

Frances Melinda Carver. CHARACTERIZATION OF RHESUS MONKEY
NATURAL KILLER CELLS MEDIATING LYTIC ACTIVITY TO RAJI.

(Under the direction of Judith M. Thomas, PhD) Department
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Natural killer (NK) cells are naturally occurring and are a first line defense mechanism against viral infections and oncogenesis. They mediate lytic activity to tumor and virally transformed target cells. Human and non-human primate NK cells mediate lysis of various tumor cells including T cell lymphomas and the myeloid leukemia, K562. However, resting human NK cells do not mediate activity to B cell lymphomas. This study describes a previously unreported lymphocyte population in the rhesus monkey mediating strong NK activity to the human B cell lymphoma derived cell line, Raji, and characterizes the functional, morphological, and phenotypic characteristics of this effector cell population.

Natural killer cell lytic activity of unstimulated cells from rhesus monkeys were detected against Raji, Daudi, RPMI-1788, and Molt human cell lines, the first three of which were EBV positive. The strong lytic activity to Raji was significantly higher than to any other cell line tested. Potential explanations for the activity of Raji are discussed as are the possible relevance to low incidence of EBV related diseases in rhesus monkeys. Analysis of in vitro functional parameters revealed that NK cells lytic to

Raji are sensitive to pH and temperature changes and are slightly affected by decreasing serum concentrations. Lysis of Raji and K562 occurs very rapidly; significant lysis is observed after five minutes of incubation although maximum lysis requires four hours. Morphological characteristics were found to include Sephadex nonadherence and enrichment in the low density, LGL enriched fraction of Percoll.

Prostaglandins are recognized as downregulators of intact and nonadherent human and murine NK cells. Addition of PGE₂ to intact rhesus NK cells significantly decreased activity to Raji and K562, although Sephadex passed cells were insensitive to the inhibitory effects of PGE₂. Cold target inhibition studies in which K562 partially blocked the activity to Raji targets suggested the existence of at least two populations of rhesus monkey NK cells. One population may recognize only Raji and a second may recognize both K562 and Raji.

Studies of the phenotypic characteristics revealed enhanced lytic activity in the ERFC⁺ subpopulation. Analysis by MAbs revealed that essentially all of the activity to Raji was contained in the CD16 (Leu 11b) subpopulation while only a portion of the cells expressed CD2 (9.6) and CD8 (OKT8) receptors. Depletion of CD4 (OKT4), HLA-DR (OKIa) or MAC-1 positive cells variably increased activity to Raji. Alloantigen specific CTL generated in vivo were depleted of Fc positive cells and

assayed for lytic activity to their specific target and to Raji and K562. These experiments revealed a functional separation of CTL and NK activity indicating that the cell population mediating lysis of Raji is not a CTL.

These findings suggest that the effector population mediating NK activity to Raji and K562 have similar phenotypic characteristics. However, they appear to differ in their functional characteristics and in their regulation by PGE_2 .

CHARACTERIZATION OF
RHESUS MONKEY NATURAL KILLER CELLS
MEDIATING LYTIC ACTIVITY TO RAJI

A Thesis
Presented to
the Faculty of the Department of Biology
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Master of Science in Biology

by
Frances Melinda Carver

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LIST OF ABBREVIATIONS

ADCC	Antibody Dependent Cell Mediated Cytotoxicity
BSA	Bovine Serum Albumin
CD	Antibody Cluster Group
CMV	Cytomegalovirus
CR	Complement Receptor
CTL	Cytotoxic T Lymphocyte
EBV	Epstein-Barr Virus
ERFC	E Rosette Forming Cell
FCS	Fetal Calf Serum
FcIgG	Fc Fragment of IgG
HBSS	Hanks Balanced Salt Solution
IFN	Interferon
IL-2	Interleukin 2
LGL	Large Granulated Lymphocyte
LMC	Lymphocyte Mediated Cytotoxicity
LU	Lytic Unit
MAb	Monoclonal Antibody
MHC	Major Histocompatibility Complex
NK	Natural Killer
NKCF	Natural Killer Cytotoxic Factor
PBMC	Peripheral Blood Mononuclear Cell
PGE	Prostaglandin E
PMN	Polymorphonuclear Neutrophil
SRBC	Sheep Red Blood Cell

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INTRODUCTION

During the search for a suitable target to detect cell mediated cytotoxic activity of antibody coated target cells (antibody dependent cell mediated cytotoxicity, ADCC or K cell activity) in the rhesus monkey, Macaca mulatta, investigators in this lab examined a panel of normal human cells and several human tumor cell lines including the B cell lymphoma, Raji. Raji was derived from a patient with Burkitts lymphoma and is Epstein-Barr virus (EBV) positive but does not shed virus (1). This cell line was used routinely as a target in human K cell assays when presensitized with anti-human lymphocyte antibody. The observed lytic activity mediated by human effectors was antibody dependent as it is widely recognized that normal human lymphocytes do not mediate cytotoxic activity to Raji in the absence of antibody or superinfection (2-5). Naturally occurring cells mediating lysis of tumor or virally transformed target cells (7,17) in the absence of antibody, presensitization, or Major Histocompatibility Complex (MHC) restriction are termed natural killer (NK) cells (12,22). In contrast to Raji targets, K562 is a well established target of human (6,7) and monkey NK activity (8-10). This cell line was derived from a patient with chronic myeloid leukemia in blast crisis (11) and lacks class I and class II major histocompatibility antigens (12).

The experiments utilizing rhesus monkey effector cells resulted in the surprising, previously unreported finding that normal rhesus peripheral blood mononuclear cells (PBMC) mediated high lytic activity to Raji in the absence of antibody and without any known prior sensitization. The goal of the research detailed below was to define the in vitro functional parameters and cell surface antigenic phenotype of the rhesus monkey effector cell population mediating lytic activity to Raji.

The in vitro functional parameters of this effector population were determined by varying the following assay conditions: sensitivity to lysis of a panel of human target cells, effector/target ratio, time kinetics, incubation temperature, pH of culture media, percentage and type of serum, and regulation by prostaglandin E₂ (PGE₂). Cold target inhibition experiments were designed to determine if the population of NK cells mediating lysis of Raji could be distinguished from effectors lytic to K562.

The phenotypic characterization of the NK effector cell mediating lysis to Raji included the following: removal of the adherent cell population, enrichment/ depletion of T cells, and complement mediated depletion of lymphocyte subpopulations identified by a panel of cross reactive anti-human monoclonal antibodies. The induction, detection and selective depletion of subpopulations of antigen specific cytotoxic T lymphocytes (CTL) were utilized to distinguish

between cells mediating lysis of Raji and K562 from allospecific CTL effector populations.

REVIEW OF LITERATURE

Definition of Natural Killer Cells

Natural killer (NK) cells were first described in humans about 13 years ago during studies of natural cell-mediated cytotoxicity. Several investigators reported that lymphocytes from normal individuals reacted against some tumor cells or tumor derived cell lines (13-15). NK cells were subsequently described in most lab animals including mice (16,17), rats (17), guinea pigs (18), dogs (19), and monkeys (8-10,20,21). The natural killer cell is generally described as a naturally occurring cytotoxic lymphocyte mediating MHC non-restricted, non-specific (12,22) lytic activity of tumor derived or virus infected cells (7,17) in the absence of known prior sensitization (12,22).

Cells mediating NK activity are functionally distinct from cytotoxic T cells (CTL) as CTL activity requires presensitization and is specific for the MHC antigens of the sensitizing cell (22). In contrast to cells mediating NK lysis, human CTL activity is mediated by cells lacking the receptor for the Fc fragment of IgG (FcIgG) but expressing the pan T cell surface marker, CD3, present on all mature human T cells (23-25,133). The CD8 receptor on CTL is present in high density as opposed to low density expression on NK cells (26,133).

Lytic activity mediated by NK cells occurs in the absence of any known antibody (17,27). However, effector

cells mediating antibody dependent cell mediated cytotoxicity (ADCC) require targets sensitized with antibody of the IgG class to which the effector cells bind via their receptors for FcIgG (27-31). Cells mediating ADCC and NK activity may be contained within the same population as both types of cytotoxicity are mediated by cells expressing receptors for FcIgG (30). Bradley and Bonavida have shown that natural killing and ADCC can be mediated by the same effector cell (147). Attempts at phenotypic differentiation of cells mediating NK activity and cells mediating ADCC have been unsuccessful (26,30,44). Both effector populations appear to share surface receptors as defined by panels of monoclonal antibodies (MAbs). A major distinguishing characteristic of cells mediating ADCC is the requirement for a functional Fc receptor (2,31). In contrast, although NK activity is highly enriched in the Fc positive population, blockage of the receptor does not inhibit NK function (2,31).

The activity of natural killer cells is regulated by soluble products of activated cells. NK lytic activity is increased by exposure of effector cells to any of the three types of interferon (IFN), alpha, beta or gamma (32,33). Alpha and beta IFN are products of leukocytes (Type I) and fibroblasts, respectively, and are secreted in response to viral infection (34). Gamma IFN (Type II) or immune IFN is produced by lymphocytes following activation by antigen or

mitogen (35). Interferon enhances NK activity by several methods: 1) by increasing the number of NK cells able to bind to the target, 2) by increasing the kinetics of lysis, 3) by increasing the recycling ability (55,143), and 4) by inducing expression of receptors for interleukin 2 (IL-2). The enhancing effect appears to be greatest in response to gamma IFN (32). Lysis of NK resistant target cells, such as EBV positive B cell lymphomas, occurs following exposure of NK cells to interferon (32,36). In contrast, pretreatment of NK sensitive target cells with IFN can confer resistance to lysis (36).

Several of the prostaglandins, PGE_2 , PGE_1 , and PGI_2 , downregulate NK activity (37,38). Prostaglandins are the products of monocytes which may be involved in the regulation of NK cells (39). Removal of adherent cells, which are rich in monocytes, resulted in increased NK activity (37). Culture supernatants obtained from isolated monocytes activated by polyinosinic acid-polycytidylic acid (poly I:C) are rich in prostaglandins and cause suppression of NK activity (37). This effect is reversed by the addition of indomethacin, a cyclooxygenase inhibitor which blocks the production of prostaglandin E.

Phenotypic Characterization of Human NK Cells

As described later in this study, the phenotypic characterization of rhesus monkey NK cells has been done

utilizing panels of anti-human monoclonal antibodies (MAbs). Therefore, a summary of human NK cell phenotypic characterization is pertinent to this work. Natural killer cells were originally described as nonadherent, nonphagocytic, surface Ig negative lymphocytes expressing receptors for FcIgG (6,27,40,41). The work of Unkeless et al. revealed that NK cells and polymorphonuclear neutrophils (PMN) expressed Fc receptors (FcR2) which bind only complexed IgG and were distinct from the Fc receptors on human monocytes (FcR1) which bind both monomeric and complexed IgG (42). Confirmation of the similarity of Fc receptors on PMN and NK cells comes from the reactivity pattern of monoclonal antibodies, Leu 11 and similar antibodies directed against the FcR2 receptor (31,43,44).

NK cells have a distinctive morphological characteristic which is the presence of large azurophilic granules in the cytoplasm as revealed by Giemsa staining (2). Percoll density gradient fractionation of mononuclear cells revealed that virtually all NK activity was contained in a low density subpopulation of lymphocytes with large azurophilic cytoplasmic granules. This population of cells was termed large granular lymphocytes (LGL) and is highly enriched for lymphocytes expressing receptors for FcIgG (2,45,46).

The lineage of NK cells has been debated in the literature for some time. There are conflicting data

implicating either a myelomonocytic or a lymphocytic lineage for NK cells. The myelomonocytic lineage hypothesis derived from observations showing that MAbs which exhibited specificity for the CR3 receptor (OKM1, MAC-1) on human myeloid cells and macrophages (131,132) also bound NK cells (47,48). However, more recent findings have shown that MAb recognizing the CR3 receptor also bind to T lymphocytes (49,50) indicating that this receptor is not restricted to cells of a myelomonocytic lineage. Froelich and Bankhurst have shown that E rosette positive and negative lymphocytes are recognized by OKM1 and reported that both subpopulations appeared to mediate NK activity (50). Griffin et al. examined the binding pattern of a monoclonal antibody, Leu 19 (NKH-1, N901) (51). This antibody was reactive with a subset of LGL which includes the natural killer cell but did not react with B cells, monocytes or granulocytes. Additional evidence refuting a myelomonocytic lineage of NK cells comes from examination of NK cells with a panel of MAb recognizing cells of this lineage. These findings revealed a negative binding pattern except for the MAbs directed against the FcIgG receptor and the CR3 receptor (52).

Although human NK cells share several surface markers with T cells, the possible T cell lineage of NK cells has been in contention. Several investigators revealed that a large proportion of NK activity is recovered in the population of cells expressing low affinity receptors for

sheep red blood cells (SRBC), now termed the CD2 receptor (2,6,44,50,52,53). This receptor is expressed on both mature and immature T cells. All mature human T cells also express a 22-29 KD membrane associated molecule termed the CD3 receptor which is complexed to the T cell receptor (Ti). The CD3 receptor is recognized by OKT3, Leu 4 and similar MAbs. NK cells lack both the CD3 and the Ti receptors (47,51,55,133).

The Ti receptor is composed of two chains, alpha and beta (54,60,61). The recent emergence of techniques enabling examination of the genes controlling the CD3/Ti complex has revealed that as immature T cells pass through the cortex of the thymus, genes encoding the variable, diversity and junction domains of the Ti molecule undergo rearrangement. Signals provided by thymic epithelial cells induce the gene rearrangements which result in the expression of the alpha:beta heterodimer, and consequently the acquisition of immunocompetence (54,60,61). Thus, mature T cells emerge from the cortex expressing the alpha:beta heterodimer. The expression of the alpha:beta heterodimer is now considered irrefutable evidence of T cell lineage (55,61).

Saito et al. have recently described a second form of the T cell receptor (Ti) composed of a heterodimer termed gamma:delta (160). It appears that the gamma:delta form of the T cell receptor precedes the expression of the

alpha:beta heterodimer in T cell ontogeny (161). T cells express either the alpha:beta or gamma:delta heterodimer. The role of the cell population expressing the gamma:delta heterodimer has yet to be defined although the cell appears to be capable of mediating the same functions, cytolysis and lymphokine production, as the cell expressing the alpha:beta heterodimer (161).

Mature CD3⁺ T cells and the majority of NK cells share expression of the CD2 receptor. Subsets of CD3⁺ effector cells and NK cells also express the CD8 receptor, and share a sensitivity to regulation by IL-2 and have similar lytic mechanisms. Grossman and Herberman have proposed that it is the expression of the alpha:beta heterodimer which is the point of divergence between T cells and NK cells (159). Following expression of the T cell receptor, T cells are subject to different regulatory signals from NK cells or other nonrearranged cells. In contrast to NK effector cells, effector T cells require prior sensitization and are MHC-specific.

Using cDNA probes for the T cell receptor, Lanier et al. showed that human NK cells and granulocytes express the germ line DNA sequence for the T cell receptor, i.e., they have not rearranged the alpha:beta genes (55). Furthermore, the CD3⁻, NK cell enriched population failed to develop expression of CD3 following several days incubation in IL-2. These findings led to the conclusion that NK cells are not

of the T cell lineage. Ritz et al. have examined the Ti genes of CD3⁺, IL-2 dependent NK cell clones and also described a germ line configuration of the genes controlling the alpha:beta heterodimers (56). However, examination of an IL-2 dependent, non-MHC specific, cytotoxic T cell clone did reveal rearranged Ti-beta genes. To explain these findings, Lanier et al. have proposed that non-MHC specific cytotoxic activity against NK sensitive cell lines is mediated by two distinct cell types (55). One population is composed of mature T cells (CD3⁺) with the expected gene rearrangement, and the second contains CD3⁻ lymphocytes not in the T cell lineage as evidenced by germ line configuration of the T cell genes. The former cell population was termed non-MHC-specific cytotoxic T cells and the latter as NK cells.

Non-MHC-specific cytotoxic T cells and NK cells are believed to recognize targets via different receptors. Non-MHC-specific cytotoxic T cells utilize the surface CD3/Ti complex to recognize their target cells (56,134). Lytic activity can be blocked by the addition of CD3 directed MAbs (56). MHC-specific CTL also use the CD3/Ti receptor for target recognition and are, therefore blocked by anti-CD3 MAbs (134). In contrast, NK cells recognize target cells by using a "NK target" structure that remains to be identified and are not blocked by anti-CD3 MAbs (56,134).

Much effort has been devoted to identification of

surface markers restricted to or highly selective for NK cells. NK cells are contained within the subpopulation of cells expressing receptors for FcIgG (CD16) (44,46,58,59,157). Depletion of this subpopulation almost totally abrogates NK activity in man and various animal species (8,16,18,19,44,157). A majority of human CD16⁺ cells coexpress the CR3 (Leu 15) receptor (44,52). The MAbs, OKT8 and Leu 2, bind to a receptor (CD8) expressed by T cells mediating suppressor/ cytotoxic cell functions (26,57). A subset of NK cells also expresses the CD8 receptor as depletion of CD8 cells decreases NK activity by 30-50% (26,58,59). The CD8 receptor is expressed in high density on CTL in contrast to low density expression on NK cells (23,26,133). The MAb Leu 7 (HNK-1) binds to most NK cells, although some cells lacking the Leu 7 receptor mediate NK activity at 10-40% of normal levels (29,59). Leu 19 has been shown to be reactive with a subpopulation of LGL containing the NK population (51,55) and also appears to recognize non-MHC specific CTL as described by Lanier et al. (55). However, Leu 19 does not appear to recognize MHC-specific CTL (134). Human NK cells do not express receptors recognized by monoclonal antibodies OKT4 (CD4, recognizes helper T cells) (44,58,59), Coulter B1/Leu 12 (recognizes B cells) (44,59), or OKIa (recognizes a monomorphic determinant on the HLA class II DR molecule) (44,58,59). A subset of MHC-specific CTL which are also Class II

restricted express CD4 in contrast to non-MHC-specific CTL which do not express CD4 (134).

In contrast to NK cells, MHC-specific CTL and non-MHC-specific cytotoxic T cells are CD3⁺ and CD16⁻ and are effectively blocked by anti-CD3 MAbs (56,134,135). Although all three effector cell populations express the CD2 receptor, only NK cells are not blocked by anti-CD2 MAbs (134) although this may vary between individuals (25). Non-MHC-specific and MHC-specific CTL can be phenotypically distinguished by the presence or absence of the Leu 19 receptor (134).

In Vivo Role of Cells Mediating NK Activity

NK cells are believed to be an important first line defense mechanism against viral infections (62-69,139) and are also thought to be involved in surveillance of malignant cells (70-73), immunoregulation of hematopoiesis (74-77), and rejection of allografts (78-80). Various approaches have been used to establish the role of NK cells as a first line defense mechanism against viral infections. Genetic associations, reconstitution experiments, in vivo NK cell depletions, and interferon levels have been examined for correlation with the incidence of viral infection. Susceptibility to viral infection in different strains of mice correlated with the level of NK activity; strains resistant to viral infections displayed higher levels of NK activity (138). Normally NK cell deficient beige mice are

highly susceptible to murine cytomegalovirus (MCMV) and resistance to MCMV can be transferred by bone marrow cells from mice with normal levels of NK activity (136). Antibody to ganglio-n-tetraosylceramide (asialo GM1), a receptor originally found on murine NK cells (81), selectively depleted NK activity in vivo (82). In vivo depletion of NK cells by treatment with anti-asialo GM1 antibody resulted in high virus titers following injection of MCMV (64). Resistance to Herpes Simplex I virus (139) and mouse hepatitis virus (64) have also been shown to depend on NK cells.

NK activity is augmented by interferon (IFN) (142) and reduced by corticosteroids (141), agents known to increase and decrease resistance to viral infections, respectively. Welsh et al. demonstrated that NK cells become activated during the early stage of viral infections (140). Interferon is secreted by leukocytes (Types I and II) in response to viral infections (34) and is responsible for the activation of NK cells during virus infections enabling these cells to increase in number and to recycle more rapidly (65). Bukowski et al. found increased levels of NK activity correlated with increased levels of interferon in mice following MCMV infections (64). The in vivo virus titer in NK cell depleted mice was high despite the presence of high interferon levels. Minato et al. found that interferon can activate pre-NK cells to differentiate into

mature NK cells (148). They depleted mature NK cells and lost all NK activity to virus infected target cells. Treatment of the NK depleted cells with interferon converted pre-NK cells into mature cytotoxic NK cells as determined by expression of an NK associated receptor and the reappearance of lytic activity.

The majority of the studies of NK activity in humans with viral infections have involved transplant patients or patients with AIDS. Transplant recipients are notoriously susceptible to life threatening viral infections, frequently caused by herpes viruses, including cytomegalovirus (CMV) (83-84) and Epstein-Barr virus (EBV) (85-88, 154). EBV is the etiologic agent of infectious mononucleosis and Burkitt's lymphoma in man (154). Furthermore, increased incidences of Kaposi's sarcoma and spontaneous lymphomas, related to CMV (89,91) and EBV infections (85-87,90-92), respectively, have been reported in renal transplant patients. Starr et al. reported that transplant recipients have impaired NK activity to CMV infected fibroblasts up to two years after transplantation (63). Ramse et al. found decreased NK activity to K562 in renal transplant patients could be restored to supranormal levels by addition of interferon in vitro (93). Kelly et al. compared the effects of in vitro versus in vivo treatment with alpha interferon on NK activity (84). They found that in vivo treatment failed to increase NK activity. Quinnan et al. studied NK

activity to K562 and CMV infected target cells in bone marrow transplant recipients (94). Recovery from life threatening CMV infections correlated with normal levels of NK activity to both targets. Patients who succumbed to CMV had depressed NK activity.

Human patients with AIDS have dramatically increased incidences of viral infections including CMV and EBV and their associated malignancies, Kaposi's sarcoma and non-Hodgkin's lymphoma, respectively (91,95). NK lytic activity to K562 and CMV infected fibroblast target cells in AIDS patients is depressed although treatment with IL-2 in vitro returns activity to normal levels (96). However, only one of six patients had increased NK activity when the effectors were treated with beta interferon.

The population of rhesus monkey natural killer cells mediating lytic activity to Raji, an EBV positive human B cell lymphoma, may be of particular interest due to the extremely low incidence of clinical symptoms of EBV, CMV and herpes virus infections, in rhesus monkeys following inoculation of leukocytes obtained from patients with infectious mononucleosis (97). However, in a subsequent study they did report the successful in vitro transformation of rhesus, baboon, and human lymphocytes by an EBV-related, monkey-derived herpes virus (98). Levine et al. combined immunosuppression or immunostimulation treatments and EBV inoculation of 45 rhesus monkeys (99). Only 37% of these

animals had a positive seroconversion while none showed any clinical symptoms of disease.

The rhesus monkey colony at East Carolina University contains an average 25% of monkeys who have antibody titers to EBV but none who have shown clinical symptoms of EBV associated diseases (infectious mononucleosis or Burkitts lymphoma). The low incidence of antibody titers to EBV in rhesus monkeys is in contrast to the greater than 90% incidence of anti-EBV titers in man (154). More than 95% of these monkeys also have antibody titers to CMV but remain asymptomatic. The incidence of CMV infections in monkeys given allografts and immunosuppressive agents is not increased, unlike human transplant patients who have high infection rates (100-102). Thomas et al. failed to find any definitive clinical evidence of CMV or EBV infections in rhesus monkeys given allografts and anti-thymocyte globulin despite severe leukopenias (103) and hyporesponsive polyclonal mitogen responses (104).

The spontaneous occurrence of malignant lymphomas in rhesus monkeys is very low. Rangan et al. described the isolation of a B lymphotropic EBV-like herpes virus from a malignant lymphoma originating in a rhesus monkey who appeared to have no underlying disease state (105). Except for this one isolated report, the few reports of lymphomas in rhesus monkeys involve animals receiving immunosuppressive drugs or having simian acquired

immunodeficiency syndrome (SAIDS). Reitz et al. described the development of lymphomas in several cynomolgus monkeys (Macaca fascicularis) receiving high doses of Cyclosporin which may have resulted in oversuppression of the immune system (146). This conclusion may be supported by the findings of Thomas et al. who failed to find any evidence of lymphomas in immunosuppressed rhesus monkey renal transplant recipients treated with various combinations of azathioprine, prednisone, Cyclosporin, or anti-thymocyte globulin (ATG) (103,144,145). The preponderance of malignant lymphomas reported in rhesus monkeys are found in monkeys with simian AIDS (SAIDS). Monkeys who develop SAIDS have an increased incidence of a Kaposi-like sarcoma and other symptoms of immunodeficiency characteristic of human AIDS (106,107). In contrast to decreased NK activity in patients with AIDS (96), Nerurkar et al. found normal levels of NK activity in monkeys with SAIDS during the early stages of the disease (108). However, the observed NK activity declined in the later stages of the disease.

NK-Like Activity of Transformed or Presensitized Cells in Non-Human Primates

Much of the work describing the in vitro characteristics of primate NK cells has involved the use of transformed or presensitized lymphocytes as the source of effector cells. Johnson and Jondal described an in vitro

transformed, marmoset (genus Saguineus) T cell line which is cytotoxic to human T cell lines. However, the marmoset cell line failed to lyse the EBV positive human B cell lines, Daudi and Raji (109). Kiyotaki et al. also described herpes virus transformed marmoset lymphoid lines mediating substantial cytotoxicity to Molt, a T cell lymphoma, weak cytotoxicity to K562, and no activity to Raji (110). These NK cell lines expressed antigens recognized by T12 (pan T cell), CD8, CD2, and Leu 19 (NKH-1). An owl monkey (genus Aotus) transformed cell line which kills the human cell lines, Molt 4 and K562, was developed by Rabin et al. (111). Work done by Lazda and Baram described the NK activity of sensitized rhesus monkey lymphocytes stimulated in vitro with keyhole limpet hemocyanin (112). The target was Rous Sarcoma transformed marmoset fibroblasts.

NK Activity of Nontransformed, Unstimulated Nonhuman Primate Cells

Limited information about the characteristics or function of nontransformed, unstimulated, nonhuman primate NK cells is available. Bertram and Pearson have described the function and phenotype of owl monkey PBMC mediating lysis of EBV positive lymphomas obtained from a marmoset and an owl monkey (20). The cells mediating this NK activity were characterized as E rosette forming T cells expressing receptors for FcIgG. In 1981, Clark and Sturge compared the NK cell activity of pigtailed and longtailed macaques,

baboon, marmoset, squirrel monkey and human cells to a panel of human and monkey cell lines (21). Baboon NK cells were shown to mediate lytic activity to both human B and T cell lines, although cytotoxic activity to the B cell line was more pronounced.

Lotzova et al. found enriched NK activity to K562 in the large granular lymphocyte (LGL) fraction of rhesus PBMC (113). Savary et al. have characterized NK cells in the rhesus monkey mediating lytic activity to the human cell lines K562, Daudi and CEM as nylon wool nonadherent and enriched in the LGL subpopulation (9). The lytic activity was potentiated by exposure of the effector cells to interleukin 2, a well recognized activator of NK activity. Utilizing K562 as a target for their studies, Gengozian et al. found significant but reduced NK activity in the bone marrow compared to PBMC of rhesus monkeys (8). They also reported enrichment of NK activity in the LGL fraction, expression of receptors for FcIgG, plastic nonadherence, and an OKT11 positive/ OKT4 negative surface phenotype to be characteristics of this effector population.

The experiments reported here were designed to determine phenotypic and functional properties of rhesus monkey NK cells which mediate lysis of the human B cell line Raji. The in vitro functional parameters of this effector population were determined by varying the following assay conditions: sensitivity to lysis of several human target

cells, effector/target ratio, time kinetics, incubation temperature, pH of culture media, percentage and type of serum, and regulation by PGE₂. Cold target inhibition experiments were designed to determine if the population of NK cells mediating lysis of Raji could be distinguished from effectors lytic to K562.

The phenotypic characterization of the NK effector cell mediating lysis to Raji included the following: removal of the adherent cell population, enrichment/ depletion of T cells, and complement mediated depletion of lymphocyte subpopulations identified by a panel of cross reactive anti-human monoclonal antibodies. The induction, detection and selective depletion of subpopulations of antigen specific CTL were utilized to distinguish between cells mediating lysis of Raji and K562 from allospecific CTL effector populations.

MATERIALS AND METHODS

Animals

Normal, unsensitized, disease free 3-10 kg male rhesus monkeys (Macaca mulatta) were obtained from Litton Bionetics, Yemassee, SC and conditioned in our colony for at least three months.

Effector Cells

Peripheral blood mononuclear cells (PBMC) obtained from heparinized blood samples were isolated on a ficoll hypaque density gradient (Pharmacia, Piscataway, NJ, s.g. 1.077) as previously described (114). The cells at the interface were washed x 3 and resuspended to 1.25×10^6 per ml in culture medium (RPMI 1640 containing 2 mM L-glutamine, GIBCO, Grand Island, NY).

Target Cells

The EBV positive human lymphoblastoid B cell line Raji (1) served as target cells in all assays. Other human cell lines included in selected assays are: K562, a myeloid leukemic cell line (12); MOLT 4, a T cell lymphoma (150); Daudi, an EBV positive B cell lymphoma (149) and RPMI-1788, an in vitro EBV transformed cell line. Raji and Daudi are in vivo derived from Burkitt's lymphomas (1,149). Normal human PBMC (cultured for 18 hours at 37°C in 10% FCS in RPMI) were also used as targets in some experiments. Raji was obtained from ATCC, Rockville, MD and K562, MOLT 4 and

Daudi were generously provided by Dr. Hilel Koren of Duke University. RPMI-1788 was obtained from Associated Biomedics, Buffalo, NY. The cells were maintained at 37°C in culture medium containing 3.5% FCS and 100 units penicillin and 100 mg streptomycin per ml in 5% CO₂. All cell lines were checked to ensure against mycoplasma contamination. Approximately 2×10^6 target cells were washed x 2 and incubated for one hour at 37°C with 175 uCi sodium ⁵¹chromate (⁵¹Cr, New England Nuclear, Boston, MA). The tagged cells were washed x 3 and resuspended to 1×10^5 cells/ ml in culture medium.

NK Cytotoxicity Assay

The assay was performed utilizing a modification of the previously described ⁵¹chromium release assay for detection of in vivo generated CTL activity in rhesus monkeys (114) based on the original method of Brunner et al. (115). Briefly, effector and target cells were mixed in round bottom microtiter plates (Costar,) at E/T ratios of 25:1 to 3.12:1 in doubling dilutions. The assay was performed in triplicate in 0.20 ml RPMI 1640 containing 2 mM L-glutamine and 10% FCS. The cultures were centrifuged and incubated at 37°C for four hours in 5% CO₂. In the time kinetic studies, culture time was begun as plates were placed at 37°C. After dispersal of the cell pellet and subsequent centrifugation at 1000 rpm for 7 minutes, the supernatants were collected

with a supernatant collection system (Skatron, McLean, VA). Radioactivity in the supernatant was measured in a Beckman Gamma Counter. Spontaneous release determined in culture medium without effector cells was 10-15% of the maximum release. Maximum release of ^{51}Cr was determined by adding 5% Liquinox solution in place of effector cells. The percentage of cell mediated lysis was calculated by the formula:

% lysis =

$$\frac{\text{CPM experimental release} - \text{CPM spontaneous release}}{\text{CPM maximum release} - \text{CPM spontaneous release}} \times 100$$

Calculation of Lytic Units

The number of lytic units was calculated from linear dose response curves and represents the number of effector cells required for 50% lysis of the target cells. The results are expressed as number of lytic units per 10^7 effector cells.

Lymphocyte Mediated Cytotoxicity (LMC)

LMC or cytotoxic T cell (CTL) activity was tested as described previously (114). PBMC were obtained from nonimmunosuppressed rhesus monkeys who had received an allogeneic kidney transplant five days previously and used as effectors in a four hour ^{51}Cr release assay. Targets were PBMC obtained from the transplant donor and an unrelated control and cultured at 1×10^6 / ml of 10%

FCS/RPMI at 37°C for 48 hours. The target cells were labeled with ^{51}Cr and washed as described above. Effector and target cells were combined in a 96 well microtiter plate at an E/T ratio of 50:1 in 200 μl of culture media containing 10% FCS and incubated at 37°C for 4 hours. The supernatants were harvested and results calculated as described above for the NK cytotoxicity assay.

Assay for Anti-EBV and Anti-CMV Antibodies

Antibody titers to EBV and CMV were determined by Dr. R L. Heberling, Southwest Foundation for Biomedical Research, San Antonio, Texas, using a Dot Immunoassay (DIA) as previously described (137). Seropositivity was defined by comparing the experimental serum sample to positive and negative controls. The intensity of the color related directly to the amount of antibody present.

Removal of Adherent Cells

Adherent cells were depleted on Sephadex G-10 (Pharmacia, Piscataway, NJ) according to the method of Ly and Michel (116) with minor modifications (117). PBMC suspended at $2 \times 10^7/\text{ml}$ in RPMI containing 2.5% BSA (Sigma, St. Louis, MO) were applied to a 5 ml column of Sephadex G-10 which was equilibrated with BSA/RPMI. After 45 minutes at 37°C, effector cells were eluted with 20 ml of warm medium and washed x 2 with RPMI before use.

Fractionation of Cells with Receptors for Sheep Red Blood Cells (SRBC)

The method of Bentwich et al. (118) for T cell enrichment/ depletion by rosetting with SRBC was used with minor modification. SRBC were washed x 3 with phosphate buffered saline (PBS) and a 0.25 ml aliquot of packed SRBC was mixed with 50 units of neuraminidase (GIBCO). The cells were incubated at 37°C for 30 minutes, washed x 3 with PBS, and resuspended to 2% suspension. For rosette formation, 1 ml each of SRBC and Sephadex nonadherent PBMC at 1×10^7 / ml in RPMI medium without serum were combined, centrifuged at 200 x g, incubated at 37°C for five minutes followed by a 1 hour incubation on ice. The pelleted cells were gently resuspended, layered on Ficoll-hypaque, and centrifuged at 650 x g for 30 minutes in the cold. The non-rosetting cells (T depleted) were harvested at the interface. The SRBC were removed from the pelleted rosetting cells (T cell enriched) by hypotonic lysis with deionized water followed by recovery in an equal volume of 2x media. The T enriched and depleted fractions were washed x 2, viability counts performed and cell concentration corrected. The number of T cells present were quantitated by rerosetting with SRBC and B cell counts were performed by determination of Coulter B1 positive cells as described below. The Coulter B1 monoclonal antibody recognizes a surface determinant of B cells not expressed on T cells or monocytes. Cells expressing receptors for FcIgG

were quantitated by indirect fluorescence as described below using a monoclonal antibody (Leu 11b) which recognizes the Fc receptor.

Depletion of Lymphocyte Subpopulations with Monoclonal Antibodies and Complement

The panel of monoclonal antibodies (MAb) and their specificities are listed in Table 1. All MAb were complement fixing and have been shown to cross react with rhesus monkey PBL (8,119-121). The complement depletion method is based on that of Zarling et al. (122). Clone 9.6 (Lyt3) MAb was obtained from New England Nuclear, Boston, MA. The OK series of MAbs were kindly donated by Ortho Diagnostics, Raritan, NJ, and the Leu antibodies were given by Becton Dickinson, Mountain View, CA.

Sephadex nonadherent PBMC were resuspended to 1×10^7 /ml in HBSS (Flow Laboratories, McLean, VA) containing 0.25% BSA, 2 mM L- glutamine, 0.025% sodium azide, and a 1:20 dilution of MAb. After a 30 minute incubation at 4°C, the cells were washed x 1 with HBSS. An aliquot was removed for staining with goat anti-mouse IgG FITC (Fab₂ fragments) and fluorescent positive cells were quantitated on a Becton Dickinson FACS fluorescent cell analyzer. The remaining cells were resuspended at 1×10^7 /ml in a 1:10 dilution of prescreened low-tox rabbit complement (Accurate, Westbury, NY) and incubated at 37°C for 1 hour, centrifuged, and again resuspended in a

1:10 dilution of complement. After a 30 minute incubation at room temperature, an aliquot of cells was removed for viability determination by trypan blue exclusion. The cells were washed x 3, corrected to 1.25×10^6 viable cells /ml, and assayed for cytotoxic activity as described above. Controls included untreated cells, antibody coated cells, and cells treated only with complement.

Discontinuous Density Gradient Centrifugation on Percoll

Discontinuous density gradient centrifugation on Percoll (Pharmacia) was performed on PBMC suspensions according to the technique of Ulmer and Flad with minor modifications (123). A stock isotonic Percoll solution (SIP) was prepared by mixing 54 ml Percoll, 5.38 ml 10x Ca/Mg free HBSS (Flow), 0.273 1 N HCl, and 0.6 ml 1 M HEPES buffer (Research Organics, Cleveland, OH). The osmolarity of the SIP and HBSS was corrected to 300 meq/L. The specific gravity (s.g.) of SIP was adjusted by adding HBSS per ml of SIP according to the formula: stock SIP s.g.- experimental s.g. / experimental s.g.- s.g. HBSS. To form the gradient, a peristaltic pump was used to underlayer five ml of each Percoll solution (s.g. 1.062, 1.068, 1.074, and 1.080) into a 50 ml glass siliconized test tube. Two ml of Sephadex passed lymphocytes at 1×10^7 per ml of 10% FCS/ HBSS were added to the top of the gradient and then centrifuged at 4°C for 20 minutes at 750 x g. Four distinct

bands and a pellet were harvested, washed x 2 with RPMI, counted and assayed for effector activity. Recovery of the cells averaged 65% and viability by trypan blue exclusion exceeded 95%. Cytocentrifuge smears were stained with May-Gruenwald Giemsa stain for morphologic analysis.

Cellular Competitive Inhibition Assay

The cold target blocking assay was performed using the method of Tai et al. (124) with minor modifications. Briefly, unlabeled tumor cells (blockers) were added in 50 ul of culture medium to wells containing 100 ul of Sephadex nonadherent PBMC effector cells. Following the addition of 50 ul ^{51}Cr tagged target cells, the plates were incubated for 4 hours at 37°C and supernatants harvested as described for the NK cytotoxicity assay. The E/T ratio was held constant at 25:1. Only the blocker cell number, and therefore, the blocker to target ratio (B/T), was varied. A control set of three wells without blocker cells was included to determine cytotoxic activity in the absence of competitive inhibition. The results were calculated for each B/T ratio as follows:

Percent inhibition of lysis =

$$\frac{\text{mean percent control lysis} - \text{mean percent test lysis}}{\text{mean percent control lysis}}$$

The optimal E/T ratio of 25:1 and B/T ratios of 25:1, 5:1 and 1:1 were used.

Treatment of Effectors with PGE₂

Unfractionated or Sephadex nonadherent effectors were seeded into microwells and prostaglandin E₂ (PGE₂, Sigma, St. Louis, MO) was added at the concentrations indicated in the results section. Serial dilutions of PGE₂ were prepared in culture media from a stock solution of 1.41×10^{-2} M PGE₂ in 95% ethanol. The cells were incubated in the presence of media or PGE₂ at room temperature for 60 minutes prior to the addition of target cells. The PGE₂ was present for the entire assay.

Statistical Analysis of Data

Statistical significance was tested using a paired t-test with Statgraphics computer software (STSC, Inc., Rockville, MD).

RESULTS

Sensitivity of Raji to Lysis by Rhesus NK Cells

Examination of rhesus PBMC lytic activity against a panel of human cells composed of normal lymphocytes and tumor cell lines reveals that these nonhuman primates have effector cells mediating kill to all cell lines tested including the B cell lymphoma, Raji. Results are given in Figure 1. The observed lytic activity was highest to Raji and significantly higher ($62.5\% \pm 7.6$ SD, $p < 0.001$), than to K562 ($45.9\% \pm 7.9$ SD), or MOLT ($41.3\% \pm 5.4$ SD). The cytotoxic activity to Raji was an unexpected finding because these results are in contrast to the NK activity of normal human cells which are cytotoxic to K562 and MOLT but do not mediate lysis of Raji (2-5). Human effector cells assayed for lytic activity to Raji and K562 confirmed these findings (data not shown). As shown in Figure 2, maximum lysis of Raji occurred at an E/T ratio of 25:1 and significant lysis was found at an E/T of 3.12:1. E/T ratios of 50:1 did not further increase cytotoxicity. Analysis of these results as lytic units yielded a linear relationship of effector to target cell numbers. Rhesus monkey PBMC have almost twice as many lytic units of activity ($LU_{50}/10^7$) to Raji than to K562, 114 ± 34 SD and 80 ± 20 SD $LU_{50}/10^7$ effectors, respectively. The lytic activity of rhesus NK cells to normal human PBMC was less than 20% of the activity to Raji,

19.2 LU₅₀/10⁷ effector cells vs. 114 LU₅₀/10⁷ effector cells, respectively.

Because information describing optimal conditions for NK activity to Raji has not been reported, a set of experiments was designed to assess the effects of various culture conditions on this effector population. The goals of these experiments were to 1) define optimal conditions for lytic activity to Raji vs. K562, and 2) determine if the effector cells mediating kill of K562 and Raji are contained within the same population.

Time Kinetics of NK Cytotoxicity to Raji and K562

A linear relationship between culture time and lysis of Raji and K562 target cells as LU₅₀/10⁷ effector cells is shown in Figure 3, a and b. Peak lysis of Raji and K562 occurred at 4 hours (71.3 and 77.4 LU₅₀/10⁷, respectively) of culture time with a decrease at 18 hours. Lysis of Raji was significantly higher at 4 hours than at 2 hours or less of incubation time ($p < 0.025$). In contrast to cytotoxic activity to Raji, no significant difference in lysis of K562 was observed at 2 hours of incubation versus 4 hours, although the lysis of K562 at two hours is borderline nonsignificant ($p = 0.054$). Comparison of the CPM after only 5 minutes of incubation to the spontaneous release CPM revealed significant lysis ($p < 0.025$) to both Raji and K562 (data not shown). Spontaneous release values were lowest (2%) at 15 minutes and highest (20%) at 18 hours for both

targets. Effector activity to Raji and K562 appear to require 4 hours of incubation to attain maximum lytic activity. Therefore, all subsequent experiments utilized a 4 hour culture time.

Temperature Dependency of NK Cytotoxicity

As shown in Table 2, NK cell mediated lysis of both Raji and K562 are clearly temperature dependent. Lytic activity to both Raji and K562 is significantly decreased at 20°C and 30°C. Because maximum cytolytic activity to both K562 and Raji occurred at 37°C, an incubation temperature of 37°C was used for all subsequent experiments.

Effect of pH on Lytic Activity

Lytic activity to K562 and Raji was assayed utilizing culture media with three different pH values, 6.5, 7.2, and 8.0. Results are expressed as mean $LU_{50}/10^7$ effector cells $\pm$ SD in Figure 4. Lytic activity to Raji appears to be highly sensitive to changes in pH. A large decrease in the pH from 7.2 to 6.5 caused a significant increase in kill to Raji ($p < 0.025$). Increasing the pH from 7.2 to 8.0 resulted in a significant decrease in cytotoxic activity to Raji. In contrast, the lytic activity to K562 was minimally altered over the pH range. Thus, only the cytotoxic activity to Raji appears to be significantly affected by changes in the culture pH. A pH of 7.2 was used for all experiments.

Effect of Serum Concentration and Type on NK Activity

Optimal NK mediated cytotoxicity of Raji and K562 occurred in the presence of 10% FCS as shown in Figure 5, a and b, respectively. Statistically nonsignificant linear decreases in $LU_{50}/10^7$ occurred as the FCS concentration was decreased. To determine if FCS was essential for NK activity, cytotoxic activity to Raji and K562 was assayed in the presence of 0.5% BSA (equivalent to the average concentration of BSA in 10% FCS) or 10% gamma globulin free newborn calf serum (GGF). Rhesus effector cells assayed in the presence of BSA versus FCS resulted in a nonsignificant decrease in lytic activity to Raji, and no decrease was observed in the activity to K562. Cytotoxic activity assayed in the presence of 10% GGF serum also resulted in a slight, nonsignificant decrease in lysis of Raji with no difference in activity to K562. Since 10% FCS gave optimal lytic activity to Raji and K562, this concentration was utilized in all subsequent assays.

Cytotoxic Activity to Raji Does Not Correlate with EBV Seropositivity

Of 24 rhesus monkeys tested for antibody to EBV, only 25% were seropositive. Lymphocytes from these 5 seropositive animals and the 18 seronegative animals were compared for NK activity to Raji. No difference was observed in activity; $156 LU_{50}/10^7$ cells $\pm$ 12 SD and $152 LU_{50}/10^7$ cells $\pm$ 58 SD, respectively.

Enrichment of Cells with NK Activity to Raji by Density Gradient Centrifugation

Fractionation of rhesus nonadherent PBMC by Percoll discontinuous density centrifugation resulted in the formation of 4 distinct bands and a pellet. As shown in Figure 6, fraction 1 (1.062 s.g.) contained significantly decreased NK activity to Raji and was very low in % LGL (2%). The greatest enrichment of % LGL and NK activity to Raji was found in fraction 2 (1.068 s.g.). This fraction contained 85% LGL and exhibited a six fold increase of NK activity to Raji compared to the unfractionated population. Significantly less activity was mediated by cells isolated in fractions 3 and 4. Negligible activity was found in the pellet. These findings associate NK activity to Raji with a relatively light cell population which is significantly enriched in LGL.

NK Activity to Raji and K562 is Retained in the Sephadex Nonadherent Fraction

Removal of adherent cells by passage over Sephadex G-10 columns caused a significant augmentation in NK activity to both Raji ($p < 0.005$) and K562 ($p < 0.05$), although the increase in lysis of Raji was greater. Results are shown in Figure 7. The plastic and nylon non-adherence of rhesus NK cells cytotoxic to K562 have been reported (8,9,21). Monoclonal antibody analysis of unfractionated and Sephadex passed cells is given in Table 3. The contamination of

monocytes as defined by binding of Leu M3 in the nonadherent cells was very low. E rosette forming T cells (CD2) were enriched by Sephadex treatment. The augmentation of NK activity in the nonadherent fraction suggested a nonmonocytic, nonadherent lymphocyte population mediates NK activity to Raji. Hence, a Sephadex G-10 nonadherent fraction was used for phenotypic characterization of the rhesus NK cell mediating lysis of Raji.

T Cells Expressing E Rosette Receptors Mediate NK Activity to Raji

The rosetting procedure was done in the absence of serum to eliminate the potential formation of rosettes by cells via receptors for FcIgG. Results are shown in Table 4. The T cell enriched/ depleted fractions contained a mean 95% and 4% ERFC, respectively. The presence of B cells as determined by indirect fluorescent binding of Coulter B1 was 4% in the former and 30% in the latter. The ERFC enriched fraction mediated increased lytic activity to Raji compared to the unfractionated population. Effector cells depleted of ERFC had reduced activity to Raji. Therefore, the population mediating lytic activity to Raji is enriched for ERFC.

Effect of PGE₂ Addition on NK Cytotoxicity

Three physiological concentrations of PGE₂ were added to either unfractionated or Sephadex nonadherent effector

cells 60 minutes prior to the addition of target cells. Results are presented in Table 5. Treatment of unfractionated rhesus NK effectors with 10^{-5} M PGE_2 resulted in significant decreases in lytic activity to Raji ($p < 0.05$) and K562 ($p < 0.025$). Lytic activity to Raji was also sensitive to PGE_2 at 10^{-7} M, and, in contrast, activity to K562 was insensitive. Sephadex nonadherent effector cells mediating activity to Raji and K562 were resistant to the inhibitory effect of PGE_2 . The addition of PGE_2 to cultures containing intact PBMC appears to induce the adherent cells to downregulate NK activity to Raji and K562 since removal of these cells on Sephadex eliminates the decrease in NK activity. There also appears to be differential sensitivity to the downregulating effects of PGE_2 between the effectors mediating lysis of Raji versus K562 which may indicate the presence of two different effector populations or the expression of target recognition receptors may be differentially affected.

Effect of Cold Target Inhibition on NK Activity

Cold target inhibition experiments were done to determine if lytic activity to both K562 and Raji are mediated by the same cell population. Lytic activity to Raji was assayed alone or with either unlabeled K562 or Raji (blocker cell) at three blocker:target ratios. Results are expressed as % inhibition in Table 6. The mean % inhibition

in lysis of Raji targets by unlabeled Raji was 72.8% versus 40.2% inhibition by K562 at a 25:1 blocker:target ratio. This difference is statistically significant ($p < 0.005$). At a B:T ratio of 5:1, although the difference was not statistically significant ($p > 0.05$).

If the effector population mediating lysis of Raji and K562 are completely overlapping, the % inhibition by K562 should not be statistically different from Raji. The results of these experiments argue against this hypothesis. Rather the data suggest there are at least two populations of NK effector cells, one population recognizing both K562 and Raji, and a second population recognizing Raji but not K562. Alternatively, rhesus may have only one population of NK cells which are able to focus on Raji to the partial exclusion of K562.

Surface Phenotype of NK Cells

The panel of antibodies and their specificities used in the phenotypic characterization studies are listed in Table 1. As shown in Table 7, treatment of Sephadex passed PBMC with clone 9.6 (CD2) MAb and complement caused a consistent, significant decrease ($p < 0.01$) in NK activity to Raji ranging between 43% and 77% of control activity. Complement mediated lysis of OKT11 (CD2) positive cells yielded an average 55% of complement control activity which is a borderline non-significant reduction ($p = .059$). The inclusion of more animals would most likely result in a

statistically significant decrease in activity. These findings agree with the results of the E rosette depletion experiments and confirm that a subpopulation of cells expressing the CD2 phenotype mediates NK cell activity to Raji.

Treatment of effector cells with Leu 11b and complement abolished cytolytic activity to Raji. Removal of OKT8 (CD8) positive effector cells caused significant reductions ($p < 0.050$) to 49% - 75% of complement control values. In contrast, OKT4, OKIa, and MAC-1 treatment variably increased NK lytic activity.

These findings indicate that NK cell lysis of Raji targets is mediated by a population of cells expressing FcIgG receptors (CD16). Additionally, the NK cell population appears to be partially contained within the CD2 population and the CD8 subpopulation.

Functional Differentiation of NK and CTL Activity

To determine if the phenotypic properties of NK cells could be distinguished from the phenotypic properties of antigen specific cytotoxic T lymphocytes, the effects of depleting CD2 and CD16 positive cells on CTL and NK activity were compared in the same experiment. PBMC were obtained from nonimmunosuppressed animals five days after transplantation of a renal allograft and treated with Leu 11b and complement to deplete cells expressing the FcIgG

receptor or with 9.6 MAb and complement to deplete CD2 positive T cells. Intact and depleted cells were assayed for NK cytotoxic activity to Raji and K562 and for CTL activity to the specific allograft donor's target cells. Results are shown in Table 8. Removal of FcIgG bearing cells resulted in an 80-90% reduction of cytotoxic activity to Raji and K562 contrasted to a slight increase in CTL activity to the allogeneic donor targets. The depletion of CD2 positive cells lowered the CTL activity by 80-90% and the NK activity by 30-50%. These results suggest that the cell population mediating lytic activity to Raji expresses a phenotype distinct from that of MHC-specific, alloantigen activated CTL.

Figure 1: Natural killer cell activity of rhesus monkey PBMC against various human targets. Data were pooled from NK assays performed in 25 normal rhesus monkeys and are expressed as the mean percent cytotoxicity $\pm$ SD obtained at an effector to target cell ratio of 25:1 using a 4 hour ^{51}Cr release assay.

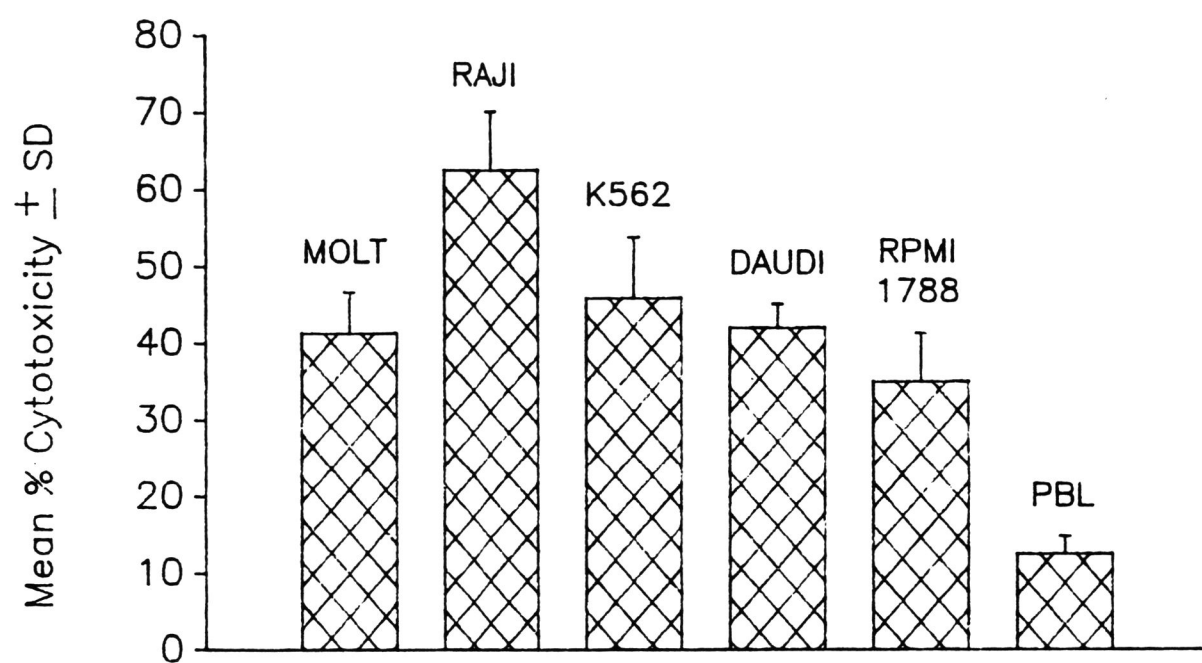


Figure 2: Cytotoxic activity of rhesus monkey natural killer cells. NK activity was tested against 3 human targets: Raji, K562, and normal PBL. Data were pooled from NK assays performed in 15 normal rhesus monkeys and are expressed as the mean % cytotoxicity $\pm$ SD obtained at 5 E/T ratios. The $LU_{50}/10^7$ effector cells $\pm$ SD for each curve are also given.

* $p < .025$, ** $< .001$ versus K562

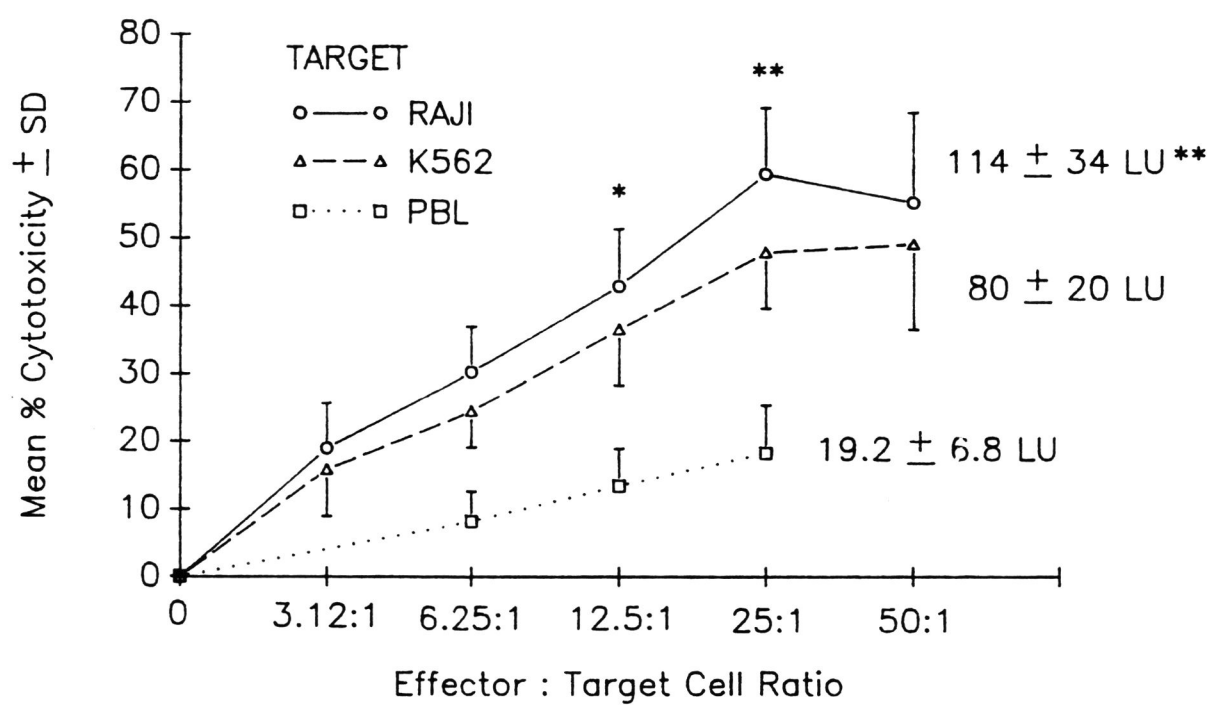


Figure 3: Time kinetics of NK lytic activity. Cytotoxic activity to (a) Raji and (b) K562 is expressed as the mean LU₅₀/ 10⁷ effector cells $\pm$ SD of 4 experiments.

* p< .010, ** p< .025, *** p< .050 versus 4 hours

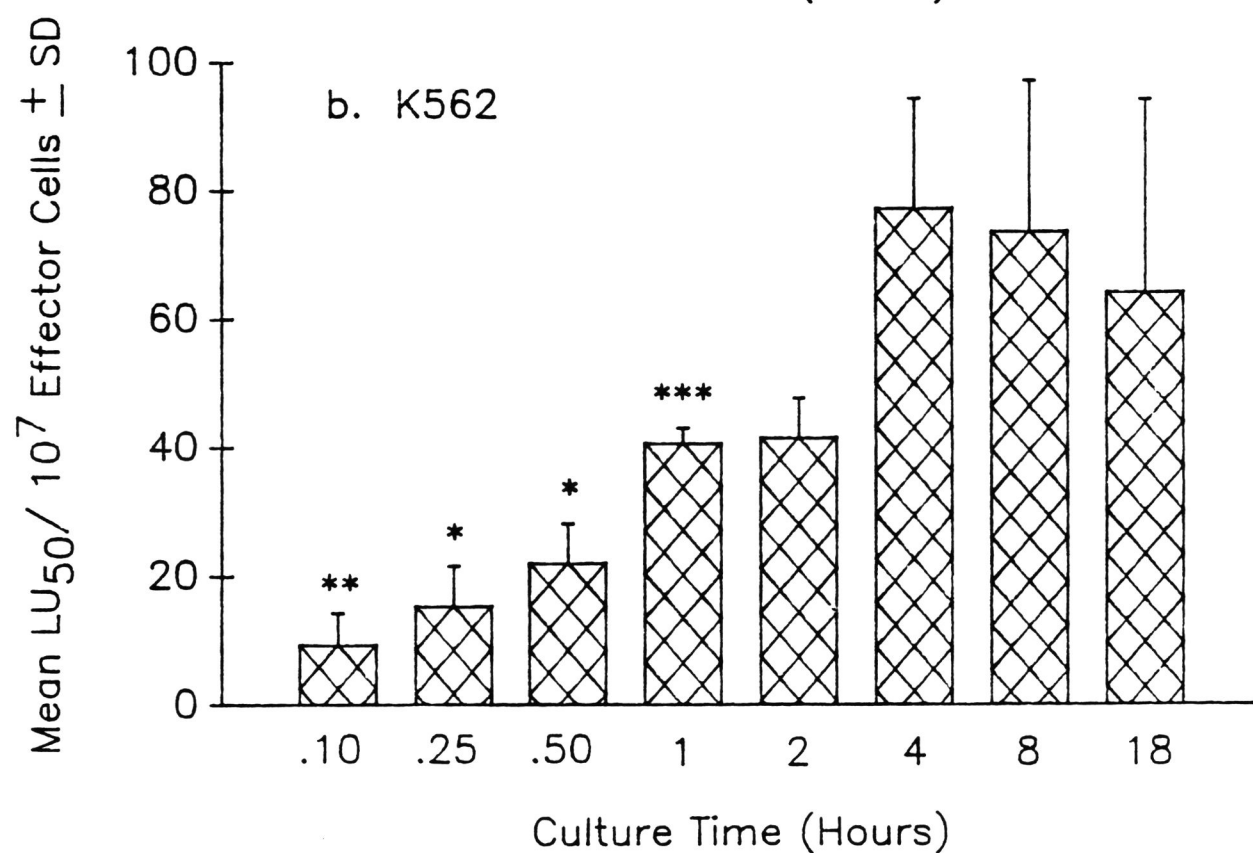
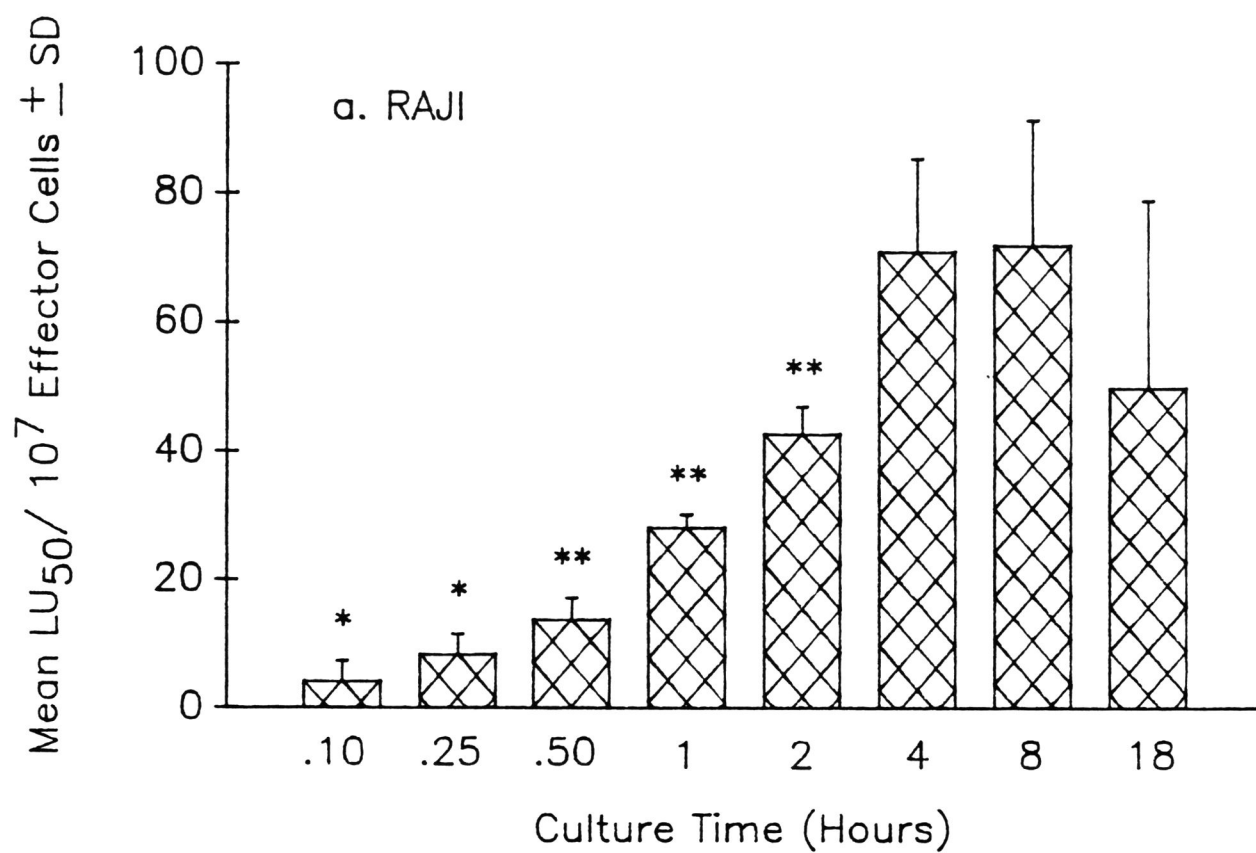


Figure 4: Effect of culture pH on NK lytic activity.
Culture media was pH adjusted prior to resuspension of effector and target cells. Data are expressed as mean LU₅₀/ 10⁷ effector cells $\pm$ SD of 4 experiments.
* p< .025 versus 7.2 pH

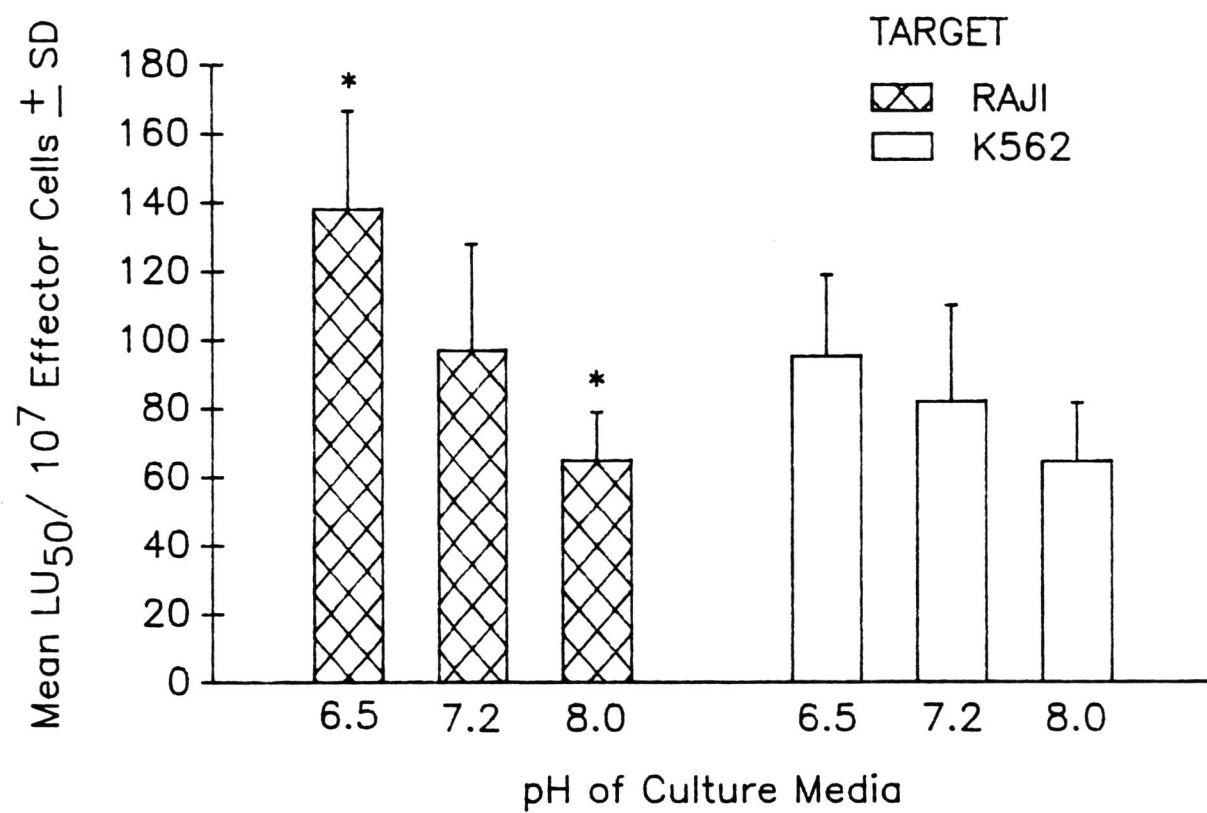


Figure 5: Effect of different serum concentrations on NK activity. Cytotoxic activity to a) Raji and b) K562 were assayed. Serum percentages are final concentrations prepared in RPMI 1640 as described in methods. Data are expressed as the mean $LU_{50}/10^7$ effector cells $\pm$ SD of 4 experiments.

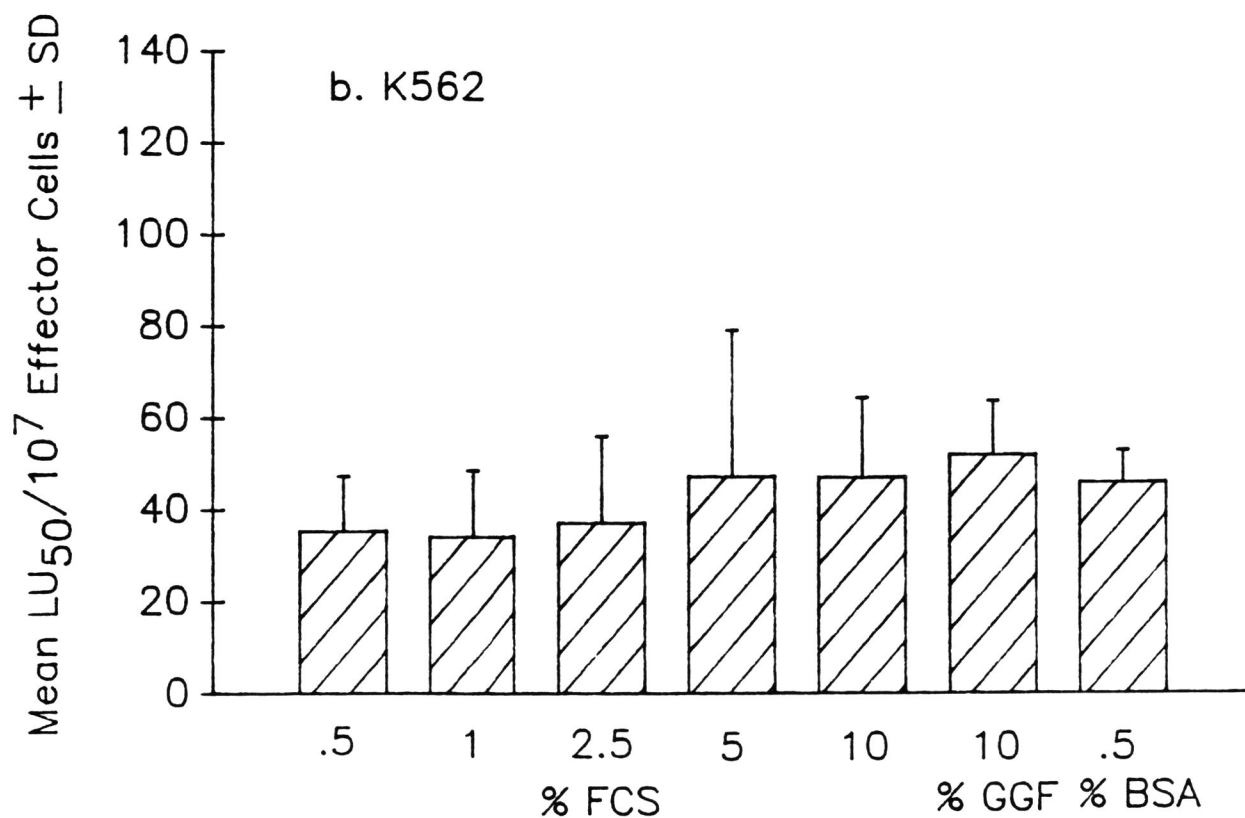
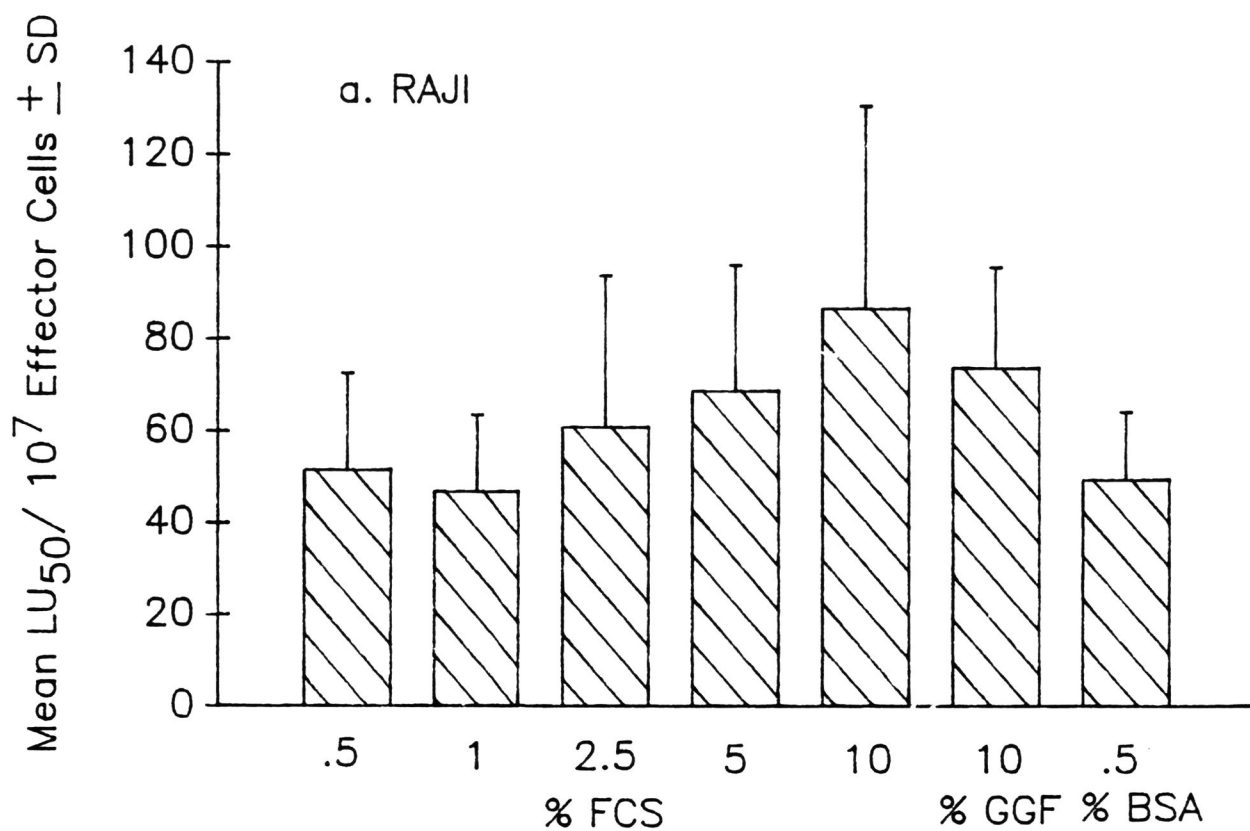


Figure 6: Percoll fractionation of NK cells mediating lytic activity to Raji. Results are expressed as mean $\text{LU}_{50} / 10^7$ effector cells $\pm$ SD (bars) and as mean % LGL $\pm$ SD (line) of 3 experiments. The fraction number corresponds to the Percoll gradients described in the methods section.

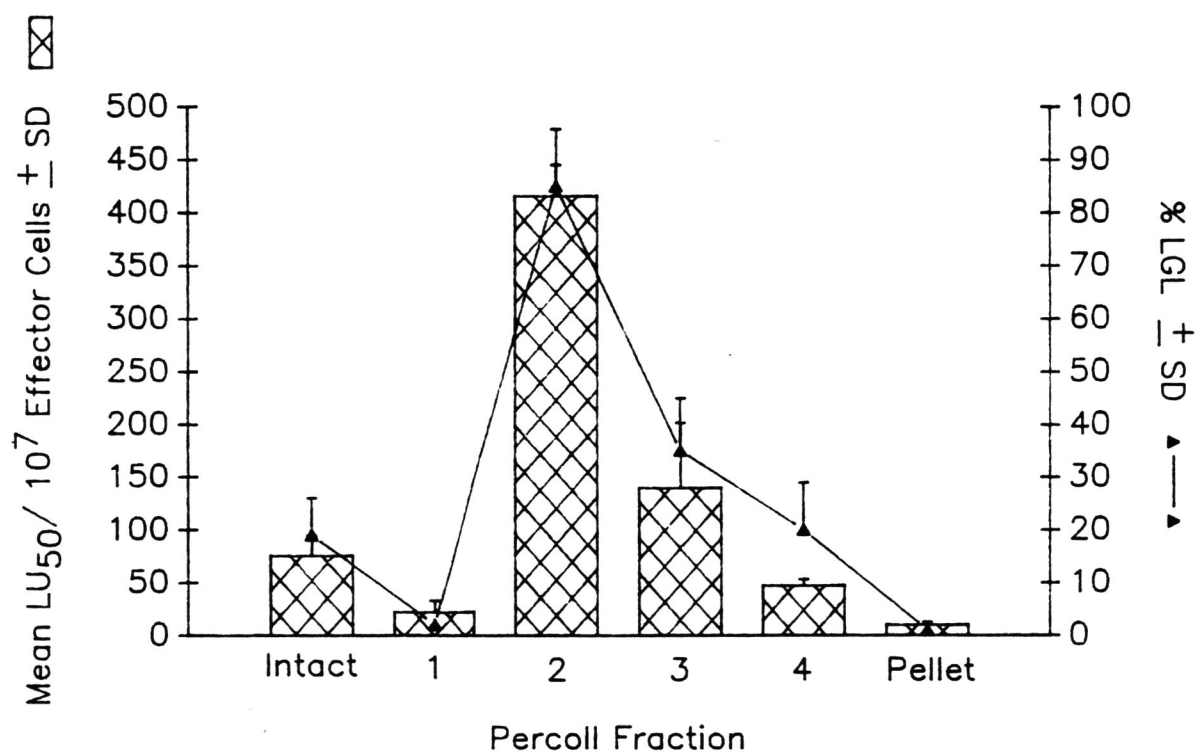


Figure 7: NK activity is enhanced by removal of adherent cells. PBMC were passed over Sephadex G-10 to remove adherent cells as detailed in methods. Data are expressed as the mean ISD of 3 experiments.

* $p < 0.050$ versus unfractionated.

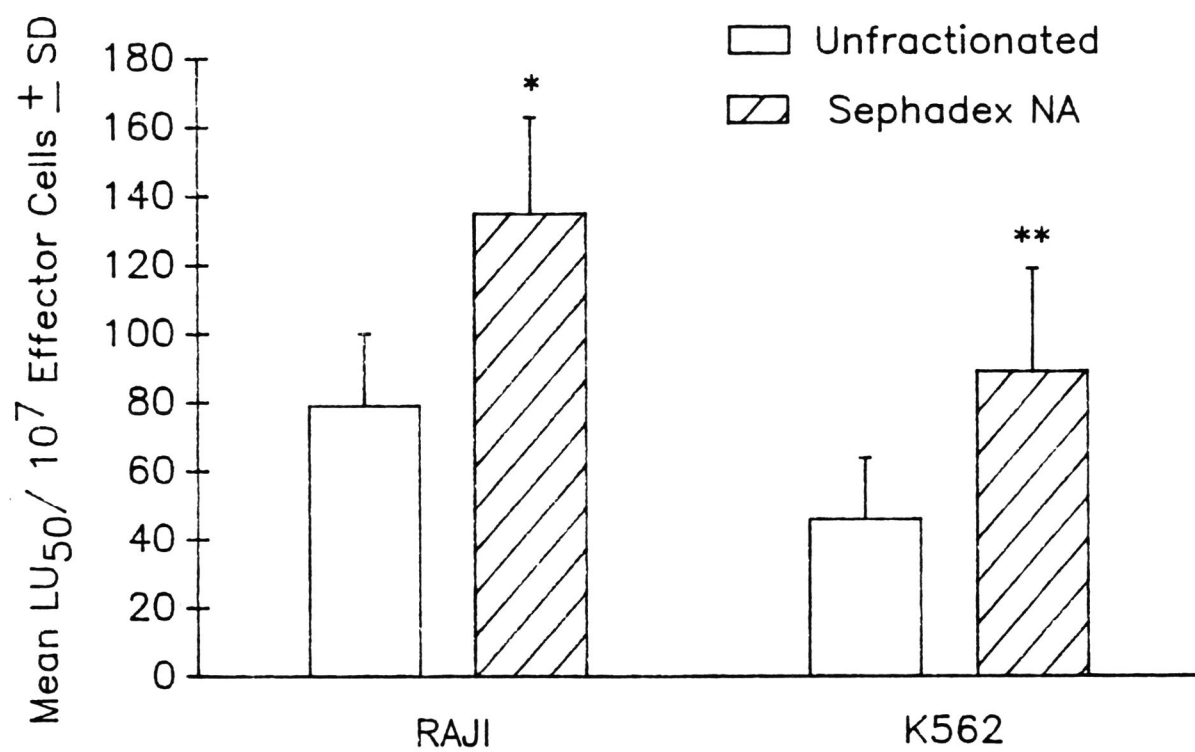


TABLE 1

CD NOMENCLATURE FOR HUMAN DIFFERENTIATION
ANTIGENS IDENTIFIED BY ANTI-HUMAN MONOCLONAL
ANTIBODIES (Mab) USED IN THIS STUDY

MAb	CD Cluster	Major Cell Reactivity
clone 9.6/ OKT11	CD2	E rosette receptor on T cells
OKT4	CD4	Helper/ Inducer T Subset
OKT8	CD8	Suppressor/ Cytotoxic T Subset
MAC-1/ OKM1 (CR3)	CD11	C3bi Receptor
Leu 11b	CD16	FcIgG Receptor
OKIa	ND ^a	DR
Leu M3	ND	Monocytes

^aND, not defined

TABLE 2

SENSITIVITY OF NK ACTIVITY TO DIFFERENT CULTURE TEMPERATURES

Temperature	LU ₅₀ / 10 ⁷ Effector Cells ^a $\pm$ SD	
	Target	
	Raji	K562
20°C	64.3 $\pm$ 26 ^b	41.4 $\pm$ 14 ^b
30°C	78.3 $\pm$ 33 ^b	51.2 $\pm$ 25 ^c
37°C	174.1 $\pm$ 64	100.5 $\pm$ 11

^a Data expressed as the mean $\pm$ SD of 4 experiments.

^b $p < .025$ vs. 37°C

^c $p < .050$ vs. 37°C

TABLE 3

CHANGES IN LYMPHOCYTE SUBSETS AS DETERMINED BY
MONOCLONAL ANTIBODY ANALYSIS FOLLOWING SEPHADEX PASSAGE

MAb	% Fluorescence Positive	
	<u>Unfractionated PBMC</u>	<u>Sephadex Passed PBMC</u>
9.6	61,80,71,72,68,64	82,80,76,81,72,75
OKT11	70,69,75,65	74,80,75,82
OKT4	34,32,38,29	40,40,36,42
OKT8	36,34,41,30	37,23,40,50
OKIa	15,20,12,16,21	12,9,11,8,7
MAC-1	15,10,9,11	8,8,10,7
Leu 11b	20,21,12,18,15	15,12,10,12,9
OKM1	15,19,22,25,20	10,14,11,12,7
Leu M3	13,16,15,10	1,3,2,0

TABLE 4

ENRICHMENT OF NK ACTIVITY TO RAJI IN THE ERFC POPULATION

Exp.	Effector	E:T Ratio			%T ^b	%B ^c	%Fc-IgG ^c
		12.5:1	6.25:1	3.12:1			
1	Intact	45 ^d	40	35	82	16	13
	T Enriched	72	64	56	94	3	3
	T Depleted	ND	18	8	4	73	ND
2	Intact	61	57	49	85	15	19
	T Enriched	83	63	39	91	5	6
	T Depleted	ND	10	4	5	80	ND
3	Intact	55	33	20	79	10	19
	T Enriched	84	68	52	92	4	8
	T Depleted	ND	21	14	10	83	ND

ND, Quantity insufficient to assay.

^a ERFC enrichment/ depletion performed by combining equal volumes of PBMC and neuraminidased sheep RBC and incubating at 4°C for 1 hr. Rosetted and nonrosetted cells were separated by centrifugation on ficoll.

^b Determination of T cells done by rerosetting with SRBC.

^c Purity of the resulting fractions was determined by binding of relevant MAb as described in methods.

^d Data expressed as % cytotoxicity of single experiments.

TABLE 5

LOSS OF SENSITIVITY TO PGE₂ BY SEPHADEX PASSED NK CELLS

Effector	Target	Mean % Cytotoxicity $\pm$ SD ^a			
		Media	PGE ₂ Concentration ^b		
			10 ⁻⁵	10 ⁻⁷	10 ⁻⁹
Unfractionated	Raji	34.2 $\pm$ 4.5	21.2 ^c $\pm$ 3.7	24.8 ^c $\pm$ 1.4	32.4 $\pm$ 3.5
SDx NA	Raji	34.7 $\pm$ 10.4	34.0 $\pm$ 6.8	40.7 $\pm$ 4.1	38.9 $\pm$ 12.4
Unfractionated	K562	20.4 $\pm$ 3.4	12.5 ^d $\pm$ 2.9	20.3 $\pm$ 8.6	20.7 $\pm$ 7.7
SDx NA	K562	32.8 $\pm$ 9.6	23.5 $\pm$ 7.8	29.7 $\pm$ 16.5	28.6 $\pm$ 14.9

^a Lytic activity was assayed at an E/T ratio of 25/1. Data are expressed as the mean % cytotoxicity $\pm$ SD of 4 experiments.

^b Concentration of PGE₂ expressed as molarity of culture. Effectors were pretreated for 30 minutes prior to addition of targets.

^c $p < .050$ vs. media control

^d $p < .025$ vs. media control

TABLE 6

COMPARISON OF RAJI AND K562 CELLS AS COLD TARGET
INHIBITORS OF NK LYSIS OF ^{51}Cr TAGGED RAJI CELLS

Exp. #	% INHIBITION ^a					
	Cold Inhibitor (RAJI) Target Cell (RAJI)			Cold Inhibitor (K562) Target Cell (RAJI)		
	25:1	5:1	1:1	25:1	5:1	1:1
1	80 ^c	50	18	49	19	9
2	67	22	6	24	5	3
3	66	12	0	25	7	0.8
4	74	11	0	58	14	0
5	72	27	4	45	5	0
X $\pm$ SD	71.8 ^b ± 5.1	24.4 ± 14.1	5.6 ± 6.6	40.2 ± 13.4	10 ± 5.6	2.56 ± 3.4

^a % inhibition = $\frac{\text{control kill} - \text{kill with inhibitors}}{\text{control kill}} \times 100$

^b $p < 0.005$ versus % inhibition when K562 is the inhibitor at 25:1 ratio.

^c Effector to ^{51}Cr tagged target cell ratio is 25:1 for all points.

TABLE 7

**PHENOTYPIC CHARACTERIZATION OF NK CELLS MEDIATING LYTIC ACTIVITY
TO RAJI AS REVEALED BY MONOCLONAL ANTIBODY TREATMENT^a**

Monoclonal Antibody	LU ₅₀ / 10 ⁷ Effectors ^b Treatment of Effectors			% Immuno- fluorescence Staining
	Antibody & Complement	Complement Control	Antibody Alone	
Lyt3 (9.6)	27,74,41,107**	70,110,109,140	65,120,140	76,80,81,72
OKT11	30,46,62	69,60,140	78,75,135	74,80,75
OKT4	109,96,128,110	68,57,110,120	82,138	40,36,40
OKT8	52,68,60*	68,108,120	67,104,126	32,39,29,40
OKIa	98,95,73	66,85,69	65,101	12,9,11
MAC-1	94,91,145	69,66,120	83,121	3,2,4
Leu 11b	0,0,0,0**	69,66,85,120	86,77,139	14,10,17,12

^a Data from three or four experiments for each parameter tested.

^b NK activity tested at three effector/target ratios and data analyzed as LU₅₀/10⁷ effector cells.

*p < 0.05, **p < 0.01 versus complement control

TABLE 8

CELLS MEDIATING NK ACTIVITY CAN BE
DIFFERENTIATED FROM IN VIVO GENERATED CTL

Exp.	Assay ^c	Target	% Lysis ^a			
			Effector Treatment ^b			
			Untreated	RC'	Leu 11b & RC'	9.6 & RC'
1	LMC	Transplant Donor	43.6	49.7	45.9	7.9
	NK	RAJI	55.2	61.4	8.8	40.6
	NK	K562	39	34.5	3.6	17.2
2	LMC	Transplant Donor	31.5	34.2	39.6	6.4
	NK	RAJI	54.2	57.2	9.8	37.1
	NK	K562	28.1	41.7	5.7	27.5

RC', Rabbit Complement

^a Data are expressed as results of two separate experiments.

^b Effectors were obtained from the recipient on day 5 post-kidney transplant and prepared as described in methods.

^c LMC and NK assays were performed as described in methods.

DISCUSSION

The experiments described here were undertaken to define the optimal in vitro culture conditions for analysis of rhesus monkey NK cell activity to the human EBV positive B cell line Raji and to define the phenotypic characteristics of the effector cell population. Rhesus monkey PBMC are highly cytotoxic to Raji, significantly higher than to K562, a well established target of human NK activity (6,7). In sharp contrast, normal human PBMC lack NK cytotoxicity to Raji, a well documented finding (2-5). This distinction between human and rhesus NK cells has not been previously appreciated since rhesus NK cells mediating lytic activity to Raji have not been reported. However, Savary et al. (9) found naturally occurring effector cells in rhesus monkeys recognizing Daudi, another human B cell lymphoma and Clark and Sturge (21) reported NK activity to Raji in baboons.

Our lab has confirmed the activity to Raji by baboons and also demonstrated that NK activity to Raji is mediated by cynomolgus monkeys (Macaca fascicularis) and squirrel monkeys (Saimiri sciureus), (JT and MC unpublished observations). Thus, the recognition of EBV transformed targets seems to be present among several nonhuman primate species and the trait appears to have been lost in the evolution of man. The evolutionary significance of this finding is entirely speculative. The pathologic potential

of EBV infection in humans (35-38,90,92,154) and certain other nonhuman species, e.g. marmosets and gibbons (98,155,156), is contrasted by the relative resistance of macaques to EBV induced illness (97,99,103). Since NK cells are believed to be a first line defense mechanism against viral infection (62-69) and oncogenesis (70-73), this association is intriguing and merits further investigation.

Since rhesus monkeys are known to be resistant to EBV infection (97,99,103), the possibility had to be considered that the cytotoxicity to Raji might be due to a specific ADCC and/or specific cell mediated cytotoxic T effector mechanism. Several experiments were done to address this issue. First, we wanted to know if the FCS used in the assay might be a source of antibody. The cytolytic activity of rhesus effectors to Raji was assayed in FCS versus gamma globulin free calf serum (GIBCO). Effector activity assayed at the optimal E/T ratio of 25:1 was identical in the two types of serum (46 ± 5.9 SD and 45 ± 8.1 SD, respectively). Furthermore, the NK activity of normal human PBMC assayed to Raji in 10% FCS was negative, confirming a well documented finding (2-5).

While this finding ruled out exogenous serum as a possible source of antibody to EBV infected cells, it did not address the possibility that rhesus PBMC might be secreting antibody in vitro. The rhesus monkey colony at East Carolina University contains 25% EBV seropositive

animals. To determine if EBV seropositivity was correlated with NK activity to Raji, we compared NK activity to Raji in animals known to have positive versus negative antibody titers to EBV. No statistical difference was observed in the lytic activity of PBMC from seropositive versus seronegative animals. Furthermore, human effector cells failed to mediate lytic activity to Raji which had been pretreated with rhesus serum (data not shown). Blazar et al. also addressed this question in humans and found that PBMC obtained from seropositive and seronegative individuals failed to lyse Raji (4).

One phenotypic characteristic of antigen specific CTL is the absence of the receptor for FcIgG (CD16) on human and murine cells (24,127,152). If activity to Raji is mediated by NK cells and not by CTL cells, removal of the Leu 11b subpopulation would be expected to result in significantly reduced activity to Raji while leaving allospecific CTL lysis essentially intact. This is exactly what we found. Depletion of cells expressing receptors for FcIgG (CD16) from in vivo generated alloantigen specific CTL left anti-donor allospecific cytolytic activity intact and effectively depleted lysis of Raji and K562. Although only a subpopulation of human NK cells expresses the E rosette receptor (CD2), most human antigen-specific CTL possess an easily demonstrable CD2 receptor (24). In the data presented here, depletion of CD2 positive cells reduced

antigen-specific CTL activity by 80% and reduced NK activity by only 30-40%. These results suggest that the population of cells mediating lysis of Raji is not an antigen specific memory CTL.

No ready explanation is available to explain why rhesus NK cells kill Raji or what target determinant on Raji cells is being recognized. One distinct possibility is the recognition of EBV viral determinants by rhesus effectors. Only a small fraction of EBV positive Raji cells enter the viral cycle at one time and therefore only a fraction of the cells express other than the EBV nuclear antigen (4). Blazar et al. have demonstrated that human NK activity to superinfected Raji is positive in contrast to nonsuperinfected cells (5). Unlike human NK cells, monkey NK cells may recognize the EBV nuclear antigen or other undefined viral determinants on Raji and lyse these target cells. However, the studies of Clark and Sturge on baboon NK cell activity to Raji, suggested that target structures other than viral determinants are being recognized (21).

Natural killer cytotoxic factor (NKCF), is secreted by human and rodent NK cells following interaction of effector cells with the target. NKCF is believed to participate in the mechanism of target cell destruction (125). Secretion of small preformed pools of NKCF and simultaneous de novo production occurs in response to stimulation or activation of the effector cells by both NK sensitive and insensitive

target cells (158). Interferon appears to increase the amount of NKCF produced (158). Although NK resistant target cells stimulate production of NKCF, these targets are not sensitive to the factor. Rhesus monkeys and baboons may respond to Raji and secrete a NKCF-like factor which is lytic to Raji targets. Ortaldo et al. have described the production of a factor by a herpesvirus transformed owl monkey line cytotoxic to the human T cell line Molt (10). This factor failed to mediate lysis of K562. Johnson has described similar factors produced by Herpesvirus transformed marmoset NK-like cell lines which mediate lysis of K562, Molt and other NK sensitive cell lines (151). The factor appears to be preformed in the cell and is released in response to stimulation by the target cell.

Epstein et al. reported the secretion of low amounts of interferon by Raji in the original description of the cell line (1). However, recent studies of Blazar et al. failed to find interferon in culture supernatants of normal or superinfected Raji cells (5). Alpha interferon can enhance human NK activity, although increased amounts are required to achieve the same degree of enhancement as gamma interferon (32). Marmoset lymphoblastoid interferon has been shown to enhance the activity of monkey NK cells to K562 (126). Rhesus NK cells may be exquisitely sensitive to very tiny amounts of interferon or they may constitutively secrete low levels which can be rapidly increased. Kurvano

et al. have shown the interaction of human NK cells with EBV infected B cells induces the production of alpha interferon which activates NK cells (127). The addition of exogenous gamma interferon had no effect. Further experiments are planned to test for interferon production in the rhesus/Raji culture supernatants.

A set of experiments was designed to determine if optimal conditions for effector lysis of Raji and K562 were the same. Assays were performed by varying culture conditions including culture time, temperature, pH, percent and type of serum. Lytic activity to Raji and K562 yielded slightly different time kinetic curves and different sensitivity patterns to changes in culture pH. Lytic activity to both Raji and K562 was very rapid; significant lysis above background occurred after five minutes of incubation. Johnson has described a preformed NKCF-like factor in marmoset NK cells (151). The presence of a similar factor in rhesus NK cells could explain the observed rapid lysis of Raji and K562 targets. NK mediated cytotoxicity to Raji appeared to be more sensitive to changes in culture pH and in % FCS. These results suggest a differential sensitivity of NK cells mediating cytotoxicity to Raji and K562 to changes in culture conditions. Alternatively, the effect of pH, kinetics, or serum concentration may result from changes in the sensitivity of the target cell to lysis.

Addition of PGE_2 to unfractionated cells resulted in a significant decrease in lytic activity to Raji and K562. However, Sephadex passed NK cells in the monkey appear to be resistant to the downregulating effects of prostaglandin. Human NK cells are known to be sensitive to the downregulating effect of the prostaglandins. Koren et al. reported that PGE_1 and PGE_2 could suppress NK activity of both unfractionated and nonadherent populations (37). Their studies were done utilizing plastic nonadherent or nylon column passed cells. Leung et al. assayed unfractionated and plastic nonadherent/ nylon passed human PBMC for NK activity in the presence of PGE_2 and found a 50% decrease in lytic activity in both cell populations (128). If the cells were incubated 18 hours and then assayed, they found a 30% decrease in NK activity. Addition of alpha or beta interferon to the cultures at the beginning of the 18 hour incubation resulted in a loss of sensitivity of PGE_2 downregulation at the time of assay.

The results reported here were done utilizing Sephadex passed cells which may remove a subpopulation required for PGE_2 suppression. Alternatively, as discussed above, rhesus lymphocytes may constitutively secrete low levels of interferon. The removal of adherent cells may eliminate a cell population that regulates interferon production. If so, this might result in a very rapid increase in interferon levels, which might override or block the downregulating

effects of PGE₂.

NK activity to Raji appears to be more sensitive to the downregulating effects of PGE₂ as evidenced by a significant reduction in activity when 10⁻⁷ M PGE₂ is present in the cultures. This apparent distinction between effectors mediating lysis of Raji and K562 could indicate the presence of two different effector populations mediating kill to only one target. Alternatively, expression of the surface receptor(s) recognizing Raji and/or K562 are differentially affected by the concentration of PGE₂ added.

The cold target inhibition studies yielded data to support at least two populations of NK cells mediating activity to Raji. One population appears to recognize Raji and not K562, the second recognizes both Raji and K562. Work done by Tai et al. characterizing the polyclonality of human NK cells describes several populations of NK cells with different target specificities (124). Other investigators have also described the polyclonality of human NK cells by selective depletion of NK cells on target cell monolayers (129) and by MAb depletion (130). Inhibition by an unlabeled target cell autologous to the labeled target generally results in greater than 90% inhibition.

In the results reported here, the addition of unlabeled Raji to labeled Raji targets resulted in a maximum of 71% inhibition of ⁵¹chromium release. These findings may be the result of nonoptimal conditions for detecting inhibition of

rhesus NK activity. An increased blocker to target ratio may yield more information. If the effector population mediating lysis of Raji and K562 are completely overlapping, the % inhibition by K562 should not be statistically different from Raji. The results of these experiments argue against this hypothesis. Rather the data suggest there are at least two populations of NK effector cells, one population recognizing both K562 and Raji, and a second population recognizing Raji but not K562. Alternatively, rhesus monkeys may have only one population of NK cells which are able to focus on Raji to the partial exclusion of K562. Methodologies such as cross absorption of effector cells onto target monolayers and subsequent detection of lytic activity of the nonadherent cells might also clarify the existence of these two populations in the monkey and develop methods to isolate them.

The reactivity of the monoclonal antibodies (MAb), OKT11 and 9.6 recognizing the CD2 receptor, with rhesus T cells has been described (8,120). Complement depletion of this subpopulation from Sephadex passed cells resulted in a significant (mean 45%) decrease in NK activity to Raji. Gengozian et al. found a similar pattern in rhesus NK cells directed against K562. (8). In contrast to the findings reported here, they did not adjust for cell viability and loss following depletion of CD2 positive cells. If dead cells were removed and corrected for cell viability, the NK

activity to K562 remained at normal levels. In their study, Gengozian et al. used intact PBMC for their depletion studies in contrast to the findings reported here which involved the use of Sephadex nonadherent cells. Secondly, they removed dead cells by centrifugation through ficoll in contrast to this report which did not use ficoll density gradients to recover viable cells. Our method used three washes which removed 90-95% of the dead cells. Several investigators have depleted the CD2⁺ fraction from human PBMC and assayed NK activity of the CD2⁻ fraction (122,130). Removal of the human CD2⁺ population resulted in a 50% decrease in lytic activity to K562. Our results show a similar pattern and suggest a significant fraction of the NK activity to Raji is mediated by a CD2⁺ population.

Depletion of the ERFC⁺ fraction by rosetting with SRBC yielded a 60-70% decrease in NK lysis to Raji. West et al. enriched/ depleted ERFC and found 90% of human NK activity resided in the ERFC⁺ pellet and only 25% was in the ERFC⁻ band (6). In contrast, Froelich and Bankhurst reported approximately equivalent numbers of LU₅₀/10⁷ cells in the ERFC⁺ and the ERFC⁻ fractions (50). They found 25% of the ERFC⁻ cells were OKT11 and only 55% of the ERFC⁺ cells were identified by OKT11. These results may be partially explained by the rosetting methodology. In their system, PBMC were rosetted at 4⁰ C for 8-12 hours in the absence of serum. The rosettes may not have been optimally stable

resulting in ERFC⁺ cells in the ficoll band which should contain ERFC⁻ cells. The investigators did not report the % ERFC⁺ cells in the fractionated subpopulations. In the early report of rosetting methodology, Jondal and Pross utilized a one hour incubation on ice to enrich the ERFC subpopulation (153). Thus, Froelich and Bankhurst have deviated significantly from established techniques.

After ERFC depletion of rhesus PBMC, we found 30-40% of the baseline lytic activity remained in the ERFC depleted fraction compared to 40-70% of complement control activity following complement depletion of CD2 positive cells with monoclonal antibody. These results might suggest that there may be subpopulations of rhesus monkey T cells expressing different epitopes of the E-rosette receptor such that a particular CD2 specific MAb may recognize a fraction of ERFC⁺ T cells. The findings of Ortaldo et al. support such a hypothesis (58). In their studies, LGL depleted of high affinity ERFC (rosetted at 29⁰ C) were assayed for 9.6 and OKT11 binding patterns. They found a high percentage of 9.6⁺ cells and very low numbers of OKT11⁺ cells.

There are other explanations for the discordant results obtained with ERFC depletion and MAb depletion of rhesus PBMC. The rhesus NK cell population mediating lytic activity to Raji may be composed of two subpopulations, one found in the CD2⁺ population and the second contained in the CD2⁻ fraction. Very low density expression of the CD2

receptor on a fraction of rhesus NK cells could yield the findings reported here. In such a case, complement mediated cytotoxicity would not be expected to effectively kill all the CD2⁺ cells. Similar studies using a mixture of the various CD2 MAb and/or removal of CD2⁺ cells by panning or sorting may provide insight into the resolution of this question.

Of the MAb tested, only the depletion of cells expressing the CD16 (FcIgG) receptor by treatment with Leu 11b and complement caused almost total abrogation of NK activity to Raji. Gengozian et al. reported that depletion of Leu 11b cells from rhesus PBMC significantly reduced lytic activity to K562 (8). Other investigators have reported enrichment of monkey NK cells in the FcIgG receptor positive population by rosetting methodology (111,112).

Treatment of nonadherent PBMC with OKT8 and complement resulted in a 25-50% decrease in lytic activity to Raji while similar treatment with OKT4, OKIa or MAC-1 caused a variable increase in NK activity. In contrast to the work of Gengozian et al. who found rhesus NK activity in both the complement receptor positive and negative populations (8), these findings described here failed to reveal any decrease in NK activity in MAC-1 depleted cells. This difference may be explained by the depletion methodology used. Gengozian et al. used complement/antibody coated SRBC monolayers to remove cells bearing complement receptors. Alternatively,

the effector population mediating activity to Raji is phenotypically nonidentical to the cell recognizing K562.

These depletion studies define the phenotypes of the rhesus NK cell mediating activity to Raji as $CD16^+$, $CD4^-$, $OKIa^-$, and $CD11^-$. A subpopulation of NK effectors appears to be $CD2^+$, $CD8^+$. Work done by Gengozian et al. described a similar phenotype for the rhesus NK cell population lytic to K562 (8).

In summary, the NK population mediating lytic activity to Raji appears to share many phenotypic characteristics with the population mediating kill of K562. Both NK populations are nonadherent and are enriched in a low density fraction containing most of the LGL. Phenotypically, both effector populations are CD16 positive and contain CD2 and CD8 subpopulations.

The depletion of CD16 positive cells dramatically decreased NK activity but failed to decrease the cytotoxic activity of in vivo generated allospecific CTL. Depletion of CD2 positive cells reduced the cytolytic activity of allospecific CTL by >90% and NK activity by 50%. Thus, the NK and CTL population of rhesus monkeys are phenotypically distinct.

Inhibition studies disclosed the possible existence of two populations of cells mediating kill to Raji since K562 could block approximately 50% of the activity to Raji. The downregulating effect of exogenous PGE_2 suggested 1)

adherent cells contribute an essential role in the downregulation of NK activity by PGE_2 , and 2) the effector populations and/or the expression of target recognition structures may be differentially affected by the concentration of PGE_2 . Variations in culture conditions revealed 1) the lytic process by which rhesus effector cells kill both Raji and K562 occurs rapidly as shown by the kinetic studies and 2) the effector cells mediating lytic activity to Raji are particularly sensitive to changes in culture pH.

The previously unreported finding that resting rhesus NK cells mediate high lytic activity to the human EBV positive cell line, Raji, may offer a potential in vitro assay system to study an immunological defense mechanism which may help explain the low incidence of EBV and related diseases in rhesus monkeys. The studies of optimal culture conditions and phenotypic characterization provide a basis for further investigations of these effector cells.

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