

Natural Cytotoxicity to Murine Cytomegalovirus-Infected Cells Mediated by Mouse Lymphoid Cells: Role of Interferon in the Endogenous Natural Cytotoxicity Reaction

GERALD D. LEE AND ROBERT KELLER*

Department of Microbiology, Michael Reese Hospital and Medical Center, Chicago, Illinois 60616

Received 1 April 1981/Accepted 19 August 1981

Lymphoid cells from unstimulated normal C57BL/6J mice were shown to lyse murine cytomegalovirus (MCMV)-infected syngeneic mouse embryo fibroblasts but not uninfected mouse embryo fibroblasts. This cytotoxicity by mouse effector cells was not restricted to MCMV-infected syngeneic cells since MCMV-infected xenogeneic rat heart fibroblasts were also lysed. Characterization of the effector cells mediating this cytotoxicity against MCMV-infected cells indicated that the effector cells are similar to described natural killer (NK) cells mediating lysis of tumor cells and virus-infected cells. Because of the described augmentation of NK activity by interferon, we examined the role of interferon in the NK reaction. Although low levels of virus-induced interferon were detectable in supernatants of MCMV-infected mouse embryo fibroblasts, no interferon was detectable in supernatants of MCMV-infected rat heart fibroblasts, a target significantly more sensitive to NK cytotoxicity than infected mouse embryo fibroblasts. We were able to augment the NK reaction against MCMV-infected cells by *in vitro* treatments with interferon. However, the amounts of interferon required for augmentation were significantly greater than the amounts generated by infected target cells. *In vitro* interferon-stimulated NK cells retained selective cytotoxic activity since they continued to remain incapable of lysing uninfected target cells. MCMV-infected rat heart fibroblasts induced more interferon and were also more susceptible to NK activity than MCMV-infected mouse embryo fibroblasts. In spite of this difference in interferon-inducing capacity, there was no augmentation of cytotoxicity of MCMV-infected mouse embryo fibroblasts when mouse splenocytes were cocultivated with both target cells. Finally, when production of interferon in the NK reaction was inhibited by the addition of actinomycin D, no reduction of NK activity was seen. Our findings suggest that native mouse NK cells can discriminate between MCMV-infected cells and uninfected cells, this ability leading to the selective lysis of the virus-infected cells. Furthermore, although we could demonstrate augmentation of NK activity by interferon, interferon activation of NK cells may not be a necessary precondition for the development of endogenous NK activity.

Cytotoxic lymphocytes reactive against a number of tumor cell lines have been shown to occur in normal nonimmune humans (11, 21, 23, 25, 30, 34), mice (6, 7, 8, 10, 13, 27, 35), and rats (19, 28). This natural cell-mediated cytotoxicity is mediated by lymphocytes which lack the surface marker characteristics of either mature B or mature T cells. Moreover, these cells do not exhibit properties associated with monocytes and macrophages. The effector cell involved has been termed the natural killer (NK) cell.

Findings from this laboratory have demonstrated that human peripheral blood lymphocytes obtained from normal nonimmune donors were capable of lysing cytomegalovirus-infected

human fibroblasts but did not produce cytolysis of uninfected human fibroblasts (3). This reaction occurred in the absence of specific cytomegalovirus antibodies. Peripheral blood lymphocytes demonstrated similar levels of lytic activity against two continuous human cell lines, HEp-2 and Chang, as well as P2 HR-1, an Epstein-Barr virus-transformed lymphoid cell line. On the basis of limited characterization studies, it appeared that the cells active against the virus-infected cells were similar to the effector cells mediating natural cytolysis of cell lines derived from human tumors.

In this report we describe and characterize a similar natural cytotoxicity by nonimmune mouse lymphoid cells against target cells infect-

ed with another member of the cytomegalovirus group, murine cytomegalovirus (MCMV). The effector cells mediating cytotoxicity of MCMV-infected cells do not have surface marker characteristics of a conventional B or T cell, a finding consistent with those described for the NK mouse tumor system.

Because of the recently described stimulatory effects of interferon in natural cytotoxicity, we have examined the role of interferon in the mediation of the NK cytotoxicity of MCMV-infected cells.

MATERIALS AND METHODS

Mice. C57BL/6J mice were purchased initially from the Jackson Laboratory, Bar Harbor, Maine, and were maintained in our laboratory. Both male and female mice 1 to 2 months of age were used in the experiments. BALB/c athymic nude mice were purchased from Sprague-Dawley, Salem, Ohio.

Cell cultures. Primary C57BL/6J mouse embryo fibroblast (MEF) cultures were prepared by trypsinization of C57BL/6J embryos. The cells were seeded and grown in flasks (Lux Scientific Corp., Newbury Park, Calif.). Only primary, first and second passages were used in the experiments. Rat heart fibroblasts (RHF) were established from beating rat heart cell cultures prepared as described by McCarl and Margosian (18). The primary rat heart cultures were trypsinized and passaged until monolayers of fibroblasts were established. The growth medium used for maintenance of these cells and in the experiments was minimal essential medium containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, 100 μ g of streptomycin per ml, and 25 μ g of amphotericin B per ml. The RL δ 1 leukemia cell line was obtained from Ronald Herberman, National Cancer Institute, Bethesda, Md. These cells were cultured in vitro in RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, 100 μ g of streptomycin per ml, and 25 μ g of amphotericin B per ml.

Virus. The Smith strain of MCMV obtained from the American Type Culture Collection was used throughout these experiments. Virus was prepared by infection of MEF monolayers. When most of the cells displayed cytopathic effect, the cells and media were freeze-thawed once; they were then centrifuged to remove cell debris, and the supernatant was collected. Samples of virus containing 10% dimethyl sulfoxide were stored at -70°C . The titer of the virus was determined by a plaque titration method.

MCMV infection of target cells. For infection of MEF or RHF cells with MCMV, 1.6×10^6 cells were seeded in 75-cm² plastic flasks 1 day prior to infection with virus. An inoculum of MCMV giving a multiplicity of infection of 2.0 to 3.0 was then added to the cell cultures, absorbed for 1 h at 37°C , and then removed. Maintenance medium was added to the cultures, and the cells were utilized 18 h later.

Uninfected control cell cultures were prepared in a similar manner.

Cytotoxic assay. Cytotoxicity was determined by a ⁵¹Cr-release assay. Infected and uninfected target cells

were harvested by trypsinization and washed with phosphate-buffered saline. These cells (5×10^6) in 0.5 ml of phosphate-buffered saline containing 5% fetal calf serum and 100 μ g of glucose per ml were then incubated with 100 μ Ci of Na₂⁵¹CrO₄ (Amersham/Searle, Arlington Heights, Ill.) for 1 h at 37°C . After being washed three times with medium, 10⁴ target cells in 0.1 ml were dispensed into wells of a Micro Test II plate (Falcon Plastics, Oxnard, Calif.). The dispensed cells were incubated at 37°C in 5% CO₂ for at least 1.5 h to allow the cells to adhere. After addition of effector cells in a 0.1-ml volume, the plate was incubated for 18 h at 37°C in 5% CO₂. When RL δ 1 leukemia cells were used as targets, the effector cells were added immediately, and the plate was incubated for 4 h at 37°C in 5% CO₂ on a rocker (5 cycles per min).

After incubation, the plate was centrifuged, and 0.1-ml amounts of supernatants were removed and counted in a gamma counter. Total releasable radioactivity was determined by lysing target cells with 1% Triton X-100. Spontaneous release was determined from target cells incubated with medium alone. The percentage of ⁵¹Cr released by effector cells (referred to as percent specific lysis) was calculated by the following formula:

$$\frac{E \text{ cpm} - \text{spontaneous release cpm}}{\text{total lysis cpm} - \text{spontaneous release cpm}}$$

where *E* is the supernatant from target cells incubated with effector cells. All experiments were performed at least in triplicate and in some cases in quadruplicate.

Preparation of effector cells. Single-cell suspensions of mouse splenocytes were obtained by passage of minced spleens through a stainless steel-wire mesh. Contaminating erythrocytes were lysed by treatment with Tris-buffered 0.75% ammonium chloride, pH 7.2, for 4 min at 5°C . Since ammonium chloride lysis has been reported to be toxic to NK cells (34) in a few experiments, a 30-s distilled water treatment was substituted to lyse the erythrocytes. However, no significant increases in cytotoxic activity of effector cells were noted.

Peripheral blood lymphocytes were prepared from heparinized blood by the Ficoll-Hypaque gradient technique (2).

Nylon-wool-column passage. Nylon-wool-column passage was performed by the method of Julius et al. (12).

Anti-theta treatment of spleen cells. A 2.5-ml volume of a 1:10 dilution of AKR/J anti-C3H/HeJ ascites cells (Litton Bionetics, Inc., Kensington, Md.) was incubated with 5×10^7 spleen cells for 30 min at room temperature. After centrifugation, the supernatant was poured off, and 3.0 ml of a (1:15) dilution of rabbit complement (Low-Tox; Accurate Chemicals & Scientific Corp., Hickville, N.J.) was added. After a further incubation of 45 min at 37°C , the spleen cells were centrifuged; they were then washed three times with growth medium, and viability was determined with a trypan blue dye exclusion test. This treatment was found to lyse 30 to 35% of spleen cells and also to reduce the blastogenic response to concanavalin A by 80% when compared with controls.

MCMV antisera. Mice received an intraperitoneal injection of 10⁶ PFU/ml of MCMV. At 3 weeks, they received an equivalent booster injection, and they were bled 2 weeks later. Antisera were tested by an

indirect immunofluorescence assay and demonstrated strong reactivity at a 1:80 dilution. The indirect immunofluorescence test was conducted with MEF cells grown on glass slides, infected with MCMV, and acetone fixed 20 h postinfection. For the immunofluorescence test, the infected cells were allowed to react with dilutions of serum for 30 min at 37°C. After being washed twice in phosphate-buffered saline, the cells were allowed to react with an appropriate dilution of fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin (Meloy Laboratories, Springfield, Va.) for 30 min at 37°C. The slides were then washed with phosphate-buffered saline, mounted in buffered glycerol, pH 8.0, and examined.

Interferon assay. The quantification of antiviral activity of interferon was performed as described by Lvovsky and Levy (16). Antiviral activity was measured by the inhibition of the cytopathic effect of vesicular stomatitis virus on L-929 monolayers. Antiviral units were expressed as the reciprocal of the dilution causing a 50% inhibition of the cytopathic effect. All assays included a titration of a mouse interferon standard (reference no. 6002-904-511, National Institute of Allergy and Infectious Diseases, Bethesda, Md.). Interferon titers were expressed in terms of the reference standard. Interferon induced in RHF by polyinosinic-polycytidylic acid was found to have equal antiviral activity when assayed on either RHF or L cells. Therefore, all subsequent assays for rat interferon were conducted on L cells.

Interferon preparations. Mouse interferon made from mouse fibroblasts was purchased commercially (Calbiochem, La Jolla, Calif.). Lots 940048 and 940065 were used in our experiments.

RESULTS

Cytotoxic activity of lymphoid cells from non-immune mice against MCMV-infected fibroblasts. Spleen cells obtained from 1- to 2-month-old unimmunized C57BL/6J mice expressed natural cytotoxicity against MCMV-infected syngeneic fibroblasts (MCMV-MEF), but not against uninfected MEF. Figure 1 shows a representative experiment. The levels of MCMV-MEF cytolysis varied from experiment to experiment, ranging from 8 to 23% at a 100:1 effector cell to target cell ratio. In contrast to the cytotoxicity levels seen against the MCMV-MEF, uninfected MEF were not significantly lysed by spleen cells, even at a 100:1 effector to target ratio. It has been reported that mice infected with MCMV are stimulated for NK activity directed against MCMV-infected cells (22). However, these findings differ from our observations in that cytotoxic cells were not detected in spleens of uninfected mice.

In light of this, we investigated the possibility that our findings were due to NK stimulation by MCMV infection. It is known that mice, particularly in the wild but also as part of breeding colonies, may become chronically infected with MCMV (17). To detect any NK stimulation due to inapparent MCMV infection, we tested mice

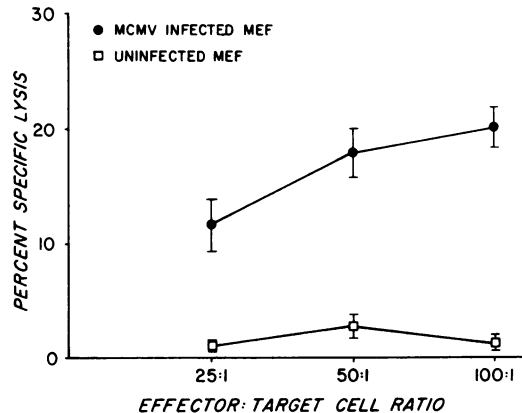


FIG. 1. Natural cytotoxicity of normal C57BL/6J mouse spleen cells against MCMV-MEF (●) and against uninfected fibroblasts (□).

randomly for antibody to MCMV by an indirect fluorescent-antibody procedure. No antibody was detected. In addition, we attempted repeatedly to isolate MCMV from salivary glands, a sensitive indicator of chronic MCMV infection (24). In no case was MCMV recovered from experimental animals. Therefore, the selective lysis of MCMV-infected cells appears to reflect endogenous NK activity and not an activation of NK activity by chronic infection.

MCMV, in contrast to other members of the cytomegalovirus group which demonstrate limited host-range specificity (14), has been found capable of infecting a wide range of cells. In the course of other studies, we observed that MCMV could produce a productive infection in the xenogeneic RHF (unpublished data). This observation permitted us to examine the question of whether the cytotoxic reaction by mouse lymphocytes was species restricted. As can be seen in Fig. 2, MCMV-infected xenogeneic fibroblasts (MCMV-RHF) were readily lysed by murine lymphocytes and, unexpectedly, the level of lysis was significantly greater than that observed with MCMV-infected mouse cells.

Characterization of the effector cell population. We examined the surface membrane characteristics of the cytotoxic effector cells. Nylon-wool-column absorption treatment resulted in the removal of 85% of the lymphocytes bearing surface immunoglobulin as determined by immunofluorescence. However, as shown in Table 1, the nonadherent cell population showed no loss of activity and actually had slightly greater cytotoxic activity than did untreated cells. Deletion of theta-bearing cells also failed to remove cytotoxic activity. There was no significant loss of cytotoxic activity when anti-theta- and complement-treated spleen cells were compared

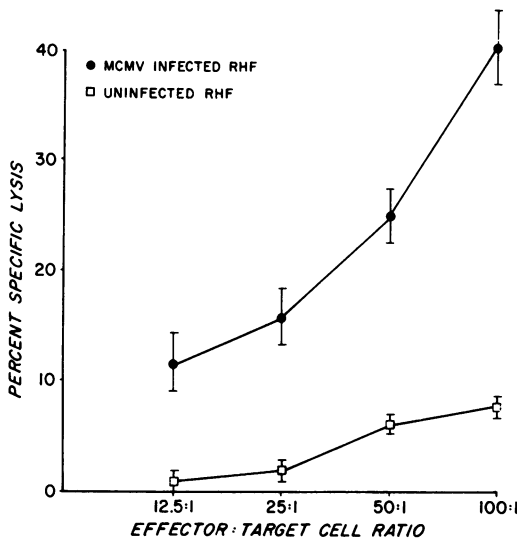


FIG. 2. Natural cytotoxicity of normal C57BL/6J mouse spleen cells against MCMV-RHF (●) and against uninfected RHF (□).

with spleen cells receiving no treatment or treatment with complement alone (Table 1). The cytotoxic activities of spleen cells from nude mice are shown in Table 2. As can be seen, the levels of activity of splenic cells from athymic mice were similar to those observed with C57BL/6J mice. Moreover, splenic lymphocytes from both C57BL/6J mice and athymic nude mice were effective in expressing cytotoxicity against a known NK-sensitive murine leukemia target cell.

Role of interferon in NK cytotoxicity of MCMV-infected cells. It has been established that interferon enhances endogenous NK activity (5, 31). Because of this it was crucial to resolve whether the NK activity noted in these experiments was due simply to nonspecific activation of NK cells responding to virally induced interferon present in the reaction mixtures or whether NK activity was due to the recognition and response of NK cells to virally modified cell surface membranes. To distinguish between these two possible mechanisms, we compared the levels of interferon necessary for NK augmentation to the levels of interferon elaborated by virus-infected cells.

To assess the amount of interferon produced by the target cells as a result of viral infection, we prepared infected and noninfected cells and incubated them for 18 h. After incubation, the supernatants were harvested and assayed for interferon (Table 3). Only in the supernatant of MCMV-MEF were low levels of interferon, i.e., 8 U, detected. No interferon was detected in supernatants of MCMV-RHF even though these

cells had been shown to be more sensitive to NK-mediated lysis than were the MCMV-MEF.

We also assayed for interferon production when the target cells were incubated in the presence of mouse splenic effector cells. As can be seen in Table 3, interferon was detected only when the effector cells were cultured with the NK-sensitive target cells, i.e., MCMV-MEF, MCMV-RHF, and RL δ 1. In contrast, no interferon was detected when the splenocytes were incubated with the uninfected fibroblasts. These findings confirm reports by others that mouse splenic cells are induced to generate interferon only when cultured in the presence of NK-susceptible tumor target cells (31).

Although only low levels of interferon were detected in supernatants of MCMV-infected cell cultures, it may have been possible that such low levels of interferon might have been sufficient to activate NK cells. Therefore, we investigated the cytotoxic ability of splenocytes activated in vitro with interferon. Splenocytes from both C57BL/6J and CBA/J mice were preincubated with various concentrations of interferon (4) and assayed for cytotoxic activity. The CBA/J mice were included because of their greater interferon-induced NK activity (4). As shown in Table 4, we found that the NK reaction against MCMV-RHF and RL δ 1 could be augmented only by additions of relatively high concentrations of interferon. When only 100 U of interferon was used, however, no augmentation was seen. Therefore, it appears that the low levels of virus-induced interferon, i.e., 8 U, found in the supernatants of MCMV-MEF cell cultures would not be sufficient to mediate NK stimulation. The addition of interferon had no effect on cytolysis of uninfected cells. Even when 2,000 U of interferon was added, a concentration which augmented cytotoxicity against the NK-susceptible cells MCMV-RHF and RL δ 1, no cytolysis of uninfected RHF cells was seen.

As shown in Table 3, mouse splenocytes were

TABLE 1. Characterization of splenic effector cells mediating natural cytotoxicity of MCMV-infected cells^a

Treatment	Specific lysis of MCMV-MEF (%)	
	100:1	50:1
Untreated	18.3 \pm 2.9	8.0 \pm 0.9
Nylon column	26.6 \pm 3.2	9.0 \pm 1.6
Untreated	17.7 \pm 1.3	12.7 \pm 1.6
Complement	16.0 \pm 0.6	14.9 \pm 1.8
Anti-theta + complement	14.5 \pm 2.7	11.2 \pm 1.2

^a Effector to target cell ratios of 100:1 and 50:1 were used. Each number represents the mean percentage of specific lysis \pm 1 standard error.

TABLE 2. Cytotoxic reaction of spleen cells from normal C57BL/6J and BALB/c nude mice against MCMV-infected cells and a mouse leukemia cell line^a

Target cell	Effector cells			
	C57BL/6J		BALB/c nude	
	100:1	50:1	100:1	50:1
MCMV-MEF	10.5 ± 1.0	7.6 ± 2.1	12.8 ± 0.9	5.4 ± 2.2
MEF	4.6 ± 2.8	2.5 ± 1.6	4.2 ± 0.6	2.0 ± 1.8
MCMV-RHF	27.1 ± 0.5	22.8 ± 1.3	21.4 ± 0.9	19.4 ± 0.3
RHF	12.4 ± 0.8	6.6 ± 0.6	5.5 ± 0.3	5.2 ± 0.2
RL δ 1	16.3 ± 0.8	9.9 ± 0.3	28.8 ± 0.3	11.0 ± 0.1

^a Effector to target cell ratios of 100:1 and 50:1 were used. Each number represents the mean percentage of specific lysis \pm standard error.

induced to elaborate interferon when cultured with MCMV-infected target cells. Considerably more interferon was induced by mouse lymphocytes cultured with infected RHF than with infected MEF. We have shown that MCMV-RHF were substantially more sensitive to NK-mediated cytotoxicity than were MCMV-MEF, suggesting that the increased elaboration of interferon may have resulted in greater NK stimulation and therefore greater cytotoxicity. Because of this we examined whether splenocyte-generated interferon (or other soluble factors) could stimulate lysis.

In preliminary experiments (not cited) in which we used the supernatants from mixtures of virally infected cells cultured with mouse splenocytes, we were unable to augment NK activity. However, it was possible that humoral NK activators may have been labile and may have been inactivated during the collection procedure. Therefore, we examined NK cytotoxicity by mouse splenic cells of ⁵¹Cr-labeled MCMV-MEF of low NK susceptibility cocultivated with an equal number of unlabeled highly NK-susceptible MCMV-RHF. If the greater NK susceptibility of MCMV-RHF was due to non-specific activation of NK cells by interferon or other soluble factors, it would be expected that cocultivation of MCMV-RHF with MCMV-MEF would have resulted in augmented NK activity against the MCMV-MEF. As shown in Table 5, even though the MCMV-RHF were readily lysed, the presence of the same cells within the reaction did not enhance the cytotoxicity of the less susceptible MCMV-MEF. Therefore, the greater susceptibility of MCMV-RHF (as well as the relative insensitivity of MCMV-MEF) to NK lysis does not appear to be related to their ability to induce interferon or another NK activator(s) in mouse splenocytes.

To further examine the role of interferon, we studied the effect of actinomycin D, an antimetabolite that inhibits interferon production (15),

on the endogenous NK activity. Accordingly, we measured interferon production and NK cytotoxicity of splenic effector cells when cultured with 18-h-old MCMV-infected RHF targets in the presence of actinomycin D. As can be seen in Table 6, treatment with actinomycin D reduced interferon production by greater than 95%. However, no loss of NK cytotoxic activity was observed under the same conditions. In other experiments not shown, the same concentrations of actinomycin D had no effect on NK cytotoxicity of RL δ 1 cells.

DISCUSSION

In this study the effector cell present in unstimulated mice that mediates lysis of MCMV-infected cells has been characterized as a population of lymphocytes similar if not identical to mouse NK cells, described by others, which are

TABLE 3. Development of interferon in MCMV-infected cell cultures in the presence or absence of mouse splenic effector cells^a

Cell	Interferon detected (U)	
	Media	50E ^b
MEF	<4	<4
MCMV-MEF	8	60
RHF	<4	<4
MCMV-RHF	<4	400
RL δ 1	<4	20
Media		<4

^a Eighteen-hour-old infected or noninfected target cells were prepared as described in Materials and Methods. One-milliliter samples containing 2.8×10^5 cells were seeded into 35-mm plastic tissue culture dishes and allowed to attach for 1 h. After this, 1.0 ml containing 1.4×10^7 effector cells (50E) or 1.0 ml of medium alone was added. The supernatants from these cultures were harvested after 18 h of incubation at 37°C and assayed for interferon as described in Materials and Methods.

^b 50E, 50:1 effector to target cell ratio.

TABLE 4. Augmentation of NK cytotoxicity by mouse interferon^a

Effector cells	Interferon (U)	Specific lysis (%)		
		RHF	MCMV-RHF	RL δ 1
C57BL/6J	None	3.9 \pm 0.8	11.4 \pm 0.9	7.3 \pm 0.2
	2,000	2.2 \pm 1.2	14.8 \pm 0.2 ^b	11.3 \pm 0.6 ^c
	100	3.3 \pm 0.6	11.7 \pm 0.5	7.5 \pm 0.6
CBA/J	None	4.5 \pm 0.8	10.1 \pm 0.5	2.6 \pm 0.3
	2,000	5.2 \pm 1.7 ^d	15.1 \pm 1.1 ^c	10.9 \pm 0.8 ^c
	1,000	6.7 \pm 1.7 ^d	13.7 \pm 0.2 ^c	7.7 \pm 0.5 ^b
	100	4.6 \pm 0.9	9.0 \pm 1.8	3.5 \pm 0.5 ^d

^a C57BL/6J splenic effector cells were prepared as described in Materials and Methods. The cell concentration was adjusted to 10⁸ cells per ml, and 0.2-ml samples were incubated with 0.2-ml volumes of either medium containing interferon or medium alone for 1 h at 37°C. After this, the cells were diluted to 5 \times 10⁶ cells/ml, added to MCMV-infected and uninfected RHF as well as the mouse leukemia cell line RL δ 1, and assayed for NK cytotoxicity at an effector to target cell ratio of 50:1.

^b $P < 0.05$.

^c $P < 0.01$.

^d Not significant.

active against a variety of target cells including tumor cells and certain virally infected cells (1, 9, 10, 13, 22, 26, 33).

It has been established that interferon can stimulate NK reactivity both in vitro and in vivo (5, 31). Since the susceptible target cells in our system were virus-infected cells, it was conceivable that the virus infection had induced the production of interferon, which in turn could have activated nonspecific NK activity.

Our results do not indicate this to be the case. First of all, only very small amounts of interferon could be detected from the supernatants of infected cells, in agreement with reports by others showing that MCMV is a poor inducer of interferon (20). Furthermore, no interferon was detected from supernatants of infected RHF, in spite of the greater susceptibility of these cells to NK-mediated cytotoxicity. In addition, attempts to augment the NK reactivity by preincubation of splenocytes with interferon were successful only when large amounts of interferon were added, significantly more than was generated by viral infection of the target cell.

TABLE 5. NK cytotoxicity of MCMV-MEF when cultured in the presence of MCMV-RHF^a

Target cells	Specific lysis (%)
MCMV-MEF	12.2 \pm 1.0
MCMV-MEF ^b + MCMV-RHF	12.3 \pm 0.8
MCMV-RHF	52.5 \pm 1.0

^a Cytotoxicity of MCMV-MEF by splenocytes was measured when cultured in the presence of the MCMV-RHF. Labeled MCMV-MEF (10⁴ cells per well) were mixed with an equal number of unlabeled MCMV-RHF, and the cytotoxicity by splenic effector cells (100:1) was assayed.

^b ⁵¹Cr-labeled.

When splenocytes from nonimmune mice were incubated with virus-infected cells, interferon was readily detected. However, no interferon was detected from supernatants of similar splenocytes cultured with noninfected fibroblasts. These results confirm the report by Santoli et al. (26) that only NK-sensitive target cells were able to induce interferon production by NK cells.

If interferon induced as a result of the reaction of NK cells with MCMV-RHF were responsible for the greater level of NK cytotoxicity seen against this target, it would be reasonable to expect enhancement of the killing of MCMV-infected mouse cells in the cocultivation experiments cited in Table 4. That is to say, if the interferon generated was sufficient to activate greater cytotoxic activity of NK cells against the infected rat cell target, these same effectors should have had increased cytotoxic activity against the MCMV-MEF in the cocultivation experiments. Moreover, we found that splenocyte interferon production during the NK reaction could be inhibited by actinomycin D. In spite of a greater than 95% loss of interferon in the reaction mixture, no reduction of NK cytotoxicity was seen. Therefore, on the basis of these findings, it appears unlikely that the NK-mediated cytotoxicity of virus-infected cells is due to nonspecific activation by induced interferon, as has been suggested by others (32). This interpretation is supported by the findings of Djeu et al. (4) that the presence of interferon antiserum does not decrease the spontaneous levels of NK cytotoxicity of mouse tumor cells. However, we cannot exclude the possibility that the endogenous NK activity observed in our experiments may have resulted from preactivated NK effector cells stimulated by interferon in vivo.

TABLE 6. Effect of actinomycin D on interferon production and NK cytotoxicity of MCMV-RHF

Effector cell treatment ^a	Interferon (U) ^b	Specific lysis (%)	
		100E ^c	50E ^c
Medium	100	25.3 ± 2.5	16.6 ± 0.8
Actinomycin D, 0.5 µg/ml	<4	27.4 ± 1.3	17.3 ± 0.8
Actinomycin D, 0.25 µg/ml	8	25.0 ± 0.6	16.0 ± 1.9

^a Splenic effector cells were prepared as described in Materials and Methods and adjusted to 10⁷ cells per ml. Samples of cells were incubated with medium containing 0.5 or 0.25 µg of actinomycin D per ml for 2 h at 37°C. The pretreated effector cells were then diluted in medium containing the same concentrations of actinomycin D and were assayed for NK activity in the presence of the inhibitor.

^b Supernatants from 50:1 effector-target cell mixtures along with an interferon control were dialyzed against phosphate-buffered saline to remove actinomycin D, which would interfere with the interferon assay. No loss of interferon activity was detected in interferon control preparations after similar dialysis.

^c Effector to target cell ratios of 100:1 and 50:1.

In discussing the selective lysis of MCMV-infected cells, it becomes necessary to differentiate between the cytotoxicity reported after *in vivo* activation with a number of potent interferon inducers (5, 33) and the low endogenous levels normally found in unstimulated animals. Thus, stimulated NK cells obtained after LCMV infection demonstrate a broad indiscriminate spectrum of cytolysis directed against both normal, uninfected primary fibroblasts and tumor cells (33). However, it appears that, in contrast to interferon-stimulated NK cells (29), mouse NK cells from normal, unstimulated mice display some selectivity in their cytolytic activity against tumor cells (10, 13). Similarly, our findings indicate that endogenous NK cells express selective recognition and lysis of MCMV-infected cells while sparing uninfected cells. Moreover, our findings suggest that this selective process of NK-mediated cytolysis is not necessarily predicated on prior interferon activation of NK effector cells.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health General Research Support grant 5476 from the Division of Research Resources.

LITERATURE CITED

- Anderson, M. J. 1978. Innate cytotoxicity of CBA mouse spleen cells to Sendai virus-infected L cells. *Infect. Immun.* 20:608-612.
- Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human peripheral blood. *Scand. J. Clin. Lab. Invest.* 21(Suppl. 97):77-89.
- Diamond, R. D., R. Keller, G. Lee, and D. Finkel. 1977. Lysis of cytomegalovirus-infected human fibroblasts and transformed human cells by peripheral blood lymphoid cells from normal human donors. *Proc. Soc. Exp. Biol. Med.* 154:259-263.
- Djeu, J. Y., J. A. Heinbaugh, H. T. Holden, and R. B. Herberman. 1979. Augmentation of mouse natural killer cell activity by interferon and interferon inducers. *J. Immunol.* 122:175-181.
- Gidlund, M., A. Örn, H. Wigzell, A. Senik, and I. Gresser. 1978. Enhanced NK cell activity in mice injected with interferon and interferon inducers. *Nature (London)* 273:759-761.
- Greenberg, A. H., and J. H. L. Playfair. 1974. Spontaneously arising cytotoxicity to the P-815-Y mastocytoma in NZB mice. *Clin. Exp. Immunol.* 16:99-110.
- Haller, O., R. Kiessling, A. Örn, K. Kärre, K. Nilsson, and H. Wigzell. 1977. Natural cytotoxicity to human leukemia mediated by mouse non-T cells. *Int. J. Cancer* 20:93-239.
- Herberman, R. B., M. E. Nunn, and H. T. Holden. 1978. Low density of Thy 1 antigen on mouse effector cells mediating natural cytotoxicity against tumor cells. *J. Immunol.* 121:304-309.
- Herberman, R. B., M. E. Nunn, H. T. Holden, and D. H. Larvin. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. *Int. J. Cancer* 16:230-239.
- Herberman, R. B., M. E. Nunn, and D. H. Lavin. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. I. Distribution of reactivity and specificity. *Int. J. Cancer* 16:216-229.
- Jondal, M., and H. Pross. 1975. Surface markers on human B and T lymphocytes. VI. Cytotoxicity against cell lines as a functional marker for lymphocyte subpopulations. *Int. J. Cancer* 15:596-605.
- Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived lymphocytes. *Eur. J. Immunol.* 3:645-649.
- Kiessling, R., E. Klein, and H. Wigzell. 1975. "Natural" killer cell in the mouse. I. Cytotoxic cells with specificity for mouse Molony leukemia cells. Specificity and distribution according to genotype. *Eur. J. Immunol.* 5:112-117.
- Kim, K. S., and R. I. Carp. 1971. Growth of murine cytomegalovirus in various cell lines. *J. Virol.* 7:720-725.
- Levy, H. B., D. Axelrod, and S. Baron. 1965. Messenger RNA for interferon production. *Proc. Soc. Exp. Biol. Med.* 118:384-385.
- Lvovsky, E., and H. B. Levy. 1976. Interferon assay of high sensitivity. *Proc. Soc. Exp. Biol. Med.* 153:511-513.
- Mannini, A., and D. N. Medearis, Jr. 1961. Mouse salivary gland virus infections. *Am. J. Hyg.* 73:329-343.
- McCarl, R. K., and S. S. Margossian. 1969. Restoration of beating and enzymatic response of cultured rat heart cells to cortisol acetate. *Arch. Biochem. Biophys.* 130:321-325.
- Nunn, M. E., J. Y. Djeu, M. Glasser, D. H. Larvin, and R. B. Herberman. 1976. Natural cytotoxicity of rat lymphocytes against syngeneic Gross virus-induced lymphoma. *J. Natl. Cancer Inst.* 56:393-399.
- Okie, H. K., J. M. Easton, D. V. Ablashi, and S. Baron. 1975. Murine cytomegalovirus: induction of and sensitivity to interferon *in vitro*. *Infect. Immun.* 12:1012-1017.
- Peter, H. H., R. F. Eife, and J. R. Kalden. 1976. Spontane-

- ous cytotoxicity (SCMC) of normal human lymphocytes against a human melanoma cell line: a phenomenon due to a lymphotoxin-like mediator. *J. Immunol.* **116**:342-348.
22. Quinnan, G. V., and J. Manischewitz. 1979. The role of natural killer cells and antibody dependent cell mediated cytotoxicity in murine cytomegalovirus infection. *J. Exp. Med.* **150**:1549-1554.
 23. Rosenberg, E. B., J. L. McCoy, S. S. Green, F. C. Donnelly, D. F. Siwarski, P. H. Levine, and R. B. Herberman. 1974. Destruction of human lymphoid tissue-culture cell lines by human peripheral lymphocytes in ⁵¹Cr-release cellular cytotoxicity assays. *J. Natl. Cancer Inst.* **52**:345-352.
 24. Ruebner, B. H., T. Hirano, R. Slusser, J. E. Osborn, and D. N. Medearis, Jr. 1966. Cytomegalovirus infection. Viral ultrastructure with particular reference to the relationship of lysosomes to cytopathic inclusions. *Am. J. Pathol.* **48**:971-989.
 25. Saksela, E., T. Imir, and O. Makela. 1977. Spontaneous augmentable cell-mediated cytotoxicity with limited target cell specificity in human blood. *Eur. J. Immunol.* **7**:126-130.
 26. Santoli, D., G. Trinchieri, and F. S. Lief. 1978. Cell-mediated cytotoxicity against virus-infected target cells in humans. I. Characterization of the effector lymphocyte. *J. Immunol.* **121**:526-531.
 27. Sendo, R., T. Aoki, E. A. Boyse, and C. F. Buafu. 1975. Natural occurrence of lymphocytes showing cytotoxic activity to BALB/c radiation-induced leukemia RL δ 1 cells. *J. Natl. Cancer Inst.* **55**:603-609.
 28. Shellam, G. R. 1977. Studies on a Gross-virus-induced lymphoma in the rat. V. Natural cytotoxic cells are non-T cells. *Int. J. Cancer* **19**:225-235.
 29. Tal, A., R. C. Burton, and N. L. Warner. 1980. Differential natural killer cells reactivity against T cell lymphomas by cells from normal or stimulated mice. *J. Immunol.* **124**:1705-1711.
 30. Takasugi, M., M. R. Mickey, and P. I. Terasaki. 1973. Reactivity of normal lymphocytes from normal persons on cultured tumor cells. *Cancer Res.* **33**:2898-2902.
 31. Trinchieri, G., and D. Santoli. 1978. Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Enhancement of natural killer cell activity by interferon and antagonistic inhibition of susceptibility of target cells to lysis. *J. Exp. Med.* **147**:1314-1333.
 32. 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**:2491-2497.
 33. Welsh, T. M., Jr., 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.
 34. West, W. H., G. B. Cannon, H. D. Kay, G. D. Bonnard, and R. B. Herberman. 1977. Natural cytotoxic reactivity of human lymphocytes against a myeloid cell line: characterization of effector cells. *J. Immunol.* **118**:355-361.
 35. Zarlring, J. M., R. C. Nowinsky, and F. J. Bach. 1975. Lysis of leukemia cells by spleen cells of normal mice. *Proc. Natl. Acad. Sci. U.S.A.* **72**:2780-2784.