Non-Specific Natural Cytotoxic Factor Released from Bovine Peripheral Blood Lymphocytes

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ABSTRACT. Natural cytotoxicity against bovine leukemia cells (PC-3 cells) was found in bovine peripheral blood lymphocytes (PBL), and in non-adherent cells but not in adherent cells to nylon-wool column. Natural cytotoxic cells (NCC), which have natural cytotoxic activity, are found in T cell-rich fraction. When NCC were cocultured with PC-3 cells, natural cytotoxic factor (NCF) was released rapidly from NCC, and dose-response curve for NCF was almost linear induction. Cytotoxicity against PC-3 cells by NCC or NCF was increased with an increment of incubation period. Cytotoxicity against K562 cells, CL-1 cells, M1 cells or EL-4 cells by NCF was almost the same level as that against PC-3 cells, but that against those cell lines by NCC was not found. NCF activity in culture fluid from NCC cocultured with K562 cells or CL-1 cells was lower than that from NCC cocultured with PC-3 cells. —KEY WORDS: bovine, calf, natural cytotoxic cell, natural cytotoxic factor.

Potentiality of natural cytotoxicity in the peripheral blood lymphocytes (PBL) was observed in various kinds of animal species, such as humans [17, 19], mice [1, 7], rats [14], pig [18], bovine [3, 5, 11, 13], and canine [10]. Previous studies demonstrated that natural cytotoxic cells (NCC) activity in bovine PBL was low or even absent against various tumor cell lines [11] or cells infected with viruses [3]. In the human and mice, natural killer (NK) cells play a major role in potentiating natural cytotoxicity in PBL [1, 7, 17, 19]. NK cells in humans and mice released natural killer cytotoxic factor (NKCF) in culture supernatant when cocultured with target cells for given periods [20–22]. These results indicated that NCC activity in bovine PBL might be related with natural cytotoxic factor released from NCC. However, little is known about the nature of natural cytotoxic factor (NCF) released from bovine NCC. Recently evidences showed that NKCF consisted of protein [2] or lipid [4] and its molecular weight ranged from 5,000 to 42,000 [2].

The present paper reported that calf of cattle PBL had NCC activity against bovine leukemia cells (PC-3 cells) but not other target cell lines, and NCF released from NCC was found to be non-specific cytotoxicity against various target cell lines.

MATERIALS AND METHODS

Animals: All experiments were performed on normal male and female calves or cattle, and these animals were not infected with BHV-1.

Chemicals: 51Cr-sodium chromate (1 mCi/ml; 3.7×10^7 Bq/ml) was obtained from New England Nuclear Research Product, U.S.A.

Target cells: PC-3 cells derived from bovine lymphosarcoma of calf [9], K562 cells from human leukemia, CL-1 cells from canine leukemia, M1 cells from mice leukemia and EL-4 from mice leukemia were maintained in RPMI-1640 (Nissui Co., Japan) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, U.S.A.), 0.3 g/l of glutamine and 60 mg/l of kanamycin at 37°C in a humidified atmosphere containing 5% CO2. PC-3 cells were kindly provided by Professor Oki of Nihon Veterinary and Zoology University, and M1 cells by Dr. Hozumi of Institute of Saitama Gan center. PC-3 cells express bovine T-cell lymphoma associated antigen in cell membrane and herpesvirus-like particles in the extracellular spaces and the intracellular vacuoles [12].

Effector cells: Effector cells used were prepared from normal male and female cattle. The mononuclear cells were isolated from heparinized blood by Leucoprep (Becton Co., U.S.A.) centrifugation at 2800 r.p.m. for 30 min. For the removal of macrophages, mononuclear cells were incubated for 1 hr at 37°C in a 60 mm plastic petri dish (Falcon Co., #3002, U.S.A.), and then non-adherent cells were obtained as PBL. PBL (5–10×10^7) were further incubated for 60 min at 37°C, passed through nylon-wool column, and non-adherent lymphocytes were used as effector cells. Nylon-wool attached cells were collected by elution with 0.02% Na~2~EDTA solution (adherent lymphocytes). The yields of non-adherent cells were usually 0.5–1.0×10^6/ml.
of blood.

Preparation of NCF: Effector cells \((5 \times 10^6)\) in 1 ml of medium were cultured with \(1 \times 10^5\) target cells in 1 ml of medium for the given periods at 37°C. After incubation, culture supernatant as an NCF sample was collected by centrifugation at 2500 r.p.m. for 10 min. The supernatant was stored at \(-20\)°C before used.

Cytotoxic Assay: The cytotoxic assay against target cells has been previously described in detail [10]. Briefly, \(1 \times 10^6\) target cells were labeled with 100 μCi (3.7 MBq) of \(^{51}\)Cr-sodium chromate for 1 hr at 37°C in 0.3 ml of medium. After being washed three times, \(1 \times 10^5\) cells in 100 μl of medium were transferred into the well in a 96-well microplate (Falcon Co., #3076 U.S.A.). Various numbers of effector cells in 100 μl of medium were added to triplicate wells to give the effector: target (E/T) ratio ranging from 25:1 to 100:1. For cytotoxicity of NCF, various concentrations of culture supernatant in 100 μl of medium were added to triplicate wells in a 96-well microplate to give a range from 0 to 25%. After incubation at 37°C for 4 hr, each supernatant was collected and counted by a gamma scintillation counter (LB-951G, Berthold, Germany). The percent cytotoxicity (PC) was calculated as follows:

\[
PC = \frac{A - C}{A - B}
\]

where A is cpm for the max release from target cells, B is cpm for the spontaneous release from target cells, and C is cpm for the release from target cells during incubation with different numbers of effector cells or various concentrations of NCF sample.

RESULTS

Cytotoxicity of lymphocytes against PC-3 cells: Natural cytotoxic activity was observed in PBL and non-adherent lymphocytes fraction, but not in adherent lymphocytes fraction as shown in Fig. 1. The cytotoxicity of non-adherent lymphocytes against PC-3 cells was increased with an increment of the E/T ratio ranging from 25:1 to 100:1, and the natural cytotoxic activity in non-adherent lymphocytes was higher than that in PBL.

Kinetic of NCF release in culture supernatant: PBL was cocultured with PC-3 cells at the E/T ratio of 50:1 for the given period, and then culture supernatant was collected by centrifugation. The yield of

![Fig. 1. Natural cytotoxicity against PC-3 cells by various cell population fractionated from calf peripheral blood lymphocytes. PC-3 cells were cocultured with PBL (○), non-adherent lymphocytes (●) or adherent lymphocytes (△) at the E/T ratio ranging from 25:1 to 100:1 for 4 hr. All effector cells were prepared from one of calves.](image)

![Fig. 2. Kinetic of NCF released from stimulated PC-3 cells. Briefly, 5×10⁶ cells/ml of PBL was cocultured with 1×10⁵ cells/ml of PC-3 cells for the given period, and then culture supernatant was collected by centrifugation at 2,800 r.p.m. for 10 min. Natural cytotoxicity in culture supernatant was tested by ⁵¹Cr release assay as described in Materials and Methods.](image)
NCF was tested at 25% dilution of culture supernatant as shown in Fig. 2. NCF activity was increased with incubation period ranging from 0–120 min, and that reached plateau at over 120 min.

Dose-response of natural cytotoxicity by NCF: Figure 3 shows the cytotoxic effect of various concentrations of NCF sample, which was collected from culture supernatant of PBL, cocultured with PC-3 cells for 4 hr. Cytotoxicity of NCF was increased with an increase in concentration, and the dose-response curve of cytotoxicity by NCF was seemed to be almost linear.

Kinetics of cytotoxicity by NCC or NCF: Figure 4 shows time-dependency of NCC or NCF cytotoxic effect on PC-3. Cytotoxicity of NCC or NCF against PC-3 cells was increased with an increment of incubation time. Cytotoxicity curve by NCC was seemed to be almost linear, and that by NCF during the experimental period from 0 to 120 min was also seemed to be linear and reached plateau.

Cytotoxic activity of NCC or NCF against various target cells: Table 1 shows the specific cytotoxicity of NCC or NCF against various target cell lines. NCC activity at the E/T ratio of 50:1 and NCF activity of 25% dilution of culture supernatant were detected by 51Cr-release assay for 4 hr at 37°C. Cytotoxicity of NCC against PC-3 cells were higher than that against other cell lines. Cytotoxicity of NCF collected from NCC cocultivated with PC-3 cells for 4 hr at 37°C was found against all target cell lines, and the NCF activities against each target cell line were almost the same. The release of NCF from NCC was tested under NCC cocultured with PC-3 cells or K562 cells or CL-1 cells for 4 hr at 37°C. NCF activity in culture fluid from NCC cocultivated with PC-3 cells was higher than that from NCC cocul-

![Fig. 3. Dose-response curves of natural cytotoxicity by NCF. Target cells were treated with various concentrations of NCF for 4 hr. Each point was obtained from 3 independent experiments. Error bars mean standard deviation.](image)

![Fig. 4. Kinetics of cytotoxicity by NCC or NCF against PC-3 cells. Labeled PC-3 cells were cultured with PBL at 50:1 (C) as the E/T ratio or 25% of NCF (●) for the given period. The detail was described in Materials and Methods. Each point was obtained from 2 to 3 independent experiments. Error bars mean standard deviation.](image)

<table>
<thead>
<tr>
<th>Target cells</th>
<th>% Cytotoxicity</th>
<th>Release of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NCC</td>
<td>NCF</td>
</tr>
<tr>
<td>PC-3</td>
<td>35.1±1.9a</td>
<td>52.7±3.9</td>
</tr>
<tr>
<td>K562</td>
<td>4.7±2.4</td>
<td>55.5±8.9</td>
</tr>
<tr>
<td>CL-1</td>
<td>6.8±3.6</td>
<td>51.4±8.0</td>
</tr>
<tr>
<td>M1</td>
<td>6.8±2.9</td>
<td>54.7±6.7</td>
</tr>
<tr>
<td>EL-4</td>
<td>4.3±3.8</td>
<td>46.4±9.2</td>
</tr>
</tbody>
</table>

a) Mean±SD.
b) Not done.
tured with K562 or CL-1 cells.

DISCUSSION

Cytotoxicity of bovine PBL against PC-3 cells was higher than that against K562 cells and CL-1 cells which were sensitive to cytotoxicity of human and canine PBL, respectively. PC-3 cells were expressed as herpesvirus-like particles in extracellular spaces and intracellular vacuoles [12]. Cytotoxic effect was found in bovine PBL obtained from BHV-1 free calves or cattle against PC-3 cells. This might be due to natural cytotoxicity of PBL. Antigen independent cytotoxicity against bovine leukemia cells (PC-3 cells) was found in PBL and in non-adherent lymphocytes which were considerably T cell-rich fraction, but not in adherent lymphocytes. Previous studies indicated a comparatively low or even no natural cytotoxicity in bovine PBL when assayed in vitro against various allogeneic and xenogeneic tumor targets [11] and against bovine cells infected with lytic or non-lytic viruses [3]. However, natural cytotoxic activity against PC-3 cells was found to be higher than the other target cells. Palmer et al. [15] demonstrated that BHV-1 glycoproteins played a major role in the cytolytic response by bovine NK-like cells and these proteins were recognized by those effector cells. Therefore, high sensitivity of PC-3 cells against NCC might be due to infection with herpesvirus.

The natural killer cell, which was mainly composed of large granule lymphocyte, played a dominant role in causing natural cytotoxicity in mice [1, 7], human [17, 19] and rat [14]. Macrophages were removed from mononuclear fraction using the plastic adherent method. Therefore, natural cytotoxicity in calf or bovine PBL or non-adherent lymphocytes might be due to natural killer cells, not to macrophage. Natural killer cells released natural killer cytotoxic factor (NKCF) when effector cells were cocultured with target cells [6, 21, 22]. Graves et al. [6] demonstrated that the release of human NKCF was rapid, and that reached plateau over 2 hr [6]. Our result showed that release of NCF from calf or bovine PBL was also rapid, and reached plateau about 2-hr incubation.

Cytotoxicity of NCC or NCF was found to be dependent on incubation time with target cells, and an initial slope of time-dependence curve for NCF was found to be steeper than that for NCC. These results suggested that cytotoxicity of NCF was more direct than that of NCC, and NCC mediated cytotoxicity might be related to the release of NCF from activated NCC. Wright et al. [22] demonstrated that cytotoxicity by NK cells might be related with NKCF as results of NKCF release from NK-sensitive or -insensitive cell lines [22]. Our results showed that cytotoxicity of NCC was seemed to be specific cytotoxicity against target cells, but that of NCF was not. The release of NCF from cocultured NCC with PC-3 was more rapid than that with other target cells. Therefore, specific cytotoxicity of calf or bovine NCC may be corresponded to the specific recognition site or binding site which might be related to expression of individual proteins in the target cell surface. Two similar models for NK cells mediated cytotoxicity have been proposed; recognition and binding of the NK cell to a sensitive target cell, NKCF generated and released from the stimulated NK cells [8, 16]. The mechanisms of cytotoxicity by NCC might be including the release of NCF.

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REFERENCES


