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Natural Cell-Mediated Cytotoxicity of Bovine Mononuclear Cells Against Virus-Infected Cells†

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The ability of mononuclear cells (MC) from peripheral blood of normal cattle to lyse a variety of cells was tested in a ^{51}Cr-release microcytotoxicity assay. Several types of bovine cells infected with parainfluenza 3 virus (PI3V) were susceptible to natural cytotoxicity. Bovine cells infected with infectious bovine rhinotracheitis virus or noncytopathogenic bovine viral diarrhea virus, uninfected bovine cells, and human cell lines MOLT-3, HSB-2, K562, and U-937 were not susceptible. The period of time that target cells need to be infected with PI3V to achieve maximal cytotoxicity was determined. Target cells were infected with PI3V and MC, added 1 h later. After the addition of effector cells, significant levels of cytotoxicity were recorded at 17 h. Maximal cytotoxicity occurred 22 to 24 h postinfection. To define the optimal time that MC must be present, cells were infected with PI3V for a total of 24 h, and MC were left in contact with target cells for various time intervals. Maximal cytotoxicity was recorded when effector cells were present for 20 h, suggesting that a period of activation was needed to stimulate effector cell function. Removal of adherent mononuclear cells on Sephadex G-10 columns did not reduce the low level of cytotoxicity against uninfected target cells, but markedly reduced the level of cytotoxicity against PI3V-infected cells. The effector cell was nonphagocytic and nonadherent. These characteristics and the fact that target cell lysis was independent of genetic restriction indicate that effector cells are similar to natural killer cells described in other species.

Recent recognition of natural cytotoxicity has substantially altered the modern concepts concerning the potential mechanisms of resistance against tumor growth and viral infection. Natural cytotoxicity is present in the absence of apparent disease or deliberate immunization (2, 26) and is directed against neoplastic (23) and virus-infected cells (2, 26). Most work dealing with natural cytotoxicity has been done in human beings and mice where natural cytotoxicity is mediated by natural killer cells. Natural killer cells have been identified as nonadherent, nonphagocytic lymphocytes with receptors for the Fc portion of immunoglobulin G (IgG) (13) and are neither conventional B nor T lymphocytes (reviewed in reference 22). Natural cytotoxicity is not genetically restricted with respect to effector and target cell, nor is it antigen specific as is cytotoxicity mediated by T lymphocytes (reviewed in reference 14). Initial studies suggested that natural cytotoxicity in mice was directed against endogenous C-type-virus-associated antigens (13), but recent evidence indicates that natural cytotoxicity is present against a wide range of specificities, including xenogeneic (1, 12) and normal cells (19). However, some degree of selectivity must exist since some target cells are highly sensitive to cytolysis, whereas others are not.

Natural cytotoxicity has been described in several species other than humans and mice (1, 5, 20, 27). Work with natural cytotoxicity in cattle has been limited to a few recent reports. Bovine mononuclear cells (MC) from peripheral blood leukocytes of normal calves have been reported to be cytotoxic for squamous cell carcinoma cells (A. H. Cochrane, R. J. F. Markham, S. J. Kleinschuster, and C. C. Muscoplat, Abstr. 61st Annu. Meet. Conf. Res. Workers in Animal Disease, 1980, no. 255, p. 47). Also, antibody-independent cellular cytotoxicity by lung lavage cells has been demonstrated in cattle against parainfluenza type 3 (PI3V)-infected target cells (29) and infectious bovine rhinotrachei-

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tis virus (IBRV)-infected targets (M. J. P. Lawman, M. Campos, C. R. Rossi, and H. S. Jones, unpublished data). Furthermore, peripheral blood leukocytes from calves during lethal infection with *Theileria parva* have been found to be cytotoxic for allogeneic lymphoblastoid cells infected with *T. parva* and xenogeneic cells (YAC-1 mouse tumor cells) (10).

Because natural cytotoxicity against tumor cells is present before tumor development and increases early after certain infections (2, 4, 33), natural cytotoxicity may be a first line of defense against tumor cells and viral infections.

The purpose of this investigation was to analyze the ability of peripheral blood MC from normal cattle for their ability to mediate natural cytotoxicity, using a ^{51}Cr-release microcytotoxicity assay.

MATERIALS AND METHODS

Target cells. Human cell lines MOLT-3, HSB-2, K562, and U-937 (obtained from C. M. Balch, Department of Surgery and Microbiology, University of Alabama in Birmingham) were maintained in suspension in RPMI 1640 containing 10% heat-inactivated (56°C, 0.5 h) fetal bovine serum (FBS) and 2 μg of gentamicin per ml (gentamicin sulfate; Shering Corp., Kenilworth, N.J.). Primary bovine cell cultures obtained from whole embryos (BE cells), bovine testicles, and a permanent cell line (GBK) were maintained as monolayer cultures in Eagle minimal essential medium (MEM) with 10% FBS, 200 U of penicillin per ml, 10 μg of streptomycin per ml, and 100 μg of neomycin per ml. Cultures were incubated at 38°C.

Viruses. The Cooper strain of IBRV (4×10^7 50% tissue culture infective doses per ml), a herpesvirus, the SF-4 strain of PI3V (4×10^6 50% tissue culture infective doses per ml), a paramyxovirus, and a noncytopathogenic strain of bovine viral diarrhea virus (NC-BVDV) (untitrated), a pestivirus, obtained from persistently infected GBK cells, were used as inocula in the microcytotoxicity assay.

Serum neutralization test. A standard serum neutralization technique previously described by Rossi and Kiesel (24) was used to determine the presence of antibody against PI3V or IBRV in serum from the animals, as well as from the FBS used in this experiment. Briefly, a drop (0.025 ml) of serial twofold dilutions of the serum was incubated with a drop (0.025 ml) containing 100 to 200 50% tissue culture infective doses of PI3V or IBRV at 38°C for 1 h. A drop (0.025 ml) containing 300,000 cells per ml was added to each well, and the cultures were incubated at 38°C in a humidified atmosphere of 95% air and 5% CO_2. Cytopathic effects were read 4 and 6 days later.

Animals. Cattle (12 to 48 months old), free of serum-neutralizing antibodies to IBVR and PI3V, were used.

Preparation of effector cells. Blood was withdrawn by venipuncture with 0.15 M sodium citrate (1 ml/10 ml of blood) as anticoagulant. Buffy coat cells were obtained by centrifugation of whole blood for 20 min at 400 × *g*. MC were obtained by placing a mixture of buffy coat cells and autologous plasma on Histopaque (Sigma Chemical Co., St. Louis, Mo.), density 1.077 g/cm^3, and centrifuging at 600 × *g* for 12 min. Cells recovered from the interface were 98 to 100% mononuclear, 5 to 9% phagocytic as determined by latex ingestion (18), and 95 to 97% viable as determined by trypan blue exclusion.

Adherent cells were removed from MC on a Sephadex G-10 (Pharmacia Fine Chemicals, Piscataway, N.J.) column by the technique described by Jerrells et al. (16). Briefly, a column was made in a 10-ml plastic syringe barrel plugged with a small piece of nylon wool at the bottom and covered by 6 to 8 ml of Sephadex G-10. The column was washed with 50 ml of RPMI 1640 containing 20% FBS and incubated at 38°C for 15 min before the addition of MC. A 2-ml amount of MC adjusted to 3×10^7 to 5×10^7 cells per ml was placed on the column. The column was incubated at 38°C for 30 min and stirred with a Pasteur pipette every 10 min during the incubation period. Nonadherent cells were eluted with 40 ml of prewarmed RPMI 1640 containing 20% FBS. Effluent nonadherent mononuclear cells (NAMC) were more than 95% viable and had less than 0.01% phagocytic cells.

Cytotoxicity assay. Microcytotoxicity assays were performed in round-bottom microtiter plates at 38°C in a humidified atmosphere of 95% air and 5% CO_2. Bovine cells were seeded at 1×10^4 to 1.5×10^4 cells per well. After cell attachment, medium was removed, and 100 μl of RPMI 1640 containing 1 μg of gentamicin per ml, 2 mM L-glutamine, 10% FBS, and 1.5 to 2 μCi of sodium chromate ($Na_2{}^{51}CrO_4$; New England Nuclear Corp., Boston, Mass.) was added to each well and incubated overnight. The ^{51}Cr-labeled medium was removed, and 100 μl of RPMI 1640 containing 10% FBS was added to each well for further incubation (2 to 4 h). The plates were finally washed two times with Hanks balanced salt solution. Virus suspensions (25 μl) were added to each well, and microtiter plates were incubated for 1 h. Viral inocula were removed, and effector cells (100 μl) were added to each well at various dilutions to obtain different effector-to-target-cell ratios. Control wells consisting of virus-infected and noninfected target cells without effector cells were prepared at the same time to determine the amount of spontaneous ^{51}Cr release. Eight wells were treated with 100 μl of 3% Triton X-100 (Sigma) to determine maximum releasable ^{51}Cr. The assay was incubated for 18 h unless otherwise indicated. At the termination of the assay, the supernatant was collected with the Titertek harvesting system (Flow Laboratories, McLean, Va.), and the amount of ^{51}Cr released was determined in a Beckman 5500 counting system (Beckman Instruments Inc., Irvine, Calif.). Spontaneous release for uninfected cells and IBRV- and NC-BVDV-infected cells was always less than 1% per h; for PI3V-infected cells it was always less than 1.5% per h.

Human target cells were labeled in suspension with 1 ml of RPMI 1640 containing 10% FBS and 100 μCi of $Na_2{}^{51}CrO_4$ for 1.5 h at 38°C. Cells were washed three times in Hanks balanced salt solution and suspended at 1×10^5 cells per ml. A 100-μl amount of cell suspension was incubated with various concentrations of effector cells in a final volume of 200 μl. Microtiter plates were incubated for 4, 10, or 18 h. Wells without effector cells were used as controls.

Specific cytotoxicity was calculated as follows:

$$\text{Percent specific cytotoxicity} = \frac{\text{effector cell release} - \text{medium control release}}{\text{total releasable } ^{51}\text{Cr} - \text{medium control release}} \times 100$$

RESULTS

Natural cytotoxicity. MC from six adult cattle lysed PI3V-infected BE cells but did not lyse uninfected cells. The degree of cytotoxicity was dependent on the ratio of effector to target cells used (Table 1).

MC from yearling calves produced greater lysis of uninfected than of IBRV-infected BE cells. There was no difference in the degree of cytotoxicity in NC-BVDV-infected and uninfected BE cells. Calf MC, as adult MC, produced greater lysis of PI3V-infected than uninfected BE cells (Table 2). Cytotoxicity against PI3V-infected GBK and bovine testicle cell targets was also evaluated. There was greater cytotoxicity to PI3V-infected than to uninfected GBK or bovine testicle cells (Tables 3 and 4), and only MC from one calf (no. 82) were not cytotoxic against PI3V-infected bovine testicle cells (Table 4). IBRV-infected GBK or bovine testicle cells were not susceptible to lysis (Table 3). MC from eight animals showed no natural cytotoxicity against human cell lines K562, U-937, MOLT-3, and HSB-2.

Effect of contact time among effector cells, PI3V, and target cells on natural cytotoxicity. To determine the optimal length of time that PI3V must be present on target cells, BE cells were infected, and 1 h later MC were added. The assay was terminated at 4, 12, 17, and 23 h after infection. At 17 h of infection, low but significant levels of cytotoxicity were recorded. Cytotoxicity increased and reached its highest level 23 h after infection (Fig. 1). At 24 h of infection with PI3V, spontaneous release of ^{51}Cr due to PI3V was too high for cell-mediated cytotoxicity to be observed.

To determine the time required for MC to cause significant lysis of PI3V-infected cells, BE cells were infected with PI3V at different intervals to complete 24 h of infection before harvesting; MC were added to each group at the same time, and MC were maintained in contact with PI3V-infected BE cells for 8, 12, 16, and 20 h. Although no cytotoxicity was found after 8 h of contact of MC with PI3V-infected BE cells, cytotoxicity was evident at 12 h. Maximal cytotoxicity was found at 20 h (Fig. 2). Further incubation times were not done because the previous results showed that spontaneous release produced by PI3V alone was more than 60%.

Natural cytotoxicity of NAMC. MC and NAMC from Sephadex G-10 columns produced the same level of lysis on uninfected BE cells.

TABLE 1. Natural cytotoxicity of MC from adult cattle against PI3V-infected BE cells[a]

Virus	% Specific ^{51}Cr release for:											
	Cow 132		Cow 714		Bull 1		Cow 191		Cow 197		Cow 715	
	100:1[b]	25:1	100:1	25:1	100:1	25:1	100:1	25:1	100:1	25:1	100:1	25:1
None	−2.0	−7.0	−2.1	−6.0	−1.7	−5.3	−1.4	−1.3	−0.1	−2.0	−2.4	−2.0
PI3V	27.8	22.7	37.0	−0.2	28.3	14.9	16.6	14.3	22.0	−1.2	7.2	3.0

[a] BE cells were infected with PI3V for 1 h. MC were added, and supernatants from cultures were harvested 18 h later.
[b] Effector-to-target-cell ratio.

TABLE 2. Natural cytotoxicity of MC from yearling cattle against PI3V-, IBRV-, and NC-BVDV-infected BE cells[a]

Virus	% Specific ^{51}Cr release for:											
	Calf 84		Calf 89		Calf 95		Calf 13		Calf 15		Calf 107	
	100:1[b]	25:1	100:1	25:1	100:1	25:1	100:1	25:1	100:1	25:1	100:1	25:1
None	29.0	8.4	9.2	7.0	12.0	8.2	3.6	1.5	2.7	1.9	9.4	0.7
IBRV	16.0	−3.0	−0.09	−4.0	3.8	−2.0	−7.0	−8.6	−6.3	−6.8	−0.4	−7.0
PI3V	36.5	4.0	20.3	−0.5	36.0	−0.9	19.0	5.3	17.0	−1.6	26.0	22.0
NC-BVDV	26.0	4.1	1.7	−1.0	8.1	7.7	1.8	1.4	7.1	3.2	10.0	8.7

[a] BE cells were infected with PI3V, IBRV, and NC-BVDV for 1 h. MC were added, and supernatants from cultures were harvested 18 h later.
[b] Effector-to-target-cell ratio.

TABLE 3. Natural cytotoxicity of MC from normal donors against IBRV- and PI3V-infected GBK cells[a,b]

Virus	% Specific ^{51}Cr release for:											
	Cow 132		Cow 191		Cow 367		Cow 714		Cow 715		Bull 1	
	100:1[c]	25:1	100:1	25:1	100:1	25:1	100:1	25:1	100:1	25:1	100:1	25:1
None	3.5	2.1	3.4	0.6	2.5	2.0	1.5	1.2	0.9	1.2	2.0	2.0
IBRV	0.4	1.7	0.9	0.7	1.2	−0.6	1.9	0.1	−0.5	−1.2	−0.8	0.5
PI3V	5.8	5.8	25.9	2.7	4.8	4.1	−1.5	2.4	4.9	−0.8	6.4	2.2

[a] Cell line persistently infected with NC-BVDV.
[b] GBK cells were infected with PI3V for 1 h. MC were added, and supernatants from cultures were harvested 18 h later.
[c] Effector-to-target-cell ratio.

But after removal of adherent cells, NAMC lysis of PI3V-infected cells was reduced 43 and 80% at effector-to-target-cell ratios of 100:1 and 25:1, respectively (Fig. 3). There was no significant difference in killing when FBS that had been and had not been heat inactivated was used in the assay; also, no antibody against PI3V or IBRV was found in the FBS used in these experiments.

DISCUSSION

Using virus-infected cells as targets, we demonstrated that PI3V-infected cells could be used as target cells to measure natural cytotoxicity of bovine MC. At 12 h after contact of MC and PI3V-infected cells, significant levels of cytotoxicity were recorded. As the time of contact between effector and target cells was increased, cytotoxicity increased and reached a maximum at 20 h of contact time (Fig. 2). The long time of MC-target cell incubation needed to demonstrate cytotoxicity suggests that cells mediating natural cytotoxicity need to be activated during the incubation procedure. These results are consistent with the work in human beings by Santoli et al. (25, 26), who found that virus-infected cells were lysed more efficiently after long incubation times with lymphocytes from normal donors. Because removal of adherent cells on Sephadex G-10 columns reduced, but did not eliminate, the cytotoxic capabilities of MC preparations against PI3V-infected cells without affecting the cytotoxicity of uninfected cells, it is possible that macrophages were involved as cytotoxic effector cells or activators of other cells mediating natural cytotoxicity or both. Activation of effector cells in viral systems has been attributed to interferon production during the assay (25, 28, 32). This boosting ability of interferon in natural cytotoxicity has also been demonstrated with either purified interferon preparations or interferon inducers (7, 9, 11, 15). The cell involved in interferon production varies among assay conditions (21, 31, 33). However, the central role of the macrophage in modulating natural cytotoxic activity by interferon production has been demonstrated by several investigators (6, 8, 17, 30). In the bovine system, Stott et al. (29) reported that cells obtained by lung lavage, identified as

TABLE 4. Natural cytotoxicity of MC from normal donors against PI3V-infected bovine testicle cells[a,b]

Virus	% Specific ^{51}Cr release for:					
	Calf 13		Calf 82		Calf 84	
	100:1[c]	25:1	100:1	25:1	100:1	25:1
None	1.2	−5.9	−2.4	−5.6	1.5	1.7
PI3V	12.0	−6.1	−5.4	−6.7	33.8	14.4

[a] Cell line persistently infected with NC-BVDV.
[b] GBK cells were infected with PI3V for 1 h. MC were added, and supernatants from cultures were harvested 18 h later.
[c] Effector-to-target-cell ratio.

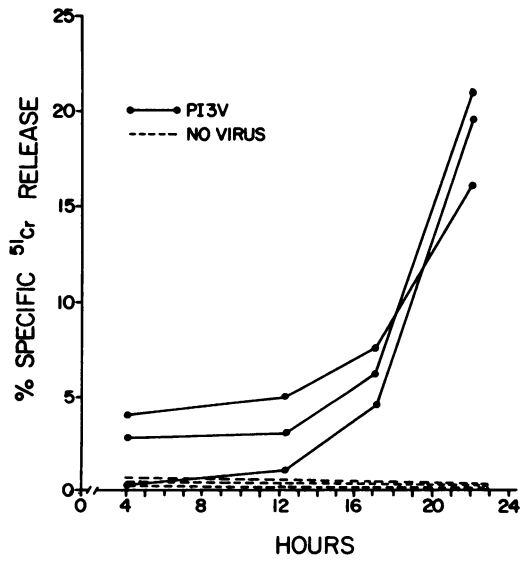

FIG. 1. Effect of contact time among effector cells, PI3V, and target cells on natural cytotoxicity. BE cells were infected, and 1 h later MC were added. Samples were assayed for released ^{51}Cr at 4, 12, 17, and 23 h after infection.

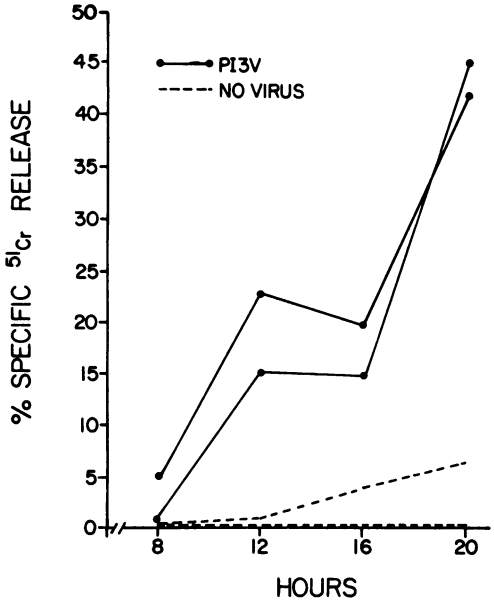

FIG. 2. Effect of contact time among effector cells, PI3V, and target cells on natural cytotoxicity. BE cells were infected with PI3V for a period of 24 h before the termination of the assay. Supernatants were harvested at 8, 12, 16, and 20 h after the addition of MC and assayed for released ^{51}Cr.

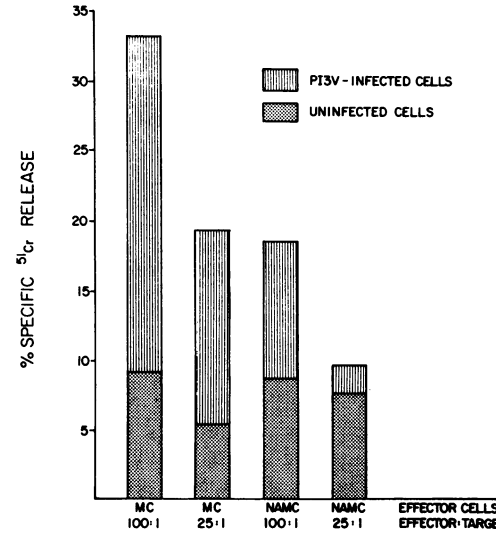

FIG. 3. Natural cytotoxicity of NAMC. Sephadex G-10 columns were used to remove adherent cells from the MC preparation. The effluent cells (NAMC) were compared with MC for their ability to mediate cytotoxicity against PI3V-infected cells.

alveolar macrophages, were cytotoxic for PI3V-infected targets in a long-term cytotoxicity assay; however, cytotoxicity was not consistently abolished when adherent cells were removed. The possibility that antibody was produced during the incubation time in our experiments and, therefore, was involved in an alternative cytotoxic mechanism against PI3V-infected cells seems unlikely since the amount of free virus present in our assay should have competed for antibody and prevented antibody attachment to viral antigens on cell membranes of infected cells. Also, unpublished observations in our laboratory have demonstrated that antibody-dependent cellular cytotoxicity mediated by MC against PI3V-infected cells is inefficient, even in the presence of high concentrations of antibody. Armerding and Rossiter (3) have shown that antibody produced during the incubation of effector and influenza virus-infected target cells in 12-h assays, plus complement present in unheated FBS, produces lysis of target cells. That complement present in FBS and antibody produced during incubation were not responsible for lysis of PI3V-infected cells in our assays was shown by the fact that heat inactivation of FBS did not reduce the amount of lysis. Furthermore, no serum-neutralizing antibody to PI3V was found in sera from experimental animals nor in the FBS used for culture.

NC-BVDV did not alter the susceptibility of uninfected cells. Whether this was due to a low amount of viral antigen or due to another factor(s) was not determined. Our results, in which normal cells were lysed to a greater degree than IBRV-infected cells, were similar to those of Welsh and Hollenbeck (32), who used normal mouse lymphocytes on herpes simplex virus-infected Vero cells.

Because considerable genetically unrestricted cytotoxicity to PI3V-infected target cells was found in nonadherent, nonphagocytic cells from bovine peripheral blood leukocytes obtained from antibody-free cattle, it is likely that the cytotoxicity detected was due to a cell similar to natural killer cells found in mice and human beings.

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