Release of Natural Killer Cytotoxic Factor (NKCF) from Canine Natural Killer (NK) Cells Stimulated with Cytoplasmic Membrane of Target Cells

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ABSTRACT. The presence of canine natural killer cytotoxic factor (NKCF) and the mechanism of the release of the NKCF by canine natural killer (NK) cells were studied. The cytotoxicity in the culture supernatant of effector cells cultured with target cells was dependent on the count of NK cells. Therefore, this suggests that the cytotoxic factor in this culture supernatant is NKCF. The NK-sensitive live target cells stimulated the release of NKCF from NK cells, but the NK-insensitive target cells did not. Moreover, NKCF was also released from NK cells stimulated with either killed target cells or their cytoplasmic membrane as well as live target cells. These findings suggest that the structure of the cytoplasmic membrane of target cells is involved in the recognition, binding and the release of NKCF by NK cells.—**KEY WORDS:** NK, NKCF, target cytoplasmic membrane.

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Natural killer (NK) cells, a subpopulation of lymphocytes derived from an unimmunized host, lyse certain tumor cell lines and virus-infected cells without major histocompatibility complex (MHC) restriction. In humans, mice and rats, the natural killer cytotoxic factor (NKCF) has been reported to play a major role in the mechanism of NK cell-mediated cytotoxicity [2, 4, 11, 16, 19]. However, the mechanism of NKCF release from NK cells has not been clear in humans, mice and rats. In dogs, the even presence of NKCF has not been demonstrated. In the present study, the presence of canine NKCF and the factors involved in the release of NKCF from canine NK cells were examined.

The peripheral blood lymphocytes (PBLs), and T-cell and B-cell subpopulations derived from clinically healthy three to four-year-old male and female dogs were used as effector cells. These subpopulations were prepared as described previously in detail [8]. The T-cell subpopulation was then fractionated by centrifugation through a discontinuous density gradient of Percoll (s.g.=1.130±5, Sigma Chemical Co., U.S.A.) with the modifications of the method described by Timonen and Saksela [17]. CL-1 cells derived from canine leukemia were used as canine NK-sensitive target cells (%cytotoxicity=44±13.9) [7] and PC3 cells from bovine lymphosarcoma, kindly provided by Dr. Y. Oki, Nihon Veterinary and Zoology University, were used as canine NK-insensitive target cells (%cytotoxicity=4.0±2.9). PC3 cells express the bovine T-cell lymphoma associated antigen in the cell membrane and herpesvirus-like particles in the extracellular spaces and the intracellular vacuoles [10]. The target cells were divided into three groups; the live (untreated) CL-1 cells or PC3 cells, the killed CL-1 cells or PC3 cells treated by freezing and thawing, and the cytoplasmic membranes of CL-1 or PC3 cells. The viability of CL-1 or PC3 cells was confirmed by the trypan blue exclusion test [13]. The cytoplasmic membrane was prepared by the method described by Koizumi *et al.* [6]. In PBLs, and T-cell and B-cell subpopulations, the effector: target (E/T) ratio was 25:1. In the case of cells from the Percoll fractionation, the E:T ratio was 2.5:1. The NKCF was collected by the

method of Kosaka *et al.* [8] and by modification of the method described by Reiter *et al.* [14]. Briefly, 1×10^5 /ml of target cells were co-cultured with either 5×10^6 /ml or 1×10^6 /ml of effector cells alone or together with 100 µg/ml of membrane protein. After incubation for 4 hr, the culture supernatant was collected by centrifugation and used as NKCF samples. The cytotoxic assay has been described previously in detail [15]. The percent cytotoxicity was calculated by the standard formula [8, 15], and expressed as the mean value of quadruplicate cultures. Morphological observation of the effector cells was performed as described previously [15]. Large granular lymphocytes (LGL) have already been reported as morphological NK cells [1, 8, 15, 17, 18, 20]. The number of LGL per 100 cells in T-cell fractions separated by a discontinuous percoll gradient centrifugation was compared.

The cytotoxicity of PBL, the T-cell subpopulation and the B-cell subpopulation was $28 \pm 2\%$, $32 \pm 8\%$, and $-2.5 \pm 4\%$, respectively, when their cytotoxicity against NK-sensitive CL-1 cells was measured by a ⁵¹chromium (⁵¹Cr) release assay. Canine NK cell-mediated cytotoxicity was found to be the highest in the T-cell subpopulation which was fractionated into the 35–40% percoll density by a discontinuous gradient centrifugation (Fig. 1). Furthermore, enrichment of LGL was observed in the cell fraction with the 35–40% percoll density (Fig. 1). The supernatant was collected effector cells co-cultured with target CL-1 cells. The cytotoxicity of the culture supernatant in PBL was $25 \pm 6\%$, that in the T-cell subpopulation was $38 \pm 8\%$, and that in the B-cell subpopulation was $6 \pm 4\%$. The cytotoxicity of culture supernatants in each fractionated effector cells also showed a similar tendency to that of NK cells (Fig. 1). Therefore, PBLs cytotoxicity was mediated by the NKCF released from NK cells. The cytotoxic activity of the NKCF obtained from PBLs co-cultured with the killed CL-1 cells was similar to that of the NKCF of NK cells co-cultured with the live CL-1 cells (Fig. 2). On the other hand, the NKCF obtained from PBLs cultured without target cells or with the killed PC3 cells showed low cytotoxicity (Fig. 2). These findings

Fig. 1. Natural cytotoxic activity of the cells and NKCFs in T-cell subpopulations fractionated using a discontinuous percoll gradient, and number of large granular lymphocytes (LGL) per 100 cells in each fraction. Cytotoxicity (%) of the cells and NKCFs are shown by closed circles and open circles, respectively. Each bar shows the mean of the relative number of LGL in each fraction. Each point shows the means of % cytotoxicity. Short vertical bars represent mean standard errors.

suggest that the release of NKCF from NK cells is required for the stimulation of live or killed target cells.

The release of NKCF from NK cells was observed at 5 min after co-culture with CL-1 target cells, and increased with incubation time (data not shown). Thus this induction may occur at the early stage of NK cell-mediated cytotoxicity. Moreover, the NK-sensitive CL-1 cytoplasmic membranes could induce the release of NKCF from NK cells, however, the NKCF obtained from NK cells co-cultured with the PC3 cytoplasmic membranes showed the low cytotoxicity (Fig. 3). Reiter *et al.* [14] also reported that the release of human NKCF from NK cells was induced by the NK-sensitive target cell (K562 or U-937) membrane, but not by the NK-insensitive target cell (GASH-P) membrane. Therefore, the release of NKCF from canine NK cells might be related to the binding site on the target cell surface. In the NK cell-mediated cytotoxicity, NK cells may recognize and bind to target cells, which stimulate the NKCF release

Fig. 2. Release of NKCF from PBLs stimulated with live or killed target cells. PBLs were co-cultured with either live CL-1 cells, live PC3 cells, killed CL-1 cells, or killed PC3 cells at a E/T ratio of 50:1 for 4 hr. The culture supernatants were collected as NKCF samples. The CL-1 cells were co-cultured with 20% of each NKCF sample for 4 hr. Cytotoxicity (%) of NKCFs against live or killed target cells are shown by open bar or closed bar. Refer Fig. 1 for explanation of each point.

from NK cells, and then, give rise to cell lysis [5]. An NKCF-inducing receptor was found to be localized on the cytoplasmic membrane of NK-sensitive target cells [14]. The existence of receptor(s) responsible for NK cells has also been suggested by other investigators [3, 14]. Therefore, the receptor(s) relevant to NK cell recognition and binding might exist in the cytoplasmic membrane of target cells, and stimulate the release of NKCF from canine NK cells.

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Fig. 3. Release of NKCF from PBLs stimulated with live CL-1 cells or PC3 cells and cytoplasmic membranes of CL-1 cells or PC3 cells. PBLs were co-cultured with live CL-1 cells (○) or PC3 cells (□) at a E/T ratio of 50:1 or 100 μ g protein/ml of cytoplasmic membranes of CL-1 cells (●) or PC3 cells (■) for 4 hr. The culture supernatants were used as NKCF samples. Refer Fig. 1 for explanation of each point.

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