low concentrations of theta antigen and rapidly losing cytotoxic activity during in vitro culture (8, 9, 11, 21, 24). The NK cell activated during the LCMV infection lyses nearly every continuous cell line as well as certain primary cells (12, 16, 24, 25). Activation of mouse NK cells is mediated by interferon, which is induced by the viral infection (5, 7, 21). Macrophages also are activated during the LCMV infection, and although these activated macrophages readily degrade the bacterium *Listeria monocytogenes*, no evidence has been presented showing that these macrophages can be cytotoxic to nucleated mammalian cells (2).

Both specific and nonspecific cellular responses may play a role in virus infections. Here we present a detailed examination of cytotoxic cells and interferon appearing in the spleen at various times during LCMV infection. We identify a population of interferon-activated NK cells whose properties are such that they may be confused with cytotoxic T cells or macrophages and whose activity appears to be mediated by interferon produced by T cells.

MATERIALS AND METHODS

Viruses. The Armstrong strain of LCMV and the Indiana strain of vesicular stomatitis virus were used in these studies (21). Defective interfering (DI) LCMV was produced in BHK-21/13S cells persistently infected with LCMV (22).

Cells. Cells were propagated either in monolayers in Eagle minimal essential medium (MEM) supplemented with 10% heat (56°C, 30 min)-inactivated fetal bovine serum and antibiotics or in suspension in RPMI medium, similarly supplemented. Monolayer cell lines used were: BHK-21/13S, a baby hamster kidney cell line; B10.Br ($H\text{-}2^k$), a methylcholanthrene-induced tumor cell line from B10.Br mice; C3H-F ($H\text{-}2^k$), a secondary culture prepared from C3H/St mouse embryonic fibroblasts; J774 ($H\text{-}2^d$), a continuous macrophage line; L-929 (L) ($H\text{-}2^k$); a continuous liver fibroblast line; F-9, a teratoma line which expresses no detectable *H-2* antigens (20); HeLa, a human epithelial line; and Vero, a continuous African green monkey kidney cell line. Suspension cultures were: EL-4 ($H\text{-}2^b$), mouse lymphoblastoid line; Raji, a human B-cell line; P815 ($H\text{-}2^d$), a mouse mastocytoma line known to be highly sensitive to macrophage-mediated cytotoxicity (4); YAC-1 ($H\text{-}2^a$), a T-cell lymphoma line known to be highly sensitive to NK cell cytotoxicity (11); and YAC-8, an NK cell-resistant variant adapted in vivo from the YAC-1 line (R. Kiessling and M. Hansson, unpublished data).

Mice. C3H/St normal mice and BALB/c WEHI and CBA athymic nude/nude mice were bred in the vivarium of the Scripps Clinic. Male mice 4 to 12 weeks of age were used for the experiments, with age-matched mice in individual experiments. Mice were infected intraperitoneally with 2×10^4 plaque-forming units of LCMV and examined every 2nd day until the 14th day postinfection (21). The levels of NK cell activity varied between experiments, as expected, because 4-week-old mice have higher NK cell levels than do 12-week-old mice (9, 11, 21).

Preparation of effector cells. Spleens were excised from sacrificed animals, and splenocytes were removed from the capsule by mincing with forceps. Peritoneal cells were isolated by lavage of the mouse peritoneal cavity with MEM containing 20 U of heparin per ml (25). Leukocytes were prepared by exposure to 0.83% NH_4Cl, which lysed the erythrocytes. For overnight incubations, spleen leukocytes were cultured at 1×10^7 to 2×10^7 cells per ml in MEM containing 0.01 M HEPES (*N*-2-hydroxylethylpiperazine-*N'*-2-ethanesulfonic acid; Calbiochem, La Jolla, Calif.) buffer. Peritoneal leukocytes were incubated overnight in various concentrations (5×10^4 to 5×10^5) in flat-bottom microtiter wells. After vigorous washing of the resulting monolayers after 16 h of incubation, the adherent cells were further identified as macrophages by uptake of zymosan and by morphology from staining with Wright stain (4). All assays and incubations of spleen or peritoneal leukocytes were done in HEPES-MEM medium.

Microcytotoxicity assays. The microcytotoxicity assay was that described previously (21). Target cells (1×10^4) labeled with sodium ^{51}Cr (New England Nuclear Corp., Cambridge, Mass.) were added to each well. Assays were run for 16 to 20 h unless otherwise stated. A modification of this technique was used for peritoneal cell targets. Radiolabeled peritoneal cells were dispersed into flat-bottomed 15-mm plastic wells, washed to remove nonadherent cells, and used in 14-h cytotoxicity assays as described (25). Results listed are from quadruplicate samples with standard errors less than 10% of the mean.

Interferon assays. Crude mouse fibroblast interferon was purchased from Calbiochem. Our assay for interferon was a microassay using L-929 cells as the targets and vesicular stomatitis virus as the challenge virus. Interferon titers in this assay agreed with National Institutes of Health standards ± a factor of 2.

RESULTS

Cytotoxic spleen cells cultured from mice infected with LCMV. Spleen leukocytes from mice infected with LCMV were tested for cytotoxicity directly or after overnight culture at 10^7 spleen cells per ml. Fresh spleen cell cytotoxicity against uninfected L cells could be detected during the first 8 days postinfection but not thereafter (Fig. 1). When spleen cells were cultured overnight at 37°C before use in the assay, no cytotoxicity was observed against L cells for the first 4 days postinfection, but significant activity was noted later in the infection, peaking at the

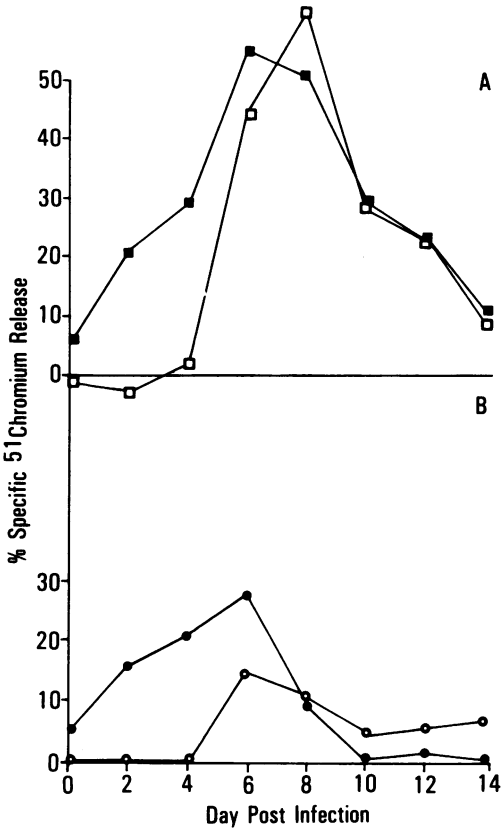

FIG. 1. *Cytotoxicity of LCMV-activated spleen cells. (A) L(LCMV) cell target cells: (■) fresh spleen cells; (□) spleen cells incubated overnight. (B) L cell target cells: (●) fresh spleen cells; (○) spleen cells incubated overnight.*

6th day. This early (day 1 to 4) cytotoxic cell activity has been identified as NK cell mediated, and the activity of these NK cells is characteristically lost in culture by incubation at 37°C for as short a time as 5 h (21, 24). Thus, the cytotoxic cell appearing on day 6 failed to show the unstable properties of cultured NK cells.

L cells infected with LCMV were also used as targets in order to survey the virus-specific cytotoxic T-cell response under these conditions of infection (Fig. 1). For the first 4 days postinfection, the killing against L(LCMV) targets was similar to that against uninfected L-cell targets for both fresh and cultured spleen cells. Thereafter, a high level of culture-stable virus-specific cytotoxicity was noted on the 6th to 8th days postinfection, with a gradual tapering off on succeeding days. In other experiments we have shown the peak in this virus-specific killing to usually occur on the 7th day postinfection. This virus-specific lysis of L cells is reported to be mediated by *H-2*-restricted cytotoxic T cells, which are relatively stable in culture (21, 24, 27).

Cytotoxic peritoneal cells from mice infected with LCMV. The intraperitoneal infection of C3H/St mice with LCMV resulted in a marked increase in the number and size of peritoneal cells. Whereas only small increases in cell number were observed during the first 4 days postinfection, approximately 4- and 25-fold increases occurred on the 6th and 8th days postinfection, respectively (Table 1). The macrophage population similarly expanded, and there was, as found by morphological examination, an increase in size of the macrophages. Adherent macrophages on days 10, 12, and 14 differed in appearance from macrophages earlier in the infection in that they were heavily vacuolated. LCMV antigens could be detected by immunofluorescence antibody technique in the cytoplasms of adherent peritoneal cells during the first 6 days of infection. Thereafter they could not be detected. When peritoneal cells were used as cytotoxic effectors, a sharp peak in the killing of uninfected L or F-9 targets was produced by overnight cultured cells taken from mice 6 days postinfection (Fig. 2). Little killing of uninfected target cells was noted before or after that time period. Activity in the cultured nonadherent peritoneal cell fraction was significantly greater than in the adherent fraction (note the different effector-to-target ratios). Adherent peritoneal cells lysed LCMV-infected L-cell targets at higher levels than uninfected targets, and lysis against virus-infected L cells was also produced by adherent peritoneal cells taken from mice 8 to 14 days postinfection. This apparent virus-specific lysis peaked on the 8th day postinfection. Although histological examination indicated that the monolayers on day 8 postinfection were populated by greater than 85% macro-

TABLE 1. *Peritoneal cells from LCMV-infected mice*

Day postinfection	Avg cell no.[a] (no. of mice tested)	% Adherent cells[b] with LCMV antigens	Macrophage morphology
0	2.7×10^6 (11)	<1	Normal
2	2.5×10^6 (3)	16	Normal
4	3.8×10^6 (6)	13	Normal
6	1.2×10^7 (6)	11	Enlarged[c]
8	7.1×10^7 (10)	<1	Enlarged
10	1.1×10^7 (6)	<1	Enlarged, vacuolated
12	8.2×10^6 (6)	<1	Enlarged, vacuolated
14	6.4×10^6 (5)	<1	Enlarged, vacuolated

[a] Composite data derived from three separate experiments.
[b] Adherent peritoneal cell monolayers were fixed in ether-alcohol and stained with a guinea pig anti-LCMV antibody conjugated to fluorescein isothiocyanate, as described (23).
[c] Enlarged refers to a noticeable (twofold) increase in the diameter of adherent macrophages stained with Wright stain.

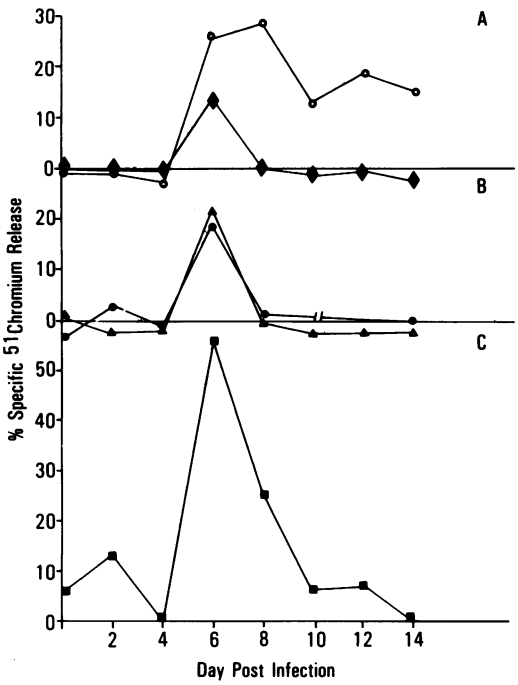

FIG. 2. *Cytotoxicity of LCMV-activated peritoneal cells. Peritoneal cells were cultured overnight and separated into plastic-adherent (A and B) and nonadherent (C) fractions before testing against target cells. (A)* (○) *L(LCMV) cells;* (◆) *uninfected L cells. (B)* (△) *F-9 cells infected with LCMV;* (●) *uninfected F-9 cells. (C)* (■) *Uninfected F-9 cells. Effector-to-target cell ratios for adherent peritoneal cell effectors were 50:1 (A and B) and for nonadherent effectors were 25:1 (C).*

phages, several experiments clearly showed that the virus-specific killing was due to contaminating cytotoxic T cells, similar to those in the spleen cell system. First, the virus-specific peritoneal cell killing appeared *H-2* restricted, as shown for cytotoxic T cells by Zinkernagel and Doherty (27). Table 2 demonstrates killing only against virus-infected targets expressing $H\text{-}2^k$ antigens. Second, antibody to theta antigen plus complement markedly reduced the virus-specific killing, whereas exposure of cultured adherent peritoneal cells to 125 μg of silica per ml for 4 h did not significantly affect the cytotoxicity (data not shown). Third, when 10^6 day 8 spleen (T) cells were added to normal macrophage monolayers (5×10^5/microtiter well) and subsequently washed 2 h later, significantly more T-cell activity remained associated with macrophage monolayers than with control plastic surfaces (e.g., macrophage monolayer, 32% lysis; plastic surface, 5.1%; unwashed spleen cells, 47%).

Characterization of cultured day 6 cells from spleen cells. Figures 1 and 2 revealed the presence of cytotoxic cells which behaved differently from NK cells, which are reported unstable in culture, and from T cells, which classically should not lyse uninfected syngeneic cells. The following series of experiments was done to characterize the cultured day 6 cells isolated from spleens.

(i) Target cell specificity. The cultured day 6 cells could lyse most tested types of target cells, whether they be syngeneic, allogeneic, or xenogeneic to the effector cell (Table 3). Fresh day 6 cells lysed the same targets (data not shown). Lysis was even noted against cultures of C3H/St fetal fibroblasts, against SWR/J adherent peritoneal cells, and against F-9 cells, which express no detectable *H-2* antigens (20). This pattern of lysis correlated with that of activated NK cells. Furthermore, whereas cytotoxic activity was high against YAC-1 cells, known to be very sensitive to NK cell-mediated lysis, the activity was very low against YAC-8 cells, an NK-resistant variant which is sensitive to lysis by alloimmune cytotoxic T cells (R. Kiessling and M. Hansson, personal communication). Similarly, cultured day 6 peritoneal cell killing was observed against all tested targets, i.e., L, B10.Br, J774, F-9, P815, and YAC-1 cells. Cultured peritoneal cells lysed YAC-1 targets at levels two- to threefold higher than levels at which they lysed P815 targets, known to be particularly sensitive to macrophage-mediated cytotoxicity.

It could be argued that on the 6th day postinfection mice are generating specific antiviral immune effector mechanisms such as antibody or T cells at a time when the virus is not yet completely eradicated. Therefore, virus from the effector cell population could theoretically infect the targets and render them sensitive to virus-specific antibody-dependent complement-mediated cytotoxicity or cytotoxic T-cell mecha-

TABLE 2. *H-2-restricted virus-specific killing elicited by adherent peritoneal cells*[a]

Target	*H-2*	LCMV infection	% Specific ^{51}Cr release
L-929	*k*	No	−0.85
L-929	*k*	Yes	32.
B10.Br	*k*	No	−2.1
B10.Br	*k*	Yes	32.
J774	*d*	Yes	3.6
F-9	None	Yes	−1.0

[a] Adherent peritoneal cells taken from mice 8 days postinfection and cultured overnight were tested for cytotoxic activity against various target cells, infected or uninfected with LCMV, at effector-to-target ratios of 50:1.

TABLE 3. *Target cell specificity of incubated day 6 spleen cells*

Target cell	Effector/ target cell ratio	*H-2*	% Specific ^{51}Cr release			
			Expt 1, fresh cells		Expt 2, cultured cells	
			Day 3	Control	Day 6	Control
L	100	*k*	33	2.2	23	0.0
L(LCMV)	100	*k*	27	−2.1	72	−1.0
C3H fibroblast	100	*k*	30	ND[a]	16	−4.0
F-9	100	None	75	11	43	−5.0
SWR/J peritoneal	50	*q*	24	−3.0	35	6.9
Raji	100	Xenogeneic	25	1.5	23	2.3
YAC-1	25	*a*	46	18	38	5.6
EL-4	100	*b*	44	17	33	0.2
Vero	100	Xenogeneic	40	11	21	−1.9
YAC-8	25	*a*	2.0	−2.4	−1.7	−5.2

[a] Not determined.

nisms. To control for this, we infected F-9 cells with DI LCMV before the cytotoxicity assay. Cells infected with DI LCMV before standard virus challenge do not synthesize LCMV proteins and are completely resistant to virus-specific cytotoxic effector mechanisms (22, 23). F-9 cells thus treated were as susceptible to lysis by the cultured day 6 cells as were uninfected F-9 cells, indicating that a virus-specific effector to target interaction was probably not involved.

To discriminate between a "nonspecific" NK cell-like killing mechanism and an allospecific cytotoxic T-cell killing associated with polyclonal activation, unlabeled YAC-1 cells were added to assay wells in cold target competition assays. The YAC-1 (*H-2*^{a}) cells competed effectively against the killing of uninfected YAC-1 and MC57G (*H-2*^{b}) cell targets, but not against LCMV-infected L cells (Fig. 3). In contrast, YAC-8 cells, which bind NK cells poorly but have normal levels of *H-2* antigens, competed poorly in this system. Since the YAC-1 (*H-2*^{a}) targets blocked the killing of *H-2*-unrelated (MC57G) targets, it can be concluded that a mechanism other than allospecific T-cell killing was involved. The failure of YAC-1 cells to block LCMV-specific T-cell killing shows that blocking is not a simple function of steric hindrance. LCMV-infected L cells did, however, effectively block the virus-specific killing. Other experiments indicated that YAC-1 but not YAC-8 cells block the killing of uninfected L cells (data not shown).

(ii) Physical properties. The physical properties of the nonspecifically cytotoxic cultured day 6 cells were compared with those of the cytotoxic T cells in the same cell preparation and to fresh day 3 cells, whose cytotoxic activity appeared to be exclusively NK-cell mediated. The nonspecific cultured day 6 cells were more sensitive to treatment with antibody to theta antigen and complement than were fresh day 3

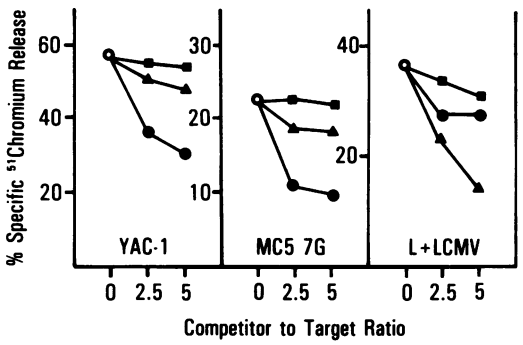

FIG. 3. *Cold target competition by YAC-1 and YAC-8 cells. Day 6 cells at an effector-to-labeled-target ratio of 50:1 were exposed to targets for 5 h in the presence of unlabeled competition target cells:* (●) *YAC-1;* (■) *YAC-8;* (△) *L plus LCMV. In this experiment the cytotoxicity against uninfected L cells was <5%, indicating that the L plus LCMV targets were being lysed predominantly by cytotoxic T cells.*

NK cells (Table 4), though the sensitivity of LCMV-specific cytotoxic T cells in the day 6 preparations to this treatment was much greater. Table 2 lists a representative experiment on anti theta sensitivity. These differences in susceptibility were quite reproducible. In five experiments the fresh day 3 cell activity was reduced an average of 34 ± 13%, in four experiments the day 6 cell activity was reduced 60 ± 33%, whereas in three experiments the specific T-cell killing was reduced 89 ± 9.7% (some of the residual killing of L(LCMV) targets is probably due to NK and not T cells). Neither cell type was reduced in activity by depletion of phagocytes with iron filings and a magnet, indicating that phagocytic macrophages were not the effectors. The effector cells passed through columns of glass wool and nylon wool, ruling out macrophages and polymorphonuclear leukocytes as effectors. Slight reductions were noted

in the activities of the day 3 NK and the nonspecific day 6 cells after passage through these columns, whereas the T-cell activity was often enriched. Thus, with the exception of stability in culture and a slightly enhanced sensitivity to anti-theta serum, the day 6 cells in these experiments behaved like NK cells. The nonspecific adherent and nonadherent peritoneal cell cytotoxicity had sensitivity to anti-theta serum and complement similar to that of cultured day 6 spleen cells (data not shown) if peritoneal cells were treated before the overnight culture. Further, adherent peritoneal cell cytotoxic activity was not reduced by treatment with 125 μg of silica per ml.

TABLE 4. *Physical properties of fresh day 3 and cultured day 6 cells*[a]

Treatment	% Specific ^{51}Cr release		
	Fresh day 3 L cells	Cultured day 6 cells	
		F9	L(LCMV)
Control	52	26	48
Complement	49	26	43
Anti-theta + complement	31	7.0	−5.0
Control	36	23	58
Iron filings + magnet	34	21	59
Control	23	43	72
Glass-wool	19	32	81
Control	27	17	57
Nylon-wool	19	7	58

[a] Fresh day 3 or cultured day 6 spleen cells were treated as described previously (2) and tested for cytotoxicity after treatment. Effector/target cell ratios: fresh day 3, 100:1; cultured day 6 on F-9 cells, 200:1; fresh day 6 on L(LCMV) cells, 50:1. The cultured day 6 cell killing against the two targets was done with the same effector preparations. In each experiment three effector-to-target cell ratios were tested, but only one is listed.

(iii) Absence from nude mice. In a series of experiments, when BALB/c nude or CBA nude mouse spleen and peritoneal cells 6 days postinfection were incubated overnight and tested for cytotoxicity against L or F-9 targets, cytotoxicity was usually nonexistent and never greater than 10% at effector-to-target ratios of up to 100:1 (Table 5). In contrast, fresh effector cells from nude mice had levels of cytotoxicity comparable to and sometimes higher than those of C3H or nude/+ controls (Table 5; 2). No culture-stable cytotoxic activity against F-9 cells could be detected with BALB/c or CBA nude effectors at 2, 4, 6, or 8 days postinfection, indicating that we were not missing the peak in stable cytotoxic activity. These experiments suggest that T cells play a direct or indirect role in the development of the cytotoxicity of the nonspecific cultured day 6 cell.

(iv) Kinetics of killing. Fresh day 2 to 3 and day 6 spleen cells were examined more closely for their ability after incubation in vitro to lyse target cells and for the time kinetics required for lysis. If the spleen cells were first cultured at 37°C before admixture with targets,

TABLE 5. *Cytotoxicity mediated by day 6 cells from nude mice*

Expt no.	Mouse strain	Effector cell type	Target cell	% ^{51}Cr release			
				200:1	100:1	50:1	25:1
1	C3H/St	Fresh spleen	F-9	90		66	
	BALB/c nude/+	Fresh spleen	F-9	39		13	
	BALB/c nude/nude	Fresh spleen	F-9	54		23	
	C3H/St	Cultured spleen	F-9	65		26	
	BALB/c nude/+	Cultured spleen	F-9	16		7.1	
	BALB/c nude/nude	Cultured spleen	F-9	−3.0		0.0	
2	C3H/St	Fresh spleen	L		17		2.7
	CBA nude/nude	Fresh spleen	L		36		11
	C3H/St	Cultured spleen	L		31		14
	CBA nude/nude	Cultured spleen	L		7.4		−0.49
3	C3H/St	Adherent PC[a]	L			17.4	
	CBA nude/nude	Adherent PC	L			−1.4	
	CBA nude/+	Adherent PC	L			9.3	
4	C3H/St	Adherent PC	F-9			16	
	BALB/c nude/nude	Adherent PC	F-9			−1.3	
	BALB/c nude/+	Adherent PC	F-9			21	

[a] PC, Peritoneal cells.

the ability of both day 2 and day 6 effectors to kill F-9 cells declined with time (Fig. 4). However, the decline in activity occurred at a faster rate in the day 2 population. This suggests that the day 6 population is either intrinsically more stable or that some other factor(s) maintains activity in culture.

The time course of lysis by fresh day 6 spleen cells contrasted markedly with that of the day 2 spleen (NK) cells (Fig. 5). Most cytotoxicity by the day 2 cells occurred within the first 8 h after admixture, whereas most of the cytotoxicity elicited by the day 6 cells occurred after the initial 8-hr period. Adherent day 6 peritoneal cells similarly required a 16- to 22-h assay period for efficient cytotoxicity. These results indicate either that the day 6 cells require a long time to lyse targets or that something during the culture process caused cells to remain cytotoxic or become newly cytotoxic. The variations in the cytotoxicity curves were not due to the synthesis of lymphotoxins, since the culture fluids alone did not lyse L cells in 16-h assays.

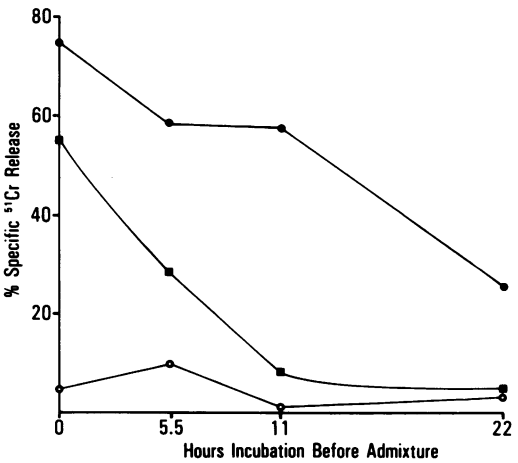

FIG. 4. *Inactivation of cytotoxic cell activity in culture. Spleen cells were cultured at 37°C for various times and then exposed to F-9 targets in a 20-h cytotoxicity assay. Data given are at effector-to-target cell ratios of 100:1. Symbols:* (●) *day 6 cells;* (■) *day 2 spleen cells;* (○) *control cells.*

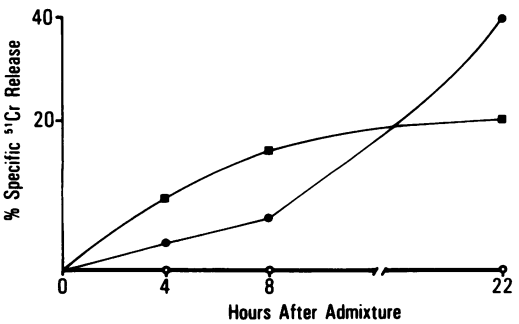

FIG. 5. *Target cell lysis as a function of time after admixture. Symbols:* (■) *day 2 spleen cells;* (●) *day 6 spleen cells;* (○) *control spleen cells. Results are plated at 100:1 ratios on F-9 cell targets.*

Interferon synthesis by cultured spleen cells. Since interferon is an important modulator of NK activity (5, 7, 13, 21), we sought to determine whether there was a correlation between the cultured day 6 cell activity and interferon levels. We had reported earlier that interferon levels in spleen homogenates from 2- or 3-day-infected C3H/St mice were considerably higher than those from 6-day-infected mice and confirm this here (Table 6) (21). However, when spleen leukocytes were cultured for 36 h (about the time for the standard culture process plus the cytotoxicity assay), the day 6 cultures produced much higher levels of interferon than day 2 or 3 cultures (Table 6). Whereas in eight experiments, 2×10^7 day 2 or 3 spleen cells produced an average of 21 interferon units (IFU) per ml of culture fluid, day 6 cells in six experiments synthesized an average of 391 U/ml. Similar results were found with peritoneal cell populations (e.g., D3 peritoneal cells, 5 IF U/ml; D6 peritoneal cells: 128 IF U/ml). BALB/c nude/+ mouse spleen cells also produced significant levels of interferon 6 days postinfection, but athymic BALB/c nude/nude mice produced very low or undetectable levels (Table 6).

To identify the class of interferon produced in spleens in vivo and by spleen cells cultured after LCMV infection, 10% tissue homogenates in MEM and cell culture fluids were adjusted to pH 2 with 1 N HCl and incubated at 37°C for 3 h. This treatment markedly reduces the activity of interferon type II (immune interferon) but does not substantially affect the activity of interferon type I (18). As reported earlier (15), the interferon present in spleens early in infection was resistant to acid treatment (Table 6), suggesting that it is interferon type I. However, interferon produced by the cultured cells either early (day 2) or later (day 6) in the infection was acid labile and therefore probably of the type II variety (18).

When either day 3 or day 6 cells were treated with monoclonal antibody to thy 1.2 antigen plus complement before incubation, virtually no interferon was produced, indicating that the interferon production was T-cell dependent (Table 7). Interferon produced by day 6 spleen cells from BALB/c nude/+ mice also was eliminated by anti-Thy 1.2 treatment. In these experiments no significant levels of interferon were produced by day 11 cells from C3H/St mice or by day 6 cells from nude/nude mice.

TABLE 6. *Interferon production in vivo and in vitro*[a]

Expt no.	Sample	Original titer	Interferon titer after treatment	
			Control	pH 2
1. C3H/St	Day 2 spleen homogenate	256	32	24
	Day 6 spleen homogenate	32	2	<1
	Day 2 culture	8	8	1
	Day 6 culture	1,024	256	32
	Interferon type I		2,048	1,024
	Interferon type II		128	16
2. C3H/St	Day 3 culture		64	2
	Day 6 culture		128	32
3. BALB/c	Day 6 culture (nude/nude)		1	0
	Day 6 culture (nude/+)		32	2

[a] Spleens were excised from LCMV-infected mice and cut in half. Leukocytes were extracted from one half and cultured for 40 h, and the culture fluid was tested for interferon. The remaining spleens were frozen at −70°C, thawed, homogenized by using 1 ml of MEM/half spleen, and cleared of cell debris by low-speed sedimentation. For pH inactivation studies, the spleen homogenates in experiment 1 were further diluted 1:5 in MEM. Some samples were adjusted to pH 2 with 1 N HCl, incubated for 3 h at 37°C, and then neutralized with 1 N NaOH before use.

Spleen cells from C3H/St mice were examined for their abilities to produce interferon after cell fractionation (Table 7). At day 2, interferon was produced by cells adherent to nylon-wool but not by nylon nonadherent cells. In contrast, not only was more interferon produced by day 6 cells, but both nylon-wool-adherent and nonadherent cells produced significant quantities of it.

Activation of cultured cells with interferon. The high level of interferon production by the cultured day 6 cells could possibly account for an NK cell activation in vitro. To test the feasibility of this hypothesis, control, day 2, and day 6 spleen cells were tested for cytotoxicity, incubated overnight, and tested for cytotoxicity again with and without treatment with an exogenous source of mouse fibroblast interferon (Table 8). For these studies the highly NK-sensitive YAC-1 cell line was used for targets (we have found that interferon activation of NK cells is most easily demonstrated with this target cell). As expected, day 2 and day 6 cytotoxic cell activity was considerably elevated over control activity (Table 8), though with the YAC-1 target significant killing also occurred with control effector cells (see Table 2). In this experiment L cells were also included as controls to demonstrate more clearly the typical cytotoxicity pattern of fresh harvests as opposed to cultured spleen cells. After incubation overnight there were major reductions in control and day 2 cytotoxicity, but day 6 killing remained elevated, again as expected. Interferon greatly augmented the cytotoxicity of cultured control and day 2 cells but did not affect the levels of the cultured day 6 cells. This experiment indicates that the cultured control and day 2 cells contained inactive cytotoxic cells which could be activated by interferon. In contrast, the day 6 cultures appeared to have no detectable inactive NK cells susceptible to interferon activation. This is consistent with the hypothesis that the NK cells in the day 6 cultures are already activated by the interferon produced in such cultures.

DISCUSSION

This paper describes the generation of a cytotoxic cell that sharply and transiently appears in the spleen and peritoneum of mice 6 days after infection with LCMV. Several lines of evidence strongly suggest that this cytotoxic activity is predominantly mediated by NK cells, rather than by T cells or macrophages, with which it may be confused. Day 6 cells are stable when compared with NK cells but not as stable as cytotoxic T cells or macrophages are reported to be in vitro. Although it is more sensitive to anti-theta treatment than endogenous NK cells, it is less sensitive to elimination by this treatment than LCMV-specific cytotoxic T cells. Herberman et al. (8) have reported that LCMV infection increases the sensitivity of NK cells to anti-theta serum. Our results suggest that this increase in sensitivity to theta serum may at least in part reflect a dependence on T-cell-dependent interferon which activates the NK cells and has the effect of stabilizing them in culture. The cytotoxic activity associated with monolayers of peritoneal macrophages also appears NK cell mediated. Activated NK cells are more adherent to various surfaces (R. Kiessling, E. Eriksson, L. A. Hallenbeck, and R. M. Welsh, submitted for publication) and could contaminate macrophage monolayers, as LCMV-specific cytotoxic T cells clearly did in this study (Table 2) and in previously documented studies (14). More cytotoxicity was associated with nonadherent than adherent peritoneal cells, and the nonspecific cytotoxicity was reduced by about 50% by anti-theta treatment but not reduced by silica.

In other respects day 6 cells behaved classically like activated NK cells. Target specificities were identical to those of activated NK cells and contrasted to those reported for allospecific cytotoxic T cells and for activated macrophages which, for instance, lyse P815 cells much better than YAC-1 cells. Even cells expressing no *H-2* antigen (F-9 cells) were lysed by the day 6 effector. Furthermore, NK-sensitive YAC-1 cells ef-

TABLE 7. *Characterization of interferon-producing spleen cells*[a]

Expt no.	Effector cell	Treatment	IFU/2 × 10^7 cells
1	Day 2 C3H/St	None	8
		Nylon-wool passed	0
		Nylon-wool adherent	64
	Day 6 C3H/St	None	1,024
	Day 6	Nylon-wool passed	512
	Day 6	Nylon-wool adherent	512
2	Day 3 C3H/St	MEM	32
		Complement	32
		Anti-theta + complement	0
	Day 6 C3H/St	MEM	128
		Complement	128
		Anti-theta + complement	4
	Day 11 C3H/St	MEM	0
		Complement	0
		Anti-theta + complement	2
	Day 6 nude/+	MEM	32
		Complement	32
		Anti-theta + complement	1
	Day 6 nude/nude	MEM	1

[a] For experiment 1, spleen cells were fractionated by the nylon wool technique, readjusted to 2 × 10^7 cells/ml, and incubated for 36 h before assay of culture fluids for interferon. For experiment 2, 2 × 10^7 spleen cells were treated with MEM or monoclonal anti-theta serum and complement and then incubated for 40 h without readjusting the cell number.

TABLE 8. *Interferon induction of cytotoxic activity of cultured spleen cells*[a]

Effector cell	% Specific ^{51}Cr release from given target cell		
	L-929, 100:1	YAC-1	
		50:1	12:1
Control	4.9	41	15
Cultured	1.4	12	2.5
Cultured + IF	ND[b]	48	27
Day 2	22	72	31
Cultured	1.5	12	3.4
Cultured + IF	ND	37	16
Day 6	25	96	46
Cultured	36	60	46
Cultured + IF	ND	59	36

[a] C3H/St mouse spleen cells were harvested from uninfected mice and from mice 2 and 6 days postinfection with LCMV. They were tested for cytotoxicity directly against L and YAC cells or after first culturing the effector cells overnight. To one set of 3 × 10^7 cultured effector cells was added 2 × 10^4 IFU in 1 ml of MEM for 2 h. The interferon (IF)-treated cells were tested for augmentation of cytotoxicity against YAC-1 targets. This type of short-term activation is usually insufficient to activate cytotoxicity against L cells in our hands.

[b] ND, Not determined.

fectively inhibited the ability of the day 6 cells to lyse heterologous targets. The resistance of cultured day 6 spleen cells to iron filings and magnet treatment clearly distinguished them from phagocytic macrophages, and their passage through glass or nylon wool columns excluded either macrophages or polymorphonuclear leukocytes as effectors. The pattern of partial retention through these columns was markedly similar to that of NK cells but different from that of cytotoxic T cells (Table 4). It should, however, be pointed out that these data do not exclude the possibility that allospecific T cells or cytotoxic macrophages may be responsible for a small proportion of the observed lysis.

Kinetic experiments suggested either that the cultured day 6 cells took a long time to lyse targets or that a substance was activating cytotoxic cells during the culture. The latter hypothesis was supported by the data indicating that much interferon is produced in the day 6 cultures but not in the day 2 or 3 cultures. Although interferon could reactivate cultured control or day 2 cells, it could not reactivate cultured day 6 cells, presumably because they were already activated by the interferon secreted in the culture process. We have not been able to activate

NK cells effectively with the levels of interferon produced in the day 6 cultures, but this may be because there is a continual production of interferon over a 16-h period in contrast to a short-term 1- to 2-h exposure, or alternatively because of enhanced interferon effects associated with cell-to-cell contact between interferon-producing and -responding cells. Undefined factors in addition to interferon may also play a role in maintaining NK cells. It should be noted, however, that the presence of interferon is of paramount importance to lyse the spectrum of cells examined, since many of them are sensitive to lysis only by interferon-activated and not by endogenous NK cells (25).

The absence of this effector cell from nude mice suggests a T-cell-related control of its function. The peak in the nonspecific culture-stable cytotoxic activity correlates well with the advent of the cytotoxic T-cell response. Interferon with the acid-labile properties of type II (immune) interferon, known to activate NK cells (13), is produced at high levels in culture concomitant with the nonspecific cytotoxic cell. Little or no interferon is made after treating day 2 or day 6 cells with anti-theta serum and complement. Immune interferon is reported to be made by T cells (6, 26), and our results are therefore consistent with that observation. Nylon wool adherence studies indicated that interferon was not made by nonadherent cells on day 2 or 3 but was made by nonadherent cells on day 6. This, however, could be explained by a macrophage requirement for T-cell generation early (day 2) but not later (day 6) in the infection. The time after infection in which high levels of interferon are generated in vitro suggests that the interferon may be produced by T cells during their clonal expansion, but this has not yet been proven in this system.

Peritoneal macrophage activation, as loosely defined by an increase in the number, size, and vacuolation of peritoneal macrophages, did not occur in nude mice. Certain types of macrophage activation have been shown to be T-cell dependent (1), and this may be the case in the LCMV system. Activated macrophages also secrete interferon (19), so a T-cell-dependent process may also influence macrophages to secrete interferon in vitro, activating endogenous NK cells or stabilizing already active ones.

The 6th day of infection in this model of the LCMV infection is the pivotal day of the infection. It marks the last day in which viral antigens can be found in peritoneal macrophages, the first day of massive infiltration of macrophages and other cells into the peritoneum, and the first day in which cytotoxic T cells can be detected in significant numbers. Although the interferon levels in the whole spleen have declined by the 6th day from a peak at day 2 or 3, interferon production in vitro is considerably higher at that time period. This could be explained if the spleen homogenate interferon detected on day 2 or 3 is made mostly by nonlymphoid cells, such as epithelial cells and fibroblasts, whereas the interferon synthesized in the day 6 cultures is made by lymphoid cells as a consequence of the immune response to the infection. Since the interferon production and NK cell activation only occur in vitro when some cells express viral antigen, we hypothesize that specific T cells responding to viral antigens may secrete interferon and activate NK cells. This mechanism may play a role in the T-cell-dependent immunopathology of the LCMV infection in vivo.

Several reports have claimed that nonspecific cytotoxic T cells are generated in mixed lymphocyte cultures, and recent evidence has suggested that the nonspecific cytotoxicity associated with an allospecific cytotoxic T-cell response may be due to NK cells bearing high concentrations of theta antigen (10). Our work in the LCMV system described here may be an in vivo corollary to such NK cell generation in mixed lymphocyte cultures.

Another major technical point ascertained from this study is that during a viral infection the cytotoxicity obtained in vitro may not be a direct reflection of the cytotoxicity in vivo. In short-term assays with fresh effectors, which should best approximate in vivo conditions, natural cytotoxicity is much higher with day 2 or 3 cells than with day 6 cells. As cells incubate for longer times in culture, however, proportionally more activity is associated with day 6 cells. This probably reflects the disparate in vivo versus in vitro interferon production. In human studies, peripheral blood leukocytes from vaccinated individuals display higher antibody-mediated cytotoxicity against virus-infected cells concomitant with a peak in in vitro antibody production 7 days after vaccination (17). Our results suggest that interferon production in vitro and resulting nonspecific NK cell activation should probably also parallel this peak in specific immune-cell proliferation.

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