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in Canine Lymphoid Malignancies

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NATURAL KILLER CELL ACTIVITY IN CANINE LYMPHOID MALIGNANCIES

By

Rose Esther Raskin

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ABSTRACT

NATURAL KILLER CELL ACTIVITY
IN CANINE LYMPHOID MALIGNANCIES

By

Rose Esther Raskin

The research objective was to evaluate natural cell-mediated cytotoxicity as an immunosurveillant mechanism in spontaneously occurring canine lymphoma. Specific goals involved morphologic, cytochemical and immunologic characterization of an effector cell population containing canine natural killer (NK) cells. Functional studies were performed in healthy and lymphoma dogs.

Peripheral blood lymphocytes isolated by routine density gradient centrifugation and nylon wool adherence resulted in low numbers of large granular lymphocytes (LGL) that morphologically resembled NK cells in people and rodents. Ultrastructurally, binding lymphocytes were LGL with membrane bound granules in close association with target cell membranes. These binding interactions were similar to those of other species. Lymphocytes had villous projections with minimal binding while extensive contact involved smooth or flattened surfaces.

Cytochemically, effector cell populations were negative for granulocytic markers but focally positive for acid

phosphatase and non-specific esterases.

Direct immunofluorescent staining for surface immunoglobulins was generally negative while cells bearing Fc receptors for IgG (Fc_G) were present up to 26% of the cells.

Overall NK cell activity in healthy dogs was measured as percent cytotoxicity in 12 and 16 hour chromium release assays using canine thyroid adenocarcinoma cells as targets. A wide range of normal activity existed but levels from individual dogs were generally constant. Percent cytotoxicity correlated poorly with numbers of LGL but well with Fc_G receptor positive cells. Morphologic features were less reliable in predicting NK cell activity levels than immunologic markers.

The relative frequency of active binding and cytotoxic NK cells was determined using a single cell binding assay. No correlation was found between percent dead binding cells and percent cytotoxicity in healthy dogs.

Untreated lymphoma dogs had significantly lower percent cytotoxicity but normal numbers of active binding and cytotoxic effector cells compared to controls. Glucocorticoid treated dogs had no change in NK cell activity or in percent live or dead binding. It appeared that canine NK cells were glucocorticoid resistant. In contrast, combined chemotherapy protocols resulted in marked inhibition of NK cell activity but effector cell numbers

were unaffected. Thus, immune dysfunction in both untreated and combined chemotherapy treated canine lymphoma patients involved defective recycling of NK cells.

To my parents, brother and special friends whose loving support and encouragement was essential in achieving this goal.

To my cats, D.J. and Paws whose purring and affection restored my sanity each evening and whose annoying morning antics got me up on time each day.

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ACP	acid phosphatase
ADCC	antibody dependent cellular cytotoxicity
ANAE	alpha naphthyl acetate esterase
ANBE	alpha naphthyl butyrate esterase
CAE	chloroacetate esterase
CHOP	cyclophosphamide, doxorubicin, vincristine prednisone
COP	cyclophosphamide, vincristine, prednisone
CPM	counts per minute
CTAC	canine thyroid adenocarcinoma
CTL	cytolytic T lymphocyte
E:T	effector to target cell ratio
Fc_G	Fc portion of immunoglobulin G
Fc_GR	receptor for Fc_G
Ig	immunoglobulin
K	killer
LCFC	lytic conjugate forming cells
LGL	large granular lymphocyte(s)
LU	lytic units
MEM	minimal essential medium
NCI	National Cancer Institute
NK	natural killer
NKCF	natural killer cytotoxic factor
PAS	periodic acid-Schiff
PBS	phosphate buffered saline
PLL	poly-L-lysine
RI	refractive index
SCBA	single cell binding assay
SEM	standard error of the mean
WHO	World Health Orgnaization

INTRODUCTION

Several specific and nonspecific mechanisms are involved in the immunosurveillance of tumor cells (Trainin & Essex, 1982; Wheelock & Robinson, 1983). Specific reactions include cytolytic T-lymphocytes or tumor specific antibodies with or without complement activation. Macrophages and antibody dependent cellular cytotoxicity may act in both a specific and nonspecific manner. Natural killer (NK) cells act rapidly within hours, nonspecifically and spontaneously against a wide variety of targets. They do not require prior sensitization, presence of antibody or complement and show no restriction related to the major histocompatibility complex (MHC) (Herberman & Ortaldo, 1981; Trinchieri & Perussia, 1984).

NK cells lack many of the characteristics of T lymphocytes (e.g., thymic dependency, T cell surface markers) or of B lymphocytes (e.g., surface immunoglobulin) or of macrophages (e.g., adherence, phagocytosis) (Herberman & Ortaldo, 1981; Trinchieri & Perussia, 1984). NK cell activity has been closely related to a subpopulation of lymphocytes, morphologically identified as large granular lymphocytes (LGL) (Timonen et al., 1979a,b). In vivo, NK

cell activity has been associated with early tumor cell elimination prior to metastasis as well as participation in cytolysis of virus infected cells, graft rejection, hematopoietic stem cell regulation and other general immunoregulatory functions (Herberman, 1983; Trinchieri & Perussia, 1984).

NK cells have been well characterized in humans and mice and to a lesser extent in other species. Characterization of these effector cells in the dog has been infrequent with reference only to their surface markers (Ringler & Krakowka, 1985; Betton, 1980). One aim of the present study was to further characterize the morphologic, cytochemical, immunologic and functional aspects of these cells in healthy dogs.

In beige mice, an animal homologue of the human Chediak-Higashi syndrome, NK cell activity is known to be profoundly reduced. Lymphoid malignancies grew faster and at higher frequencies in this mouse strain than in normal littermates (Karre et al., 1980). Studies in people with lymphoma or leukemia have demonstrated reduced NK cell activity (Tursz et al., 1982; Nasrallah & Miale, 1983; Kay & Sinkovics, 1984). This activity increased during remission of disease (Yoda et al., 1983; Lotzova et al., 1986) and with administration of biological response modifiers such as interferon or interleukin 2 (Hooper et al., 1986). Evaluation of the significance of these changes in NK cell

activity during the course of disease or treatment must be coupled with the effect of chemotherapeutic agents commonly used to treat these conditions. Early studies indicate that cytoreductive chemotherapy produces either increases or decreases in NK cell activity depending upon the level of immunocompetence of patients prior to treatment (Braun & Harris, 1986). Due to the close relationship between NK cell activity and lymphoproliferative malignancies in other species, a second aim of this study involved the evaluation of this activity in untreated and treated cases of canine malignant lymphoma. If *in vitro* measurement correlates well with *in vivo* activity, then NK cell assays may be beneficial in determining response to treatment or prognosis for survival.

It is hoped that future applications of this current work would aid in the understanding and treatment of canine and human lymphomas. Canine lymphoma is a frequent, spontaneously occurring disease that clinically and pathologically resembles its human counterpart (Crow, 1982; Carter *et al.*, 1986). Treatment protocols for lymphoma are similar for the two species. Therefore it is possible that the canine disease could assist future studies involving biological response modifiers for the treatment of lymphoma.

CHAPTER 1

NATURAL CELLULAR CYTOTOXICITY AGAINST TUMORS: A REVIEW OF THE LITERATURE

NATURAL CELLULAR CYTOTOXICITY AGAINST TUMORS: A REVIEW OF THE LITERATURE

Historical Perspective

During the 1960's many investigators pursued the premise that cell mediated mechanisms were involved in tumor defense. Researchers sought to demonstrate that specific tumor associated antigens provided the recognition mechanism for this process. Hellstrom et al. (1967, 1968, 1971) using colony inhibition and visual cytotoxicity assays demonstrated both humoral and cell mediated activity of lymphoid cells against murine and human induced and cultured tumor cells. Several years later a similar demonstration of tumor associated antigens was made in dogs with lymphoma (Warren et al., 1975). These specific cell mediated processes were considered at the time the major mechanism of tumor defense.

During this time, experimental observations began to indicate that besides specific lymphocyte mediated cytolysis, non-specific cytolytic processes were occurring. These had been originally considered to be technical errors or artifacts. This involved reactivity by cells of normal

controls during trials investigating the cellular activity of tumor patients. Rosenberg et al. (1972) was among the first to report this reactivity by normal human cells against fresh, noncultured leukemia cells. He used identical twins in which one had leukemia for a genetically controlled study. Lymphocyte mediated cytotoxicity by cells of the nonleukemic twin was demonstrated against leukemia cells from the diseased twin. This activity was attributed to possible prior sensitization to leukemia associated antigens. Soon after, other reports followed that supported the occurrence of cytotoxicity by normal cells. Takasugi et al. (1973) noted that lymphocytes from normal individuals possessed higher reactivity against tumor cell lines than those of tumor patients having the same histologic cell type as the target cells. This suggested prior sensitization was not a prerequisite for reactivity. Kay & Sinkovics (1974) similarly found that healthy human subjects possessed lymphocytes that could reduce the number of cultured tumor cells measured in a test system. This study suggested the existence of immunosurveillance in people.

Further characterization of this process continued using a variety of tumor targets, most of which gave similar results. These experiments suggested the presence of a natural or spontaneous cytolytic process which neither required prior immunologic sensitization or the presence of antibody or complement. De Vries et al. (1974) demonstrated

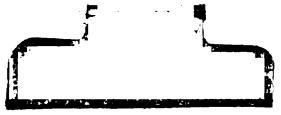
that the non-T cell fractions from normal individuals and melanoma patients were more cytotoxic than the T cell fractions or total lymphoid population. Initially these cells were classified as "null cells" i.e., lacking characteristic markers of either T or B lymphocytes (Herberman et al., 1979). Later studies would indicate that they share some of the markers identifying T cells and myelomonocytic cells (Trinchieri & Perussia, 1984).

Simultaneous to the human studies, similar observations of natural cytotoxicity were reported in rats and mice. The term "natural killer cell" was generally accepted when Kiessling et al. (1975a, 1975b) and Herberman et al. (1975a, 1975b) independently characterized the first NK cells, those of the mouse spleen.

Assay Methods

Early investigations of cell mediated cytotoxicity utilized visual assays of colony inhibition and microcytotoxicity to evaluate tumor systems. Cell numbers were calculated following visual inspection. Detection of colony reduction or lysis of tumor cells by these methods was tedious and time consuming.

Brunner et al. (1968) developed a sensitive quantitative radiolabel technique which involved chromium-51 release in a microcytotoxicity assay to measure cell death.



The chromium release microcytotoxicity assay has since become the standard testing procedure. This involves placing a fixed number of radiolabelled tumor cells together with variable numbers of effector cells into tubes or microtiter plates. When killed or lyzed, the target cells release their radiolabel into the supernatant which can be measured as counts per minute (cpm) by a gamma counter. A direct relationship exists between the amount of radiolabel released and the number of effector cells present since increases in effector to target cell ratios (E:T) produce an increase in the amount of Cr^{51} measured.

A qualitative calculation of percent cytotoxicity can be used to define natural killer activity within various situations. Another commonly used calculation of NK cell activity is that of lytic units. These are semi-quantitative and require the use of multiple E:T ratios. The units are mathematically derived and define the number of cells necessary to produce a predetermined percentage of cytolysis.

The radiolabel release method measures the overall activity of the cells and considers their recycling ability (Pross et al., 1986). Because cytotoxic lymphocytes are not destroyed during cytolysis, they may cause death of additional target cells through a stage in the process termed recycling.

A second commonly used assay for cellular cytotoxicity

studies is the single cell binding assay (SCBA). The advantage of this method is the prevention of cell recycling. This is accomplished by allowing effector cells and targets to form conjugates which can then be immobilized in a medium such as agarose (Grimm & Bonavida, 1979; Timonen et al., 1982) or poly-L-lysine (Vargas-Cortes et al., 1983). This simple method allows the direct microscopic visualization of cell binding and cytolysis. Calculations such as percent live binding or percent cytolysis are determined by counting the number of cell conjugates having target cells that respectively, have not or have stained with trypan blue, a dye that labels dead cells. A high correlation was found between lytic conjugate forming cells (LCFC) frequency i.e., the percentage of lymphocytes bound to dead targets and NK cell cytotoxicity defined by the chromium release assay, suggesting that LCFC are NK cells (Rubin et al., 1982). However, Rubin et al. (1982) noted a low correlation between total conjugate forming cells and NK cytotoxicity implying that all lymphocytes that bound to target cells were not NK cells. The SCBA provides a semi-quantitative estimate of the frequency of NK cells active against the target cell tested (Pross et al., 1986). Combination of the results from chromium release and single cell binding assays provides information about the recycling and target binding capacity of NK cells (Ullberg & Jondal, 1981).

Target Cells

The target cells used in natural killer cell assays have been variable but all were identified by the ability of NK cells to spontaneously lyze these tumor or virus-infected cells. Although this activity is not entirely species specific, optimal response is obtained with allogeneic target cells. For human studies, K562 cells, an erythroleukemic cell line derived from a chronic myelogeneous leukemia case in blast crisis, has become the standard target. Mouse studies involve YAC-1, a cultured murine lymphoma induced by Maloney leukemia virus.

Various cell lines including heterologeous K562 and Vero (African Green monkey kidney) or homologenous canine kidney, melanoma, thyroid adenocarcinoma (CTAC) and canine distemper virus infected cells have been used in cell mediated cytotoxicity studies in dogs (Betton, 1980; Shek et al., 1980; Krakowka, 1983; Ringler & Krakowka, 1985). Of all of these, the CTAC appears to be the most sensitive for use in dog studies (Krakowka, 1983). It was cloned from a single cell and is characterized by adherence and an epithelioid syncytial growth pattern (Kazna, 1964). Use of this cell line requires trypsinization which could affect target antigens (Pross et al., 1986). However, this problem has not been encountered.

Cytolytic Effector Cells

There are several types of naturally occurring lymphocyte effector cells involved in the immune response to tumor cells (Trainin & Essex, 1982). These may be subdivided into those requiring major histocompatibility complex (MHC) antigens for target cell recognition and those that are non-MHC restricted. The MHC-restricted lymphocytes possess the T cell antigen receptor with rearrangement and transcription of the genes for it confirming the T cell origin of these cells (Marrack & Kappler, 1986; Lanier et al., 1986). The cytolytic T lymphocyte (CTL) requires a class I MHC antigen and prior sensitization to the target cell for recognition to occur. The cytolytic process is slow requiring several days such that it is thought to constitute the second line of tumor defense (Herberman, 1983).

The second group of cytolytic lymphocytes are non-MHC restricted and are frequently classified by function. This involves the requirement of antibody for target cell recognition. The killer (K) cell of antibody-dependent cellular cytotoxicity (ADCC) has Fc receptors for the IgG molecule which bind to the antibody coated target cells (Djeu, 1982). The NK cell also has Fc receptors but antibody is not necessary for target cell binding to occur. Treatment to remove antibody does not reduce cytolysis by NK cells of sensitive targets. The K and NK cells share in common some

surface markers and the formation of pores that induce target cell damage (Muchmore, 1986). Additional evidence to suggest these are the same cell involve the demonstration of a single effector cell binding to and killing both NK and ADCC targets (Bradley & Bonavida, 1982). On the other hand, there is a temporal difference in their appearance in gnotobiotic pigs. ADCC was fully expressed by 2 weeks whereas NK cell activity was not apparent until 6 weeks of age. However, it is also possible that NK and ADCC activity are mediated by the same cell at different stages of maturation (Muchmore, 1986).

The non-MHC restricted lymphocytes may also be classified by surface markers. Lanier et al. (1986) proposed dividing these cells into two groups. The most abundant effectors of non-MHC-restricted cytotoxicity would be distinct from T cells and include NK cells and those with NK-like activity. A smaller group would include those possessing T cell receptor gene rearrangements.

Cytolytic Mechanisms and Regulation

The main stages of the lymphocyte mediated cytolytic process include: (i) recognition and binding of target cells; (ii) activation and release of cytotoxic factors; and (iii) target cell lysis and effector cell recycling (Henkart, 1985). Recognition mechanisms were characterized

for the various effector cells in the previous section. Target recognition receptors for K cells involves functional Fc receptors which require antibody to be present. Unlike CTLs, the T cell receptor is not involved in NK cell recognition mechanisms as gene transcripts of the receptor are lacking (Tutt et al., 1986). A possible recognition mechanism may involve laminin, a membrane glycoprotein, since it has been identified on NK cells and receptors for it have been reported occurring on NK sensitive target cells. Blockage of these receptor sites on target cells or anti-laminin treatment of NK cells has resulted in reduced binding and cytolytic activity (Hiserodt et al., 1985). Other target cell antigens proposed to attract NK cells include those involved in the differentiation process as mature cells are insensitive to NK lysis and immature cells are more susceptible (Herberman, 1985). Storkus et al. (1987) reported an inverse correlation between NK conjugate or binding formation and the expression of MHC class I antigens on the surface of target cells. This expression may be increased by agents such as interferon (Hansson et al., 1980). Much research is presently directed towards answering this question.

Binding of NK cells to targets is necessary for cytolysis to occur, and has been shown to require Mg^{2+}. A Ca^{2+} dependent activation stage follows which is sensitive to a number of pharmacologic agents (Trinchieri & Perussia,

1984). The ion involvement lends support to a secretory process triggered by membrane receptors as the mechanism of cytolysis (Henkart, 1985). Targan and Newman (1983) reported that binding alone does not trigger or activate the lytic event. Using a monoclonal antibody against the T-200 glycoprotein, they were able to fully inhibit NK cell activity following conjugate formation.

Several of the post-binding stages are similar for the process of cytolysis by NK, K and CTL cells (Henkart, 1985; Bonavida & Wright, 1986; Muchmore, 1986). Following activation, granule exocytosis occurs. The extent to which these granules perform in the cytolytic process is not fully understood. Ultrastructurally, these granules orientate towards the target cell, fuse with each other and release their contents in the space between the two cells (Henkart, 1985).

Two types of soluble factors are associated with the granules. One has been termed pore forming proteins and consists of perforins or cytolysin (Marx, 1986). These effect polymerization of proteins forming a tubular structure that inserts into the target cell membrane. This has been viewed ultrastructurally as thickened rings on the cell surface akin to the complement attack complex (Henkart, 1985). A protein resembling perforin has recently been isolated from the cytoplasmic granules that is immunochemically related to the ninth component of human

complement (Zalman et al., 1986). The second type of factor is a glycoprotein termed NK cytotoxic factor (NKCF). The similarities between NKCF mode of activity and that observed in NK cell reactions suggest that the factor is closely involved in NK cell activity (Bonavida & Wright, 1986). It is suggested that NK cell resistant targets are such because they lack receptor sites for NKCF (Kiyohara et al., 1986).

The last stage in the cytolytic process involves target cell lysis and recycling of the effector cell. Target cell death may be related to colloid osmotic lysis due to the release of small ions or to the insertion of pore forming proteins into cell membranes (Henkart, 1985). This final step, termed lethal hit, is independent of the effector cell. The NK cell is not destroyed in the cytolytic process and may recycle to kill again following a refractory period (Timonen et al., 1982).

Regulation of NK cell cytolytic activity is mostly associated with interferon and interleukin-2 (Djeu et al., 1979; Henney et al., 1981). Interferon is known to activate pre-NK cells to recognize and bind targets, increase binding rate and reduce the refractory period for recycling of NK cells (Timonen et al., 1982). In addition, it increases NKCF release (Bonavida & Wright, 1986). Interleukin-2 has also been shown to booster NK cell activity, possibly due to increased NKCF release (Bonavida et al., 1986).

CHAPTER 2

MORPHOLOGIC, CYTOCHEMICAL AND IMMUNOLOGIC CHARACTERIZATION OF CANINE NATURAL KILLER CELLS

MORPHOLOGIC, CYTOCHEMICAL AND IMMUNOLOGIC CHARACTERIZATION OF CANINE NATURAL KILLER CELLS

INTRODUCTION

Natural killer (NK) cells have been defined in terms of their function of spontaneous cytotoxicity to a wide variety of targets including virus-infected cells, tumor cells and some poorly differentiated cells (Herberman, 1983). NK cells along with cytotoxic T lymphocytes, specific antibodies, macrophages and antibody dependent cellular cytotoxicity killer lymphocytes represent the major mechanisms involved in anti-tumor immunity (Trainin & Essex, 1982).

The morphology of cells associated with this functional activity has been well described in humans and rodents (Ferrarini et al., 1980; Reynolds et al., 1981; Timonen et al., 1979a,b; Saksela et al., 1982; Gastl et al., 1983). There is strong correlation between NK cell activity and cells which are termed large granular lymphocytes (LGL) (Timonen et al., 1979a; Savary & Lotzova, 1986). Up to 80% of LGL can function as NK cells (Timonen et al., 1982). In Wright's or Giemsa stained preparations these cells are medium to large sized lymphocytes having variably numbered

small azurophilic stained cytoplasmic granules.

The LGL has been identified to a variable extent in peripheral blood, bone marrow, lung, intestine and major lymphoid structures such as lymph nodes, spleen and thymus (Savary and Lotzova, 1986). They may be found in peripheral blood smears of normal human subjects between 2-6% of the total leukocyte population (Timonen et al., 1981).

Ultrastructurally, the granules most resemble the lysosomal structures seen in granulocytes (Grossi & Ferrarini, 1982). However, others report parallel tubular arrays in association with these granular structures (Huhn et al., 1982; Zucker-Franklin et al., 1983; Payne, 1984). Despite disagreement regarding morphology, it is generally regarded that the granules contain cytotoxic factors that contribute significantly to the cytolytic process (Henkart, 1985; Bonavida & Wright, 1986).

Binding between NK cells and tumor targets is necessary as the initial step in cytolysis. This activity usually involves close physical contact between the two cells (Roder et al., 1978; Hiserodt & Beals, 1985). It is thought that orientation of the granules occurs towards the target surface followed by release of their lytic factors (Henkart, 1985). Circular lesions have been observed ultrastructurally on the surface of the target cell due to polymers similar to those of the complement system (Podack & Dennert, 1983; Zalman et al., 1986). It is possible that these circular

lesions or pores provide a mechanism for target cell death through loss of ions or insertion of cytotoxins.

Cytochemically, the granules stain similarly to lysosomes being strongly positive for acid phosphatase. Alpha naphthyl acetate esterase is weakly positive while another nonspecific esterase, alpha naphthyl butyrate esterase, is negative in humans and rodents (Ferrarini & Grossi, 1986). In addition, chloroacetate esterase, a granulocytic marker stain has been reported positive in some human LGLs (Heumann et al., 1983). However, other granulocytic marker stains such as peroxidase, are negative (Huhn et al., 1982).

NK cells are heterogenous with respect to their surface markers. They are not considered purely B- or T-lymphocytes or myelomonocytic in origin although they share in common some of the markers found on these cell types. This suggests their origin is different but related to these cell lines. Characteristic immunologic features associated with human or rodent NK cells include non-adherence, non-phagocytosis, absence of surface immunoglobulin (Ig), presence of receptors for the Fc portion of IgG (Fc_G) and a tendency to form low affinity rosettes with sheep erythrocytes (Trinchieri & Perussia, 1984).

Most information known about canine NK cells has been associated with functional or immunologic marker studies (Betton & Gorman, 1978; Betton, 1980; Shek et al., 1980;

Krakowka, 1983; Ringler & Krakowka, 1985a,b; Savary & Lotzova, 1986). There is one account of the light microscopic appearance (Savary & Lotzova, 1986) and none that involve cytochemical staining of the effector cells.

The purpose of this report will be to further characterize the morphologic, cytochemical and surface marker features of the effector cells associated with NK cell activity in the dog. These peripheral blood lymphocytes will be evaluated for their light and electron microscopic features. In particular, the percentage of LGL in fresh blood and in isolated lymphoid cell populations will be determined. LGL will be evaluated ultrastructurally to assess the composition of the granules. Electron microscopy will also be used to visualize the cell membrane interaction between effector and target cells. In addition, routine cytochemical stains will be applied to the effector cell population. The results of these studies will be compared to those found in other species, notably rodents and people.

MATERIALS AND METHODS

Animals and lymphocyte isolation

A total of 18 healthy appearing adult pure and mixed breed dogs of both sexes were used. These had been housed in the Veterinary Clinical Center at Michigan State University. A standard lymphocyte isolation procedure to obtain canine

peripheral blood NK cells (Krakowka, 1983) was used with slight modification. Twenty milliliters of heparinized whole blood were collected from each subject, seven of which were sampled more than once. Blood samples were incubated at 37 C for 1 hour with carbonyl iron filings to assist in the removal of phagocytic cells. This blood was then diluted 1:5 with saline and layered over Ficoll-Hypaque solution (RI 1.3550). This solution is a high molecular weight polymer of sucrose (Ficoll) with sodium diatrizoate used to separate mononuclear cells from granulocytes and erythrocytes. Mononuclear cells were recovered by centrifugation of this solution at 450 g for 18 minutes. Contaminating red cells were removed by hypotonic shock lysis and remaining cells were washed with saline. Cells were resuspended in minimal essential medium (MEM) supplemented with 10% (v/v) fetal calf serum, 1% (v/v) glutamine and 1% (v/v) penicillin-streptomycin. Adherent cells were removed by incubation at 37 C for 1 hour in a 10 ml syringe packed with 0.75g nylon wool fiber (Fenwal Lab., Deerfield, IL). Non-adherent cells were eluted using 15 ml of warm MEM without additives. The final cell suspension was adjusted to 5.0 X 10^6 cells/ml. This population of isolated lymphocytes demonstrated spontaneous cytotoxicity in routine NK cell assays (data not shown). Therefore these effector cells contained a functional NK cell population.

Light microscopic studies

To evaluate the percentage of LGL in the initial peripheral blood sample, smears were made from each sample prior to further processing. The morphologic appearance of the effector cell population was also examined from slide preparations made from 5.0 X 10^5 cells with a cytocentrifuge (Shandon Southern Instruments Inc, Sewickley, PA). All slides were air-dried. One blood smear and one cytospin slide were stained with a modified Wright-Giemsa stain (Hema-tek, Ames Co, Elkhart, IN). Cytochemical staining was performed on additional cytospin preparations which included the following stains: peroxidase, naphthol AS-D chloroacetate esterase (CAE) (Sigma Diagnostics, St. Louis, MO), alpha napthyl acetate esterase (ANAE), alpha napthyl butyrate esterase (ANBE), alkaline phosphatase, acid phosphatase (ACP), periodic acid-Schiff (PAS) and Sudan black B. The techniques for these stains have been fully described elsewhere (Barka & Anderson, 1962; Jain, 1986). Cell differentials were performed on a minimum of 100 cells.

Preparation of cells for electron microscopy

The ultrastructural morphology of the effector cells alone and those involved in target cell interactions were examined following processing. Canine thyroid adenocarcinoma (CTAC), a cell line maintained as monolayer cultures (Kasza, 1964), was kindly provided by Dr. Steven Krakowka (The Ohio

State University). The target cells were trypsinized prior to use and concentrations were adjusted to 1.0 X 10^6 cells/ml. Non-adherent effector cells isolated as previously described were also adjusted to a concentration of 1.0 X 10^6 cells/ml. An equal amount (1 ml) of each cell suspension was placed together, pelleted and allowed to incubate at 37 C for variable times. These times included: 0.5, 2, 8, 10, 12 and 16 hours.

Samples containing isolated lymphocytes (5.0 X 10^6 cells) and 1:1 mixtures of lymphocytes and target cells were processed similarly following pellet formation. Karnovsky's fixative (pH 7.3) was added to the pellet and left for 2 hours. Samples were rinsed in Zitterquist wash and post-fixed in 1% osmium tetroxide. Following routine dehydration, preparations were embedded in DDSA + Araldite 502. Thin sections were stained with uranyl acetate and lead citrate and examined in a transmission electron microscope (Zeiss EM 952).

Immunofluorescent staining of effector cells

A direct immunofluorescent procedure was applied to cell suspensions to detect surface staining. Fluorescein conjugated $F(ab')_2$ fragments were used that included goat anti-dog-IgM and goat anti-dog-IgG (Cooper Biomedical Inc, Malvern, PA) for the detection of surface IgM and IgG respectively. In addition, to detect the presence of Fc

receptors for IgG, fluorescein conjugated Fc fragment for dog IgG (Jackson Immunoresearch Laboratories Inc, Avondale, PA) was used. Dilutions (1:10) were made of the antibodies and 0.1ml of each was added to 1.5 X 10^6 cells of the non-adherent lymphocyte population. Incubation for 1 hour at 4 C was followed by 2 phosphate buffered saline (PBS) washes. Cells were resuspended in PBS, then transferred to a glass slide and examined under a fluorescent microscope. Mononuclear cells were counted and positive cells were expressed as a percentage of the total number.

RESULTS

Isolation and light microscopic studies

Twenty-six samples from the 18 dogs were evaluated for the proportion of lymphocytes and LGL present in peripheral blood smears and cytocentrifuge preparations of isolated non-adherent mononuclears. Data is presented in Table 1. Four isolation samples with total lymphocyte populations below 80% were contaminated to a moderate degree with neutrophils and eosinophils. Monocytes accounted for less than 1% in each of the lymphocyte isolated preparations.

Morphologically, the LGL was recognized by a larger size (10-15 um) compared to other well-differentiated lymphocytes. The cytoplasm was moderately abundant and pale blue with Romanowsky-type stains. Present within the

Table 1 - Frequency of lymphocytes and LGL in peripheral blood smears and mononuclear isolations for 26 samples taken from healthy dogs.

	Peripheral Blood	Isolation Samples
Lymphocytes (%)		
Mean	33.6	90.1
Range	21-54	64.5-99
LGL (%)		
Mean	1.8	6.4
Range	0-5	0.5-16.5
LGL/Lymphocytes (%)		
Mean	5.7	6.9
Range	0-18.5	0.7-16.8

cytoplasm to one side of the nucleus were variably numbered small red azurophilic staining granules (Figures 1 and 2).

Cytochemical staining of the effector cell population was negative with peroxidase, Sudan black B and leukocyte alkaline phosphatase. One preparation had rare focal granular staining by chloroacetate esterase accounting for 0.5% of all lymphocytes in that sample. All other isolations were negative for that stain. The mean and (range) percentage of positive staining lymphocytes are listed for the following stains: PAS 6.5% (4-9), ANAE 10.5% (5-19), ANBE 23.0% (1-53) and ACP 23.7% (3-55). The pattern of staining for the latter 3 stains was generally focal rather than diffuse (Figures 3-5).

Ultrastructural Studies

The morphology and binding interaction of LGL in dogs was compared to that in other species in terms of the presence and appearance of organelles or cytolytic structures in addition to the manner of cell membrane involvement. The LGL was characterized by a round to ovoid shape with a smooth surface or more frequently irregular by the presence of several short thin pseudopod projections (Figure 6). The nucleus was also round to ovoid with rare deep clefts (Figure 7). Nucleoli were occasionally noted but often the chromatin stained densely and peripheralized to the nuclear margins. Frequently a shallow nuclear membrane

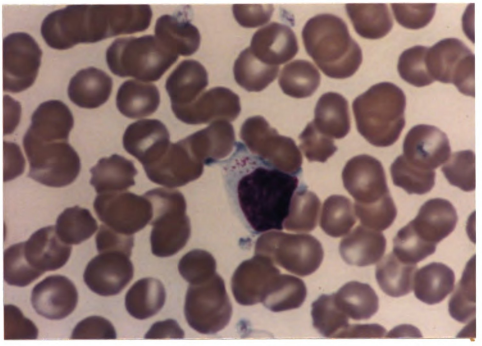


Figure 1 - Granular lymphocyte present in the peripheral blood from a clinically healthy dog. Wright's stain.

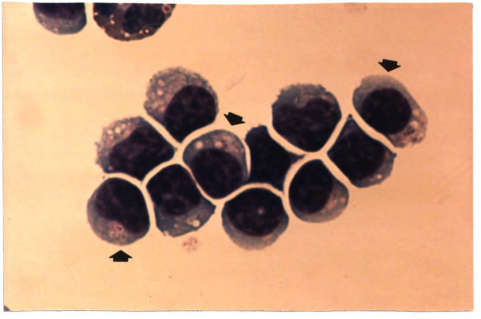


Figure 2 - Cytocentrifuge preparation of isolated non-adherent mononuclear cells with three granular lymphocytes (arrows). Wright's stain.

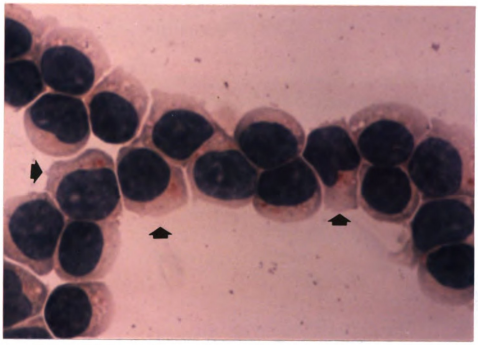


Figure 3 - Weak focal staining of isolated lymphocytes with alpha naphthyl acetate esterase (arrows).

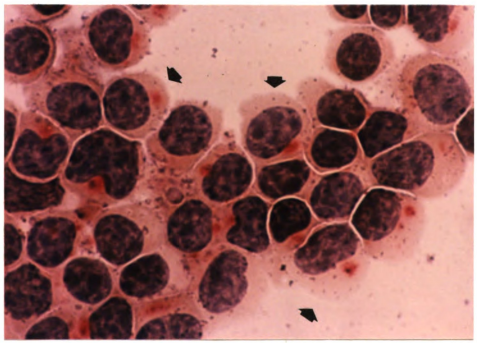


Figure 4 - Strong focal paranuclear staining of isolated lymphocytes with alpha naphthyl butyrate esterase (arrows).

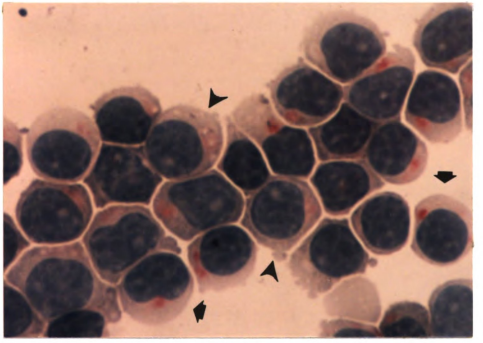


Figure 5 - Focal (arrows) and diffuse (arrowheads) granular staining of isolated lymphocytes with acid phosphatase.

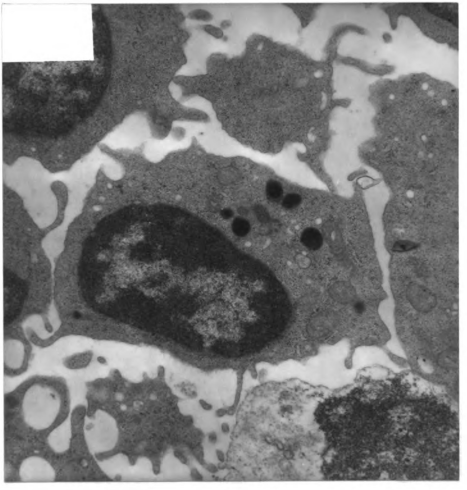


Figure 6 - Granular lymphocyte with several short pseudopod projections. Lead citrate-uranyl acetate stain; X 18,360.

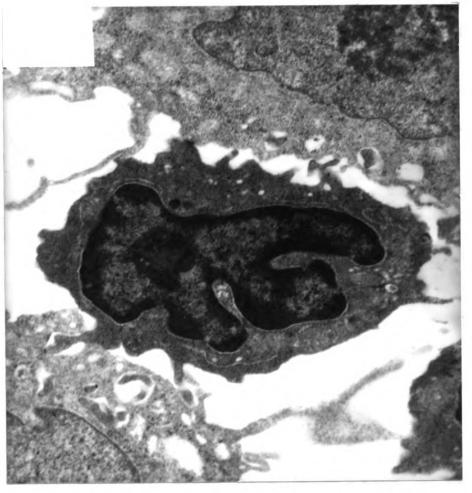


Figure 7 - Deep clefted nucleus in a granular lymphocyte in contact with target cells. Lead citrate-uranyl acetate stain; X 18,360.

indentation was present. At this site or to one end of the cell were gathered most of the cytoplasmic organelles (Figure 8). These included the golgi apparatus, mitochondria, centrioles, vacuoles, rough endoplasmic reticulum and dense granules (Figures 9-12). Mitochondria were often large, round and few in number (2-6). The granules were found occasionally scattered throughout the cytoplasm or more frequently located all to one side. They varied in number (3-7), size and density. Most were membrane bound and several were strongly osmiophilic and homogeneous in density (Figures 6 and 10). However granules with variable density and myelin figure formation were common (Figures 9, 10 and 12). Rarely noted in long incubation preparations (16 hours) were cytoplasmic structures which were not membrane bound. These consisted of a cluster of granular densities resembling ribosomes or glycogen granules that were arranged in chains (Figure 13). Occasionally a disparity existed between the frequency of LGL observed under light microscopy and that seen ultrastructurally with the latter being more sensitive.

The morphology of the binding between lymphocytes and target cells was investigated at various incubation times. Few attachments were present at 30 minutes or 2 hours. As early as 30 minutes, broad attachments were noted with increased focal density where the cell membranes touched (Figure 14).

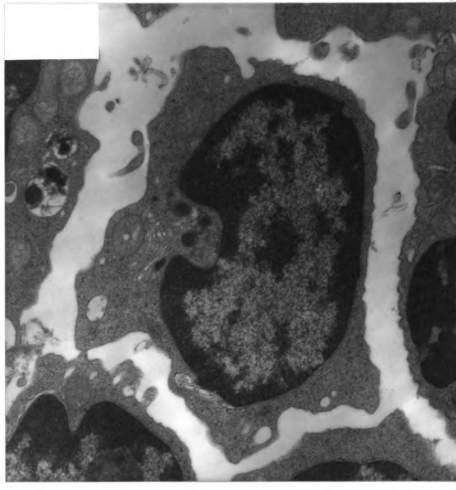


Figure 8 - Granular lymphocyte with cytoplasmic organelles found within the indented portion of the nucleus. Lead citrate-uranyl acetate stain; X 18,360.

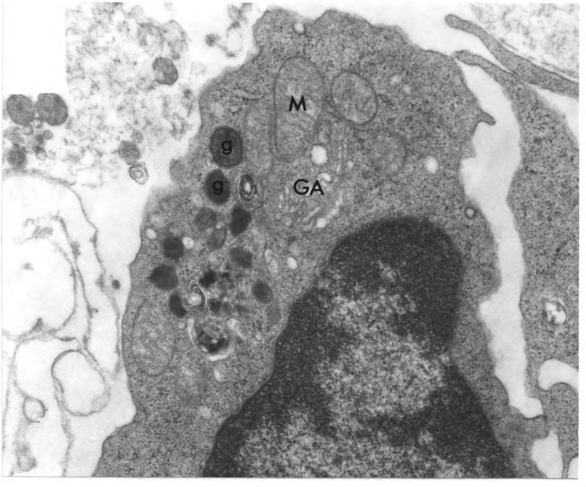


Figure 9 - Gathered to one end of the cytoplasm are several mitochondria (M), golgi apparatus (GA) and variably dense granules (g). Lead citrate-uranyl acetate stain; X 31,500.

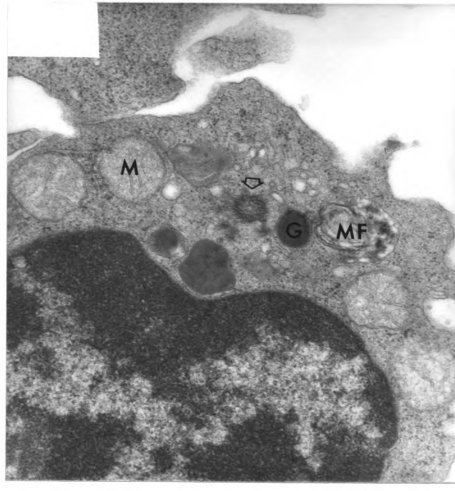


Figure 10 - High magnification of granular lymphocyte with mitochondria (M), centriole (arrow), membrane bound granules (G) and myelin figure (MF). Lead citrate-uranyl acetate stain; X 36,720.

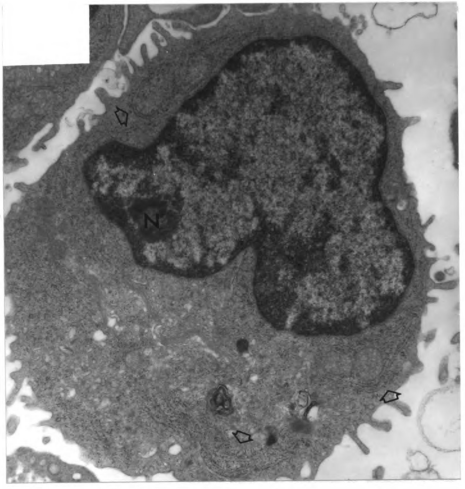


Figure 11 - Granular lymphocyte with irregularly shaped nucleus, prominent nucleolus (N) and abundant rough endoplasmic reticulum (arrows). Lead citrate-uranyl acetate stain; X 18,360.

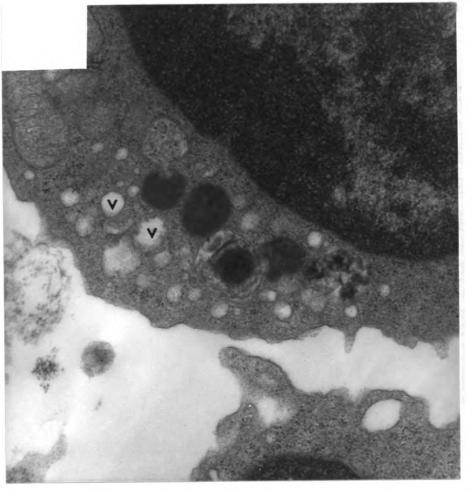


Figure 12 - High magnification of granular lymphocyte with variably dense granules and numerous vacuoles (V). Lead citrate-uranyl acetate stain; X 36,720.

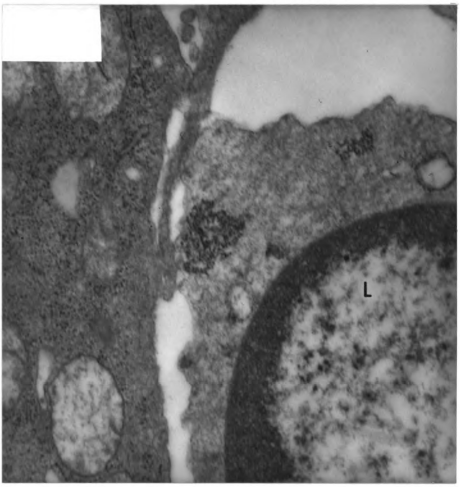


Figure 13 - Possible aggregation of ribosomes or a lysed granule within the cytoplasm of a lymphocyte (L) in contact with a target cell. Lead citrate-uranyl acetate stain; X 36,720.

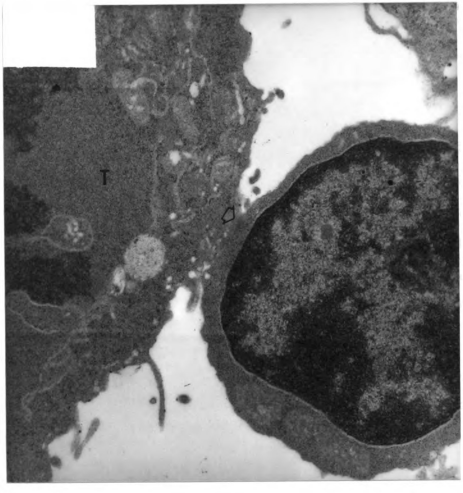
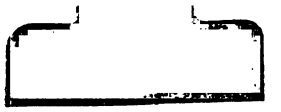


Figure 14 - Lymphocyte bound to tumor target cell (T) with increased focal density where cell membranes touch (arrow). Incubation of 30 minutes. Lead citrate-uranyl acetate stain; X 18,360.



The cell surface varied with the degree of binding present between effector and target cells. Generally, cells with extensive binding had smooth membrane surfaces with few projections (Figure 15). Cells with no or minimal contact frequently had long finger-like pseudopodia (Figures 16-18). This form of interaction was the most common observed. Both granular and agranular lymphocytes were found attached to targets but the former cell type predominated. Occasionally more than one cell bound to the tumor cell (Figure 19). Granules were often in various locations within the cytoplasm when binding involved minimal attachment (Figures 20 and 21). However with extensive adherence, granules appeared to be positioned more frequently in the cytoplasm facing the target cells (Figures 15, 22 and 23). This was subjectively determined while viewing microscopic material. Occasionally coalescence of several granules was noted (Figure 23). Evidence of epicellular pore-like structures or the intercellular tubular material that has been observed in other species were not found during these studies.

Immunofluorescent staining studies

The results are presented in Table 2. Surface IgG and IgM were infrequent in that positive cells generally accounted for less than 5% of the mononuclear population. A relatively larger proportion of mononuclear cells stained positive for receptors of the Fc portion of IgG. Neutrophils

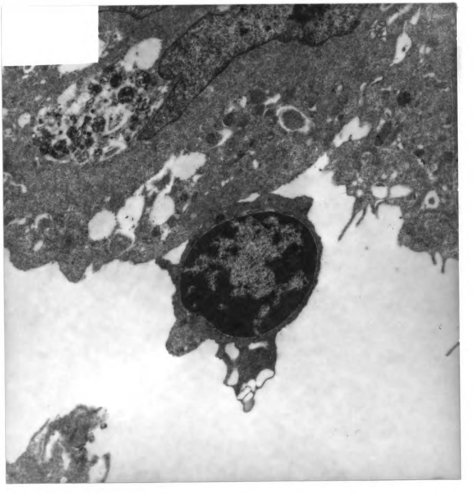


Figure 15 - Granular lymphocyte with smooth cell surface bound extensively to a target cell. Cytoplasmic granule located immediately adjacent to the target cell membrane. Incubation of 10 hours. Lead citrate-uranyl acetate stain; X 10,200.

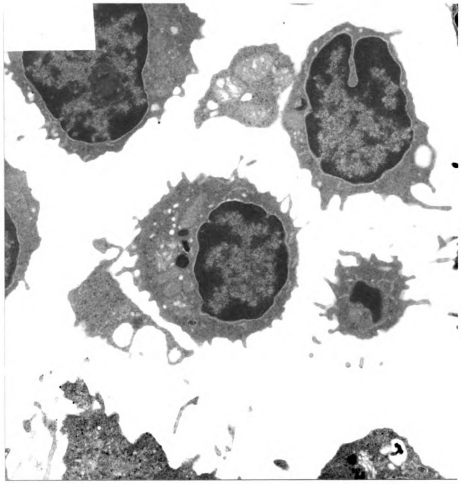


Figure 16 - Isolated granular lymphocytes with numerous short finger-like surface projections. Incubation of 30 minutes. Lead citrate-uranyl acetate stain; X 10,200.

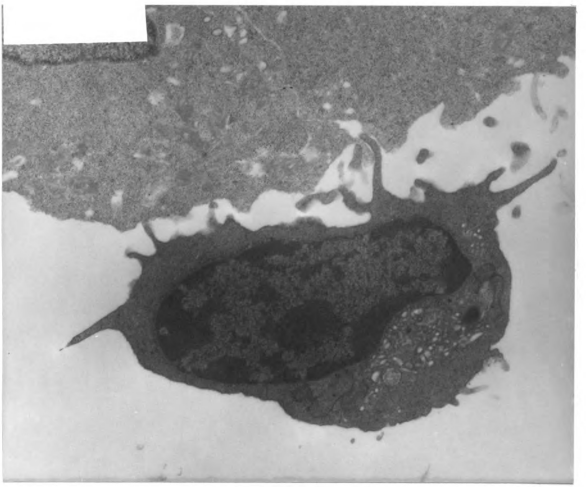


Figure 17 - Granular lymphocyte with long pseudopod surface projections minimally touching a target cell. Incubation of 12 hours. Lead citrate-uranyl acetate stain; X 18,260.

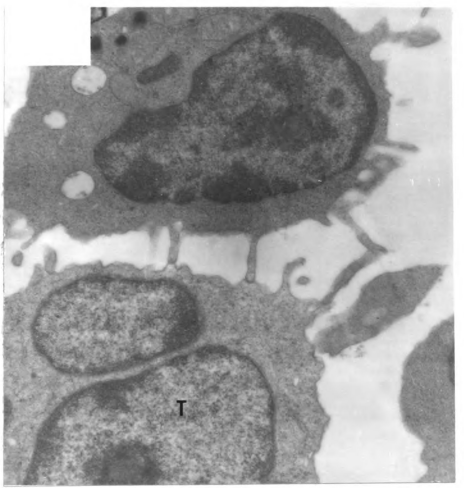


Figure 18 - Granular lymphocyte with minimal binding to a tumor target (T) via pseudopod projections of the cell membrane. Incubation of 16 hours. Lead citrate-uranyl acetate stain; X 16,110.

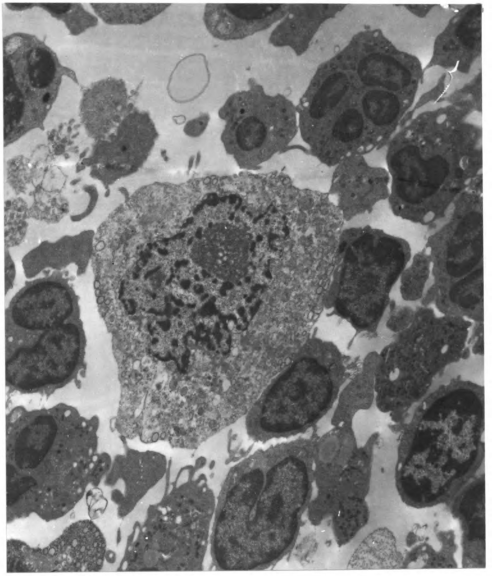


Figure 19 - A granular and an agranular lymphocyte binding to the same tumor target cell. Incubation of 16 hours. Lead citrate-uranyl acetate stain; X 6,593.

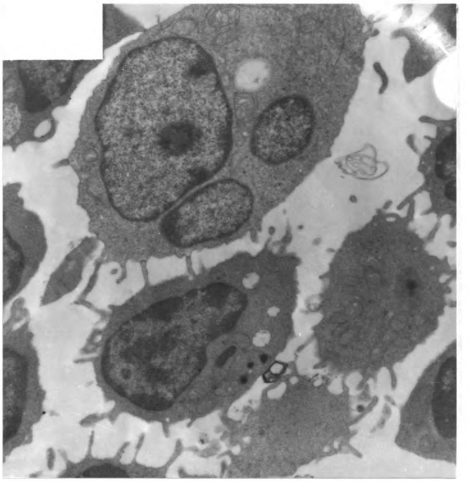


Figure 20 - Cytoplasmic granules located to a side furthest from the target cell in a lymphocyte with minimal binding. Incubation of 16 hours. Lead citrate-uranyl acetate stain; X 8,950.

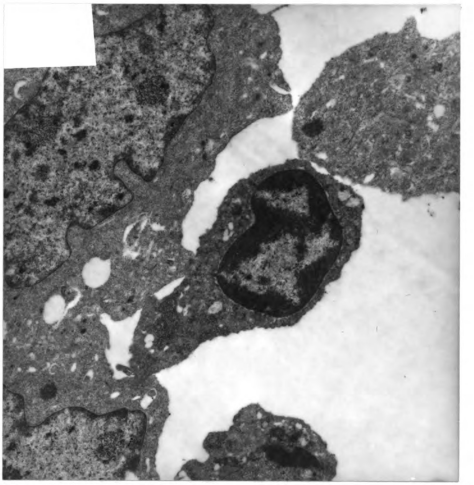


Figure 21 - Granules are present between the lymphocyte nucleus and the target cell to which the lymphocyte has minimal attachment. Incubation of 2 hours. Lead citrate-uranyl acetate stain; X 27,450.

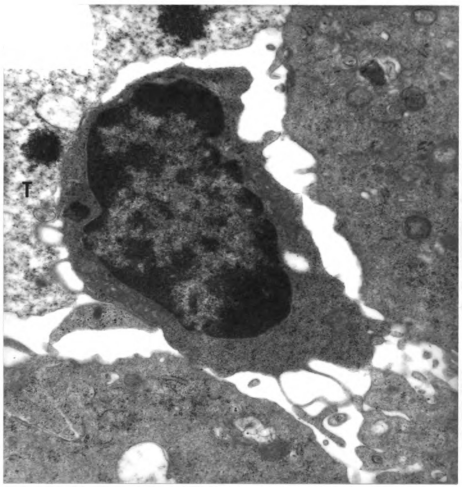


Figure 22 - Granule placement directly adjacent to target cell membrane (T) in a lymphocyte with extensive binding. Incubation of 2 hours. Lead citrate-uranyl acetate stain; X 18,360.

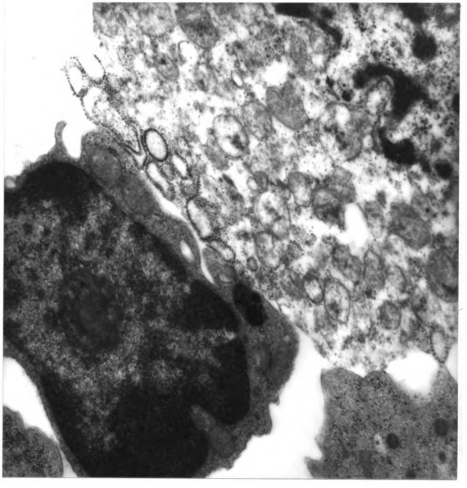


Figure 23 - Coalescence of several granules and their location immediately adjacent to the target cell to which the lymphocyte has extensively attached. Incubation of 16 hours. Lead citrate-uranyl acetate stain; X 25,704.

Table 2 - Frequency of positive staining lymphocytes with surface immunoglobulins (Ig) and Fc receptor for IgG (Fc_GR) as related to the percentage of LGL per isolated lymphocytes.

Dog	IgM (%)	IgG (%)	Fc_GR (%)	LGL (%)
Spot	5	10	19	8.4
Frankie	6	10	26	6.8
Spice	1	4	2	8.2
Annie	2	2	6	4.2
Mean	3.5	6.5	13.3	6.9
SEM	1.2	2.1	5.6	1.9

acted as positive controls for the latter stain (Figure 24). The percentage of positive mononuclears however, represented only a minor population of the total mononuclear cells.

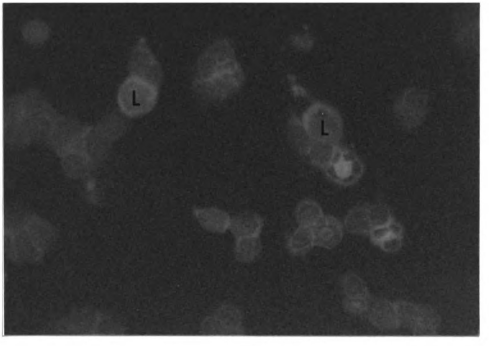


Figure 24 - Positive staining of 2 lymphocytes (L) and 2 neutrophils. Direct immunofluorescence staining for Fcreceptor of IgG; X 40.

CONCLUSIONS

These studies indicated that LGL were present normally in canine peripheral blood and generally accounted for 6% of all lymphocytes. This percentage could be increased up to 17% following routine NK cell isolation procedures.

Morphology of LGL with Wright's stain was similar to that observed in other species. Numerous azurophilic staining granules were eccentrically placed within the cytoplasm of a large lymphocyte that measured 10-15 μm).

The isolated effector cell population was heterogeneous with variable cytochemical and surface marker staining. This population contained canine NK cells as verified by the demonstration of spontaneous cytolysis of tumor target cells (data not shown), but these cells likely represented only a minor portion of the total population.

Cytochemically, positive staining of effector cells resembled that in other species. The effector cells stained negative for those stains that identify granulocytic markers (Sudan black B, peroxidase, alkaline phosphatase, PAS and CAE). They stained positive for ANAE, ANBE and ACP in a variable percentage of the population. Generally ANAE was the weakest of the three and all demonstrated a focal pattern of stain distribution. The variability in staining indicated a mixed population of T cells with up to 55% having a focal staining pattern and a much smaller

population with diffuse (ACP) staining.

Immunologically, the percentage of effector cells with surface immunoglobulins was similar to other species. However, cells with Fc_G receptors did not predominate as has been reported in rodents and people. Immunofluorescent staining with surface antibodies indicated the majority of cells were negative for IgG and IgM. Presence of receptors for Fc_G was found only in a small number of effector cells (up to 26%).

Ultrastructurally, LGL and their binding interaction with target cells were similar to that observed in rodents and humans with the exception that no pore-like or ring structures were demonstrated by these studies. The granules were generally membrane bound, osmiophilic staining structures resembling the lysosomes of myelomonocytic cells. Binding interactions varied from villous interdigitations with minimal contact to a smooth and extensive membrane adherence. Approximately 75% of the cells binding to tumor targets involved granular lymphocytes which is similar to most species. The location of the granules during these interactions was variable but generally found on the side of the nucleus closest to the target cell. This orientation was particularly apparent where binding was extensive suggesting a direct role of the granules in cytolysis, a process which has been shown to require intimate contact between effector and target cells.

DISCUSSION

Since the LGL is reported to mediate NK cell activity in many species, an attempt was made to determine their frequency in canine peripheral blood, isolate and characterize these cells morphologically, cytochemically and immunologically and finally to relate their appearance in binding interactions with target cells. The frequency of LGL in peripheral blood was similar to that reported in people being generally less than 5% of leukocytes (Timonen et al., 1981).

The type of isolation procedure utilized may affect the yield of LGL. The use of nylon wool adherence following Ficoll-Hypaque gradient centrifugation increased the LGL purity (data not shown) by removing neutrophils, monocytes and some B cells. Reynolds et al. (1981) demonstrated a similar increase in purity in rats using nylon wool adherence. However there are suggestions that activated NK cells have an enhanced adherence to nylon wool or plastic (Herberman, 1985b). This could presumably remove some functional NK cells.

Purification procedures used in this study were less extensive than those used for other species. Discontinuous Percoll gradient centrifugation or flow cytometry for specific NK markers are additional methods commonly used to isolate NK cells. Simpler and less sensitive techniques were

used in this study. This may account for the marked difference in the yield of LGL, 15% in the dog compared to 60-90% for rodent and human cells (Reynolds et al., 1981; Timonen et al., 1981; Hiserodt & Beals, 1985). However, a canine study using nylon wool adherence and discontinuous Percoll gradients yielded 15% LGL as the greatest concentration of any fraction (Savary & Lotzova, 1986). This suggests the canine granular lymphocytes have more variable cellular densities than human or rodent cells and that dog LGL may not be readily purified by adherence or density methods. Similarly, porcine NK studies (Yang et al., 1987) using plastic adherence and discontinuous Percoll gradients produced fractions abundant in non-granular small to medium sized lymphocytes (4.5 - 6.0 μm). Therefore these procedures will yield a variety of cell types responsible for NK cell activity.

Morphologically, the canine LGL most closely resembled those of non-human primates and people (Savary & Lotzova, 1986). Cell size was similar to that of people (12-15 μm) and rats (10-14 μm). The size and numbers of azurophilic granules were similar to those in people and primates (Savary & Lotzova, 1986). However, mice and rats appear to have more abundant and less localized cytoplasmic granules than other species (Reynolds et al., 1981; Savary & Lotzova, 1986).

Cytochemical staining overall indicated only a small

proportion of the isolated lymphocytes stained positive. Similar to human and rodent studies, stains such as peroxidase, Sudan black B, leukocyte alkaline phosphatase and PAS were negative. One human study found positive staining in a majority of LGL with chloroacetate esterase, a granulocytic marker (Heumann et al., 1983). This could not be supported by this study as only rare positive staining resulted. This finding is in agreement with the majority of human and rodent reports.

Rat and human studies indicate ANBE is negative in NK cells. The presence of focal ANBE staining in this study in up to 53% of the lymphocytes suggests a mixed population of T cells and non-T cells. A similar focal appearance was noted in ACP and ANAE stained canine preparations. The focal or globular pattern seen with ANBE, ANAE or ACP has been associated with T cells in people and rodents (Manconi et al., 1979; Bozdech & Bainton, 1981; Yang et al., 1982). Specifically, this focal staining pattern with ACP or ANAE in people corresponds to T non-G lymphocytes or T cells with Fc receptors for IgM (Manconi et al., 1979; Ferrarini et al., 1980). These cells have little or no NK cell activity (Ferrarini & Grossi, 1986).

A diffuse fine granular staining with ACP was present in a small number of isolated canine lymphocytes. Human T cells with Fc receptors for IgG and third population cells which lack B or T cell markers but possess receptors for

Fc_G, stain in a diffuse or paranuclear granular pattern with ACP and ANAE (Ferrarini et al., 1980; Huhn et al., 1982). The third population cells are primarily responsible for NK cell activity.

Information regarding staining pattern by nonspecific esterases (ANAE, ANBE) and ACP has not been well worked out in dogs. Extrapolation of the significance of these patterns from other species to this study suggests that the isolated lymphocytes consisted of a small population of T cells and non-T cells having Fc_G receptors. However a larger proportion of the cells were likely T cells with IgM receptors.

Ultrastructurally, there is strong similarity between the dog and other species in the types of organelles present within the cytoplasm. The majority of granules were lysosomal in nature being membrane bound with dense internal staining. One cell had a granule-like structure which resembled the parallel tubular arrays reported in people. However, this cluster of granular chain-like densities likely represented an accumulation of glycogen or ribosomes. Their frequency was rare compared to a more common occurrence of parallel tubular arrays in people.

Ultrastructurally, the interaction between target cells and granular or agranular canine lymphocytes was analogous to that seen in rats, pigs and humans, particularly in the early binding stages. The pore-like structures found on the

surface of target cells in human and murine studies (Henkart & Henkart, 1982; Podack & Dennert, 1983) were not observed in this study. The intercellular cylindrical or ring-like structures reported by Hiserodt and Beals (1985) were occurring naturally or produced within 15 minutes after the addition of calcium. Their absence in the dog may be due to differences in isolation procedures, incubation times, fixation or microscopic resolution.

The addition of calcium was also associated with a change in the cell surface characteristics which occurred 5-15 minutes following calcium pulsing (Hiserodt & Beals, 1985). Prior to its addition and after 30 minutes, microvillous projections were present. This morphologic change which was also observed with Fc bearing lymphocytes adhered to antigen-antibody complexes, may be necessary in the cytolytic process (Alexander & Henkart, 1976). Similar to these studies, cell surface appearance in the dog changed from villous to smooth with increased attachment of lymphocytes to target cells.

The location of granules on the side of the nucleus closest to the target cell is in agreement with the majority of reports which also suggest this orientation is important for the cytolytic process to occur. The exocytosis of granule contents releases potent cytotoxic substances (Henkart, 1985).

The observations made at various incubation periods

indicated few time-related differences relative to the pattern of attachment of lymphocytes to target cells. This may be due to the recycling nature of NK cells. Once cell death has been initiated by the NK cell, it can attach to other target cells and begin again the cytolytic process. The preparation of cells for electron microscopy can disrupt the interaction between targets and effector cells, particularly in the prebinding or postbinding stages of cytolysis. Manipulation of the cytolytic rate with serial examinations performed within 1 hour of incubation would be more helpful in evaluating this process.

Since binding is necessary for cytolysis to occur, one can conclude that the cells most frequently bound to targets are responsible for NK cell activity. This study indicated that approximately 75% of binding lymphocytes contained cytoplasmic granules. Other evidence to support the LGL as the canine NK cell comes from data which demonstrates parallelism between the frequency of canine LGL and NK cell activity. Percoll fractions with larger numbers of LGL produced increased cytotoxicity in lytic units as measured in a chromium release assay (Savary & Lotzova, 1986).

Immunologic marker studies in dogs indicate the NK cell variably rosettes with human and guinea pig erythrocytes (Betton, 1980; Ringler & Krakowka, 1985). To additionally support the T cell origin, Ringler and Krakowka (1985) demonstrated the expression of Thy-1, a T cell marker of

mice on a majority of the canine NK cells.

Furthermore, Ringler and Krakowka (1985) demonstrated that 39.0% of binding lymphocytes rosetted with IgG coated ovine erythrocytes indicating the presence of Fc receptors for IgG. They also showed that depletion of these cells significantly reduced NK cell activity. The present study using a direct immunofluorescence method detected 13.3% IgG receptor positive cells. A low proportion (6.5%) of positive cells stained by direct immunofluorence for surface IgG was also found in this study. This compares to (1%) by Ringler and Krakowka (1985). The variation in positive cell percentages may be explained by differences in the techniques and the population of the lymphocytes sampled. The addition of nylon wool adherence in the present study may have removed some of the Fc_G receptor positive cells as well as granulocytes and monocytes. Ringler and Krakowka's evaluations were performed on lymphocytes bound to dead targets while the present study assessed a total lymphocyte population.

There is a direct relationship between human LGL and IgG receptor positive cells (Gastl *et al.*, 1983). Enrichment procedures for cells having Fc_G receptors have produced 90% purity of LGL (Timonen *et al.*, 1981). The percentage of LGL per lymphocytes in this canine study did not correlate well with the percentage of positive Fc_G receptor cells ($p > .05$) using correlation analysis. However, there was significant

correlation between the percentage of Fc_G receptor positive cells and NK cell activity (data not shown).

It is concluded from this study that several morphologic, cytochemical and immunologic features of canine NK cells resemble those described for other species including humans and mice. These involve the confirmation of the presence of LGL in peripheral blood and in NK effector cell populations. In addition, morphologic studies suggest the LGL may mediate NK cell activity in the dog. Effector cells used in the NK cell assays were weakly positive for ANAE, ANBE and ACP. Furthermore, they involve a small population of Fc_G receptor positive cells.

CHAPTER 3

NATURAL KILLER CELL ACTIVITY IN NORMAL DOGS

NATURAL KILLER CELL ACTIVITY IN NORMAL DOGS

INTRODUCTION

Natural killer (NK) cell activity is a spontaneous nonspecific cell mediated cytotoxicity reaction which acts independently of the humoral defense mechanisms. In vivo, NK cell activity has been attributed to early tumor cell elimination, cytolysis of virus infected cells, graft rejection, hematopoietic cell regulation and general immunoregulatory functions (Trinchieri & Perussia, 1984; Wheelock & Robinson, 1983). NK cell activity has been recognized in almost every species including: invertebrates, fish, amphibians, sheep, cattle, pigs, chickens, dogs, cats, rodents, non-human primates and humans (Savary & Lotzova, 1986; Yamamoto et al., 1985; Yang et al., 1987; Sharma & Coulson, 1979; Betton, 1980; Tompkins et al., 1983; Kiessling et al., 1975b; Reynolds et al., 1981; Altman & Rapp, 1978; Gengozian et al., 1986; Timonen et al., 1979b).

Features used to identify the cells responsible for NK activity include: nonadherence, nonphagocytosis, receptors for Fc portion of immunoglobulin (Ig) G, some T cell and myelomonocytic surface markers, absence of surface

immunoglobulin, cytolysis of NK cell-sensitive cell lines, and the presence of azurophilic cytoplasmic granules (Trinchieri & Perussia, 1984; Gastl et al., 1984; Betton, 1980; Kiessling et al., 1975b; Ringler & Krakowka, 1985a; Timonen et al., 1975a). The frequency of the large granular lymphocytes (LGL) has been closely associated with NK cell activity in rodents and humans (Reynolds et al., 1981; Gastl et al., 1983).

Reports evaluating NK cell activity in dogs have been few in number. Immunologic studies involving erythrocyte rosetting; presence of Thy-1, surface Ig, or Fc receptors for IgG; adherent and phagocytic properties have been reported in the dog (Betton, 1980; Shek et al., 1980; Ringler & Krakowka, 1985a). Investigation of NK functional activity has included conventional and gnotobiotic dogs (Savary & Lotzova, 1986; Krakowka, 1983; Betton, 1980), canine distemper virus infected dogs (Krakowka, 1983; Shek et al., 1980; Ringler & Krakowka, 1985b) and dogs with spontaneous neoplasia (Betton & Gorman, 1978; Warren et al., 1975). Reports involving morphology of canine NK cells are limited (Savary & Lotzova, 1986).

The aim of this work was to evaluate NK cell activity from healthy adult dogs through concurrent functional and light microscopic studies. The relationship between frequency of LGL present in effector cell populations and NK cell activity was explored using both chromium release and

single cell binding assays.

MATERIALS AND METHODS

Animals

Eighteen clinically healthy pure and mixed breed adult dogs were used. There were 3 intact males, 6 intact and 9 ovariohysterectomized females. Seven dogs were evaluated 2-3 times providing a total of 26 samples.

Effector and target cell isolations

The isolation of effector and target cells was performed by the method described in Chapter 2.

Light microscopic studies

Determination of the frequency of LGL was accomplished by the procedure described in Chapter 2. LGL were distinguished by the presence of numerous small red cytoplasmic granules located focally at one end of a large lymphocyte. Percent LGL refers to their frequency in the total isolated mononuclear population.

Chromium release microcytotoxicity assay

Following isolation of target cells, 100 μCi of sodium chromate (^{51}Cr) in saline (New England Nuclear, Boston, MA) was added to approximately 5 X 10^6 cells and allowed to

incubate at 37 C for 90 minutes. Final target cell suspension was adjusted to 5 X 10^4 cells/ml in supplemented MEM. Fifty or 100 µl of the effector cell suspension were placed in a 96 round bottom well microtiter plate (Flow Lab., Inc, McLean, VA) along with 100 µl of the target cell suspension for effector to target cell ratios (E:T) of 50:1 or 100:1. Wells containing 100 µl of target cells alone were used to measure spontaneous release of the radiolabel. Maximum release was defined by lysis of 100 µl of target cells with 10% Triton X-100, a cell detergent. Plates were centrifuged 200 g for 2 minutes at 4 C. Samples were read immediately and after 12 or 16 hours incubation in a 37 C humidified 5% CO_2 /95% air environment. Supernatants were collected from wells using a Skatron harvesting set (Sterling, VA) and read in a gamma counter (TM Analytic, Inc, Elk Grove Village, IL). All tests were performed minimally in triplicate. Cytotoxicity was measured using counts per minute (CPM) of radioactive release. In the following formula, percent cytotoxicity equals:

$$\frac{\text{CPM (effectors + targets) - CPM (spontaneous release)}}{\text{CPM (maximum release) - CPM (spontaneous release)}} \times 100$$

Single cell binding assay

Preparation of poly-L-lysine (PLL) coated coverslips were modified from the technique described by Vargas-Cortes

et al. (1983). Poly-L-lysine hydrobromide (Sigma Chemical Co, St. Louis, MO) was diluted with buffered Hanks solution to a concentration of 2 µg/ml and 1 ml was placed on each glass coverslip (22 mm X 22 mm) used. Coverslips were incubated for 45 minutes at room temperature, rinsed with Hanks solution and allowed to dry before use.

One hundred microliters of cell suspensions concentrated to 1 X 10^6/µl were used for both effector cells and targets. Targets alone and an E:T of 1:1 were pelleted and allowed to incubate 15 minutes at 37 C. The supernatant was then replaced with 0.5 ml serum free MEM and the cells gently resuspended. The cell mixture was plated upon the PLL coated coverslips and allowed to incubate 20 minutes at room temperature to insure adherence. Samples were read immediately and after 12 and 16 hours incubation in a 37 C humidified 5% CO_2 environment. To detect dead cells, coverslips were stained with 0.1% trypan blue in Hanks solution, then rinsed to remove any excess. Coverslips were then inverted onto glass slides and the number of conjugates per 200 live lymphocytes was counted. The percentage of live or dead conjugates with effector cells bound to targets was then calculated.

Immunofluorescent staining of effector cells

A direct immunofluorescent procedure was applied to cell suspensions to detect surface staining. To detect the

presence of receptors for the Fc portion of IgG (Fc_G), fluorescein conjugated Fc fragment for dog IgG (Jackson Immunoresearch Laboratories Inc, Avondale, PA) was used. A dilution (1:10) was made of the antibody and 0.1ml was added to 1.5 X 10^6 cells of the non-adherent lymphocyte population. Incubation for 1 hour at 4 C was followed by 2 phosphate buffered saline washes. Cells were resuspended in phosphate buffered saline, then transferred to a glass slide and examined under a fluorescent microscope. Mononuclear cells were counted and positive cells were expressed as a percentage of the total number.

Statistical analysis

Arcsin transformation of percentage data was performed prior to analysis. Paired and unpaired Student's t tests (two tailed) were used to compare incubation times, cell ratios and sex differences. Relationships between frequency of LGL, percent cytotoxicity, percent live and dead cell binding and percent Fc_G receptor positive cells were determined by Pearson's correlation analysis.

RESULTS

Table 3 lists the NK cell activity in healthy dogs as measured by the chromium release assay. There was no significant difference ($p > .05$) between the 12 and 16 hour

Table 3 - NK cell activity in 26 samples from 18 healthy dogs.

	12 Hours		16 Hours	
	50:1	100:1	50:1	100:1
Males: (3)				
Mean	26.3	35.0	23.5	40.2
SEM	9.7	10.3	13.1	11.7
# of Samples	3	3	3	3
Intact Females: (6)				
Mean	14.4	17.5	19.3	18.6
SEM	4.3	5.2	4.9	4.7
# of Samples	10	8	10	8
Neutered Females: (9)				
Mean	16.6	24.8	18.1	24.5
SEM	2.3	3.4	1.9	4.4
# of Samples	13	12	13	12
TOTAL:				
MEAN	16.9	23.6	19.2	24.5
SEM	2.3	2.9	2.4	3.3
# OF SAMPLES	26	23	26	23

Data expressed as percent cytotoxicity at 12 and 16 hours of incubation with 50:1 and 100:1 (E:T).

incubation periods for effector to target cell ratios of 50:1 or 100:1. Sixteen hour incubation generally was higher in NK cell activity than 12 hours (Figure 25). Significant differences were present between 50:1 and 100:1 (E:T) at 12 hours ($p < .0005$) and at 16 hours ($p < .005$) demonstrating a positive dose response effect. Relative to sex differences, there was a suggestion of highest activity in males, followed by ovariohysterectomized females, with intact females having the lowest activity (Figure 26). However this was not statistically significant ($p > .05$). In 5 dogs with repeated samplings over 1-3 months, 3 had relatively stable activities (Figure 27).

The data from 26 samples indicate the following mean (SEM) for percent LGL: 6.9 (0.9), percent cytotoxicity: 16.9 (2.3) at 12 hours with E:T of 50:1, percent live binding: 8.4 (1.2) at 12 hours and percent dead binding: 1.0 (0.2) at 12 hours. When compared by correlation analysis, no significant difference ($p > .05$) could be found between these variables. There was significant positive correlation ($p < .05$) between the 4 dogs evaluated for percent Fc_G receptor positive cells and percent cytotoxicity (Table 4). In these same dogs, negative correlation ($p < .02$) was present between percent Fc_G receptor positive cells and percent live binding. No correlation could be found between percent Fc_G receptor positive cells and percent LGL.

Figure 25 - Percent cytotoxicity in healthy adult dogs at various effector to target cell ratios for 12 and 16 hours of incubation. Number of samples tested per incubation time is given in parentheses.

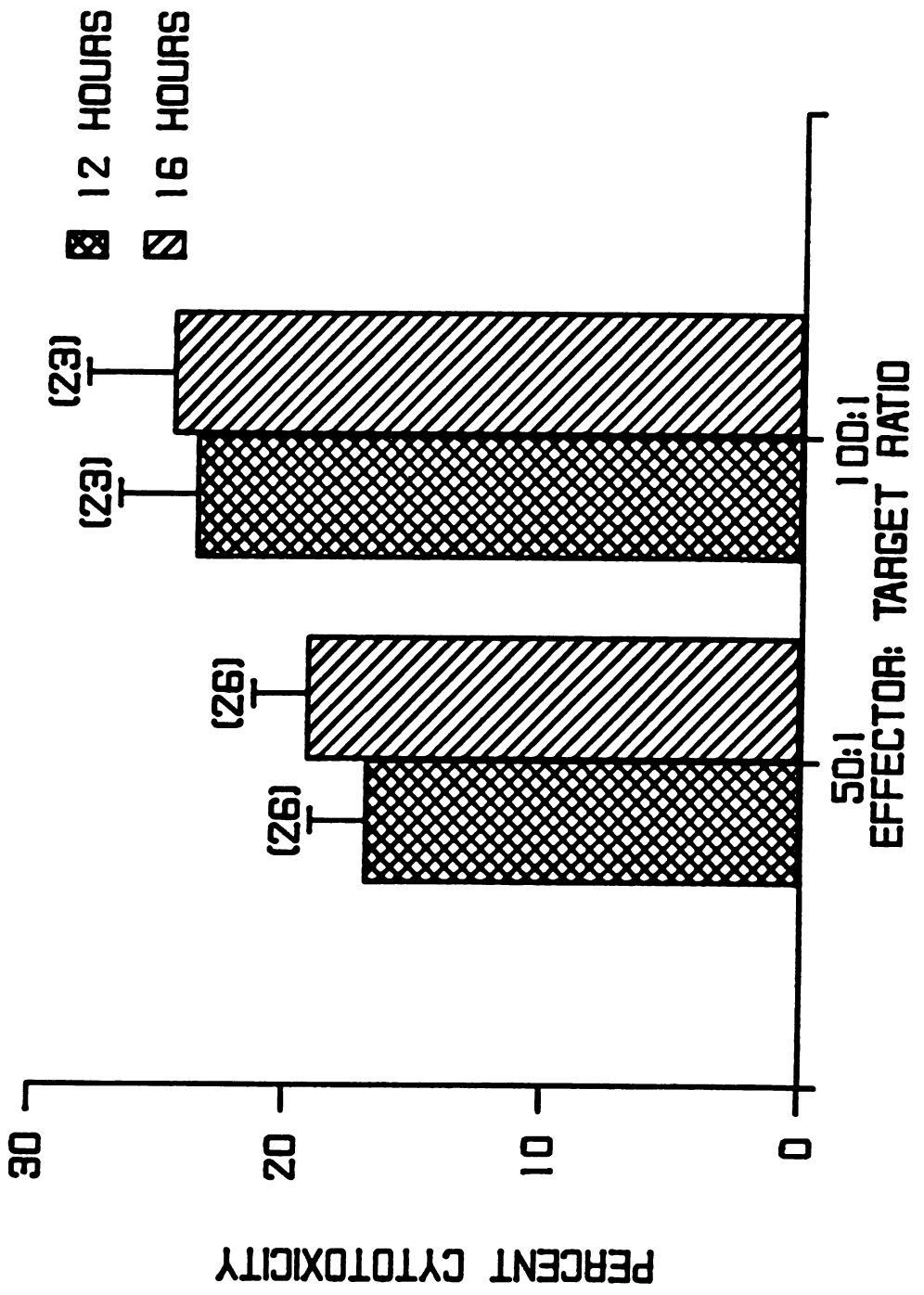

12 HOURS
16 HOURS
(23)
(23)
(26)
(26)
50:1
100:1
EFFECTOR: TARGET RATIO
PERCENT CYTOTOXICITY
0
10
20
30

Figure 25

Figure 26 - Comparison of percent cytotoxicity at various incubation times and effector to target cell ratios for males, intact and neutered females. Number of samples per group is given in parentheses.

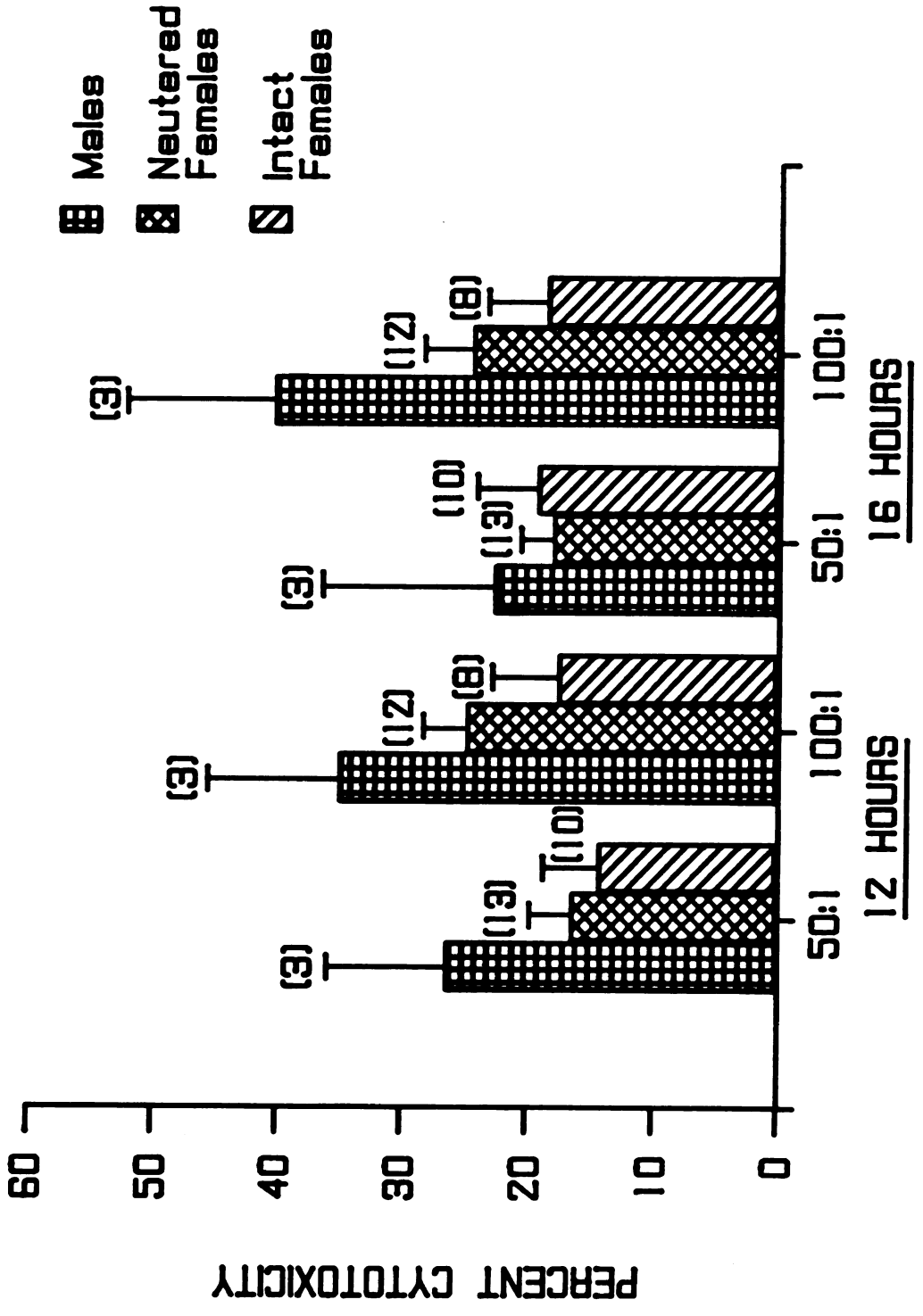

Males
Neutered Females
Intact Females
PERCENT CYTOTOXICITY
0
10
20
30
40
50
60
(3)
(13)
(10)
(3)
(12)
(8)
50:1
100:1
12 HOURS
(3)
(13)
(10)
(3)
(12)
(8)
50:1
100:1
16 HOURS

Figure 26

Figure 27 - Samplings of 5 dogs taken twice over 1-3 months. Percent cytotoxicity determined at 12 hours incubation with various effector to target cell ratios. Paired symbols identify individual dogs.

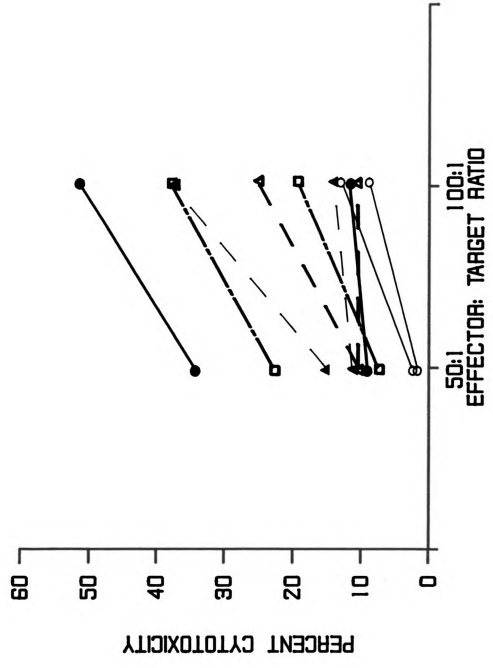

PERCENT CYTOTOXICITY
60
50
40
30
20
10
0
50:1
100:1
EFFECTOR: TARGET RATIO

Figure 27

Table 4 - Frequency of Fc_G receptor positive cells and correlation to percent cytotoxicity, percent live or dead cell binding and percent LGL.

	Dogs				Correlation Coefficient	Significance
	1	2	3	4		
% Fc_G Receptor:	19	26	2	6	-	-
% Cytotoxicity:						
50:1 (12)	7.6	10.5	8.1	9.0	0.444	NS
100:1 (12)	19.3	25.0	9.7	11.8	0.990	$p < .05$
50:1 (16)	20.6	19.0	6.2	10.6	0.961	$p < .05$
100:1 (16)	15.3	23.3	13.3	13.0	0.833	NS
% Cell Binding:						
Live (12)	3.5	1.5	9.0	8.0	-0.981	$p < .02$
Dead (12)	0.5	0.5	0	2.0	0.236	NS
% LGL:	8.4	6.8	8.2	4.2	0.155	NS

Number in parentheses indicates hours of incubation.
NS = not significant

CONCLUSIONS

This study indicated no significant difference in NK cell activity in healthy dogs between 12 and 16 hours incubation. However, increased cell concentrations demonstrated significantly higher NK cell activities. Too few individuals were available per group but there was nonetheless a definite trend towards higher NK cell activity in males compared to females.

Similar to other species, percent cytotoxicity varied widely within the total group of 26 canine samples. However, healthy individuals tended to maintain the same level of activity over time.

An unexpected finding was that neither frequency of LGL nor percent dead binding correlated well with NK cell activity. The percentage of cells possessing Fc_G receptors did significantly and positively correlate with percent cytotoxicity but negatively with percent live binding. While cell isolation procedures and quantitation of NK cell activity may account for some of the discrepancy, it appears that identification of the canine NK cell is more closely related to the presence of immunologic markers than to mere morphologic criteria.

DISCUSSION

Use of percent cytotoxicity to evaluate NK cell activity has been shown to be a qualitative method (Pross et al., 1986). Studies in people suggest this method produces a large degree of variability with a broad range of activity found in repeatedly tested normal individuals (Pross & Baines, 1982). However, Gengozian et al. (1986) found individual rhesus monkeys could be separated into low and high responder categories. Within these groups, twice repeated samples from individuals over time produced a relatively constant level of activity using percent cytotoxicity.

Two canine studies indicate a similar variability in NK levels. Ringler & Krakowka (1985a) reported levels of NK activity in a small population of adult dogs that ranged from 12.4% to 51.5% using CTAC targets with 30:1 (E:T) at 16 hours of incubation. Betton (1980) measured percent cytotoxicity in 10 dogs over 2-3 weeks and found considerable differences in activity. In comparison, the present study utilizing percent cytotoxicity to measure NK cell activity, also found a wide range within the entire population. However, stable activity was evident in most of the individuals that were sampled twice over time.

To reduce some of the variability in NK cell activity, mathematical calculations from several E:T may be performed

to express this activity in terms of lytic units (LU) or relative cytotoxicity. Lytic units refers to the activity per effector cells necessary to produce lysis of a predetermined percentage of target cells. Calculation of a ratio between the LU of an individual as compared to that of normals run concurrently gives a more accurate and quantitative measurement of NK cell activity (Pross & Baines, 1982). Since 3 or more E:T are necessary to accurately derive LU, this calculation could not be attempted in the present study.

The variability of NK cell activity in humans has been attributed to age, sex, drugs, exercise and disease (Pross & Baines, 1982; Brahmi et al., 1985; Huwyler et al., 1985; Lipinski et al., 1982). Age differences in people involve lower values at birth which gradually increase towards middle age and decrease mildly in the elderly (Pross & Baines, 1982). Since this study involved only adult dogs, this comparison could not be made. The only reference to age in dogs is by Ringler & Krakowka (1985b) who evaluated pups from 1 to 6 weeks of age. They found activity present as early as 1 week of age that minimally fluctuated over the 6 weeks.

Relative to sex differences, Pross & Baines (1982) reported that the mean activity in each age group tested including neonates, was higher for males than females. Sex-related differences were not found in 23 gnotobiotic pups

evaluated from 1-6 weeks of age (Ringler & Krakowka, 1985b). The results from the present study indicated a trend similar to people with males having the highest activity. However this was based on a small number per group and hence was not statistically significant. Between intact and ovariohysterectomized females, differences were much less pronounced. Since dates of the surgery were unknown and most were sampled once, little significance can be attributed to this observation. Estrogens have been shown to inhibit NK cell activity in mice (Seaman et al., 1978). It is therefore possible that this hormone is responsible for the differences seen between males and females in this study.

Other dog studies indicate activity increases over time, particularly when CTAC targets are used (Krakowka, 1983; Savary & Lotzova, 1986). Results from this study using 8 (data not shown), 12, and 16 hours of incubation also showed a gradual increase in NK activity. However there was no significant difference between the 12 and 16 hour incubation periods for either E:T (50:1 or 100:1) tested. Therefore evaluations of NK cell activity in the dog against CTAC targets may use either 12 or 16 hour incubation times.

Several human and rodent studies suggest strong association between the number or percentage of LGL present or binding to targets and NK cell activity, measured in percent cytotoxicity or LU (Timonen et al., 1979b; Reynolds et al., 1981; Gastl et al., 1983). Gastl et al. (1984)

reported this finding was particularly evident in malignant and nonmalignant disease states. Despite the association, Reynolds et al. (1981) felt LGL alone would not predict NK cell activity since not all LGL have cytolytic function against certain targets. They found that only 10-30% of LGL formed conjugates with target cells.

The present study could find no significant correlation between the frequency of LGL seen by light microscopy and percent cytotoxicity. Savary and Lotzova (1986) evaluated several Percoll fractions in the dog relative to percent LGL and NK cell activity that was measured in LU. They found parallelism between the highest levels for each variable with peaks occurring in fraction 3 which consisted of approximately 45% Percoll. Comparison of this work to the present study is impossible due to differences in LGL isolation and measurement of activity. In addition, the number of animals tested was not indicated in Savary and Lotzova's report. It is also possible that Percoll fractions which separate cells on the basis of density may select for a different functionally active LGL or nongranular lymphocyte. There is information to suggest that the cell's activity may be linked to granule maturation which could affect cell density. Furthermore, recent porcine studies indicate that the cell mediating its NK cell activity is primarily nongranular (Yang et al., 1987). Since percentages of LGL following isolation procedures are much lower in dogs

compared to rodents and people, it is necessary that additional studies be conducted in the dog to confirm or deny the role of LGL in NK cell activity.

While the frequency of LGL may correlate well with activity levels in some studies, the number of binding cells may not as well (Ullberg & Jondal, 1981; Reynolds et al., 1981; Rubin et al., 1982; Gastl et al., 1984). There is some disagreement as Roder et al. (1978) reported that the frequency of target binding conjugates did correlate well to percent lysis. It is well known that agranular lymphocytes may also participate in effector to target cell binding. Therefore it seems unlikely that there would be strong correlation. This conclusion was supported by the present study.

On the other hand, the percentage of dead or lytic effector-target cell conjugates has been highly correlated to NK activity for certain targets (Ullberg & Jondal, 1981; Rubin et al., 1982). This observation could not be supported by this study which may reflect differences in technique and longer incubation periods which were required in the canine analysis.

Instead of morphologic features to establish the identity of the NK cell, functional or immunologic markers have been more widely used in humans and rodents. Discussion of all these markers is out of the scope of this report. However, the marker that identifies the receptor for the Fc

portion of IgG was evaluated in this study. Ringler and Krakowka (1985a) demonstrated marked reduction in NK activity following depletion of Fc_G receptor positive cells. In addition, they found 39% of functional canine NK cells possessed this marker. Using a different technique, this study demonstrated that up to 26% of isolated lymphocytes were Fc_G receptor positive.

The importance of this marker in evaluating NK cell activity is additionally supported by human studies which highly correlate Fc_G receptor positive cells with target binding cells or cytotoxicity (Ullberg & Jondal, 1981; Timonen et al., 1979b). While the present study demonstrated significant correlation between Fc_G receptor positive cells and percent cytotoxicity, it established a significant negative correlation with live conjugate formation. Since only 4 dogs were tested, it would be premature to be definitive about this data. Additional studies are clearly necessary before the role of Fc_G receptor positive cells in NK cell activity is established for the dog.

It can be concluded from this study that certain similarities and differences exist between the NK cell activity of various species and that in normal or healthy adult dogs. Similarities involve the presence of spontaneous or natural cell mediated cytotoxicity against tumor target cells, a wide range of normal NK cell activity, minimal variation in activity among individual dogs over time, sex

differences in activity and a significant correlation between Fc_G receptor positive cells and percent cytotoxicity. Unexpected results were the lack of significant correlation between LGL frequency or percent dead binding and NK cell activity.

CHAPTER 4

NATURAL KILLER CELL ACTIVITY IN UNTREATED AND TREATED CASES OF CANINE LYMPHOMA

NATURAL KILLER CELL ACTIVITY IN UNTREATED AND TREATED CASES OF CANINE LYMPHOMA

INTRODUCTION

Lymphoma is the most frequent hematopoietic neoplasm in dogs accounting for 15% of all malignancies (Priester & McKay, 1980). Affected dogs are usually middle to older aged and sex predilection is not evident (Rosenthal, 1982). Several breeds appear to be at high risk including boxers, basset hounds, airedale terriers, Scottish terriers, bulldogs, Saint Bernards, labrador retrievers and bull mastiffs (Rosenthal, 1982; Onions, 1984). Elevated reverse transcriptase activity has been associated with lymphoma cell cultures but definitive proof of a viral etiology is lacking (Madewell, 1985).

Classification of canine lymphoma has involved their anatomic distribution, immunologic features and cytologic or histologic type (Couto, 1985). The most common form of anatomic presentation is multicentric characterized by generalized lymphadenapathy which progresses to organomegaly and terminally into leukemia. Immunologic studies demonstrated most canine lymphomas were of B cell origin

(Holmberg et al., 1976; Onions, 1977). Classification of histologic cell types have been adapted to those described in people with the most recent being the NCI Working Formulation (Carter et al., 1986).

Prognosis has been related to clinical staging, presence of paraneoplastic syndromes, sex and histologic cell type (Crow, 1982; Cotter, 1983; Gray et al., 1984; Couto, 1985; MacEwen et al., 1987). Survival without treatment is short being about 2 months. Glucocorticoids alone provide a survival range between 2-6 months. Combined chemotherapy protocols may extend this range to 1.5 years with a median survival of 6-7 months (Crow, 1982; Couto, 1985).

The role of NK cells in tumor immunosurveillance has been suggested indirectly by studies of in vitro NK cell activity correlated to growth of transplantable tumors in experimental animals. For example, beige mice with deficient NK activity, develop more rapid and frequent tumors compared to normal mice (Karre et al., 1980). On the other hand, nude athymic mice with higher than normal NK activity, have increased resistance to tumor growth (Warner et al., 1977).

The in vivo role of NK cells in the prevention of spontaneous tumors has been derived indirectly from observations that individuals with genetic immune deficiencies, such as Chediak-Higashi with low NK cell activity, have a high risk of developing lymphoid

malignancies (Roder et al., 1980; Abo et al., 1982). The same is true for transplantation patients receiving large doses of immunosuppressant drugs that reduce NK cell activity (Lipinski et al., 1982). In contrast, normalization of NK cell activity occurs during periods of disease remission (Stupp et al., 1978; Yoda et al., 1983).

An inverse in vivo relationship between NK cell activity and progression of disease can be derived from experiments in mice which deplete the NK cell population through specific NK antibodies (asialo GM_1, NK1.1) or drugs (estradiol, cyclophosphamide) allowing increased tumor growth and hematogenous metastasis (Hanna & Burton, 1981; Hanna & Schneider, 1983; Barlozzari et al., 1983; Hanna, 1987; Seaman et al., 1987). Restoration of the depleted NK cell population produced decreased lung metastasis and evidence to confirm the NK cell's role in host defense against early metastasis (Hanna & Fidler, 1980; Hanna, 1986).

The malignancies most commonly associated with decreased NK cell activity in various species involve hemolymphatic conditions. These include lymphoid or myeloid leukemias, Hodgkin's and non-Hodgkin's lymphoma and bovine leukosis (Behelak et al., 1976; Stupp et al., 1978; Ziegler et al., 1981; Hawrylowicz et al., 1982; Tursz et al., 1982; Laroche & Kaiserlian, 1983; Nasrallah & Miale, 1983; Kay & Zarling, 1984; Yamamoto et al., 1984; Jezewska et al., 1985;

Fontana et al., 1986; Fujimiya et al., 1986; Hooper et al., 1986). It is therefore possible for a similar association to exist between canine lymphoma and reduced NK cell activity.

If NK cell activity plays an important role in the progression of canine lymphoma, then it is desirable for drug protocols that treat the condition, to enhance that activity. Several investigators have reported in contrast, an inhibition of NK cell activity by glucocorticoids, cyclophosphamide and combination protocols presently used for the treatment of lymphoproliferative disorders (Stupp et al., 1978; Oehler & Herberman, 1978; Parrillo & Fauci, 1978; Hochman & Cudkowicz, 1979; Riccardi et al., 1981; McGeorge et al., 1982; Bray et al., 1983; Ballas, 1986; Braun & Harris, 1986).

The purpose of this study was to investigate the presence of natural cytotoxic mechanisms in spontaneous untreated cases of lymphoma in dogs. In addition, the effects of glucocorticoids and combined chemotherapy protocols used to treat these patients would also be evaluated relative to NK cell activity. The use of concurrent chromium release and single cell binding assays would help determine whether the disease and treatment effects are associated with low NK cell numbers and/or altered NK cell function.

MATERIALS AND METHODS

Animals

A total of 11 adult canine lymphoma patients were evaluated. These were placed into 3 groups depending upon prior treatment. The groups were as follows: I - untreated (n=7), II - glucocorticoid-treated (n=5), III - combined chemotherapy-treated (n=4). The combined chemotherapy protocols involved COP (cyclophosphamide, vincristine and prednisone) or CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone). Five of these dogs were placed in 2 groups (Table 5). A summary of the clinical data for these cases including sex, breed, age, clinical staging (WHO classification), histologic cell typing (Carter et al., 1986) and specific treatment may be found in Appendix B. A total of 11 clinically healthy control dogs were used for concurrent or paired evaluations of lymphoma patients. A larger comparison population, namely "all controls", consisted of the above 11 paired controls plus 15 additional clinically healthy dogs.

Effector and target cell isolations

The isolation of effector and target cells has been previously described in Chapter 2.

Chromium release microcytotoxicity assay

Measurement of recycling ability or overall function of

NK cells was performed by the method described in Chapter 3.

Table 5 - The group placement of 11 canine lymphoma patients (I = untreated, II = glucocorticoid-treated, III = combined chemotherapy-treated)

Dog Number	Group I	Group II	Group III
1	X *	X	
2	X *		X
5	X		X
6	X		
8		X	X
9		X	X
10		X	
11	X		
12		X	
14	X		
17	X		

* Chromium release data unavailable for 100:1 effector to target cell ratios

Single cell binding assay

Methods to evaluate the frequency of target binding and cytotoxic cells in effector cell populations were discussed previously in Chapter 3.

Statistical analysis

Arcsin transformation of percentage data was performed prior to analysis. A split-plot factorial analysis of variance was used to determine the between and within group interactions of the 3 lymphoma conditions relative to cell ratios and incubation times. Additionally, a paired

Student's t test (one-tailed) was used to compare mean differences between lymphoma patients and their concurrently measured controls. Significant differences were considered to be $p < .05$.

RESULTS

Table 6 lists the NK cell activity of canine lymphoma patients as measured by the chromium release assay for various incubation times and effector to target cell ratios (E:T). Significant differences ($p < .05$) were found between paired controls and Group I patients at 16 hours and an E:T of 100:1. No significant differences were found between paired controls and prior glucocorticoid-treated lymphoma dogs. Significant differences were present between controls and COP or CHOP treated lymphoma patients at various incubation times and E:T. Figure 28 illustrates the mean and standard error differences in percent cytotoxicity between paired controls and lymphoma patients at 16 hours and 100:1 (E:T).

The live and dead target cell binding activity by lymphocytes in lymphoma dogs for 12 and 16 hours of incubation is listed in Table 7. No significant differences were found between paired controls and lymphoma dogs of any group. The mean and standard error differences for controls and lymphoma patients measured as percentage of live and

Table 6 - NK cell activity* in canine lymphoma patients versus paired and all controls.

	12 HOURS		16 HOURS	
	50:1	100:1	50:1	100:1
GROUP I (untreated)				
Controls:				
Mean	10.8	15.5	11.9	23.0
SEM	2.3	5.7	2.2	8.9
Number	7	5	7	5
Patients:				
Mean	7.7	10.0	12.1	14.6 [a]
SEM	2.4	2.8	2.9	5.2
Number	7	5	7	5
GROUP II (glucocorticoid-treated)				
Controls:				
Mean	17.0	25.7	22.1	25.3
SEM	7.3	7.3	6.2	8.5
Number	5	5	5	5
Patients:				
Mean	15.3	24.1	21.5	26.8
SEM	5.9	7.5	9.6	9.9
Number	5	5	5	5
GROUP III (combined chemotherapy-treated)				
Controls:				
Mean	13.5	13.4	10.8	12.3
SEM	6.0	3.7	3.4	2.5
Number	3	4	4	4
Patients:				
Mean	3.9 [a]	5.1 [b]	2.2	3.5 [c]
SEM	2.3	1.8	1.3	1.2
Number	3	4	4	4
ALL CONTROLS				
Mean	16.9	23.6	19.2	24.5
SEM	2.3	2.9	2.4	3.3
Number	26	23	26	23

* Data expressed as percent cytotoxicity at 12 and 16 hours of incubation with 50:1 and 100:1 (E:T).
a = $p < .05$
b = $p < .01$
c = $p < .025$

Figure 28 - NK cell activity in percent cytotoxicity (mean plus standard error) for canine lymphoma patients and paired controls. Horizontal area with vertical hatching represents the mean +/- 1 standard error for all controls.

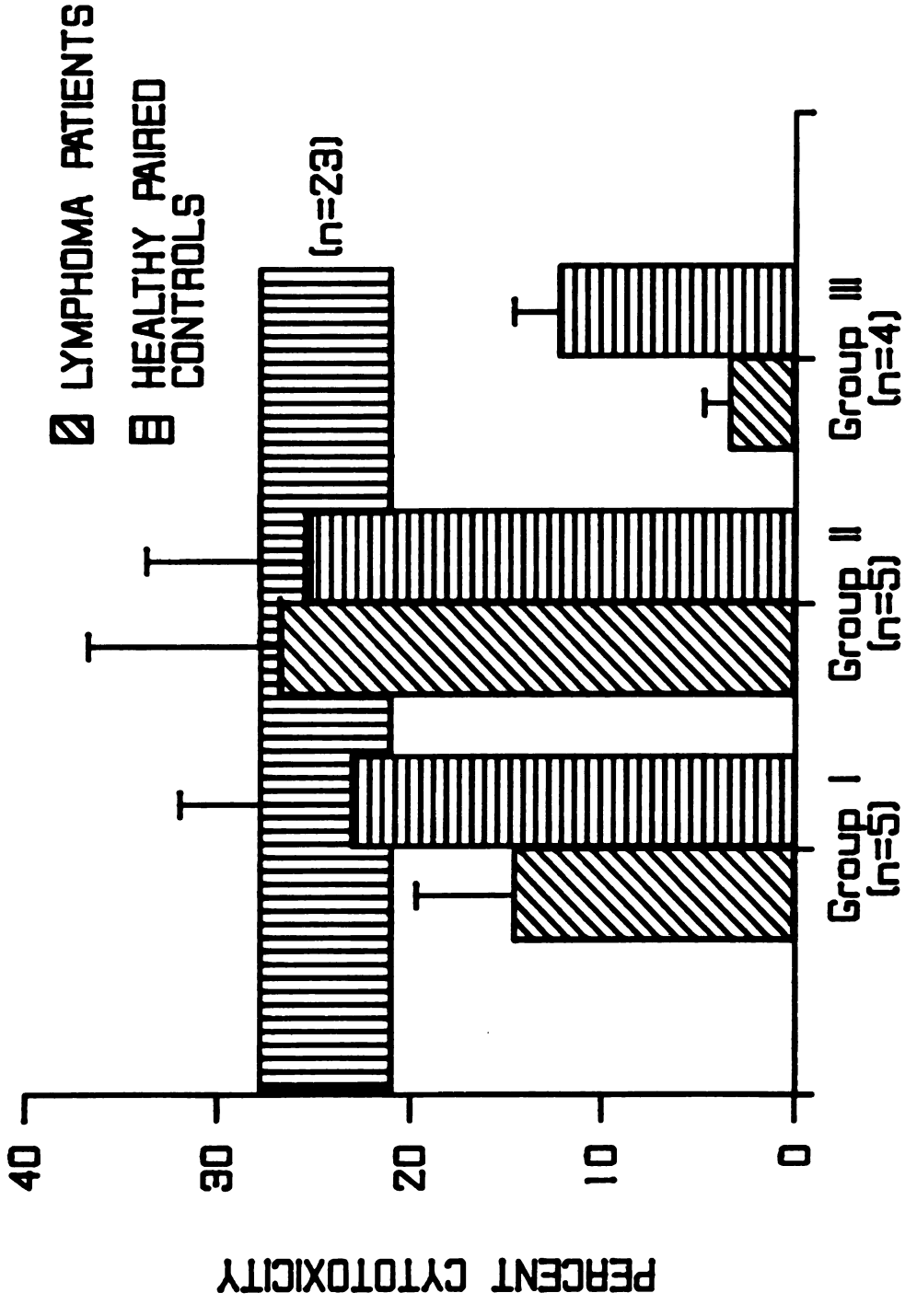

LYMPHOMA PATIENTS
HEALTHY PAIRED CONTROLS
(n=23)
PERCENT CYTOTOXICITY
40
30
20
10
0
Group I (n=5)
Group II (n=5)
Group III (n=4)

Figure 28

Table 7 - Single cell binding activity* in canine lymphoma patients versus paired and all controls.

	12 HOURS		16 HOURS	
	Live	Dead	Live	Dead
Group I (untreated)				
Controls:				
Mean	15.9	0.9	15.2	1.0
SEM	4.4	0.6	4.6	0.4
Number	7	7	6	6
Patients:				
Mean	14.7	0.4	14.3	0.2
SEM	6.2	0.2	4.5	0.2
Number	7	7	6	6
Group II (glucocorticoid-treated)				
Controls:				
Mean	11.5	3.4	14.0	2.8
SEM	3.4	2.3	3.0	1.3
Number	4	4	4	4
Patients:				
Mean	10.5	0.5	16.3	0.3
SEM	1.2	0.3	2.1	0.3
Number	4	4	4	4
Group III (combined chemotherapy-treated)				
Controls:				
Mean	26.0	3.3	24.0	2.3
SEM	5.7	1.6	5.2	1.3
Number	4	4	4	4
Patients:				
Mean	29.5	0.5	31.0	1.8
SEM	3.6	0.3	9.5	1.4
Number	4	4	4	4
ALL CONTROLS				
Mean	19.0	2.0	21.2	1.2
SEM	3.8	0.2	2.8	0.2
Number	23	23	24	24

* Data expressed as number of live or dead target cells bound per 200 lymphocytes at 12 and 16 hours of incubation.

dead targets bound to lymphocytes at 16 hours incubation are illustrated in Figure 29. Although patients generally had reduced binding to dead targets compared to controls suggesting fewer cytotoxic cells, the differences were not statistically significant.

Table 8 indicates the effects of combined chemotherapy in relation to the initial or pre-treatment levels of NK cell activity. The data suggested an association between relative initial levels and the relative degree of reduction in NK cell activity seen with combined chemotherapy. For example, high initial NK cell activity compared to all controls is not protective to the decrease in NK activity. The lowest initial activity, on the other hand had the least reduction following combined chemotherapy.

CONCLUSIONS

The results of this study were similar to human studies and indicated a significant reduction in NK cell activity for untreated lymphoma patients when compared to their paired controls and measured in percent cytotoxicity. No significant differences were found between lymphoma patients and their controls relative to the relative frequency of live target binding cells or cytotoxic binding cells in effector cell populations. Despite individual effectiveness, these active NK cells were less cytotoxic overall than

Figure 29 - Percent live target binding to lymphocytes (mean plus standard error) for canine lymphoma patients and paired controls. Horizontal area with vertical hatching represents the mean +/- 1 standard error for all controls.

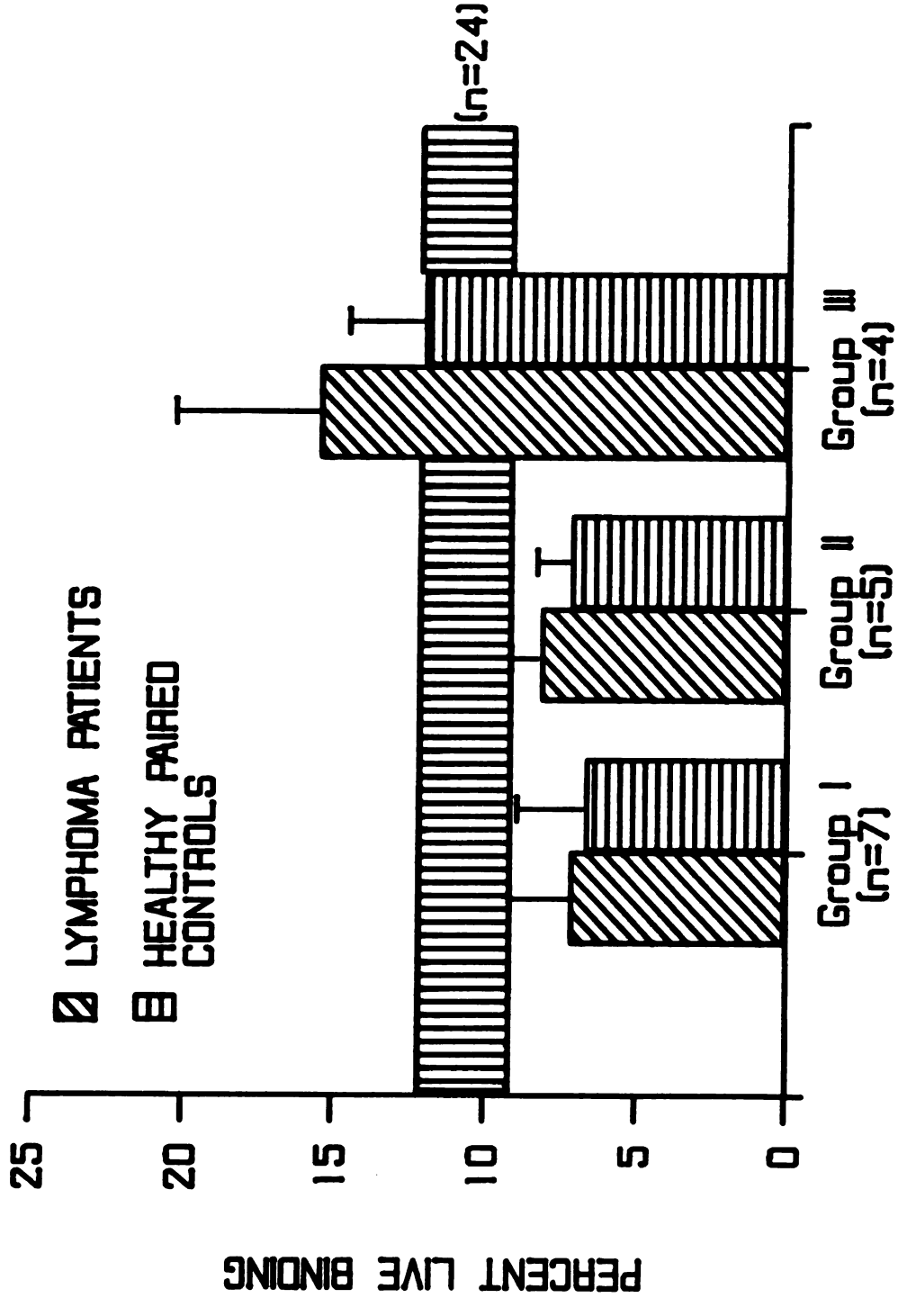

PERCENT LIVE BINDING
25
20
15
10
5
0
LYMPHOMA PATIENTS
HEALTHY PAIRED CONTROLS
Group I (n=7)
Group II (n=5)
Group III (n=4)
(n=24)

Figure 29

Table 8 - Degree of reduction in NK cell activity following combined chemotherapy relative to initial levels as compared to the initial NK cell activity levels relative to normal controls.

Dog #	Therapy Before Testing	Relative Initial [a]	Degree of Reduction [b]
5	None	-72%	25%
2	None	-31% [c]	61%
8	Glucocorticoids	+16%	85% [d]
9	Glucocorticoids	+139%	98%

a = mean difference in % cytotoxicity compared to all controls for 4 experiments unless otherwise stated
b = mean difference in % cytotoxicity for 4 experiments unless otherwise stated
c = mean of 2 experiments
d = mean of 3 experiments

controls. Therefore it appears that a mechanism of immune dysfunction in canine lymphoma involves an inability of NK cells to recycle.

Different effects from treatment were seen depending upon the type used. Dogs having received glucocorticoids prior to testing had normal NK cell activity, suggesting canine NK cells are steroid-resistant. COP or CHOP treated patients, on the other hand, had marked inhibition of NK cell activity, the mechanism of which appears similar to untreated lymphoma dogs. Neither treatment significantly depressed individual effector cell function suggesting these drugs were not preferentially cytotoxic to NK cells.

DISCUSSION

Unique insight into the likely nature of the NK cell dysfunction in canine lymphoma is permitted by the concurrent use of chromium release and single cell binding assays. These procedures when performed together have helped to determine the mechanism of the defect for other disease conditions such as, Chediak-Higashi, Hairy cell leukemia and advanced solid epithelial malignant tumors (Abo *et al.*, 1982; Fontana *et al.*, 1986; Steinhauer *et al.*, 1982). The presence of decreased NK cell activity in untreated canine lymphoma as measured by a chromium release assay agrees with all known reports involving human lymphoma (Lipinski *et al.*,

1982; Tursz et al., 1982; Hawrylowicz et al., 1982; Gastl et al., 1984). However, in none of these reports were single cell target binding and chromium release assays performed together.

In the present canine study using single cell binding assays, the relative frequency of effector cells bound to live or dead target cells was the same in untreated lymphoma patients as compared to their controls indicating active NK cells were present to the same degree in the effector cell population used for the assay. Therefore the decrease in chromium release from dead target cells could not be attributed to relatively low numbers of active NK cells. Individual cells in the chromium release assay were able to detach from dead target cells and bind to additional targets thereby accomplishing multiple cytotoxic reactions. This ability to "recycle" has been determined to be defective in certain solid tissue malignancies when decreased NK cell activity is unrelated to the frequency of effector cells (Steinhauer et al., 1982). It could be concluded that canine lymphoma also involves a recycling defect.

The exact mechanism for decreased NK cell activity in lymphoma is unknown but possible explanations, in addition to recycling defects, include depressed levels of produced or available interferon (Hawrylowicz et al., 1982). They reported only half of the cases responded to pretreatment of in vitro interferon suggesting low levels of it alone could

not account for all the differences. In vivo studies (Foon, 1986) show again only partial response to interferon.

Other cytokines or biological response modifiers, such as interleukin 2 have demonstrated correction of deficient NK cell activity against fresh human lymphoid leukemias (Lotzova et al., 1987). This would suggest a relationship between low amounts of interleukin 2 available and NK cell defects. Specifically this cytokine is known to affect the rate of cell lysis and release of cytotoxic factors from NK cells (see Chapter 1).

The relationship of interleukin 2 and NK cell activity in human leukemias relative to cytolytic rate may help to explain percent cytotoxicity differences between controls and untreated lymphoma dogs. The cytolytic rate relative to recycling involves the following conditions: 1)rate and ability of attachment by effector cells to target cells, 2)availability of cytotoxic factors with the cell, 3)rate of release of these factors by effector cells and 4)rate of detachment and movement towards other target cells. The single cell binding assay results indicated conditions 1 and 3 were satisfied to a normal degree. The combined effect in having many slower moving NK cells or those with lower quantities of cytotoxic factors would help to explain why the only statistically significant difference between the above 2 groups occurred at the longest incubation period (16 hours). As time evolved, fewer numbers of competent

cytotoxic cells would reach their targets. The difference in cytolytic rate in untreated lymphoma supports a possible role of interleukin 2 in the treatment of canine lymphoma. Additional studies, using *in vivo* or adoptive transfer of interleukin 2 cultured or activated effector cells, are clearly necessary to better define the mechanism of immune dysfunction in canine lymphoma.

Investigation into the absolute number of NK cells present would contribute much to further understanding the disease mechanism, however these were not determined due to the lack of a practical marker for this canine cell type. An earlier study (Chapter 3) showed that cells which stained positive by direct immunofluorescence for the Fc_G receptor correlated well with canine NK cell activity. This could be used for future studies as one such marker, but more specific ones are necessary. Monoclonal antibodies that are specific for murine and human NK cells include asialo GM_1, NK1.1, NKH-1 and Leu-11. Of the studies evaluating NK cell numbers in human lymphoma, one report (Baumann *et al.*, 1986) quantitated circulating NK cells by flow cytometry using the monoclonal antibody NKH-1 (Leu-7). They found in previously treated lymphoma patients, reduced NK cell numbers compared to controls. In addition, Baumann *et al.* (1986) reported a correlation between NKH-1 positive cells and the clinical course of these patients. No simultaneous assay for NK cell activity was performed in this study to verify a low overall

activity existed. One report argued that the cell marker Baumann et al. used only detects a portion of the active NK cell population and may not truly reflect the entire population (Foa et al., 1986).

To contrast the report of reduced NK cell numbers in lymphoma, Gastl et al. (1984) found when compared to controls, an increase in the absolute number of large granular lymphocytes (LGL) present in non-Hodgkin's lymphoma. These cells have been closely correlated with NK cell activity in species other than dogs (Chapter 3). Gastl et al. (1984) concluded that "defects other than the simple reduction of effector cells are involved in this malignancy-associated impairment of NK function".

Effects of glucocorticoids on NK cell function are variable as indicated in the literature. This may well explain the lack of inhibition or augmentation in cytotoxic activity or target binding ability when compared to controls for canine patients treated with glucocorticosteroids. Many early rodent and human studies (Oehler & Herberman, 1978; Hochman & Cudkowicz, 1979; Parrillo & Fauci, 1978; Bray et al., 1983) noted transient in vivo and in vitro reductions in NK cell activity with hydrocortisone and dexamethasone. Bray et al. (1983) concluded that the drug in vitro blocked an early membrane signaling event necessary for activation of the lytic mechanism.

A recent human study (Katz et al., 1984) using in vivo

and *in vitro* corticosteroids on normal human subjects, found on the other hand, increased NK activity which was attributed to a selective depletion of noncytoxic cells from circulation and a relative increase in the frequency of target binding cells. Also, single cell binding and cytolysis were not affected by *in vivo* corticosteroids.

To further characterize the unresponsiveness of LGL to corticosteroids, Katz *et al*. (1985) examined corticosteroid receptor numbers and affinity but found no differences. In addition, they concluded that corticosteroid receptor characteristics bore more resemblance to those of neutrophils and monocytes than T cells which had lower receptors sites per cell and a lower dissociation constant.

An earlier study by Onsrud and Thorsby (1981) demonstrated a change in NK cell activity over time following *in vivo* administration of corticosteroids to human subjects. Activity was increased in samples collected at 4 hours while decreased for samples at 24 hours. Levels returned to normal at 48 hours. Parallel variations were found in the relative percentage of positive cells for the Fc_G receptor, but not in their absolute numbers. This suggests the effects of corticosteroids are due to a reversible time dependent redistribution of cells other than NK cells.

The variation in reported effects for glucocorticoids may suggest the time factor is most critical. Incubation

periods used in the present canine study were relatively long. A study involving shorter time periods might help to fully explain the observed effects in dogs.

This study in dogs agreed with human and rodent studies which reported reductions in NK cell activity due to combined chemotherapy protocols or to their component drugs alone. It has been shown with *in vivo* administration of cyclophosphamide alone in normal rodents that a transient dose dependent decrease in NK cell activity was produced (Mantovani *et al.*, 1979; Hanna & Fidler, 1980; Riccardi *et al.*, 1981; Ballas, 1986). Robinson & Heath (1968) reported that cyclophosphamide depressed levels of interferon in the intact animal but not *in vitro*. Since interferon inducers will not boost the NK activity of cyclophosphamide-treated mice (Hanna & Fidler, 1980), this suggests the relationship to interferon is only a secondary response and not the primary mechanism of cyclophosphamide-induced inhibition. The depressant effects on NK cell activity were short lived, returning to normal levels by 7-12 days (Mantovani *et al.*, 1978; Riccardi *et al.*, 1981; Ballas, 1986). The drug reduced the number of positive Fc_G receptor cells (Hunninghake & Fauci, 1976). Permanent effects on NK cell maturation and activity were not seen with cyclophosphamide administration as adoptive transfer i.e., reconstitution with activated spleen and bone marrow cells could remove the cyclophosphamide induced effects of enhanced pulmonary

metastasis of tumors (Hanna & Fidler, 1980; Riccardi et al., 1981).

Adriamycin (doxorubicin) given alone produced no change in NK activity but analogs of it resulted in marked decreases of activity (Mantovani et al., 1978).

The use of combined chemotherapy to treat acute lymphoblastic leukemia in children, acute myelogenous leukemia in adults and several solid tissue tumors in people resulted in changes in NK cell activity (McGeorge et al., 1982; Stupp et al., 1978; Braun & Harris, 1986). The drugs used in common with the present canine study included vincristine, prednisone, cyclophosphamide and doxorubicin. Two of the above studies involved cyclophosphamide which has already been shown to suppress NK cell activity. The third study utilized vincristine and prednisone along with 6-mercaptopurine and methotrexate (McGeorge et al., 1982). These investigators demonstrated marked depression in NK cell activity receiving this combined therapy but not for patients in remission whose treatment had been discontinued for at least 6 months. Therefore, combined chemotherapy appears to produce no long term effects on NK cell function.

One report related the effects of chemotherapy to NK cell activity levels prior to treatment in patients with solid tissue tumors (Braun & Harris, 1986). They demonstrated that patients with low pre-treatment NK cell activity levels had augmentation of that activity with

chemotherapy. On the other hand, patients with normal pre-treatment NK cell activity later developed depressed activity following chemotherapy. The study concluded that the effects of combined chemotherapy depended in part on pre-treatment levels which in turn were dependent upon glass adherent cell (monocyte) regulation of NK cell activity.

The present canine study had similar results regarding pre-treatment levels and chemotherapy effects. The initial pre-treatment NK cell activity related directly to the degree their activity decreased as a result of treatment. Initial activity which was much higher than controls, was associated with a high degree of reduction in activity following treatment, causing treatment values to be quite low. An important aspect to further explore is whether the levels of NK cell activity measured during treatment have any bearing on the response of the patient to these drugs or to overall survival.

The role of NK cell numbers or activity has been correlated to prognosis in human lymphoma and acute myelogenous leukemia (Baumann et al., 1986; Stupp et al., 1978). Additional studies are necessary to determine this relationship in canine lymphoma.

In summary of this chapter, the study indicated depression of NK cell activity in canine lymphoma patients compared to normal controls. It was concluded for untreated canine lymphoma that the mechanism of immunologic

dysfunction relative to the NK cell defect involves recycling activity rather than a deficiency of active binding or cytotoxic effector cells. Treatment affected only NK cell activity measured as percent cytotoxicity, not the frequency of NK cells but this effect depended upon the type of treatment. Short or long term glucocorticoid therapy did not influence NK cell activity as measured in a 12 or 16 hour incubation chromium release assay. However, more aggressive therapy involving cyclophosphamide, vincristine, prednisone and frequently doxorubicin did significantly depress NK cell activity.

SUMMARY

Three aspects of canine NK cell activity were covered in this study. These involved: 1) morphologic, cytochemical and immunologic characterization of the NK effector cell population; 2) recycling and single cell target binding capability of NK cells from healthy and lymphoma dogs; and 3) treatment effects on NK cell function in lymphoma dogs.

Morphologic features at both light and electron microscopic levels were similar to those observed in other species. The majority of binding cells were large lymphocytes having several lysosomal granules within their cytoplasm. In binding to targets, NK cell surface features changed from irregular villous interdigitations to smooth and extensive tight junctions. Cytoplasmic granules were oriented most frequently between the NK cell nucleus and target cell membrane, supporting their role in cytolysis.

The effector cell population assumed to be NK cells was cytochemically heterogenous with variable numbers of cells staining focally with acid phosphatase and alpha naphthyl butyrate esterase.

Immunologic characterization of the effector cell population confirmed earlier canine studies which found a

majority of cells staining negative for surface immunoglobulins. Cells staining positive for the Fc receptor of IgG were found more frequently, but still involved a minor portion of the population.

NK cell function was measured in both chromium release and single cell binding assays to evaluate overall activity and relative frequency of active binding and cytotoxic cells, respectively. The population of healthy dogs studied had a wide range of NK cell activity, but individual dogs generally maintained constant levels over time. There was no correlation between the number of large granular lymphocytes and NK cell activity or percent dead target cell binding. However cells positive for Fc_G receptors correlated positively to NK cell activity and negatively to percent live target binding. It was concluded that immunologic markers related better to levels of NK cell activity than morphologic features.

The NK cell activity for untreated and combined chemotherapy treated lymphoma dogs was significantly less than that of healthy controls. The relative frequency of active binding or cytotoxic NK cells was not affected in any of the lymphoma groups. It was concluded that the immune defect in untreated and combined chemotherapy treated lymphoma dogs relative to NK cell function involved decreased recycling ability to lyse multiple target cells. Therapy with glucocorticoids had little effect on either

overall activity or cytotoxic binding cell numbers which suggested the canine NK cell may be steroid-resistant.

APPENDICES

APPENDIX A

NATURAL KILLER CELL ACTIVITY IN NORMAL DOGS

Table 9 - NK cell activity* of 26 samples from 18 healthy dogs (raw data).

	12 Hours		16 Hours	
	50:1	100:1	50:1	100:1
Males:				
	19.6	35.1	25.5	43.4
	45.4	52.7	45.1	58.7
	13.9	17.1	0	18.6
Mean (SEM)	26.3 (9.7)	35.0 (10.3)	23.5 (13.1)	40.2 (11.7)
Intact Females:				
	34.5	51.3	30.2	50.6
	9.0	11.8	10.6	13.0
	2.9	6.1	5.0	12.6
	37.0	NT	47.3	NT
	13.4	23.3	22.4	13.3
	28.1	NT	41.3	NT
	2.6	12.8	8.1	9.7
	1.8	8.8	8.4	18.4
	8.1	9.7	6.2	13.3
	6.9	15.8	13.2	18.2
Mean (SEM)	14.4 (4.3)	17.5 (5.2)	19.3 (4.9)	18.6 (4.7)
Neutered Females:				
	5.0	5.5	4.5	6.2
	10.5	10.6	6.0	7.0
	10.5	25.0	19.0	23.3
	16.6	NT	19.6	NT
	25.4	22.5	19.1	11.5
	15.4	37.8	21.8	57.5
	11.5	14.1	13.3	19.6
	33.7	42.9	24.4	33.7
	14.3	25.1	18.2	23.5
	22.9	37.8	26.7	41.0
	7.6	19.3	20.6	15.3
	15.3	21.5	14.8	17.2
	27.5	35.6	27.4	38.0
Mean (SEM)	16.6 (2.3)	24.8 (3.4)	18.1 (1.9)	24.5 (4.4)
TOTAL MEAN	16.9	23.6	19.2	24.5
SEM	2.3	2.9	2.4	3.3

* Data expressed as percent cytotoxicity at 12 and 16 hour incubations with 50:1 and 100:1 (E:T).
NT = not tested

Table 10 - Comparison between percent LGL, percent cytotoxicity and percent live or dead binding in 26 samples from 18 healthy dogs.

% LGL	% Cytotoxicity[1]	% Live Binding[2]	% Dead Binding[2]
12.6	19.6	0.5	0.5
2.6	45.4	4.5	0.5
0.7	13.9	3.5	2.5
2.3	34.5	10.0	1.5
4.2	9.0	8.0	2.0
7.1	2.9	9.0	0.5
13.5	37.0	21.5	1.5
0.6	13.4	2.0	0
4.6	28.1	13.0	1.5
6.4	2.6	9.5	1.0
15.6	1.8	10.5	0
8.2	8.1	9.0	0
5.8	6.9	9.0	5.5
5.4	5.0	13.0	0
7.8	10.5	17.5	2.0
6.8	10.5	1.5	0.5
1.0	16.6	1.5	0
4.0	25.4	21.0	1.0
6.2	15.4	4.5	0.5
12.9	11.5	11.0	0
4.4	33.7	2.0	1.5
9.7	14.3	17.5	1.5
10.1	22.9	0	1.5
8.4	7.6	3.5	0.5
2.1	15.3	12.0	0
16.8	27.5	5.5	0.5
TOTAL MEAN (SEM)			
6.9 (0.9)	16.9 (2.3)	8.4 (1.2)	1.0 (0.2)

1 = Percent cytotoxicity determined at 12 hours incubation and 50:1 (E:T).
2 = Binding assay determined at 12 hours incubation.

APPENDIX B

NATURAL KILLER CELL ACTIVITY IN UNTREATED AND TREATED CASES OF CANINE LYMPHOMA

Table 11 - Summary of clinical data for canine lymphoma patients.

Dog/Clinic # Sex; Age Breed	Histologic Cell Type[1]	Date Tested	Group #	Clinical Staging[2]	Prior Test Treatment
1/533714 F/S; 4 y Bull Mastiff	Small Noncleaved	12/19/86	I	V a	None for 2 weeks
		4/15/87	II	V b	Prednisone (3 weeks)
2/533698 M; 5 y Ches Bay Retr	Immuno-blastic	12/19/86	I	V b	None
		2/4/87	III	"	COP (week #7)
5/534655 F/S; 8 y Gold Retr	Immuno-blastic	2/12/87	I	IV a	None
		4/10/87	III	"	CHOP (week #9)
6/514375 M; 8 y Lab Retr	Diffuse Large	2/16/87	I	III a	None
8/535013 F; 7 y Bull Mastiff	Unknown	3/3/87	II	IV b	Prednisone (3 months)
		3/23/87	III	"	CHOP (week #3)
9/535241 M/C; 6 y Beagle	Immuno-blastic	3/11/87	II	V a	Prednisone (1 week)
		4/6/87	III	"	CHOP (week #4)

Table 11 (continued)

Dog/Clinic # Sex; Age Breed	Histologic Cell Type[1]	Date Tested	Group #	Clinical Staging[2]	Prior Test Treatment
10/535392 M; 11 y Gold Retr	Lympho-blastic	3/24/87	II	V b	Prednisone (4 d)
11/535695 M/C; 6 y Boxer	Lympho-blastic	4/8/87	I	V a	None
12/535860 F/S; 7 y Boxer	Unknown	4/10/87	II	III a	Dexamethasone (10 d); Methylprednisone acetate (3 d before)
14/512947 F; 8 y Afghan	Unknown	5/28/87	I	IV a	None
17/536938	Unknown	6/2/87	I	III a	None

1 = cell types according to NCI Working Formulation
2 = stages according to WHO classification
(COP-cyclophosphamide, vincristine, prednisone; CHOP-cyclophosphamide, vincristine, doxorubicin, prednisone)

Table 12 - NK cell activity* in untreated canine lymphoma patients versus paired controls.

	12 HOURS			
	CONTROLS		PATIENTS	
	50:1	100:1	50:1	100:1
	16.6	NT	20.3	NT
	16.6	NT	10.0	NT
	10.5	10.6	7.2	7.3
	15.4	37.8	5.6	20.3
	11.5	14.1	1.3	5.2
	2.9	6.1	7.7	10.8
	1.8	8.8	1.7	6.2
Mean	10.8	15.5	7.7	10.0
SEM	2.3	5.7	2.4	2.8
Number	7	5	7	5
	16 HOURS			
	14.3	NT	25.1	NT
	14.3	NT	15.3	NT
	6.0	7.0	2.8	6.2
	21.8	57.5	17.3	34.8
	13.3	19.6	6.7	11.6
	5.0	12.6	10.5	12.9
	8.4	18.4	7.1	7.5
Mean	11.9	23.0	12.1	14.6
SEM	2.2	8.9	2.9	5.2
Number	7	5	7	5

* Data expressed as percent cytotoxicity at 12 and 16 hours of incubation with 50:1 and 100:1 (E:T)
NT = Not Tested

Table 13 - NK cell activity* in glucocorticoid-treated canine lymphoma patients versus paired controls.

	12 HOURS			
	CONTROLS		PATIENTS	
	50:1	100:1	50:1	100:1
	9.0	11.8	5.2	20.7
	13.4	23.3	20.6	28.5
	45.4	52.7	33.5	47.0
	10.5	25.0	16.6	23.0
	6.9	15.8	10.6	1.3
Mean	17.0	25.7	15.3	24.1
SEM	7.3	7.3	5.9	7.5
Number	5	5	5	5

	16 HOURS			
	CONTROLS		PATIENTS	
	50:1	100:1	50:1	100:1
	10.6	13.0	11.8	17.5
	22.4	13.3	22.0	26.6
	45.1	58.7	57.5	63.3
	19.0	23.3	11.9	22.6
	13.2	18.2	4.3	4.1
Mean	22.1	25.3	21.5	26.8
SEM	6.2	8.5	9.6	9.9
Number	5	5	5	5

* Data expressed as percent cytotoxicity at 12 and 16 hours of incubation with 50:1 and 100:1 (E:T)

Table 14 - NK cell activity* in combined chemotherapy treated canine lymphoma patients versus paired controls.

	12 HOURS			
	CONTROLS		PATIENTS	
	50:1	100:1	50:1	100:1
	25.4	22.5	7.9	9.1
	6.9	15.8	3.6	6.7
	5.0	5.5	NT	3.1
	8.1	9.7	0.1	1.3
Mean	11.4	13.4	3.9	5.1
SEM	4.7	3.7	2.3	1.8
Number	4	4	3	4

	16 HOURS			
	CONTROLS		PATIENTS	
	50:1	100:1	50:1	100:1
	19.1	11.5	0	0.5
	13.2	18.2	4.3	6.4
	4.5	6.2	4.4	4.0
	6.2	13.3	0	3.2
Mean	10.8	12.3	2.2	3.5
SEM	3.4	2.5	1.3	1.2
Number	4	4	4	4

* Data expressed as percent cytotoxicity at 12 and 16 hours of incubation with 50:1 and 100:1 (E:T)
NT = Not Tested

Table 15 - Single cell binding activity* in untreated canine lymphoma patients versus paired controls.

	12 HOURS			
	CONTROLS		PATIENTS	
	Live	Dead	Live	Dead
	21	0	17	0
	18	1	11	0
	22	0	10	0
	9	1	10	1
	35	4	50	1
	3	0	1	0
	3	0	4	1
Mean	15.9	0.9	14.7	0.4
SEM	4.4	0.6	6.2	0.2
Number	7	7	7	7

	16 HOURS			
	CONTROLS		PATIENTS	
	Live	Dead	Live	Dead
	11	2	12	0
	14	0	15	0
	18	2	19	0
	2	0	NT	NT
	36	0	33	0
	6	1	4	0
	6	1	3	1
Mean	13.3	0.9	14.3	0.2
SEM	4.3	0.3	4.5	0.2
Number	7	7	6	6

* Data expressed as number of live or dead target cells bound per 200 lymphocytes at 12 and 16 hours of incubation.
NT = Not Tested

Table 16 - Single cell binding activity* in glucocorticoid-treated canine lymphoma patients versus paired controls.

	12 HOURS			
	CONTROLS		PATIENTS	
	Live	Dead	Live	Dead
	18	11	7	0
	3	1	12	0
	9	1	11	1
	4	0	NT	NT
	16	4	12	1
Mean	10	3.4	10.5	0.5
SEM	3.1	2.0	1.2	0.3
Number	5	5	4	4

	16 HOURS			
	CONTROLS		PATIENTS	
	Live	Dead	Live	Dead
	17	6	19	0
	20	0	19	0
	6	3	10	1
	16	0	NT	NT
	13	2	17	0
Mean	14.4	2.2	16.3	0.3
SEM	2.4	1.1	2.1	0.3
Number	5	5	4	4

* Data expressed as number of live or dead target cells bound per 200 lymphocytes at 12 and 16 hours of incubation.
NT = Not Tested

Table 17 - Single cell binding activity* in combined chemotherapy treated canine lymphoma patients versus paired controls.

12 HOURS

	CONTROLS		PATIENTS	
	Live	Dead	Live	Dead
	18	0	24	0
	26	0	33	1
	18	11	23	1
	42	2	38	0
Mean	26.0	3.3	29.5	0.5
SEM	5.7	1.6	3.6	0.3
Number	4	4	4	4

16 HOURS

	CONTROLS		PATIENTS	
	Live	Dead	Live	Dead
	17	1	17	0
	23	1	25	0
	17	6	23	6
	39	1	59	1
Mean	24.0	2.3	31.0	1.8
SEM	5.2	1.3	9.5	1.4
Number	4	4	4	4

* Data expressed as number of live or dead target cells bound per 200 lymphocytes at 12 and 16 hours of incubation.

APPENDIX C

DETAILED TECHNIQUE PROCEDURES

CANINE LYMPHOCYTE ISOLATION

MATERIALS (2 Samples)

4 - 10ml tubes of heparinized blood
Nalgene .20M filter 115ml (Nalge Co; #120-0020 Rochester,NY)
4 - sterile 50 ml plastic centrifuge tubes with plug seal cap (Corning #25330)
sterile saline
60 ml filtered Ficoll-Hypaque (R.I. = 1.3550)
28-32, (13ml) 17 X 100mm sterile plastic Falcon tubes with caps (#2057 Oxnard, CA)
sterile deionized water
sterile 2xEPS
sterile MEME with 10% FCS, 1% glutamine, 1% pen/strep
sterile MEME without additives
sterile disposable 10ml syringe
0.75g sterile scrubbed nylon fiber (3 denier,3.81 cm, type 200 - #4C2906 Fenwal Laboratories, Deerfield, IL)
sterile 3 way stopcock
hemacytometer
37 C incubator (5% CO_2)
sterile Pasteur pipettes
10 ml sterile pipettes
parafilm
sterile Petri dish or beaker
sterile forceps
sterile carbonyl iron powder
timer

PROTOCOL

1. incubate each 10ml tube of blood following addition of sterile carbonyl iron for 1 hr at 37 C

2. dilute each 10ml tube of blood with saline to 45ml of 50ml tube

3. place 3ml Ficoll-Hypaque into 10 Falcon tubes for each patient and layer gently 9ml of diluted blood over it; 1800 RPM (450 G) for 18 min (BEGIN STEP #8)

4. remove lymph layer by Pasteur pipette and place into 4 Falcon tubes per each patient; fill tube with saline; mix; 1500 RPM (300 G) for 8 min

5. pour off supernatant; fill tube halfway with deionized water; wait 6 sec; agitate 6 sec; add 2xEPS to top; 1000 RPM (140 G) for 8 min

6. pour off supernatant; add saline and combine tubes into 2 per each patient; 1500 RPM (300 G) for 8 min

7. pour off supernatant; resuspend into 5 ml MEME with additives; fill hemacytometer and differentiate granulocytes/lymphocytes

8. attach stopcock and hydrate sterile nylon wool in Petri dish with MEME without additives; place into syringe; fill with 10 ml warm MEME without additives keeping medium above column and allowing a moderate drip rate (use Pasteur pipette)

9. add lymphocyte suspension slowly while eluding the media with the stopcock; once the suspension is completely added, close the stopcock; cover top with Parafilm; allow to incubate 1 hr at 37 C (BEGIN TARGET CELL ISOLATION)

10. elute the non-adherent cells by slowly adding 15 ml warm MEME without additives while keeping the level of media above the nylon column

11. fill hemacytometer; resuspend in complete media and adjust to 5 x 10^6 cells/ml (100 cells/5 hemacytometer squares) REMEMBER TO AVERAGE COUNT TO 5 SQUARES!

Save cell suspension for cytospin preps (minimum of 6) for Wright's, chloroacetate esterase, naphthyl butyrate esterase, and acid phosphatase staining (3-4 drops of lymph suspension/cytospin container, 1000 RPM for 1-5 min)

TARGET CELL ISOLATION and LABELLING

STV (trypsin) (filter periodically)
Chromium-51
1ml tuberculin syringe with 25g needle
37 C water bath
sterile MEME with additives
Triton X-100 solution
sterile tissue culture 96 U shaped well plates with lids (Linbro Titertek #76-013-05, Mc Lean, VA)
supernatant collection system with harvesting frames and macrowell tube strips (Skatron, Inc Harvesting Set #7075, Sterling, VA)
dispensing pipette (200 µl)

PROTOCOL

1. add 3ml STV to culture flask with target cells; pour out; add fresh 3ml STV and allow to incubate 20 min at 37 C

2. split cells passing some into new flasks with remainder placed in 10ml complete MEME; count cells/5 squares

3. use 10 ml of cell suspension (concentration of approximately 10 cells/5 squares); save some unlabelled targets for single cell binding assay

4. pellet target cells at 1500 RPM (300 G) for 10 min

5. Cr labelling calculated as follows:

$$\text{Activity} \times 2\ \text{mCi} \times 1000 = \text{units } \mu\text{Ci needed}$$

$$\frac{\text{units } \mu\text{Ci needed}}{2\ \text{ml}} = \frac{100\ \mu\text{Ci}}{x}$$

6. add Cr and separate cells through syringe and needle; incubate cells for 90 min in 37 C water bath (PREPARE PLL COVERSLIPS)

7. add 5 ml cold MEME to target cell mixture; 1500 RPM for 10 min; discard supernatant

8. add 2 ml cold MEME to target cell mixture; agitate cells gently by pipette; incubate in ice bath for 30 min (BEGIN VIABILITY TESTING AND SCBA)

9. make 10% triton solution by adding 0.5ml Triton X-100 to 5ml MEME

10. determine cell count in the 2ml; pellet cells at 1500 RPM for 10 min; remove supernatant and adjust count to 1 cell/5 squares with additional complete MEME; pellet; add fresh medium especially if frequent aspirations were necessary to obtain proper count

11. fill 2 U shaped well plates with labelled targets, triton, and effector cells; centrifuge at 4 C at 1000 RPM (200 G) for 2 min with blank plate

12. plates read at 0, 12, and 16 hours using supernatant collection system with a gamma counter

Gamma Counter:
TM Analytic, Inc Model 1185
Elk Grove Village, IL

Chromium-51 settings:
Base 5.6; Window 1.6
Attenuator: Coarse (inside) 4 (green)
Fine (outside) 25 (red)
Coarse 900; Fine 3.6
Wide Differential
Set time to minutes; 1 min
High voltage - on
Test - off
Master - on
Auto Stop
Count Twice

Setting Up Trays

lymphocytes or effector cells:
100 cells/5 squares = 5 X 10^6/ml

target cells:
1 cell/ 5 squares = 5 X 10^4/ml

	100:1	50:1	spont	max
lymphs (min 2ml)	100 µl	50 µl	0	0
targets (min 10ml)	100 µl	100 µl	100 µl	100 µl
triton (min 2ml)	0	0	0	100 µl

NOTE: total amount per well = 200 µl or .2 ml

SAMPLE TRAY DIAGRAM FOR CONTROL AND PATIENT

Os	Os	Om	Om	12s	12s	12m	12m	16s	16s	16m	16m
-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-
-	-			-	-			-	-		

12C 50	12C 100	12C 100	12P 50	12P 100	12P 100	16C 50	16C 100	16C 100	16P 50	16P 100	16P 100
-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-

SAMPLE COLLECTION ORDER

O hour - Max(6) + Spont(8)

12, 16 hours - Max(6) + Spont(8) + P 100(8) + C 100(8) + P 50(4) + C 50(4)

PREPARATION OF POLY-L-LYSINE COATED COVERSLIPS
(Modified from Vargas-Cortes J Immunol Meth, 1983)

35 mm plastic petri dishes with lids (9)
22 mm X 22 mm glass coverslips
poly-L-lysine hydrobromide (# P6282 5 mg; MW 90,000; degree of polymerization 430; Sigma Chemical Co, St. Louis, MO
sterile water
HEPES buffered Hank's pH 7.3

PROTOCOL FOR MAKING PLL MIXTURES

1. add 50 ml sterile distilled water to 5mg lyophilized PLL producing 0.1 mg/ml (keep frozen)

2. prepare HEPES buffered Hank's (pH 7.3)
   - 450 ml sterile distilled water
   - 50 ml 10X (conc) Hanks Balanced Salt Solution
   - 1.5 ml 7.5% Na bicarbonate solution
   - 26.3 ml sterile HEPES buffer (pH 7.2)

3. add 1 ml PLL mixture to 49 ml HEPES buffered Hank's solution; place 10ml in 5 Falcon tubes (2 µg/ml) and freeze extra tubes

PROTOCOL FOR COVERSLIP COATING

1. keep 1 tube refrigerated and place 1 ml (about 15-20 drops) to coat coverslip placed in petri dish; rock to cover completely

2. incubate at room temperature for 45 min

3. wash well with HEPES buffered Hanks; lift coverslip off dish and allow to dry before using

VIABILITY TESTING; SINGLE CELL BINDING AND CYTOTOXIC ASSAY

filtered 0.1 % trypan blue in HEPES buffered Hank's
9 dried PLL coated coverslips
12 conical plastic tubes (1.25 ml)
Pasteur pipettes
MEME without additives (serum free)
HEPES buffered Hank's solution
1% formaldehyde in PBS
glass slides
hemacytometer

PROTOCOL

1. pellet isolated lymphocyte population (1500 RPM X 8 min); add serum free MEME to 5 X remaining volume to produce 20 cells/5 squares or 1 X 10^6 cells/ml

2. pellet (unlabelled) target cell suspension (1500 RPM X 10 min); add serum free MEME to produce concentration of 20 cells/5 squares or 1 X 10^6 cells/ml

3. place 100 µl of target cell suspension in 10 conical tubes for: 1 viability, 3 target control, 6 E:T mixtures; place 100 µl of control/patient effector cell suspensions in 8 tubes for: 2 X 1 viability, 2 X 3 E:T mixtures (1:1). Total tubes = 12.

4. test viability by adding equal volume (100 µl) of cell suspension and trypan blue; leave stain for 5-15 min; count % dead on hematocytometer

5. pellet remaining 8 tubes in microfuge (Fisher) at low speed 1500 RPM (200 G) for 5 min

6. incubate tubes at 37 C for 15 min; suction off supernatant

7. resuspend pellet with <u>one</u> suction by Pasteur pipette in serum free MEME (0.5 ml/tube)

8. plate mixture onto PLL coated coverslips; incubate 20 min at room temperature to allow cell attachment (0 hr); remove 1 target control and 1 each of E:T coverslips for initial testing

9. incubate remainder coverslips for additional 12 and 16 hours at 37 C in 5% CO_2

10. following incubation 0, 12, 16 hour samples are tested for:
    % spontaneous dead (target controls)
    % conjugate E:T binding/200 lymphocytes (live and dead)

11. coverslips are prepared as follows:
    wash dropwise with HEPES buffered Hanks or serum free MEME
    stain with 0.1% trypan blue (few drops) for 5 min
    wash/fix slides (several drops) 1% formaldehyde for 5 min
    invert coverslips onto glass slides; drain excess fluid

PROTOCOL FOR ELECTRON MICROSCOPY SAMPLES

1. place equal volumes of effectors and targets (E:T = 1:1) into small falcon tube. BEST TO USE >1 ML OF EACH FROM SCBA WITH SERUM FREE MEME

2. centrifuge 1000 RPM (140 g) for 5 min

3. incubate at 37 C for 15 minutes minimally to allow cell binding

4. suction off supernatant leaving 0.5 ml; resuspend gently

5. pipette mixture into plastic microfuge tubes and incubate at 37 C for desired time e.g., 10, 12, 16 hours

6. replace medium with Karnovsky's fixative leaving for 2 hours minimally

7. pellet at high speed for 30 seconds

8. ready for EM processing

IMMUNOFLUORESCENCE STAINING - REAGENTS AND EQUIPMENT

sterile PBS and 0.02 % NaN_3:
filter 1 ml sodium azide solution (100mg/ml) and add to 500 ml sterile PBS

PBS + 0.02 % NaN_3 + 5 % FCS (complete PBS):
add 20 ml FCS to 380 ml of above solution

reconstitute lyophilized antisera - FITC conjugated using sterile distilled water to volume suggested by manufacture; aliquot 50 µl into plastic microfuge vials and freeze

Fluorescein Conjugated $F(ab')_2$ Fragment Goat Anti-Dog IgM (Mu Chain Specific) (Cooper Biomedical Inc, Cat #1305-0201, 2 ml, Malvern, PA)

Fluorescein Conjugated $F(ab')_2$ Fragment Goat Anti-Dog IgG (Heavy & Light chains specific) (Cooper Biomedical Inc, Cat #1305-0081, 2 ml, Malvern, PA)

Fluorescein Conjugated ChromPure Dog IgG, Fc Fragment (Jackson Immunoresearch Laboratories, Inc; Cat #004-1008, 1.1 ml, Avondale, PA)

dilutions made with PBS + sodium azide solution:
1:100 50 µl to 5.0 ml PBS - use for smears
1:10 50 µl to 0.5 ml PBS - use for live cells

acetone for slide fixation
coplin jar
wooden applicator sticks
dark moist chambers for slide incubation
buffered glycerol to preserve slides
coverslips for cells and smears
glass pipettes

TECHNIQUE FOR IMMUNOFLUORESCENCE STAINING - CELLS (sIg)

1. place 0.3ml of lymphocytes (100/5 squares) and 0.1ml FITC-Antibody at 1:10 dilution into small falcon tubes

2. incubate 4 C for 1 hour (minimal time = 30 minutes)

3. wash twice with 1 ml PBS + FCS + NaN_3; use refrigerated centrifuge at 1500 RPM (500 G) for 5 min following washes

4. add 0.1 ml of complete PBS to pellet

5. transport to fluorescent scope unexposed to light

6. place 1 drop to glass slide, coverslip and read

7. place heat filter on top of FITC filter in field diaphragm

8. place red suppressor filter in condenser holder

9. swing out auxillary lens

10. use #53 filter to detect green fluorescence; 0 filter for total blue count of cells; do 100 cell differential

TECHNIQUE FOR IMMUNOFLUORESCENCE STAINING - SMEARS (cIg)

1. fix slides 30 seconds in acetone; air dry

2. wash 3 changes for 15 min total in "complete" PBS

3. place wooden stick applicator across slide, lay edge of coverslip on stick and fill area below with antisera (1:100); 300 µL NEEDED

4. incubate in moist chamber in refrigerator overnight (24 hours)

5. wash 3 changes for 15 min total in "complete" PBS

6. mount coverslip on glycerol coated area and place into moist chamber

7. transport to fluorescent microscope and read

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VITA

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