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HETEROGENEITY OF LYMPHOKINE-ACTIVATED KILLER CELLS: ROLE OF INTERLEUKIN-2 AND INTERLEUKIN-4

By

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ABSTRACT

The addition of Interleukin-2 to a culture of blood lymphocytes increases the ability of Natural Killer (NK) cells to kill target cells, including target cells that were previously resistant to NK cells. This phenomenon is known as the generation of Lymphokine Activated Killer cells (LAK). Recent data suggest that the LAK phenomenon is not restricted to NK cells. T cells may also show LAK activity.

Our main objective was to determine if the LAK activity is restricted to NK cells or if it is also detectable in T cell subsets, especially in CD3+ T cells that are also negative for CD4 and CD8 cell surface markers.

To achieve our objective we first implemented methods for cell separation and culture of isolated subsets. We were able to obtain highly purified subsets of NK cells, helper/inducer (CD3+4+8-), suppressor/cytotoxic (CD3+4-8+) and double negative (CD3+4-8-) T cells. The double negative T cells are now known to be a subset of $\gamma\delta$-T cells. Highly purified subsets were obtained using antibody (sheep anti-mouse IgG) coated magnetic particles and monoclonal antibodies. The purified subsets were tested for LAK activity against a standard target cell (K562), a NK resistant/LAK sensitive target cell (HTB 58), and a NK/LAK resistant target cell. We found NK cells develop LAK activity after IL-2 stimulation and that two T cell subsets (cytotoxic/suppressor and double negative) consistently showed LAK activity (3 experiments). The helper/inducer subset showed LAK activity in 2 of 3 experiments. Interleukin 4 had an inhibitory effect on the proliferative activity of IL-2 stimulated T cells and was not used in the LAK experiments.

In conclusion we have found that the LAK function is a property of an heterogeneous group of lymphocyte subsets that includes NK cells and T cell subsets.

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ABBREVIATIONS

CD:	Cluster of Differentiation (cellular antigens)
^{51}Cr:	Chromium 51
con-A:	Concanavalin-A
CTL:	Cytotoxic T Lymphocyte
E+:	Cells forming Rosettes with Sheep Red Blood Cells
E-:	Cells not forming Rosettes with SRBCs
E:T ratio:	Effector to Target Ratio
IFN:	Interferon (alpha, beta or gamma)
IL:	Interleukin (IL-1, IL-2, IL-3, IL-4)
IL-2R:	Interleukin-2 Receptor
Ig:	Immunoglobulin
LAK:	Lymphokine-activated Killer Cell
LGL:	Large Granular Lymphocyte
MHC:	Major Histocompability Complex
MNC	Mononuclear Cell
MW	Molecular Weight
NAC:	Non-adherent Cell
NK:	Natural Killer
NT:	Not Tested
PBL:	Peripheral Blood Lymphocyte
PHA:	Phytohaemagglutinin
PMA:	Phorbol Myristate Acetate
SRBC:	Sheep Red Blood Cell
^{89}Sr:	Strontium 89
TCR:	T cell Receptor

^{3}H-Tdr:	Tritiated thymidine
Ti	T cell antigen receptor idiotype
TNF:	Tumour Necrosis Factor

REAGENTS

Na Citrate:	Sodium Citrate - blood anticoagulant (Fisher Chemicals)
PBS:	Phosphate-buffered Saline, pH 7.4 (Sigma)
FICOLL-HYPAQUE:	Pharmacia
IMDM:	Iscove's modified Dulbecco's medium (Sigma)
Sephadex-G10:	Pharmacia
FCS:	Foetal Calf Serum (HyClone)
BCS	Bovine Calf Serum
SRBC:	Sheep Red Blood Cells (Cedarlane Labs.)
AET:	2-Aminoethyl isothiouronium Bromide (Sigma)
^{3}H-Tdr:	Tritiated Thymidine (Amersham)
^{51}-Cr:	Chromium 51 (Amersham)
HCl:	Hydrochloric Acid (BDH Chemicals)
BIOMAG:	Magnetic Particles (Advanced Magnetics)
BSA:	Bovine Serum Albumin (Sigma)

I. INTRODUCTION

NATURAL KILLER (NK) CELLS

Natural Killing is the term coined to describe the cellular cytotoxicity observed *in vitro* against a particular cell target by a donor with no previous (known) sensitization (1). Despite early, nonspecific indications of the phenomenon of Natural Killing, the first real evidence for this appeared soon after the development of the in vitro ^{51}Chromium (^{51}Cr)-release assay (2,3). At that time, this background cytotoxicity was recognized as being a true phenomenon which could not be accounted for by cytotoxic T-lymphocyte-mediated lysis alone (2,3). Natural Killer cells are CD3 negative (T Cell receptor negative) large granular lymphocytes, and they express CD16 (FCRgIII) and CD56 receptors. They mediate cytolytic reactions that do not require expression of class I or class II MHC molecules on the target cells. Activated T cells may also express "NK-like" or non-MHC restricted cytolysis. This occurs in the apparent absence of disease, and without prior sensitization or deliberate immunization (4).

LYMPHOKINE-ACTIVATED KILLER (LAK) CELLS

Rosenberg et al (5) were the first to describe that lymphoid cells, after exposure to Interleukin-2 (IL-2), are able to lyse NK-resistant tumour cells *in vitro* and such cells would not lyse normal cells. Because these cells were incubated in the lymphokine IL-2 for several days, the resulting effector cells were termed Lymphokine-Activated Killer (LAK) cells. The only operational difference between Natural Killing and Activated Killing is the fact that the latter case involves

pretreatment of the effector cells with lymphokine(s) (6) and that LAK cells are able to kill NK resistant target cells. Both NK and LAK cells can be distinguished from other cytotoxic cells by the differential expression of cell surface markers (7). Such markers will be discussed later.

TARGET CELLS

Although much heterogeneity exists among candidate cells, human NK cells have traditionally been defined according to results with the target cell K562 (4). The choice of one defining target cell line, K562, has the major advantage of permitting results from various sources to be compared. The K562 cell line was derived from the pleural effusion of a patient with chronic myeloid leukaemia in blast crisis (8). The cell line was reported to carry the Philadelphia chromosome, to lack surface and intracellular immunoglobulin and T cell antigens (8). The K562 cell line is very sensitive to NK cells present in normal peripheral blood lymphocytes. K562 cells, like the Daudi cell line, lack B_2-microglobulin expression and do not express human histocompatibility antigens (8). It is mainly this property which made the cell line a choice for use in cytotoxic assays involving NK cells. Characteristics of the NK-sensitive thymocyte, for example, which include many characteristics typical of immature cells, seem to indicate an NK preference for immature cell targets in both human and murine systems (9). There is also a high degree of NK sensitivity seen in fetal bone marrow cells, as compared to the more NK-resistant adult bone marrow target cells (9). These results have been demonstrated in normal allogeneic as well as syngeneic systems (9). When target cells for both NK and LAK activity are compared, three groups are observed. First, target cells sensitive to both NK and LAK cells (K562). Second, target cells

resistant to NK cells but sensitive to LAK cells (such as HTB 58 used in this thesis) and finally target cells resistant to both NK and LAK cells (such as Daudi).

CHARACTERISTICS OF NK CELLS

Natural Killer (NK) cells are present in all individuals, they respond to various stimuli (resulting in increased activity), they do not require prior antigenic stimulation, and the recognition of target cells is not restricted by the Major Histocompatibility Complex (MHC) (10). Morphologically, the NK cell is a large, granular lymphocyte (LGL) containing azurophilic granules in the cytoplasm (11). This morphology gave rise to the term LGL, although investigators were unaware of the extent of the heterogeneity of the NK cell population. The cytoplasm is relatively abundant, and, due to their large size and density, the cells have been separated using Percoll gradient centrifugation techniques. The NK cells will be found in the lower density fractions, approximately 37.5% v/v Percoll (11). Cells with LGL morphology have been shown to participate in or mediate many functions in addition to tumouricidal activities. These cells produce a gamut of lymphokines, regulate immune responses, contribute to graft-versus-host disease, participate in organ allograft rejection, and, in addition to still other functions, play an important role in host defence against microbial and viral attacks (12,13). LGLs produce measurable amounts of IL-2 upon phorbol myristate acetate (PMA) and Concanavalin-A (con-A) stimulation, and this production was attributable to CD3-, CD2+ LGLs (12) (CD2 is a surface receptor for sheep erythrocytes). The CD2 structure is not physically linked to the CD3-TCR and T cell activation through CD2 would appear to represent an antigen-independent pathway (14). LGLs can also secrete IL-1 (13), and can exert positive or negative regulatory action upon B cells (12). They have also been shown to secrete interferon alpha and gamma (12). To date, no cell type other than T cells has been shown to produce IL-2 (15).

CLASSIFICATION OF NK CELLS

Cell surface markers

In humans, NK cells share many T cell-related as well as monocyte-related markers such as CDllb, CD7, CD8 and CD2 (16). However, the marker that NK cells consistently express is the low affinity Fc receptor for IgG, FcRIII (7) (Table 1) . The receptor in question, defined by the antigenic marker CD16, has a MW of 55-70 kDa, and is expressed on neutrophils (in high density), NK cells and a minor population of T cells, eosinophils, and tissue macrophages, but only a few are present on monocytes (17). For practical purposes CD16 is used to identify NK cells in lymphocyte surface phenotyping analysis. The activation of CDl6+ NK cells results in the expression of low molecular weight IL-2 receptor (p55), and in the production of IFN and TNF (17). IL-2 and CDl6 ligands act synergistically to activate NK cells, and anti-CDl6 monoclonal antibody treatment enhances NK cell activity (17). The CDllb antigen, found hitherto exclusively on the surface of cells of the myeloid lineage, and now appearing on the surface of NK cells (18), gave rise to the proposal that NK cells might be of myeloid lineage, and not of T cell lineage, but the issue remained speculative (18). Based on the expression of many T cell-associated markers, Kaplan (19) hypothesized that NK cells represent pre-thymic T cells, which express germ-line, gene-encoded receptors for self/non-self recognition.

TABLE 1

TABLE OF HUMAN LEUKOCYTE ANTIGENS (7,12,17,19)

ANTIGEN	SIMILAR CLONES	ANTIGEN MW	MAJOR REACTIVITY
CD2	OKT11, T11		E Rosette Receptor Associated
CD3	OKT3	45-50 K	Pan-T cells (75% PBLs)
CD4	OKT4	22-28 K	Helper-Inducer T cells (45% PBLs)
CD8	OKT8	55 K	Suppressor/Cytotoxic T cells (28% PBLs)
CD11b	Mo.1	32-34 K	T, NK, monocyte granulocyte
CD14	Mo.2		85% of monocytes, macrophages
CD16	—	55K	Fc R_{III} receptor NK, neutrophils
CD57	Leu 7 HNK1	55-70 K	
CD56	Leu 19 NKH1	110 K	cell adhesion molecule
		220/135 K	

TABLE 2

TABLE OF PHENOTYPIC CHARACTERISATION OF CYTOTOXIC EFFECTORS (20)

ANTIGEN	T CELLS MHC restricted	T CELLS nonMHC restricted	NK CELLS
CD3	+++	+++	-
CD4	++	-	-
CD8	++	-	+
CD16	-	+	+++
CD11b	-	-	+
CD2	+	+	+
CD56	-	-	++
CD5	+	-	-
ßTCR	+++	+	-
TCR	+	+++	-

The T cell Receptor (TCR)

The antigen receptor expressed on the surface of most mature T lymphocytes is known as the Ti (T idiotype) heterodimer, which is composed of a + b subunit chains (20) (Table 2). These chains comprise both constant and variable regions, which determine antigen specificity. The Ti structure, on the surface of immune specific

T cells, is associated with 3 noncovalently linked, invariable CD3 peptide chains known as γ, δ and ϵ (20). This Ti-CD3 complex distinguishes mature T cells from NK cells, which do not have the Ti-CD3 complex and associated molecules such as CD4 and CD8. Some NK cells express CD8 at low antigenic density (20).

T cell antigen Receptor gene rearrangement

The TCR genes must be rearranged to produce a functional transcript. The genomes of both mouse and man contain two *B* chain genes, both of which can be rearranged and expressed (21). Most thymocytes and all functionally competent, mature T cells rearrange and express Cβ genes (21). This event can therefore be used as an unequivocal marker to discriminate T from non-T lymphocytes. Early evidence suggesting that NK cells may arise from T cells comes from the fact that some cultured NK-like clones expressed a truncated form of the T cell receptor β-chain message, and some clones expressed clonally-restricted 90 kDa heterodimeric cell surface antigen recognition structures analogous to those detected on CTL cell lines (22). However, Allan Mowat (23) cited evidence showing that only CD3+ cells rearranged Vβ genes, whereas true, classical NK cells do not. Using a Cβ cDNA as a probe, Lanier and his group analysed the genomic DNA of CD16+ NK cells and T cells from peripheral blood (21). The Cβ genes were found to be in germline configuration in NK cells (and in granulocytes) whereas T lymphocytes showed rearrangement of the Cβ genes (21). NK cells are therefore fundamentally different and distinct from T lymphocytes in both lineage and nonself recognition (21).

γδ receptor-bearing T cells

A minor population of CD3+ T lymphocytes lacks the expression of the ß-TCR. These T cells, which constitute approximately 2% of peripheral blood T cells, are CD4-, CD8- and clones of these cells display MHC-nonrestricted cytolytic activity, like true NK cells (22). These novel T cells also bear FcRIII(CD16) receptors and mediate Antibody-Dependent Cellular Cytotoxicity, but their expression of CD16 is lower than in true CD3- NK cells (20). A subset of CD3+, CD4-, CD8- thymocytes was also shown to express the IL-2 receptor constitutively, but it lacked spontaneous (NK) cytotoxicity (24). These thymocytes were shown to display CTL activity after incubation in IL-2 -containing culture medium (24). The activity of this particular subset could be blocked with anti-OKT3 monoclonal antibody, demonstrating that these cells mediate cytotoxic activity via their T3 receptor complex, a complex absent in natural killer clones (24). These cells were found not to express Leu 7, Leu ll or CD11b antigens which are associated with true NK cells (24). This same CD3+, CD4-, CD8- cell subset was later isolated from mouse peripheral blood, and investigators also reported that these double-negative cells expressed a novel T cell receptor structure composed of γ- and δ-chains (25). It was suggested by the authors that these TCR-γδ-expressing cells may reflect a lack of dependence upon CD4 and CD8 accessory molecules in their recognition of target structures (25).

MHC RESTRICTION

NK and LAK cells are MHC-unrestricted cytotoxic cells as opposed to cytotoxic T cells that are MHC-restricted. MHC-restriction means that the target antigen has to be presented by a MHC molecule identical to the MHC of the effector cytotoxic T cells. NK and LAK sensitive target cells are killed regardless of their MHC make up.

NATURAL KILLING IS A PROPERTY OF AN HETEROGENEOUS LYMPHOCYTE POPULATION

Before the issue of Natural Killing heterogeneity was resolved, Lanier and his group were stating that NK cells could unequivocally be distinguished from T and B cells, and they further suggested that the development of NK cells was dependent upon intact bone marrow (26). Certain phenotypic characterizations were cited in this study: the two populations, which were clearly separable into CD3+ and CD3- subsets, differed at certain surface markers:most of the CD3- large granular lymphocytic cells express the CD16 antigen, whereas except for rare instances CD3+ T cells are Fc receptor negative (26). Further, certain antigens (CD2, CD7, CD8, CD11, Leu7 and Leu 19) present on T and myeloid cells and also expressed on NK cells were dismissed as evidence for lineage classification since there was no reason to assume that these antigens must be restricted to a single cell lineage (26). The authors also proposed that earlier studies which seemed to indicate that NK cells were acquiring CD3 in culture were in fact demonstrating the faster rate of growth of contaminating T cells as compared to NK cells (26).

SPECIFICITY OF NK ACTIVITY

Although early investigations led researchers to believe that NK cytotoxic activity was nonspecific (or non-selective) later evidence tended to favour the theory that NK cells did indeed display a pattern of selectivity in their reactions against a variety of target cells, indicating a yet-to-be elucidated recognition mechanism between killer cell and susceptible targets (27). The first suggestion of individual patterns of NK specificity came from studies whereby cancer patients and normal donors exhibited markedly different NK reactions to NK-sensitive targets (28). These studies and others involving inhibition of cytotoxicity led to the idea that many recognition structures may be involved in NK killing.

ORIGINS OF NK CELLS

Bone Marrow

Studies in mice have demonstrated that NK cells are uniquely dependent upon an intact bone marrow for their differentiation. These studies involved the use of the isotope ^{89}Sr which destroyed the bone marrow, resulting in a selective loss of NK cell function while B cell, T cell and macrophage cellular functions were unaltered because there is a large enough reservoir of these three cell types in secondary lymphoid organs and in the body, which does not appear to be the case with NK cells (26). Unlike human fetal bone marrow, adult bone marrow cells which are HNK-1 positive (Leu-7) also coexpress the T cell-associated antigens CD3 and CD8 (29). Interestingly, these cells, while morphologically resembling LGLs, contain few azurophilic granules. Adult spleens were found to exhibit substantial levels of NK activity, and 10% of splenocytes express HNK-1 (29). Many instances of

cord blood NK activity (against K562) have been documented, while in young and old adults, no differences in cytolytic activity have been observed (29). In humans, as in rodent systems, the highest levels of NK activity have been demonstrated in peripheral blood and spleen tissues (29).

Thymus

Contrary to earlier reports, it is now agreed that no NK activity is seen in the thymus (30). However, thymocytes are capable of developing LAK activity after culture with rIL-2 (30). The active precursor for this LAK activity is a CD3- lymphocyte (30). These thymic LAK cells bear a strong resemblance to LAK cells derived from peripheral blood. In addition, cytotoxic activity correlated well with the expression of the NKH-1 surface marker (30). Uncultured thymocytes are devoid of NKH-1 positive lymphocytes (30). Interestingly, thymic LAK cells were reported to be CD16-, which represents the major difference between PBL-LAK cells and thymic LAK cells (30).

Nude mouse

To further investigate the role of the thymus, congenitally athymic mice, known as nude mice (strain Balb/c) (31), were studied. T cell precursors are present in the bone marrow, but very few T cells have been detected in T cell compartments in nude mice (31). However, some aspects of the defence system may actually be more developed in nude mice than in normal mice, such as NK activity (31). NK activity in the spleens of nude mice was completely abrogated in the presence of anti-asialo GM1 (the murine NK cell marker) and complement (32). It is known that anti-asialo GM1 does not affect T or B cells (32). Over 90% of

nude mouse spleen cells are asialo GM1+ and NK activity correlates positively with the expression of this marker (32). Since mature thymocytes and peripheral T cells in mice do not express asialo GM1, murine Thy-1$^+$, asialo GM1+ cells are considered to be distinct from mature T cells (33). Therefore, murine NK cells would appear to be normal cells which are independent from T cells (33).

ROLE OF LYMPHOKINES AND LYMPHOKINE RECEPTORS

Interferons (IFN) alpha, beta and gamma have been found to substantially increase the activity of NK cells, both in separated LGL populations and unseparated lymphoid cells, without the need for any accessory help (34). It is also known that NK cells can themselves produce high levels of IFN upon exposure to various tumour cell lines, as well as viruses, mitogens and Bacille-Calmette-Guerin (BCG) (34,16). This clearly indicated a regulatory role for NK cells, in addition to their postulated role as immune surveillance cells. Thymic hormones have been demonstrated to augment NK activity vs K562 in the CD3+, CD14+ cell-depleted LGL fraction (34). The mechanism at work remains unknown. Thymic hormones by themselves do not induce *de novo* synthesis of either IFN or IL-2, but will modulate the effects of these in addition to IL-2 receptor expression induced by mitogen (34). The p70 chains of the IL-2 receptor have been shown to be expressed in equal numbers on the surfaces of human NK cells and resting, high-density T lymphocytes (35). However, NK cells proliferated strongly in response to much lower amounts of IL-2 than were required for T cells, and T cells additionally required the presence of monocytes (35). It would appear that the T-cell activation is required for the synthesis of p55 chains, which, together with the p70 chains, form high-affinity IL-2 receptors which seemingly transmit a stronger proliferative signal (35).

ORIGIN OF LYMPHOKINE-ACTIVATED KILLER CELLS

Natural killer cells and lymphokine-activated killer cells display different characteristics. In a report (36), reactivity with the monoclonal antibody CD11b is absent in the activated population, there are less Fc receptor-bearing cells, and more activated killers adhere to nylon wool.

Before the effector cells of lymphokine-activated killer cell activity were described, investigators had distinguished this population of cells from cells displaying Natural Killer activity (37,38). It was optimistically suggested that LAK cells might have a role in defending the host against NK-resistant tumours, based on the premise that LAK cell lysis of fresh tumour cells involved recognition of an antigen distinct from the determinant involved in the NK-mediated lysis of target cells (39). Other studies have demonstrated that LAK cells had the same characteristics as NK cells. For the most part LAK effectors in human blood, like the majority of NK effectors, were LGLs with the CD3-, CD16+ phenotype (39).

LAK CELLS AND THE TREATMENT OF CANCER

When it became apparent that LAK cells could lyse a wide spectrum of NK-resistant tumour targets, their potential as immune cells with *in vivo* as well as *in vitro* antitumour reactivity was tested in the murine system (5) In mice, it was found that adoptive transfer of IL-2-treated lymphocytes could mediate the regression of established lung and liver metastases in different strains of mice (5). Human LAK cells can be generated in large numbers from peripheral blood lymphocytes incubated in IL-2. Human LAK cells will not attack normal tissues, and can be infused in large numbers with restricted toxic side effects (5). There are also several

theoretical advantages to this form of treatment:the transfer of immune cells should not cause immunosuppression in the recipient, and does not even require host immunocompetence (5). Further, this therapy could be combined with other therapies because its morbidity is low while its specificity is high (5).

The Cytotoxic Event

Whatever the differences in recognition structures of NK cells and T cells, work was continued in the area of the cytotoxic event itself. An important finding was that of a pore-forming protein termed perforin (or alternatively cytolysin) which was isolated from the granules of LGL cytotoxic cells which include NK cells and cytotoxic T cells (40). This finding appeared to correlate with the morphology of these cells, but later findings which suggested the need for the presence of IL-2 to induce perforin production clouded the issue somewhat (40).

Effect of monocytes

As a consequence of the discovered effects of prostaglandins on NK cell activity, researchers looked at the possibility of monocyte interference in NK cell assays. Monocyte contamination in these assays was found to be much less critical than at first thought. Monocytes left after adherent-cell depletion processing of the peripheral blood lymphocyte preparation, were ineffective in causing significant lysis (41), and since LGLs themselves can produce IL-l, this by-product of the presence of monocytes would be unlikely to interfere with NK assay results.

II. OBJECTIVES

From the review of the literature several questions became evident. Do T cells display LAK activity? If they do, which T cell subsets have LAK activity? Among the T cell subsets, what is the contribution of double-negative T cells (CD3+4-8-) to LAK activity? To answer such questions we set up several goals.

Our first goal was to achieve satisfactory separations of the cell populations from human peripheral blood by various procedures which we evaluated. The first set of experiments therefore consisted of the investigation of cell separation methods. Our second goal was to study the cytolytic activities of the resultant populations with and without modulation by lymphokine(s), and make concise statements concerning the heterogeneity of the mediators of the NK and LAK phenomena. Thus, the second set of experiments consisted of functional assays using the cell populations obtained by different methods of cell separation. This last set included NK and LAK assays. Theoretically, there are many advantages in the potential application of the LAK cell system in a clinical setting. If the antitumour activity demonstrable in vitro can be applied *in vivo*, there exists the potential for a fine degree of specificity in the ensuing immune reactions (5).

In summary our objectives were:

1. To develop methods of separation of T cell subsets.
2. To determine if LAK activity is restricted to NK cells or if it is also detectable in T cell subsets, including double negative (CD3+4-8-) T cells.

III. METHODS

PREPARATION OF LYMPHOCYTES (MNCs)

Mononuclear cells (MNCs) were separated from the peripheral blood of normal laboratory donors as follows: blood (120 ml) was collected in 3.8% Sodium Citrate to a final concentration of 10% v/v (1 part Citrate, 9 parts blood). This blood was aliquoted into 50 ml centrifuge tubes. In later experiments, whole units of blood with anticoagulant were obtained courtesy of the Ottawa Red Cross, and were aliquoted into 250 ml centrifuge bottles. Undiluted blood samples were centrifuged at 1500 rpm for 15 minutes, the plasma layer and the buffy coats were collected separately, and the remaining blood was diluted with PBS-10%FCS to its original volume before being re-centrifuged for another round of plasma and buffy coat collection. The buffy coats were pooled and then re-diluted with PBS-10% FCS, layered over Ficoll Hypaque, and the mononuclear cells isolated by density gradient centrifugation at 1800 rpm for 25 minutes at 22 ^{o}C. The MNCs were washed three times with IMDM-10% FCS before being resuspended in the same medium.

ADHERENT CELL DEPLETION

Mononuclear cells were depleted of adherent cells (ie monocytes, accessory cells, etc.) by passage through Sephadex G-10. Sephadex G-10 which had been washed four times with sterile saline to remove fine, non-settling particles, was allowed to swell overnight. The Sephadex G-10 was resuspended in saline such that the saline represented 50% of the estimated bed volume of the Sephadex. This

slurry was then autoclaved for 30 minutes and was allowed to cool before use. The slurry was stored at 4 ^{o}C until needed. Sephadex G-10 columns were made by removing the plug from 10 ml syringes, and equipping the "columns" with stopcocks. With the stopcock in the closed position, a nylon wool plug was added to the tip of the column, and Sephadex G-10 slurry was added slowly, allowing the slurry to pack. With the stopcock now in the open position, excess saline was drained from the packed column, while care was taken not to allow the column to dry. Cells were put through Sephadex columns at a concentration of 1 x 10 ^{7} cells/ml in IMDM-20%FCS and 10 ^{7} cells were used for every ml of packed Sephadex slurry in the column. Non-adherent cells were eluted from the column using pre-warmed IMDM-10%FCS at 37 ^{o}C.

Alternatively, mononuclear cells were adherent-cell depleted by resuspension in IMDM-20% FCS, followed by incubation on glass Petri dishes for two rounds of 45 minutes at 37 ^{o}C. After incubation, non-adherent cells were washed 4 times with IMDM.

SHEEP ERYTHROCYTE ROSETTING

Sheep erythrocyte (SRBC) rosetting was used to separate T cells from non-T cells. Sheep erythrocytes were first treated with aminoethyl isothiouronium bromide (AET) to enhance SRBC-T cell interactions, and were then washed with cold saline and resuspended in IMDM-20% FCS at a concentration of 10% v/v. Mononuclear cells were resuspended in IMDM-20% FCS at a concentration of 3 x 10 ^{6} cells/ml, and equal volumes of cells and SRBC (diluted to 2% v/v in IMDM-20%FCS) were mixed and centrifuged at 1100 rpm for 5 minutes. This mixture was incubated at room temperature for 30 minutes. To separate E+ (rosetted) from E- cells, the rosettes were gently resuspended, underlayered with cold Ficoll-Hypaque,

and centrifuged for 25 minutes at 1800 rpm. The interphase cells (E-) were harvested and washed, and the E+ cells isolated by lysing the sheep erythrocytes in the cell pellet. E+ and E- cells were washed thrice and resuspended in IMDM-10% FCS.

SUBSET SEPARATION BY PANNING WITH MONOCLONAL ANTIBODIES

Petri dishes (100 x 15 mm) were coated with a goat anti-Mouse IgG antibody (Jackson Lab) (10 ug/ml) and were incubated overnight at 4 ^{o}C before decanting the antibody solution. Dishes were then washed with phosphate-buffered saline and blocked for one hour with PBS-1%FCS. In the meantime, lymphocytes to be panned were stained with the appropriate mouse monoclonal antibody for 30 minutes, washed twice with PBS-5% FCS, and resuspended in the same medium. Cells were poured onto antibody-coated dishes, at 3 ml/dish, incubated for 40 minutes at 4 ^{o}C, swirled to redistribute the cells, and incubated for 30 minutes. Non-adherent cells were removed by aspiration of the supernatant using a pipette, plates were washed 5 times with PBS, while adherent cells were recovered by flushing the dishes with 25 ml of PBS-1% FCS using a 30 ml syringe equipped with a 25g needle.

IMMUNOFLUORESCENCE STAINING

Mononuclear cells to be stained were resuspended in PBS-5%FCS at a concentration of 2 x 10 ^{7} cells/ml. Using a Pipetman (Gilson), 50 ul of this cell solution were dispensed into 96-well microtiter plates (Linbro) and wells were "stained" with monoclonal antibodies at the appropriate concentration. Plates were then refrigerated at 4 ^{o}C for a 30 minute incubation. After incubation, plates were

centrifuged at 1000 rpm for 3 minutes, the supernatants were aspirated, and plates were washed 3 times with PBS-5%FCS. If indirect immunofluorescence was performed, a secondary antibody was used in the appropriate wells, as described above. When cells had been washed, 10 ul of 2%-paraformaldehyde was added to each well and the cells were fixed for 5-10 minutes. Wells were filled with PBS, and plates were centrifuged at 1000 rpm for 3 minutes, the supernatants were aspirated, and plates were washed 3 more times. To mount cells on microscope slides, 1 or 2 drops of PBS was added to each well, and 1 drop of this cell solution was placed on a microscope slide, allowed to dry, and viewed under the fluorescent microscope (Leitz) using the appropriate mounting fluid.

PROLIFERATION ASSESSMENT USING ^{3}H-TDR UPTAKE ASSAY

Lymphocytes at 5 x 10^{4}/ml in IMDM-10% BCS were dispensed into flat-bottomed, 96 well microtiter plates (Linbro) in triplicate volumes of 100 ul/well. Lymphokines (IL-2, IL-4) to be assayed were added in the proper dilutions to the wells and plates were incubated at 37 ^{o}C for 2 and 5 or 3 and 6 days, at which time cultures were pulsed with 50 ul diluted ^{3}H-thymidine (25 Ci/mmol,1.0 mCi/ml). Four hours later all cultures were harvested (using a Titertek cell harvester) onto glass fiber filters, and radioactivity incorporated into DNA was measured by liquid scintillation.

^{51}Cr RELEASE ASSAY FOR CYTOTOXICITY

Target cells (K562, Daudi, HTB 58) were washed and labelled with 100uCi ^{51}Cr per 10^{6} cells. These were incubated for 1 hr at 37 ^{o}C, washed and re-incubated for 30 minutes at room temperature in IMDM-10% FCS. Cells were

washed twice, resuspended in IMDM-10% FCS, and 5000 target cells dispensed per well in a V bottom microtiter plate (Linbro).

Lymphocyte effector cells were cultured with or without lymphokine(s) at 37 ^{o}C for 2 days, were harvested and resuspended at 4 x 10^{6} cells/ml in IMDM-10% FCS. Effector to target ratios used were 80:1, 40:1, 20:1, 10:1, 5:1 and 1:1. Briefly, effector cells were added to triplicate target cell wells to obtain appropriate ratios, plates were centrifuged at 1000 rpm for 2 minutes, and incubated at 37 ^{o}C for 4 hours. After incubation, plates were re-centrifuged for 10 minutes at 1000 rpm, and 150 ul of supernatant from each well was transferred to tubes and counted. Spontaneous release of radioactivity was measured by replacing effector cells with IMDM-10% FCS, and total release of cytotoxicity by lysing target cells with 1N HCl. Percent lysis was determined using the following formula:

$$\% \text{ Lysis} = \frac{\text{test cpm - spontaneous cpm} \times 100}{\text{total cpm - spontaneous cpm}}$$

CELL SUBSET DEPLETION USING ANTIBODY-COATED MAGNETIC PARTICLES

Cell subsets were depleted by immune rosetting with monoclonal antibodies and magnetic particles coated with goat antiMouse IgG (BioMag, Advanced Magnetics). The magnetic particles (slurry) were allowed to settle and the supernatant discarded. The slurry was washed thrice with PBS, with intermittent vigorous vortexing to break clumps of particles. The slurry was resuspended at 50% of its original volume in sterile PBS. 0.5 ml of slurry was used for every 10^{7} stained lymphocytes.

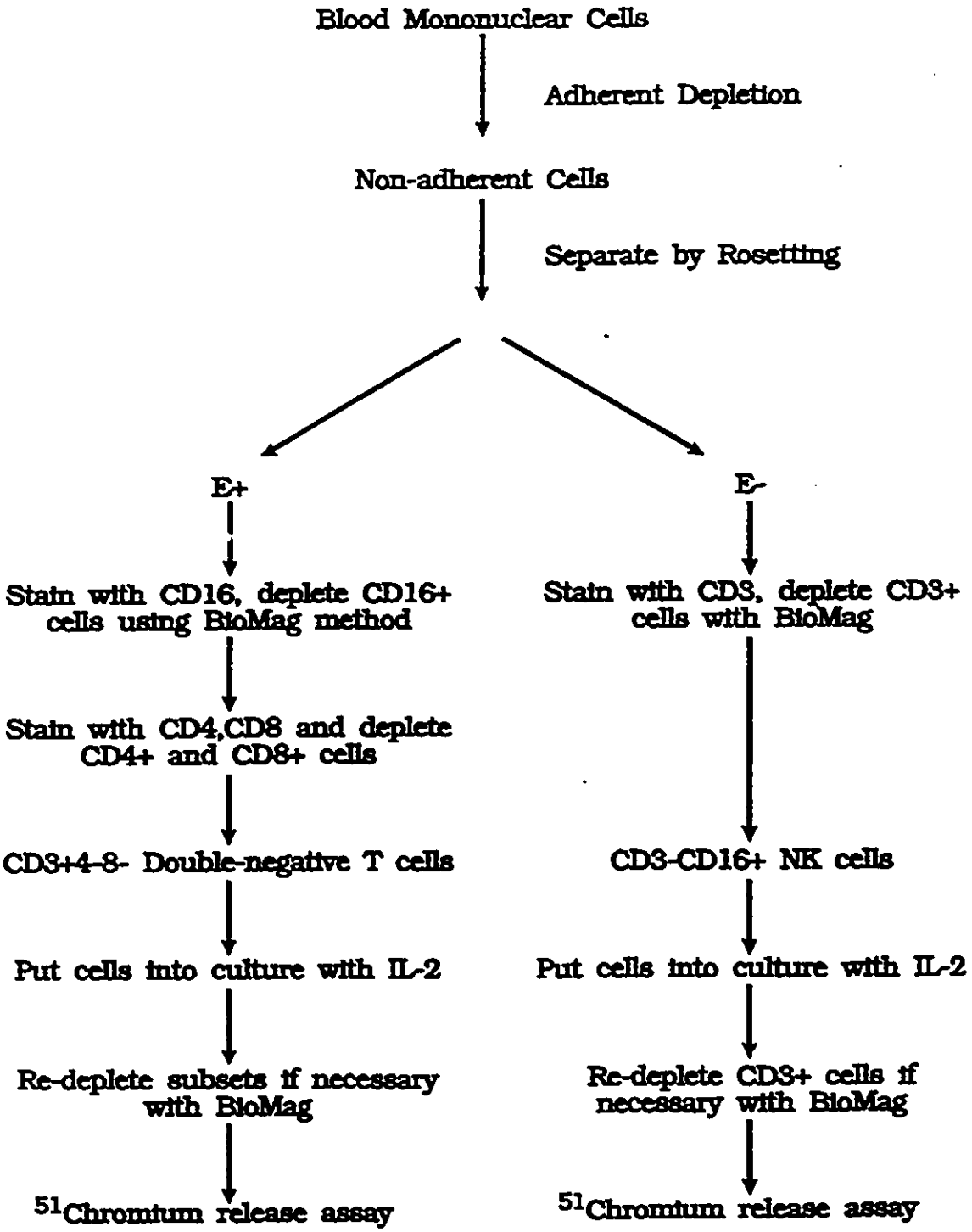
Lymphocytes were separated, on the basis of their reactivity with sheep red blood cells, into T cells (E+) and non-T cells (E-). The E+ cells were stained first

with 250 ul of OKT4 ascites fluid (1:2 dilution) for every 10^7 cells, incubated for 30 minutes at 4 ^oC, and washed 3 times with IMDM-1% BSA. Cells were resuspended at 10^7/ml, and 0.5 ml of the BioMag slurry was added per ml of stained cells. The mixture was alternatively centrifuged for 5 minutes at 500 rpm and incubated for 10 minutes at 4 ^oC, (repeated twice) for a total of 30 minutes, transferred to a T25 flask and placed horizontally over the BioMag magnet for 10 minutes. Unbound cells were aspirated and cultured, and bound cells were also cultured because it was discovered that, in the presence of 10-15% FCS, bound cells spontaneously uncouple from BioMag after 2-3 days. This procedure was repeated with OKT8 for the E+ cells, and with a mixture of OKT4 and OKT3 for the E- cells.
The following flow chart (Fig 1) illustrates the steps used to isolate and culture NK cell and T cell subsets.

GENERATION OF LAK CELLS

Separated cells were cultured and maintained in growth medium consisting of Iscove's medium, 10% fetal calf serum, gentamicin, and 200 U/ml of recombinant IL-2 (Cetus). Generation of LAK requires at least 24 hours' exposure to IL-2. IL-2 was titrated in preliminary experiments and 200 U/ml gave saturating concentrations.

FLOW CHART


Blood Mononuclear Cells
Adherent Depletion
Non-adherent Cells
Separate by Rosetting
E+
E-
Stain with CD16, deplete CD16+ cells using BioMag method
Stain with CD4,CD8 and deplete CD4+ and CD8+ cells
CD3+4-8- Double-negative T cells
Put cells into culture with IL-2
Re-deplete subsets if necessary with BioMag
51Chromium release assay
Stain with CD3, deplete CD3+ cells with BioMag
CD3-CD16+ NK cells
Put cells into culture with IL-2
Re-deplete CD3+ cells if necessary with BioMag
51Chromium release assay

IV. RESULTS

CELL SEPARATION EXPERIMENTS

Monocyte depletion using Sephadex G-10 columns

The yield of mononuclear cells from 120 ml of blood from normal laboratory donors ranged from 8×10^7 to 2.5×10^8 cells. In early experiments, mononuclear cells were depleted of adherent cells by passage through a Sephadex G-10 column, and the recovery of non-adherent cells ranged from 24.4 to 37.0 percent as shown in Table 3. The non-adherent cells were tested in the NK cell activity assay. These cells were used in separation experiments by panning.

TABLE 3

CELL RECOVERIES AFTER DEPLETION WITH SEPHADEX G-10

Exp't #	Unseparated MNC	After Sephadex[a]	%Recovery[b]
1	8.0×10^7	1.94×10^7	24.4%
2	8.25×10^7	3.08×10^7	37.0%
3	8.0×10^7	2.9×10^7	36.4%

a = number of cells recovered after Sephadex step
b = number of cells recovered as a percentage of input cells

Separation of T cells by "panning" with an anti-CD3 antibody

Further separation of the non-adherent cells was achieved by panning with an anti-CD3 monoclonal antibody. Two populations were obtained, T cells and non-T cells. Phenotyping (by immunofluorescence) of the two populations, CD3+ and CD3-, was performed to check the efficacy of the separation, by determining its composition in terms of cell phenotype. Improper panning, which results in incomplete population separation, was indicated by high positive values using a monoclonal antibody marker to stain depleted populations (data not shown). The cell recovery was low and the two separated populations contained many contaminating cells. Phenotyping included such markers as CD3, CD4 and CD8 for T cells, Mo2 (CD14, Leu M3) for monocytes, B1 (CD20, Leu 16) for B cells and Leu 11a (CD16) for cells of the NK subset.

Separation of non-adherent cells by sheep erythrocyte (E) rosetting

An alternative method was sought to separate T cells from non-T cells. Sheep erythrocyte rosetting, when performed after depleting the mononuclear cells of adherent cell contaminants by passage through Sephadex G-10 columns, was found to yield a better separation of lymphocyte populations, and was additionally favoured when the sheep erythrocytes were treated with 2-aminoethyl isothiouronium bromide (AET). Phenotypically, there was less contamination by CD3+ cells in the E- (non-T) cell population, and cell recoveries were close to 100%. However as shown in table 4, both fractions contained a significant number of CD16+ NK cells.

TABLE 4

COMPARISON OF 2-AMINOETHYL-ISOTHIOURONIUM BROMIDE-TREATED AND UNTREATED SHEEP ERYTHROCYTES ON T CELL SEPARATION

	Post-Rosetting Phenotypes			
Marker	E_{AET} +	E_{AET} -	E+	E-
CD3	81	4	90	59
CD16	18	24	15	33

Separation of non-adherent cells using magnetic particles and monoclonal antibodies

Our previous experiments showed that panning and rosetting techniques alone can yield information from which only limited conclusions may be drawn. Thus another technique was perfected and introduced, which permitted a much finer degree of separation of lymphocytes. This approach is based on the fact that cells possess antigenic markers recognizable by monoclonal antibodies, which in turn can be recognized by secondary antibodies. The secondary antibodies employed in these experiments are goat antiMouse IgG (heavy and light chains) and they are coated onto magnetic particles called BioMag. These antibody-coated particles will recognize and bind to mouse-derived monoclonal antibodies used to stain the lymphocytes to be separated.

In order to use the BioMag particles successfully, titration experiments had to be performed which would yield the optimal working conditions for the secondary antibody. To this end, experiments were done using non-adherent cells obtained from blood mononuclear cells from normal donors. Equal numbers of these cells were aliquoted into tubes (500 ul/tube) and increasing amounts of BioMag slurry was added. The resulting depletion of OKT8+ cells was determined by phenotyping (by immunofluorescence) the depleted lymphocyte populations.

As shown in Tables 5 and 6 , the magnetic particle/antibody method gave excellent separation of CD8+ cells with depletion to less than 1% of CD8+ cells. As shown later, using this method it is possible to isolate or deplete various lymphocyte subsets such as NK cells (CD16+), CD8+ cells, CD4+ cells, and to enrich for CD3+4-8- T cells (double negative T cells).

TABLE 5

MAGNETIC PARTICLE TITRATION: EXPERIMENT 1

# Cells used	Volume	Slurry[a]	$CD8^+$ cells	%Recovery[b]
8.8×10^6	500 ul	--	28.8 %	NT
8.8×10^6	500 ul	100 ul	10.6 %	NT
8.8×10^6	500 ul	200 ul	6.9 %	NT
8.8×10^6	500 ul	300 ul	9.3 %	NT
8.8×10^6	500 ul	400 ul	6.5 %	NT

a = concentrated suspension (50% v/v) of BioMag beads
b = number of cells recovered as a percentage of original cells
NT= not tested

TABLE 6

MAGNETIC PARTICLE TITRATION: EXPERIMENT 2

# Cells used	Volume	Slurry[a]	$CD8^+$ cells	%Recovery[b]
5.0×10^6	500 ul	–	18.7 %	84 %
5.0×10^6	500 ul	250 ul	<0.1 %	42 %
5.0×10^6	500 ul	500 ul	0 %	26 %
5.0×10^6	500 ul	1000 ul	0 %	40 %

a = concentrated suspension (50%) of BioMag beads
b = number of cells recovered as a percentage of original cells

Thus, it was determined that the most effective and economical depletion of stained cells, as well as the highest cell recoveries occur when 500 ul of BioMag slurry are added to a maximum of 10^7 lymphocytes/ml. The technique of BioMag separation was used in all subsequent experiments to deplete lymphocyte subsets.

FUNCTIONAL ASSAYS

NK cell activity in non-adherent cells

When the cells had been passed through a Sephadex G-10 column, and phenotyped, they were used for the NK assay (Table 7). The target cell was the classical NK cell-determining cell line, K562, and these unstimulated effector lymphocytes demonstrated a range of baseline values, from 25 to 63 percent specific lysis. The ratio of effector cell to target cell was kept at 80:1 for these early experiments:

TABLE 7

NK CELL ASSAY*

Exp. No.	Target	E:T Ratio	^{51}Cr CPM Spon	Total	Test	% Lysis#
1	K562	80:1	80	1074	708	63
2	K562	80:1	349	847	476	25

** 1 donor per experiment

* ^{51}Chromium release assay
\# calculated according to formula on page 20.

Lymphokine-activated killer cell (LAK) activity mediated by the E+ and E- cell subsets

The percentage lysis mediated by LAK cells derived from E+ and E- cell populations from a normal donor's mononuclear cells was determined for two different cell lines, K562 (which is NK sensitive) and HTB 119 (which is a relatively NK resistant human small cell lung carcinoma cell line). Mononuclear cells were separated by Ficoll-Hypaque centrifugation, adherent-cell depletion, rosetting and BioMag subset depletion, as described, except that the adherent-cell depletion was carried out on glass dishes (45 minutes at 37 ^{o}C in 20% FCS/IMDM) instead of by the Sephadex G-10 method. The cell recovery after Ficoll-Hypaque centrifugation was 2.39 x 10 ^{8}, after adherent-cell depletion, 1.2 x 10 ^{8}, and 100% following sheep erythrocyte rosetting. The separated E+ cell fraction was processed with CD16 antibody and BioMag to remove CD16+ contaminating cells, and the E- cell fraction was treated with anti-CD3 monoclonal antibodies and BioMag to remove as

many of the CD3+ cells as possible. Cells of both fractions were then put into culture, with and without the addition of recombinant IL-2 (200 U/ml), and were incubated at 37 ^{o}C for 2 days. After 2 days, the cells were used as effectors and were tested for lymphokine-activated killer activity, and they were also phenotyped so that a relationship was established between LAK activity and cell phenotype.

The data in table 8 indicates that IL-2 increases spontaneous cytotoxicity for both targets and in both fractions (E+ and E−). However, the phenotypic analysis showed that the E+ fraction was contaminated with NK cells at a percentage similar to the E− fraction, thus we can not conclude that the LAK effect was mediated by T cells. We can also deduce that depletion of CD16+ cells using magnetic particles was not successful.

TABLE 8

CYTOTOXICITY OF THE E+ AND E- CELL FRACTIONS

			% Lysis		
Fraction	Target	E:T ratio	no IL-2	with IL-2	%increase[a]
E+	K562	20:1	12.4	21.4	73
E+	HTB119	20:1	21.9	37.9	73
E-	K562	20:1	6.9	40.1	481
E-	HTB119	20:1	31.8	44.1	39
Phenotype					
E+ CD3			68	n.d.	
CD16			15	n.d.	
E- CD3			5	11	
CD16			21	17	

n.d. = not done
a = increase in cytotoxic activity above that of control (no IL-2)

Studies of the effect of IL-2 and IL-4 on LAK activity

It had been reported in the literature (42) that the lymphokine interleukin-4 (IL-4) was capable of synergizing with IL-2 to enhance cellular proliferation. Experiments were therefore performed using mononuclear cells and non-adherent mononuclear cells (processed through Sephadex G-10 columns as previously described) to investigate the effect of IL-4 alone and with IL-2 (Fig. 2). Different concentrations and combinations of the lymphokines were used.

Mononuclear cells were obtained (as previously described) from whole donor blood by Ficoll-Hypaque centrifugation. An additional step of Sephadex G-10 depletion was used to obtain non-adherent lymphocytes. Both subsets of cells were cultured in 96-well plates with the appropriate lymphokine(s) added, for 3 and 5 days at 37 ^{o}C. ^{3}-H-Thymidine was then added to each well, plates were re-incubated for four hours, and then cells were harvested and radioactivity was assessed.

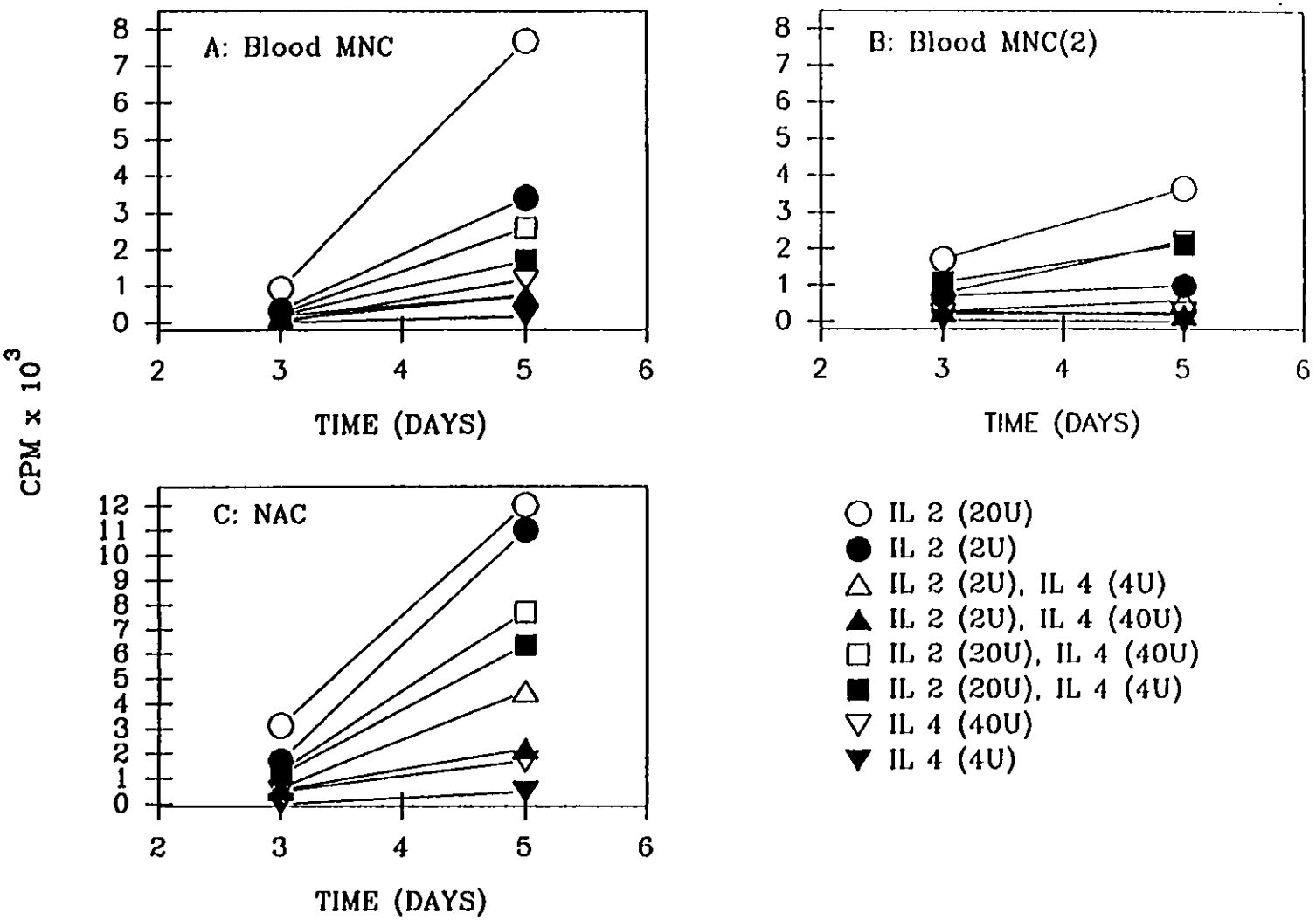
Results are expressed as CPM of radioactivity, which reflects cellular proliferation. As expected, highest proliferative rates were seen when mononuclear cells were treated with IL-2 at its highest concentration (20 U per well) (Figures. 2A and 2B). Unexpectedly, the addition of IL-4 at either concentration had a markedly inhibitory effect on the IL-2 induced proliferation, and IL-4 alone could not induce proliferation above baseline levels. The same pattern was observed with adherent-cell depleted lymphocytes, although the rates were much higher (Figure 2C). In all cases proliferation was higher on Day 5 than on Day 3.

Figure 2.-

Effect of IL4 on IL2 induced proliferation of activated T cells:

(A) and (B): Blood mononuclear cells (MNC) were activated with 1% phytohemagglutinin (PHA) for 3 days. On day 3 the cells were washed extensively and subcultured at 2×10^5 cells/ml in the presence and/or absence of various concentrations of IL2 and IL4. Both experiments showed that maximal proliferation of T cell blasts was obtained with 20 U/ml of IL-2. The addition of both concentrations of IL4 (4 and 40 U/ml) inhibited the proliferative response of IL2 stimulated T cell blasts. The concentration of IL4 that we used induced very little or no proliferative response. The optimal stimulatory capacity of the IL4 that we used was 200 U/ml (data not shown) and was about half of the maximal proliferation induced by an optimal concentration of IL2 (data not shown).

(C) Blood MNC were depleted of adherent cells by incubation on plastic. The non-adherent cells (NAC) were then tested as described for (A) and (B) with similar results.


A: Blood MNC
B: Blood MNC(2)
C: NAC
CPM x 10^3
TIME (DAYS)
TIME (DAYS)
TIME (DAYS)
IL 2 (20U)
IL 2 (2U)
IL 2 (2U), IL 4 (4U)
IL 2 (2U), IL 4 (40U)
IL 2 (20U), IL 4 (40U)
IL 2 (20U), IL 4 (4U)
IL 4 (40U)
IL 4 (4U)

Spontaneous cytotoxic activity of T cell subsets and NK cells in culture

Once the preparatory experiments on lymphocyte separation had been completed and the optimal conditions established for maximal lymphokine stimulation with IL-2, more extensive subset separations were undertaken to further subdivide and purify the lymphocyte fractions.

To do these more extensive experiments we used whole blood units from the Red Cross. Units of blood (500 ml) were processed in the same manner as previously smaller quantities (120 ml). Mononuclear cell yields ranged between 14.9×10^7 and 58.2×10^7 lymphocytes per unit of blood.

To obtain a pure NK population, E- cells were depleted of contaminating T cells using OKT3. To remove contaminating cell subsets from the larger E+ fraction, stepwise depletions were performed (See Fig. 1). First, NK cells were removed using CD16. This resulted in complete or near-complete elimination of CD16+ cells, as would be expected since they constitute a small percentage of peripheral lymphocytes and were additionally enriched in the E- fraction in the previous step. Next, the CD4+ and CD8+ cells were depleted to yield double-negative T cells (CD3+, CD4-, CD8-). It was generally easier to eliminate CD8+ cells (using OKT8) than CD4+ cells (using OKT4) since there are fewer CD8+ cells present in the E+ fraction.

It was necessary to monitor the cell numbers and the phenotypes which resulted after each depletion step. This was achieved by counting and phenotyping (by immunofluorescence) cells after each successive depletion. During the phenotyping of cells separated using BioMag particles, an extra marker, Goat anti-Mouse, was used to detect stained lymphocytes which had not bound to the BioMag Goat anti-Mouse particles, but which had bound to the primary antibody.

Results of post-separation subset phenotypes are given in Table 9. CD16+ cells and CD3+4-8- cells were phenotyped immediately after separation, whereas CD3+8+ cells were phenotyped after they had uncoupled from the BioMag particles (2-3 days later, as described in Methods).

When the presence of contaminating cells was sufficiently reduced, subsets were cultured with or without IL-2 (200U/ml) for 2 days, and were used for cytotoxic assays, to determine whether double-negative (CD3+, 4−, 8−) cells displayed cytotoxic activity comparable to that of natural killer cells and CD3+8+ cells. Phenotypes determined on the day of the cytotoxic assay are presented in Table 10.

In all experiments, depletion of CD16+ cells from CD3+ cell subsets could be achieved to less than 5% (Table 10). CD4+ cells, which constitute a larger percentage of mononuclear cells than do CD16+ cells, were somewhat more difficult to deplete, but in most cases they were reduced to a final concentration of 10% or less for the cytotoxic assays.

CD8+ cells were more difficult to deplete from CD3+ cell populations, and as a result this subset was re-depleted prior to the cytotoxic assays. Final concentrations were 5% or less for the CD3+, double-negative cell subset (Table 10).

TABLE 9

Phenotypes of Cell Fractions after Biomag Separation Experiments

	Exp. 1				Exp. 2				Exp. 3			
Cell Fraction	D3	D4	D8	D16	CD3	CD4	CD8	CD16	CD3	CD4	CD8	CD16
$CD16^+$	46	7	25	18	45	0	22	9	34	4	39	25
$CD3^+8^+$	74	4	36	0	-	-	-	-	85	12	49	2
$CD3^+4^-8^-$	46	7	52	0	64	4	15	0	78	10	17	0

TABLE 10

Phenotypes of Cell Fractions on the day of Cytotoxic Assay

		Exp. 1				Exp. 2				Exp. 3			
Cell Fraction	IL2	CD3	CD4	CD8	CD16	CD3	CD4	CD8	CD16	CD3	CD4	CD8	CD16
CD16+	-	8	5	40	57	5	2	59	69	10	8	49	63
	+	2	1	31	45	0	3	47	63	14	8	34	48
CD3+4-8+	-	93	8	56	1	--	--	--	--	93	9	60	0
	+	90	71	15	3	--	--	--	-	99	18	64	0
CD3+4-8-	-	90	8	5	3	95	10	0	2	95	3	0	0
	+	93	4	0	2	96	15	2	2	98	9	4	0

Results of the cytotoxicity assays (Table 11, Figures 3, 4 and 5), expressed as percentage lysis values following a 4-hour ^{51}Chromium release assay, indicate that NK cells, cytotoxic/suppressor and double negative T cells all have similar LAK activity. The target cells used were the NK-sensitive line K562, the NK-resistant line Daudi, and the NK-resistant line HTB 58. When treated with IL-2, cytotoxic effector cells displayed an increase in lysis rates against K562, and no increase in lysis rates against Daudi. Cells in all subsets were capable of lysis against HTB 58 targets in the presence of IL-2, thus displaying lymphokine-activated killer cell activity. The helper/inducer subset (CD3+4+8-) was also tested and it showed LAK activity in 2 of 3 experiments. Because we did not use Lytic Units to measure cytotoxic activity, we cannot compare the potency of the lytic activity of the various lymphocyte subsets.

TABLE 11

Cytotoxic Activity of NK cells ($CD16^+$), double negative T cells ($CD3^+4^-8^-$) and $CD8^+$ T cells

Cell Fraction	Target Cell	Cytotoxic activity at 40:1 Effector:Target ratio (%)					
		Exp.1		Exp.2		Exp.3	
		- IL2	+ IL2	- IL2	+ IL2	- IL2	+ IL2
$CD16^+$	K562	32	48	33	47	42	60
	HTB 58	18	52	17	36	21	69
	Daudi	22	19	11	20	18	28
$CD3^+4^-8^-$	K562	28	59	30	45	27	51
	HTB 58	22	42	14	32	10	44
	Daudi	15	21	11	17	12	33
$CD8^+$	K562	28	50	ND	ND	22	51
	HTB 58	21	31	ND	ND	7	39
	Daudi	14	22	ND	ND	5	4

ND = Not Determined

Figure 3.

Lymphokine Activated Killer (LAK) activity of isolated T cell subsets and NK cells (exp. 1)

Fresh blood mononuclear cells (MNC) were separated into T cell subsets (double negative T cells "CD3+4-8-" and suppressor/cytotoxic T cells "CD3+4-8+") and NK cells (CD3-16+) using monoclonal antibodies and magnetic particles as described in Methods. Cell subsets were stimulated with PHA for 3 days and then cultured with IL-2 (20U/ml). Before doing the LAK assay, the cells were phenotyped for cell surface markers and then depleted from unwanted cell subsets as described in Methods.
The figures show results for 3 cell subsets:

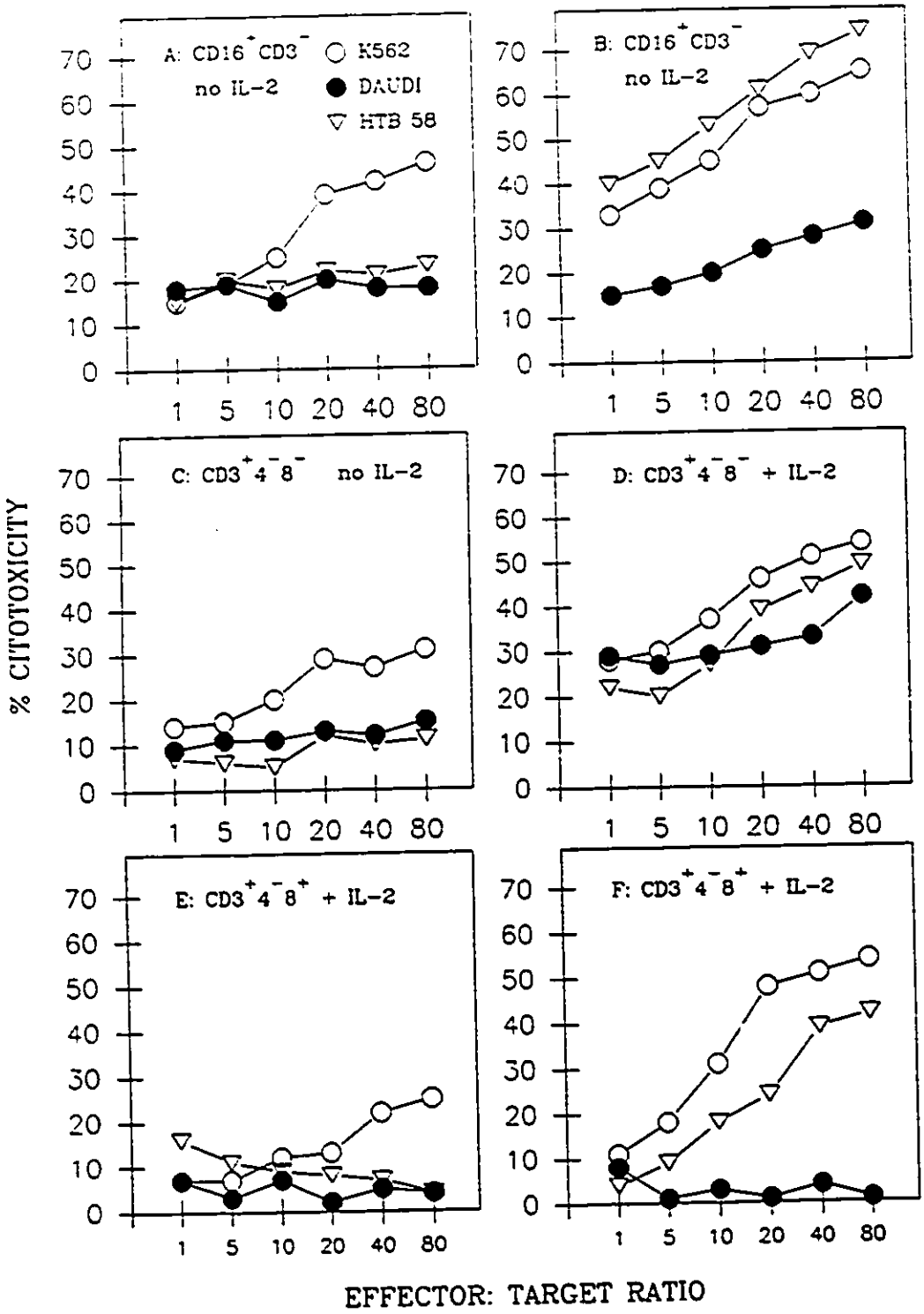
figures 3A and 3B CD3-16+ subset

figures 3C and 3D CD3+4-8- (double negative T cells)

figures 3E and 3F CD3+4-8+ subset

In some experiments (A,C,E) IL-2 was removed from the culture of effector cells 48 hours before the LAK assay.

The figures show that IL-2 increased the cytotoxicity against NK and LAK sensitive cell lines (K562, HTB58) but not against the LAK resistant cell line (DAUDI). The increase in cytotoxic activity is not restricted to classical LAK cells (CD3-16+) but also is present in double negative T cells and CD8+ T cells


A: CD16+CD3-
no IL-2
K562
DAUDI
HTB 58
B: CD16+CD3-
no IL-2
C: CD3+4-8-
no IL-2
D: CD3+4-8-
+ IL-2
E: CD3+4-8+
+ IL-2
F: CD3+4-8+
+ IL-2
% CITOTOXICITY
0
10
20
30
40
50
60
70
1
5
10
20
40
80
EFFECTOR: TARGET RATIO

V. DISCUSSION

Lymphokine Activated Killer cells were first described by Rosenberg et al (37) as cells that developed spontaneous killer activity against target cells that were previously resistant to NK cells. LAK cells were generated after the inclusion of Interleukin-2 in the culture medium. This *in vitro* phenomenon acquired potential clinical significance when the same group of investigators showed that transfusion of *in vitro* generated LAK to mice carrying experimental tumors induced remission of the tumors in a significant number of cases. Such observations have led to testing human LAK as a way to treat human cancer (5).

The origin of LAK cells remained obscured for some years but recently it was established that NK cells are the precursors of LAK cells (39). However, recent data from several laboratories suggested strongly that the LAK phenomenon is not an exclusive property of NK cells and it can also be found among activated T cells. It was not very clear which of the T cell subset had this property or if the LAK activity was the result of long term cultures of cloned T lymphocytes. It is of special interest that a novel T cell subset, the so-called double-negative T cells ($CD3^+4\text{-}8\text{-}$), now known to be a subset of $\gamma\delta$-T cells, have significant non-MHC restricted cytotoxicity.

In our project the goal was to establish if activated, polyclonal cultures of purified T cell subsets have LAK activity. We first developed methods for isolation of T cell subsets. Such subsets were maintained in culture and then tested for LAK activity.

CELL SEPARATION METHODS

In the clinical research laboratory NK cell activity is usually measured on mononuclear cells previously depleted of adherent cells (monocytes) by passing through a nylon wool (43) or Sephadex G-10 columns (44). The purpose is to avoid non-specific killing mediated by monocytes. However, recent data indicate that the presence of monocytes may not significantly add to the NK cell activity of the sample (45). In normal fresh samples, the contribution by T cells is also minimal; however, when activated T cells are used non-specific killing activity may be observed (26). The contribution of $\gamma\delta$-T cells to the total NK cell activity has not been determined but it is likely to be small. Our first objective was to evaluate various cell separation procedures and to confirm that activated T cells do have IL-2 induced non-specific cytotoxic activity and that $\gamma\delta$-T cells (double-negative or CD3+4-8- cells) have NK cell activity. Thus our main objectives were to evaluate and/or develop cell separation techniques that would permit the measurement of NK/LAK cell activity by various lymphocyte subsets.

In our initial experiments we used common separation procedures such as Sephadex G-10 columns, rosetting with sheep erythrocytes and panning with monoclonal antibodies. In our hands these methods alone and in combination did not yield pure populations of cells for studies of NK/LAK function. Although the panning method has been used by many investigators, in our hands it was not successful, besides it is laborious and time-consuming, and not suitable for processing large numbers of cells. For those reasons we chose not to evaluate and/or improve further the panning method. The alternative technology to separate cells available to us was the Fluorescence Activated Cell Sorter (FACS

440). However such a method is also time-consuming and not easy to implement unless the Flow Cytometry lab has extensive and continuous experience with cell sorting, and such conditions were not met by the Flow Cytometry lab at the University.

In general, the present technology for cell separation has reached its limits, however there is a demand for a simpler cell separation technology. The most common methods for cell separation have so far been the combination of gradient centrifugation (46) combined with panning techniques (47), complement-mediated cell lysis with monoclonal antibodies (48), killing of specific cell subsets using immunotoxins (48), cell depletion after sheep erythrocyte rosetting (49) or rosetting with ox red cells covered with antibodies (50).

These techniques, in many variations, have been applied by investigators in studies that required highly purified cell subpopulations. New cell separation tools have to meet the following requirements: they must be reliable, fast, easy to handle, cost effective and adaptable to several applications. Magnetic particles bound to antibodies is a method tha: met such requirements. In our project we used an indirect approach to cell labelling: first we labelled the cells with a monoclonal antibody, and secondly the magnetic beads coated with an anti-mouse IgG antibody was added. It is not possible to direct label cells with monoclonal antibody-coated magnetic beads. In conclusion, the results that we presented indicate that the antibody-coated magnetic beads are a simple and efficient method of cell separation. In our experiments they were used to separate NK cells and T cell subsets (CD3+4+8- and CD3+4-8+).

DOUBLE NEGATIVE T LYMPHOCYTES

The expression of CD4 and CD8 has been used to follow the differentiation pathway of thymocytes and blood lymphocytes. In the thymus most cells coexpress CD4 and CD8 while mature thymocytes segregate into subsets expressing CD4 or CD8. A small subset of thymocytes lacking both CD4 and CD8 was reported and thought to be precursors of cells that will differentiate into CD4 and CD8 positive thymocytes (24). CD3+4-8- thymocytes were found to be responsive to IL-2 and to have cytolytic activity (24) perhaps in contradiction with their precursor role. In a subsequent report Lanier et al described a small subset of human blood T cells that express neither CD4 nor CD8 (double negative T cells) (51). In our study of more than 20 normal donors, such populations comprised about 3% of blood lymphocytes. Morphological analysis of purified double negative T cells showed that they were large lymphoblasts with abundant cytoplasm containing azurophilic granules. Thus, morphologically the double negative T cells are similar to the large granular lymphocytes originally described as NK cells and phenotypically they are a subset of LGLs different from NK cells. The double negative cells isolated by Lanier et al (51) were also cytotoxic in response to IL-2, capable of killing NK-sensitive and NK-insensitive tumour target cells without MHC restriction.

Recently it has been recognized that double negative T cells belong to a subpopulation of T cells that carry the recently-recognized gamma-delta T cell antigen Receptor ($TCR_{\gamma\delta}$) (52). Double negative T cells constitute about 60% of $TCR_{\gamma\delta}$ T cells. The function of $TCR_{\gamma\delta}$ T cells still remains to be determined (58).

The work reported in this thesis was done before the discovery of $TCR_{\gamma\delta}$ T cells. Our objective was to confirm and extend the observation of Lanier et al (51) and also to establish if CD4 and CD8 subsets had a significant non-MHC-restricted cytotoxic activity. Our results confirm that double negative T cells have non-MHC-

restricted cytotoxic activity against NK-sensitive and insensitive target cells. However, CD4+ and CD8+ T cell clones also expressed non-MHC-restricted cytotoxic activity. In conclusion, it appears that non-MHC restricted cytolytic activity is a property shared not only by NK and LAK cells but also by all T cells once they have been activated and induced with IL-2. The significance of such a function under normal physiological and in pathological conditions is unknown. The functional role of $\gamma\delta$-T cells is also not understood as such cells are clearly able to kill target cells without MHC restriction.

THE EFFECT OF IL-4 ON IL-2 INDUCED PROLIFERATIVE ACTIVITY

Although initial reports suggested that IL-4 enhanced the proliferation activity of IL-2 (42) as well as enhanced their cytolytic function (42) we found that IL-4 inhibits significantly IL-2 induced cell proliferation. Our results have been confirmed by several recently published reports (54,55,56). Nagler et al (56) reported that recombinant IL-4 effectively blocked IL-2 dependent proliferation of NK cells. IL-4 does not have an effect on the cytotoxic activity of resting NK cells but IL-4 is capable of inhibiting in a dose-dependent manner the IL-2 induced LAK activity against NK cell-resistant tumour cell lines (54,56). IL-4 appears to act on NK cell precursors but not on already committed killer cells (54). No change in IL-2 receptor expression was observed (54). Although IL-4 prevents the activation of LAK cell precursors, it does not inhibit the generation of antigen-specific cytotoxic T cells in mixed leukocyte cultures (54).

Our results together with results from several other laboratories suggest that the therapeutic effect of IL-2 treated lymphocytes may not be due to the generation of LAK cells from NK cells but it may be due to the expansion of cytotoxic T cells with or without tumor specific activity (reviewed in 57). This hypothesis is supported

by the observation that tumor infiltrating lymphocytes are more effective anti-cancer agents than peripheral blood lymphocytes (or splenocytes in mice) in the same patient. The majority of cells in tumor infiltrating lymphocytes cultures are T cells rather than NK cells. This represents a conceptual change with increased focus on T cells as better tools for this type of immunotherapy. Whether the LAK activity of T cells or some as yet undefined tumor specific killing activity is responsible for the antitumor effect remains to be determined.

FIRST GENERATION OF ADOPTIVE IMMUNOTHERAPY

The use of LAK and IL-2 as a form of adoptive immunotherapy for the treatment of cancer was originally proposed by Rosenberg (5). It has had limited success although in some cases the results were encouraging such as in malignant melanoma and renal cell carcinoma (57). The variable clinical response to IL-2 and LAK cells is perplexing but perhaps not surprising because we still do not know the mechanism of action of both IL-2 and LAK cells (57). In patients with the same type of tumor and treated in similar clinical conditions, the tumor burden will decrease in only some patients after adoptive immunotherapy.

An additional problem for this type of therapy is severe toxicity (58). The variable clinical response has not permitted the definition of which features of cancer patients may permit prediction of the therapeutic outcome (57). The most common observations during therapy are IL-2 dependent lymphocytosis and increased *in vivo* LAK cell activity (57). However, increased LAK activity has been associated with good response in one (59) but not in other studies (60,61).

Our results, together with that of other investigators (39,57) have established that LAK activity is neither generated from a unique precursor nor mediated by a unique effector. Although NK cells are the major subpopulation to respond to IL-2

in vitro and become LAK cells, it is possible that a small percentage of activated T cells might also be expanded and thus contribute to the activity of LAK cells. Thus, the first generation of adoptive immunotherapy has proven to be effective in a minority of patients with cancer and in an unpredictable fashion.

SECOND GENERATION OF ADOPTIVE IMMUNOTHERAPY

Phenotypic analysis of tumor-infiltrating lymphocytes (TILs) has revealed the presence of CD3+ T cells but not CD3-16+ NK cells. This suggests that lymphocyte traffic to the tumor site consists essentially of T cells. In a comparative study of the ability of radiolabelled TILs and LAKs to home to the tumor sites, it was found that TILs localized to the tumor site in 4 of 7 cases and that LAKs went to the tumor site in only 1 of 7 cases (62). Thus TILs are better that LAKs in targeting of the tumor mass. It also has been shown that cytolytic T cells may coexist with LAK cells and show a more restricted capacity to lyse tumor cells (63). Studies in mice also suggest that the antimetastatic effect of LAK cells was due to the activity of cytolytic T cells reacting against the tumor (63).

Thus, it appears that in TILs the major subpopulation responsible for the anti-tumor activity is cytotoxic T lymphocytes perhaps reacting against tumor-restricted antigens. In consequence, the cellular basis for a successful adoptive immunotherapy of cancer may be TILs because they are tumor-specific and are able to traffic to the tumor site and target to the metastatic lesions. If the hypothesis is correct, then there is a very important corollary. The ability of a particular tumor to be sensitive to adoptive immunotherapy will be dependent o the ability of the tumor to present antigen. Thus, the surface antigenic density of class I MHC antigens will be an important property of tumors. Equally important will be that the tumor may be able to process self-antigens that are not recognized as such by cytotoxic T cells.

Perhaps such antigens are fetal antigens for which the tolerization that T cells learn in the thymus has not happened. Identification of such antigens will not be easy because they are the results of antigen processing by proteolysis and consist of peptides of less than 20 amino acids. Anti-melanoma T cell responses have been found to be mediated by T cells and are restricted by MHC (63).

Both hypotheses predict that a clinical response occurs only in those patients whose tumor burden expresses sufficient amounts of class I MHC antigents as well as tumor restricted antigens (short peptides) that are not recognized as self antigens. Thus, measurement of class I MHC antigens in the tumor burden as well as the *in vitro* assay of TILs to kill autologous tumor cells in an MHC-restricted fashion may predict the outcome of TIL adoptive immunotherapy.

THIRD GENERATION OF ADOPTIVE IMMUNOTHERAPY

The infusion of Tumor Necrosis Factor to mice with cancer leads to rapid shrinking ot tumors. Preliminary experiments in humans have been unsuccessful due to the excessive toxicity of TNF when given intravenously. To avoid the systemic effects of TNF and to achieve larger doses of TNF in the tumor, Rosenberg has proposed to package the gene for TNF inside TILs that can target to the tumor cells. The cloned gene for TNF is attached to a retrovirus rendered unable to replicate itself. The engineered virus is used to infect the TILs in culture thus inserting its genes and the TNF gene into the TILs own DNA. Approval for such clinical trials has recently been granted. Such trials will determine if TILs can be used to target therapeutic agents.

In conclusion, it appears that TILs and IL-2 are a far better combination than LAKs and IL-2 in the treatment of cancer. The umpredictability of the clinical response may be due to the fact that the cellular mechanism underlying a clinical response is the ability of the tumor to present antigen in an MHC-restricted fashion. Tumors that do not express surface class I MHC antigen and/or do not have tumor-restricted antigens will not be susceptible to adoptive immunotherapy.

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