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Ultrastructure and Cytochemical Staining Characteristics of Canine Natural Killer Cells

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ABSTRACT ***Background*: The purpose of this work was to describe the ultrastructure and cytochemical staining characteristics of canine peripheral blood lymphocytes with natural killer (NK) cell activity, with comparison made to non-NK lymphocytes.**

***Methods*: Canine lymphocyte populations evaluated for ultrastructure, cytochemical staining, and NK function (by ^{51}chromium release assay) included: peripheral blood lymphocytes; lymphocytes from band 1 (NK-enriched), band 2, and the pellet of a 45/50% percoll gradient; lymphocytes from the supernatant fluid (non-conjugated lymphocytes) and pellet (lymphocytes conjugated to tumor cell targets) of a 17% percoll gradient; and null ($CD4^-CD8^-$) and $CD4^-CD8^+$ lymphocytes.**

***Results*: NK activity was concentrated in band 1 lymphocytes of the 45/50% percoll gradient with further enhancement of activity occurring in sorted null cells. Canine NK cells were 5.5 to 6.5 μm in diameter with a reniform (kidney bean shape) nucleus, and electron-dense cytoplasmic granules. NK cells (percoll band 1 cells and null cells) had larger cell and nuclear area, and less round nuclei when compared to non-NK lymphocytes. The overall cytochemical staining (chloracetate esterase, peroxidase, sudan black B, naphthyl acetate esterase, naphthyl butyrate esterase periodic acid-Schiff stain, and acid phosphatase with and without tartrate) pattern was similar in all the lymphocyte populations evaluated.**

***Conclusions*: This work confirms the usefulness of a 45/50% percoll gradient in obtaining a NK-enriched fraction of canine lymphocytes, and shows further enhancement of NK activity in sorted $CD4^-CD8^-$ cells. The ultrastructure of canine NK cells is similar to that reported for human NK cells, but is different from that of other canine peripheral blood lymphocytes. Standard cytochemical staining does not discriminate canine NK cells from other lymphocytes.**

Key words: NK cell, Dog, Ultrastructure

Natural killer (NK) cells are a population of lymphocytes which lyse a variety of tumor cells in vitro, and which are postulated to play a major role in host resistance to tumor growth and metastasis in vivo (Herberman and Ortaldo, 1981; Trinchieri, 1989; Anderson, 1992). In humans, lymphocytes with NK activity have been identified as large granular lymphocytes recognized by azurophilic granules in the cytoplasm, reniform (kidney bean shape) nuclei, and high cytoplasmic to nuclear ratio (Timonen et al., 1981; Neighbour et al., 1982; Kang et al., 1987). NK activity has been measured by ^{51}chromium-release assay in peripheral blood lymphocytes from several species including the dog (Knapp et al., 1993). Although the morphology of canine lymphocytes possessing NK activity has been reported briefly (Loughran et al., 1985; Knapp et al., 1993), a complete description of canine NK cells has not been published. We report here the ultrastructure and cytochemical staining characteristics of canine

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lymphocytes exhibiting NK cell activity, with comparison made to lymphocytes without NK activity. These findings are important in veterinary and comparative immunology in which several canine models of human diseases are under investigation.

MATERIALS AND METHODS

Subjects

Blood was obtained from 12 healthy, mixed breed dogs. All dogs were vaccinated against common infectious diseases and were free of disease at the time of use. The care and use of the dogs was approved by the Purdue University Animal Care and Use Committee.

Lymphocyte Isolation and Percoll Enrichment of NK Cell Populations

Whole blood from the dogs was collected by venipuncture into EDTA(K_3) vacutainer tubes (Becton-Dickenson, Rutherford, NJ). Mononuclear cells were isolated by centrifugation over a two-layer ficoll/hypaque gradient (specific gravity of two layers: 1.066/1.119) (Histopaque 1.119, Sigma Chemical Co., St. Louis, MO) as previously described (Wunderli and Felsburg, 1989; Knapp et al., 1993). Isolated mononuclear cells were resuspended in "incubation medium" (RPMI 1640 supplemented with 10% fetal bovine serum and 1% L-glutamine (Sigma Chemical Co.), and were incubated in 75 cm^2 culture flasks (at a density of less than 50×10^6 cells per flask) at 37°C (5% CO_2) for 1 hour. The nonadherent cells (lymphocytes) were collected.

A 45/50% percoll gradient was used to obtain a population of canine lymphocytes with maximal NK activity as previously described (Knapp et al., 1993). Briefly, percoll (Sigma Chemical Co.) was diluted with 1.5 M saline to yield 90% percoll, and was further diluted with RPMI 1640 medium (without serum) to yield the specific concentrations (45 and 50%) needed. Four milliliters of 45% percoll were carefully layered onto 4 ml of 50% percoll in a 15 ml centrifuge tube. Isolated lymphocytes in incubation medium were layered onto the percoll gradients (maximum of 50×10^6 cells in 2.0 ml media), and centrifuged in a fixed angle rotator at 500*g* for 30 minutes. The band of lymphocytes at the interface of the media and 45% percoll layer was removed by pasteur pipette, and the cells were washed twice in PBS. These lymphocytes (NK-enriched lymphocytes) were used in conjugate studies, were sorted to obtain null cells as described below, and were evaluated by light and electron microscopy. NK activity was measured by ^{51}chromium release assay (Knapp et al., 1993). Lymphocytes from band 2 (cells at the interface of 45/50% percoll layers) with minimal to moderate NK activity, lymphocytes from the pellet (small lymphocytes without NK activity), and lymphocytes not fractionated by gradient were also evaluated for comparison.

Conjugate Studies

Studies were performed to evaluate NK cells that formed conjugates with NK-sensitive tumor target cells (canine thyroid adenocarcinoma, CTAC) (Kasza, 1964; Krakowka, 1983; Knapp et al., 1993). Conjugation is considered a key step in NK cell killing of target cells (Bonavida and Wright, 1986). NK-enriched lymphocytes (percoll band 1 cells) and CTAC target cells at E:T (effector:target) ratios of 5:1 and 25:1, were allowed to attach in normal incubation medium during a 20-minute period at 37°C and 5% CO_2. The cell suspension was then gently layered onto a 17% percoll gradient in a 15 ml centrifuge tube, and centrifuged (SBR) at 40*g* for 8 minutes (Leibnitz, 1988). The pellet (lymphocyte/target cell conjugates) and the supernatant (nonconjugated cells) were examined with light and electron microscopy.

Isolation of Canine Null ($CD4^-CD8^-$) Lymphocytes

The NK-enriched lymphocyte fraction was washed with Hanks balanced salt solution with 1% L-glutamine and 1% bovine serum albumin (HBSS/G/BSA, Sigma Chemical Co.), labeled with mouse anti-canine CD8 (a gift of D.H. Gebhard, North Carolina State University, Raleigh, NC) (Gebhard and Carter, 1992), and incubated for 30 minutes at 4°C. The lymphocytes were washed with HBSS/G/BSA and labeled with FITC-conjugated goat anti-mouse IgG (Caltag Laboratories, San Francisco, CA) to label the CD8 positive cells. After washing with HBSS/G/BSA, mouse anti-canine CD4 (a gift of D.H. Gebhard, North Carolina State University, Raleigh, NC) (Gebhard and Carter, 1992) was incubated with the cells for 30 minutes at 4°C and the cells washed with HBSS/G/BSA. The $CD4^+$ cells were labeled with phycoerythrin-conjugated goat anti-mouse IgM (Tago Immunochemicals, Burlingame, CA). After washing, the cells were analyzed and sorted on an EPICS Elite flow cytometer (Coulter Corporation, Hialeah, FL). Controls consisted of unlabeled cells, and cells incubated with secondary reagents only (CD8 [IgG-FITC] and CD4 [IgM-PE]). The lymphocyte fraction was discriminated using forward angle light scatter (FALS) and 90° light scatter (90LS) to separate any contaminating neutrophils. The null fraction ($CD4^-CD8^-$) and $CD4^-CD8^+$ fraction were evaluated for ultrastructure and NK activity by ^{51}Cr-release assay (Knapp et al., 1993).

Ultrastructural Studies

Electron microscopy was used to examine peripheral blood lymphocytes (not fractionated by percoll gradient); lymphocytes from band 1 (NK-enriched), band 2, and the pellet of the percoll gradient; the supernatant (nonconjugated) and pellet (conjugated) lymphocytes from the conjugate studies; and the sorted $CD4^-CD8^+$ and null ($CD4^-CD8^-$) lymphocytes.

Cell pellets of the different isolated fractions were fixed in 3% phosphate buffered glutaraldehyde (pH 7.3) overnight. After rinsing in buffer, cells were post-fixed in 1% osmium tetroxide-1.5% potassium ferrocyanide in phosphate buffer for 1.5 hours at 4°C. Systematic photography of the thin sections at a magnification of 2,600 diameters was performed on a JEOL JEM-100CX transmission electron microscope. The negatives were photographically enlarged 2.5 times and the positive image digitized via a CCD camera (Hitachi HV-C11U, Tokyo, Japan) and a Frame grabber (Oculus TCX, Coreco, St Laurent, Canada). Cells were evaluated for overall morphology including granularity. Cell and nuclear size (area and perimeter), and cell and nuclear shape factor were assessed using image analysis soft-

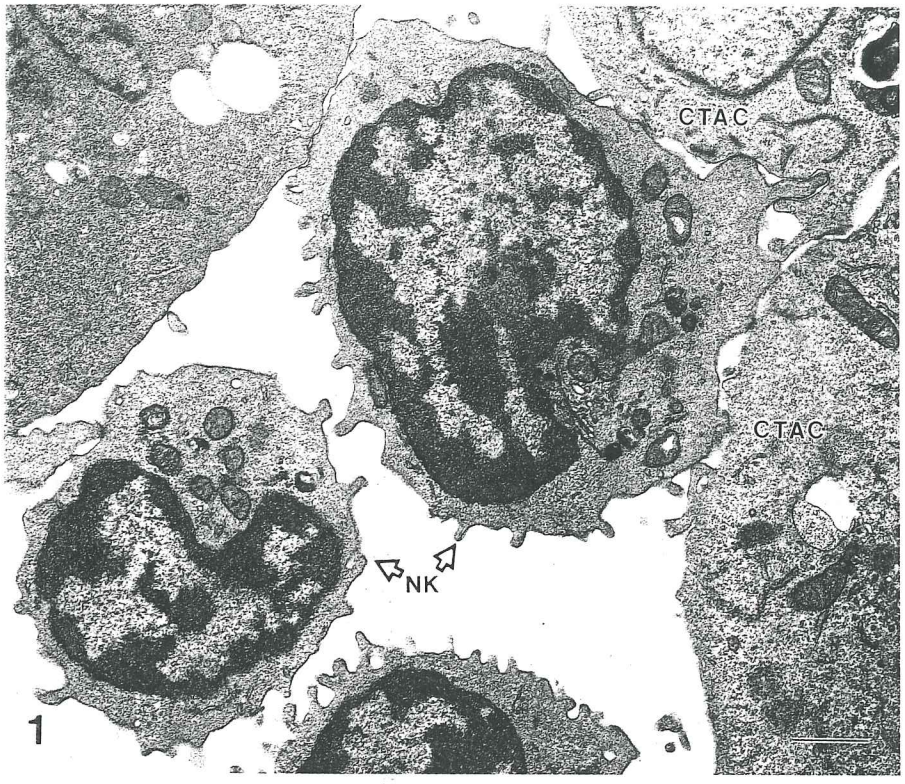

Fig. 1. Conjugate lymphocyte (NK cell) with CTAC target cell. Note interdigitation of NK cell and CTAC target cell. CTAC, canine thyroid adenocarcinoma cell; NK, natural killer cell. Bar = 1 μm.

ware (Optimas 4.02, Edmonds, WA). The shape factor was determined by the formula: $4\Pi X$ area/perimeter^2.

Cytochemical Staining

Cytochemical staining was performed on cytocentrifuge prepared slides of: peripheral blood lymphocytes (not fractionated by percoll gradient); of lymphocytes from band 1, band 2, and the pellet of the percoll gradient; and of cells from the supernatant (nonconjugating) and pellet (conjugating) of the conjugate studies. The following stains were applied: acid phosphatase with and without tartrate, chloracetate esterase, naphthyl acetate esterase, naphthyl butyrate esterase, peroxidase, sudan black B, and periodic acid-Schiff (Sigma Chemical Co.). For each lymphocyte fraction, 500 lymphocytes were evaluated and the percentage of negative, weakly positive or positive cells was determined based upon characteristic staining patterns described by the manufacturer of the stain. Peripheral blood smears from clinically normal dogs were used as controls for all stains and staining batches.

Statistical Analysis

Comparison between conjugating and non-conjugating lymphocytes was made using an unpaired, Student's two-tailed t-test. Statistical comparisons between the peripheral blood lymphocytes, percoll band 1 cells, percoll band 2 cells, $CD4^-CD8^+$ cells, and $CD4^-CD8^-$ cells were made using the Kruskal-Wallis nonparametric ANOVA test, followed by Dunn's multiple comparisons test (Instat, Graphpad, San Diego, CA).

RESULTS

^{51}Chromium release assays confirmed previous findings (Knapp et al., 1993) that lymphocytes from band 1 of the percoll gradient had greater NK activity than other cells from the percoll gradient and from peripheral blood lymphocytes (data not shown). The NK activity was further enhanced by isolation of null ($CD4^-CD8^-$) cells from other cells in the percoll band 1. At an effector:target ratio of 50:1, the percent specific lysis by the percoll band 1 cells (NK-enriched lymphocytes) was 20% compared to 45% by the null cells.

Similar ultrastructure was observed for percoll band 1 lymphocytes, null cells, and lymphocytes which formed conjugates with CTAC cells. Lymphocytes which formed conjugates with CTAC target cells were 5.5 to 6.5 μm in diameter with a reniform (kidney bean shape) nucleus (Fig. 1). Cross-sections through the center (as indicated by the presence of centrioles or Golgi complex) of the lymphocytes usually revealed one to three electron-dense cytoplasmic granules which varied in density (Figs. 2,3). The percentages of cells containing electron dense granules and reniform shape nuclei were determined (Table 1).

The cell and nuclear areas of the different lymphocyte fractions are reported in Figure 4. Both the cell area and nuclear area of percoll band 1 cells, percoll band 2 cells, and null cells were significantly greater

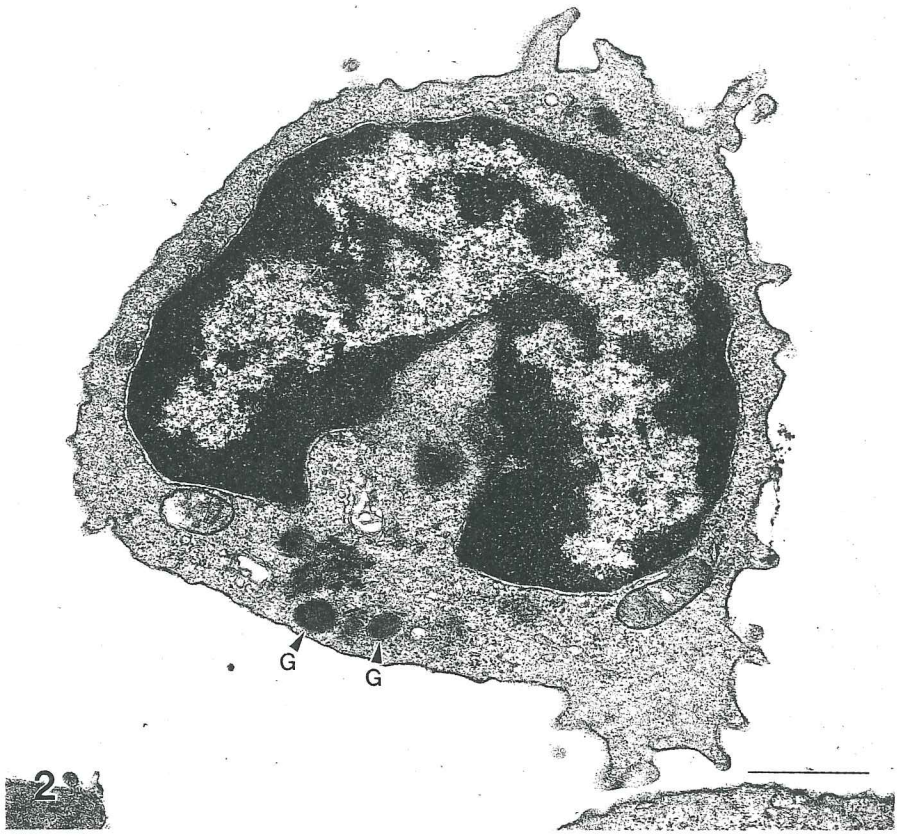

Fig. 2. NK cell. Depending upon the plane of section, one to three electron dense granules 0.3–0.35 μm diameter could be observed in the cytoplasm on the concave side of the reniform nucleus. G, granule. Bar = 1 μm.

($P < .001$, Dunn's multiple comparisons test) than that of peripheral blood lymphocytes. Similarly, the cell area of percoll band 1 cells, of percoll band 2 cells, and of null cells was significantly greater ($P < .05$, Dunn's multiple comparisons test) than that of $CD4^-CD8^+$ cells, with the difference between null cells and $CD4^-CD8^+$ being most significant ($P < .001$). The nuclear area of null cells was also significantly greater ($P < .05$, Dunn's multiple comparisons test) than that of the $CD4^-CD8^+$ cells.

The cell and nuclear shape factor of the different lymphocyte fractions are reported in Figure 5. A shape factor of 1 represents a perfect circle. Numbers less than 1 indicate less roundness. The cell shape factor of percoll band 1 cells, percoll band 2 cells, and of the null cells was significantly less (less round) ($P < .01$, Dunn's multiple comparisons test) than that of peripheral blood lymphocytes. The nuclear shape factor was not significantly different between percoll band 1 and 2 cells, null cells, of peripheral blood lymphocytes. The nuclear shape factor of band 1 cells was significantly smaller (less round) than that of $CD8^+$ cells ($P < .05$).

The cell area and shape, and nuclear area and shape of conjugating and nonconjugating lymphocytes are reported in Figure 6. Although overall cell area and shape were not different, the nuclei of the conjugating lymphocytes were less round than the nuclei of the nonconjugating lymphocytes when the plane of section was near the center of the cell, as indicated by the presence of centrioles or Golgi complex ($P < .006$, two-tailed t-test).

After cytochemical staining, lymphocytes from all fractions were negative for chloracetate esterase, peroxidase, and sudan black B stains. Weakly positive focal staining of lymphocytes from different fractions was noted for naphthyl acetate esterase, naphthyl butyrate esterase, and periodic acid-Schiff stains. Positive staining of lymphocytes with acid phosphatase ranged from 10–30% without tartrate and from 2–13% with tartrate. Differences between peripheral blood lymphocytes and cells from the percoll gradient, and between conjugating and nonconjugating lymphocytes were not observed. The overall cytochemical staining pattern was similar in all the lymphocyte populations evaluated (peripheral blood lymphocytes; percoll band 1, band 2, and pellet lymphocytes; and conjugating and nonconjugating lymphocytes).

DISCUSSION

The purpose of this work was to describe the ultrastructure and cytochemical staining characteristics of canine peripheral blood lymphocytes demonstrating NK cell activity, with comparison made to non-NK cells. This work confirms previous findings (Knapp et al., 1993) that centrifugation of canine peripheral blood lymphocytes on a 45/50% percoll gradient maximizes

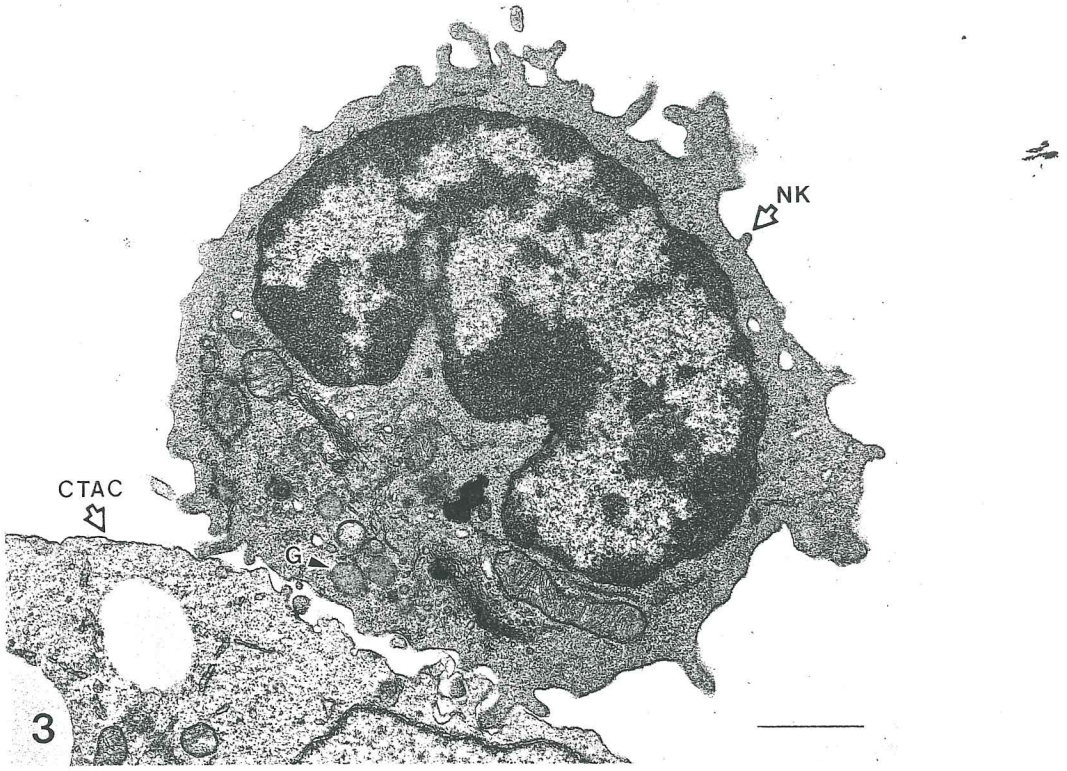

Fig. 3. Conjugate lymphocyte (NK cell) with CTAC target cell. Some lymphocytes had granules that were less electron dense than those in Figure 2. CTAC, canine thyroid adenocarcinoma cell; G, granule; NK, natural killer cell. Bar = 1 μm.

TABLE 1. Comparison of different lymphocyte fractions

Lymphocyte fraction	Reniform shaped nucleus (%)	Electron dense granules (%)
Peripheral blood lymphs	27.1	15.3
Band 1 of percoll	44.4	25.6
Band 2 of percoll	40.8	20.8
$CD4^-CD8^+$	35.2	17.6
$CD4^-CD8^-$	35.5	26.4

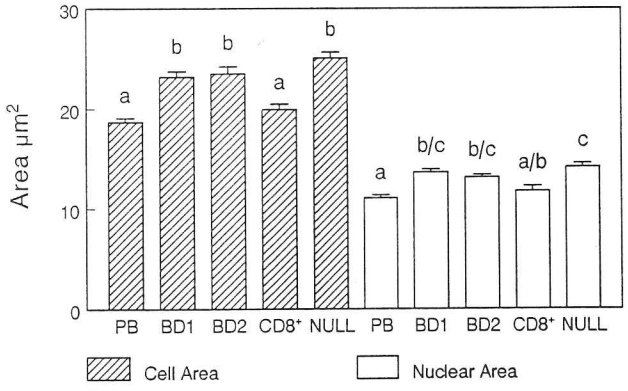

Fig. 4. Comparison of lymphocytes fractions. Groups with the same letter are not statistically different. The cell area of BD1, BD2, and null cells was significantly greater than that of PB lymphocytes ($P < .001$). The nuclear area of BD1, BD2, and null cells was significantly greater than that of PB ($P < .001$). The $CD8^+$ nuclear area was significantly smaller than that of null cells ($P < .05$). PB, peripheral blood lymphocytes; BD1, lymphocytes from band 1 of the percoll gradient; BD2, lymphocytes from band 2 of the percoll gradient; $CD8^+$, $CD4^-CD8^+$ lymphocytes; null, $CD4^-CD8^-$ lymphocytes.

NK activity to band 1 cells. Here we demonstrated further enhancement of NK activity in null ($CD4^-CD8^-$) cells. Nylon wool enrichment was not used in these experiments because this process did not increase NK activity in canine lymphocyte populations in earlier work (Knapp et al., 1993).

Electron microscopy showed canine NK cells to be 5.5 to 6.5 μm in diameter with a reniform nucleus and electron-dense cytoplasmic granules. This morphology is similar to that of human NK cells which have been reported to be 5–8 μm in diameter with abundant cytoplasm, reniform nuclei, and electron-dense cytoplasmic granules (Timonen et al., 1981; Neighbour et al., 1982). The canine NK cells studied here had cytoplasmic granules which varied in electron density. This variation in density has also been observed in human NK cells (Neighbour et al., 1982). The ultrastructure of canine NK cells (percoll band 1 cells and null cells) differed from that of non-NK cells. NK cells had a larger cell area, a larger nuclear area, and a less round nucleus when compared to non-NK lymphocytes. Although these features were readily visualized with

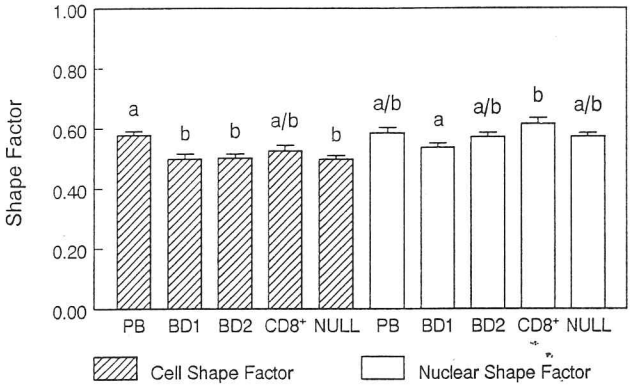

Fig. 5. Comparison of lymphocyte fractions. Groups with the same letter are not statistically different. The PB shape factor is larger (more round) than that of BD1 ($P < .01$), BD2 ($P < .001$), and than null cells ($P < .001$). The nuclear shape factor of BD1 cells is significantly smaller (less round) than that of $CD8^+$ cells ($P < .05$). PB, peripheral blood lymphocytes; BD1, lymphocytes from band 1 of the percoll gradient; BD2, lymphocytes from band 2 of the percoll gradient; $CD8^+$, $CD4^-CD8^+$ lymphocytes; null, $CD4^-CD8^-$ lymphocytes.

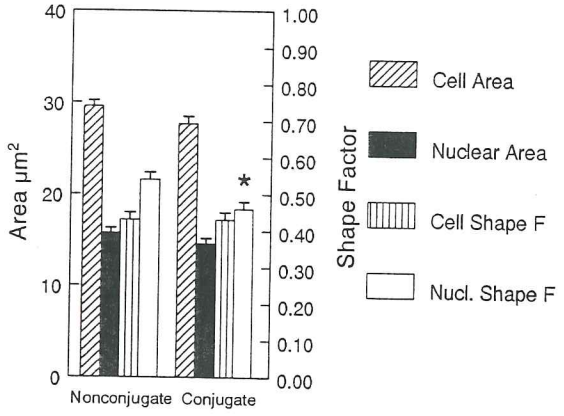

Fig. 6. Comparison of nonconjugate and conjugate (conjugated to CTAC target cells) lymphocytes. There was no difference in the cell and nuclear areas or the cell shape factor between the nonconjugate and conjugate lymphocytes. The nuclear shape factor for the conjugate lymphocytes (*) was significantly lower ($P < .006$) than the nonconjugate lymphocytes. Cell Shape F, cell shape factor; Nucl. Shape F, nuclear shape factor; *significant difference between groups.

electron microscopy, they were difficult to consistently detect using light microscopy.

Cytochemical staining was performed to determine if any specific staining characteristics would help differentiate NK cells from non-NK lymphocytes. Unfortunately, cytochemical staining was not useful in discriminating any of the lymphocyte populations studied. Although monoclonal antibodies are useful in identifying and sorting human NK cells (Zarcone et al., 1992), monoclonal antibodies specific for canine NK cell surface antigens are not presently available.

The mechanism of target cell killing by NK cells has been divided into two clearly defined steps: (1) target recognition and binding of the NK cell to the target cells, and (2) target cell lysis (Herberman et al., 1986). Because this process starts with conjugation of the NK cell to the target cell, we studied the morphology of lymphocytes which formed conjugates with the NK sensitive CTAC target cells. The conjugating cells were found to have a different nuclear shape factor (less round) than the nonconjugating cells. This is consistent with the reniform shape of the NK cell nucleus. Other differences (such as cell and nuclear size) were not noted. This may be due to prior selection of large cells by percoll gradient centrifugation. When we compared percoll band 1 cells to peripheral blood lymphocytes, the percoll band 1 cells (NK-enriched) did have larger cell area and nuclear area.

In conclusion, this work confirms that canine NK activity is enriched in lymphocytes separated by a 45/50% percoll gradient. Further enhancement occurs when the percoll-enriched fraction is sorted to obtain the $CD4^-CD8^-$ cells. The ultrastructure of canine NK cells is different from that of other peripheral blood lymphocytes. Canine NK cells are similar in morphology to human NK cells. Cytochemical staining is not useful in discriminating NK lymphocytes from other lymphocytes.

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LITERATURE CITED

Anderson, S.K. 1992 Molecular cloning of human and murine natural killer cell tumor recognition proteins. In: NK Cell Mediated Cytotoxicity: Receptors, Signaling and Mechanisms, E. Lotzová, ed., CRC Press, Boca Raton, pp. 1–14.

Bonavida, B., and S.C. Wright 1986 Role of natural killer cytotoxic factors in the mechanism of target-cell killing by natural killer cells. J. Clin. Immunol., *6:*1–8.

Gebhard, D.H., and P.B. Carter 1992 Identification of canine T-lymphocyte subsets with monoclonal antibodies. Vet. Immunol. Immunopathol., *33:*187–199.

Herberman, R.R., and J.R. Ortaldo 1981 Natural killer cells: Their roles in defenses against disease. Science, *214:*24–30.

Herberman, R.B., C.W. Reynolds, and J.R. Ortaldo 1986 Mechanism of cytotoxicity by natural killer (NK) cells. Annu. Rev. Immunol., *4:*651–680.

Kang, Y.H., M. Carl, P.M. Grimley, S. Serrate, and L. Yaffe 1987 Immunoultrastructural studies of human NK cells: I. Ultracytochemistry and comparison with T cell subsets. Anat. Rec., *217:* 274–289.

Kasza, L. 1964 Establishment and characterization of canine thyroid adenocarcinoma and canine melanoma cell lines. Am. J. Vet. Res., *25:*1178–1185.

Knapp, D.W., R.R. Leibnitz, D.B. DeNicola, J.J. Turek, R. Teclaw, L. Shaffer, and T.C.K. Chan 1993 Measurement of NK activity in effector cells purified from canine peripheral lymphocytes. Vet. Immunol. Immunopathol., *35:*239–251.

Krakowka, S. 1983 Natural killer cell activity in adult gnotobiotic dogs. Am. J. Vet. Res., *44:*635–638.

Leibnitz, R.R. 1988 Development of a canine natural killer cell assay and characterization of the effector cells. Masters thesis, Purdue University, West Lafayette, IN.

Loughran, T.P. Jr., H.J. Deeg, and R. Storb 1985 Morphologic and phenotypic analysis of canine natural killer cells. Evidence for T-cell lineage. Cell. Immunol., *95:*207–217.

Neighbour, P.A., H.S. Huberman, and Y. Kress 1982 Human large granular lymphocytes and natural killing: Ultrastructural studies of strontium-induced degranulation. Eur. J. Immunol., *12:* 588–595.

Timonen, T., J.R. Ortaldo, and R.B. Herberman 1981 Characteristics of human large granular lymphocytes and relationship to natural killer and K cells. J. Exp. Med., *153:*569–582.Trinchieri, G. 1989 Biology of natural killer cells. Adv. Immunol., *47:*187–376.

Wunderli, P.S., and P.J. Felsburg 1989 An improved method for the isolation of enriched canine peripheral blood mononuclear cell and peripheral blood lymphocyte preparations. Vet. Immunol. Immunopathol., *20:*335–344.

Zarcone D., G. Ceruti, C. Tenca, G. Arancia, W. Malorni, F. Iosi, and C.E. Grossi 1992 Human NK cell adhesion molecules (CAMs). In: NK Cell Mediated Cytotoxicity: Receptors, Signaling and Mechanisms. CRC Press, Boca Raton, FL, pp. 77–83.