INVOLVEMENT OF TRANSFERRIN RECEPTORS IN HUMAN NATURAL KILLER CELL SPECIFICITY

by

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Ph.D. thesis by Alan Lazarus
short title;

TRANSFERRIN RECEPTORS IN HUMAN

NATURAL KILLER CELL SPECIFICITY

Dedicated to my mother and father.

ABSTRACT

Ph.D Alan H. Lazarus Microbiology & Immunology

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Natural Killer (NK) cells are able to recognize and lyse tumor cells without prior immunization or sensitization. The initial events leading to target cell lysis by NK cells involves a poorly defined recognition and binding phase. It has been hypothesized however that human NK cells may recognize transferrin receptors as target structures on tumor cells.

To determine the possible involvement of transferrin receptors in human NK cell specificity, a correlation study between transferrin receptor expression and competitive activity for NK cell mediated lysis was undertaken. We have determined that the level of transferrin receptors expressed by different populations of K562 cells correlated well with their level of competitive activity for NK cell mediated lysis.

To investigate if these transferrin receptors could be recognized and bound by NK cells, a solid phase receptor binding assay was developed. As a model system, it was demonstrated that nitrocellulose immobilized transferrin retained its specific functional receptor binding capacity. This technique was quantitative and

proved to be sufficiently sensitive to specifically detect nanogram quantities of transferrin receptor protein. Binding was assessed using an ELISA based system.

Human PBL were fractionated by discontinous Percoll density centrifugation, bound to nitrocellulose, and evaluated for transferrin receptor binding capacity. A sample aliquot of cells from each Percoll fraction was retained to assess NK cell activity. It was observed that there was no positive relationship between NK cell activity and transferrin receptor binding capacity in these Percoll fractionated cells.

These findings indicate that while transferrin receptors may be involved in human NK cell specificity, they do not support a role for transferrin receptors in a high affinity mechanism between NK cells and tumor target cells.

RESUME

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LE ROLE DU RECEPTEUR DE LA TRANSFERRINE POUR LA SPECIFICTE DES CELLULES "NATURAL KILLER"

Les cellules "Natural Killer" (NK) sont capables de tuer certaines cellules cancéreuses en l'absence d' anticorps et en l'absence d' immunization préalable. La destruction de la cellule cible par les cellules NK implique une phase initiale de reconnaissance et d' interactions specifiques dont le mecanisme d'action demeure énigmatique. Une hypothése actual suggère que la structure reconnue par le cellule NK serait le récepteur de la transférrine qui se trouve présent à la surface des cellules cancéreuses.

Dans un premier temps, j'ai entrepris d'examiner la relation qui existe entre l'expression du recepteur de la transférrine et l'activité cytotoxique des cellules NK. Cette étude, utilisant diverses populations de la cellule cible K562, a démontrée que l'activité NK est préférentiellement dirigé vers les cellules cancéreuses ayant le plus grand nombre du récepteur de la transférrine.

Pour faire suite a cette étude, j'ai analysé d'une facon plus directe l'interaction du récepteur da la transférrine avec les cellules NK humaines. Pour accompli

ceci, une technique en phase solid permettant de détecter l'interaction du récepteur de la tranférrine avec la transférrine a été developpé. Cette methode, ou la transférrine se trouve fixée sur une membrane de nitrocellulose, permet de quantifier jusqu'au nanogramme le récepteur de la transférrine. Cependant lorsque les cellules NK sont utilisées comme source du ligand, aucune interaction avec le récepteur da la transférrine est observée.

Ces resultats indiquent qu'il est peux probables que le récepteur de la transférrine soit impliqué dans une interaction à haute affinitée entre la cellule NK et la cellule cancéreuse cible. Il n'est cependant pas exclus que le récepteur de la transférrine joue un rôle dans la selection des cellules qui sont sensible à l'action des cellules NK.

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CLAIM OF CONTRIBUTION TO KNOWLEDGE

1. Development of a rapid and efficient microtechnique for the analysis of functional transferrin receptors on tumor cells.
2. Demonstration that K562 cells subjected to trypsin or heat pretreatment lose the ability to bind transferrin.
3. Demonstration that the growth phase of K562 cells correlates with their expression of transferrin receptors.
4. Demonstration that K562 cell transferrin receptors could be modulated by several means and that the level of expression of transferrin receptors on these cells correlated with their competitive activity against control K562 cells for NK cells.
5. That transferrin receptor expression in genetically different cell lines does not correlate with competitive activity against control K562 cells for NK cells.
6. Demonstrated that a subpopulation of NK cells possess a transferrin like epitope on their plasma membrane.
7. That K562 cell lysates could be frozen, thawed, and sonicated and retain the ability to specifically compete with parent K562 cells for NK cells.
8. That these lysates retained immunologically and functionally detectable levels of transferrin receptors.

9. That transferrin can be immobilized onto nitrocellulose and retain specific receptor binding ability as demonstrated by a novel immunoblotting technique.

10. That there is no relation between NK cell activity and apparent Tf receptor binding capacity in percoll fractionated PBL.

MANUSCRIPTS AND AUTHORSHIPS

This thesis encompasses the text from 2 published manuscripts and 2 other manuscripts in preparation. All of the work presented here was independently conducted by the author under the supervision of Dr. Malcolm G. Baines with the exception of the reticulocyte studies and electron microscopy studies which were performed in collaboration with Mr. Kevin Glasgow and Ms. Anna Camparina respectively.

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LIST OF ABBREVIATIONS

ADCC	Antibody dependent cell-mediated cytotoxicity
aGM_1	Ganglio-N-tetraosylceramide
ALK	Activated Lymphocyte Killer
AK	Activated (Anomolous) Killer
anti-Tf	Polyclonal antisera raised against human serum transferrin
Apo-Tf	Iron free human serum transferrin
B-Tf	Bovine serum transferrin
BSA	Bovine serum albumin
CMV	Cytomegalovirus
cpm	Counts per minute
CTL	Cytotoxic T Lymphocyte
DAG	Diacyl glycerol
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbant assay
E/T	Effector to target cell ratio
FACS	Flourescence activated cell sorter
FcR2	Plasma membrane receptor for aggregated IgG
GVHD	Graft versus host disease
HSA	Human serum albumin
H-Tf	Human serum transferrin
IAK	Interferon Activated Killer
IAK	Interleukin Activated Killer
IFN	Interferon
IL-2	Interleukin 2

IP_3	Inositol triphosphate
KCIL	Killer cell independent lysis
LAK	LymphoKine Activated Killer
LGL	Large Granular Lymphocyte
Lt	Lymphotoxin
MHC	Major histocompatibility complex
MLC	Mixed lymphocyte culture
NA	not available
NC	Naturally Cytotoxic
NK	Natural Killer
NKCF	Natural killer cell cytotoxic factor
NS	Natural Suppressor
PBS	Phosphate buffered saline
PHA	Phytohemagglutinin
PIP_2	phophitidyl inositol diphosphate
$_r$IL-2	recombinant interleukin 2
S.E.M.	standard error of the mean
sIg	surface membrane immunoglobulin
SSEA-1	Stage specific embryonic antigen-1
TBS	Tris buffered saline
Tf	transferrin
Ti	T cell receptor
TNF	Tumor necrosis factor
TPA	12-O-tetradecanoylphorbol-13-acetate
TTBS	TBS with Ø.5% tween 2Ø

I. LITERATURE REVIEW

Natural Killer (NK) cells were first discovered about 15 years ago when it was observed that lymphocytes from unprimed normal individuals had lytic activity against some tumor cells [reviewed in Herberman and Holden, 1978]. Since their initial discovery, NK cells have been shown to exist in a variety of species including mice, rats, pigs and humans. They are found in the peripheral blood, spleen, liver, lymph nodes, lungs, intestine, thymus and in almost all sites of inflammation. They are able to lyse a variety of transformed as well as non-transformed cells without prior immunization or sensitization. Thus, these cells represent a population distinct from B cells, T cells, and myeloid type cells.

1. NK cell function

1.1 **Control of tumor growth.** NK cells have been implicated as a primary line of defence against neoplastic disease. This is based on several lines of evidence as briefly outlined below. First, NK cells have been shown to be able to lyse tumor cells in vitro. Second, other studies have demonstrated that nude mice, which are deficient in T cell dependent responses but display normal levels of NK activity, developed 3-methylcholanthrene

induced tumors with a very low incidence (Stutman, 1974). This implies that NK cells were the major effector mechanism inhibiting the tumor growth. Nude mice have also been shown to inhibit the in vivo growth of other types of tumors, including the B16 melanoma cell line (Giovanella et al, 1974). A third source of evidence comes from studies involving mice with the beige mutation. These mice are selectively deficient in NK cell activity, while their T cell, B cell and myeloid functions are normal (Roder and Duwe, 1979). Tumor cells injected into these mice display an increased growth rate, faster induction time and increased metastatic spread when compared to those injected into control mice (Talmadge et al, 1980; Karre et al, 1980). Fourth, humans suffering from a genetic disease known as Chediak Higashi syndrome, have depressed NK function (Roder et al ,1980; Katz et al, 1982). These individuals also have an increase in malignancies associated with this disease (Dent et al, 1966). Fifth, NK cells have been shown to inhibit tumor metastases (Warner and Dennert, 1982; Hersey et al, 1982). Sixth, IL-2, which augments NK cell activity (Grimm et al, 1982; Lotzova et al, 1987), has recently been shown to produce a partial remission of liver and lung metastases (Laferniere et al, 1985; Mazunder et al, 1984) and intraperitonial cancer (Ottow et al, 1987) as well as some late cancer in humans (West et al, 1987; Rosenberg et al, 1987). The preceeding evidence suggests that NK cells are important as a primary line of defence against

neoplastic growth.

1.2 Control of viral infection. NK cells have also been implicated as effectors against viral infection [reviewed in Welsh, 1986]. These cells have been shown to bind and lyse some virally infected cells, but not their uninfected counterparts. This phenomenon is not restricted to infection by only one type of virus. The list of viruses which cause cells to become susceptible to NK cell mediated lysis includes murine cytomegalovirus (CMV) (Lee and Keller, 1982), human CMV (Diamond et al, 1977), mumps virus (Harfast et al, 1975), sendai virus (Weston et al, 1981), vesicular stomatitis virus (Moller et al, 1985), and herpes simplex virus. (Munoz et al, 1983; Ching and Lopez 1979; Fitzgerald et al, 1982; Fitzgerald et al, 1983). In the CMV system, additional evidence to support the contribution of NK cells in anti-viral defence includes the following: 1) Beige (NK deficient) mice are very susceptible to CMV infection (Shellam et al, 1981); 2) in vivo depletion of NK cells with antibodies to the glycolipid antigen aGM-1 (found on virtually all murine NK cells) renders mice more susceptible (500 fold higher virus titer) to CMV infection (Bukowski et al, 1983); 3) It has been shown through experiments involving the adoptive transfer of nonimmune adult lymphocytes into susceptible five day old mice that the cell population which conferred protection was composed "phenotypically" of NK cells (Bukowski et al,

1985). In addition, interferon (IFN) which has demonstrated antiviral activity (Issacs and Lindenma, 1957) and is produced early during viral infection, is a potent inducer of NK cell activity (Targan and Stebbing, 1982)(Schmidt et al, 1987; Ochoa et al, 1987; Testi et al, 1986; Marini et al,1986). Thus IFN activation of NK cells may be an important secondary antiviral action of this protein.

1.3 Regulation of differentation and immune function.

NK cells are also thought to be capable of regulating immune function. They have been shown to be lytically active against hematopoietic stem cells (Hansson et al, 1982; Holmberg et al, 1984) thymic and fetal thymic cells (Nunn et al, 1977; Hansson et al, 1981) and bone marrow cells (Hansson et al, 1981). They can also suppress the generation of cytotoxic T lymphocytes (CTL) (Gilbertson et al, 1986), possibly by their action on accessory cells (Abruzzo and Rowely, 1983; Shah et al, 1985). It has also been postulated that NK cells may be the effector cells in the hybrid resistance to bone marrow grafts (Okumura et al, 1982; Lotzova et al, 1982; Kumar et al, 1979). Recent evidence also indicates that they are one of the pathogenic effector cells in graft versus host disease (GVHD) (McGuinness and Baines, 1988). In addition, they are phenotypically very closely related to natural suppressor (NS) cells which as the name implies are capable of suppressing in vitro immune responses in an MHC and antigen independant manner [reviewed in Strober,

1984]. These NS cells do not however possess any lytic potential (even though they possess granules and the general morphology of NK cells or LGL's) and therefore their relationship to NK cells or to an NK lineage is unknown.

NK cells have also been shown to mediate other functions not associated with lysis. They are capable of producing various lymphokines (Kasahara et al, 1983) and are able to hydrolyse serum amyloid A protein (Zucker-Franklin, 1984). Thus NK cells may regulate the immune system in a variety of different ways.

1.4 Control of microbial growth. There is a limited amount of information concerning the role of NK cells in the control microbial disease. The growth of the yeast-like organism Cryptococcus Neoformans has been shown to be inhibited in vitro by NK cells (Hidore and Murphy, 1986). There is also some evidence that in vivo clearance of C. Neoformans and Paracoccidioides Brasiliensis can be mediated by NK cells (Hidore and Murphy, 1986; Jimenez and Murphy, 1984). A recent report using the organism Candida Albicans showed that this organism could block lysis of K562 cells but that NK cells could not directly lyse this organism despite exhaustive attempts by the authors (Zunino and Hudig, 1986). Thus the importance of NK cells in the control of yeast infections requires further investigation. While there are some indications that NK cells may exhibit a negative effect on bacterial growth,

the evidence suggesting this is indirect (Skamene et al, 1982; Cheers and Macgeorge, 1982).

2. Mechanism of killing

The mechanism through which an NK cell kills a target cell is complex and involves several well defined stages [reviewed in Herberman et al, 1986; Henkart, 1985; Trinchieri and Perussia, 1984; Roder and Pross, 1982]. The current model of NK cell "cytotoxicity" involves the secretion of a lytic factor by the NK cell which, in turn, binds and lyses the target cell. This model is supported by early studies which showed that the lysis of tumor cells by NK cells could be blocked by agents that inhibited secretion (Carpen et al, 1981; Roder et al, 1978; Roder et al, 1979; Weissmann et al, 1975). In addition, pretreatment of NK cells with Sr^{++}, which causes premature degranulation, inhibits the subsequent lysis of tumor cells (Neighbour and Herberman, 1982). It was also observed that, upon incubation of NK cells with tumor cells, the Golgi apparatus of the NK cell (which also plays an important role in secretion) polarizes toward the area of contact between the NK cell and its target, presumably to focus secretory function on the target cell. (Carpen etal, 1982).

Since these earlier findings, much information has been obtained on the mechanism of NK cell mediated lysis

and the lytic process has been arbitrarily broken down into 5 stages [reviewed in Herberman, 1986]:

1) binding of NK cell to target cell
2) Activation of NK cell by target cell
3) release of lytic factor(s)
4) binding of lytic factor(s) to target cell
5) target cell lysis.

2.1 Binding (stage I). The initial event leading to target cell lysis involves a specific Mg^{++} dependent binding or contact between the effector lymphocyte and the target cell. The binding may be via antibody bound to the target cell, in which case the interaction between effector and target is termed antibody dependent cell-mediated cytotoxicity (ADCC). The recognition of the target cell can also occur in the absence of specific antibody through the direct recognition of the target cell membrane by the NK cell. Both methods of recognition appear to be equivalent (ie. necessary but not sufficient) from the viewpoint of lytic activity. Elucidation of the manner in which an NK cell recognizes its target forms the basis of this thesis and will therefore be discussed in greater detail later.

2.2 NK cell activation (stage II). The events occurring at this stage are very poorly understood. While signal transduction must occur across the membrane of the NK cell in order to activate effector function, the nature of

this signal is still unknown.

2.2.1 Early steps: Although a sequence of events has not been formally proposed for the activation of NK cells, it is thought to be similar to that seen in T lymphocytes. Signal transduction in T cells involves the following steps [reviewed in Isakov et al, 1987; Linch et al, 1987; Gelfand et al, 1987]. Upon interaction of the T cell with "major histocompatibility complex (MHC) + antigen", the CD-3 molecule which is juxtaposed to the T cell receptor (TcR) activates phospholipase C (possibly via a G protein). Phospholipase C then hydrolyses phosphatidyl inositol diphosphate (PIP_2), yielding two products; diacyl glycerol (DAG) and inositol triphosphate (IP_3). IP_3 is released into the cytoplasm where it mobilizes Ca^{++} from the endoplasmic reticulum (within 1 min). DAG along with Ca^{++} now activates protein kinase C which is translocated to the plasma membrane (approximately 10 min after activation). Protein kinase C has then been shown to phosphorylate the Na^{++}/H^{+} exchanger which is thought to cause an increase in intracellular pH.

This sequence of events is still somewhat hypothetical and how the T cell distinguishes between a signal for growth and killing is not clear.

The general mechanism for signal transduction in T cells is illustrated because, in the author's opinion, there is very good preliminary evidence to suggest that that NK cells utilize a mechanism very similiar to that of T cells.

Bonavida's group has shown that a Ca^{++} influx (induced by the Ca^{++} ionophore A18237) along with direct activation of protein kinase C (via the phorbol ester TPA) was sufficient to induce natural killer cell cytotoxic factor (NKCF, a lytic product secreted by NK cells) release (Graves et al, 1986). Jondal and coworkers (1987) have also shown that the same treatment can induce NK cells to be nonselectively cytotoxic for tumor target cells. Similar results have been reported for cytotoxic T lymphocytes (CTL)(Berrebi et al, 1987; Lancki et al, 1987). In addition, Seaman et al (1987) using a rat cell line with NK cell activity have demonstrated that incubation of these cells with the NK sensitive YAC-1 cell line, but not with the NK insensitive EL-4 cell line, results in an increase in IP_3 (a major mediator of T cell signal transduction). In addition to the above mentioned similarites to T cell mediated signal transduction, NK cells may utilize a phospholipid methylation induced cyclic nucleotide balance as a means of signal transduction (Zimmerman et al, 1978; Hoffman et al, 1981; Kiyohara et al, 1985; Hirata and Axelrod 1980; Roder et al, 1979; Katz et al, 1982; Katz et al, 1982b).

2.2.2 Late steps. Immediately following signal transduction, it has been observed by electron microscopy that the microtuble organising center and the Golgi apparatus position themselves between the nucleus of the NK cell and the area of membrane contact between the NK

cell and the target cell (Carpen et al, 1982; Kupfer et al, 1983; Saksela et al, 1982).

2.3 Release of lytic factor(s) (stage III). The granules of NK cells as well as other cytotoxic lymphocytes contain cytolytic factors (Frey et al, 1982; Hacket et al, 1982; Kay and Zarling 1984; Henkart et al, 1984; Walhin and Perlman, 1983). These granules contain acid phosphatase, inorganic trimetaphosphatase, uronic acid, hexose, proteoglycans, and arylsulfatase as well as several other proteins. They do not possess protease, peroxidase, or other common lysozomal enzymes (Grossi et al, 1982; Frey et al, 1982; Millard et al, 1984; Schmidt et al, 1985; Zucker-Franklin et al, 1983). Agents that inhibit the release of granules also inhibit NK cell activity (Katz et al, 1982; Weissmann et al, 1975).

Several granule-derived factors have been implicated in mediating the lytic event by NK cells. The interrelationship of these factors is currently unknown and they are therefore discussed independently.

2.3.1 Perforin. The granules of LGL have been purified and shown to contain a pore forming protein called perforin or cytolysin. This protein can cause membrane lesions in target cells 1-16 nm in diameter (Young et al, 1986; Henkart et al, 1984) and has been shown to exhibit sequence homology with the ninth component of the complement system (Zalman et al, 1987). It is capable of

lysing target cells quickly and nonspecifically. The pore forming ability of this protein is pH, temperature and Ca^{++} (but not Mg^{++}) dependent (Young et al, 1986; Young et al, 1987). This correlates with NK cell mediated lysis which is also pH, temperature and Ca^{++} dependent at a late stage of the lytic process (Glasgow et al, 1986; Hiserodt et al, 1982). Thus, one possible mechanism of NK cell mediated lysis of tumor cells, is the introduction of large "complement-like" pores in the tumor cell membrane causing colloid osmotic lysis.

2.3.2 Natural killer cell cytotoxic factor (NKCF).

NKCF is produced by LGL upon incubation with target cells and is also postulated to be involved in the lytic event [reviewed in Herberman et al, 1986]. The protein(s) is capable of binding to and lysing tumor cells that are normally sensitive to NK cell mediated lysis but not NK insensitive target cells (Wright et al, 1983; Farram et al, 1983). Thus, it is specific for NK sensitive targets. There is an excellent correlation between agents that inhibit NKCF function (such as phosphorylated sugars, monensin, PGE_2, NH_4Cl...) and those that inhibit NK cell mediated lysis of tumor cells (Ortaldo et al, 1985; Hiserodt et al, 1983). A variant of the cell line Yac-1, where the only observed defect was an inability to bind NKCF, was also found to be resistant to NK cell-mediated lysis (Wright and Bonavida, 1983). A factor discounting the importance of NKCF in the lytic event is that it takes

40 hours to maximally lyse target cells whereas NK cells can lyse a target cell in 30 min and reach a killing plateau in about 4-6 hours. This sharp contrast can be rationalised by arguing that an NK cell deposits its lytic factor(s) in the small area of contact between itself and its target, whereas NKCF measured in vitro is diluted in the tissue culture well and is therefore not "focused" toward the target cell.

2.3.3 Lymphotoxin (Lt). This lytic factor was originally shown to be produced by splenocytes stimulated with phytohemagglutinin (PHA) (Granger et al, 1978). It has since been shown to possess lytic activity against the NK sensitive cell line K562 (Weitzen et al, 1983) although its spectrum of lytic activity appears to be much broader than that of NK cells (Peters et al, 1986; Wright and Bonavida, 1987). It has been postulated that Lt is produced by NK cells based on the ability of antibodies to Lt to inhibit NK cell-mediated lysis (Weitzen et al, 1983). However, in another study, purified populations of human LGL were not demonstrated to produce Lt (Peters et al, 1986). This same study indicated that the LGL population did however produce tumor necrosis factor (TNF), a related lytic protein produced by myeloid cells. TNF is produced by naturally cytotoxic cells (NC) in mice (Ortaldo et al, 1986; Jadus et al, 1986) and a human counterpart for NC cell activity has been suggested (Rola-Pleszczynski and Lieu, 1983). Thus it is possible that it is the "NC" cells that are producing TNF in the study by

Peters and co-workers (1986) rather than true NK cells since NC activity is also found in NK cell populations [reviewed in Hacket et al, 1986]. There is currently no other evidence to indicate that NK cells produce TNF.

2.3.4 Which lytic factor do NK cells utilize? The roles played by NKCF, Lt, and perforin/cytolysin in the lytic process are not necessarily mutually exclusive. Lt has a 30% sequence homology with TNF_{α}. In addition, TNF_{β}, Lt, and NKCF all have roughly similiar kinetics of action and Bonavida now suggests that NKCF shares some similarities with TNF (Wright and Bonavida, 1987).

It appears that perforin and cytolysin may be the same molecule [Marx, 1986] and that these molecules are responsible for introducing pores in the target cell membrane. These "pores" once formed may serve two functions: 1) to lyse the cell by colloid osmotic lysis 2) to allow access of other granule contents into the target cell.

In speculation, the other factors, NKCF and Lt, may then be released into the target cell cytoplasm (through the pores?) and cause their cytopathic effects. This theory is supported by studies demonstrating target cell nucleic acid damage following lysis by either NK or T cells. It was shown that both NK and CTL induce target cell deoxyribonucleic acid (DNA) damage as part of the lytic event (Gromkowski et al, 1986; Russel et al, 1982). The actual transfer of endonucleases by the NK cell to the

target cell does not seem likely since the pattern of nuclease damage is dependant on the target cell and not the effector cell (Gromkowski et al, 1986). Thus the activation of target cell endonucleases by NK cell derived cytolytic factors may be occuring. It remains to be determined if NKCF or Lt can activate endonucleases themselves.

This theory helps to explain why NKCF and Lt require extended periods of time to lyse target cells; purified NKCF or Lt would not have direct access to the target-cell interior. This may also be why in vitro purified NKCF requires endocytosis and energy production (Deem et al, 1986) to effect cytotoxic damage.

2.4 Binding of lytic factors to target cell (stage IV).

In the case of perforin, lesions in the target cell membrane can be visualized when purified perforin is incubated with target cells (Young et al, 1986) as well as when NK cells are incubated with target cells (Podack and Dennert, 1983). In order for lesions to occur, it appears that the perforin protein(s) must go through 2 stages;

1) assembly

2) insertion into membrane.

Ca^{++} is an absolute requirement at this stage of lysis by granule-derived perforin (Young et al, 1986) and there is one report that Ca^{++} is specifically required for pore or tubule formation (Henkart et al, 1984). This study also

demonstrated that pore formation can occur in the absence of target cell membrane indicating that the assembly phase may be independent of membrane insertion.

It has been postulated that NK sensitive tumor cells have specific receptors for NKCF. Although there is no direct evidence in the literature to substantiate this claim, it appears to be a popular theory among those working on NKCF (Wright et al, 1983; Herberman et al,1986; Graves et al, 1986; Hiserodt et al, 1983). The most compelling indirect evidence to support the presence of binding sites has come from a study on NK resistant mutants. The major factor contributing to their resistance appeared to be the inability of these cells to compete for NKCF mediated lysis (Wright et al, 1983). It was therefore assumed that these cells were specifically deficient in NKCF receptors.

Farram and Targan (1983) have reported that NKCF can only be absorbed with NK sensitive cell lines and not with NK resistant cell lines. In addition, glutaraldehyde treatment of target cells removes their ability to be lysed or to compete for NKCF binding in the above assay system (Deem et al, 1984).

It has been suggested that carbohydrates may be involved in NKCF binding to target cells. One early study suggested that the lytic factor may bind to a target cell via mannose-6-phosphate receptors (Forbes et al, 1981). There have been numerous studies on the inhibition of NK

cell activity by carbohydrates (Stutman, 1980; Pohajdak et al, 1986; Kornbluth et al, 1985; Haubeck et al, 1985; Werkmeister et al, 1984; Pohajdak et al, 1984; Kornbluth et al, 1984; Werkmeister et al, 1983; Vose et al, 1983; Nose et al, 1987; Chambers and Oeltmann 1986; Werkmeister et al, 1985; Young et al, 1981). The effect of inhibition of N-linked glycosylation by both genetic means (Haubeck et al, 1985; Pohajdak et al, 1984; Pohajdak et al, 1986) and by tunicamycin treatment (Pohajdack et al, 1986; Kornbluth et al, 1985; Nose et al, 1987) have also been studied. In general, these studies suggest that carbohydrates are likely involved in a late phase of the lytic event, possibly via NKCF binding. Mannose-6-phosphate which has the greatest NK inhibitory activity, and functions after the Ca^{++} dependant stage (Werkmeister et al, 1984) does not appear to be specific for NK cell mediated lysis (Werkmeister et al, 1983) and may not (as originally thought) inhibit by occupying a specific receptor (Haubeck et al, 1985).

Thus while it is highly probable that NKCF "binds" to the target cell to affect lysis, this binding is not necessarily specific for a receptor, and may be a consequence of a nonspecific uptake mechanism (Deem et al, 1986). Perhaps future studies using Scatchard type analysis could provide direct evidence for the involvement of a receptor in NKCF function.

There is little additional specific information available about Lt and therefore a dissection of how this

factor lyses its target cell is not appropriate.

2.5 Target cell lysis (stage V). This stage of the lytic event is very poorly understood, especially since it is not known which of the cytolytic molecules or combination thereof is responsible for target cell lysis. What is known about this stage of lysis is that the target cell can be lysed even if the NK cell is prematurely detached from the target cell (Hiserodt et al, 1984; Hiserodt et al, 1983). This final lytic event is called killer cell independant lysis (KCIL) and occurs after the Ca^{++} dependent phase (Hiserodt et al, 1982) [recall: perforin assembly is dependant upon Ca^{++}]. This stage is sensitive to the effects of papain whereas NKCF activity is not (Hiserodt et al, 1983), suggesting that NKCF alone is not sufficient for lysis.

3. Phenotype

3.1 Morphology. NK cells are large granular lymphocytes (LGL) that have a high cytoplasmic:nuclear ratio, slightly indented nucleus, and roughly six to twelve azurophilic granules in their cytoplasm. Due to this morphology, they are often directly referred to as LGL. Approximately 15-18% of human peripheral blood lymphocytes (PBL) possess LGL morphology, and about 1/3 of these LGL are capable of lysing K562 target cells. The current state of the art

in "medium to large" scale purification of NK cells, in fact, relies on their large size. This method involves density gradient centrifugation on percoll as the last phase of separation of LGL from smaller B and T lymphocytes (Timonen et al, 1982). However, the LGL population purified in this manner is not solely composed of by NK cells. Only between 12 to 30 percent of LGL are capable of binding to and lysing the NK susceptible target cell line K562 (Bradley and Bonavida, 1982; Bonavida et al, 1984; Timonen et al, 1982). Analysis of the LGL population by flourescence activated cell sorting (FACS) analysis using the Leu-19 and NKH-1 antibodies which have been shown to label the entire pool of NK cells (based on subsequent killing of K562) confirm that the purity of percoll separated LGL is only in the range of 32-42 percent (Lanier et al, 1986). Thus the actual purity of "highly enriched" NK cells is not exceptional.

3.2 Surface phenotype. Several proteins have consistently been identified on the surface of LGLs effecting natural killer cell activity. These include IgG FcR2 (Lanier et al, 1986; Lanier et al, 1983; Perussia et al, 1983; Perussia et al, 1983b; Rumpold et al, 1982) and an epitope with unknown function designated NKH-1 (Griffin et al, 1983). NK cells do not express surface immunoglobulin (Jondal and Pross, 1975; Bakacs et al, 1977; Pross et al, 1979) or the T cell receptor (Tutt et al, 1987; Moingeon et al, 1987; Ang et al, 1987; Borst et

al, 1987; Lanier et al, 1986). The possession of other epitopes by NK cells is arbitrary and often depends on the criteria used for the definition of an NK cell. In this thesis we define an NK cell as an immediately ex vivo lymphocyte which can kill the cell line K562 (ie. kill in an MHC unrestricted fashion without prior activation). Likewise, an LGL is a lymphocyte whose final purity is derived from the low buoyant density fraction of a percoll gradient containing the larger, less dense cells.

The surface phenotype of NK cells has been studied by several different methods and each method yields results which must be interpreted seperately.

In order to simplify a discussion on the phenotype of an "NK cell", the relevant studies have been grouped into 3 different catagories. 1) direct studies on NK cells 2) analysis of LGL populations highly enriched in NK cells and 3) studies on cloned (IL-2 driven) NK-like cells.

3.2.1 NK cells. In general, it can be seen from Table I that NK cells comprise a phenotypically heterogenous group of cells. The only markers that have been consistently found on the surface of these cells are the FcR for IgG and the NKH-1/Leu-11 antigen. These 2 surface markers have been assessed by FACS techniques and the results show that the entire pool of NK active cells reside in these positively selected fractions (Griffin et al, 1983; Lanier et al, 1986; Perussia et al, 1983a; Perussia et al, 1983b; Perussia et al, 1984; Lanier et al 1983). The majority of NK cells simultaneously express

Table I.

Phenotypic analysis is based on NK activity (ie. either FACS sorting or antibody depletion studies followed by analysis of NK lytic function.

NA- not available.

MAG- myelin associated glycoprotein

CRP- C-reactive protein

1)Lanier et al, 1983

2)Perussia et al, 1983

3)Griffin et al, 1983

4)West et al, 1977

5)Pross et al, 1977

6)Zarling et al, 1981

7)Ault et al, 1981

8)Beller et al, 1982

9)Abo and Balch, 1981

10)Abo and Balch, 1983

11)McGarry et al, 1983

12)Clayberger et al, 1986

13)Baum et al, 1987

14)James et al, 1981

TABLE I

Phenotype of Human NK Cells in Peripheral Blood

Antigen*	(% Positive)	Old Name	Reference
CD-16	(>95)	FcR2	(1,2)
NKH-1	(>95)		(3)
CD-2	50	E-Rec;T11	(4,5,6)
CR-3	50	Mac-1/OKM-1	(6,7,8)
MAG	(>60)	HNK-1	(9,10,11)
KC-1	(>60)		(12)
CRP	(>50)		(13,14)

* Phenotype analysis is based on NK activity (i.e. either FACS sorting or antibody depletion studies.

HNK-1 MAG (myelin associated glycoprotein) (11)
CRP (C-Reactive Protein)

both the NKH-1 and FcR markers (Lanier et al, 1986) and a further 35 percent of these cells express the CD-8 antigen, commonly found on T (cytotoxic/suppressor) cells (Lanier et al, 1986; Abo et al, 1982; Perussia et al, 1983c).

3.2.2 Percoll-enriched Large Granular Lymphocytes. A significant percentage of LGL (see Table II) have also been shown to express the FcR and NKH-1 antigens. In addition these cells exibit relatively high NK activity (Timonen et al, 1982). With respect to other markers, it can be seen that these cells also comprise a very heterogenous group. Phenotypic analysis of LGL must always be interpreted with extreme caution because, for example, while it has been shown that NK cells do not generally express the CD-3 antigen, (Table I), as many as 47% of percoll-enriched LGL can express this T cell marker (Lanier et al, 1986).

3.2.3 Cloned NK-like cells. There is now a considerable amount of evidence that NK-like cells can be maintained and cloned in IL-2 (Lanier et al, 1985; Allavena and Ortaldo, 1984; Talmadge et al, 1986; London et al, 1986)

Native NK cells were originally described as unstimulated and unprimed lymphocytes that could lyse tumor cells in a short time period without MHC restriction. Upon the discovery that lymphokines could be used to culture and clone PBL with NK-like activity, the definition of an NK cell has become less clear cut.

It is now well documented that PBL incubated in IL-2

Table II

Phenotypic analysis is based on studies with percoll enriched human peripheral blood lymphocytes.

NA- not available

1)Lanier et al, 1986a

2)Lanier et al, 1983

3)Abo and Balch, 1983

4)Ortaldo et al, 1981

TABLE II

Phenotype of Percoll Enriched Human LGL

Antigen	(% Positive)	Old name	Reference
NKH-1	(42)		(1)
CD-16	(32)	FcR	
CD-2	NA	E-Rec/T11	(2)
CR-3	NA	Mac-1/OKM-1	
HNK-1	NA		(3)
CD-8	(21)		(4)
OKT-10	(62)		
LYT-3	(40)		
CR-3	(67)		
aGM-1	(68)		
MHC II	(35)		
TA-1	(88)		
3A1	(59)		
5A12	(50)		

give rise to cells able to lyse tumor cells with a similiar, albeit broader, specificity than endogenous non-activated NK cells [reviewed in Herberman et al, 1987; Hersey and Bolhuis, 1987]. These IL-2 driven lymphocytes are termed lymphokine activated killer (LAK) cells (Grimm et al, 1982; Rosenstein et al, 1984). Cells with NK-like activity have also been referred to as activated lymphocyte killers (ALK), activated killers (AK), anomalous killers (AK), interferon activated killers (IAK) or interleukin activated killers (IAK) (Dalm et al, 1986) [reviewed in Klein, 1982].

NK-like activity has also been derived from cloned antigen-specific CTL (Brooks, 1983). When murine CTL clones that were originally highly antigen-specific and had the phenotype of a mature CTL (i.e. Ig^-, $Thy\text{-}1^+$, $Ly\text{-}2^+$) were incubated in high concentrations of crude IL-2, they gained the ability to lyse NK sensitive but not resistant tumor cells (Brooks, 1983). Thus the ability to lyse NK target cells appears to be attainable by T-lymphocytes.

Studies on the LAK cell precursor have corroborated Brooks finding that "non-NK cells" can give rise to NK-like activity. Percoll fractionation of human PBL, human bone marrow, murine splenic lymphocytes, and murine bone marrow cell populations, has shown that both NK enriched and NK depleted fractions contain LAK precursors (Ballas et al, 1987; Ortaldo et al, 1986; Rustoven et al, 1986;

Merluzzi et al, 1986; Lotzova et al, 1987; Migliorati et al, 1987). In addition, NK-like activity can be elicited by a variety of means (i.e. IL-2, IFN, PHA, and MLC conditions) and the final phenotype mediating the NK-like activity is similiarly heterogenous [reviewed in Hersey and Bolhuis, 1987]. Both NK cells and LAK cells are therefore heterogenous and at present the relationship between these two cell populations is difficult to resolve. The advantage of using cloned NK-like cells is that they can be analysed individually for a variety of markers. The phenotype of some of these clones is illustrated in Table III. It can be observed from this table that these cloned NK-like cells have many characteristics of T lymphocytes. Interestingly however, although the CD-2 antigen is found in a majority of these clones there does not appear to be any single T cell specific marker which is consistently associated with these cloned NK-like cells. It therefore appears that killing of K562 cells probably does not involve any of the T cell markers analysed in table III.

The FcR is not consistently found on all NK-like clones. This is somewhat surprising since virtually all endogenous NK cells express this marker. This finding may indicate that not all NK cells are capable of ADCC, or that the FcR and NK "recognition structure" are expressed independently of each other. The relationship between cells able to mediate NK activity and ADCC was originally unclear. However it now appears from single cell studies

Table III

1)Hercend et al, 1983

2)Schmidt et al, 1985

3)Hercend et al, 1984

4)Pawlec et al, 1982

5)Brenner et al, 1987

6)Moingeon et al, 1987

7)Nowill et al, 1986

8)Moingeon et al, 1986

TABLE III

Phenotype of human (cloned) NK-Like Cells

Clone	CD-2	CD-3	CD-4	CD-8	CD-16	NKH-1	HNK-1	Tf Rec	Tc Rec alpha	Tc Rec beta	Tc Rec gamma	Reference
JT1	-	-	-	-		+						(1,2,3)
JT3	+	-	-	-		+						
JT9	+	-	-	+		+			+	+		
JT10	+	+	-	+		+			+	+	-	
JTB18	+	-	-	-		+						
CNK5	+	-	-	-		+						
JTA17	+	+	-	-		+						
JTA19	+	+		+		+						
C23-10	+	+	-	+			-	+				
C23-29	+	-	+	+			-	+				
C23-1	+	+	-	-			-	+				
C23-2	+	+	-	-			-	+				
C23-24	+	+	+	-			-	+				
12-1			-	+								(4)
12-9			-	+								
29-15	+		+	-								
30-3			-	+								
38-33			+									
38-37			+									
PBL-C1	+	+	-	-					-	-	+	(5)
F6C7	+	+		+		+					+	(6,7,8)

on endogenous NK cells that the same lymphocyte can mediate both events (Bradley and Bonavida, 1982). While not all NK cells necessarily mediate ADCC, it appears that the FcR is required for ADCC (see Table III) and that all of the clones that possess the FcR can kill via an ADCC mechanism.

In summary, NK cells comprise a heterogenous group of LGL which can express a variety of surface markers. At the 1988 International Workshop, it was decided to define NK cells as a population of large granular lymphocytes which possess the CD-16 and NKH-1 surface markers and kill tumor cells in a non-MHC requiring manner. They are distinct from T cells in that they do not express the CD-3 antigen and do not express the alpha, beta, gamma, or delta chain of the T cell receptor.

4. Specificity

NK cells have been shown to recognize and lyse a wide variety of cells, including malignant as well as nonmalignant cells. Initially it was thought that NK cells were nonselective and nonspecific because a wide variety of tumor cells were recognized and lysed by them. In later studies, using monolayer adsorption and competition studies, it was shown that NK cells recognized and lysed some tumor cells while leaving others intact [reviewed in Ortaldo and Herberman, 1982]. Thus the search

for the "NK target structure" developed. Although a great deal of effort has gone into the study of NK specificity, exactly what NK cells recognize in either the murine or human system is still unknown.

4.1 Target cell spectrum. NK cells have been shown to be lytically active against certain virally infected cells (Biron and Welsh, 1982; Stein-Streilein et al, 1983; Habu et al, 1984), hematopoetic stem cells (Hansson et al, 1982; Holmberg et al, 1984) thymic and fetal thymic cells (Nunn et al, 1977; Hansson et al, 1981), bone marrow cells (Hansson et al, 1981), and tumor cells (see chapter III). Tumor cells manifest the highest degree of sensitivity to both recognition and killing by NK cells and for this reason, most of the work on NK cell specificity has been carried out on tumor cells.

Early studies analysed NK specificity by several methods;

1) **Direct cytotoxicity:** NK cells (usually PBL) are incubated with ^{51}Cr-labelled tumor cells, and the extent of tumor cell lysis is assessed by the amount of ^{51}Cr released.

2) **Cold competition analysis:** This assay is similar to the direct cytotoxicity assay with the exception that an unlabelled competitor cell is included in the assay. The decrease in ^{51}Cr released by the labelled target is a measure of the relative concentration of competitive target structure added.

3) **Monolayer adsorption studies:** Tumor cells are immobilized onto a plastic dish, NK cells are allowed to bind to these immobilized cells and then the nonadherent cells are removed. The resulting nonadherent NK cells are then measured for their ability to lyse a panel of tumor target cells. This type of analysis allows one to determine the similarity between different target cells, and to determine if all NK cells share the same specificity.

The results of these studies indicated that:

1) NK cells are capable of recognizing a variety of tumor cells.
2) NK cells are capable of recognizing at least seven specificities.
3) Not all tumor cells contain the same determinant(s) recognized by NK cells.
4) The pattern of specificity demonstrated by NK cells indicated that these cells were polyclonal in nature (ie. all NK cells do not recognize the same determinant).

Other studies also showed that target cell MHC class I and class II molecules were not required for recognition by NK cells (see section 4.6.5 of this chapter). Also, possible in vitro artifacts such as fetal calf serum (Pross et al, 1978), mycoplasma contamination, or C-type virus particles were not related to NK susceptibility

[reviewed in Pross and Baines, 1980].

4.2 Target cell characteristics. It has proven difficult to identify one particular target cell phenotype that consistently correlates with NK susceptibility. While some leukemic cells such as K562 and Molt-4 are particularly sensitive to NK mediated lysis, several other leukemic cells are not recognized or lysed by NK cells. Furthermore K562 and Molt-4 are derived from different cell lineages, K562 being an erythroleukemic cell line while Molt-4 is of T cell lineage. Some nonleukemic tumor cells have also been shown to be susceptible to the effects of NK mediated lysis (Yoo et al, 1982).

NK cells are capable of lysing allogeneic, syngeneic, and xenogeneic tumor cells (Stern et al, 1980; Karre et al, 1982), although fresh in vivo derived tumor cells appear to have the most resistant phenotype. This may be due to in vivo selection pressures [Herberman and Holden, 1978].

The degree and state of differentiation of a tumor target cell also appears to be important. Tumor cells induced to differentiate become somewhat resistant to the effects of NK cell mediated lysis (Gidlund et al, 1981; Werkmeister et al, 1982; Torsteinsdottir et al, 1984) (Chapter III section 4.2). Murine cells transformed with the cellular Harvey ras oncogene (less differentiated) or rat cells transformed with the viral Kirstene ras oncogene become sensitive to NK mediated lysis (Trimble et al,

1986; Johnson et al, 1987). Since a tumor cell represents an aberrant cell from the viewpoint of differentiation, it is not surprising that NK cell recognition and lysis correlates inversely with degree of differentiation of the target.

4.3 Carbohydrate. Several studies have investigated the possibility that carbohydrates may serve as target structures for NK cells. (Stutman, 1980; Pohadjak et al, 1986; Kornbluth et al, 1985; Haubeck et al, 1985; Werkmeister et al, 1984; Pohadjak et al, 1984; Kornbluth et al, 1984; Werkmeister et al, 1983; Vose et al, 1983; Nose et al, 1987; Chambers et al, 1986; Werkmeister et al, 1985; Young et al, 1981), and it is well known that tumor cells display profound differences in plasma membrane carbohydrate antigens [reviewed in Hakomori, 1984]. Based on initial studies that simple sugars could inhibit killing of tumor cells by NK cells, it was suggested that NK cells recognized a carbohydrate structure on the surface of tumor cells (Stutman, 1980). With the exception of one study where it was shown tha N-acetyl-D-glucosamine and beta-gentabiose specifically inhibited NK conjugate formation (Werkmeister et al, 1983), it has been clearly demonstrated in subsequent studies that the stage of killing affected by simple sugars (as well as glycosylation inhibitors) is not at the level of conjugate formation but rather at a late phase in the lytic event

(see section 2.4 of this chapter). The inhibition of NK activity by simple sugars appears to be nonspecific in that ADCC, NC, macrophage, and CTL mediated lysis mechanisms were also affected by some of the sugars tested (Werkmeister et al, 1983; Stutman, 1980).

Evidence that sialic acid levels may be important in NK recognition is found in reports that the levels of sialic acid on the target cell inversely correlate with the ability of these cells to be lysed (Yogeeswaran, 1982). Sialic acid removal from the target cell similiarly increases the ability of that cell to be lysed by NK cells (Pross et al, 1978). Since sialic acid carries a net negative charge, it is possible that this negative charge is soley responsible for the observed change in target cell lysability. Thus the role of carbohydrate in NK cell recognition remains unclear.

4.4 Target cell membrane lipids. In spite of the many differences between membrane lipids in normal and transformed cells [reviewed in Yogeeswaran, 1983], very little attention has been given to the possible role of membrane lipids as target structures recognized by NK cells. This may be because lipid heterogeneity is perceived to be less extensive than the heterogeneity of other membrane components such as proteins or carbohydrate determinants. On a molar basis, lipids are the major constituents of the plasma membrane, and the physical state of a membrane is largely determined by its lipid

composition (Stubbs, 1983). Lipid composition is also known to be an important factor contributing to cell-cell adhesion (Poste et al, 1976; Margolis et al, 1984) which is an important factor in NK recognition and binding. Lipid modification of tumor cells can increase their sensitivity to NK attack (Yoo et al, 1982) and murine lymphoma target cell asialo GM_2 expression was found to be a prerequisite for NK attack (Young et al, 1981). In a very recent study, it was dertermined that purified GM_2 could inhibit conjugate formation between human Nk cells and K562 cells (Ando et al 1987). This same study also showed that a correlation existed between target cell GM_2 levels and sensitivity to NK cell mediated lysis. The authours have therefore postulated that GM_2 may be a target stucture on tumour cells recognized by human NK cells.

The stage specific embryonic antigen-1 (SSEA-1) has been implicated as a possible target structure (Harris et al, 1984). Antibodies to SSEA-1 block the competitive activity of target cells displaying this antigen. However, there was no apparent correlation between SSEA-1 levels on the tumor cells studied and their competitive activity (Harris et al, 1984). Thus these antibodies may have blocked NK mediated lysis via steric hindrance or some other non-specific effect.

4.5 Antibody dependent cell-mediated cytotoxicity.

Virtually all endogenous NK cells possess the Fc receptor

for aggregated IgG (FCR2; CD-16), and cloned NK-like cells that have an FcR2 can kill via an ADCC type mechanism (see Table III). Lymphocytes able to kill via ADCC were originally termed Killer (K) cells. NK and K cells are morphologically indistinguishable. They possess the same tissue distribution, are both nonphagocytic and nonadherent, have similiar radiation sensitivity, and are phenotypically identical [reviewed in Herberman and Holden, 1978]. In single cell studies using "highly purified" LGL, the same effector cell was shown to be able to lyse both an NK target cell and an antibody coated cell (ie. ADCC target) (Bradley and Bonavida, 1982). In addition, since CD-16$^+$ FACS sorted lymphocytes contain virtually 100% of the NK as well as ADCC activity (Perussia et al, 1983), these two activities probably come from the same cell.

It was originally thought that NK specificity could be attributed soley to natural antibody arming of K cells (Koide and Tagasugi, 1977). Although this appears to be a very plausible idea, it has since been shown that the FcR2 (CD-16) on NK cells does not bind uncomplexed monomeric immunoglobulin and that fresh NK cells can lyse tumor cells in the absence of serum (Pross et al, 1978). Furthermore, in vitro propagated NK-like cells maintain their ability to lyse NK susceptible target cells for several generations (Allavena et al, 1984; Roberts et al, 1985). Many NK-like clones do not possess Fc receptors (Table III), and trypsin treated NK cells incubated in

serum do not consistently regain cytotoxicity against tumor cells (Kay et al, 1978). It therefore seems highly unlikely that NK specificity is a result of natural antibody coating of NK cells.

4.6 Membrane proteins. Membrane proteins have been implicated as target structures on tumor cells recognized by NK cells in both the murine and human systems. The removal of membrane proteins from target cells with the proteolytic enzyme trypsin both renders the target cell less susceptible to lysis by NK cells (Jondal and Pross, 1975; Chapter III section 4.2) and to an even greater extent decreases the ability of that target cell to compete (chapter III section 4.2). Pretreatment of the NK cell itself with proteolytic enzymes also decreases its ability to lyse a target cell (Pross et al, 1978). This indicates that the NK cell "recognition structure" may also be a membrane protein.

Several studies have sought to specifically solubilize target cell membrane proteins. These studies demonstrate that the resultant membrane protein extracts can inhibit NK cell mediated killing of the parent tumor cell line in both the murine and human systems (Roder et al, 1979a; Roder et al, 1979b; Foster et al, 1985; Obexer et al, 1983; Henkart et al, 1986). Thus it appears likely that target cell membrane proteins may be determinants recognized by NK cells.

4.6.1 Virally encoded antigens. Since NK cells can kill some virally infected cells, it has been suggested that virally encoded antigens may be specifically recognized by NK cells. One hypothesis is that NK cells may simply recognize C-type viral particles on the surface of in vitro cultured tumor cells and that the recognition of tumor cells in vitro is therefore an artifact (Kiessling et al, 1975; Herberman et al, 1975). In one study it was shown that the major retroviral glycoprotein gp 71 inhibited NK cell mediated cytolysis (Lee et al, 1977). On the other hand, infection of a resistant tumor cell line with retrovirus did not lead to any increase in susceptibility (Kiessling et al, 1978) and no correlation could be shown between target cell susceptibility and retroviral antigen expression (Becker et al, 1976). It is therefore difficult to reconcile how retrovirally encoded proteins could be target structures on NK susceptible tumor cells.

There are some preliminary results which indicate that HSV-1 antigens may be involved in NK specificity; human Fab fragments from HSV-1 seropositive but not seronegative individuals can block lysis of HSV-1 infected WISH cells (Bishop et al, 1984). The subpopulation of NK cells able to mediate lysis of HSV-1 infected cells however does not appear to overlap with the population of NK cells which lyse K562 cells in humans (Bishop et al, 1984; Fitzgerald et al, 1983).

4.6.2 Laminin receptor. Hiserodt et al have suggested that the laminin receptor may be a murine NK cell target structure (Hiserodt et al, 1985a; Hiserodt et al, 1985b). They reported that NK and NC sensitive tumor cell lines can bind ^{125}I-labelled laminin and that exogenous laminin pretreatment of the target cell blocked its NK sensitivity and competitive activity. They have also shown that murine splenic NK cells may express a cell surface epitope immunologically crossreactive with laminin (Hiserodt et al, 1985b) and that NK effector cells (but not alloimmune CTL) pretreated with a polyclonal anti-laminin sera have diminished ability to bind and lyse Yac-1 tumor target cells. Thus it appears that Hiserodt's group has provided a considerable amount of evidence implicating a laminin-laminin receptor mechanism in the murine NK recognition process.

In some preliminary studies by this author, however, we were unable to decrease murine splenic NK activity for Yac-1 targets by competition with commercially obtained laminin (ICN biochemicals; Cleveland Ohio), or with a rat monoclonal antibody specific to the P_1 fragment (Serotec laboratories) of murine laminin (data not shown). In the case of the monoclonal antibody treatment, preliminary data suggests that roughly 5-10% of spleen cells react with the antibody in histological sections (data not shown).

4.6.3 T-cell receptor (Ti). The Ti is a well documented surface membrane protein found on mature T-

lymphocytes and plays a major role in the recognition of antigens in association with the products of MHC. The Ti consists of 2 disulfide linked chains; α and β. Both these chains contain both variable and constant domains (Acuto et al, 1983; Kappler et al, 1983; Mcintyre and Allison, 1983). Thus the basic structure and sequence of the Ti molecule resembles that of the immunoglobulin gene superfamily (Hood et al, 1985; Novotny et al, 1986).

Since NK cells and T cells have many similarities and have even been hypothesized to belong to a common lineage, they may also share a roughly similiar type of recognition system. Several studies have focused on the possibility that NK cells use or possess some form of the Ti (Yanagi et al, 1985; Biron et al, 1987; Ritz et al, 1985; Moingeon et al, 1986; Moingeon et al, 1987; Tutt et al, 1986; Tutt et al, 1987; Lanier et al, 1986; Lanier et al, 1986b; Lanier et al, 1987; Alarcon et al, 1987; Yokoyama et al, 1987; Ang et al, 1987; Alessandro et al, 1985; Reyonalds et al, 1985; Nakanishi et al,1987; Brenner et al, 1987; Kishara et al, 1987). Analysis of Ti related genes has however, indicated that endogenous NK cells do not express any functional transcripts of Ti α or β (Borst et al, 1987; Reynolds et al, 1985; Lanier et al, 1986b; Tutt et al, 1987; Tutt et al, 1986; Kishihara et al, 1987). Utilizing the antibody WT31 which is thought to react with a framework epitope of the human Ti α,β structure, NK cells have been confirmed not to express any Ti α,β

structure (Spitz et al, 1985; Alarcon et al, 1987; Moingeon et al, 1987; Moingeon et al, 1986). In vivo stimulated NK cells were also found to be devoid of any functional Ti $\alpha, \beta, \gamma, T3\delta$, or $T3\epsilon$ genes (Biron et al, 1987).

The Ti γ chain has been found on NK-like cells in several systems thus far (Moingeon et al, 1987; Alarcon et al, 1987; Borst et al, 1987; Ang et al, 1987; Brewer et al, 1987) and antibodies directed to the Ti associated molecule CD-3 (Koning et al, 1987; Brenner et al, 1987) do inhibit killing of NK targets by these clones (Hercend et al, 1983). A monoclonal antibody "NKF1" raised against a fetally derived NK-like clone is thought to recognize the Ti γ chain (Moingeon et al, 1986) and this antibody also blocks NK-like effector function by some clones (Moingeon et al, 1986).

While some NK cells may express rearranged functional transcripts of the Ti γ chain (Moingeon et al, 1986 Lanier et al, 1986), the vast majority of peripheral blood NK cells do not have any detectable rearranged Ti γ chain message (Tutt et al, 1987; Lanier et al, 1986). NK specificity for tumor cells does therefore not depend on the expression of the Ti $\alpha, \beta, (\gamma \pm \delta)$ structures and one would have to conclude that these structures are probably not involved in NK recognition of tumor cells. Whether or not these Ti structures are involved in some other form of MHC unrestricted killing, such as NC, or a discrete population of NK cells reactive with virally infected cells (Bishop et al, 1984; Fitzerald et al, 1983) remains

to be determined.

4.6.4 TNK_{tar}. Hercend's group has derived a monoclonal antibody (anti-TNK_{tar}) against a 140 Kd membrane acivation antigen present on tumor cells. This antibody blocks cytotoxic function by some cloned NK-like cells (Hercend et al, 1984). This inhibitory effect appears to be at the target cell level because preincubation of target cells with this antibody also inhibits their ability to compete for lysis by these NK-like clones (Moingeon et al, 1985). The TNK_{tar} antigen appears to be the same activation related protein as that recognized by the antibody 4F2 (Moingeon et al, 1985). This antigen was found on all in vitro tumor cell lines tested, as well as on normal lymphocytes and monocytes (Hercend et al, 1984). The density of TNK_{tar}/4F2 increases upon lymphocyte activation (Hercend et al, 1984).

According to Hercend's group, only NK-like clones which express a CD-3 associated 90 Kd membrane protein (Hercend et al, 1983) recognize this TNK_{tar}/4F2 antigen (Hercend et al, 1983; Moingeon et al, 1985). In addition, antibodies against the 90 Kd protein also block effector function (Hercend et al, 1984). However, the clone Jt9 used in this study also expressed a complete 1.3 Kb form of Ti b (Ritz et al, 1985) and the relationship between this Ti β gene product, the 90 Kd molecule, and CD-3 antigen is uncertain. The relevence of these findings to $CD\text{-}3^-$ peripheral blood NK cell specificity also remains to

be determined.

4.6.5 MHC encoded gene products. It seems highly unlikely that NK cells recognize any MHC encoded antigens. In general, NK cells do not lyse normal syngeneic, allogeneic, or xenogeneic cells. Antibodies to MHC class I or class II antigens do not block NK-like killing of tumor cells (Hercend et al, 1983) and tumor cells which are among the best targets for NK cells, either express no or low MHC encoded antigens (Stern et al, 1980; Cikes et al, 1973; Stern et al, 1973).

In fact, it has been suggested that NK cells may recognize a "lack of MHC" (Karre et al, 1986). The basis of this theory is that MHC encoded gene products are actually an inhibitory signal. Once a normal cell has evolved into a tumor cell and lost MHC expression, the cell no longer possesses this inhibitory signal and thus it is lysed by the NK cell. One major obstacle to this theory is that RBC which do not express any MHC encoded products should therefore be lysed by the host's own NK cells. This would obviously be somewhat undesirable and does not appear to occur.

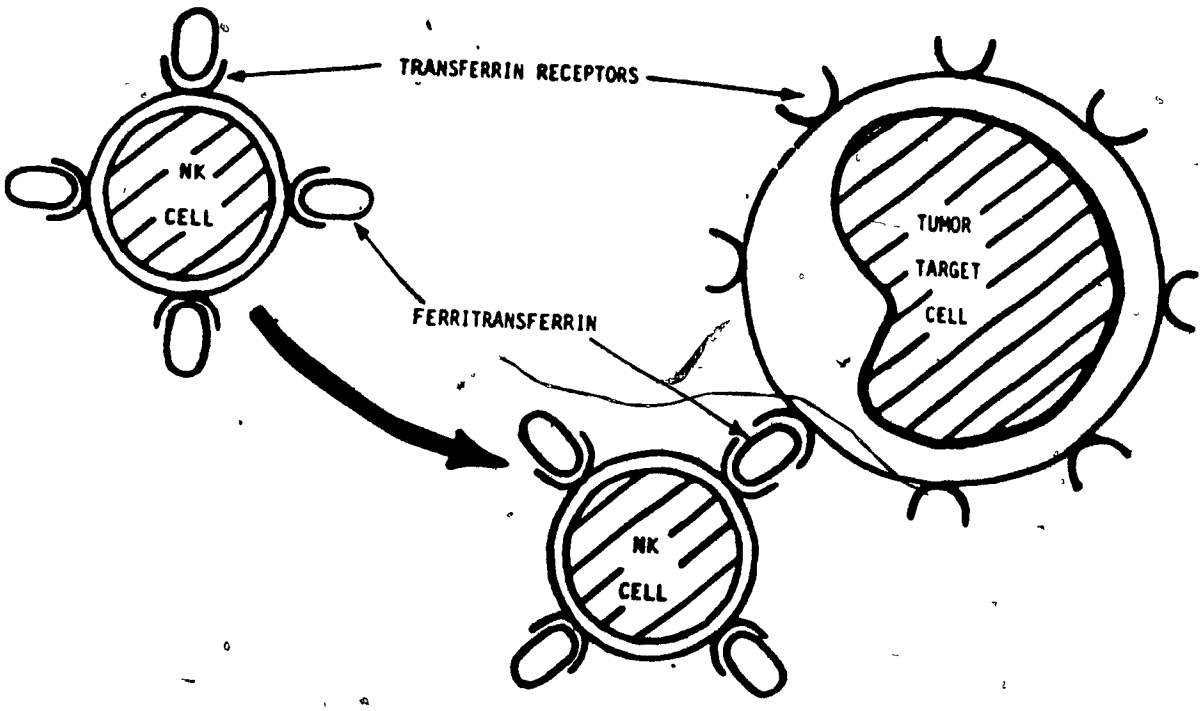
The "lack of MHC" hypothesis, was suggested because murine lymphoma cells selected for low/nil H-2 expression proved less malignant than wild type lymphoma cells when injected into syngeneic hosts. This tumorigenicity was not due to an intrinsic inability to grow in vivo. In addition, NK cell susceptibility inversely correlates with

H-2 expression in these variants as well as in the well known murine NK target Yac-1 (Piontek et al, 1985; Karre et al, 1986). This theory is consistent with reports that interferon treated tumor cells increase their MHC expression (Pionteck et al, 1985) and at the same time become resistant to NK attack (Welsh et al, 1981; Piontek et al, 1985). Storkus and co-workers (1987) have also demonstrated an inverse relationship between MHC class I antigen expression and target cell susceptibility to human NK cell activity. The F_1 hybrid resistance model, in which NK cells are thought to lyse normal semiallogeneic bone marrow cells may also be explained by this "lack of MHC" theory (Cudkowicz and Bennett, 1971; Carlson et al, 1980).

4.6.6 Tf receptor. In 1983 it was suggested that the Tf receptor may serve as a target structure recognized by NK cells (Baines et al, 1983; Vodinelich et al 1983). Since then, the involvement of Tf receptors in NK specificity has been a very controversial topic.

Initial studies in this laboratory showed that the normal serum iron binding protein Tf, could block both NK cell activity and conjugate formation between PBL and K562 cells at physiological concentrations (Baines et al, 1983). The effect of Tf on NK cell activity was also confirmed by Bierman and co-workers (1984) as well as by Philips (1986). Borysiewicz and co-workers (1986) showed that transferrin could inhibit NK cell mediated lysis of

Fig 1. Model of Human Natural Killer Cell recognition of tumor cells via transferrin receptors.



Taken from Baines et al, 1983

virally infected cells.

In vivo, human serum Tf is partially saturated with iron and only this iron saturated form of the protein can bind to its receptor at physiological pH (Klausner et al, 1983; Dautry-Varsat et al, 1983). The iron free form of Tf (apo-Tf) can bind to the Tf receptor only at low pH (Klausner et al, 1983)(Dautry-Varsat et al, 1983). Apo-Tf has also been shown to inhibit conjugate formation (Baines et al, 1983). Since apo-Tf retains its inhibitory capacity, it is possible that the inhibition observed by either Tf or apo-Tf does not involve receptor participation. Studies by M. Caldwell in this department, however, show that apo-Tf may become partially saturated with iron under the conditions of the assay since RPMI-1640 contains significant amounts of free contaminating iron as shown by atomic absorption spectroscopy (M. Caldwell, personal communication).

Pretreatment of PBL or LGL with either intact or F(ab)'2 fragments of rabbit antibodies against human serum Tf also blocks NK cell activity. However, when target cells on the other hand were pretreated with antibodies against Tf, no effect was seen (Baines et al, 1983; Alarcon et al, 1985).

The Tf receptor is composed of 2 identical subunits of 90 Kd each and it is thought that each subunit is capable of binding 1 molecule of Tf. There are disulfide linkages near the membrane to which the Tf binding domains are distal linked regions [reviewed in Ward, 1987 and

Trowbridge et al, 1984]. Trypsin cleavage of the plasma membrane bound Tf receptor liberates a 70 Kd fragment which retains the ability to bind Tf (Omary and Trowbridge, 1981; Schneider et al, 1982). Vodenlitch and co workers (1983) have shown that this Tf receptor fragment but not HLA-B fragments can specifically block NK cell activity towards K562 cells.

Taken together the above results suggest that the transferrin receptor is a target structure on tumor cells recognized by NK cells. Furthermore, the inhibition studies on NK cells with anti-Tf antibodies indicate that the NK cell may also possess Tf receptors to which Tf is bound. This Tf receptor-Tf complex on the NK cell was postulated to be the recognition structure utilized by NK cells to bind tumor cell Tf receptors (see Fig.1).

Based on the above model, antibodies against the Tf receptor should block NK cell activity at the target cell level. However, antibody blocking studies on target cells has provided confusing and contradictory results. The results are confusing because NK cells are capable of ADCC. Thus sensitizing a target cell with antibody should predispose that cell to attack by an ADCC mechanism especially since it has been shown that murine monoclonal antibodies can be recognized by human NK cells (Ortaldo et al, 1987; Park and Brahmi, 1984; Herlyn et al, 1985). Most antibody studies against Tf receptors have either blocked NK activity or had no measurable effect (see Table IV).

Table IV

1)Vodinelich et al, 1983

2)Dokhelar et al, 1984

3)Storkus et al, 1986

4)Golightly et al, 1984

5)Baines, M.G. (unpublished observations)

6)Perl et al, 1986

TABLE IV

Inhibition of NK Cell Activity with Monoclonal Antibodies to the Tf Receptor

Monoclonal Antibody	Effect on NK Cell Activity	Reference
B3/25	Decrease	(1)
B3/25	Nil	(2)
5E9*	Nil	(3,4,5)
L 01.1	Decrease	(6)
42.6	Nil	(2)
OKT-9	Nil	(2)

*Pretreatment of effector cells with this Antibody also had no effect.

Another possible interpretation of the results of target cell treatment with antibody is that the epitopes of the Tf receptor recognized by these antibodies does not overlap with the epitope involved in NK recognition.

If Tf receptors are target structures on tumor cells recognized by NK cells, then the numbers of Tf receptors on the tumor cells should correlate with their susceptibility to lysis. Since a major aspect of this thesis is to determine the relationship between Tf receptor expression and NK sensitivity, an analysis of this aspect of NK specificity will be deferred until chapter III.

II. A RAPID AND EFFICIENT MICROTECHNIQUE FOR THE ANALYSIS OF FUNCTIONAL TRANSFERRIN RECEPTORS ON TUMOR CELLS.

1. Abstract

A simple and efficient method for the analysis of the affinity and number of functional transferrin receptors on human tumor cells is described. The technique is designed to utilize microtitration equipment, and is suitable for easy comparison of up to eight different cell preparations per assay. Using this technique, five established cell lines were evaluated for functional Tf receptor expression. The control erythroleukemic cell line K562 posessed 3.28×10^5 functional Tf receptors per cell ($\pm$ 3.69×10^4, S.E.M.) Kd=9.0×10^{-9} M-1. Trypsin and heat pretreated cells were compared to control erythroleukemic K562 cells from the same culture to determine both the effects of receptor removal and cell viability on the assay. Trypsin and heat pretreatment of these K562 cells severely decreased receptor function as indicated by Scatchard analysis as well as by time course and cold competition analysis respectively. Whereas the affinity of trypsin treated receptors on cells was similiar to control values, heat killed cells displayed an altered cellular affinity for transferrin-I-125 underscoring the importance of utilizing cells of high viability in receptor assays.

2. Introduction

Transferrin receptors are ubiquitously present on tumor cells in high numbers (Larrick and Cresswell, 1979; Faulk et al, 1980; Schindelman et al, 1981; Sutherland et al, 1981). They are a principle method through which these cells, as well as normal cells acquire iron (Aisen, 1983). The analysis of Tf receptors as a possible marker for malignancy (Faulk et al, 1980; Schindelman et al, 1981), and as a differentiation marker (Tei et al, 1982; Testa et al, 1982; Yeh et al, 1982; Horton et al, 1983), has recently gained wide attention. In addition, the Tf receptor has been implicated as a target structure for Natural Killer cell mediated killing of human tumor cells (Baines et al, 1983; Vodinelich et al, 1983). The findings that the receptor is structurally and functionally related to the human melanoma- associated antigen p97 (Brown et al, 1982) and that transferrin itself (a normal serum iron binding protein) exibits sequence homology with an avian oncogene product (Goubin et al, 1983) gives further credence to its role as a possible target structure on tumor cells. Many studies have quantitated the expression of this receptor through the use of antibodies directed against different epitopes on the Tf receptor (Faulk et al,1980; Lebman et al, 1982; Yeh et al, 1982; Horton et

al, 1983). A major drawback in the use of antibodies to quantitate the receptor, is that the number of functional Tf receptors often differs significantly from the number of immunologically detectable receptors (Frazier et al, 1982). Whether there is a similiar diversity between different monoclonal antibodies recognizing different Tf receptor epitopes or not, is a matter of speculation. Scatchard analysis of functional Tf receptors using iron saturated transferrin as a ligand thus eliminates this extra parameter. The described microtechnique is ideal for simultaneously comparing Tf receptor on several different cell preparations. This should prove to be useful because discrepancies exist in the literature on comparative Tf receptor estimation (Frazier et al, 1982; Testa et al, 1982; Klausner et al, 1983a), and thus comparisons between different authors are difficult to interpret. The method makes use of equipment normally used in laboratories which measure cytotoxicity by the chromium release assay (Jondal and Pross, 1975; Pross and Jondal, 1975) and is similarly fast and economical.

3. Materials and Methods

3.1 Cell culture. The erythroleukemic human tumor cell line K562 (Lozzio and Lozzio, 1975) as well as RAJI, MOLT-4 and HL-60, were grown in suspension culture and maintained at 37°C in a humid environment in 5% CO_2. The

cells were cultured in RPMI-1640 containing 10% heat inactivated fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin-G, 100 ug/ml streptomycin sulfate, 10 mM HEPES and 2 mg/ml sodium bicarbonate. The cell line MeWo which is a tissue culture monolayer was grown in the same media as above with the addition of 2-mercaptoethanol at a final concentration of $4.29 \times 10E-^{5}M$. These cells were harvested from culture by scraping the cells from flasks with a bent glass pipette, the cells were then vigorously resuspended to break up clumps. All cell lines were assayed 3-4 days post feeding.

3.2 Radiolabelling of Tf. Apotransferrin (Sigma; St. Louis, MO.) was saturated with iron, as previously described (Holbein, 1981). Batches were periodically verified to be >92% saturated by comparing the optical absorbance ratio at 465/280 nm to a standard regression line (Holbein, 1981). Iron saturated transferrin was then iodinated using the method of Hunter and Greenwood (1962). Briefly, 200 ug of chloramine T (BDH; Montreal,Que.) was added to 10 mg of iron saturated transferrin and 2 mCi of carrier free ^{125}I as NaI (Amersham, IMS.300; Oakville, Ont.) in a total volume of 500 ul of 10 mM (Ca^{++} and Mg^{++} free) phosphate buffered saline (PBS). After 15 minutes, the reaction was stopped with 250 ug of sodium metabisulfite and the free ^{125}I was then immediatly separated from transferrin-I-125 on a sephadex G-25 column. A 5 ul aliquot of each fraction was then

subjected to precipitation in a 10% trichloracetic acid and 1% bovine serum albumin mixture in a total volume of 400ul, this sample was then centrifuged at 13,100xg for 10 minutes. Typically >93% of the radioactivity was precipitable, indicating that the label was bound to the protein. Protein concentration was determined using the Biorad protein assay with iron saturated transferrin as the standard. Although transferrin is generally accepted to be a nonlabile protein, the transferrin-I-125 solution was supplemented with 1% BSA w/v as a stabilizing agent for free radicals produced by ^{125}I decay. The specific activity obtained, was usually in the range of 200,000 cpm/ug protein.

3.3 Binding assay. Specific high affinity binding of transferrin-I-125 to cells was evaluated by Scatchard analysis of binding data as detailed below. All binding assays were performed in 96 well V-bottom microtitration plates (Linbro/Titertech; Flow laboratories,inc.). RPMI-1640 (100ul) was added to all wells except column 1, using a 12-channel pipettor (Titertech; Flow laboratories,inc.). Column 1 received 200 ul RPMI-1640 plus a concentration of transferrin-I-125 capable of fully saturating cellular Tf receptor (approximatly 0.2 ug transferrin-I-125/well). The contents of column number 1 was then serially diluted in 100 ul steps to column 11. One million cells in 100 ul RPMI-1640 were then added to each row (ie.each row contained a different cell line to be compared) and the plate was transferred to a 37°C incubator and gently

agitated at a speed of 3 on a Dynatech micro-shaker II (Dynatech laboratories,inc.; Alexandria Virgina) for 45 minutes. Upon termination of the incubation, 50 ul of resuspended cells from all wells were removed and pipetted into LP/2 tubes (Luckham;Sussex, England) sealed with paraffin and counted for ^{125}I. A factor which makes this assay efficient is that LP/2 tubes (capacity=600 ul) fit perfectly into 96-well flat bottomed tissue culture plates (Flow Laboratories,inc.), thus allowing the direct transfer of cells from the assay plate to LP/2 tubes with the 12-channel pipettor. This measurement was performed to determine the total counts per minute (cpm) of transferrin-I-125 in the assay, and was denoted as 1/4 bound+free cpm of transferrin-I-125. The remaining 150 ul in the plate was then rinsed 3 times at 4°C by centrifuging the plate in an IEC DPR-6000 refrigerated centrifuge in a Cooke microtiter plate carrier (Dynatech laboratories,inc.), for 5 minutes at 500xg. The plate was then removed, inverted, and firmly but gently flicked, to cast off the supernatant. It is important that the plate be perpendicular to the floor when casting off the supernatant, to ensure that each corner of the plate recieves an identical force. The cell pellets were rinsed with 200 ul of PBS at 4°C, this rinsing step was done gently, so as not to disturb the cell pellet and increase cell loss. After the above washing procedure was carried out 3 times, the cells were removed in 200 ul of PBS, the

wells were susequently rinsed with 200 ul of PBS and both washings were placed in LP/2 tubes using the 12-channel pipettor. These cell preparations represent 3/4 of the bound cpm of transferrin-I-125 in the assay.

3.4 Cell loss. On average, 20% of the cells which should be recovered in the cell pellet are lost during the washing phase of this assay system. It has been our experience that cell loss is consistant from well to well within the same cell line, however, different cell lines may exhibit differences in the numbers of cells lost. While most cell lines were comparable, the trypsin treated cells were particularly susceptible to cell loss. To compensate for this cell loss, the cell contents of representative wells (row 12) were counted at the end of the assay for each cell line tested. Transferrin-I-125 was not added to these wells. Since $1x10^6$ cells were placed into each well, and 25% of these cells were removed before washing (to measure the bound+free fraction), then $7.5x10^5$ cells should have been recovered from each well. Since cell loss occured solely due to washing, the measurement of bound+free was obtained without error and therefore did not require correction. The number of bound cpm however varied in direct proportion to the cell loss. The values obtained in cpm for the bound fraction were corrected by multiplying the observed value of bound cpm by a cell loss correction coefficient.

3.5 Time dependent binding. This assay was done in the same plates as conventional assays, however the

concentration of transferrin-I-125 in each well was kept constant. One million cells were added to each well sequentially at 5 minute intervals. The reaction was terminated by transferring the contents of each well (using a 12-channel pipettor) to ice cold LP/2 tubes containing 400 ul of PBS. The tubes were precoated with a 0.1% bovine serum albumin solution, to keep nonspecific binding to a minimum. These tubes were then mounted in a 96-well flat bottomed tissue culture plate and centrifuged for 5 minutes at 350 xg. It should be noted that only 4 rows of tubes can be mounted on a plate and centrifuged at one time because of the design of the plate carrier. After centrifugation, 550 ul of supernatant was removed with the multichannel pipettor using narrow pipette tips (Biorad Laboratories, (Canada) Ltd.; cat. number 223-9037). Five hundred fifty ul of PBS at 4°C was then forcefully added to the tubes ensuring that the pellet was resuspended. After the fourth wash was completed, the tubes were sealed in paraffin and counted in a gamma counter. This measurement represented the amount of transferrin-I-125 bound to the cells.

3.6 Data analysis. Scatchard plots from the binding data obtained were corrected for nonspecific binding by the method of Chamness and McGuire (1975). The method was based on the assumption that one could discriminate between specific and nonspecific binding, as the the nonspecific component was directly proportional to the

availiable free ligand, whereas specific binding was proportional to receptor concentration. When the raw data was plotted by the method of Scatchard (1949), an asymptote was formed at a limiting ratio of bound/free. This limit was then used to factor out the nonspecific component for each data point on a Scatchard plot

(eq. 1).

specific bound= experimental bound- free [Lim bound/free]
$B/F \to \infty$

The limit for each assay and cell line was determined by using the well with the highest concentration of Transferrin-I-125. Since receptor saturation must be achieved to determine the limit, representative experiments were routinely analyzed to ensure that receptor saturation did occur. The molecular weight of transferrin was taken to be 80,000 Mr (Aisen, 1983) in conversions of binding data from cpm/ug to either moles bound or molarity of free ligand.

3.7 Trypsin treated cells. K562 cells were pretreated with trypsin (1:250 Gibco/Grand Island Biological Company; Grand Island, NY) by incubating approximately $3x10^7$ cells in 0.5% trypsin w/v solution in PBS for 20 minutes at 37°C. The cells were then washed 3 times at 4°C and maintained at that temperature until assayed.

4. Results

The binding of transferrin-I-125 to K562 cells was shown to be specific for transferrin at 37°C (fig.2). The addition of cold transferrin to a binding assay at 37°C effectively displaced transferrin-I-125 from the cellular Tf receptor whereas the addition of cold bovine serum albumin did not alter binding. Figure 3 shows that with uncorrected data, binding was saturable at 37°C. As mentioned in materials and methods, saturation must be achieved in each assay to mathematically factor out the nonspecific binding. Incubation of K562 cells for differing periods of time showed that the system achieved equilibrium by 30 minutes and remained static thereafter (fig.4). All subsequent assays were incubated for 45 minutes to ensure that a system at equilibrium existed. The number of functional Tf receptors on K562 cells derived by Scatchard analysis (fig.5) as averaged over fifteen different experiments was 3.28x105 Tf receptors/cell($\pm$3.69x10^4) Kd=9.0x10^{-9} M-1. When K562 cells were pretreated with trypsin, the cells did not saturate with time and, as can be seen from the Scatchard plot, bound considerably less ligand then their K562 parent cells. The affinity of these trypsin treated cells did not however differ significantly from that of K562. This shows that the Tf receptor was sensitive to the effects of trypsin and that these cells possessed few measurable Tf receptors (fig.5). K562 cells were heat

Fig 2. Specificity of binding of ^{125}I-transferrin to K562 tumor cells. 11.5 picomoles of ^{125}I-tranferrin was added to all wells and the concentration of cold competing protein was varied. ●----●; Competition by cold transferrin on K562 cells. ■---■; Competition by cold bovine serum albumin on K562 cells. O----O; Competition by cold transferrin on K562 cells pretreated at 56°C for 30 minutes. This graph illustrates that binding of the radioactive transferrin was specific for transferrin on K562 cells, while heat pretreated K562 cells did not bind ^{125}I-transferrin specifically.

fig.2

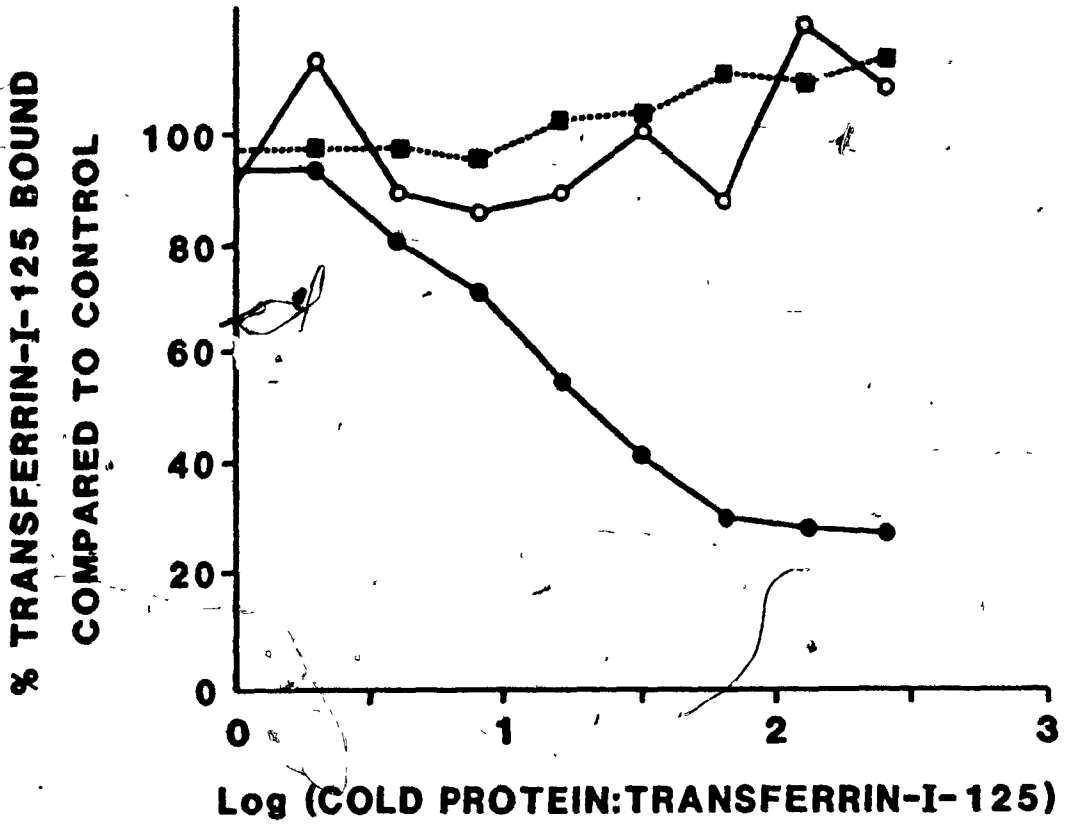

% TRANSFERRIN-I-125 BOUND
COMPARED TO CONTROL
100
80
60
40
20
0
0
1
2
3
Log (COLD PROTEIN:TRANSFERRIN-I-125)

Fig 3. Saturation of binding of ^{125}I-transferrin to K562 tumor cells. The binding of ^{125}I-transferrin to K562 cells was shown to be saturable at 37°C. Saturation was achieved at approximately 0.2 ug transferrin/ $1x10^6$ cells.

fig.3

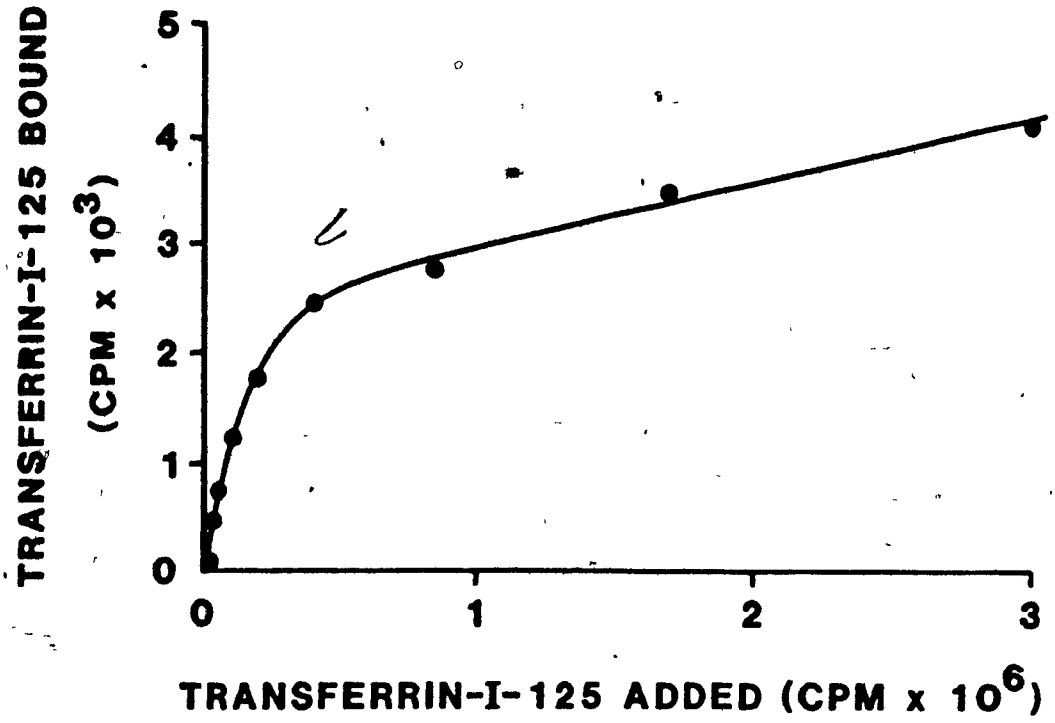

TRANSFERRIN-I-125 BOUND
(CPM x 10^3)
5
4
3
2
1
0
0
1
2
3
TRANSFERRIN-I-125 ADDED (CPM x 10^6)

Fig 4. Time dependent binding of ^{125}I-transferrin to K562 tumor cells. Binding of ^{125}I-transferrin to K562 (●), or trypsin pretreated K562 cells (▲) at 37°C as a function of time. K562 cells bound ^{125}I-transferrin in a time dependent manner whereas trypsin pretreated cells which bound low amounts of ^{125}I-transferrin did not show time dependent binding in assays when the specific activity of the ligand was decreased. This underscores the importance of using ^{125}I-transferrin which has a specific activity of at least $2x10^5$ cpm/ug protein, for cell lines posessing few receptors.

fig.4

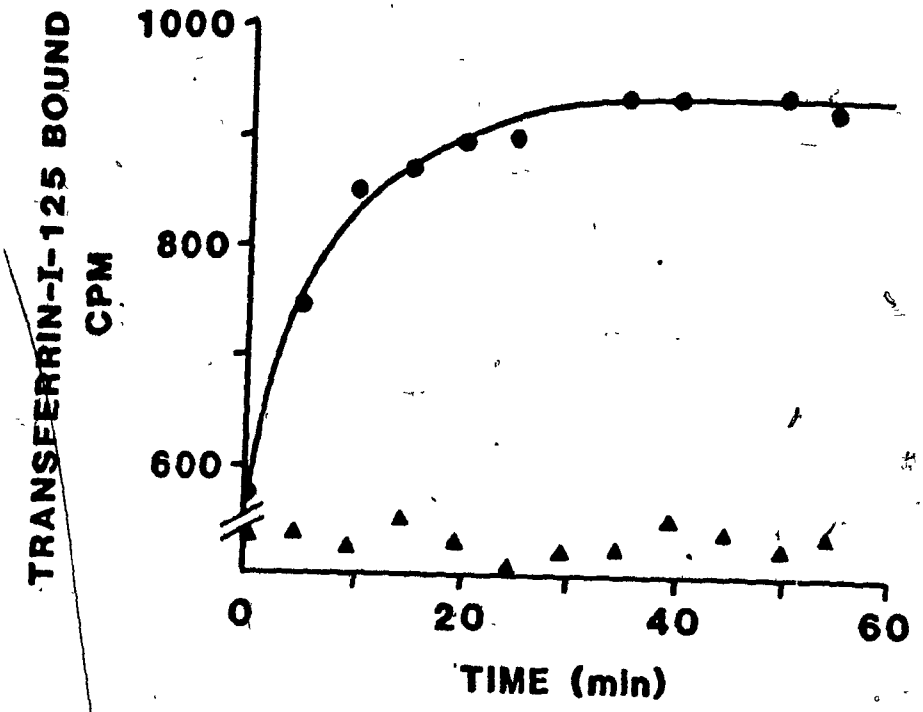
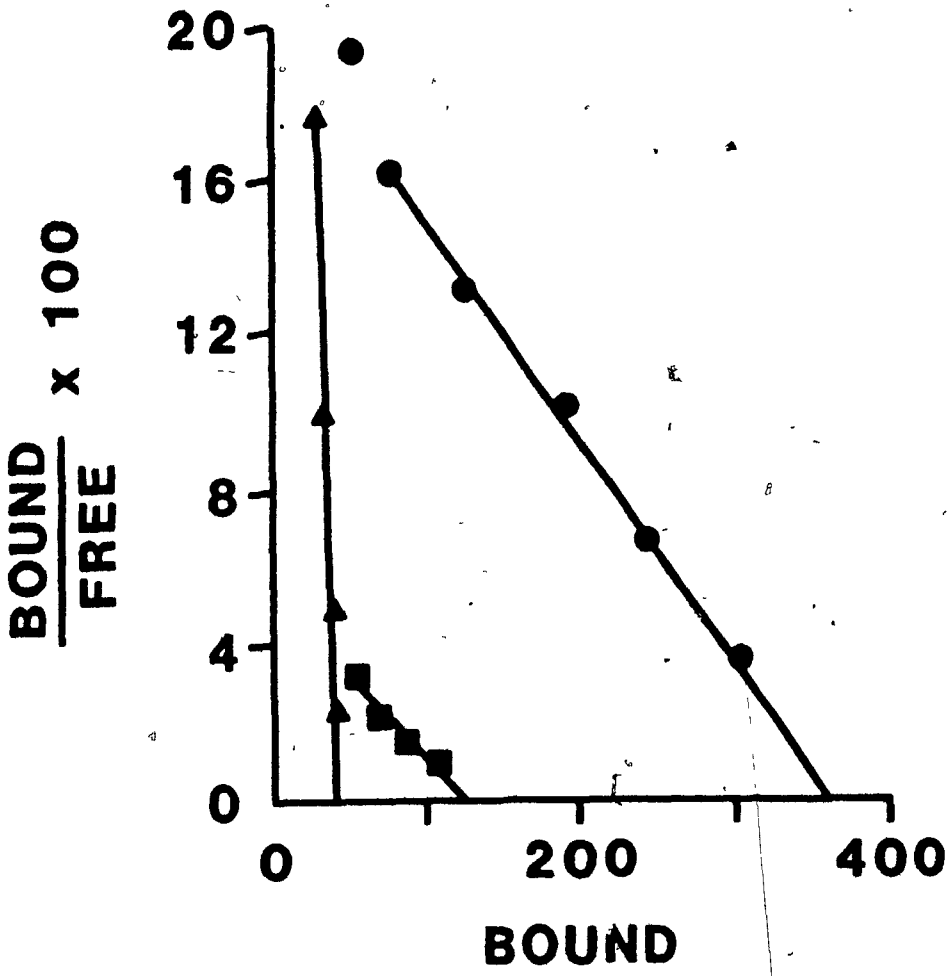


Fig 5. Scatchard analysis of cellular binding of ^{125}I-transferrin to K562 cells pretreated with trypsin or heat. Cells were incubated at 37°C for 45 minutes. Units on the absisca are femtomoles of ^{125}I-transferrin bound. ●; K562 cells maintained in tissue culture and fed 3-4 days prior to assay. ■; Trypsin pretreated K562 cells, the affinity of these cells for ^{125}I-transferrin was not significantly different from K562. ▲; K562 cells pretreated at 56°C for 30 minutes., this heat pretreatment abrogated receptor function (see text).

fig.5



pretreated at 56°C for 30 minutes to determine if receptor function would be abrogated by this treatment. Upon analysis of these heat pretreated cells, it was found that they were capable of binding very little ligand but that this binding was of a higher affinity ($K_d=4.85\times10^{-10}$ M-1 $P<.01$) than with K562 cells, (fig.5). Upon cold competition analysis it was observed that cold transferrin could not displace transferrin-I-125 from the cells, indicating that the binding of the transferrin-I-125 was not specific and therefore did not involve Tf receptors (fig.2). These heat pretreated K562 cells were typically of low viability as judged by trypan blue incorporation. Since trypan blue stains dead "leaky" cells it is highly probable that the Tf receptor estimate from the Scatchard plot shown is due to labelled ligand permeating dead cells. If these cells trapped transferrin-I-125 in a non-specific manner, then an unusually high affinity would be expected. Using this method of analysis, several common tumor cell lines were assayed for Tf receptor expression under normal growth conditions (table V). It was seen that all these established human cell lines expressed substantial numbers of functional Tf receptors. These results agree with previously published values (Haynes et al,1981; Fraizer et al, 1982; Vodenlich et al, 1983; Mattia et al, 1984) substantiating the validity of this miniaturized method of Tf receptor analysis.

TABLE V

Transferrin Receptor Expression on Human Tumor Cell Lines:

CELL LINE	TRANSFERRIN RECEPTORS/CELL	S.E.M.
K562	3.28×10^5	3.69×10^4
Trypsin Pretreated K562	4.50×10^4	1.61×10^4
RAJI	2.28×10^5	4.42×10^4
MOLT-4	1.42×10^5	3.05×10^4
HL-60	3.18×10^5	5.46×10^4
MeWo	3.09×10^5	5.38×10^4

5. Discussion

A technique has been developed to measure functional Tf receptors on tumor cells. Using the described technique the number of functional Tf receptors on K562 cells was estimated to be 3.28×10^5 Tf receptors/cell. The affinity of these receptors was 9.0×10^{-9} M-1. It should be noted that the values for receptor number obtained in this study represent the total number of functional Tf receptors on the cell lines tested. Since the Tf receptor along with its ligand is internalized within minutes (Dautry-Varsat et al, 1983; Hamilton, 1983; Klausner et al, 1983b; Lamb et al, 1983; Willingham et al, 1983), the value of Tf receptor number in these experiments includes surface membrane receptors plus the active internal receptor population. When K562 cells were pretreated with trypsin, the number of functional Tf receptors were severly decreased, however the affinity of the receptors measured remained unchanged. This result suggests that the receptor population measured was probably either an internal receptor population not exposed to trypsin but expressed later on during the receptor assay or de novo synthesized Tf receptor. Although the K562 cell line used is cultured in suspension, many tissue culture monolayers are removed from culture by treatment with this proteolytic enzyme. It would be advisable to use other methods of cell removal when analyzing surface receptors of any type. Another argument against the use of

proteolytic enzymes for harvesting cells, is that the treatment could also unveil new receptor sites, thus permitting the entry of a new parameter to the system. K562 cells pretreated at 56°C for 30 minutes showed a decreased ability to bind transferrin-I-125 but had an increased affinity for this ligand as analyzed by Scatchard analysis Kd=4.85×10^{-10} M-1. This minimal binding could not be competed with by cold transferrin, indicating that the binding observed was not specific for the Tf receptor. The average viability of these cells was typically 15% of the total cell number as measured by trypan blue incorporation. The binding of the ligand was most likely the result of transferrin-I-125 entering these dead "leaky" cells, and being trapped there, thus giving the impression of a high affinity receptor. Since the labelled ligand could not be competed for, the binding must be considered to be nonspecific and not receptor mediated (Cuatrecasas and Hollenberg 1976). From this result it can be seen that the number of dead cells present in a receptor assay could greatly affect the estimation of affinity and this error would subsequently cause an underestimate of the receptor number because the slope of the line derived from a Scatchard plot would increase as a direct result of the proportion of dead cells. Thus the cells used in Scatchard analysis should be scrupulously monitored for viability. We have also shown that multiple tissue culture cell lines (including a

tissue culture monolayer) could be simultaneously assayed with this microtechnique providing results which agree well with the published literature (Haynes et al,1981; Fraizer et al, 1982; Vodinelich et al, 1983; Mattia et al, 1984). The assay is sensitive to changes in receptor number (trypsin-K562) as well as affinity (heat-K562) and is therefore a sensitive and efficient method of Tf receptor evaluation.

III. Studies on the Mechanism of Specificity of Human Natural Killer Cells For Tumor Cells:Correlation between Target Cell Transferrin Receptor Expression And Competitive Activity.

1. Abstract

Previous studies to determine the nature of the specificity of Natural Killer (NK) cells for leukemic cells indicated that functional transferrin (Tf) receptors may be one of the determinants recognized by NK cells. To further investigate these observations, the relationship between cellular Tf receptor expression and ability to compete with a control K562 cell preparation in a standard chromium release assay was studied.

K562 cells were selected at different phases of growth by removing cells from tissue culture at one, three, and five days post feeding. Under these conditions, K562 cells respectively displayed relatively high, medium, and low numbers of Tf receptors and corresponding competitive activity against a control K562 cell preparation. K562 cells were modified by either trypsin, heat, or sodium butyrate (differentiation inducer) pretreatment. An NK resistant clone was also studied. There was a good correlation between Tf receptor expression and cold competitive activity of the above K562 cell preparations ($r=0.82$ $P<0.01$).

The different tumor target cell lines K562, Molt-4,

Raji, HL-60, and MeWo which would be expected to express different ranges of specificity did not show a significant correlation between Tf receptor expression and their cold competitive activity against ^{51}Cr labelled K562 cells.

Rabbit reticulocytes which express high numbers of Tf receptors were tested for their ability to compete with K562 cells for NK cells. These cells were able to compete with K562 cells while mature rabbit red blood cells which do not express Tf receptors did not compete well. These findings support the contention that the Tf receptor may be involved in NK cell recognition of some tumor cells.

2. Introduction

Natural Killer (NK) cells have been implicated as a primary line of defence against neoplastic disease [Herberman and Holden, 1978]. These cells have also been shown to be lytically active against certain virally infected cells (Biron et al, 1982; Stein-Streilein et al, 1983; Habu et al, 1984;), hematopoetic stem cells (Hansson et al, 1982; Holmberg et al, 1984),thymic and fetal thymic cells (Nunn et al, 1977; Hansson et al, 1981), and bone marrow cells (Hansson et al, 1981).

The mechanism through which an NK cell kills a target cell is complex and involves many well defined stages [reviewed in Trincheiri and Perussia, 1984]. The initial event leading to target cell lysis involves a specific

binding or contact between the effector lymphocyte and target cell. Several studies have been done to identify the target structures involved in this recognition event (Roder et al, 1979; Obexer et al, 1983), and the results of these studies pointed to membrane proteins, however the Mr of the reputed target structures in these two studies turned out to be different, and these proteins have yet to be characterized. One other problem with these studies was that the membrane proteins involved in both studies were treated under denaturing conditions prior to their evaluation in competition assays and thus the "target structures" were not evaluated in their natural state.

It has recently been suggested by several laboratories that the Tf receptor may be a target structure on tumor cells recognized by NK cells (Baines et al, 1983; Vodinelich et al, 1983). The findings that Tf (a normal serum iron binding protein) may be functionally and structurally related to the human melanoma antigen p97 (Brown et al, 1982) and that Tf exhibits sequence homology with an avian oncogene product (Goubin et al, 1983), gives credence to the proposal that its complimentary receptor could be a candidate as an NK cell target structure. The generalization that Tf receptors are expressed on actively dividing cells such as tumor cells (Larrick and Cresswell, 1979; Sutherland et al, 1981; Chapter 2), bone marrow cells (Horton, 1983; Lebman et al, 1982), and hematopoetic progenator cells (Lesley et al, 1984), and that NK cells have been generally shown to be active against these cell

types suggests that NK cells may have a general regulatory role on normal proliferating cells via the involvement of Tf receptors. In order to investigate this point, tumor cell lines at various stages of growth were subjected to competition assays and assays for Tf receptor number and affinity. In addition, target cells with directly altered Tf receptor expression were also used in these assays to identify the relationship between NK recognition and Tf receptor expression.

3. Materials and Methods

3.1 Tumor cells. The erythroleukemic human tumor cell line K562 (Lozzio and Lozzio, 1975), as well as Clone I (MacDougall et al, 1983), Raji, Molt-4, and HL-60 were grown in suspension culture and maintained at 37°C in a humid environment in 5% CO_2. The cells were cultured in RPMI-1640 suplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 IU/ml penicillin-G, 100 ug/ml streptomycin sulfate, and 10 mM HEPES. The cell line MeWo which is a tissue culture monolayer was grown in the same media as above with the addition of 2-mercaptoethanol at a final concentration of 4.29×10^{-5}M. These cells were harvested from culture by scraping the cells from flasks with a bent glass pipette, the cells were then vigorously resuspended to break up clumps. K562 cells grown in culture for 1, 3, and 5 days were

adjusted to a starting density of 1x10E5 cells/ml in 100% fresh media, the starting time for these cultures was staggered so that their assays were always done concomitantly. K562 cells were induced to differentiate as previously described (Werkmeister et al, 1982) in 1mM sodium butyrate (Sigma: St. louis, MO) for 5 to 25 days in tissue culture prior to assay. K562 cells were pretreated with trypsin (1:250 Gibco/Grand Island Biological Company; Grand Island, NY) to remove membrane proteins by incubating K562 cells in 0.5% trypsin (w/v) solution in Ca^{++} and Mg^{++} free phophate buffered saline (PBS) for 20 minutes at 37°C. The cells were then washed 3 times at 4°C and maintained at that temperature until assayed. Heat pretreated K562 cells were prepared by incubating K562 cells at 56°C for 30 minutes in RPMI-1640 plus 10% FBS and then washed 3 times and storing at 4°C until assayed.

3.2 Lymphocyte isolation. Heparinized blood was collected from healthy volunteers by venipuncture, and incubated with carbonyl iron (10mgm/ml) for 30 minutes at 37°C, on a rotary mixer. After incubation the blood was passed twice over a 2.5Kg magnet to deplete the adherent population of cells. The blood was then diluted with an equal volume of RPMI-1640 and the lymphocyte population was separated by buoyant density centrifugation on Ficoll/Hypaque (sp. gr.= 1.077). The cells were washed 3 times in RPMI-1640 and resuspended in RPMI-1640 + 10% FBS at 4°C until used.

3.3 Receptor analysis. Tf receptor expression was assessed on cells by a micromethod as previously described (Chapter 2). Briefly, iron saturated transferrin (Sigma; St.Louis Mo) was iodinated using the method of Hunter and Greenwood (Hunter and Greenwood, 1962). All binding assays were performed in 96 well V-bottom microtitration plates (Linbro/Titertech; Flow labs). RPMI-1640 (100ul) was added to all wells except column 1, using a 12-channel pipettor (Titertech; Flow labs.). Column 1 received 200 ul RPMI-1640 plus transferrin-I-125. Half the contents of column number 1 (100ul) was then serially diluted in 100 ul of diluent to column 11. One million cells in 100 ul RPMI-1640 were then added to each row and the plate was transferred to a 37°C incubator and gently agitated at a speed of 3 on a Dynatech micro-shaker II (Dynatech labs inc.; Alexandria Virgina) for 45 minutes. Upon termination of the incubation, 50 ul of resuspended cells from all wells were removed and pipetted into LP/2 tubes (Luckham;Sussex, England) sealed in paraffin and counted for ^{125}I. This measurement was performed to determine the total counts per minute (cpm) of transferrin-I-125 in the assay. The remaining 150 ul in the plate was then rinsed 3 times at 4°C by centrifuging the plate for 5 minutes at 500xg. The plate was then removed, inverted, and firmly but gently flicked, to cast off the supernatant. The cell pellets were then rinsed with 200 ul of PBS at 4°C. After the above washing procedure was carried out 3 times, the cells were removed in 200 ul of PBS, the wells were

susequently rinsed with 200 ul of PBS and both washings were pooled and counted for transferrin-I-125.

3.4 Cold competition studies. The ability of competing cell preparations to compete with a control K562 population was assessed in a modification of the standard chromium release assay (Pross and Jondal, 1975). Ten thousand ^{51}Cr labelled K562 cells were added to each well of a microtitration plate followed by cold competitor cells at ratios from 20/1 to 1.25/1 cold competitors to ^{51}Cr labelled K562 cells. Two hundred thousand lymphocytes were then added to these wells to give an effector to target ratio of 10/1. The spontaneous target death was derived by incubating $1x10^4$ ^{51}Cr labelled targets in the absence of lymphocytes. Replicate wells were resuspended prior to supernatant harvest to determine the maximum kill possible. The percentage of ^{51}Cr labelled K562 cells killed was calculated by formula (1):

$$\% \text{ lysis of K562 cells} = \frac{100\ (\text{test } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release})}{\text{maximum } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}} \quad (1)$$

Maximum killing by effector cells was evaluated by including wells without cold competitor cells. The 50% inhibition level was determined by dividing the percent

kill due to 110/1 effector cells without competitors by 2. Values of inhibitory units at 50 % inhibition ($IU_{50}/10^6$ cold competitors) were derived by calculating the cold competitor to ^{51}Cr labelled K562 cell ratio which gave 50% inhibition from a regression analysis of the linear portion of the dose response curve. Once the ratio of competitor to target cell ratio was known, the value of $IU_{50}/10^6$ cold competitors was calculated according to formula (2).

$$IU_{50}/10^6 \text{ cold competitors} = \frac{1\times10^6 \text{ competitors}}{(\text{competitor/target ratio})(1\times10^4 \text{ targets}) \text{ at } 50\% \text{ inhibition}} \qquad (2)$$

4. Results.

4.1 K562 cells in culture for different periods of time.

K562 cells were analysed under well defined conditions from tissue culture to investigate if a relationship existed between Tf receptor expression on K562 cells and the length of time the cells were grown in tissue culture. Cells were seeded in large tissue culture flasks at a starting density of 1×10^5 viable cells/ml in complete medium. Cells were then taken from culture after 1, 3,

and 5 days, the cells were subsequently analysed for functional Tf receptor expression by Scatchard analysis (Scatchard, 1949) (fig.6), as well as cold competitive activity against ^{51}Cr labelled K562 cells in a standard chromium release assay. As can be observed in table VI, the number of functional Tf receptors is greatest in K562 cells which have been maintained in fresh media 1 day prior to assay. As the length of incubation in tissue culture increases, a concurrent decrease in Tf receptor expression is observed with cells at day 5 exhibiting 65% less Tf receptors then day 1. Control cells were fed 3-4 days prior to assay and exhibited receptor numbers characteristic of day 5 K562 cells. These control cells were were not started in 100% fresh media, but had fresh media added to the flasks so that that their starting density would be between $0.75\text{-}2.5\times10^5$ cells/ml at the time of feeding. Inhibitory units were derived from cold competition experiments, PBL were matched against ^{51}Cr labelled K562 cells plus cold competing cells from day 1, 3, 5, and control K562 cells. A good correlation can be seen between Tf receptor expression and cold competitive activity in K562 cells when compared by this method ($r=0.97$ $P<0.01$).

Fig 6. Scatchard plot of ^{125}I-transferrin to K562 cells grown in tissue culture for 1,3, and 5 days. Cells were maintained in tissue culture at a starting density of $1x10^5$ cells/ml in fresh medium and analysed for transferrin receptor expression after the times indicated. Values for receptor number/ cell were: ■, $5.90x10^5$/cell :O, $4.74x10^5$/cell :●, $2.65x10^5$/cell.

The affinity of K562 cells remained constant over all time periods tested Kd=$9.0x10^{-9}$ M-1. The results of this experiment were repeated with almost identical results.

fig.6

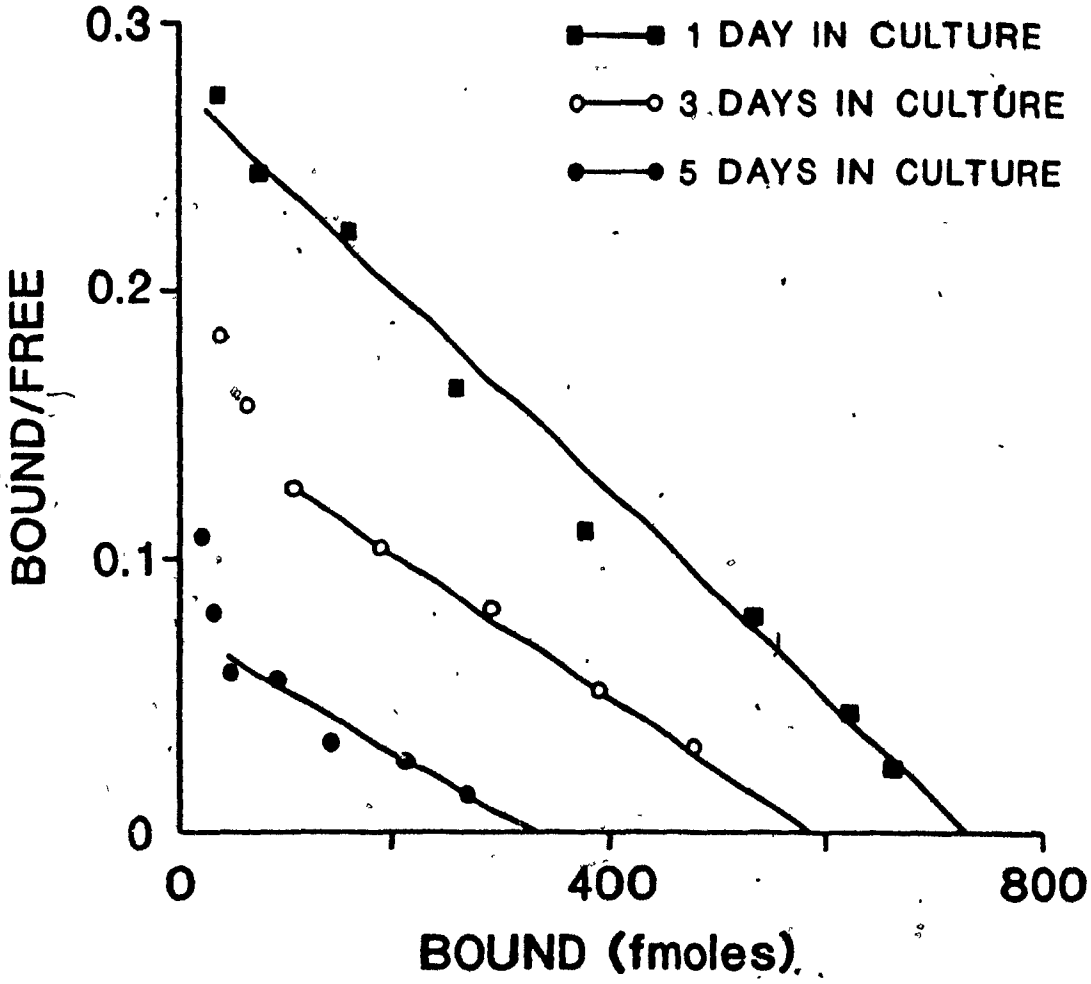

0.3
0.2
0.1
0
BOUND/FREE
0
400
800
BOUND (fmoles)
1 DAY IN CULTURE
3 DAYS IN CULTURE
5 DAYS IN CULTURE

Table VI. Relationship between transferrin receptor expression and competitive activity of K562 cells for NK cells.

TABLE VI

RELATIONSHIP BETWEEN TF RECEPTOR EXPRESSION AND COMPETITIVE ACTIVITY OF K562 CELLS FOR NK CELLS

cell type	cell density at time of assay x10^5	net doubling time in culture at time of assay (in hours)	receptors per cell x10^5	inhibitory units 50%
control K562	pooled a	37.0 b	2.97	28.74
day 1	0.95	0.0	8.11	42.07
day 3	2.85	34.8	4.66	39.68
day 5	7.40	36.2	2.77	29.76

a Cells were harvested from 3 control culture flasks at varying densities (5-8x10^5 cells/ml).

b Typical doubling time as tested in an independent experiment. The net or overall doubling time is based upon gross viable cell counts and does not take into account cell losses during culture.

4.2 Modulation of Tf receptors and competition in K562 cells. K562 cells were subjected to different treatments and analysed for both Tf receptor expression and cold competitive activity against ^{51}Cr labelled control K562 cells. The following methods of Tf receptor modulation were chosen to investigate if the relationship between Tf receptor expression and competitive activity as described in table V would continue when radically different approaches were employed to effect changes in either one of the above parameters. K562 cells have been shown to possess high levels of Tf receptors (Vodinlech et al, 1983; Chapter 2). K562 is also a very sensitive cell line to NK cell mediated cytotoxicity and was therefore arbitrarily assigned values of 100% for Tf receptor expression as well as cold competitive activity. When K562 cells modulated by different means were compared on this scale, it was shown that 3 out of 4 preparations of K562 cells demonstrated a good correlation between Tf receptor expression and ability to compete with ^{51}Cr labelled K562 cells (fig.7,8).

An NK resistant clone of K562 designated as clone I (MacDougall et al, 1983) did not show correlation between Tf receptor expression and cold competitive activity. Since Tf receptors are probably not the only target structure on K562 cells, it is possible that some other target structure has been changed or decreased on this cell line causing decreased recognition by NK cells.

Fig 7. Relationship between transferrin receptor expression and competitive activity of modulated K562 cells. All values for transferrin receptor expression and competitive activity were expressed as a percentage of that obtained with control K562 cells (lane 1). Trypsin pretreated cells were incubated with 0.5% trypsin for 20 minutes then washed. The heat pretreated cells were subjected to 56°C for 30 minutes which totally abolished transferrin receptor function, the value presented indicates nonspecific label uptake by these heat treated cells. The bars represent the overall mean of independent experiments $\pm$ 1 S.E.M..

fig.7

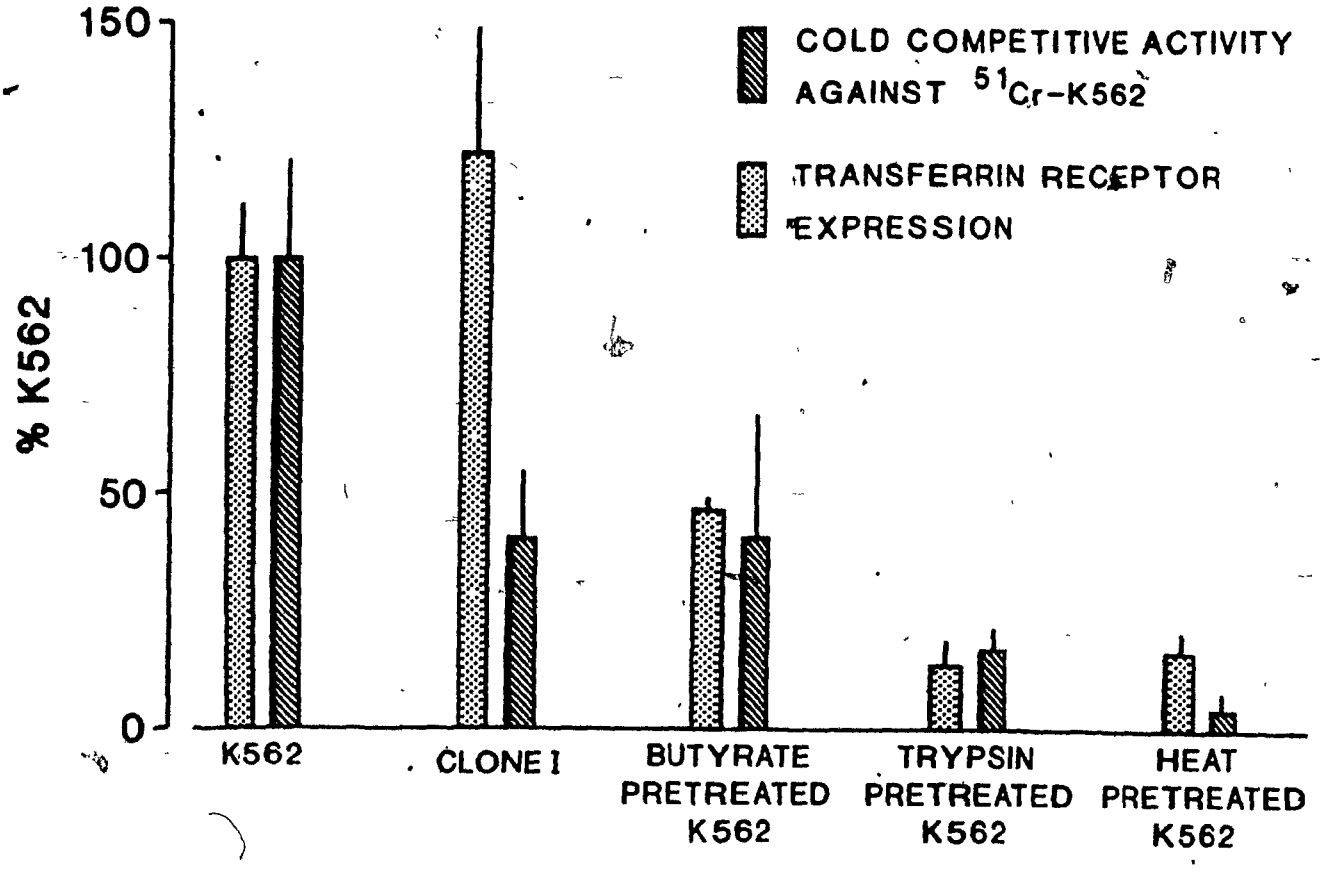
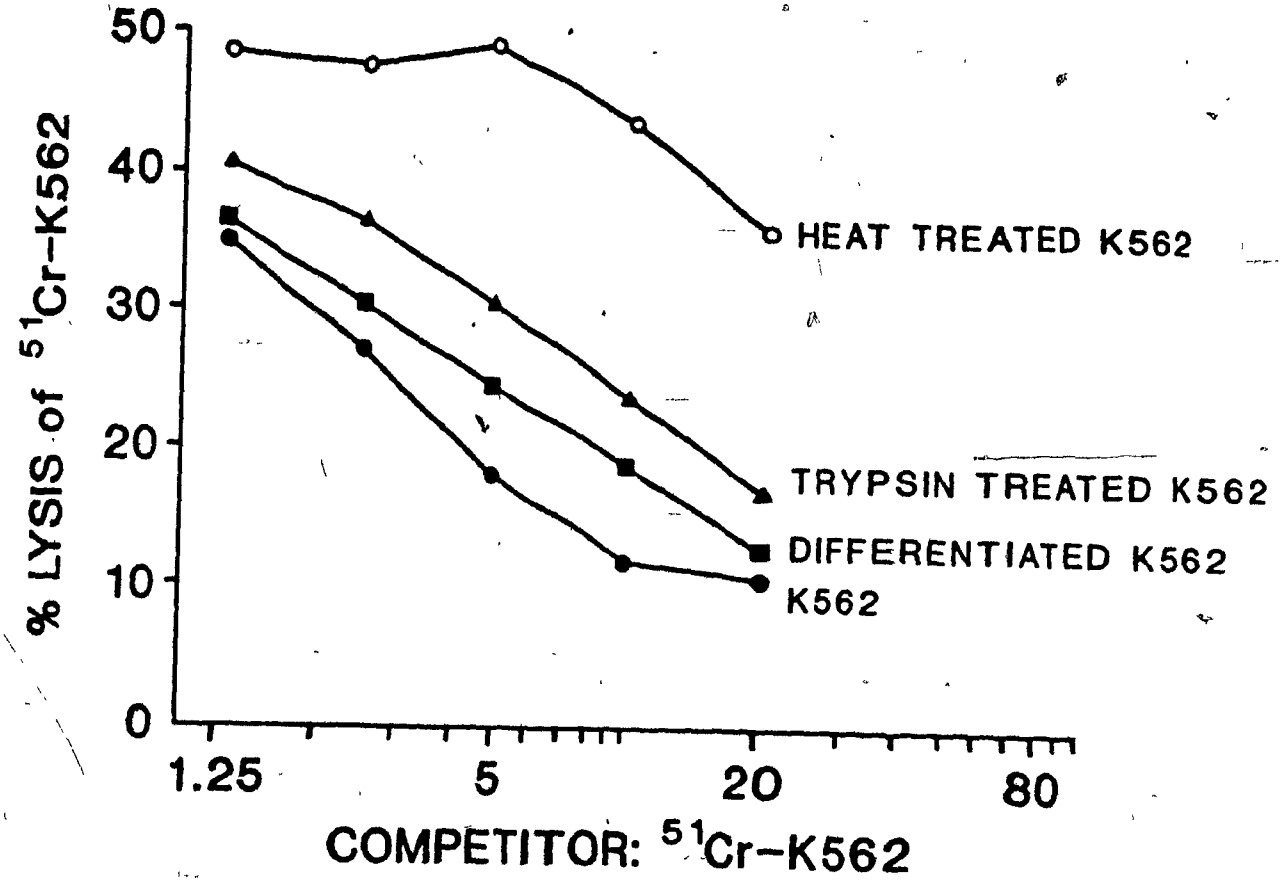

150
100
50
0
% K562
COLD COMPETITIVE ACTIVITY AGAINST ^{51}Cr-K562
TRANSFERRIN RECEPTOR EXPRESSION
K562
CLONE I
BUTYRATE PRETREATED K562
TRYPSIN PRETREATED K562
HEAT PRETREATED K562

Fig 8. Ability of modulated K562 cells to compete with control K562 cells for NK cells. The indicated cells were added to a standard chromium release assay at competitor to target ratios of 1.25, 2.5, 5, 10, and 20 to 1 using ^{51}Cr-labelled K562 cells as the targets. Heat pretreated cells were tested in an independent experiment.

fig.8


50
40
30
20
10
0
% LYSIS of ^{51}Cr-K562
HEAT TREATED K562
TRYPSIN TREATED K562
DIFFERENTIATED K562
K562
1.25
5
20
80
COMPETITOR: ^{51}Cr-K562

Since differentiation has been shown to alter Tf receptor expression, K562 cells were pretreated with 1mM sodium butyrate in tissue culture at least 5 days prior to assay to induce differentiation (Werkmeister et al, 1982). These treated cells display an intermediate level of competition as well as Tf receptor expression which is in good agreement with the results obtained by other authors for both these parameters (Werkmeister et al, 1982; Garson et al, 1983; Testa et al, 1982) (fig.7,8).

If Tf receptors are the major target structures on K562 cells then K562 cells devoid of Tf receptors should result in a population of cells with little competitive activity. Two treatments to severly decrease Tf receptor expression on competing cells were employed. The number of functional Tf receptors were reduced to 13.7% of the original number by trypsin pretreatment of K562 cells, and K562 cells pretreated at 56°C for 30 minutes displayed no receptor function as illustrated in chapter 2. When these two cell preparations were analysed for cold competitive activity it was found that both K562 subtypes competed very poorly with ^{51}Cr labelled K562 cells (fig.7,8).

4.3 Other target cell lines. Different tumor target cell lines were evaluated for Tf receptor expression, cold competitive activity, and NK suseptibility to lysis to investigate if a relationship existed between these parameters. The cell lines K562, Molt-4, MeWo, Raji, and HL-60 were compared. No correlation was evident when comparing Tf receptor expression to lytic sensitivity

(r=0.38 P>0.05), or cold competitive activity against ^{51}Cr labelled K562 cells (r=0.55 P>0.05). This indicates that the expression of Tf receptors is not a major factor when comparing target cells of different origins. The proposal that multiple recognition structures exist on target cells has been previously proposed by several authors (Obexer et al, 1983; Ortaldo and Herberman, 1982), and the results reported here corraborate this proposal.

4.4 Reticulocyte studies. It is well known that a major difference between normal reticulocytes and erythocytes is that reticulocytes possess high numbers of Tf receptors whereas mature erythocytes do not express measurable numbers of Tf receptors (Enns et al, 1981; A. Lazarus and M. Baines, unpublished data). Rabbit reticulocytes have been shown to bind human transferrin (Schulman et al, 1981) and should therefore be functional in a human system. If Tf receptors are true recognition structures on target cells, then these reticulocytes should specifically compete with K562 cells for NK cells. The ability of rabbit reticulocytes to compete with ^{51}Cr labelled K562 cells in a human NK cell chromium release assay is illustrated in table VII. Rabbit reticulocytes showed dose dependent competition whereas mature erythrocytes from normal rabbits did not compete. It is conceivable that the anemic rabbits could contain some other factor or cell type other than reticulocytes which may have caused the competitive activity. To investigate

Table VII.

Cold competition analysis of labelled K562 cells by rabbit reticulocytes. New Zealand White rabbits (2.5 Kg.), were bled via the central ear arteriole for 50 mls per day for 5 consecutive days. On the 6th day, whole rabbit blood was collected in tubes containing heparin and the blood was layered on a Ficoll-Hypaque density step gradient (specific gravity 1.077 gm/cc), and centrifuged for 30 minutes at 400 G as previously described. The mature erythrocytes and leukocytes sedimented through the density medium to the bottom of the tube and the cells at the interface were collected. The cellular content of the upper layer was found to be up to 95% reticulocytes as determined by the New Methylene Blue staining procedure. Intermediate percentages of reticulocytes were obtained by mixing together mature erythrocytes and reticulocytes to obtain the desired suspension in balanced salt solution.

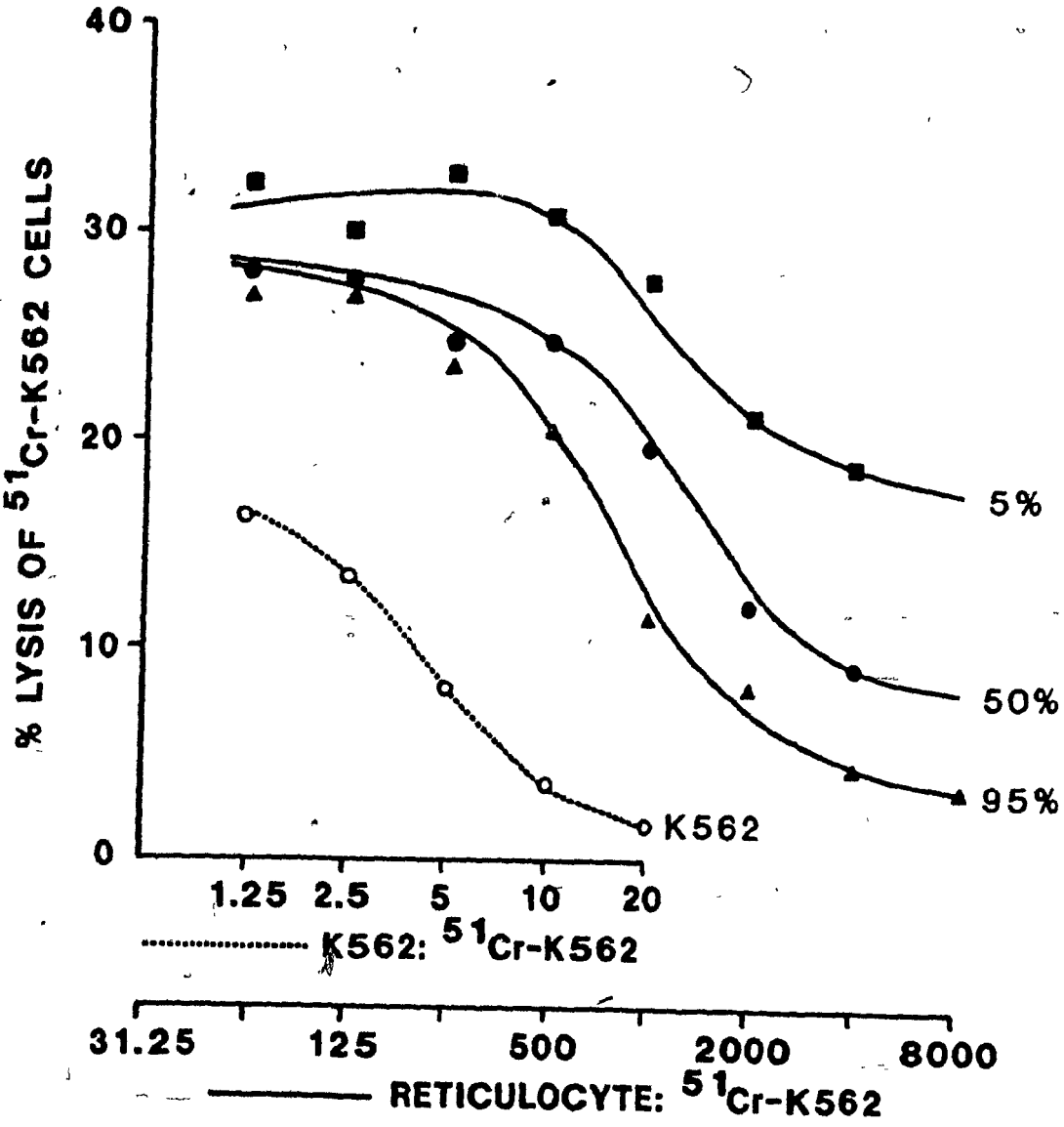
TABLE VII

COLD COMPETITION OF LABELLED K562 CELLS BY RABBIT RETICULOCYTES

cold competitor	actual % of reticulocytes in population[a]	$IU_{50}/10^9$
normal rabbit erythrocytes	>1.5	3.0
rabbit reticulocytes	82.8	105.7
K562	-	30,352.0

[a] as measured by the "new methylene blue" staining procedure.
$P \sim 0.001$ for all values of $IU_{50}/10^9$

Fig 9. Ability of rabbit reticulocytes to compete with ^{51}Cr-labelled K562 cells for NK cells. Reticulocytes were purified to 95% purity and diluted with normal autologous erythrocytes to the concentrations indicated. The ratios of reticulocytes to ^{51}Cr-K562 cells is depicted in the lower absisa. Cold K562 cells were used in the same experiment as competitor cells and the ratios of K562 cells to ^{51}Cr-labelled K562 cells is depicted in the upper absisa.

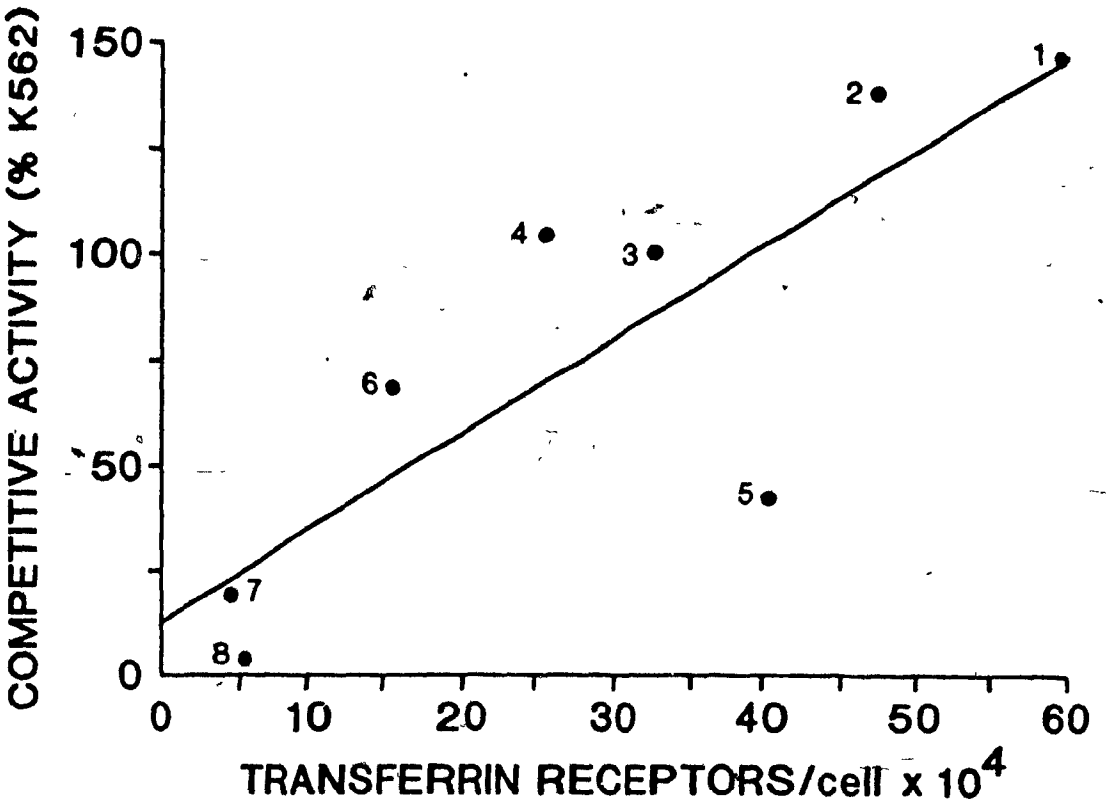

40
30
20
10
0
% LYSIS OF 51Cr-K562 CELLS
5%
50%
95%
K562
1.25 2.5 5 10 20
K562: 51Cr-K562
31.25 125 500 2000 8000
RETICULOCYTE: 51Cr-K562

this possibility, reticulocytes were enriched from anemic rabbit blood to 95% purity and then diluted to different concentrations with mature autologous erythrocytes and analysed for their competitive activity (fig.9). A good correlation between the percentage of reticulocytes in the competitive population and the ability to compete with ^{51}Cr labelled K562 cells can be seen. This indicates that the reticulocytes themselves are responsible for the competitive activity. It should be noted that although reticulocytes were competitive, the level of competition by these cells was relatively weak when compared to that of K562 cells. This could have been due to requirements for species specificity of the Tf receptor, receptor density, or the co-expression or absence of other relevant target structures.

4.5 Correlation analysis. To assess the cumulative results between Tf receptor expression and cold competitive activity in K562 cells from the data obtained, a plot of functional Tf receptor expression versus cold competitive activity was constructed (fig.10). As can be seen, a good correlation exists between Tf receptor expression and cold competitive activity in K562 cells. This further supports the contention that the Tf receptor may be one of the target structures on K562 cells. Since it has been previously shown by this laboratory that functional Tf receptor expression may be involved in NK cell recognition (Baines et al, 1983) and that the number of immunologically measurable Tf receptors can differ significantly from the number of functional Tf receptors, functional receptors were measured by Scatchard analysis on all cell preparations tested. The correlation is therefore directly between functional receptors and competition in this study.

Fig 10. Correlation analysis between transferrin receptor expression and competitive activity against control K562 cells for NK cells. K562 cells grown in culture for; (1) 1 day, (2) 3 days, (4) 5 days, (3) control K562 cells, (5) clone I cells, (6) sodium butyrate induced K562 cells, (7) Trypsin pretreated K562 cells, (8) heat pretreated K562 cells. $r=0.82$ $P<0.01$.

fig.10


COMPETITIVE ACTIVITY (% K562)
150
100
50
0
0
10
20
30
40
50
60
TRANSFERRIN RECEPTORS/cell x 10^4
1
2
3
4
5
6
7
8

5. Discussion

In this correlation study competitive activity has been compared to Tf receptor expression. We have been careful not to use "Killing" as a measure of recognition because the final lytic event is dependent on many parameters whereas competition is accepted as a direct measure of recognition (Herberman and Ortaldo, 1980). Further, the ability of K562 cells to be killed by NK cells is not a valid measure of the recognition state of that cell since it has been demonstrated by Hagner that the lytic phase of killing can be dramatically altered by the growth phase of K562 cells (Hagner, 1984). He found that K562 cells could be killed with differing degrees of efficiency depending on the proportion of target blast cells in a given culture which was directly attributable to a more effective lytic mechanism.

Hagner also showed that while the total number of conjugates between unfractionated lymphocytes and K562 cells remained constant over a range of different Tf receptor densities on the target cells, the proportion of $OKT8^+$ lymphocytes forming conjugates varied with the density of Tf receptors on K562 cells. Since it has been shown by two different investigators that a significant proportion of active NK cells carry the $OKT8^+$ phenotype (Abo et al, 1982; Perussia et al, 1983), it is possible that this subpopulation of "$OKT-8^+$ NK cells" may utilize Tf receptors as a target structure on K562 cells.

Alternatively, since the $OKT8^+$ phenotype is indicative of a T suppressor cell, it is possible that these "T suppressor cells" are capable of directly or indirectly modulating the activity of NK cells.

In this study a significant correlation between Tf receptor expression and cold competitive activity of modified K562 cells was found. It is interesting that within this correlation study there was a direct relationship between Tf receptor expression and competitive activity in K562 cells grown under identical culture conditions for different times. It is not known why K562 cells express more Tf receptors during the short term incubation, however it is possible that the cells themselves are initially passing through a stress induced lag phase of growth prior to the start of log phase growth on approximately day 1 (data not shown) to a late log phase of growth on day 5. Alternatively, it is possible that some factor in the growth media which is altered or decreased with time is contributing to the expression of Tf receptors.

To determine if the correlation between Tf receptor expression and competitive activity would remain intact when Tf receptor levels were extremely low or absent, two independent treatments were employed to severely decrease Tf receptor function. K562 cells were either heat-pretreated at 56°C for 30 minutes or treated with trypsin (0.5% for 20 minutes). In both of these treatments the

numbers of Tf receptors as well as competitive activity were severely decreased. However, when the trypsin treated cells were tested as direct targets, their sensitivity to killing was found to decrease by 21%, thus they were still relatively sensitive to killing (data not shown).

In a further attempt to effect the competitive activity of K562 cells and then monitor the expression of Tf receptors, differentiated K562 cells as well as a resistant clone of K562 cells were employed. The sodium butyrate differentiated K562 cells displayed reduced competitive activity and correspondingly reduced Tf receptor expression, however the NK resistant clone, while being a poor competitor displayed substantial levels of Tf receptors. This clone was selected by McDougal and co-workers (1983) primarily on the basis of its inability to form conjugates with NK cells. Clone I exhibits an increased level of acidic and neutral lipids (A. Sullivan and S. MacDougall, personal communication), which should contribute to an alteration in membrane charge. It has been shown that the negative charge imparted to cell membranes by sialic acid residues (Warren, 1976) may lead to a decrease in sensitivity in killing target cells by NK cells (Yogeeswaran et al, 1982). The point is germain to this study because if membrane charge is in fact an important parameter in cell to cell binding and recognition, then the effect of Tf receptor expression on Clone I cells may be dominated by this other membrane

parameter. It is interesting to note that the original clone I cell line displayed a lower number of Tf receptors and a lower doubling time than K562 cells (A. Sullivan and S. MacDougall, personal communication) however under the conditions in this laboratory, clone I cells displayed high numbers of Tf receptors and had a faster doubling time than K562 cells while retaining their degree of NK resistance. (see also appendix VI)

Baines et al (1983) found that iron saturated transferrin could inhibit killing of K562 target cells at the level of the effector cell, as well as conjugate formation between lymphocytes and target cells. In addition it was found that antibodies to transferrin itself could block effector cell function, this later parameter has since been confirmed by Alarcon and Fresno (1985) These findings specifically implicated the involvement of the Tf receptor at the functional level. To determine if "functional" Tf receptors are target structures on K562 cells as opposed to an epitope of the native Tf receptor (or a neighbouring protein), rabbit reticulocytes were tested for their competitive activity. The rational was that epitopes of the Tf receptor in rabbit reticulocytes are most likely different from Tf receptor epitopes on human tumor cells, however, rabbit reticulocytes can bind human transferrin (Schulman et al, 1981) which indicates that these reticulocytes possess Tf receptors that are functionally similiar to their human

counterparts. These reticulocytes were capable of competing with K562 cells (albeit weakly) in a dose dependent manner. Mature rabbit erythrocytes on the other hand which do not possess measurable levels of Tf receptors (Lebman et al, 1982) could not compete with K562 cells for NK cells. This further implies a role for functional Tf receptors as target structures on K562 cells. It was subsequently found that the rabbit reticulocytes and erythrocytes could not be lysed by human NK cells (data not shown). The possession of Tf receptors was therefore only involved in recognition and not a sufficient condition for target cell lysis to proceed in these cells. Thus it seems unlikly that Tf receptors are involved in the lytic phase of NK cell mediated lysis.

Work by Dokhelar and co-workers (1984) appeared to have data which contradicts the hypothesis that Tf receptors are a possible target structure on tumor cells. Upon careful scrutiny of this data, the experiment which compared K562 cells grown at either log phase or plateau phase of growth actually displayed in 3 out of 4 experiments that the log phase cells (which displayed higher levels of Tf receptors) could be killed more readily than the cells maintained in the plateau phase of growth. Although the difference in killing appeared small by utilizing a single data point, a small difference may in fact be significant. There have been several publications which have dealt with the mathematical evaluation of cytotoxicity data (Pross et al, 1981;

Callewaert et al, 1983). However, many authors continue to express differences in activity as absolute percentage cytotoxicity, and often utilizing a single data point (E/T ratio). It has been previously stated that this is not a valid means of data analysis due to the dose response nature of the cytotoxicity assay (Pross et al, 1981) and the need to utilize data from the reliable linear portion of the graph.

Different tumor cell lines were evaluated to assess the contribution of Tf receptor expression on the competitive activity of these cells. No significant correlation between these two parameters was obtained. It has been proposed by several laboratories that more than one target structure may be present on tumor cells (Phillips et al, 1980; Obexer et al, 1983) and thus the modulation of Tf receptors may not be a major parameter when comparing different cell lines. It has also been shown in cloned NK cell lines that heterogeneity exists at the level of the effector cell (Allevena and Ortaldo, 1984). Thus all effector cells may not recognize the same determinant on target cells and there may in fact be 7 or more specificities demonstrable by NK cells (Phillips et al, 1980). This would minimize the contribution of a single determinant as measured in a chromium release assay. In addition, Clone I cells displayed sufficient levels of Tf receptors to compete with K562 cells but were still somewhat resistant to NK mediated recognition. This

has implications concerning the nature of the involvement of Tf receptors in NK recognition. The recognition system employed by NK cells may be more complex than originally conceived, in that Tf receptors may only be a minor but specific target structure. Alternatively, Tf receptors may serve soley as an affinity structure between effector and target cell thus facilitating the interaction between effector and target cell.

In conclusion, there was a good correlation ($r=0.82$ $P<0.01$) between the expression of Tf receptors on K562 cells and the ability of these cells to compete in a standard chromium release assay using control K562 target cells. These results support previous studies which have indicated that Tf receptors are involved in NK cell mediated specificity of human tumor cells.

IV. TRANSFERRIN RECEPTORS IN HUMAN NATURAL KILLER CELL SPECIFICITY: ANALYSIS OF TRANSFERRIN RECEPTOR BINDING TO PERCOLL FRACTIONATED LYMPHOCYTES USING A SOLID PHASE ASSAY SYSTEM.

1. Abstract

The initial event leading to target cell lysis by Natural Killer (NK) cells involves binding between the NK cell and a target cell. It was postulated that transferrin (Tf) receptors on the tumor cell could serve as target structures for human NK cells. Treatment of PBL with antibody plus complement indicated that at least a subpopulation of NK cells expressed a Tf-crossreactive epitope. Since Tf receptors on tumor cells bound Tf with high affinity, it was possible that NK cells could use this epitope to interact with tumor cells to form a high affinity conjugate.

To investigate if these Tf receptors could be recognized by NK cells, a solid phase receptor binding assay was developed. As a model system, it was demonstrated that nitrocellulose immobilized Tf retained its specific functional receptor binding capacity. This technique was quantitative and proved to be sufficiently sensitive to specifically detect nanogram quantities of Tf receptor protein. Binding was assessed using an ELISA based system.

Human PBL were fractionated by discontinous Percoll

density centrifugation, bound to nitrocellulose, and evaluated for Tf receptor binding capacity. A sample aliquot of cells from each Percoll fraction was retained to assess NK cell activity. It was observed that there was no positive relationship between NK cell activity and Tf receptor binding capacity in these Percoll fractionated cells. In addition, analysis of the above parameters with IL-2 boosted lymphocytes did not lead to any positive relationship between NK cell activity and apparent Tf receptor binding capacity in Percoll fractionated lymphocytes.

These findings do not therefore support a role for Tf receptors in the NK cell:target cell interaction.

2. Introduction

NK cells comprise a group of MHC unrestricted lymphocytes that have the ability to lyse tumor cells without prior sensitization or immunization (Herberman and Holden, 1978). The molecules present on the tumor cell recognized by the NK cell in both the murine and human systems have not however been definitively established. The recognition structure used by the NK cell has also not been established and studies on the T cell receptor (Ti) have indicated that this molecule(s) does not appear to be involved in endogenous NK cell mediated recognition

(Reyonolds et al, 1985; Lanier et al, 1986; Tutt et al, 1986; Borst et al, 1987; Kishihara et al, 1987; Biron et al, 1987). Several molecules have been proposed as NK target structures including, the Tf receptor (Baines et al, 1983; Vodinelich et al, 1983), GM_2 (Young et al, 1981; Ando et al, 1987), SSEA-1 (Harris et al, 1984), Fc receptors (Perl et al, 1986), TNK_{tar} (Hercend et al, 1983; Hercend et al, 1984), and the laminin receptor (Hiserodt et al, 1985; Hiserodt et al, 1985). The Tf receptor is conceptually the most interesting candidate because, in contrast to the other proposed target structures, it is expressed on roughly the same spectrum of cells that are also sensitive to NK cell mediated lysis. This list includes tumor cells, bone marrow cells, fetal cells, thymic cells, and virally transformed cells.

The possible involvement of the Tf receptor in NK specificity was first suggested in 1983 (Baines et al, 1983; Vodinelich et al, 1983). Since then, however, the involvement of Tf receptors in NK specificity has been a very controversial topic. Initial studies in this laboratory showed that, at physiological concentrations, the normal serum iron binding protein Tf could block both NK cell activity and conjugate formation between peripheral blood lymphocytes (PBL) and K562 cells (Baines et al, 1983). The inhibitory effect of Tf on NK cell activity against K562 cells was also found by Bierman and co-workers (1984), and Phillips (1986), Borysiewicz and

co-workers showed similiar effects against virally infected cells (1986).

Data from antibody studies on effector cells indicated that pretreatment of PBL or LGL with either intact or F(ab)'2 rabbit antibodies raised against human serum Tf block NK cell activity (Baines et al, 1983; Alarcon et al, 1985). When target cells were pretreated with these antibodies against serum Tf, no effect was seen (Baines et al, 1983). These results were interpreted to suggest that NK cells possess Tf on their surface (Baines et al, 1983).

The Tf receptor is a homo-dimeric glycoprotein (dimer Mr=180Kd) and each dimer in turn is composed of 2 identical subunits of 95 Kd each (Schneider et al, 1982). It is thought that each subunit is capable of binding one molecule of Tf (Schneider et al, 1982). Trypsin cleavage of the plasma membrane bound Tf receptor liberates a 70 Kd fragment which retains the ability to bind Tf (Omary et al, 1981; Schneider et al, 1982; Vodinelich et al, 1983). Vodenlitch and co workers (1983) have shown that this Tf receptor fragment but not HLA-B fragments can block NK cell activity towards K562 cells.

Taken together, the above results suggest that the transferrin receptor is a target structure on tumor cells recognized by NK cells, and that NK cells utilize membrane bound Tf to interact with the tumor cell Tf receptor.

If the above proposed model is correct, then antibodies against the Tf receptor should block NK cell

activity at the target cell level. Antibody blocking studies on target cells, however, have provided confusing and contradictory results. Since murine monoclonal antibodies can be recognized by human NK cells (Ortaldo et al, 1987; Park et al, 1984; Herlyn et al, 1985), sensitizing a target cell with antibody should predispose that cell to attack by an ADCC mechanism (see chapter I). Thus antibody blocking studies at the target cell level should provide confusing results; on the one hand, antibodies to Tf receptors should sensitize the target cell to attack by ADCC while on the other hand, if Tf receptors are target structures on tumor cells recognized by NK cells, then the killing of the target cell should be blocked. Most antibody studies against NK target cells have either blocked NK activity or had no measurable effect (Vodinelich et al, 1983; Perl et al, 1986; Golightly et al, 1984; Dokhelar et al, 1984). Perhaps the use of F(ab')2 antibodies would be a better choice as a blocking agent.

If Tf receptors are target structures on tumor cells which are recognized by NK cells, then Tf receptor expression on tumor cells should correlate with target cell competitive activity. We have reported a correlation between Tf receptors on K562 tumor cells and competitive activity (chapter III) and this is supported by several other groups (Vodinelich et al, 1983; Borysiewicz et al, 1986; Newman et al, 1984) although some

disagree (Reiber et al, 1986; Phillips, 1986; Storkus and Dawson, 1986; Schurman et al, 1985; Lauzon and Roder, 1985; Dokhelar et al,1984).

To further investigate the involvement of Tf receptors in NK specificity, this study has used nitrocellulose immobilized lymphocytes as an affinity matrix to determine if NK cells bind Tf receptors. The results indicate that there is no relationship between NK cell activity and apparent transferrin receptor binding in both percoll fractionated lymphocytes and percoll fractionated IL-2 activated lymphocytes. Thus this data does not support the hypothesis that Tf receptors are involved in a high affinity association between NK cells and tumor cells.

3. Materials and methods

3.1 Tumor cells. The erythroleukemic human tumor cell line K562 (Lozzio and Lozzio, 1975), and HL-60 were grown in suspension culture and maintained at 37°C in a humid environment in 5% CO_2. The cells were cultured in RPMI-1640 suplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 IU/ml penicillin-G, 100 ug/ml streptomycin sulfate, and 10 mM HEPES. K562 (d1) cells were grown in tissue culture at a starting density of $1x10^5$ cells/ml in 100% fresh media. Control K562 cells were fed every 3-4 days and generally used on day 3.

3.2 Lymphocyte isolation. Heparinized blood was collected

from healthy volunteers by venipuncture, and incubated on tissue culture grade plastic for 1 Hr @ 37' C. The non-adherent cells were gently rinsed off and then diluted with an equal volume of RPMI-1640. The lymphocyte population was separated by buoyant density centrifugation on Ficoll/Hypaque (sp. gr.= 1.077). The cells were washed 3 times in RPMI-1640 and resuspended in 10mM Ca^{++} and Mg^{++} free PBS at 285 mOsM/Kg. The lymphocytes were then put into the bottom fraction of a discontinuous percoll gradient and centrifuged at 13,500xG for 30 min. Each lymphocyte fraction was carefully removed with a Pasteur pipette, washed 3 times in RPMI-1640, and resuspended in RPMI-1640 + 10% FBS for analysis of NK cell activity. Fresh lymphocytes that were used directly for dot-blot analysis were washed in PBS and resuspended in PBS without protein. These cells were either used immediately or frozen at -80'C until used.

3.3 Electron Microscopy. Human PBL pellets were lightly fixed in 0.02% gluteraldehyde for 5 min and then in 2.5% paraformaldehyde for 60 min. The cells were then incubated in 1% osmium tetroxide for 1 Hr, rinsed in PBS, dehydrated in acetone and embedded in epon. The blocks were then cured for 3 days at 60'C. Ultrathin sections were cut on an LKB microtome, collected on copper grids and then etched with sodium metaperiodate. The grids were then blocked in 1% BSA for 30 min and transferred to a 1/200 dilution of either rabbit anti-human transferrin or normal rabbit serum in 1% BSA for 60 min. The grids were

rinsed in PBS and incubated with protein A-gold (23nM (±6) in diameter) for 30 min at 25'C. The grids were then washed extensively in PBS, followed by H_20, and then stained with uranyl acetate and examined under a Phillips model 300 electron microscope. The percentage of positively stained cells were evaluated as follows; a field of cells were photographed at random and the resulting prints were analysed blind. Cells were evaluated for the number of gold grains observed on the outer leaflet of the plasma membrane. The mean number of colloidal-gold grains on normal rabbit serum stained lymphocytes was 8 grains/cell. The percentage of cells containing more than 8 electron dense gold grains from the normal rabbit serum controls were subtracted from the percentage of cells containing more than 8 gold grains from the anti-Tf treated cells. The expression of Tf-crossreactive epitopes on lymphocytes were also evaluated using a monoclonal antibody to TF (MoAb-Tf; Serotech laboratories inc.). Antibodies NKH-1 and MoAb-Tf were used at a 1/20 and 1/100 dilution respectively. Lymphocytes with more than 5 grains of gold were counted as positive for MoAb-Tf as well as the isotype identical NKH-1 antibody (IgG1; Coulter Electronics inc.).

3.4 Flow cytometry. Percoll fractionated human PBL were resuspended in RPMI-1640 containing 5% FBS plus 4 dilutions of anti-Tf antibody HTF-14 (1/15 to 1/500) at 4'C for 30 min. Antibody HTF-14 was a kind gift from Drs.

V. Vicklicky and J. Bartek (Czechoslovak Academy of Sciences) The cells were then washed 2 times and incubated in 10ug/ml of Fab'2 sheep anti-mouse IgG:FITC for 30 min. The cells were washed, resuspended in RPMI-1640, 5% FBS with propidium iodide and analysed for flourescence. Dead cells and RBC were gated out of the analysis by red flourescence and size respectively. Background binding was evaluated by leaving out the primary antibody.

3.5 Antibody depletion. Human PBL ($1x10^6$/ml) were incubated with a monoclonal antibody to human transferrin (HTF-14),a commercial monoclonal antibody to human transferrin (MoAb-Tf) (Serotech laboratories inc.) or anti-transferrin receptor (Becton Dickinson, Mountain View) At 4'C for 30 min. The cells were then washed and developed with 10ug/ml of a goat anti-mouse IgG at 4'C for 30 min. The cells were washed and resuspended in 10% low-tox rabbit complement (Cederlane laboratories inc., Toronto) at 37'C for 30 min. The cells were then washed and tested for residual NK cell activity without adjusting the cell concentration. Control cells recieved only the developing antibody. Complement controls cells received all reagents with the exception of the primary antibody.

3.6 Assay for NK cell activity. The ability of NK cells to lyse K562 target cells was assessed by the standard chromium release assay (Pross and Jondal, 1975). Fifty ul of ^{51}Cr-labelled K562 cells were added to each well of a V-bottom microtiter plate containing six serial dilutions

of lymphocytes in 100 ul. The plates were centrifuged at 200xG for 5 min, and then incubated @ 37'C for 4 hrs. Subsequent to incubation, the supernatants were collected for gamma counting. The spontaneous target death was derived by incubating $1x10^4$ ^{51}Cr-labelled targets in the absence of lymphocytes. Replicate wells were resuspended prior to supernatant harvest to determine the maximum kill possible. The percentage of ^{51}Cr-labelled K562 cells killed was calculated by formula (1):

$$\%\ \text{lysis of K562 cells} = \frac{100-(\text{test } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release})}{\text{maximum } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}} \quad (1)$$

Values of lytic units ($Lu_{20}/10^6$ effector cells) were derived from a regression analysis of the linear portion of the dose response curve. Once the ratio of effector to target cell was calculated, the value of $LU_{20}/10^6$ effector cells was calculated according to formula (2).

$$LU_{20}/10^6 \text{ effectors} = \frac{1x10^6 \text{ effectors}}{(\text{lymphocyte:target cell ratio at 20\% lysis})(1x10^4 \text{ targets})} \quad (2)$$

3.7 Cold competition analysis. The ability of cell lysates to compete with a control K562 population was assessed in a modification of the standard chromium release assay. Ten thousand ^{51}Cr-labelled K562 cells were added to each well of a microtitration plate followed by cold competitor cell lysates. The lysates were evaluated as cell equivalents and were added to the assay at ratios from 200/1 to 6.25/1 cell equivalents to ^{51}Cr-labelled K562 cells. Two hundred thousand lymphocytes were then added to these wells to give an effector to target ratio of 10/1. The spontaneous target death was derived by incubating $1x10^4$ ^{51}Cr labelled targets in the absence of lymphocytes. Replicate wells were resuspended prior to supernatant harvest to determine the maximum kill possible. The percentage of ^{51}Cr-labelled K562 cells killed was calculated by formula (1). Maximum killing by effector cells was evaluated by including wells without cold competitor cell lysates. The 50% inhibition level was determined by dividing the percent kill due to 10/1 effector cells without competitors by 2. Values of inhibitory units at 50 % inhibition ($IU_{50}/10^6$ cold competitor equivalents) were derived by calculating the cold competitor to ^{51}Cr-labelled K562 cell ratio which gave 50% inhibition from a regression analysis of the linear portion of the dose response curve. Once the ratio of competitors to target cells was determined, the value

of $IU_{50}/10^6$ cold competitor equivalents was calculated according to formula (2).

3.8 IL-2 boosting of NK cell activity. One million Percoll fractionated lymphocytes/ml were incubated in 25 BRMP U/ml of $_r$IL-2 (Cetus; Cellular products inc., Buffalo, N.Y.) for 18 Hr in RPMI-1640 supplemented with 10% autologous human plasma at 37°C. The lymphocytes were then washed 3 times in RPMI-1640 and resuspended in RPMI-1640 with 10% FBS. The optimal incubation time and dosage of $_r$IL-2 utilized was previously determined in an independant experiment using unfractionated PBL.

To assess the net increase in NK cell activity due soley to exogenous $_r$IL-2, net lytic unit values were derived according to formula (3).

$$\text{net increase in lytic units due to IL-2} = \text{lytic units of lymphocytes incubated with IL-2 for 18hrs} - \text{lytic units of lymphocytes incubated without IL-2 for 18hrs} \qquad (3)$$

3.9 Tumor cell lysates. The stability of specific NK target structures was assessed by subjecting NK sensitive (K562) and NK insensitive (HL-60) tumor target cells to a freeze-thaw and sonication procedure. The competitive nature of the resulting cell lysates was then evaluated. Tumor cells were removed from tissue culture at log phase growth, counted for viability using trypan blue and processed concomitantly. Cells were washed 3 times in RPMI-1640 and resuspended to $1x10^7$ cells/ml. They were

then subjected to 3 cycles of freezing in dry ice and ethanol, followed by thawing in a 37'C water bath. The lysates were then subjected to intermittent sonication @ 4'C for ca. 1 min. This treatment resulted in total cell disruption as assessed by light microscopy. Large debris and nuclei were then centrifuged out at 350xG for 5 minutes and the lysates were tested for competitive activity. Transmission electron microscopy of the cell lysates indicated that this treatment resulted in the generation of unilaminar microsomes (data not shown).

3.10 Dot-Blot. The basic dot-blot technique was performed on a Bio-dot apparatus (Bio-rad laboratories; Misissauga, Ont.). Pure 0.45 um nitrocellulose (Schleicher & Schuell; Keene, N.H.) was equilibrated in 20 mM Tris, 500mM NaCl, pH 7.5 (TBS) for 30 min prior to the application of the primary protein/lysate. The nitrocellulose was then inserted into the dot-blot apparatus and reacted with the primary protein/lysate in 100ul of TBS. The membrane was then rinsed with TBS, removed from the apparatus, and blocked overnight in 3% gelatin (Difco laboratories; Detroit MI.) in TBS at 25'C. All subsequent incubations were performed on the free membrane. The assay was performed in this manner because whole cell lysates (even when clarified @13,000 xG for 15 Min.) clogged the nitrocellulose and interferred with the results.

3.11 Tf receptor binding assay. K562 cell lysates were used as described above except that sonication was not

performed and freeze-thawed cells were resuspended at $1x10^6$ cells/ml in TBS plus 1% gelatin. These K562 cell lysates were then incubated with nitrocellulose that had been previously reacted with the primary protein or lysate. This incubation was always performed overnight, and the unbound material was then extensively washed with TTBS, and the nitrocellulose was probed for the presence of bound Tf receptors.

The extent of Tf receptor expression on blotted membranes was performed as follows: Nitrocellulose was incubated with the antibody HuLy-M9 (Australian monoclonal development Pty. Ltd; Artarmon, Australia) at a dilution of 1/5,000 in TBS with 0.5% polyoxyethylene sorbitan monolaurate (Tween 20) (TTBS) (Sigma; St Louis, MO) for 2 Hr at 25'C. The membrane was then extensively rinsed with TTBS and incubated in a 1/1,000 dilution of affinity purified alkaline phosphatase conjugated goat anti-mouse $IgG_{(H+L)}$ immunoglobulin (Jackson Immunoresearch laboratories, Inc. cat. No. 02HLEUK11; West Grove, PA) for 1 Hr at 25'C. The membrane was subsequently washed in TTBS, TBS, and then incubated in 0.3 mg/ml nitro blue tetrazolium salt 0.15 mg/ml 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt (Sigma; St. Louis, MO) in 0.1 M $NaHCO_3$, 1.0 M $MgCl_2$, pH 9.8 until the desired level of development was achieved. The reaction was stopped by immersing the nitrocellulose in H_2O Note, all immunological reagents were previously titrated for optimal working concentrations in the dot-blot assay and

all incubations were carried out in 1% gelatin in TTBS. Lymphocytes contain a small amount of endogenous alkaline phosphatase and some contain minute levels of Tf receptors. To circumvent the contribution of this background noise, binding is assesed by formula 1:

$$\text{Tf receptor binding} = \text{absorbance of Tf receptors on K562 treated lymphocytes} - \text{absorbance of Tf receptors on K562 untreated lymphocytes} \quad (1)$$

The values for net Tf receptors bound by rIL-2 activated NK cells were assessed according to formula (2):

$$\text{net Tf receptors bound} = \frac{(\text{abs. of IL-2-lymphocytes with K562 lysate treatment}) - (\text{abs. of IL-2-lymphocytes without K562 treatment})}{(\text{abs. of control lymphocytes with K562 lysate treatment}) - (\text{abs. of control lymphocytes without K562 lysate treatment})} \quad (2)$$

3.12 Lysate adsorption with Tf. K562 cell lysates at 8×10^6 cell equivelants/ml were specifically blocked (adsorbed) with or without human serum Tf at 20 mg/ml for 30 min @ 25°C. The lysates were then diluted to 1×10^6 cell equivelents/ml in TBS with 1% gelatin and used. The absorbed lysates were not centrifuged at this point because the Tf receptors are still embedded in the plasma membrane and extensive centrifugation results in lower Tf receptor yields as measured in an independant experiment (data not shown).

3.13 Dot-blot quantitation. Dot-blots were quantitated by a photoreproducion method. The darkened image produced on nitrocellulose using the dot-blot template is superimposable on a enzyme-linked immunosorbant assay (ELISA) microtiter plate reader by affixing the matrix on top of a 96 well ELISA plate. Since nitrocellulose is opaque, however, the developed membrane is first photoreproduced onto acetate (using a high quality xerox 1048) and this acetate is affixed on top of the ELISA plate and quantitated at 690nm. This method of reproduction gives a consistant result. As can be seen in figure 3 there is a good correlation between the darkness of a dot and the relative absorbance as assessed by this photoreproduction method. The two previously defined K562 cell populations, K562(control) and K562(dl) (Chapter III section 4.1) were analysed by this technique for Tf receptor expression and the results were compared to Scatchard analysis. The relative quantity of Tf receptors expressed in these two populations generally coincides. Thus the analysis of Tf receptors by this dot-blot method is a valid method of quantitatively measuring Tf receptors on nitrocellulose. While most photoreproduction methods produce an image which is ca. 2% larger that the original, it has been our experience that this enlargement is not a problem with the ELISA plate reader used (Titertech multiscan MC;Flow laboratories) as long as the acetate is carefully aligned on the plate. Alternatively, the xerox 1048 as well as most similiar

machines can be easily modified by the manufacturer to give a 0% enlargement of the original upon special request.

4. Results

4.1 Identification of an epitope on human peripheral blood lymphocytes crossreactive with human serum transferrin. Human PBL thin sections were lightly fixed and labelled with either a polyclonal sera raised against human serum Tf or normal rabbit serum. The sections were then incubated with protein A coupled to collodial gold and examined under an electron microscope (fig.11). The percentage of positively stained lymphocytes were evaluated as described in materials and methods. Figure 12 indicates that 9.9% of PBL contain a Tf-crossreactive epitope using a polyclonal antisera and roughly the same number using a monoclonal antibody raised against Tf. For comparison, the percentage of NKH-1 stained cells is presented, the frequency of positively stained cells using this monoclonal antibody was somewhat higher than expected (ie. 23%).

To characterize the nature of the Tf-crossreactive lymphocyte, human PBL were subjected to percoll fractionation, stained with the Tf specific monoclonal antibody HTF-14, and then analysed by flow cytometry. Table VIII indicates that the majority of Tf-crossreactive PBL reside in the low density fraction of percoll separated lymphocytes. The 42.5% and 45%

Fig. 11. Electron micrograph of a Tf^+ human peripheral blood lymphocyte. This cell was stained with rabbit anti-human transferrin antisera plus protein A-gold. The number of gold graines on this cell is 21. The mean number of gold grains on a NRS stained lymphocyte was 8 grains/cell. Note the large bilobed nucleus and cytoplasmic granules (Magnification X 16,400).

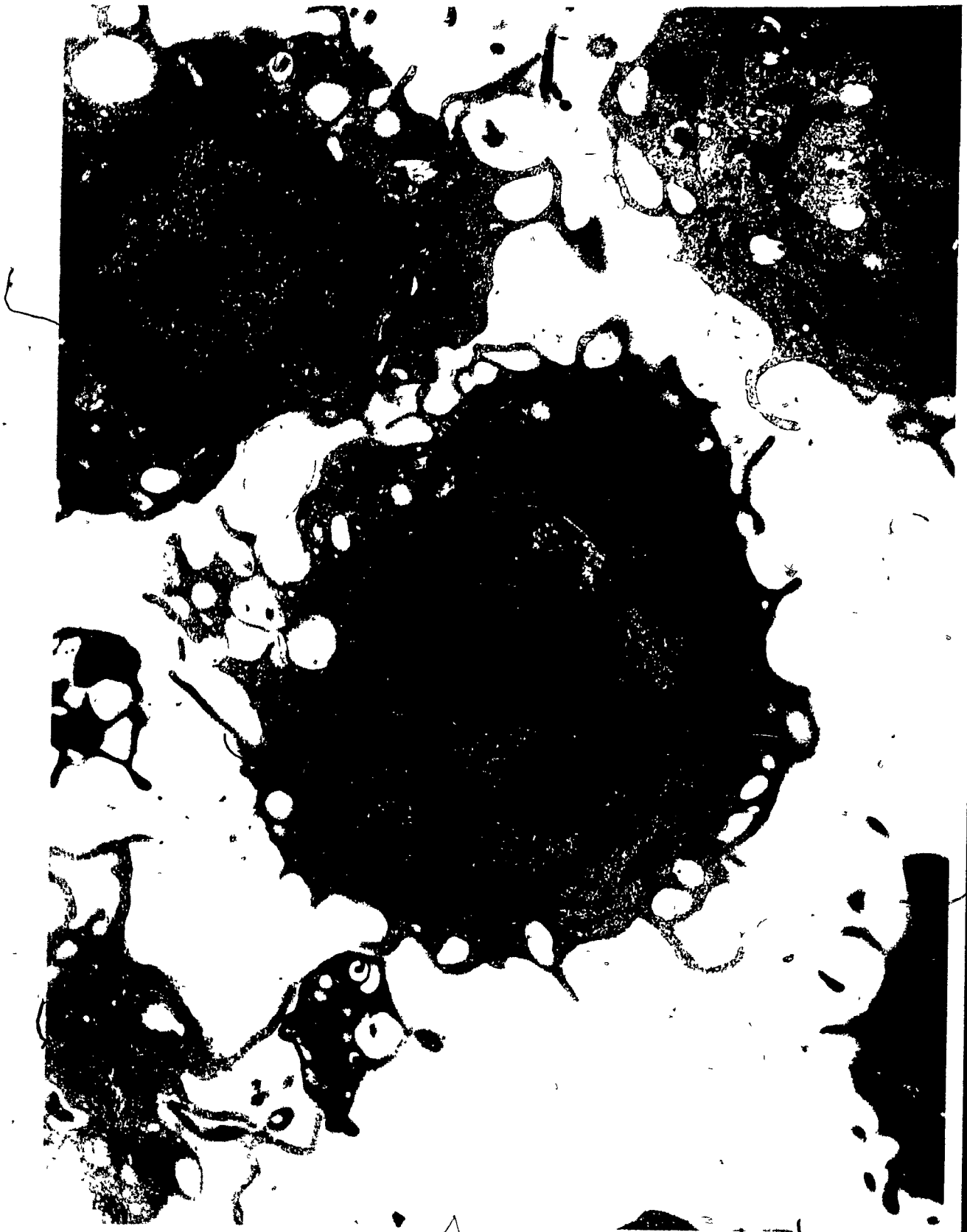


Fig. 12. Electron microscopy analysis of Tf^+ H-PBL. Tf^+ PBL were assessed by enumerating randomly sampled immuno-gold stained lymphocytes by electron microscopy. RaHuTf is a polyclonal rabbit antisera raised against human serum transferrin. Control sections for this polyclonal sera were stained with normal rabbit serum and the values obtained subtracted from data derived with the RaHuTf sera. MoAb-Tf is an anti-transferrin monoclonal antibody from serotech laboratories inc.. NKH-1 is a monoclonal antibody purshased from Coulter electronics that reacts with human NK cells. These results indicate that a small proportion of human lymphocytes contain an epitope cross reactive with human serum transferrin.

Fig. 12

Electron microscopy analysis

Percent positive staining H-PBL's

Antibody	% Positive	S.E.M.	n
RaHuTf	9.9	2.1	4
MoAb-Tf	12.5	4.2	2
NKH-1	23.3	3.3	2

Table VIII. Analysis of Tf^+ percoll fractionated PBL. Flow cytometry was used to analyse the phenotype of percoll fractionated H-PBL's. Lymphocytes isolated from the specified fraction were reacted the monoclonal antibody HTF-14 which is known to react with human serum transferrin (Viklicky and Bartek, 1984). The cells were then stained with a F(ab')2 sheep anti-mouse IgG:FITC antisera and analysed for flourescence. Dead cells (as assessed by propidium iodide uptake) were gated out of the analysis. Background flourescence was assessed by excluding the primary antibody. These results indicate that the cell displaying a Tf^+ phenotype is a low density PBL.(n=1)

TABLE VIII

FACS analysis: Tf-crossreactive lymphocytes

Percoll Fraction	Lytic units	% Kill @ 5/1	% Positive cells
42.5 %	146	60	6.9
45.0 %	173	56	1.1
50.0 %	21	21	0.6
60.0 %	20	21	0.9
H-PBL's	110	54	2.9

fractions display roughly the same level of NK cell activity, yet only the lower density 42.5% cells display a significant increase in Tf-crossreactive cells compared to unseparated lymphocytes. This may indicate that only a subpopulation of low density LGL are Tf-crossreactive.

To further analyse if NK cells contain a Tf-crossreactive epitope, antibody depletion studies were performed using 2 independent monoclonal antibodies to Tf. PBL pretreated with these antibodies plus complement displayed a decreased ability to kill K562 target cells after depletion (fig 13). These data suggest that a subpopulation of NK cells express a Tf-crossreactive epitope on the plasma membrane.

4.2 K562 lysates contain functional NK target structures. To investigate the stability of NK target structures, the NK sensitive cell line K562 was subjected to a freeze-thaw and sonication procedure. As can be observed in figure 14, K562 cell lysates retain their ability to compete with ^{51}Cr-labelled K562 cells, while the lysates generated from the NK insensitive cell line HL-60 compete poorly. Thus lysates from K562 cells appear to retain functional NK target structures.

4.3 Model system. It has been well documented that nitrocellulose is capable of irreversibly binding a wide variety of proteins. The ability of these immobilized proteins to function has not, however, been extensively studied. Human serum Tf (Calbiochem Bering; La Jolla CA), bovine Tf (Sigma; St. Louis), bovine serum albumin

Fig. 13 Antibody plus complement depletion of NK cell activity using antibodies to transferrin. Human PBL were pretreated with the indicated monoclonal antibody. The cells were then reacted with a rabbit anti-mouse IgG and incubated in rabbit complement. The cells were then tested for NK cell activity. Cells were treated with either secondary antibody alone (Control), secondary antibody plus complement alone (C'), a monoclonal anti-human transferrin (HTF-14), Cedarlane monoclonal anti-human transferrin (MoAb-TF) or anti-transferrin receptor (anti-TfR).

tig. 13

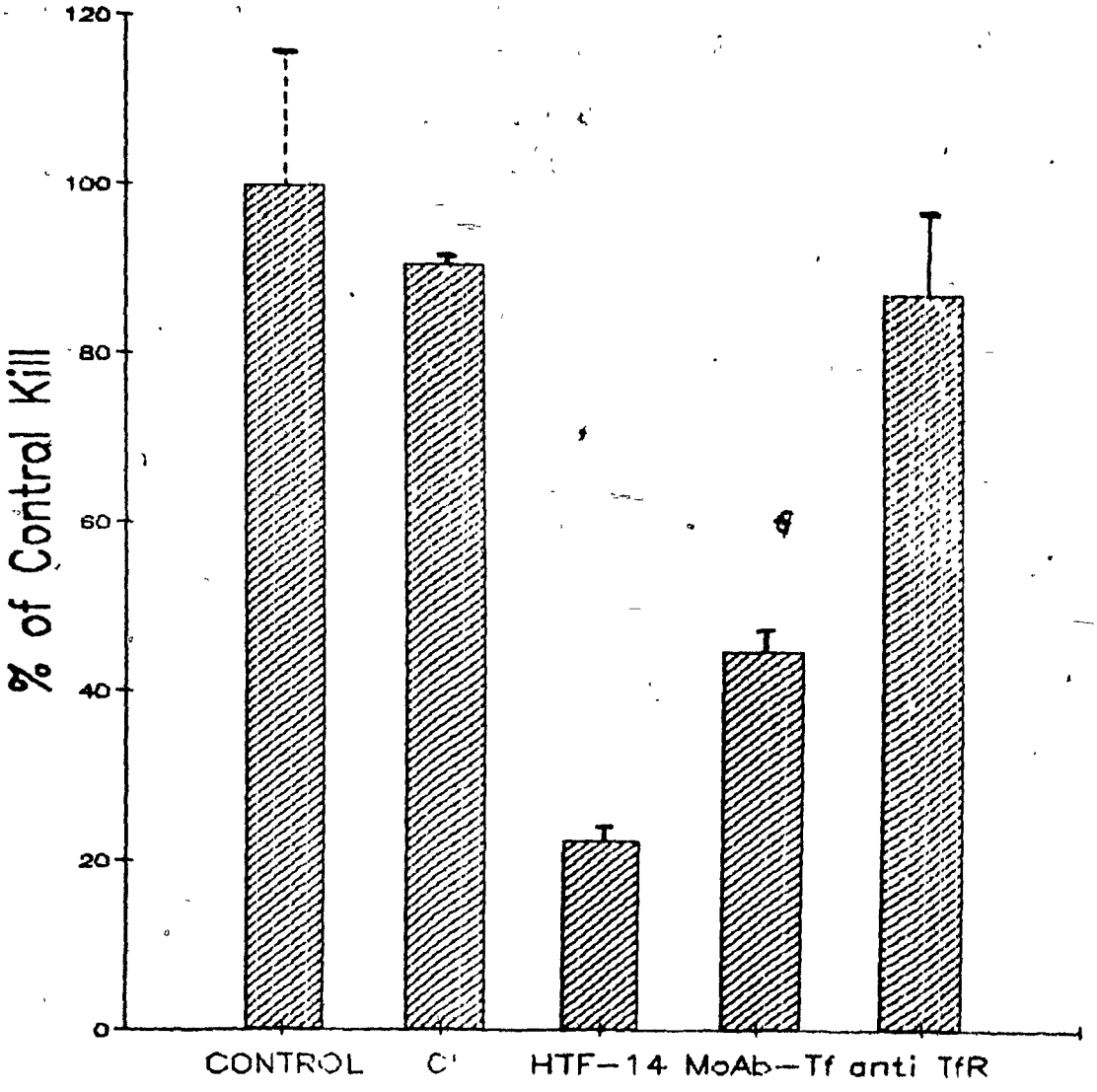
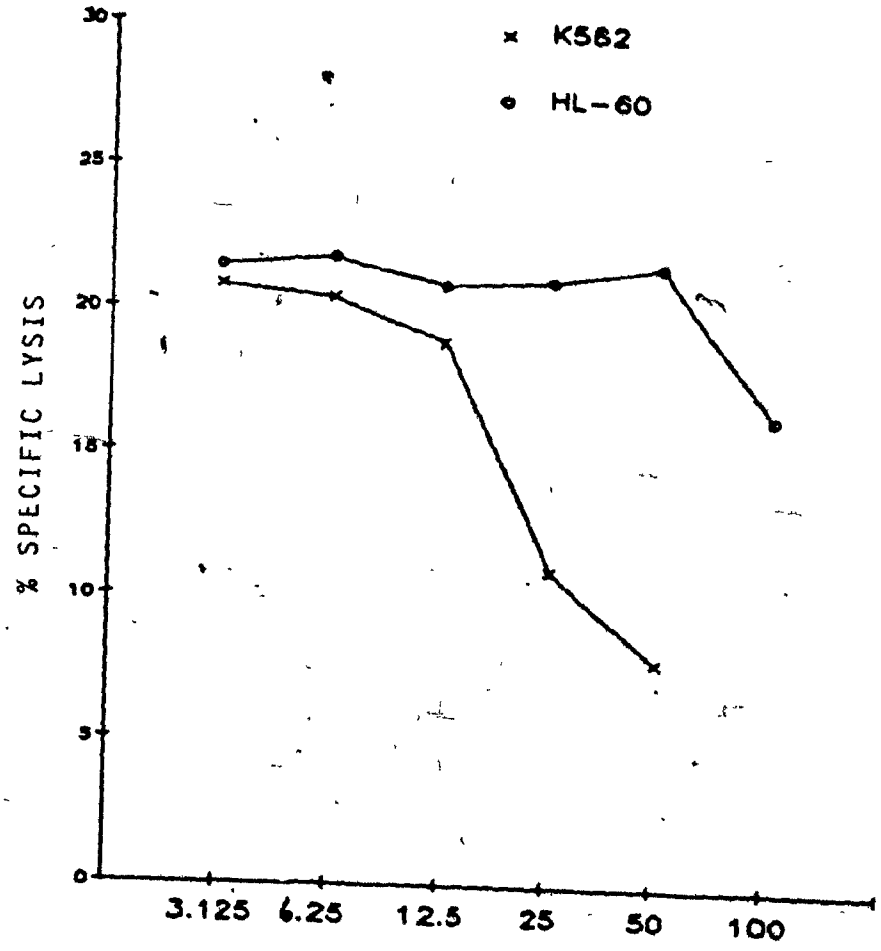

120
100
80
60
40
20
0
% of Control Kill
CONTROL
C'
HTF-14 MoAb-Tf anti TfR

Fig. 14. Ability of K562 and HL-60 cell lysates to compete with ^{51}Cr-labelled K562 cells for NK cells. The X-axis represents competitor cell equivelents to target cell ratios. The tumor cell lysates were added to a standard ^{51}Cr release asssay. X---X; competition by NK sensitive K562 cell lysates, O---O; competition by NK insensitive HL-60 cell lysates. Shown is a representitive experiment (n=6).

fig. 14


K562
HL-60
% SPECIFIC LYSIS
30
25
20
15
10
5
0
3.125
6.25
12.5
25
50
100
COMPETITOR:TARGET RATIO

(BSA)(Sigma), and human serum albumin (HSA) (Sigma) were applied to nitrocellulose. After blocking, the nitrocellulose was then incubated with K562 cell lysates, which we have shown above retain target structures. After incubation, the nitrocellulose was washed and probed for Tf receptor protein as described in materials and methods. Nitrocellulose immobilized Tf was able to bind human Tf receptors from K562 cell lysates (fig. 15). This binding was shown to be specific for human Tf because, bovine Tf, bovine serum albumin, and human serum albumin did not bind detectable levels of Tf receptor protein (fig. 16).

To ensure that the apparent binding of K562 Tf receptors was true receptor binding and not due to adherance by some nonspecific mechanism; K562 lysates were preabsorbed with human Tf (fig. 17). By pre-saturating these K562 Tf Receptor lysates with Tf, the ability of these absorbed preparations to bind to immobilized Tf were drastically reduced. Thus, the protein detected by this technique is specific for a "Tf receptor". This dot-blot technique is also sensitive to changes in Tf receptor expression in K562 cell lysates. We have previously shown that K562 cells grown in tissue culture for different lengths of time and different starting conditions, express different levels of Tf receptors (chapter 2). K562(control) and K562(dl) tumor cells were absorbed onto nitrocellulose, blocked, and then directly probed for Tf receptors. Figure 18 shows that K562(dl) cells expressed more Tf receptors than K562(control) cells. This result is in

Fig. 15. Binding of transferrin receptors by nitrocellulose immobilized human transferrin. Nitrocellulose membranes were reacted with, transferrin or bovine serum albumin. The membranes were then blocked with gelatin, and then incubated in K562 cell lysates (as a source of transferrin receptors) for 16 Hrs. The membranes were then washed and probed for bound transferrin receptors using a monoclonal anti-transferrin receptor antibody followed by an alkaline phosphatase conjugated goat anti-mouse secondary antisera. The results were then analysed by a colorimetric assay as described in materials and methods (chapter IV). (n=8)

fig. 15

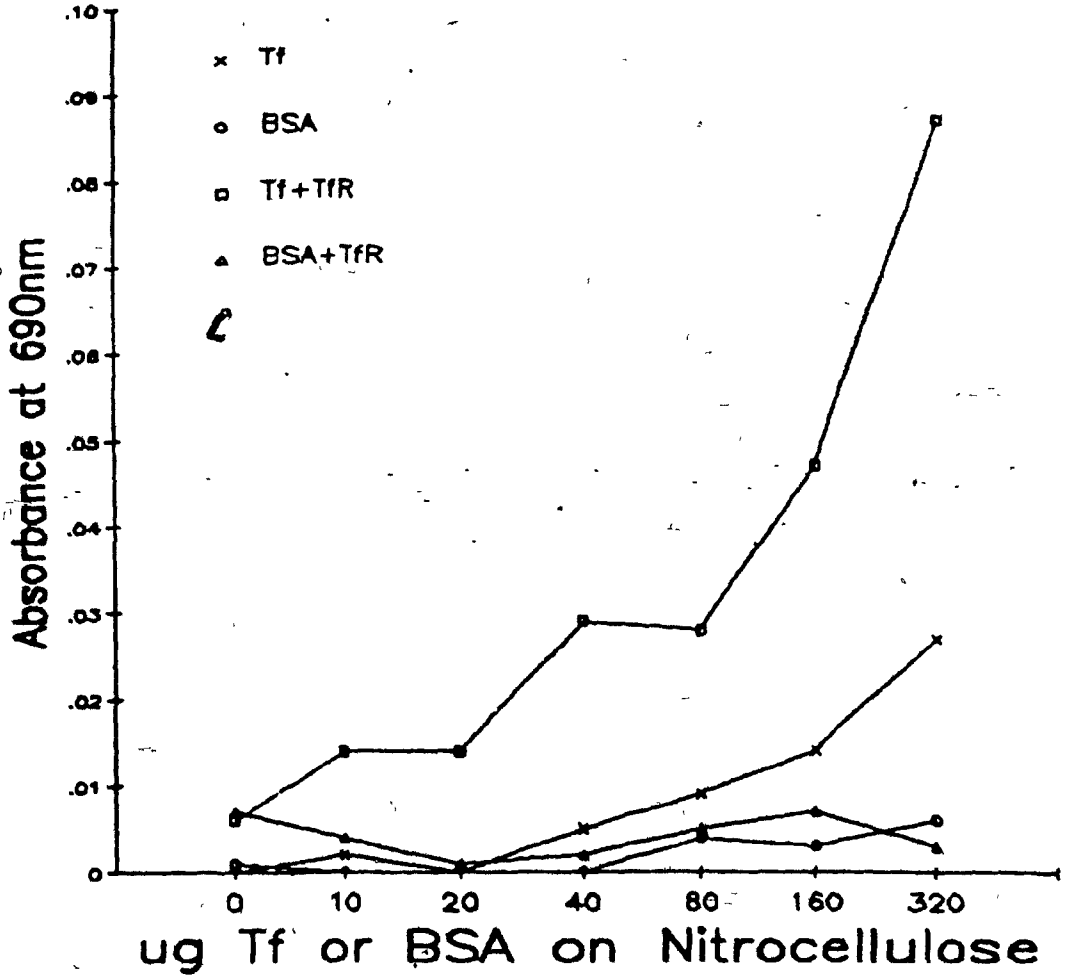

Tf
BSA
Tf+TfR
BSA+TfR
Absorbance at 690nm
.10
.09
.08
.07
.06
.05
.04
.03
.02
.01
0
0
10
20
40
80
160
320
ug Tf or BSA on Nitrocellulose

Fig. 16. Demonstration that transferrin receptor binding by nitrocellulose immobilized transferrin is specific for human transferrin. Nitrocellulose was reacted with the proteins H-Tf; human serum transferrin, B-Tf; bovine serum transferrin, HSA; human serum albumin, and BSA; bovine serum albumin. The membranes were then blocked, reacted with K562 cell lysates, and probed for bound transferrin receptors as described in figure 12. S.E.M. bars were derived from quadruplicat determinations.

fig. 16

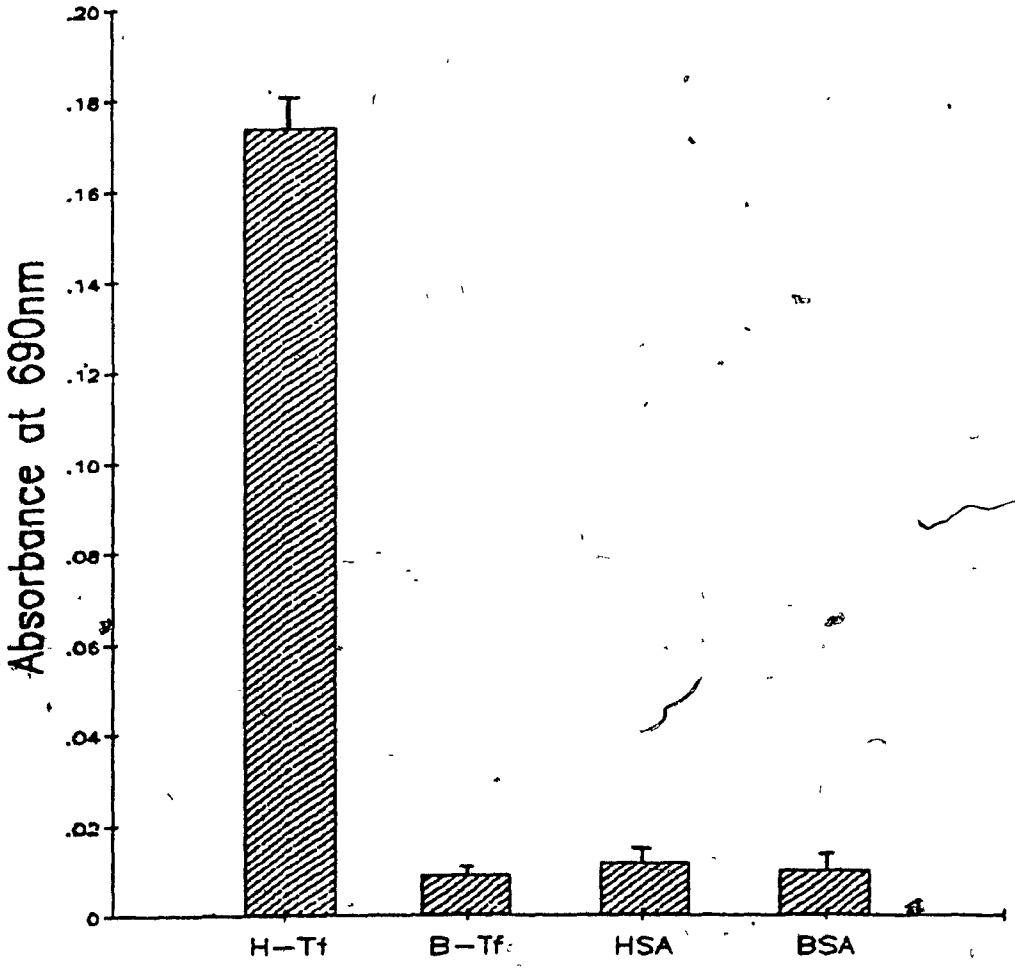

Absorbance at 690nm
.20
.18
.16
.14
.12
.10
.08
.06
.04
.02
0
H—Tf
B—Tf
HSA
BSA

Fig. 17. Specificity of binding of transferrin receptors to nitrocellulose immobilized transferrin. Nitrocellulose was reacted with Tf, and then reacted with either K562 cell lysates (TfR), or K562 cell lysates that had been preabsorbed with Tf (Blocked-TfR), control wells did not receive any lysate treatment. The menbranes were then probed for bound transferrin receptors as described in figure 12. S.E.M. bars were derived from quadruplicate determinations.

fig. 17

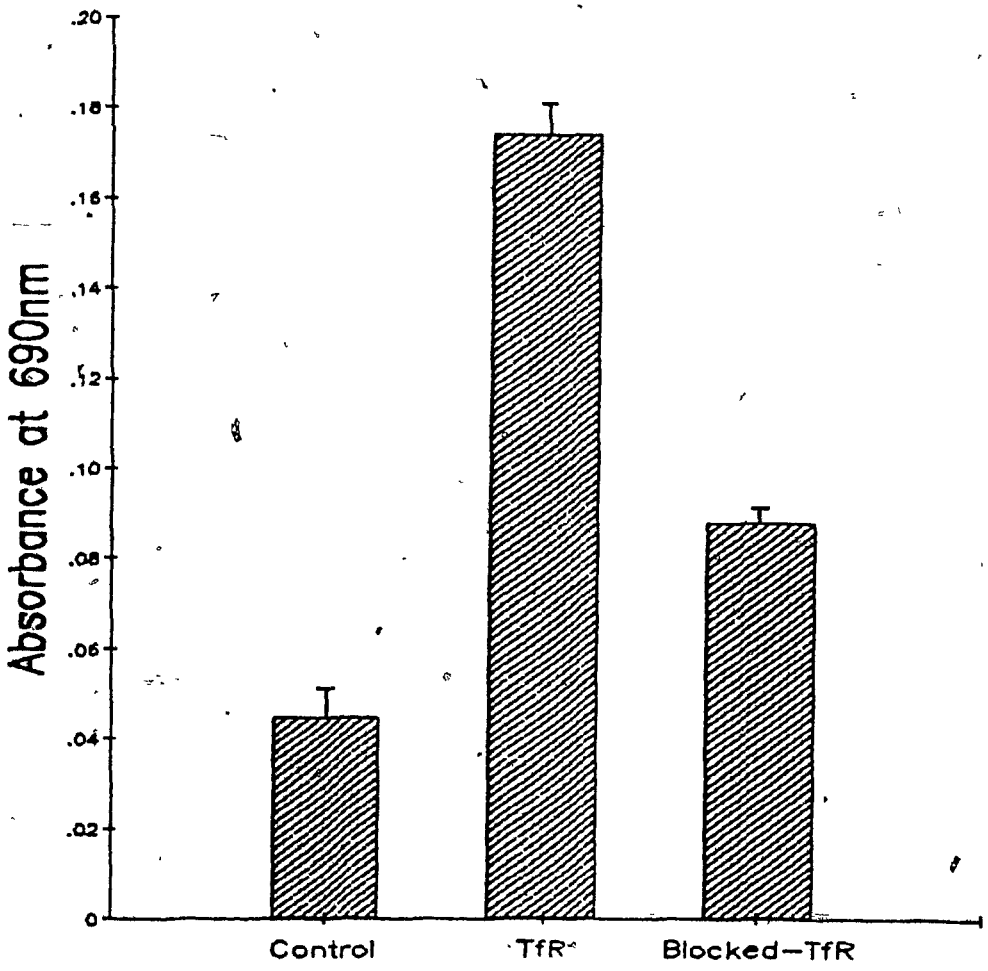
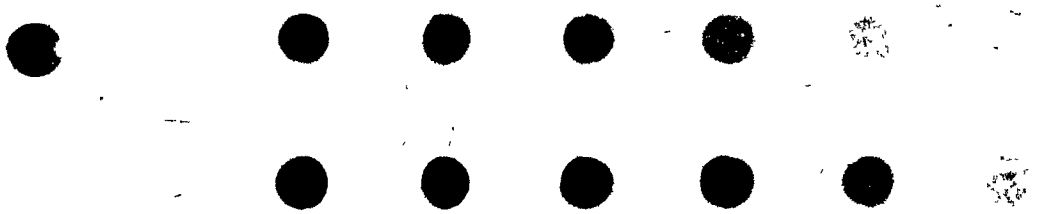
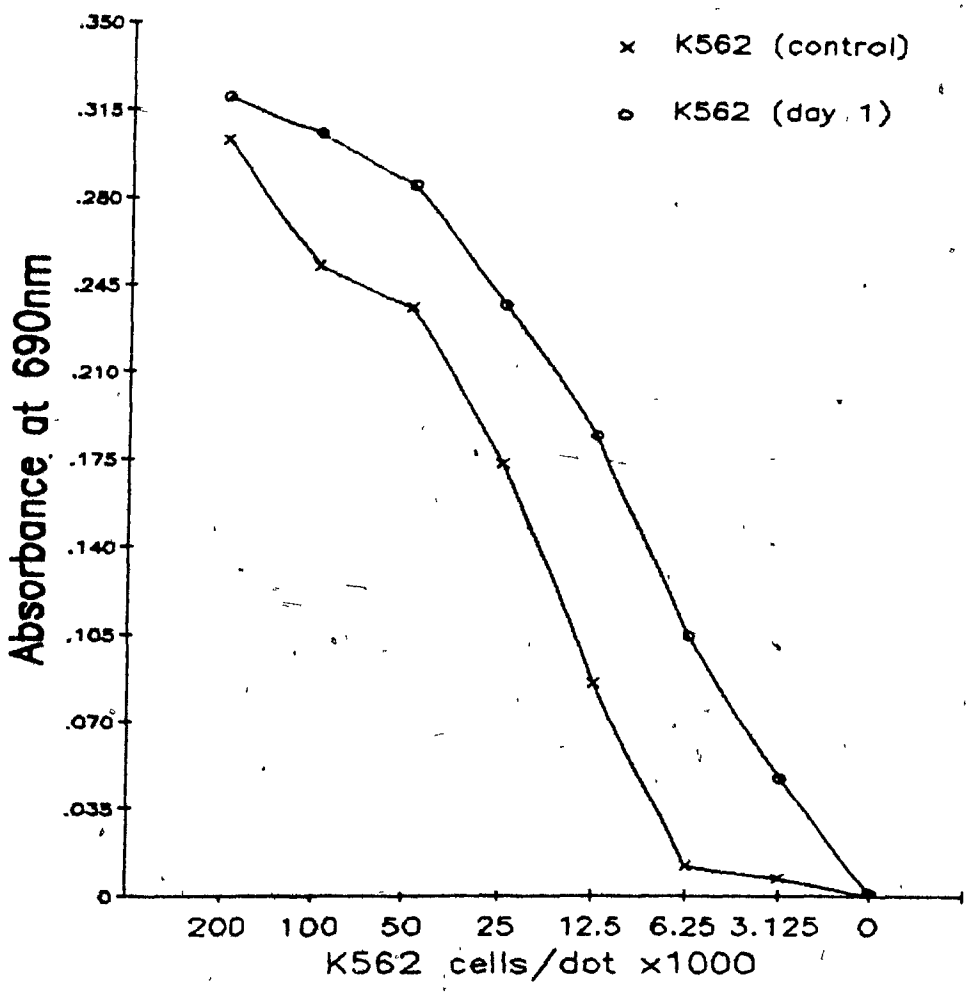

Absorbance at 690nm
.20
.18
.16
.14
.12
.10
.08
.06
.04
.02
0
Control
TfR
Blocked-TfR

Fig. 18. Photoreproductive technique for analysis of transferrin receptors correlates well with visual results. K562 cell lysates were directly reacted with nitrocellulose by adding between $2x10^5$ to $3.1x10^3$ cells/dot. The membranes were then analysed for transferrin receptors using a mouse monoclonal anti-transferrin receptor antibody followed by a alkaline phosphatase conjugated goat anti-mouse IgG immunoglobulin. The membranes were then developed and quantitated in the colorimetric assay as described in materials and methods (chapter IV). a)Photograph of nitrocellulose illustrating the visual difference between Tf receptor expression in K562 control cells (upper row) and K562 day 1 cells (lower row). b)The nitrocellulose shown above was reproduced onto acetate and the absorbance at 690nm was measured using an ELISA plate reader. (see also figure 6 and table V for comparison to Scatchard analysis)(n=7)

a



b


.350
.315
.280
.245
.210
.175
.140
.105
.070
.035
0
Absorbance at 690nm
× K562 (control)
o K562 (day 1)
200 100 50 25 12.5 6.25 3.125 0
K562 cells/dot ×1000

direct agreement with Scatchard analysis (chapter 2) and indicates

1) that cell lysates can be absorbed to nitrocellulose and specific membrane proteins detected.
2) that this dot-blotting technique is sensitive to changes in the levels of Tf receptor expressed in K562 cell lysates.

The quantity of Tf receptors detectable by this technique was 5 femtomoles/dot (1ng) of Tf receptor protein as tested in an independent experiment.

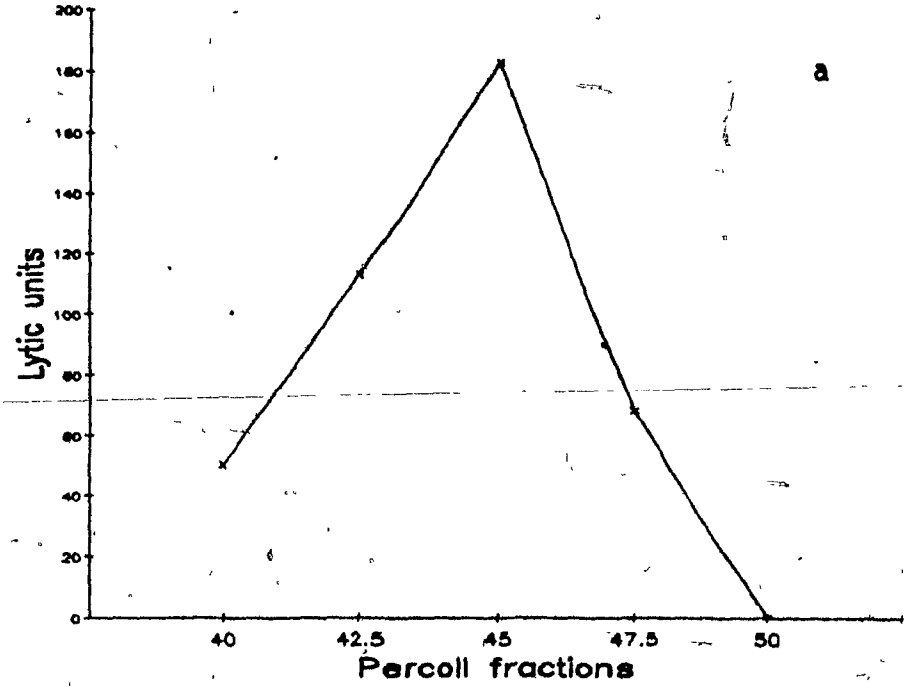
4.4 Analysis of transferrin receptor binding by percoll fractionated lymphocytes. Using the above described model system, nitrocellulose immobilized lymphocytes were tested for their ability to bind Tf receptors from K562 lysates.

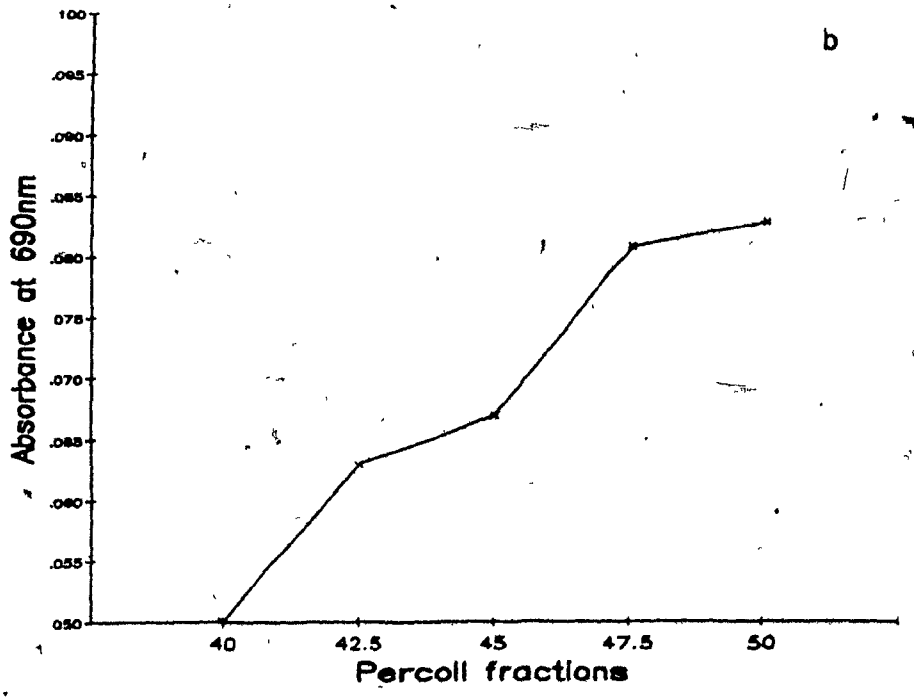
It has thus far been demonstrated that, 1)K562 lysates contain NK competitive material, 2)that these lysates contain immunologically detectable levels of Tf receptors, 3)that these receptors retain functional specificity, 4)that at least some NK cells possess a Tf crossreactive epitope and concurrent studies have shown that this epitope is immunologically detectable on nitrocellulose filters (Pannunzio, 1988) and 5) that as a model system "Tf" retains its receptor binding capacity on nitrocellulose.

Percoll separated lymphocytes were absorbed onto nitrocellulose, blocked, and then reacted with K562 cell lysates in the presence of 1% gelatin @ 37'C overnight in

Fig. 19. Comparison of lytic activity and binding of transferrin receptors by percoll fractionated human peripheral blood lymphocytes. (a) Human peripheral blood lymphocytes were fractionated on discontinous percoll density gradients from 40-50% percoll. The lymphocyte bands were collected and assessed for lytic activity against ^{51}Cr-labelled K562 cells. (b) Percoll fractionated lymphocytes from the same bands were simultaneously reacted with nitrocellulose. The nitrocellulose was then blocked, incubated in K562 cell lysates (as a source of transferrin receptors), and developed for transferrin receptor binding as in figure 16.(n=3)

fig. 19


a
Lytic units
200
180
160
140
120
100
80
60
40
20
0
40
42.5
45
47.5
50
Percoll fractions


b
Absorbance at 690nm
100
095
090
085
080
075
070
065
060
055
050
40
42.5
45
47.5
50
Percoll fractions

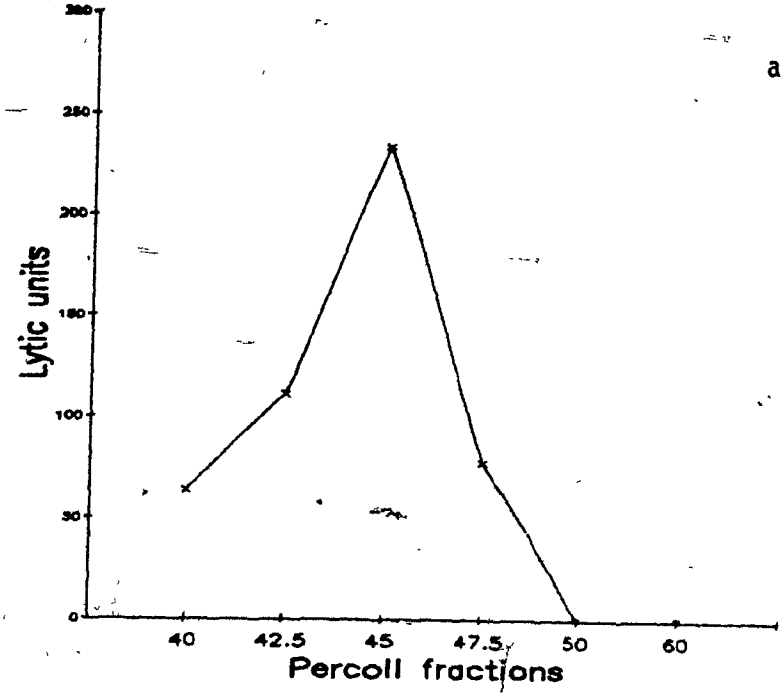
TBS. The nitrocellulose was then extensively washed and probed for Tf receptors. Figure 19 shows the relationship between the NK cell activity and the net apparent Tf receptor binding by percoll fractionated lymphocytes. As can be seen, there is no positive relationship between apparent Tf receptor binding by lymphocytes and NK cell activity (Fig. 19).

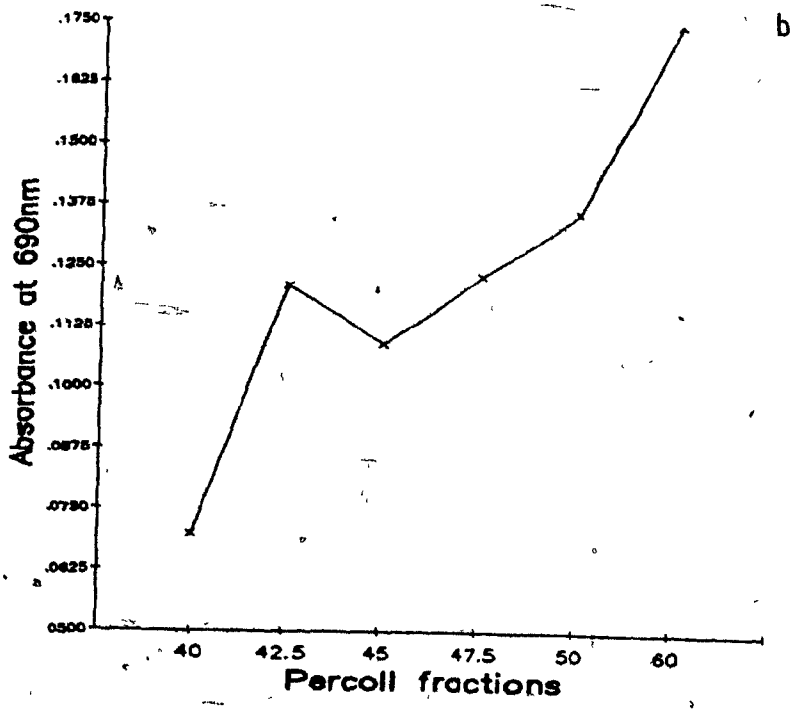
Interestingly, this experiment does however show that the small lymphocytes (depleted in NK cell activity) in the higher density bands may bind Tf receptors.

It has been well documented that IL-2 pretreatment of NK cells leads to a marked increase in NK cell activity. In an independant experiment, it was observed that the optimal in vitro dose of IL-2 for maximal NK activity was 25 BRMP Units/ml for 18 hr (data not showm). Percoll-fractionated $_{r}$IL-2 boosted PBL were thus analysed for possible Tf receptor binding activity. These cells were incubated in $_{r}$IL-2 for 18 hr @ 37'C, and the extent of Tf receptor binding was assessed in all $_{r}$IL-2-boosted percoll fractions. Figure 20 shows the results of a representative experiment. As can be observed, there is maximal receptor binding in the 60% fraction. This fraction of lymphocytes does not contain a significant number of NK cells as assessed by NK cell activity (fig. 20) or flow cytometry using the NKH-1 antibody (data not shown). Thus a "non-NK fraction" displays maximal apparent Tf receptor binding activity. There is also no relationship between apparent Tf receptor binding activity

Fig. 20. Comparison of lytic activity and binding of transferrin receptors by percoll fractionated $_{r}$IL-2 activated human peripheral blood lymphocytes. (a) Human peripheral blood lymphocytes were fractionated on discontinous percoll density gradients from 40-60% percoll. The lymphocyte bands were collected and activated in the presence of recombinant IL-2 for 18 Hrs. Activated lymphocytes were then assessed for lytic activity against ^{51}Cr-labelled K562 cells. (b) Lymphocytes from the same bands were simultaneously reacted with nitrocellulose. The nitrocellulose was then blocked, incubated in K562 cell lysates (as a source of transferrin receptors), and developed for transferrin receptor binding as in figure 16.

fig. 20


a
Lytic units
250
200
150
100
50
0
40
42.5
45
47.5
50
60
Percoll fractions


b
Absorbance at 690nm
.1750
.1625
.1500
.1375
.1250
.1125
.1000
.0875
.0750
.0625
.0500
40
42.5
45
47.5
50
60
Percoll fractions

and NK cell activity when all of the percoll fractions are compared.

The net minimal increase in apparent binding of the lymphocytes in the 42.5% band was not reproduced in any of the other experiments, and even in this experiment the small peak increase in apparent binding at 42.5% percoll did not correspond to maximal NK cell activity (ie. at 45% percoll). The net apparent binding of Tf receptors in the 60% fraction was however seen in 3 out of 4 $_r$IL-2 boosting experiments. This result indicates that there is no relationship between Tf receptor binding and NK cell activity in percoll fractionated $_r$IL-2 boosted lymphocytes.

5. Discussion

If a lymphocyte binds Tf receptors, then it must possess some "ligand" through which it can accomplish this task. Studies by Baines and co-workers (1983) in this laboratory illustrated that antibodies to human serum Tf could block NK cell function at the level of the effector cell.

To determine if NK cells possess a Tf crossreactive epitope, 3 approaches were used;

1)H-PBL's were stained with a polyclonal antiserum raised against Tf followed by protein A-colloidal gold and then examined by electron microscopy.

2)Flow cytometry was used to examine if the Tf^+ phenotype co-purified with NK cell activity on percoll fractionated H-PBL's.

3)Antibody plus complement depletion studies were performed using 2 independent monoclonal antibodies against serum transferrin.

The results of these investigations suggest that at least a subpopulation of NK cells do possess a determinant immunologically crossreactive with human serum Tf. We are not yet able to determine if NK cells simply have Tf receptors to which Tf is bound or if they possess a de facto transmembrane protein that resembles Tf such as the p97 antigen found on melanoma cells (Brown et al, 1982). Indications by both Scatchard analysis as well as antibody plus complement studies, suggest that the numbers of Tf receptors on NK cells to be minimal to nonexistant (data not shown). However, a major criticizm of this approach is that if NK cells possess Tf receptors to which Tf is bound, then Scatchard analysis will not yield meaningfull results since the receptor should be blocked. Double marker analysis for Tf and Tf receptors by flow cytometry should provide a more definitive result.

Since Tf receptors on tumor cells bind Tf with high affinity, and NK cells possess a Tf-crossreactive epitope on their surface, it was hypothesized that NK cells use this "Tf-like" epitope to interact with tumor cells to form a high affinity conjugate. This study has

used nitrocellulose immobilized percoll fractionated lymphocytes as an affinity matrix to ask if NK cells bind Tf receptors. The results indicate that, although the method used is sensitive and specific for Tf receptors, there is no relationship between NK cell activity and apparent Tf receptor binding in percoll fractionated PBL. In contrast, it appeared that Tf receptors preferentially bound to lysates from high density lymphocytes. This ability of high density non-NK cells to bind Tf receptors may be a result of the release of internal stores of Tf from T-lymphocytes. Several previous studies on T-lymphocytes have indicated that these cells possess stores of Tf (Nishiya et al, 1980b; Lum et al, 1986; Lu et al, 1986). Since these non-activated lymphocytes possess either very little or no Tf receptors (Lum et al, 1986), and our preliminary FACS analysis did not show any Tf-like protein on the surface of high density lymphocytes, we hypothesize that the "Tf-like" pool measured must therfore come from internal stores.

The level of Tf in CD-8^+ T-lymphocytes was calculated by Lu and co-workers (1986) to be 8ug of Tf/$1x10^7$ lymphocytes. Based on the molecular weights and stoichiometry of Tf-Tf receptor interaction, this is equivilent to 1 ng of Tf/12,500 lymphocytes. The detection limit of Tf receptors in our system is 1 ng of receptor protein, and we use between 3,125 and 200,000 lymphocytes/dot in our dose response curves, therefore the

level of Tf in T-lymphocytes is well within the measurable range of our dot blot system. Our results therefore corroborate previous findings that T-lymphocytes contain an internal Tf receptor binding protein and that $_{r}$IL-2 activation of these lymphocytes increases the expression of this Tf receptor binding protein (Lum et al, 1986).

Our findings indicate that NK cells possess an epitope that is immunologically crossreactive with serum Tf. Concurrently, NK cell activity appeared not to correlate with Tf receptor binding activity. This paradox may be explained by several possibilities:

1) NK cells are activated lymphocytes that possess Tf receptors to which Tf is already bound. Since it appears that Tf only has one binding site (Lazarus et al, 1987), this surface Tf is not available for exogenous receptor binding.

2) The Tf-like protein on the surface of NK cells may be an integral membrane protein such as p97 and does not bind to Tf receptors.

3) This Tf-like membrane protein is more labile than Tf and is inactivated by nitrocellulose binding.

4) The level of Tf receptor binding protein is below the detection limit of this assay.

5) The relevant epitope itself is unrelated to Tf.

6) or finally, the NK recognition structure is somehow similiar to the T cell receptor in that it does not bind free antigen but requires the Tf receptor to be associated with some other membrane complex.

While it is difficult to ascertain which of the above possibilities is correct, it seems somewhat unlikely that the protein is simply too labile to function in this system because Tf itself proved to be exceptionally stable. In addition, while it is possible that the protein of interest is a minor membrane protein and is below the detection level of this assay; dot blotting is noted for its sensitivity to small quantities of protein. Since a Tf crossreactive epitope was measured by electron microscopy which itself is a relatively insensitive method of detection, it would be hypothesized that the protein detected is present in substantial quantities.

In conclusion, NK cell activity of both percoll fractionated lymphocytes as well as IL-2 activated NK cells does not correlate with apparent Tf receptor binding. Thus the results of this study do not support the hypothesis that Tf receptors are involved in NK cell to tumor cell binding.

V. GENERAL CONCLUSIONS

The Tf receptor has been hypothesised to be a target structure on tumor cells recognized by human NK cells (Baines et al, 1983; Vodinelich et al, 1983). If this theory is correct, then there should be a correlation between Tf receptor expression and the ability to be recognized by NK cells.

It was found that when the cell line K562 was modulated for Tf receptor expression, there was a good correlation between the level of Tf receptors on K562 cells and the ability of those cells to compete with a control K562 cell preparation. It was also found that although Tf receptor expression correlated with NK competitive activity on K562, when different cell lines were evaluated there was no apparent correlation (chapter 2). It should be noted that in earlier studies when different cell lines were evaluated in checkerboard type experiments for the relatedness of target structures, it was generally concluded that different tumor cell lines could possess different target structures [reviewed in Ortaldo and Herberman, 1982]. In addition to this, nonspecific physical factors affect target cell sensitivity to lysis. These include sialic acid content and membrane charge (Yogeeswarn et al, 1982; Warren, 1976), lipid composition (Young et al, 1981; Yoo et al, 1982), membrane fluidity (Roozemond and Bonavida, 1985), and target cell metabolism (Deem et al, 1984). Thus when

diverse cell types are compared, the contribution of these nonspecific physical factors, coupled with different spectrums of specificity at both the effector and target cell level, may obscure the significance of differences in Tf receptor expression in different cell lines. Because many differences exist between different cell lines, the competitive activity of these cell lines may not necessarily correlate with the number of target structures. For example, it is known that the level of sialic acid found on a tumor cell inversely correlates with its ability to be lysed by an NK cell (Yogeeswarn et al, 1982). Thus if target cell "B" posseses more target structures than target cell "A" and target cell "B" also posseses higher levels of sialic acid than target cell "A", then target cell "B" will not necessarily compete better than target cell "A" for NK cell attention.

To further verify if a correlation between Tf receptor expression and competitive activity is relevant, a system utilizing non-tumor cells was evaluated. Not surprisingly, it was found that rabbit erythrocytes which do not possess Tf receptors did not compete with K562 cells for human NK cells. However, rabbit reticulocytes which do express Tf receptors could compete with K562 cells for human NK recognition. Thus Tf receptor expression was found to correlate with competitive activity even in this non-tumor cell system.

Several other investigators have since studied the

relationship between Tf receptor expression and NK cell mediated lysis (Newman et al, 1984; Dokhelar et al, 1984; Bridges and Smith, 1985; Lauzon and Roder, 1985; Schuurman et al, 1985; Borysiewicz et al, 1986; Phillips, 1986). However, as was mentioned in chapter 3, difficulties in data interpretation arise when Tf receptor expression is correlated with NK mediated lysis (as opposed to competition or conjugate formation).

In this correlation study, competitive activity has been compared to Tf receptor expression. We have been careful not to use "killing" as a measure of recognition because the final lytic event is dependent on many parameters whereas competition is a direct measure of recognition (Herberman and Ortaldo, 1980). Within the context of recognition and lysis, it has been well documented that in order for an NK cell to lyse the target cell, it must first bind to the tumor cell and then be activated to secrete its lytic factor. Once secreted, this factor must bind or interact with the tumor cell and only then may lysis proceed. If any step of this process is blocked then the final lytic event will not ensue. Further, the ability of K562 cells to be killed by NK cells is not a valid measure of the recognition state of that cell since it has been demonstrated by Hagner that the lytic phase of killing can be dramatically altered by the growth phase of K562 cells (Hagner, 1984). He found that K562 cells could be killed with differing degrees of efficiency depending on the proportion of target blast

cells in a given culture, this increased sensitivity to lysis was found to be attributable soley to a more efficient lytic mechanism. It can therefore be concluded on the basis of Hagner's results (as well as ours that lysis is a poor measure of "NK recognition".

Among the several studies that have compared Tf receptor expression to NK cell function, two have directly modulated Tf receptors and then correlated the resultant expression of receptors with competitive activity. In the first, mouse L cells were transfected with high M.W. DNA from a human tumor cell line (Newman et al, 1984). It was found that the expression of human Tf receptors by a particular transfected clone correlated with its competitive activity. In the second study, although the title of this manuscript suggests that data is provided which shows a lack of correlation between Tf receptor expression and NK recognition (Bridges and Smith, 1985), upon careful scrutiny of this data, the manuscript examines two separate phenomena:

1) NK recognition and lysis of virally infected HeLa cells.

2) NK recognition of K562 tumor cells.

With respect to virally infected cells, it has been previously indicated that a different subset of NK cells may recognize some virally infected cells (Fitzgerald et al, 1983) and it is quite possible that the subset of NK

cells which recognizes virally infected cells does not recognize Tf receptors. The authors do however show very elegantly that K562 cells treated with desferrol (an iron chelator) or hemin (an iron supplement) can increase or decrease respectively the expression of Tf receptors on these cells. When the Tf receptor modulated cells were tested for competitive activity, the authors interpreted their results as indicating that Tf receptor expression does not correlate with competitive activity. [In order to discuss the data by the authors their data from "table II" is presented in graphic form in appendix III.] However this does not appear to be so, as can be seen in both experiments hemin treated K562 cells (which have lower levels of Tf receptors) actually compete less well than base line K562 cells. Thus using a specific modulator of Tf receptors, they show a correlation between Tf receptor expression and competitive activity in K562 cells in 2 out of 2 experiments. It should be mentioned that their other experiment which uses hemin treated K562 cells as targets and control K562 cells as competitors has not been reproduced here because the data is too eratic to interpret.

Since Tf receptors on tumor cells bind Tf with high affinity, and it was previously shown that antibodies to Tf could block NK cell activity at the effector cell level, NK cells were examined for a possible Tf crossreactive epitope. The results indicate that a

subpopulation of NK cells possess a Tf-like epitope on their surface, this result has corrobarated by Alarcon and Fresno (1985). It was hypothesized that NK cells use this "Tf-like" epitope to interact with tumor cells and form a high affinity conjugate.

In this study we have used nitrocellulose immobilized percoll fractionated lymphocytes to examine if there is any relationship between NK cell activity and apparent Tf receptor binding in these fractions. The results indicate that although the method used is sensitive and specific for Tf receptors, there is no apparent correlation between NK cell activity and Tf receptor binding activity in the nitrocellulose immobilized percoll fractions. Thus even if Tf receptors are somehow involved in NK cell mediated lysis of tumor cells, this data does not support a role for Tf receptor involvement in conjugate formation between NK cells and tumor cells.

The mechanism via which an NK cell forms a high affinity conjugate between itself and a tumor cell is not known. There have been however several membrane proteins that have been implicated in "lymphocyte adhesion". These include; CD-2, LFA-3, CD-4, CD-8, and the well known LFA-1 molecule which shares a common β chain with p150,95 and MAC-1 [reviewed in Springer et al, 1987]. The CD-4 and CD-8 antigens have been postulated to recognize nonpolymorphic regions of MHC encoded gene products, although this has been questioned (Fleischer et al, 1986) and the CD-2 molecule appears to recognize the LFA-3

antigen (Springer et al, 1987; Shaw et al, 1986). The most relavent of these proteins may be the LFA-1 antigen. This membrane protein is found on virtually all lymphocytes and is thought to be directly involved in lymphocyte conjugate formation. Since this antigen is widely distributed on lymphocytes, it may account for the the occasional study where "NK patterned binding" is found in non-NK cells (Piontek et al, 1982).

It is possible that Tf receptors are involved in some other aspect of NK cell recognition, such as "triggering". If Tf receptors do serve as triggering molecules on the target cell, then this may explain why although NK cells do not appear to bind Tf receptors directly, Tf receptor expression is correlated with competitive activity. Any molecule that "triggers" the NK cell or, in essence, attracts the NK cell's attention, should be an excellent competitor.

In summary, there was a good correlation between the expression of Tf receptors on K562 cells and the ability of these cells to compete in a standard chromium release assay. It was also found that rabbit reticulocytes which express high numbers of Tf receptors competed with K562 cells for NK cells whereas mature rabbit erythrocyteswhich do not express Tf receptors did not compete. These results support previous studies which have indicated that Tf receptors are involved in NK cell mediated specificity for tumor cells. The involvement of Tf receptors in NK

specificity could be at either of 2 stages.

1) as an affinity structure between NK cells and tumor cells, or

2) as an activation or triggering structure.

The results from this thesis indicate that there is no relationship between NK cell activity and Tf receptor binding capacity in nitrocellulose immobilized percoll fractionated lymphocytes. This data does not therefore support the hypothesis that NK cells utilize a Tf-like molecule to bind to tumor cells. Thus if Tf receptors are involved in NK cell specificity, it is likely that they play a role in NK activation or triggering. One way to test this hypothesis would be to react tryptic peptides of the Tf receptor protein (Vodinelich et al, 1983) with LGL and monitor early events in signal transduction (see chapter 1).

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Effect of acid striping on human NK cell activity

pH	Lytic units	S.E.M.	n
7.0	14.4	4.6	4
5.5	5.2	1.6	4

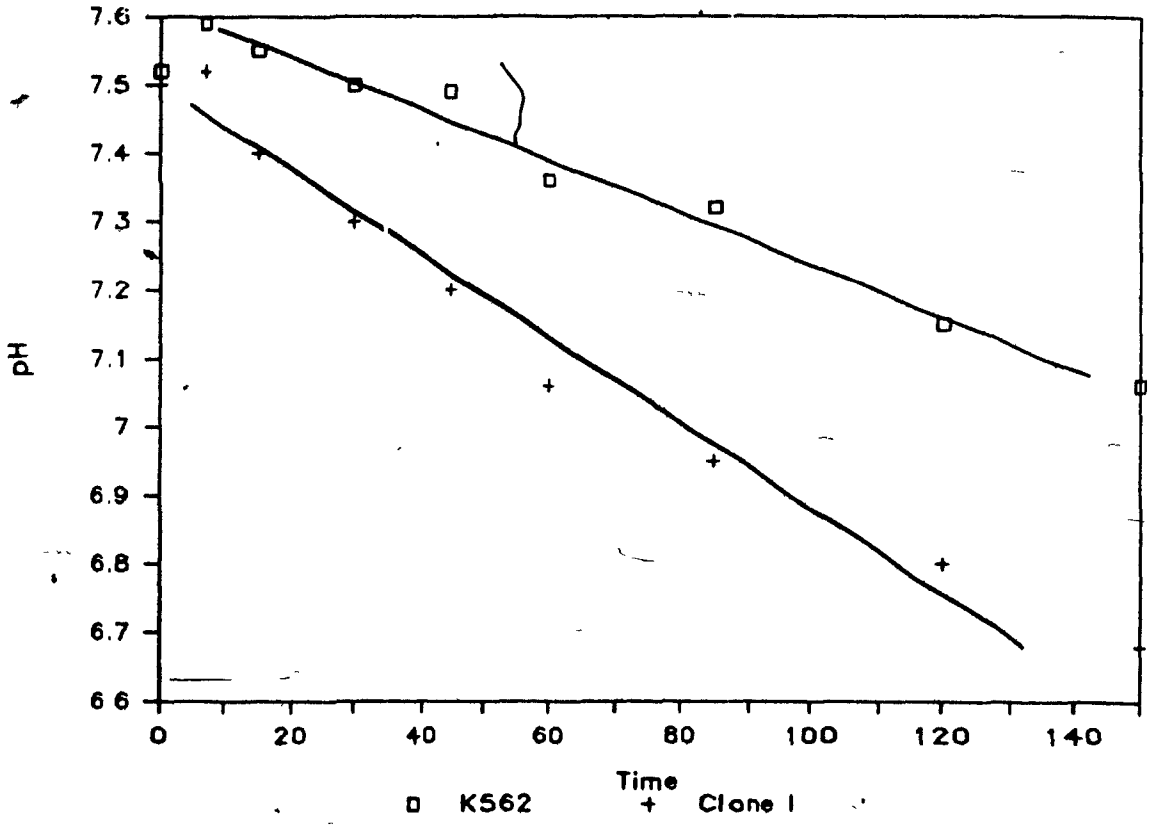
H-PBL's were pretreated at the above pH for 1 Hr at 37'C in either biscine or HEPES balanced salt solution. The lymphocytes were then washed, counted for viability using trypan blue and tested in a standard ^{51}Cr-release assay.

pH 5.5 has been shown by several investigators to strip the iron co-factor from transferrin. When the cells are subsequently adjusted to pH 7.0; transferrin dissociates from its receptor (Dautry-Varsat et al, 1983; Klausner et al, 1983).

The decrease in NK cell activity seen in the pH 5.5 treated lymphocytes may be due to the dissociation of transferrin from its receptor resulting in a reduced level of recognition. (see fig.1)

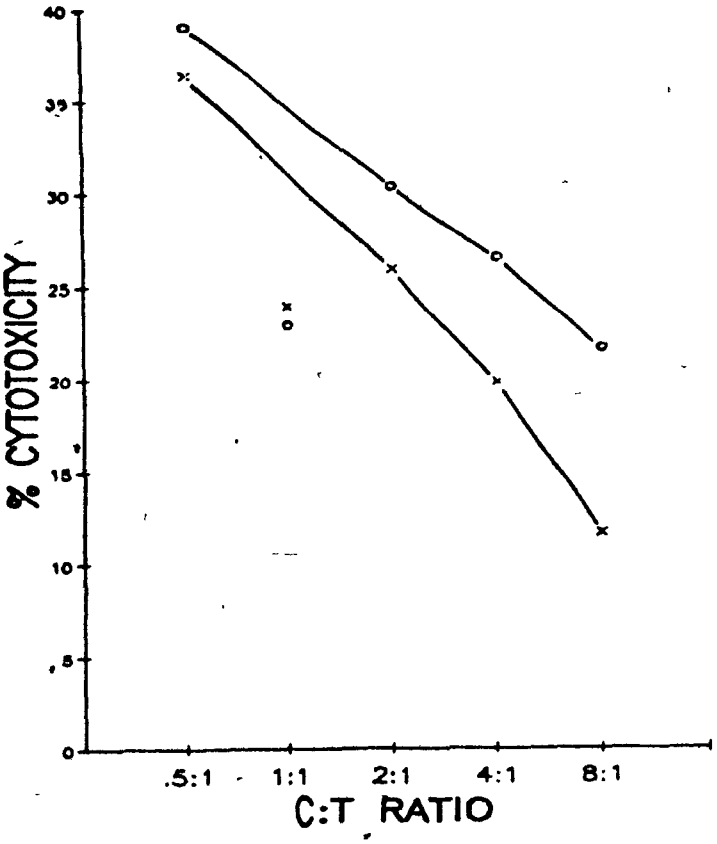
Appendix II. In the correlation studies between transferrin receptor expression and competitive activity it was observed that clone I cells were more resistant to NK mediated lysis than control K562 cells. In the previous appendix it was demonstrated that decreased pH is inhibitory for NK cell mediated lysis. Clone I cells and K562 cells were analysed for there ability to produce acidic byproducts as a possible resistance mechanism.

K562 and Clone I cells were removed from tissue culture, washed and resuspended to $1x10^7$ cells/ml in RPMI-1640 supplemented with 10% FBS. The cells were incubated in a 37'C water bath and pH readings taken at the times indicated. The slopes of the lines (calculated by linear regression) obtained for K562 and Clone I cells was $-3.49x10^{-3}$ and $-5.75x10^{-3}$ respectively; this indicates that clone I cells produce more acid byproducts than the parent K562 cell line and this may contribute to the resistance of clone I cells to lysis by NK cells.


7.6
7.5
7.4
7.3
7.2
7.1
7
6.9
6.8
6.7
6.6
pH
0
20
40
60
80
100
120
140
Time
K562
Clone I

Appendix III. Data from Bridges and Smith (1985). The data from "table II" in the above publication has been reproduced in graphical form soley for discussion (see chapter V). The experiment is a cold competition assay comparing, O——O, hemin-treated and X——X, untreated K562 competitors. The hemin treated cells possess fewer Tf receptors than the nontreated cells because they are in an iron rich environment. As can be clearly seen in both experiments, untreated cells appear to compete better than hemin treated cells.

Experiment 1



Experiment 2