The University of Manitoba

MECHANISMS OF NATURAL KILLER CELL RECOGNITION

BY

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A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfilment of the Requirements for the Degree of

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ABBREVIATIONS

ADCC	antibody dependent cellular cytotoxicity
bg/bg	beige mutant
CFC	conjugate forming cells
CGD	chronic granulomatous disease
CHO	Chinese hamster ovary
CHO-WT	Chinese hamster ovary wild type
CL	chemiluminescence
ConA	concanavalin A
$ConA^R$	concanavalin A resistant
$ConA^S$	concanavalin A sensitive
CTL	cytotoxic T lymphocyte
DIFP	diisopropyl fluorophosphate
DNP	dinitrophenol
dolichol-P	dolichol-phosphate
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
Gal	galactose
GalNAc	N-acetyl-galactosamine
$GD1_a$	ceramide-glucose-galactose (sialic acid)-N-acetyl-galactosamine-galactose (sialic acid)
$GD1_b$	ceramide-glucose-galactose (sialic acid-sialic acid)-N-acetyl-galactosamine-galactose
GlcNAc	N-acetyl-glucosamine
GT	ceramide-glucose-galactose (sialic acid-sialic acid)-N-acetyl-galactosamine-galactose (sialic acid)
IFN	interferon
IL-1	interleukin-1
LAK	lymphokine activated killer
LGL	large granular lymphocytes
LPS	lipopolysaccharide
MAF	macrophage activating factor
αMEM	alpha modified Eagles medium
NaN_3	sodium azide
NDGA	nordihydroguaiaretic acid
NK	natural killer
NK^R	natural killer resistant
NK^S	natural killer sensitive
nu/nu	nude mutant
NWC	nylon wool column
PCM	phytohaemagglutinin conditioned medium
PHA	phytohaemagglutinin
PMSF	phenylmethylsulfonyl fluoride
PNA	peanut agglutinin
Poly I:poly C	polyinosinic polycytidylic acid
PPD	purified protein derivative
PPLO	pleural pneumonia-like organism
SOD	superoxide dismutase
UDP	uridine 5-diphosphate
WGA	wheat germ agglutinin
WGA^R	wheat germ agglutinin resistant

ABSTRACT

We have examined the hypothesis that NK cells bear a lectin-like recognition system for tumors by examining target cell lines that have altered cell surface carbohydrates. Three independently selected concanavalin A-resistant ($ConA^R$) Chinese hamster ovary (CHO) cell lines (C^R-7, BC^R-2 and EC^R-1) which are defective in the synthesis of asparagine-linked oligosaccharides all showed an increased binding and cytolysis by NK cells as compared to each parental wild-type, while a revertant of the C^R-7 (RC^R-7) was near wild-type levels. These results suggested that the defect in oligosaccharide biosynthesis in these cells altered their NK reactivity. Tunicamycin, an inhibitor of asparagine-linked glycosylation, was able to reverse the NK binding to the CHO lines. The mutation rendering the C^R-7 $ConA^R$ and NK sensitive also resulted in a reduced susceptibility to lysis by activated macrophages. The C^R-7 was less tumorigenic when injected subcutaneously into nude mice compared to CHO-wild type (CHO-WT) or RC^R-7 and this could be reversed by injections of antiserum directed at the NK cell determinant asialo-GM1 which suppresses NK activity in vivo. Two other $ConA^R$ mutants (L6 and BW5147), which have a different mutation in oligosaccharide biosynthesis from the C^R-7, did not exhibit this enhanced NK reactivity. These results provide evidence for the hypothesis that a lectin-like recognition mechanism exists for NK cells and that altered carbohydrate expression on $ConA^R$ CHO mutants may be a determining factor for their reactivity with these effectors.

In a second series of experiments we have examined the hypothesis that tumor cells can stimulate a respiratory burst by human NK cells and that superoxide anion is utilized in the NK lytic mechanism. Purified

human NK cells generated a chemiluminescence (CL) response when triggered by NK^S but not NK^R tumors. Although NK reactive monoclonals could abrogate the response, the cell generating the CL was a contaminating monocyte based on the following observations: a) A monocyte specific antibody (MO2) and complement (c′) completely abolished CL; b) Nylon wool columns removed the CL producing cell; c) Addition of purified monocytes to a non-CL NK preparation could restore CL; d) Supernatants obtained from brief LGL/K562 tumor incubations could trigger CL in a purified monocyte preparation. These procedures (a, b) or the addition of superoxide dismutase had no effect on NK lytic activity. We therefore propose that CL responses are not required for NK cytolysis and originate from contaminating monocytes which were triggered by factor(s) released from tumor-activated NK cells.

INTRODUCTION

Natural Resistance

The "Immune Surveillance" theory originally postulated by Thomas (1959) suggested that the immune process that occurs during transplantation is also the mechanism for natural defense against neoplasia. Later, Burnet (1971) expanded this hypothesis by proposing that tumors are antigenic and can stimulate a rejection process by a T-dependent immune response. This theory lost favour when it was shown that thymus deficient mice did not have a higher incidence of spontaneous tumors (Rygard and Povlsen, 1976) and that athymic mice could also resist chemically-induced tumors (Stutman, 1974). Nude mice are more succeptible to oncogenesis by polyoma virus (Allison et al., 1974; Vandeputte et al., 1974). However, it is well known that T cells are involved in recognizing and attacking virus-infected cells (Doherty and Zinkernagel, 1974). Hewitt et al. (1976) also showed that murine spontaneous tumors (27 varieties) are not immunogenic. These types of results have also been reported in other species such as the rat (Middle and Embleton, 1981).

It was suggested that the mechanism for tumor rejection may be T-independent (Greenberg and Greene, 1976). During these years, a large amount of effort went into the study of these non-adaptive T-independent immune mechanisms, now referred to as "Natural Resistance". The three major mediators of a T-independent natural resistance mechanism are natural killer cells (NK) (Herberman, 1983a), natural antibodies (NAb) (Greenberg et al., 1983) and macrophages (Keller, 1983). This historical review will focus primarily on natural killer cell research.

Natural Killer Cells

Discovery

During the early studies of tumor immunogenicity in which attempts were made to determine whether or not a T cell response could be mounted against tumor cells, it became apparent that lymphocytes from a wide variety of normal animals and humans could lyse the tumors without prior sensitization (Takasugi et al.,1973; Greenberg and Playfair, 1974; Kiessling et al., 1975a,b). While many classical immunologists viewed this normal reactivity as background "noise" or even referred to it as artifactual, others postulated that these cells may have an important role in tumor elimination (Herberman, 1973). The natural killer cells fit the T-independent model since nude mice, which lack conventional T cells, had normal levels of NK cells (Herberman et al., 1975). Mice which were athymic also had normal levels of NK cells (Haller et al.,1978). It soon became apparent that all animals tested had these cells (Herberman and Holden, 1978) and that NK cells played an important role in natural resistance (Herberman, 1983b).

Since their discovery nearly 12 years ago, a great deal of research has been undertaken on NK cells. Several excellent reviews have been written (Kiessling and Haller, 1978; Roder et al., 1981a; Ortaldo and Herberman, 1984; Trinchieri and Perussia, 1984) including a recent historical review (Oldham, 1982) and multi-authored books which have covered particular areas of NK research in great detail (Herberman, 1980, 1982, 1983b).

Cell Lineage

The cell lineage of NK cells is very controversial and at the present time is unresolved. NK cells have been described as having a separate lineage (Ortaldo, 1982) unlike that of other mononuclear cells (Table 1; Table compiled from: Gidlund et al., 1980; Koo et al., 1980; Roder, 1980a; Herberman and Ortaldo, 1981; Minato et al., 1981; Ortaldo and Herberman, 1984; Trinchieri and Perussia, 1984). It is generally agreed that NK cells are bone marrow derived (Haller et al., 1977). The problem in assigning a specific lineage is that these cells have characteristics and properties associated with many types of immune cells (Table 1). Another major problem with assigning a cell lineage is that the NK population, as identified by surface markers, is heterogenous in both the mouse (Lust et al., 1981; Minato et al., 1981) and human (Zarling et al., 1981; Abo and Balch, 1982). As shown in Table 1, the major populations of human NK cells bear the markers OKM1, OKT11 and HNK-1 (Zarling and Kung, 1980; Zarling et al., 1981; Abo et al., 1982) while mouse NK cells primarily are asialo GM1, NK-1, Qa-4, and Qa-5 positive with fifty percent expressing thy-1 antigen (Koo et al., 1980; Kasai et al., 1981; Minato et al., 1981).

In vivo reactivity

A role for NK cells in vivo has been demonstrated using a number of experimental approaches. Mutant beige mice have been shown to have impaired NK activity but normal T cell reactivity (Roder and Duwe, 1979). These mice were less able to reject transplanted NK^S tumors than their normal littermates (Karre et al., 1980). Furthermore, NK sensitive (NK^S) tumors had an increased growth rate, increased induction time and

Table 1. A comparison of characteristics and markers of NK cells with leukocytes.

	NK	T	B	MØ
Characteristics:				
Size μM	12-15	9-12	9-12	16-20
Ratio cytoplasm/nucleus	high	low	low	high
Nucleus	slightly indented	round	round	markedly indented
Adherence	- (+)	-	-	+
Phagocytosis	- (+)	-	-	+
Thymus-dependent	-	+	-	-
BM-dependent	+	+	+	-
Spontaneous reactivity	+	-	-	+
ADCC	+	-	-	+
Memory response	-	+	+	-
MHC Class I (H-2K/D)	+ (-)	+	+	+
Class II (Ia)	-	- (+)	+	+
Surface Ig	-	-	+	-
C3R	- (+)	-	+	+
Acid phosphatase	+	+	?	+
Non-specific esterase	-	+	?	-
Cortisone sensitive	+	+	-	-
^{89}Sr sensitive	+	-	-	-
Silica sensitive	-/+	-	-	+
Mouse Markers:				
Lyt 1,2,3	-	+	-	-
Thy-1	- (+)	+	-	-
Asialo-GM1	+	- (+)	-	?
NK-1	+	-	-	-
Mph-1	-	-	-	+
FcR	+/- wk	- (+)	+	+
Human Markers:				
OKT3	-	+	-	-
OKT4	-	+ (-)	-	-
OKT8	- (+)	- (+)	-	-
OKT10	+ Activated	+ Activated	-	-
Leu1	-	+	-	-
HNK-1 (Leu7)	+ (-)	- (+)	-	-
N901	-	+	-	-
MO2	-	-	-	+
B73.1	+	-	-	-
Asialo-GM1	+	-	-	+
IgG FcR1	-	-	-	+
IgG FcR2	+	-	-	-
IgG FcR	-	-	+	-
C3b receptor	-	-	+	+
C3d receptor	-	-	+	-
C3bi receptor (OKM1)	+ (-)	-	-	+
sRBC receptor (OKT11)	low affinity	high affinity	?	-

( ) = small subpopulation, ? = unknown, wk = weak

an increased metastatic ability in beige mice when compared to NK resistant (NK^R) tumors (Talmadge et al., 1980).

Antibodies directed against the cell surface glycolipid ganglio-N-tetraosylceramide (asialo GM1) could effectively kill NK cells in vitro and in vivo (Kasai et al., 1981) and could also alter the growth rate or clearance of transplanted tumors in vivo (Riccardi et al., 1980; Habu et al., 1981; Kasai et al., 1981; Barlozzari et al., 1983). Cyclophosphamide was also shown to affect NK cells in vivo and its effect correlated with an increase in pulmonary metastasis which could be reversed by the adoptive transfer of NK cells (Hanna and Burton, 1981). This reconstruction of NK depressed mice was also reported by Riccardi et al. (1981). In an elegant study, Warner and Dennert (1982) also provided strong evidence for a role of NK cells in immune surveillance. In these experiments, a cloned NK line, when transferred in vivo into NK deficient mice, restored the rejection of tumor cells and bone marrow allografts in NK deficient mice. They also showed that the incidence of radiation-induced thymic leukemias could be reversed by the transfer of NK cells into irradiated mice.

Humans with impaired NK activity, resulting from diseases such as Chediak-Higashi syndrome, X-linked lymphoproliferative disease, ataxia telangiectasia and severe combined immunodeficiency disease, all have a higher incidence of lymphoid malignancies (rev. Sullivan et al., 1980; Roder et al., 1980a; Haliotis et al., 1982).

Although these results support the hypothesis that NK cells have an important role in vivo, a number of inconsistencies and discrepancies in results have hindered a firm conclusion (Riccardi et al., 1980; Chow et al., 1981; Collins et al., 1981; Gorelik and Herberman, 1981). For

example, using various radiolabelled NK^S tumor lines injected *in vivo*, it was shown that *in vivo* clearance rates did not correlate well with their sensitivity to NK lysis *in vitro* (Riccardi *et al.*, 1980). *In vivo* clearance in the lungs showed good correlations while clearance from the spleen was poorly correlated with NK splenic activity (Riccardi *et al.*, 1980). Also, NK deficient beige mice showed *in vivo* clearance of NK^S tumors as effectively as normal C57Bl/6 mice (Gorelik and Herberman, 1981). Chow *et al.* (1981) have suggested that these inconsistencies are based upon the fact that natural resistance is a heterogeneous phenomenon.

NK Reactivity

Roder and Haliotis (1980) have subdivided NK mediated cytolysis into four stages: a) recognition and binding; b) triggering and activation of the lytic mechanism; c) lethal hit; d) target destruction. This step-wise approach has been discussed by others (Hiserodt *et al.*, 1982; Quan *et al.*, 1982) and will be used here to provide a framework to organize the large amount of information that has accumulated on this subject.

I. RECOGNITION A. General Features

The recognition of tumor cells by NK cells is much less specific or restricted when compared to cytotoxic T lymphocytes (CTL). CTL recognize virus (or antigen) in conjunction with self antigens which are coded within the major histocompatability complex (MHC), and the response is referred to as being MHC-restricted (Zinkernagel and Doherty, 1974). NK cells, on the other hand, do not require this MHC restricted recognition of antigen to react with target cells. For example embryonal carcinoma

(EC) cell lines, which lack major histocompatibility antigens, cannot be effectively lysed by CTL (Zinkernagel and Oldstone, 1976) but are effective NK^S targets (Stern et al., 1980). Also, the K562 tumor, which is a highly sensitive NK tumor target (West et al., 1977), lacks class I MHC antigens which are required for CTL recognition (Andersson et al., 1979; Klein et al., 1981).

Hansson et al. (1978) have shown that NK cells recognize syngeneic, allogeneic and even xenogeneic tumor targets. In their study, however, they found that intraspecies NK reactivity is much stronger than interspecies reactivity. Since NK cells recognize several determinants on each tumor target, these results may indicate that fewer determinants are recognized on xenogeneic tumors.

B. Involvement of Antibodies in NK recognition

Takasugi and colleagues suggested that NK cells use natural antibodies to recognize tumor targets (Akira and Takasugi, 1977; Koide et al., 1977, 1978). The role of antibodies in NK recognition or serum involvement could not be confirmed by others (Herberman and Holden, 1978; Pross et al., 1978). Furthermore, NK cells have been shown to lack surface immunoglobulin (Kiessling et al., 1975b; Roder et al., 1978). They do, however, have functional Fcδ receptors (Jondal and Pross, 1975; Kay et al., 1977; Timonen et al., 1981) and can bind and kill antibody coated targets and mediate antibody dependent cellular cytotoxicity (ADCC) (Kay et al., 1977; Perussia et al., 1980). The ability of aggregated immunoglobulin (Kiessling et al., 1975a) or staphylococcal protein A (Kay et al., 1977) to inhibit ADCC but not NK mediated lysis suggests that the mechanism of recognition by these mediators is quite different. The

ability of lymphoid cells to mediate ADCC was discovered prior to NK cells and the cells mediating this lysis were named K cells (Greenberg et al., 1975). Although still controversial, it is generally accepted that NK/K cells may in fact reside in the same cell population (rev. Bolhuis, 1980; Kamiyama and Takasugi, 1980; Kay, 1980; Koren and Jensen, 1980; Perussia et al., 1980)

C. Normal Cells as Targets

In addition to tumors, NK cells can react with a wide variety of normal cells (rev. Hansson and Kiessling, 1983). Normal cells sensitive to NK mediated lysis include fetal thymocytes (Ono et al., 1977; Hannson et al., 1979, 1981), fetal and adult bone marrow cells (Kiessling et al., 1977; Nunn et al., 1977; Hansson et al., 1981; O'Brien et al., 1983), peritoneal macrophages (Welsh et al., 1979), fetal fibroblasts (Kiessling and Welsh, 1980) and antigen presenting accessory cells (Abruzzo and Rowley, 1983). Taken together, these results strongly suggest that in addition to providing a mechanism of immune-surveillance of tumors, NK cells can also regulate hematopoiesis and possibly immune functions.

D. Viral Antigens

Since NK cells can recognize and lyse virus transformed lines (e.g. YAC-1), it was suggested that endogenous C-type viruses may facilitate the mechanism of NK recognition (Herberman et al., 1975). Testing this hypothesis, Becker et al. (1976) were unable to show a correlation between murine leukemia virus (MuLv) antigen (gp71, p30, p15, or p12) expression in 11 tumor lines and their susceptibility to NK mediated lysis. The same negative results were also reported using human cells infected with murine

leukemia viruses (Kiessling et al., 1978) and with Epstein Barr Virus (EBV) infected cell lines (Clark and Sturge, 1981). Others, on the other hand, have shown that virus infection of target cells increases NK susceptibility (Nunn et al., 1977; Minato et al., 1979; Blazer et al., 1980) and that antibodies directed against viral antigens (e.g. anti gp71) can partially block NK lysis (Kende et al., 1979) but this inhibition may depend on the strain variation used (Lee et al., 1977). These discrepancies may, in part, be due to each investigator using a different virus, cell line or heterogenous NK population (of which a subset may recognize virus components?) and may also be due to virus increasing NK activity by stimulating IFN production. Most recently it was shown that hamster cells transformed by the oncogenic adenovirus Ad12 have decreased NK and macrophage susceptibility and show a high incidence of tumor susceptibility in nude mice while the nononcogenic Ad2 virus has increased NK susceptibility and show poor tumor takes (Cook and Lewis, 1984). This direct correlation between NK resistance and in vivo tumorgenicity by Ad2 virus has also been substantiated by others (Sheil et al., 1984).

E. Differentiation antigen

Since fetal thymocytes and fetal bone marrow cells are NK^S targets, while their adult counterparts are relatively NK^R (Ono et al., 1977; Hansson et al., 1981), it has been suggested that NK cells may be recognizing oncofetal or differentiation antigens (Roder, 1980b; Gidlund et al., 1981). Support for this hypothesis comes from the work of Stern et al. (1980) who reported that while embryonal carcinoma (EC) cells were NK^S, differentiated cell lines from the same tumors (endodermal cells) were NK^R. Further evidence for a NK recognition of a differentiation

antigen was obtained by using chemical agents (e.g. butyrate, TPA) which cause cells to undergo differentiation. Treatment of various NK^S cell lines (e.g. K562, U-937, HL-60) with these agents resulted in a marked decrease in NK susceptibility as measured by both lysis and competition type experiments, indicating a loss of recognition (Gidlund et al., 1981; Dokhelar et al., 1982; Werkmeister et al., 1982). Interestingly, this tumor differentiation did not alter ADDC or macrophage susceptibility (Werkmeister et al., 1982). Haliotis et al. (1984) have shown that differentiation of human melanoma cells resulted in an increase rather than a decrease in NK susceptibility. This observation was also obtained using virally transformed cells (Blazer et al., 1980; Clark et al., 1981). Differentiation of normal human lymphocytes with TPA also increased their susceptibility to autologous NK mediated lysis (Kabelitz and Kunkel, 1983). Thus, differentiation can dramatically alter tumor (or even normal cell) susceptibility to NK cells and the direction (increase or decrease) in reactivity most probably reflects the initial stage of differentiation for each cell being studied.

F. Biochemical characterization

The first biochemical characterization of NK recognition was reported by Roder et al. (1979a,b) who showed that a detergent solubilized material could be obtained from NK^S but not NK^R tumors, which was able to inhibit binding of NK-tumor conjugates. In this study, NK target structures (NK-TS) causing inhibition resided in three major molecular weight groups ranging from 130 to 240 K. The NK-TS bound to ConA, suggesting it was a glycoprotein and could also partially bind to NK cells. The 240 K molecule was shown to be unique among various tumor

lines while the 140 K had extensive cross-reactivity among the lines tested. The material had no association with other known cell surface antigens such as H-2, gp71, p30, p12 or p15E. These results supported earlier work which argued against viral determinants being involved in NK recognition (Becker et al., 1976). Somatic cell hybrids constructed from NK^S and NK^R tumor resulted in a cell which was NK^R (Roder et al., 1979b). It was concluded that the expression of NK-TS was recessive or suppressed. Roder (1980b) felt this supported the postulate that differentiation antigens, which are also recessive, may be involved in NK recognition.

Using a modification of Roder's approach, Ortaldo et al. (1983) found that detergent-solubilized material could inhibit NK-tumor binding only when the material was incorporated into synthetic lipid micelles. The isolated NK-TS was a series of glycoproteins varying in molecular weight from 30 to 165 K. The difference in molecular weights obtained by Ortaldo et al. (1983) and Roder et al. (1979a) may be due to the different cell lines used for each study and/or the use of micelles as an NK-TS carrier in the study by Ortaldo et al. (1983). Miscelles may be providing strong stabilization or support for the low molecular weight NK target structures which were not observed by Roder et al. (1979a).

NK-TS are also secreted (or shed) into the medium after 24 - 48 h of in vitro culture of NK^S tumors (Zaunders et al., 1981). The shed NK-TS used in Zaunders' study were tested by their ability to inhibit NK lysis rather than by conjugate formation. The NK-TS was shown to bind ConA and WGA, was trypsin but not neuraminidase sensitive and had a molecular weight of 120 - 140 K. Again, the differences in molecular weights for NK-TS material reported by Roder et al. (1979a) and Ortaldo et al. (1983a)

may reflect experimental differences in each study. Zaunders et al. (1981) used shed material rather than detergent-solubilized material and also tested their inhibitory material in a lysis assay rather than as NK-tumor binding conjugate assay.

G. Carbohydrate antigens

Stutman et al. (1980) first described the ability of simple monosaccharides to inhibit both murine NC and NK lysis while not inhibiting CTL lysis. Only mannose, and in some experiments galactose, were effective in inhibiting NC lysis while a wide variety of sugars could inhibit NK lysis. They postulated that NC and NK recognition involve lectin-like structures and that sugars may be inhibitory by preventing target-effector interactions. Using human NK cells, Forbes et al. (1981) observed that the phosphorylated sugars mannose-6-phosphate and fructose-6 and -1 phosphate were good inhibitors of lysis while the unphosphorylated sugars such as mannose, fucose etc. were ineffective. Differences in the ability of specific sugars to inhibit lysis depended on which tumor was used as a target. The authors also prepared a synthetic toxic compound by linking gelonin to mannose phosphate. This cytotoxic substance was taken up by tumor cells but uptake could be inhibited by mannose-6-phosphate. With this evidence Forbes et al. (1981) then postulated that a cytolytic lymphokine has a hexose phosphate moiety and that this moiety is involved in the lytic phase of NK reactivity against tumor cells.

MacDermott et al. (1981) showed that in addition to phosphorylated sugars, a wide variety of sugars (both D and L forms) including many non-physiological sugars could inhibit NK lysis but not ADCC. They suggested that sugar groups on tumor cells determine whether they are

NK^S or NK^R and that the lectin like receptor is on NK cells.

The NK lectin receptor recognition hypothesis began to lose some credibility when it was shown that the sugars, while being effective inhibitors of NK lysis, had no effect on NK binding to tumors (Forbes and Oeltman, 1982; Vose et al., 1983; Ortaldo et al., 1984).

The preliminary results of Stutman et al. (1980) did however, stimulate interest in the hypothesis that NK cells may recognize carbohydrate determinants on tumor cells. It is now well established that virus-infected cells (Warren et al., 1974) or tumor cells (Gahmberg and Hakomori, 1973) have altered glycosylation patterns on their cell surface glycoproteins (Warren et al., 1978) and glycolipids (rev. Hakomori, 1981a; Hakomori and Kannagi, 1983). Whether NK cells can recognize these altered glycosylation patterns is presently unknown.

H. Relationship to neuraminidase releaseable sialic acid

Yogeeswaran et al. (1981) observed that NK sensitivity is inversely correlated to the amount of neuraminidase releaseable sialic acid. This correlation was not found with total sialic acid content. Neuraminidase treatment of cells also partially increased NK reactivity. Biochemical differences between the NK^S and NK^R cell lines indicated an increase in expression of asialo-GM2 on NK^S and to a lesser extent GM1, GD1a, GD1b and GT. The involvement of asialo-GM2 expression and NK susceptibility was also found by Young et al. (1981). This group reported that the NK^S L5178Y (clone 27v) contained this glycolipid while it was absent in the NK^R line. Yogeeswaran et al. (1983) went on to report that IFN treatment of tumors, which was earlier shown to protect tumors against NK lysis (Trincheri and Santoli, 1978; Welsh et al., 1981), cause a 3 to 6

fold increase in neuraminidase releasable sialic acid, supporting the general hypothesis that sialic acid had some involvement in NK recognition. Neuraminidase treatment of the IFN-treated tumor targets only slightly reversed the NK resistance. The authors suggested that IFN protection of tumors from NK mediated lysis was not totally due to sialic acid masking the NK-TS.

As mentioned earlier, the differentiation of some NK^S tumors leads to a decrease in NK susceptibility. Werkmeister et al. (1983) reported that differentiated K562 had a four fold higher sialo-transferase enzyme activity. This, was reflected, in a 2 - 3 fold higher sialic acid content in glycoproteins and a 6 - 13 fold higher level in glycolypids. The differentiated K562 NK susceptibility could also be increased by neuraminidase treatment. This increase was due to an increase in binding as measured by cold-target inhibition or target binding cell assay.

Although it seemed plausible that NK cells may be recognizing glycolipids or asialo-GM2, these results were not supported by earlier work of Roder et al (1979a, b) who isolated high molecular weight NK-TS which were glycoprotein in nature rather than glycolipid. Young et al. (1981) also mention that there is evidence to point against asialo-GM2 being the NK-TS. Increasing asialo-GM2 on cells or treating NK^S asialo-GM2 positive cells with an antibody directed against that determinant had no effect on NK susceptibility. Durdik et al. (1980) also mention that many NK^S cell lines lack asialo-GM2 and that the NK-TS on tumors is sensitive to papain treatment (suggestive of a protein).

I. Correlations between NK and NAb reactivity

Gronberg et al. (1980) reported that a direct correlation existed between the ability of tumors to be lysed by NK cells and their ability to be lysed by (or bind) rabbit natural antibodies (RNAb). Gronberg et al. (1981) also showed that YAC-1 NK^R variants generated after both in vitro and in vivo selection (8 - 10 cycles) showed decreases in both NK and RNAb susceptibility. In partial accordance, Chow et al. (1981b) observed a direct correlation between murine natural antibody (MNAb) lysis and NK kill of the YAC-1 tumor. However, no correlation could be found with another tumor, the SL2-5.

Since a large proportion of natural antibodies are carbohydrate reactive and cells that are usually MNAb reactive are also NK reactive (e. g. tumors, thymocytes) (rev. Greenberg and Pohajdak, 1982; Greenberg et al., 1983) it would support the notion that, if NK cells are also carbohydrate reactive, as initially postulated by Stutman et al. (1980), both effectors may in fact recognize similar carbohydrate alterations on tumor cells. Urdal et al. (1982) have shown that the NK^S L5178Y clone 27v, but not the NK^R clone 27av, binds RNAb, supporting the initial hypothesis of Gronberg et al. (1980) that RNAb correlates with NK susceptibility. Furthermore, Urdal et al. (1982) have shown that RNAb recognizes the immunodominant sugar GalNAc on the glycolipid gangliotriosylceramide present on clone 27v. The authors, however, could not provide evidence for the involvement of this glycolipid in NK recognition.

Interferon treatment of the YAC-1 tumor results in a decrease in both NK^S and RNAb reactivity. However, the same IFN treatment of the SL2-5 tumor results in a major decrease in NK susceptibility while RNAb

reactivity increased (Greenberg and Pohajdak, 1982). These results indicate that RNAb probably is not recognizing the NK-TS per se on the SL2-5, but do not rule out recognition through altered glycosylation or the possible association of altered glycosylation with the NK reactivity of the YAC-1 and L5178Y clone 27v tumors.

J. Blood Group Antigens

Further evidence for carbohydrate involvement in NK recognition has come from Rooney and Munro (1984) who have recently shown that human NK cells can recognize both asialated autologous lymphocytes and ABO-mismatched lymphocytes (allogenic). For example, blood group O NK cells effectively kill group A lymphocytes. This reactivity could be inhibited (cold-target) by group A RBCs but not by group O RBCs. The authors suggest that neuraminidase exposes NK-TS (carbohydrate or protein component) that are masked by sialic acid. This is somewhat analogous to the removal of asialated serum glycoproteins by galactose lectin receptors of hepatocytes in the liver (Ashwell and Morell, 1974).

Recently it has been shown that monoclonal antibodies against the carbohydrate determinant (Galβ1-4(Fucα1-3)GlcNAc) of the stage specific embryonic antigen (SSEA-1) could effectively inhibit NK lysis (Harris et al., 1984). These antibodies were also capable of reducing the ability of NK^S targets to act as cold targets, thus inhibiting NK lysis of radiolabelled cells. They could also block tumors from releasing material which may inactivate NK cells. The authors suggest that they are effective competitors at the recognition stage rather than at the lytic stage. Since SSEA-1 antigens closely resemble blood group antigens (Nudelman et al., 1980) these data support the hypothesis that blood group

antigens may be important in NK recognition (Rooney and Munro, 1984). The observation that these blood group carbohydrate antigens are found on both glycolipids and glycoproteins (Hakomori, 1981b) strongly suggests that glycosylation patterns or specific carbohydrate determinants on tumor cells may play an important role in NK recognition.

K. Transferrin Receptor

Vodinelich et al. (1983) reported that the transferrin receptor which is associated with activated or proliferating cells may be the NK-TS. They showed a direct correlation between NK binding (and in some lines, lysis) and the expression of transferrin receptor as measured by the anti-transferrin antibody OKT9. Furthermore, tryptic fragments of transferrin receptor and antibody against transferrin receptor (B3/25 but not OKT9) were both able to inhibit NK lysis.

These results have been criticized by Dokhelar et al. (1984) who argued against the transferrin receptor or transferrin as being the putative NK-TS. This group has shown that cells can have low transferrin receptor and still be effective NK targets. Protein inhibitors or differentiation with hemin dramatically decreased transferrin receptor but only had minor effects on NK lysis. Also, the addition of transferrin or antibodies directed against transferrin receptor could not inhibit lysis. The lack of correlation between transferrin receptor and NK susceptibility has also been reported by Hagner (1984).

II. NK BINDING

Although NK binding and recognition of target cells may in fact be the same phenomenon, there are several physical properties associated with NK binding that do not fit the context of a "lock and key" recognition phenomenon. For example, Piontek et al. (1982) have shown that cells which do not have NK activity (e. g. thymocytes) also show an NK-like binding pattern. In other words, NK^S tumors bind to other non-NK cells better than NK^R cells. This would suggest that NK binding or even NK recognition may be accomplished through a more general binding or adhesion mechanism rather than specific recognition structures. For example, many tumors have cell surface fibronectin which has been shown to be involved in adhesion of foreign material to macrophage and monocytes (Gauperaa and Johnson, 1984). Whether NK cells can also utilize an adhesive glycoprotein on the surface of tumor cells for recognition and binding is presently unknown.

In essence there are three major methods used to assess NK binding as a separate entity distinct from NK lysis of tumor cells. NK binding assays include a single target binding conjugate assay (Roder and Kiessling, 1978; Roder et al., 1978), cold-target inhibition assay (Herberman and Holden, 1978; Herberman et al., 1979) and the tumor monolayer depletion assay (Philips et al., 1980).

NK binding is distinguishable from CTL binding in several ways. Metabolic inhibitors such as DNP, NaN_3 or low temperatures (0^oC) effectively block CTL binding but do not alter NK binding (Roder and Kiessling, 1978; Roder et al., 1980b). These early studies were later confirmed and extended by Hiseroldt et al. (1982) who showed that NK

binding was Mg^{++} dependent, Ca^{++} independent, occurred within one minute and was not altered at 4°C. It has also been shown that cytochalasin B, an inhibitor of mobility of membranes and microfilaments, was also able to inhibit NK binding (Roder et al., 1980b; Quan et al., 1982).

Initially, Roder et al. (1978) showed that nylon wool column purified murine NK cells preferentially bound NK^S tumors and not NK^R tumors. Timonen and Saksela (1980), using purified human NK cells, found that 40 - 50% of large granular lymphocytes (LGL) bound NK^S tumors and up to 80% of the binders (if activated) could effectively lyse the target cells. It soon became apparent, however, that a large number of cells that were not lysed by NK cells could in fact bind NK cells. Collins et al. (1981) showed that NK cells could bind but not lyse fibroblasts. Brooks et al. (1981) have even indicated that fibroblasts bind better than some spontaneously or chemically induced tumors. Roder et al. (1981) have also shown that NK^R clones which were selected from a mutagenized NK^S YAC tumor could bind to NK cells, as measured by cold target inhibition and the frequency of target-effector conjugates. Since many NK-tumor conjugates are not lytic (Bonavida et al., 1984; Hagner, 1984), many groups have adopted the single cell cytotoxicity assay done in agarose as originally described by Grimm and Bonavida (1979). NK lysis in this assay was shown to correlate significantly with that determined by the ^{51}Cr release assay (Rubin et al., 1982). Hagner (1984) has shown that the non-lytic conjugates do however, cause significant inhibition of target proliferation. Since metabolic inhibitors render many NK^R cells susceptible to NK lysis, it has been suggested that NK resistance may reflect a membrane repair process (Kunkel and Welsh, 1981; Hudig et al., 1981).

Bonavida et al. (1984) have suggested that NK^R tumors that conjugate with NK cells are not able to trigger the lytic activity in the NK cell. Evidence for this hypothesis comes from the ability of ConA or antibody to trigger cytotoxicity in these non-lytic conjugates. Supporting evidence for this trigger hypothesis was the observation by Targan and Newman (1983) that an antibody recognizing a glycoprotein on NK cells was able to inhibit NK lysis and cold target inhibition but not conjugate formation.

III. MECHANISMS OF LYSIS

The mechanism of target cell lysis by NK cells is very controversial and, like CTL lysis, is presently unresolved. NK lysis most probably is a multifaceted phenomenon involving a series of individual steps that trigger or activate the lytic event which leads to the lethal hit and finally to the destruction of the target cell. The "stimulus-secretion" model of NK lysis, as originally proposed by Roder et al. (1980b) and Roder and Haliotis (1980) is presently the most favoured theory and has gained wide support (Carpen et al., 1981; Quan et al., 1982). This theory has also been proposed for CTL lysis, although it is still controversial (rev. Henney, 1977, 1980). In their model, Roder and Haliotis (1980) proposed that cAMP/cGMP ratios in the NK cell may regulate cytolysis and/or trigger cytolysis. They propose that drugs or inhibitors that increase cAMP suppress NK lysis, while increasing cGMP levels augments NK lysis. This may not be a general phenomenon since induction of cAMP in other studies has been shown to affect the target recognition stage by NK cells (Ullberg et al., 1983), thus decreasing NK lytic activity.

A. Enzymes Involved in Cytolysis

Protease involvement in NK lysis was first demonstrated by Roder et al. (1978) who showed that PMSF or DIFP, potent inhibitors of serine esterases, could affect NK lytic, but not binding, activity. The involvement of enzymes in the lytic mechanism has been confirmed by others (Hudig et al., 1981; Goldfarb et al., 1982; Quan et al., 1982). Quan et al. (1982) showed that NK lysis was generally inhibited by chymotrypsin inhibitors while trypsin inhibitors, which inhibit CTL lysis (Chang and Eisen, 1980), do not affect NK lysis. Chloroquine, a drug which inhibits lysosomal enzymes, also inhibits NK lysis (Roder et al., 1980b). Recently a role for arylsulfatase in NK cytolysis has been suggested by Zucker-Franklin et al. (1983) who showed that Na_2SO_4, a competitive inhibitor of arylsulfatase, can reduce NK lysis.

Phospholipase A_2, an enzyme which generates lysophosphatidylcholine (lysolecithin), a strong detergent-like molecule from phosphatidylcholine, has been also implicated in NK lysis (Hoffman et al., 1981, 1982). In these studies, inhibitors of phospholipase A_2 (e. g. mepacrine, tetracaine, corticosteroids and Rosethal's inhibitor) were capable of significantly decreasing NK lysis. These results are similar to the observation made earlier in a study of phospholipase A_2 involvement in CTL lysis (Frye and Friori, 1975). Berke (1977) has argued that these artificial inhibitors were inhibiting conjugate formation rather than lysis and thus argued against the role of lysolecithin as a lytic moiety. However, lipomodulin, a naturally occurring phospholipase inhibitor, has been shown to inhibit NK lytic activity without an affect on binding or conjugate formation, providing strong evidence for the involvement of phospholipase

A_2 (Hattori et al., 1983). Quan et al. (1982) suggest that the phospholipase A_2 requiring step occurs during the early membrane activation event and probably not in the later lethal hit stage. Whether lysolecithin is directly involved in the lethal hit in NK cytolysis is unknown, but is worthy of future investigation.

Thus, a variety of enzyme inhibitors cause an inhibition of NK cytolysis, suggesting a role for these enzymes in this mechanism. Whether this inhibition is specific or due to other non-specific effects (e. g. toxicity) and whether these enzymes are involved in the lytic activation steps or are directly involved in the lethal hit is unknown at this time.

B. Soluble NK Cytotoxic Factors

In 1981, Wright and Bonavida reported for the first time the involvement of a soluble intermediate in the NK lethal hit mechanism and named it NK cytotoxic factor (NKCF). This material was initially produced by lectin stimulation of either murine splenocytes or human peripheral blood lymphocytes. Activity was measured after a 40 h Marbrook type culture system using a visual trypan blue viability count. Unlike rapid lysis of tumor by NK cells, the cytotoxic material was not active in a 4 h ^{51}Cr release assay. It appeared that NKCF was specific for NK^S tumors (not NK^R) and interestingly, its activity could be inhibited by the same sugars shown previously to inhibit NK lysis (Stutman et al., 1980). NKCF was produced by asialo GM1 positive cells, while only low levels could be produced by the NK deficient beige mouse (Wright and Bonavida, 1982). Later, it was shown that both NK^R and NK^S tumors could trigger NKCF production, but only NK^S tumors bind and are lysed by NKCF (Wright et al., 1983). IFN or poly I:poly C stimulation of NK cells is capable of

augmenting NKCF activity (Wright and Bonavida, 1983a) but interferon treatment of the tumor reduced their ability to trigger NKCF production. The tumor was, however, still sensitive to the lytic action of NKCF (Wright and Bonavida, 1983b). Antibodies raised against NKCF were capable of inhibiting NK lysis (Wright et al., 1983b). Continual exposure of YAC cells to NKCF resulted in the selection of a tumor which was also NK^R in the normal lytic assay (Wright and Bonavida, 1983c). Furthermore, it was shown that this NK^R YAC tumor could bind to NK cells (similar to Roder's NK^R YAC tumor, Roder et al., 1981b) and could trigger NKCF production but was not able to bind NKCF. Farram and Targan (1983) were also able to generate NKCF. However, their study differed from Wright et al. (1983a) in that NK^R tumors were not able to induce NKCF.

These studies are similar to those on lymphotoxins generated from lymphocytes which were shown to be involved in the mechanism of alloimmune CTL cytotoxicity (Ware and Granger, 1981). Interestingly, lymphotoxins have also been implicated in NK cytotoxicity (Evans, 1981; Yamato et al., 1982; Ransom et al., 1983). Lymphotoxin was shown to enhance NK cytolysis (Evans, 1981) by growth inhibition of the tumor (Ransom et al., 1983). Also, inhibition of either NK or lymphotoxin mediated lysis could be obtained by treatment with anti-lymphotoxin antisera or with various monosaccharides (Yamamoto et al., 1982; Weitzen et al., 1983).

Evidence against the role of NKCF or lymphotoxin in cell mediated cytolysis comes from the observation that innocent bystanders in CTL (Canty and Wunderlich, 1971) and NK (Roder et al., 1980b) cytolysis are not lysed. The failure of others to detect toxic material accumulating in the supernatant during CTL kill also discredits the soluble intermediate hypothesis (rev. Henney, 1977, 1980). These conflicting results might

indicate that NKCF or lymphotoxins have a very short range of activity or a short half life. However, Wagner and Brooks (1984) have recently found that mycoplasma contaminated tumors can generate a splenic cytotoxic factor which cannot be obtained in mycoplasma free cultures. They also detected NKCF-like activity using mitogen activated splenocytes but suggested that this lytic activity could be residual toxic mitogen.

These problems will only be resolved if NKCF is purified and properly characterized. Only then can we determine whether it truly is the lethal hit moiety involved in NK cytolysis.

C. Role of Granules

Unlike T cells, most NK cells have prominent granules in their cytoplasm. Granules have been found in murine (Luini, 1981; Kumagai et al., 1982), rat (Reynolds et al., 1981) and human (Timonen et al., 1981) NK cells and are often a feature of secretory cells.

Earlier, Roder and Haliotis (1980) suggested that lysosomes may be involved in NK cytolysis. In support of this idea, Carpen et al. (1981, 1982) showed that the drug monensin, which interrupts movement of vesicles and inhibits cell secretion, was capable of inhibiting NK lysis.

The possible involvement of NK granules in NK cytolysis was further studied by Neighbour and Huberman (1982) who showed that Sr^{++} caused spontaneous degranulation in 80% of NK cells and resulted in marked inhibition of lysis, although it had no effect on binding. These results are interesting since Sr^{++} does not inhibit CTL lysis (Gately and Martz, 1981). NK cells have also been shown to degranulate when they come into contact with tumor (Frey et al., 1982). These results are interesting given the study of Galli et al. (1982) who showed that a basophil

cell line having FcR for IgE was capable of lytic activity in a pattern similar to NK cells. The cell line could be stimulated by IFN and showed both granularity and some surface markers associated with NK cells. Thus, this basophil cell line showed characteristics of NK cells.

The possible involvement of granules in NK lytic activity is also suggested by studies of patients with Chediak-Higashi disease, who have both low levels of NK lytic activity (Haliotis et al., 1980) and are unable to degranulate their neutrophils. LGL from these individuals are abnormal, having a large single cytoplasmic granule (Abo et al., 1982) rather than the multiple granules seen in normal individuals. Recently, patients with chronic lymphocytic leukemia were shown to have a major decrease (75%) in azurophilic cytoplasmic granules in their LGL (although they have normal number levels), which corresponded with to depressed NK lytic activity in these patients (Kay and Zarling, 1984).

Recently, granules have been isolated, purified and characterized from NK-like cytotoxic rat tumors (Millard et al., 1984). Purified granules were capable of lysing sheep RBC. These granules contained five major proteins and were quite different from the granules obtained from mast cells, eosinophils and neutrophils in both biochemical and enzymatic features.

In an elegant study, Henkart et al. (1984) have further shown that granules obtained from normal LGL have properties similar to the granules from rat tumor LGL. In this study they showed that minute amounts (20 ng/ml) could completely lyse RBC targets in under 5 minutes at room temperature. The relative amounts of granules and targets used corresponded to the ability of one effector to effectively lyse 5 targets. Nucleated targets usually required 30-200 fold more granules. There

appeared to be no target specificity for these granules in that normal lymphocytes or RBCs were effective targets for its action, although these targets are relatively resistant to NK cytolysis. These results are different from those of Wright and Bonavida (1981) who found that NKCF could only lyse NK^S but not NK^R targets. In the same study Henkart et al. (1984) also showed that granules prepared from granulocytes, macrophage, mast cells or even T cell lysosomes did not show similar action to those prepared from LGL or LGL tumors. The lytic activity of the granule was totally dependent of Ca^{++} without a requirement for Mg^{++}. This pattern of ion dependence is similar to that observed by Hiserodt et al. (1982, 1983) for NK cell lytic activity. The strongest evidence in favour of a role for granules in NK lethal hit activity in the Henkart study was the observation that these granules were capable of forming 15 - 28 nM diameter rings in the membranes of target cells, shown by electron microscopy (EM). These results are in agreement with the finding by Podack and Dennert (1983) that a cloned NK line was capable of producing similar 16 nM lesions in target cells. In this study, Podack and Dennert found tubular complexes being assembled into the membrane, resulting in transmembrane channels. This activity could be reversed by monensin, suggesting that a secretory phenomenon was producing the lesion. In Podack and Dennert's study, RBCs were used as targets and lectin was used as a bridge to join the NK cells to the targets. This observation again suggests that the material released probably is not restricted to the lysis of NK^S cells .

Henkart et al. (1984) also found that the granules are very unstable at 37°C in calcium supplemented medium and that the lytic activity is pronase sensitive. Lytic activity can be solubilized from the insoluble

granule by 2 M KCl and is presently being purified by that group. The instability of this material also suggests that it is unlike NKCF (Wright and Bonavida, 1981) which was shown to be relatively stable (8 h to 3 days without effect) while the granule lost all activity after 30 minutes.

Whether the soluble protein from the granules is NKCF remains to be seen. It would be of considerable interest to determine on a panel of tumors if this material is similar to NKCF in lysing only NK^S and not NK^R tumors (preliminary evidence by Henkart et al., 1984, suggests not) and whether simple monosaccharides which can inhibit NKCF activity can also inhibit granule induced cytotoxicity.

D. Role of Superoxide Production and Chemiluminescence

When phagocytic cells of the myeloid lineage (e. g. polymorphs, monocytes, macrophage) are appropriately stimulated they undergo a process termed "respiratory burst" or "oxidative burst" which is involved in an oxygen-dependent killing mechanism (rev. by Babior, 1978; Klebanoff, 1980). The respiratory burst is produced by the single electron (univalent) reduction of oxygen to superoxide anion (Babior et al., 1976) which is catalyzed by the membrane bound NADPH oxidase (Patriarca et al., 1971) utilizing the pyridine nucleotide NADPH as the electron donor:

$$2O_2 + NADPH \longrightarrow 2O_2^- + NADP^+ + H^+$$

Two molecules of superoxide anion can then interact spontaneously (dismutation) or undergo catalysis by the enzyme superoxide dismutase (SOD) to form hydrogen peroxide:

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Hydrogen peroxide can either react with the superoxide anion to form hydroxyl radical by the Haber-Weiss (1934) reaction:

$$O_2^- + H_2O_2 \longrightarrow \cdot OH + OH^- + O_2$$

or with halide ions (Cl^-, Br^- and I^-) catalyzed by the enzyme myleoperoxidase (Klebanoff, 1967) to produce hypohalite ions:

$$X^- + H_2O_2 \longrightarrow XO^- + H_2O$$

Products of these reactions (O_2^-, $\cdot OH$, XO^- and H_2O_2) have also been shown to be bactericidal (rev. by Babior, 1978; Klebanoff, 1980). The products can be detected by the emission of light which is referred to as chemiluminescence (CL) (Allen et al., 1972; Cheson et al., 1976) and can also be detected by the reduction of cytochrome C or nitroblue tetrazolium dye (Baehner et al., 1976). Chemiluminescence can be amplified by the compound luminol (Allen and Loose, 1976).

The killing of tumor cells by monocytes and granulocytes was shown to involve both an oxygen-dependent, myeloperoxidase-dependent mechanism (Clark and Klebanoff, 1979; Weiss and Slivka, 1982) and a myeloperoxidase-independent mechanism involving the generation of H_2O_2 (Mavier and Edgington, 1982). In these studies catalase was effective at inhibiting tumor cytolysis while SOD showed little or no effect (Weiss and Slivka, 1982; Abrams and Brahmi, 1984).

Roder and co-workers (Roder et al., 1982; Helfand et al., 1982a) proposed the hypothesis that NK cells, like myloid cells, utilize a respiratory burst in their cytolytic pathway. It was shown that HNK-1 positive NK cells, when triggered by NK^S and not NK^R tumor, can generate a respiratory burst as measured by luminol CL or cytochrome C reduction. It was also shown that superoxide dismutase, but not catalase, was able to inhibit both NK cytolysis and CL, indicating that the superoxide anion was intimately involved in the NK lytic mechanism. These

results were different from those mentioned earlier showing that catalase, but not SOD, could inhibit tumor cytolysis by monocytes and granulocytes.

If the differentiated K562 tumor, which shows lower NK lytic susceptibility, was used as the stimulus for the NK respiratory burst, there was a decreased response as compared to the undifferentiated K562 tumor (Werkmeister et al., 1982). Werkmeister et al. (1983b) also reported that humans with low NK lytic activity, but who had normal levels of HNK-1 positive cells, had a correspondingly low NK respiratory burst when triggered by the K562 tumor. This supports the hypothesis that the respiratory burst of NK cells plays an important role in the cytolytic pathway.

These results differ from the observations of Seaman et al. (1982) who reported that catalase and SOD do not inhibit NK lysis and that oxygen radicals actually inhibit the lytic activity of NK cells. Patients with chronic granulomatous disease (CGD) lack an oxidative response but have normal levels of NK lytic activity (Kay et al., 1983; El-Hag and Clark, 1984) suggesting a dissociation between the respiratory burst and NK lytic activity. The inability of NK cells to generate a respiratory burst (CL) or to be inhibited by SOD was reported by several investigators (Hoffman and Ferrarini, 1983; Kay et al., 1983; Abrams and Brahmi, 1984; Ramstedt et al., 1984; Suthanthiran et al., 1984). The reason for these discrepancies in results, when testing the ability of NK cells to generate a respiratory burst or the ability of SOD to inhibit NK cytolysis, are not known.

Roder et al. (1982) also point out that the correlation between NK cytolysis and respiratory burst are not totally perfect, since Chediak-Higashi patients, who have low levels of NK lytic activity (Haliotis

et al., 1980) have a normal CL response. This group suggests (Helfand et al., 1982b) that the generation of superoxide anion may be involved in activation of other events (e. g. phospholipases to produce arachidonic acid) rather than being directly involved in the lethal hit mechanism. In partial support of this phospholipase involvement hypothesis, Suthanthiran et al. (1984) found that a large variety of hydroxyl radical inhibitors (e. g. DMSO, thiourea, benzoic acid) could effectively inhibit NK lysis. They suggest that the hydroxyl radicals are generated by the lipoxygenase pathway of arachidonic acid metabolism since NDGA blocked the enzyme's activity and resulted in a reduction in NK lysis . They also showed that inhibitors of the cyclooxygenase pathway (acetylsalicyclic acid or indomethacin) were ineffective in inhibiting NK lysis.

It has been postulated that the ability of tumors to stimulate the production of reactive oxygen intermediates by rat spleen suspension and by polymorphs was dependent on tumor contamination with mycoplasma (Koppel et al., 1984; Peterhans et al., 1984). These authors were unable to induce a CL response with uncontaminated tumors.

It also should be mentioned that while the respiratory burst is utilized in the kill mechanism by macrophage and polymorphs, it is not involved in CTL cytolysis (Nathan et al., 1982).

In conclusion, the ability of NK cells to generate a respiratory burst and utilize oxygen radicals (superoxide or hydroxyl) in the NK cytolysis mechanism is presently controversial.

IV. TARGET DESTRUCTION

It is generally believed that after the NK lethal hit target destruction occurs through a colloid osmotic lysis similar to that found in antibody and complement mediated lysis (Roder et al., 1980; Hiserodt et al., 1982; Henkart et al., 1984). Hiserodt et al. (1982) have shown that by dissociation of the conjugates, similar to CTL lysis, there was a killer-cell independent cytolysis stage. This event was Ca^{++} and Mg^{++} independent, was blocked at 4°C and required 30 minutes to be completed.

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CHAPTER I. AN OLIGOSACCHARIDE BIOSYNTHETIC DEFECT IN CONCANAVALIN A-RESISTANT CHINESE HAMSTER OVARY (CHO) CELLS THAT ENHANCES NK REACTIVITY IN VITRO AND IN VIVO

ABSTRACT

We have examined a concanavalin A-resistant Chinese hamster ovary cell line (C^R-7) that has a defect in the synthesis of asparagine-linked oligosaccharides and consequently an alterated expression of membrane carbohydrate. The C^R-7 mutant, which has a decreased ability to incorporate mannose into oligolipid and membrane glycoprotein and an increased membrane fucose, was more sensitive to natural killer cell lysis than the parental wild type (CHO-WT). Splenocytes mediating the lysis of the C^R-7 line were asialo $GM1^+$, nonadherent, stimulated by IFN, absent in the bg/bg mutant and co-fractionated on Percoll density gradients with cells mediating lysis of the YAC-1 murine lymphoma. The increase in NK lysis correlated with enhanced binding of NK cells to the mutant determined by adsorption on tumor monolayers, cold target inhibition and target binding analysis. A revertant of C^R-7 (RC^R-7) which showed wild-type levels of NK sensitivity was intermediate in its ability to bind or cold target inhibit NK cells. The C^R-7, CHO-WT and RC^R-7 lines were equally sensitive to hypotonic lysis and cytotoxicity by human lymphokine activated killer (LAK) cells suggesting that the mutation did not non-specifically alter membrane fragility. The NK sensitive C^R-7 line was less tumorigenic after subcutaneous injection in nude mice when compared to the parental CHO-WT or RC^R-7 lines. This decreased tumorigenicity could be reversed by the intravenous injection of antiserum directed at the NK cell determinant asialo-GM1. In conclusion, a $ConA^R$ tumor cell line with a demonstrable oligosaccharide biosynthetic defect, exhibited enhanced NK lytic sensitivity and was poorly tumorigenic in vivo, a feature which may also be a consequence of its altered NK reactivity.

INTRODUCTION

The ability of natural killer (NK) cells to lyse tumors depends partly on a poorly understood recognition mechanism which facilitates binding, and may be important in determining susceptibility to tumor lysis. Earlier studies have shown that isolated tumor NK target structures which can inhibit NK binding are glycoproteins that can bind to ConA Sepharose (1). Since simple monosaccharides have been shown to block NK lysis (2,3), and the amount of neuraminidase releasable sialic acid from tumors may correlate with NK cell reactivity (3,4), it has been suggested that membrane carbohydrates may be intimately involved in tumor cell recognition and lysis (2,6). In the following experiments, we have examined certain cell lines with altered carbohydrate on cell surface glycoproteins as a result of a mutation affecting resistance to the lectin ConA. The $ConA^R$ CHO cell line, C^R-7, has been shown to have a decreased ability to incorporate mannose into lipid, oligo-lipid and protein with a concommittant increase in cell surface fucose (7). This alteration of cell surface carbohydrate expression results from a mutation affecting the synthesis of asparagine linked oligosaccharides (8). It was of interest to determine whether this mutation had altered in vitro recognition by NK cells as well as in vivo tumorigenicity.

MATERIALS AND METHODS

Mice

Six to 8 week old male CBA/J, C57Bl/6 beige (bg/bg) and (bg/+) mice were obtained from Jackson Laboratories, Bar Harbor, Maine. Inbred homozygous BALB/c nudes (nu/nu) were obtained from Life Sciences Inc.,

St. Petersburg, Florida, U.S.A., and were housed continually in sterile cages in a horizontal laminar flow containment hood. CB7Bl/6 mice were bred at the University of Manitoba vivarium.

Cell Lines

The ConA^R cell C^R-7 line was selected from a parental wild type Chinese hamster ovary (CHO-WT) cell line for its resistance to the cytotoxic action of concanavalin A, which binds mannose and has been described in detail in previous reports (7,9,10,11). Briefly, these cells have a pleiotropic phenotype exhibiting numerous changes in membrane-associated properties due to a single genetic mutation affecting the synthesis of asparagine-linked oligosaccharides. The C^R-7 binds 2.5 times less ConA than the CHO-WT and also shows a reduced agglutinating activity with ConA (9,10). They have a decreased ability to incorporate mannose into lipid, oligo-lipid and protein, and a decreased membrane mannoprotein with a concomitant increase in cell surface fucose (7). Krag (8) has shown that ConA^R CHO cells with the above properties have reduced amounts of dolicholphosphate due to an enzyme defect involving the ability to glucosylate oligo-lipid intermediates. The C^R-7 cells are temperature sensitive as compared to the CHO-WT in that they show reduced ability to grow in culture at 39°C (10). However, they exhibit approximately wild type growth abilities at 37°C (10). The RC^R-7, a revertant isolated from the C^R-7 was selected by its ability to grow at 39°C (9). The RC^R-7 is intermediate in its ability to bind ConA or survive the cytotoxic action of ConA as compared to the wild-type or C^R-7 mutant (9).

The lines were maintained as monolayer cells at 37°C with a 5% CO_2 atmosphere growing in 10% fetal calf serum in alpha minimal essential medium (αMEM)(Flow Laboratories) supplemented with streptomycin and penicillin. Prior to NK lysis assays, tumor cells were removed by light trypsinization and placed into spinner cultures at 10^5 to 2 x 10^5/ml for 18-20 h at 37°C in a water bath.

NK Lysis Assay

This assay has been previously described (12). Briefly, CHO cells from 18 h spinner cultures were centrifuged and labelled with ^{51}Cr (100 Ci/10^7 cells) for 45-60 minutes at 37°C with agitation every 10 minutes. The cells were then washed three times in cold α MEM without serum and the concentration was adjusted to 1 x 10^5/ml in 10% medium. NK cells were obtained from spleens of normal or poly I:poly C (Sigma) stimulated CBA/J or C57Bl/6 mice. Poly I:poly C was injected intraperitoneally (100μg/mouse) 20 h prior to splenectomy. The assay was performed in triplicate in microtitre plates containing a final volume of 200 μl. Following incubation at 37°C in 5% CO_2, the plates were centrifuged, and 100 μl of supernatant was removed, counted in an LKB gamma counter, and the percentage of ^{51}Cr release was calculated.

To obtain interferon (IFN) stimulated NK cells, normal CBA/J splenocytes (10^7) were incubated for 90 minutes at 37°C with 1000 U/ml of IFN (L-cell Newcastle Disease Virus-induced obtained from Dr. C. Ogburn, Department of Microbiology, Medical College of Pennsylvania, U.S.A.). To deplete NK activity, splenocytes from poly I:poly C stimulated CBA/J mice were treated with 1/200 dilution of Rabbit anti-asialo GM1 antiserum (obtained from Dr. M. Sugi, Yamasa Shoyu Company,

Chiba, Japan) for 1 h at 37°C followed by incubation with a $^{1}/10$ dilution of rabbit complement (Cedarlane Lowtox) for 1 h at 37°C.

NK Absorption Assay

CHO cells were plated on 60 x 15 mm tissue culture petri dishes for 20 h at 37°C. Non-adherent tumor cells were then removed by washing with ∝MEM at 37°C and 2 x 10^7 CBA/J splenocytes were added. The dishes were incubated for 30 or 60 minutes at 37°C and the non-adherent splenocytes were gently removed, washed and the cell concentration re-adjusted prior to incubation with ^{51}Cr labelled C^R-7 target cells in the NK lysis assay. The number of tumor cells per plate at the time of splenocyte incubations were obtained from duplicate cultures handled as in the experimental groups. The tumor cells were removed by trypsinization and enumerated in a Coulter (Coulter Electronics, Fla.) counter. The number of CHO cells which detached from the plates during splenocyte absorption was minimal (<1%) and in cold target inhibition assays this amount of unlabelled target cells, a ratio of 0.5:1 labelled tumor, was insufficient to account for the differences in lytic sensitivity noted between the tumors (<5% inhibition).

Cold Target Inhibition

Unlabelled C^R-7, CHO-WT and RC^R-7 cells were taken from spinner cultures, washed and incubated with ^{51}Cr labelled C^R-7 target cells in the lysis assay. NK cells were then added and the experiment was carried out as indicated earlier.

Conjugate Forming Cells (CFC)

LGL obtained from Percoll gradients were labelled with fluorescein diacetate (Sigma) as described by Vose et al (13). CHO cells (4 x 10^5) were added to 10^5 fluorescein labelled LGL in a final volume of 100 μl, centrifuged at 150 x g for 5 min and incubated as a pellet for 30 min at 4°C. Cells were then resuspended either by gentle pipetting or by vortexing for 5 seconds. The percentage of fluorescent effector cells binding to tumor targets was determined by counting 400-500 effector cells on a hemocytometer using a U.V. microscope.

Preparation of Murine Large Granular Lymphocytes (LGL)

Spleens were taken from poly I:poly C stimulated CBA/J or C57B1/6 mice and erythrocytes were lysed with ammonium chloride (0.83%) (12). The cells were washed three times and 10^8 cells were applied to each column of 10 ml of compacted nylon wool then incubated for 1 h at 37°C (46). Nonadherent nylon wool column passaged cells were removed, washed and layered on Percoll (Pharmacia) density gradients (14) (10^8 cells/10 ml gradient). Discontinuous Percoll was made iso-osmolar by adjusting with phosphate buffered saline and then dilutions were prepared with 10% fetal calf serum in Fishers (Gibco, N. Y.) medium. The density of the fractions increased from top to bottom by 5.0% Percoll increments. Cells placed on the gradients were centrifuged at 500 x g for 30 minutes. The bands and pellet were removed and washed three times prior to each assay.

Generation of Lymphokine Activated Killer (LAK) Cells

The method of purifying human large granular lymphocytes (LGL) and generation of lymphokine-activated killer (LAK) cells has been

previously described (15). Briefly, buffy coats or whole blood from normal volunteers was layered upon Ficoll (Pharmacia, density 1.077) and centrifuged at 500 x g for 30 minutes. The mononuclear band was washed and incubated on autologous plasma coated petri dishes for 1 h at 37°C. The non-adherent cells were removed, washed and placed on discontinuous Percoll (Pharmacia) density gradients. The gradient was centrifuged at 500 x g for 30 minutes and then the LGL band was removed and washed three times. The LGL contained 40% HNK-1 positive cells by immunofluorescence and <1% monocytes by non-specific acid esterase staining. The cells were incubated in 10% phytohaemagglutinin (PHA) condition medium (PCM) with 10% fetal calf serum in RPMI 1640 (provided by Dr. J. Wilkins, University of Manitoba) at 37°C with 5% CO_2. After 5 to 12 days the cells were removed, washed and tested for LAK activity in a manner similar to the NK lysis assay.

Hypotonic Lysis

The CHO cells were labelled with ^{51}Cr and then incubated at 37 in 10% FCS containing ∝MEM diluted with distilled water. After specific time periods, the cells were centrifuged and the supernatants harvested and counted as described in the NK lysis assay.

Growth of CHO Lines in nu/nu Mice

The CHO lines (CHO-WT, C^R-7, RC^R-7) were grown for 18 h in spinner cultures, then washed three times in sterile medium without fetal calf serum. Cells were counted and 4 x 10^6 viable cells were injected subcutaneously (s.c.) into the lower midback of 9-10 wk old BALB/c nu/nu mice. The mice were maintained in a sterile environment throughout the experiment.

RESULTS

NK Cytolysis of the CHO Lines

In this study, we were interested in determining whether cell surface changes resulting from a defect in glycosylation, which renders cells resistant to the cytotoxic action of ConA, would alter their susceptibility to NK mediated lysis. As shown in Figure 1, the selected $ConA^R$ CHO cell line C^R-7, was a better target for NK mediated cytotoxicity than the parental wild-type (CHO-WT) cells whereas a revertant line of the C^R-7 (RC^R-7) was lysed at the same level as wild-type. While the CHO lines were susceptible to normal CBA/J NK cells in a 6 h assay, lytic activity was substantially higher following 18 h of incubation or when activated by the prior injection of poly I:poly C, a potent IFN and NK inducer. The C^R-7 mutants were consistently 2-3 times more susceptible to lysis when compared to the parental wild-type or revertant lines in assays using target cells taken from spinner cultures or left as adherent monolayers (data not shown).

The effector cells mediating lysis of the C^R-7 target phenotypically resembled NK cells (Table 1). They were asialo GM1 positive, non-adherent, IFN stimulatable and low in the bg/bg mutant (16). Natural cytotoxic (NC) cells which are primarily involved in natural cell-mediated cytotoxicity of adherent cell lines (17), similar to the CHO lines, do not carry these surface antigens nor are they affected by IFN or the beige mutation (18). Monolayers of the CHO $ConA^R$ mutant were also able to partially absorb NK cells directed against a common murine NK target, the YAC-1 lymphoma, indicating that

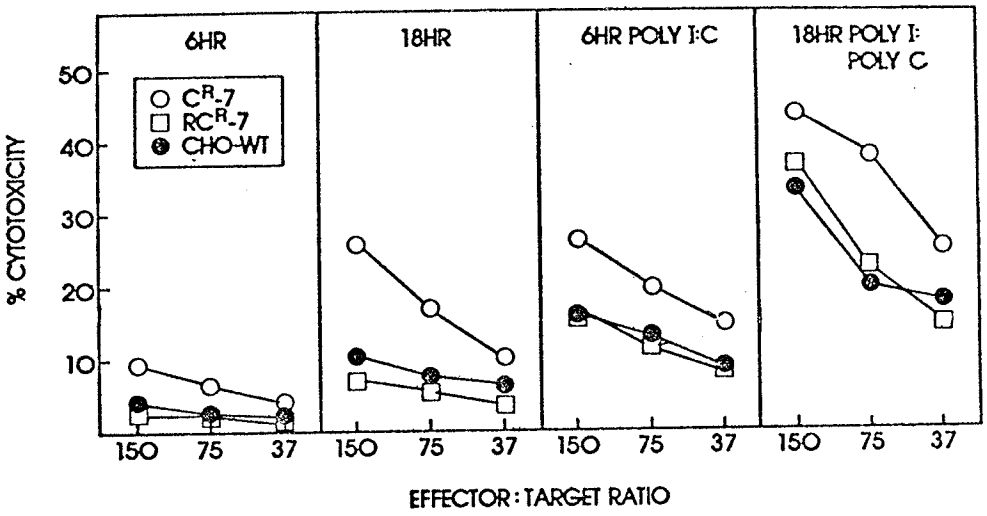

Figure 1 NK cell mediated lysis of CHO lines. The $ConA^R$ mutant C^R-7 (○) and a revertant of C^R-7 (RC^R-7)(□) were compared to the CHO-WT (●) for sensitivity to NK mediated lysis after 6 and 18 h of incubation. This experiment was performed 3 times with similar results. In this experiment and throughout the thesis, variation in levels of NK lytic activity occurred due to experimental conditions and individual differences between mice, as well as due to differences in strains of mice. Within each experimental replicate, the same similarities and differences between treatments and cell lines were found, but between replicates some differences exist, due to these random fluctuations.

TABLE 1. CHARACTERIZATION OF EFFECTOR CELLS LYSING C^R-7 MUTANT

EXPT.	EFFECTOR CELLS	% SPECIFIC LYSIS 150:1	75:1	37:1
1.	Control splenocytes	10.0	6.8	6.0
	IFN (1000 U/ml) in vitro	21.9	12.3	6.7
	Poly I:poly C (100 μg) in vivo	48.0	36.7	22.3
2.	Control splenocytes	46.4	32.4	19.1
	NWC non-adherent	37.2	26.1	20.9
3.	bg/bg splenocytes	4.8	4.3	2.2
	bg/+ splenocytes	16.7	9.1	4.5
4.	Control splenocytes	33.2	25.5	16.7
	Complement	33.6	27.1	17.3
	Anti-asiolo GM1+ complement	1.2	0.9	2.0

there was some crossreactivity of the effectors reacting with both of these cell lines (not shown).

When murine splenocytes were fractionated on discontinuous Percoll gradients (Fig.2) the LGL mediating the greatest lysis of the YAC-1 tumor were concentrated in the range of 50-55% Percoll concentration (Fraction 2 and 3). Most of the mononuclear cells, which were contained in fractions 4 and 5, exhibited little activity. Lysis of the C^R-7 and CHO-WT cells was also enriched in fractions 2 and 3, with C^R-7 kill 7 lytic units greater than CHO-WT cells in Fraction 2, and 4 lytic units greater in fraction 3.

NK Target Binding

(a) NK adsorption to CHO monolayers

Tumor cells that are sensitive to NK lysis have often been shown to have a greater ability to bind NK cells (reviewed 18,19). We first examined the possibility that enhanced lysis was due to an improved membrane interaction with the mutant by assessing the depletion of NK activity of CBA/J splenocytes on tumor monolayers. Figure 3 illustrates that the $ConA^R$ cell line C^R-7 was indeed more efficient at adsorbing splenic NK cells than the corresponding CHO-WT line after 30 and 60 minutes of incubation. The RC^R-7, which was less able to deplete NK activity than the C^R-7 line, was somewhat more effective than the wild type. By increasing the length of incubation of splenocytes on the monolayers from 30 minutes (Figure 3a) to 60 minutes (Figure 3b), the RC^R-7 NK adsorptive capacity was now intermediate between the C^R-7 and CHO-WT lines.

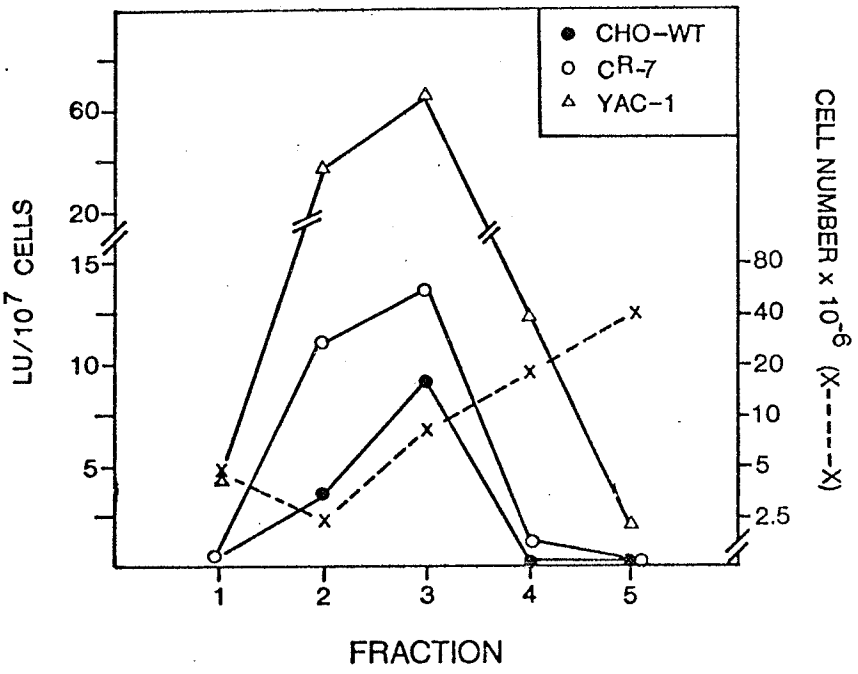

Figure 2 Percoll density gradient fractionation of large granular lymphocytes (LGL). Splenocytes were prepared and layered on a discontinuous Percoll density gradient as outlined in Methods. Concentrations of Percoll in each fraction varied by 5% increments: 1 = 45%, 2 = 50%, 3 = 55%, 4 = 60%, 5 = pellet. A lytic unit (LU) is the number of splenocytes (effectors) necessary for 30% lysis of 10^4 ^{51}Cr labelled target cells.

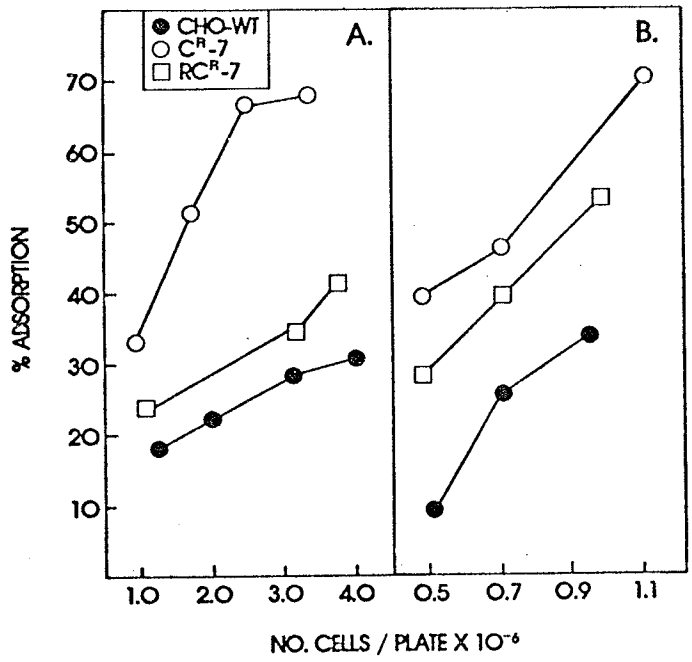

Figure 3 Adherence of NK cells to CHO monolayers. CBA/J splenic NK cells were incubated on CHO monolayers for A) 30 min or B) 60 min. The percentage reduction in NK cell activity was determined by assaying the lytic activity of the non-adherent splenocytes and comparing this to the initial population. The $ConA^R$ C^R-7 (○) removed more NK activity than the CHO-WT (●) and the revertant RC^R-7 (□). This experiment was repeated several times with similar results.

(b) Cold-Target Inhibition

An assay that has been used to assess the efficiency of NK-tumor interactions is the ability of unlabelled tumor cells to compete with labelled targets in the NK lysis assay. Although most researchers suggest that this assay indicates how well a tumor can bind to NK cells (18,19), another interpretation has been proposed that inhibition is the result of NK inactivation by certain tumors rather than simple tumor competition (20). Unlabelled C^R-7 tumor cells were clearly more effective at inhibiting NK lysis of the C^R-7 target than either the CHO-WT or RC^R-7 cells (Figure 4). The RC^R-7 line was intermediate in its inhibitory capacity, similar to the tumor monolayer adsorptions. We next proceeded to a direct target binding assay to confirm that the mutation had altered NK recognition of the C^R-7 line.

(c) Conjugate Forming Cells (CFC)

After Percoll fractionating non-adherent splenocytes, fractions containing the greatest and least lytic activity (Fraction 3 and 6, respectively) were assessed for conjugate forming cell frequency (CFC) against the three CHO lines (Table 2). Two conditions were compared, one in which conjugates were resuspended gently, and a second where vigorous vortexing would presumably leave only high affinity binding conjugates. As expected, Fraction 3 cells formed conjugates at a higher frequency than the NK depleted fraction 6, however, a significant number of rosettes were detected even in this population in spite of the total absence of NK activity. The C^R-7 line exhibited a higher frequency of CFC's than the CHO-WT or RC^R-7 lines with Percoll fraction 3 cells

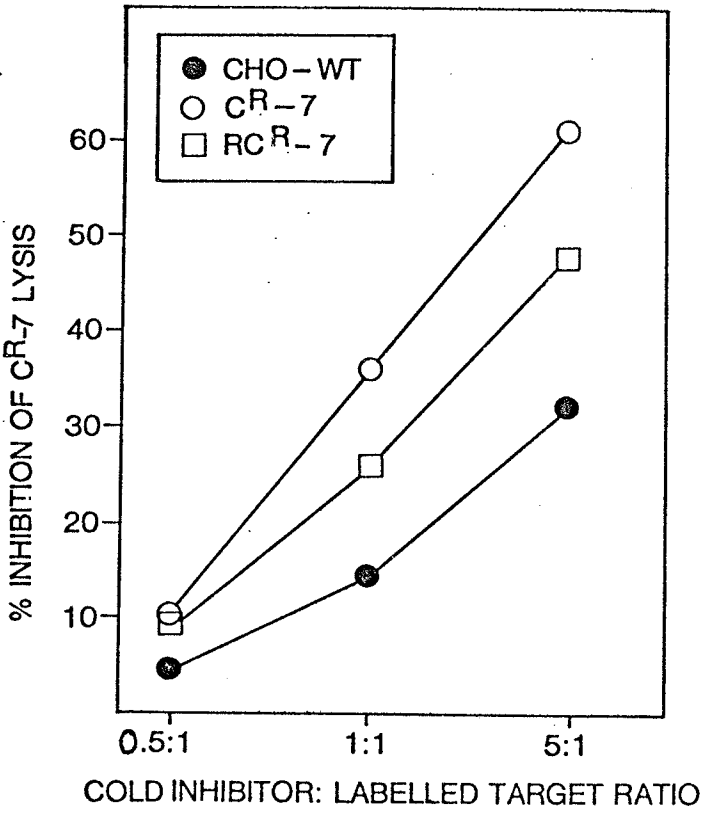

Figure 4 Cold-target inhibition of NK lysis of C^R-7. Lysis of labelled C^R-7 by poly I:poly C stimulated CBA/J NK cells at an effector to target ratio of 150:1 was inhibited by various concentrations of cold CHO-WT (●), C^R-7 (○) and RC^R-7 (□) tumor cells. This experiment was repeated an additional two times with similar results.

TABLE 2. CONJUGATE FORMING CELL (CFC) FREQUENCY IN NK ENRICHED AND DEPLETED PERCOLL FRACTIONS

PERCOLL FRACTION	LYTIC ACTIVITY	CFC AFFINITY[1]	% CFC		
			C^R-7	CHO-WT	RC^R-7
3	high	high	12.4	8.7	7.8
6	low	high	6.5	4.7	4.4
3	high	low	16.7	10.2	14.2
6	low	low	5.4	5.2	5.8

[1] High affinity CFC were counted after vigorous vortexing and low affinity CFC after gentle pipetting. This experiment was repeated once with similar results. At least 500 lymphocytes were counted to determine the CFC frequency.

using both conditions for resuspending the rosettes although, as one might predict, the frequency of CFC's surviving vigorous mixing was somewhat lower.

Lytic Sensitivity of the CHO Lines

It has been shown that NK targets can vary in their lytic susceptibility as a result of non-specific alterations in membrane fragility which can be detected by sensitivity to osmotic lysis (21). This does not appear to be the case for the C^R-7 cells since the effects of hypotonic medium on this NK susceptible mutant were very similar to both the CHO-WT and the RC^R-7 cells (results not shown).

In examining the sensitivity of the CHO lines to human NK cells, it was found that although they were resistant to enriched populations of large granular lymphocytes (LGL), they were killed by LAK cells prepared as described by Grimm et al (15) (Figure 5). The mutation for $ConA^R$ did not alter the susceptibility of the CHO cells to this effector cell.

The results from these experiments, then, would rule out the possibility that greater membrane fragility or poorer membrane repair was the determining factor for NK susceptibility of the C^R-7 mutant. In addition, it suggests that the alteration in cell mediated lytic sensitivity of the C^R-7 line is limited to NK cells.

Growth of CHO Lines in Nude Mice

It has been shown that the sensitivity of tumors to in vitro NK lysis correlates inversely with the ability of the tumors to grow in vivo (22). We tested the tumorigenicity of the CHO cell lines after

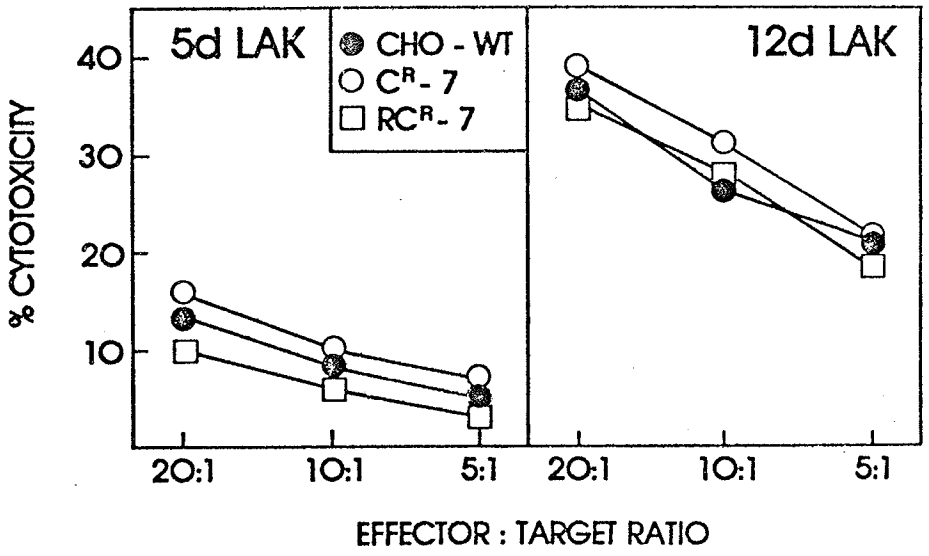

Figure 5 Lymphokine activated killer (LAK) cell lysis of CHO lines. LAK cells were generated from human NK cells cultured in 10% PHA-conditioned medium for 5 or 12 days. Lysis of the CHO-WT (●), C^R-7 (○) and RC^R-7 (□) cells was identical. Similar results were obtained with LAK cells cultured for longer periods of time.

subcutaneous injection into BALB/c nu/nu mice and found that only the CHO-WT and the RC^R-7 cells grew well (Figure 6). The differences in tumor size were clearly evident by day 10 and continued until day 19 when the animals were sacrificed for histo-pathological examination. No organ metastases were observed in these mice.

A second experiment tested the hypothesis that the poorer tumorigenicity of the C^R-7 mutant was, at least in part, the result of the action of NK cells in the nude mouse. According to the method of Kasai et al (23), nude mice were injected intravenously with rabbit anti-asialo GM1 antiserum immediately before the C^R-7 mutant was implanted subcutaneously. Antiserum treatment was repeated every three days for a total of 6 injections. The reduced latency and enhanced growth rate of the C^R-7 cells in antiserum treated mice approached that of the CHO-WT parent (Figure 7). In a repeat of this experiment, the antiserum had little influence on CHO-WT growth rates while again, increasing the tumorigenicity of the C^R-7. Normal rabbit serum was without effect (data not shown).

Discussion

In this report we present evidence that a single mutation (47) in oligosaccharide biosynthesis that rendered CHO cells resistant to the cytotoxic action of the lectin, ConA, enhanced tumor sensitivity to NK mediated lysis. Studies with murine, rat and human tumors have shown that NK susceptibility correlates well with the ability of the tumor cells to bind NK cells (18,19). However, others have shown that changes in tumor susceptibility can also result from altered membrane repair (24), fragility to osmotic lysis (21), inactivation of NK cell after binding (20), and susceptibility to soluble NK cell lytic

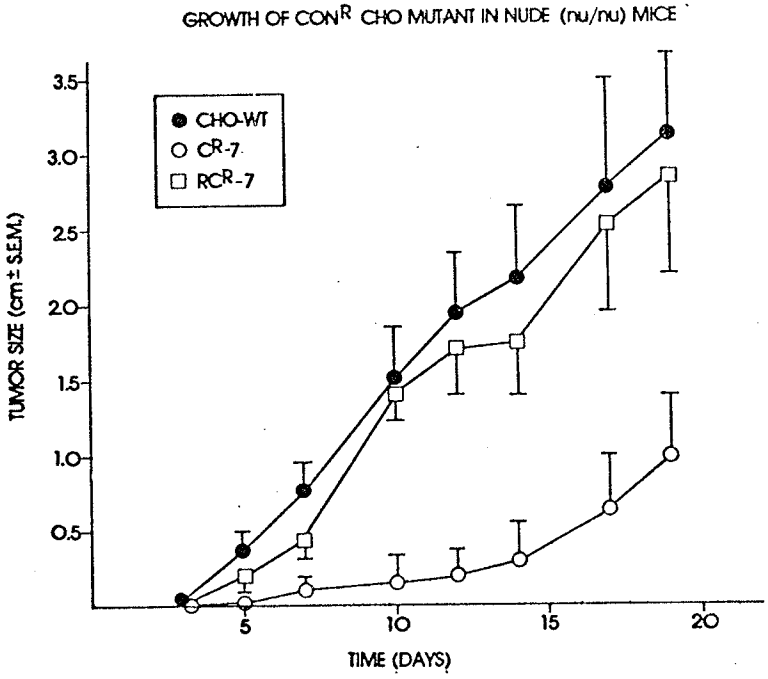

Figure 6 Growth of CHO lines in vivo. CHO cells from spinner cultures were inoculated (4×10^6) subcutaneously into the lower midback of BALB/c nu/nu mice. Tumor volume was measured three times weekly with vernier calipers. The CHO-WT (○) and RC^R-7 (□) lines grew significantly ($p<0.05$) faster and produced larger tumors than the C^R-7 (●) on days 7 through 19 (Student's t-test).

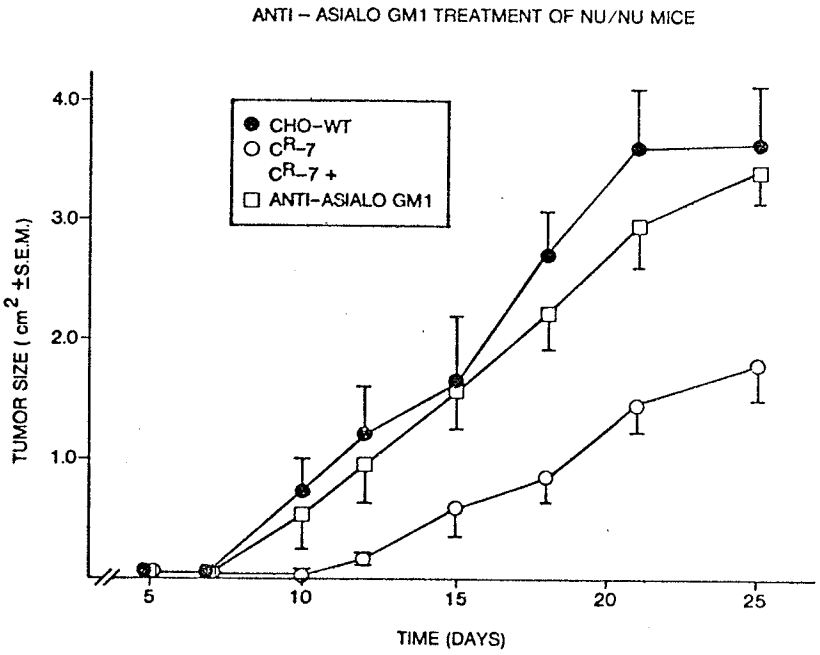

Figure 7 Effect of in vivo treatment of BALB/c nude mice with anti-asialo GM1 on C^R-7 growth rate. BALB/c nu/nu mice were pre-injected intravenously with 15 μl of anti-asialo GM1 antiserum in 100 μl of Hanks medium prior to inoculation of the C^R-7 subcutaneously followed by 10 μl injections intravenously every three days for a total of 6 injections. Antiserum treated mice developed tumors as rapidly as mice injected with the CHO-WT. The C^R-7 line grew significantly ($p<0.05$) slower than the CHO-WT or C^R-7 anti-asialo GM1 treated mice on days 18 through 25 (Student's t-test).

factors (NK lymphotoxin) (25). We believe that based on several experimental approaches, the most likely explanation for the altered lytic sensitivity of the $ConA^R$ mutant is a membrane alteration resulting in enhanced NK binding. The cold target competition and monolayer adherence assays inhibit lysis as a consequence of NK interaction with tumor. Whereas the competition experiments rely on preferential NK binding and/or inactivation, monolayer adsorptions deplete high affinity binding cells from the splenocyte pool. Although it has not been directly demonstrated, it is unlikely that the decreased lytic activity in the nonadherent splenocytes recovered from tumor monolayers was due to inactivation followed by detachment, rather than simple depletion of tumor adherent NK cells. This could only be true if the affinity of the CHO cells for NK cells was relatively weak. We subsequently tested the binding capacity of high and low NK activity LGL fractions from Percoll gradients. These experiments confirmed that the C^R-7 mutant more readily formed conjugates with NK enriched fractions and the frequency of CFC's was greater than CHO-WT and RC^R-7 lines. These conjugates were maintained even after vigorous agitation demonstrating high affinity NK-CHO tumor binding. The differences between the CHO lines was not as large in the CFC assay as those detected with the cold target inhibition or adherence techniques. These latter assays measure residual NK cell activity in a functional NK lysis assay, however, it has been shown that not all splenocytes which bind tumors in the CFC assay are lytic or are NK cells (26). This was confirmed in our experiments where cells taken from NK depleted fractions of the Percoll gradient were also able to form CFC's. It may be then, that non-NK cell interactions were obscuring the NK conjugate frequencies

even in the NK enriched fraction 3.

The revertant, RC^R-7 which was as resistant to lysis as the parental CHO-WT line, proved to be intermediate in its ability to interact with NK cells when tested in the absorption and cold target inhibition assays suggesting that it may only be a partial revertant for the NK adherence phenotype. This was, in fact, similar to its ConA phenotype in that the D_{10} value for cell survival, and Hill coefficient for ConA binding were intermediate between C^R-7 and parental CHO-WT (9). The observation that RC^R-7 lytic sensitivity is at wild type levels, then, suggests that the resistance to lysis may only be in part a consequence of the reversion of the $ConA^R$ and NK adherence phenotype. The RC^R-7 line is a revertant selected for its ability to survive at 39°C, a permissive temperature for growth of the CHO-WT, but not the temperature sensitive C^R-7 mutant (10). It is, of course, possible that a second (suppressor?) mutation has occurred during the selection of a temperature resistant variant of the C^R-7 line, which has only partly restored the NK binding and $ConA^S$ phenotype.

It has been proposed that carbohydrate determinants on tumor cells are important for NK target recognition (3). Simple monosaccharides have been shown to inhibit NK lysis of both human and murine tumors (2,3) while these carbohydrates do not inhibit lysis either by allo-sensitized cytotoxic T cells or antibody dependent cellular cytotoxicity (ADCC) (27). Vose et al (13), while confirming that monosaccharides will block NK lysis, found that they do not inhibit binding to a susceptible tumor. These authors postulated that NK cells do not use a lectin like mechanism for target recognition but that carbohydrate

inhibition is due to a post-binding lytic event. Wright and Bonavida (25) have provided further evidence to support this hypothesis. These investigators found that the lytic action of NK lymphotoxins was inhibitable by the same monosaccharides which were found to suppress cellular NK lysis. Although it was possible that the mutation affecting C^R-7 cells may have altered a saccharide "acceptor" site for NK lymphotoxin, we have not been able to confirm this. The CHO lines are not susceptible to lymphotoxins that we have been able to generate from ConA or YAC-1 stimulated splenocytes, while absorption of lymphotoxin by the C^R-7 and CHO-WT lines using a YAC-1 target has not revealed any significant differences (28).

Evidence in favor of a carbohydrate moiety involved in NK recognition comes from the correlations between the amount of neuraminidase releasable sialic acid and NK susceptibility. Yogeeswaran et al (4) have found an inverse correlation which was related to an alteration in sialylation of glycosphingolipids (gangliosides). In addition, the protective effects of IFN on the susceptibility of tumors to NK lysis was correlated with increased amounts of neuraminidase releasable sialic acid (5). Werkmeister et al (29) recently reported that butyrate induced modulation of K562 NK susceptibility also produced changes in membrane sialation of both glycoproteins and gangliosides, possibly as a result of enhanced sialo-transferase enzyme activity. Increased NK susceptibility in these experiments was accompanied by a greater frequency of CFC's.

ConA resistance of CHO cells is thought to be the result of the deficiency of an enzyme (glucosyl transferase) which glucosylates dolichol-P-P-($GlcNAc_2$)-Man_9 in the formation of asparagine-linked oligosaccharides (8). These cells consequently

incorporate less mannose into lipid and oligolipid, and cell surface labelling with mannose revealed alterations in glycosylation of membrane glycoproteins (7). Concomitant increases in membrane fucose were associated with an increased fucosyl transferase enzyme activity (7). It is of interest that glycosylation by fucosyl transferase and sialyltransferase are mutually exclusive events in the formation of asparagine-linked oligosaccharides of asialotransferrin (30). If fucosylated glycoproteins are not acceptors for sialyltransferase, then one might predict poor sialation of these glycoproteins on the C^R-7. In accord with this hypothesis we have found that the C^R-7 mutant binds wheat germ agglutinin (WGA) which recognizes GlcNAc and sialic acid, less well than CHO-WT or RC^R-7. However, measurement of neuraminidase releasable sialic acid from these cell lines has revealed only a minimal reduction in the sialation of the C^R-7 line compared to the CHO-WT. This would suggest that, if a sialation defect exists, it must be of a very limited nature, possibly effecting only a few glycoproteins. Alterations in cell surface glycoproteins in the C^R-7 mutant have been detected using the galactose oxidase-(^{3}H)borohydride technique, lactoperoxidase catalyzed iodination of surface polypeptides and metabolic incorporation of labelled glucosamine (11), in which new glycoproteins have been found on the ConA resistant mutant but not CHO-WT or RC^R-7 revertant lines. Evidence that may point to an altered N-linked oligosaccharide on these cells which participates in the NK recognition event is the ability of tunicamycin to reverse the enhanced binding of NK cells to the C^R7 mutant (31). Tunicamycin is an inhibitor of the formation of dolichol-GlucNAC. Using concentrations that decreased the incorporation of ^{3}H-mannose into C^R-7 by 80%, but did not alter

protein or DNA synthesis measured by ^{3}H-leucine and ^{3}H-TdR uptake, NK cell adhesion to tumor monolayers was reduced to wild-type levels (B. Pohajdak, A.H. Greenberg, manuscript in preparation).

NK cells can recognize and lyse a variety of tumors and also normal cells including fetal thymocytes (32), bone marrow precursors (33) and fibroblasts (20) suggesting a wide range of cell reactivities. It has generally been found (although exceptions exist) that NK lytic susceptibility correlated with the ability of tumors to bind NK cells as measured by cold target inhibition, adsorption and conjugate target binding cell assays (18,19). Piontek et al (26) have found that other non-NK cells (thymocytes and macrophage) can effectively bind tumors with the same NK specificity pattern. Although NK-tumor membrane binding is an important initial event in NK recognition, the phenomenon may be a consequence of non-specific changes in intercellular adhesiveness, rather than a specific receptor-ligand interaction. In that regard, it has been previously shown that variants of CHO cells selected for increased trypsin detachability have increased levels of hyaluronic acid (34), a glycosaminoglycan involved in cell adhesion (reviewed in 35).

NK cells may play a role in controlling tumor growth and metastasis (36, 37). We have reported that the C^{R}-7 cells which are more NK sensitive were less tumorigenic when injected subcutaneously in nude mice than the parental or revertant lines. It was also shown that intravenous injections of antiserum directed against NK membrane antigen asialo GM1 could increase the growth rate of the C^{R}-7 tumor in vivo. An increase in tumor growth following anti-asialo GM1 pretreatment of nude mice was also reported by Kasai et al (23) using the RL♂1 tumor.

This antiserum ablates NK activity and the observed enhancement of tumor growth rate is probably due to this action of the antiserum. Independent confirmation using other forms of NK deprivation will be required before this conclusion can be reached.

Alterations in metastatic and invasive behaviour of tumors selected for resistance to lectins has been reported from several laboratories (38,39,40). Melanoma (38), lymphosarcoma (40) and leukemia (39) lines which are highly metastatic can be made non-metastatic by selection of WGA lectin resistant variants. Interestingly, these nonmetastatic WGA resistant mutants are poorly sialated (41,42,43). It has been suggested, for example, that sialic acid can mask site on glycoproteins s involved in tumor adhesiveness (41,44). Finne et al (45) have shown that the non-metastatic WGA resistant B16 melanoma cells not only have decreased amounts of cell surface sialic acid but also have a concomitant increase in surface fucose, similar to the $ConA^R$ C^R-7 line. One might speculate then that some of the lectin resistant mutants with oligosaccharide changes comparable to the $ConA^R$ CHO line described here have altered NK cell reactivity, and that NK cells may therefore contribute to the altered metastatic or tumorigenic phenotype.

In conclusion, we have shown that a defect in asparagine-linked oligosaccharide biosynthesis which occurred as a result of a single mutation that renders CHO cells resistant to the cytotoxic effect of ConA, enhanced tumor reactivity with NK cells and reduced tumorigenicity in nude mice. Whether or not the altered membrane carbohydrates on the $ConA^R$ mutant are directly involved in the enhanced NK adhesiveness of the mutant is presently under study. It is suggested that alterations

in tumorigenic capacity of tumors selected for resistance to plant lectins may be a result of altered NK reactivity.

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CHAPTER II. COMPARATIVE ANALYSIS OF NK CELL AND MACROPHAGE RECOGNITION OF CONA RESISTANT CHINESE HAMSTER OVARY (CHO) CELLS

ABSTRACT

We have previously shown that the mutation rendering CHO cells $ConA^R$ resulted in an enhanced binding and susceptibility to NK cells. The $ConA^R$ C^R-7 cells as compared to the CHO-WT or reverted RC^R-7 lines have altered cell surface carbohydrate expression as detected by flow cytometry using FITC-conjugated lectins ConA, WGA, PNA and by a radiolabelled monoclonal antibody (H.8) which recognizes a β-galactose determinant. Two other independently selected $ConA^R$ CHO lines (BC^R-2 and EC^R-1) also have increased NK reactivity suggesting a similar single mutation has altered the phenotype of these cells. Tunicamycin, an inhibitor of N-linked glycosylation, was able to reverse the binding to the CHO lines. The mutation rendering the C^R-7 $ConA^R$ caused an inverse binding and killing of the line by bone marrow derived macrophage as compared to the CHO-WT or RC^R-7. Two other $ConA^R$ cell lines, the BW5147.3 and L6 myoblasts, did not have altered NK reactivity which may reflect previous observations that a different mutation is rendering these cells resistant to lectin. These results provide further evidence for the hypothesis of a lectin-like reactivity of both NK cells and macrophage recognizing carbohydrate moieties on tumor cells.

INTRODUCTION

Unlike cytotoxic T cells (CTL), little is known about the mechanisms of recognition of tumor cells by two of the major mediators of natural resistance, natural killer (NK) cells and macrophage (1-5). Both effectors share some similarity in recognition of tumor cells in that they do not require prior sensitization (6-8), can lyse across HLA or H-2 barriers (2,6,7,9,10) and can kill a wide variety of targets (11,12) even across species barriers (6,13,14). To account for this wide degree of general specificity, it has been proposed that both NK cells (15,16) and macrophages (17-19) bear a lectin like receptor reactive with carbohydrate determinants on target cells. A wide variety of simple monosaccharides have been shown to effectively inhibit both NK lysis (15,16,20) and macrophage binding (17,18) to tumor cells. Sugars which inhibit macrophage lysis are different from those that inhibit NK lysis of the same target (5). Both NK cells and macrophages can recognize and lyse allo- or xenogeneic red blood cells, especially if previously neuraminidase treated (21,22). These results support the notion that a lectin like recognition system exists for both NK and macrophage recognition. However, many investigators have shown that the monosaccharide inhibition of NK lysis had no effect on NK binding (23-25) and it is now generally believed that this inhibition occurs during a post-binding step (25). This result does not, however, rule out whether recognition based on a more complex carbohydrate is still occurring during the NK recognition step.

Brooks et al (3) have previously shown that, at least using solid tumor targets, NK cells and macrophages have similar specificities for a panel of tumors. These results do not support the earlier work of Roder

et al (2) who showed that with a panel of lymphoid targets, NK cells and activated macrophage had different specificities. Although exceptions exist, it has generally been found that an inverse relationship exists between macrophage and NK reactivity towards tumor targets (2,4). Cells resistant to NK lysis are usually macrophage sensitive and vice versa.

Recently, Nestel et al (26) have shown that the metastatic MDAY tumor, which is NK^R and macrophage sensitive, when mutagenized and selected for WGA resistant variants, results in a clone (MDW4) which is non-metastatic, NK^S and macrophage resistant. These results support the observation of an inverse relationship between NK and macrophage tumor susceptibility. Preliminary evidence from this group suggests that a carbohydrate component of cell surface N-linked oligosaccharides may be providing the alterations between the two lines required for NK and macrophage recognition and that NK cells are involved in surveillance of this tumor in vivo.

We have previously shown that a mutation in N-linked oligosaccharide biosynthesis in CHO cells, rendering them resistant to the plant lectin ConA, also resulted in increased susceptibility to NK reactivity (27). This was associated with an increas in NK binding to the $ConA^R$ mutant. In this report we will show that this mutation has also altered the tumor cells susceptibility to activated bone marrow derived macrophage.

MATERIALS AND METHODS

Cell Lines

The ConA^R cell lines C^R-7, BC^R-2 and EC^R-1 have all been described in detail in previous reports (28-31). Briefly, the C^R-7 line was selected from its parental CHO wild type line by its ability to survive 10 passages in 40 μg/ml ConA at 34°C and was then subcloned (28). The BC^R-2 was selected from its wildtype similarly to the C^R-7 except that 14 passages in ConA were used (28). The EC^R-1 was selected from its wild type line by first mutagenizing the cells for 17 h in ethylmethane sulfonate (300 μg/ml) followed by one selection in 40 g/ml ConA (28). The RC^R-7, a revertant of the C^R-7 line, was selected by its ability to grow at 39°C, a non-permissive temperature for the temperature sensitive C^R-7 line (28). The lines were grown as monolayers in alpha minimal essential medium (αMEM) (Flow laboratories) containing 10% fetal calf serum supplemented with streptomycin and penicillin and maintained at 37°C with a 5% CO_2 atmosphere. Prior to NK lysis or lectin binding analysis, tumor cells were removed by light trypsinization and placed into spinner cultures at 10^5 to 2 x 10^5/ml for 18-20 h at 37°C in a water bath.

The ConA^R BW5147.3 murine lymphoma and wild type lines were obtained from Dr. I. S. Trowbridge, Salk Institute, San Diego, U.S.A.

Mice

Six to 8 week old male CBA/J mice were obtained from Jackson Laboratories, Bar Harbor, Maine. DBA/2J and CBA/CaJ mice used in macrophage assays were 6 to 8 weeks old and were bred locally by the Health Sciences Animal Center, University of Alberta.

Lectin Binding

CHO cell lines from spinner cultures were washed once and incubated for 30 minutes in either FITC labelled ConA, WGA or PNA (Sigma, St. Louis, MO.) in 10% fetal calf serum ∝MEM containing 10 mM HEPES buffer and 0.1% azide at 4°C. Cells were then washed and analyzed by an EPIC IV Flow cytometer (Coulter Electronics, Hialeah, FL.) for fluorescence. Nonspecific binding controls consisted of including 0.2 M of the inhibiting hapten sugar (mannose, GlcNAc or galactose) to the incubation mixture and this value was subtracted to obtain specific binding.

NK Lysis

This assay has been described elsewhere (32). Briefly, CHO cells from spinner cultures were centrifuged and labelled with ^{51}Cr (100 μCi/10^7 cells) for 45-60 minutes at 37°C. NK cells were obtained from poly I:poly C (Sigma) stimulated CBA/J mice. Poly I:poly C was injected intraperitoneally (100μg/mouse) 20 h prior to splenectomy. The assay was performed in triplicate in V bottom microtitre plates containing a final volume of 200μl. Following 16-20 h incubation at 37°C in 5% CO_2, the plates were centrifuged and 100μl of supernatant was removed, counted in an LKB gamma counter and the percentage of ^{51}Cr released was calculated.

NK Adsorption and Tunicamycin Treatment of C^R-7

CHO cells were plated onto 60 x 15 mm tissue culture petri plates for 20 h. Non-adherent tumor cells were then removed, the plates were washed once with 37°C medium and 2 x 10^7 poly I:poly C stimulated CBA/J splenocytes were added and incubated for 30 minutes at 37°C.

Non-adherent cells were removed, washed and readjusted to 150:1 effector to target ratio, prior to incubation for 16-20 h with ^{51}Cr labelled C^R-7 target cells in the NK assay.

The CHO cells were treated with tunicamycin (Sigma) for 20 h as described in Results. Number plated refers to the number of cells incubated 20 h prior to the addition of drug. Final cell concentrations were obtained from duplicate cultures which were handled as in the experimental group, then completely removed by trypsinization and enumerated on a Coulter cell counter.

The effect of tunicamycin on carbohydrate, protein and DNA synthesis was determined by incorporation of (^3H)mannose, (^3H)leucine and (^3H)thymidine, respectively, into the C^R-7 cells following the procedure outlined by Irimura et al (33).

Macrophage Lysis

The methods used to obtain activated bone marrow derived macrophage have been previously described (34). Briefly, bone marrow macrophage were removed aseptically from the femur and tibia of mice with Pucks saline A solution and then cultured for 5 days in medium supplemented with 10% fetal calf serum, 10% horse serum and 10% L cell conditioned medium containing the macrophage growth factor (35). The adherent cells which were 99% macrophage by morphology were then activated for 18 h in medium containing 3 μg LPS/ml and (1% or 10%) supernatant containing macrophage activating factor (MAF). MAF was previously prepared from ConA or PPD stimulated rat splenocytes. Supernatants were millipore filtered prior to use and in the case of ConA stimulation were absorbed with Sephadex to remove residual ConA. Macrophages were removed by phosphate buffered

saline-EDTA and then washed twice in RPMI 1640 prior to addition to CHO lines and incubated for two days at various effector to target ratios. CHO lines were previously grown in a monolayer at 10^4 cells per well and were radiolabelled for 18 h with 0.5 μCi ^{125}I-iododeoxy-uridine (IUdR) per ml and washed four times prior to addition of macrophage. Following incubation, 100 μl of supernatant was removed and the amount of radioactivity was determined by a gamma counter. The percent specific ^{125}I release (lysis) was defined as:

$$\frac{\text{Experimental count - Background spontaneous release}}{\text{Total } ^{125}\text{I in target}} \times 100$$

Macrophage Adherence

Five day bone marrow macrophage were activated with 3 g/ml LPS and 10% PPD stimulated rat supernatant and were also radiolabelled with ^{125}IUdR (0.5 μCi/ml) for 18 h. Macrophage were then removed by EDTA, washed and then added to CHO monolayer cultures and incubated for 30 minutes at 37°C. Non-adherent cells were removed and the total amount of radioactivity per well was determined.

RESULTS

Membrane Oligosaccharide Alterations in the ConA^R Mutant Detected by Lectins and Monoclonal Antibody

Previous results have shown that a mutation in CHO cells which involves the enzyme that glucosylates oligolipid intermediates (36) in asparagine-linked oligosaccharide biosynthese renders the cells resistant

to the lectin ConA. This mutation also results in alterations of cell surface glycoproteins as detected by the galactose-oxidase (^3H) boro-hydride technique, lactoperoxidase catalyzed iodination of surface poly-peptides and metabolic incorporation of labelled glucosamine (30). We then determined whether there is a cell surface structural basis for the alterations in $ConA^R$ in CHO cells by FITC-lectin binding analysis, which indicates changes in lectin accessible cell surface carbohydrates. As shown in Figure 1 (upper panel), the $ConA^R$ CHO line C^R-7 binds less FITC-ConA as compared to its parental wild type (CHO-WT). This suggests that the mutant has fewer exposed mannose residues on its cell surface, which agrees with earlier observations that less membrane mannolipid and mannoprotein are synthesized in this line (31). The RC^R-7, a revertant of the C^R-7 line, was intermediate in its ability to bind FITC-ConA.

$ConA^R$ CHO lines have been previously shown to also have a concom-itant resistance to other plant lectins (32). We determined whether the C^R-7 mutant had also acquired altered binding patterns to other plant lectins. As shown in Figure 1 (middle panel), the C^R-7 also bound less FITC-WGA than the parental CHO-WT. The RC^R-7 was again intermediate in its FITC-WGA binding. This lectin primarily recognizes neuraminic acid and GlcNAc determinants (38). Of additional interest was the observation (Figure 1, lower panel) that FITC-PNA could stain, although weakly, the C^R-7 line while both the CHO-WT and RC^R-7 were nearly negative. This lectin reacts with terminal (or exposed) galactose residues, with a strong preference for the sugar sequence D-galβ1$\rightarrow$3D-GalNAc (38). Binding of the lectins could be inhibited ($>$90%) by 0.2 M of the appropriate hapten sugar and the flow cytometric analyses represent only specific sugar inhibitable binding.

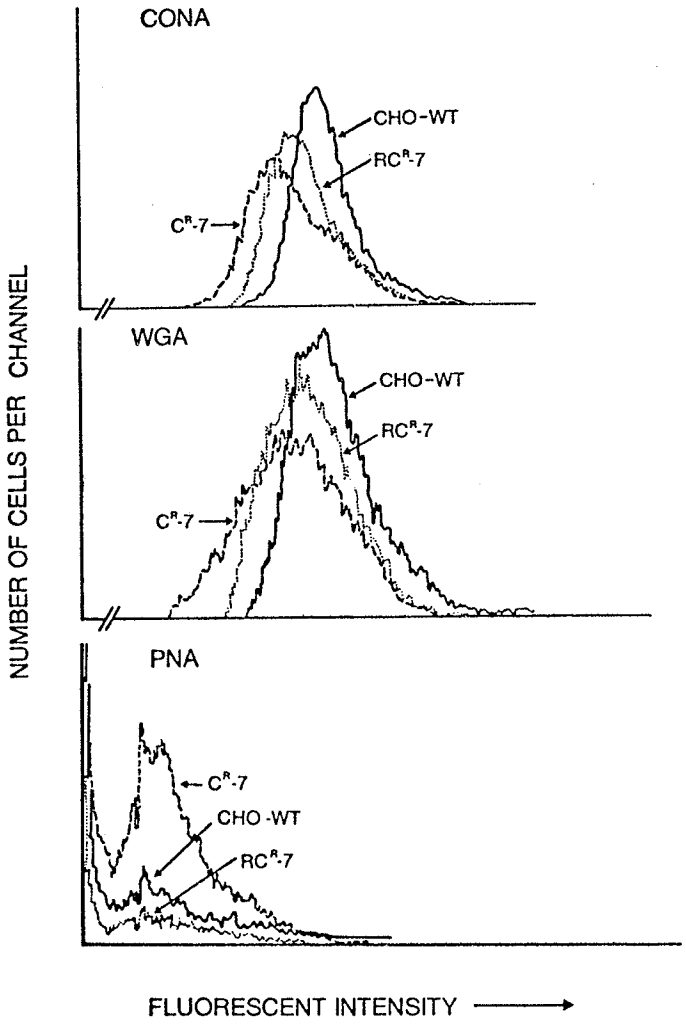

Figure 1. Flow cytometry analysis of FITC-labelled lectin binding to CHO lines. The CHO lines were treated with FITC-labelled lectins as outlined in materials and methods.

The suggestion that the C^R-7 mutant may have more exposed galactose residues using FITC-PNA was confirmed using a monoclonal antibody, 49 H.8, which preferentially reacts with phenylβ-galactoside determinants (Figure 2) (39). Addition of 50mM phenylβ-galactose, but not phenylα-galactose, to the incubation mixture effectively inhibited antibody binding. These results suggest that cells selected for resistance to ConA not only have decreased expression of the glycoconjugates reacting with the selective lectin (ConA), but also bear other oligosaccharide alterations on their cell surfaces.

NK Lysis and Binding of $ConA^R$ CHO Lines

We have previously shown that the $ConA^R$ CHO line, C^R-7, is 2-3 fold more sensitive to NK mediated lysis than its wild type or revertant (27). This increase in NK susceptibility correlated with increased NK binding to the tumor membrane (27). $ConA^R$ cells have a complex phenotype showing numerous membrane alterations (28-31,40) which could be predicted as a consequence of a single mutation in oligosaccharide biosynthesis. Our initial observation of the C^R-7 line having increased NK reactivity may, however, be due to other parameters associated with a peculiar membrane alteration on the mutant not associated with a specific single mutation. As shown in Figure 3, we have extended this observation to two other independently selected $ConA^R$ CHO lines. Both the BC^R-2 and the EC^R-1 $ConA^R$ lines are lysed more effectively by NK cells than their corresponding wild type lines. Monolayers of all three $ConA^R$ cell lines more efficiently adsorbed NK cells than their corresponding wild type lines as shown in Figure 4. These results indicate that all three independently selected mutants have the identical phenotype of increased

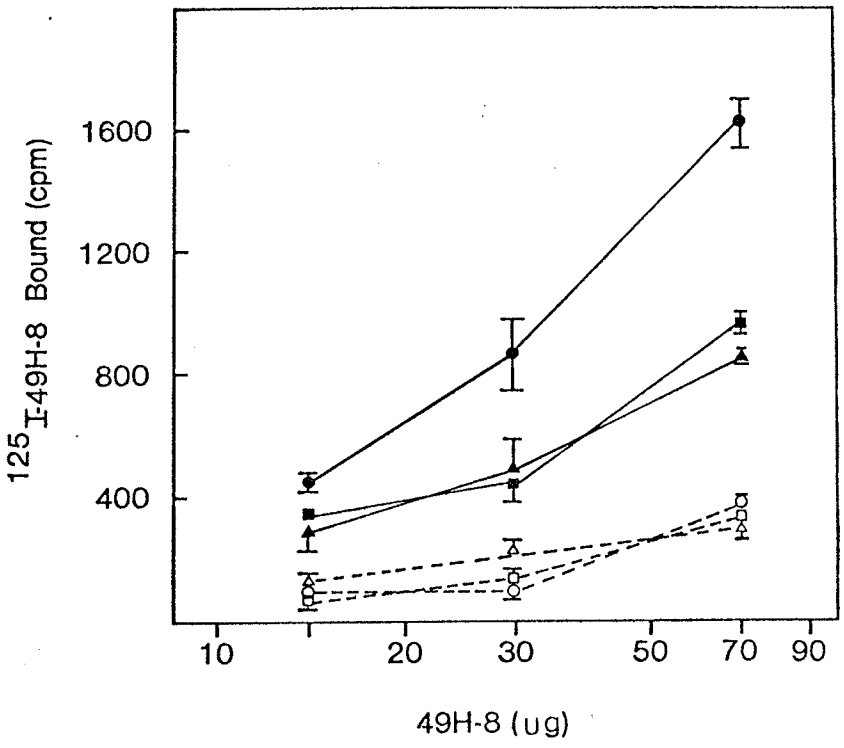

Figure 2. Binding of radiolabelled 49H.8 monoclonal antibody to CHO lines. 3 x 10^5 C^R-7 (●), RC^R-7 (■) and CHO-WT (▲) cells were incubated with 50 1 of stock ^{125}I labelled 49H.8 antibody for one hour at 4°C. The cells were then washed three times with HANK's buffer and counted in a gamma counter. As shown, binding to the C^R-7 (○), RC^R-7(□) and CHO-WT (△) could be inhibited by the hapten sugar β-D-galactose. Binding was not blocked by the inappropriate monosaccharide α-D-galactose (not shown). This experiment was performed by V. Miller.

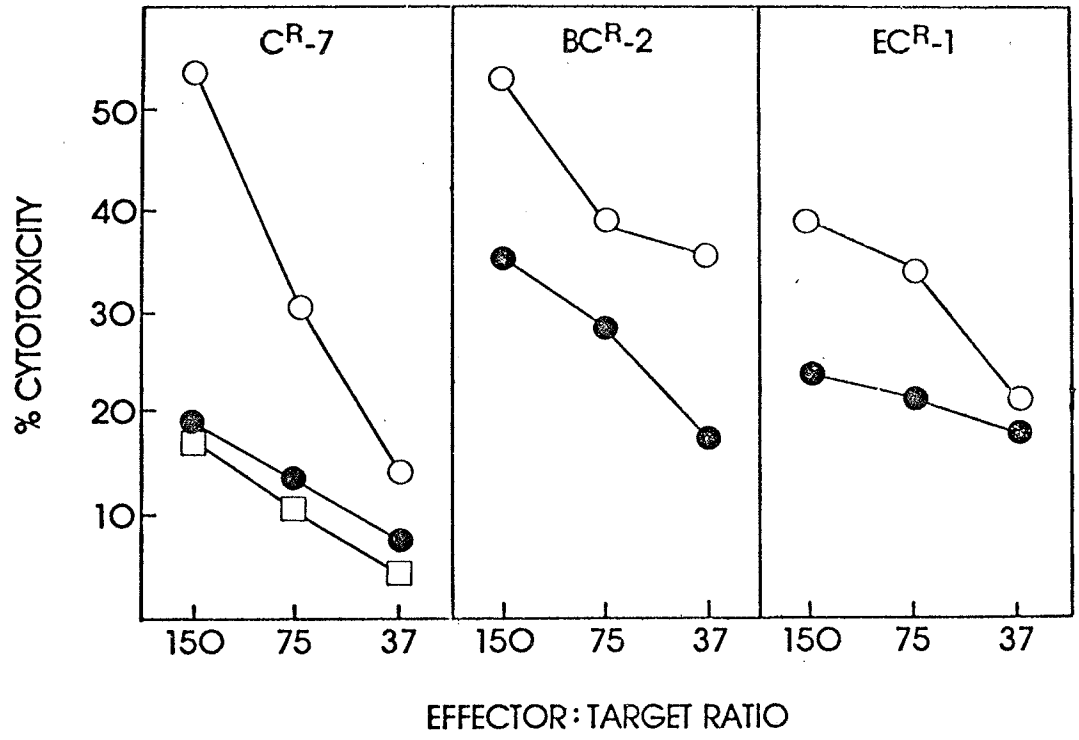

Figure 3. NK mediated lysis of three independently isolated ConA^R CHO lines. The ConA^R mutants C^R-7, BC^R-2 and EC^R-1 (○) and a revertant of the C^R-7 (RC^R-7) (□) were compared with their corresponding wild type lines (●) for sensitivity to NK mediated lysis. The NK assay was performed as indicated in methods for 18 h using poly I:poly C stimulated CBA/J splenocytes as the source of NK effectors. This experiment was repeated with similar results.

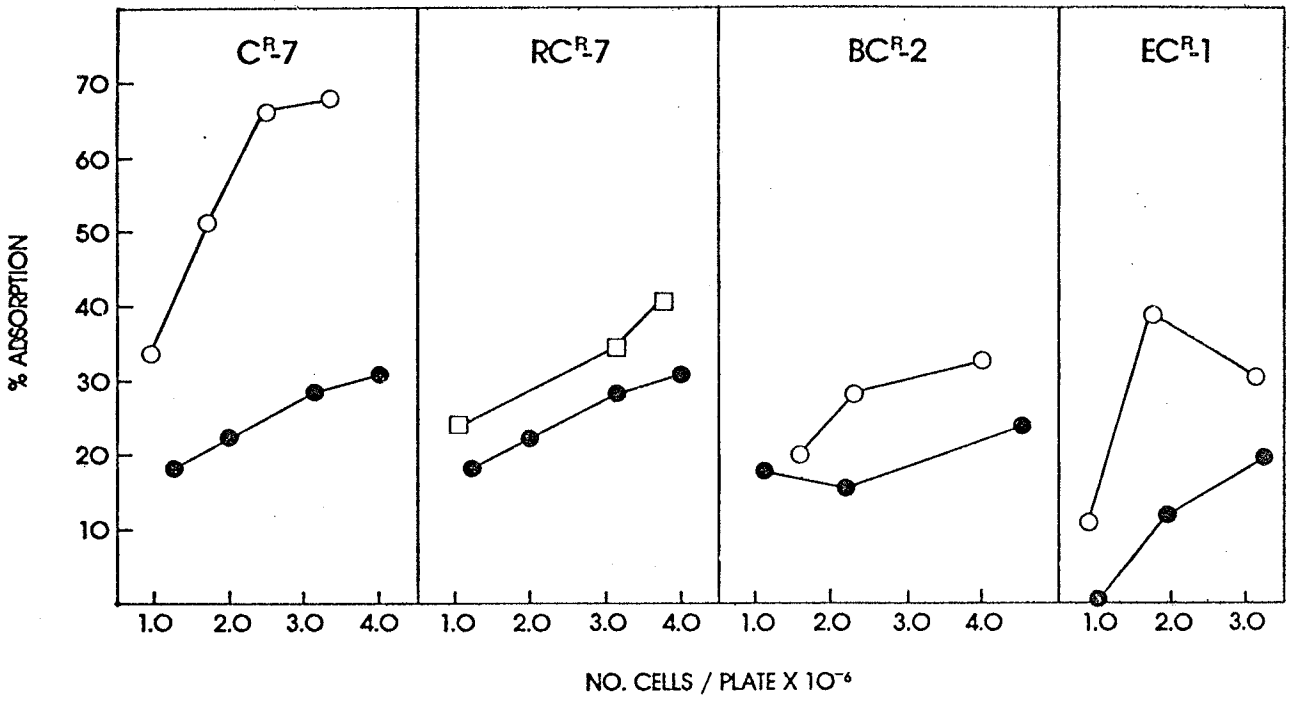

Figure 4. Adherence of NK cells to CHO monolayers. Poly I:poly C stimulated CBA/J splenocytes (2×10^7) were incubated for 1 h at 37°C with various cell concentrations of CHO lines. Non-adherent cells were gently removed, washed and tested for residual NK activity in a 16 h NK lysis assay using ^{51}Cr labelled C^R-7 as the indicator target cell. The number of CHO cells which detached was minimal (< 1%) and this amounted, as previously tested, could not account for cold target inhibition with these concentrations. As shown, the independently selected $ConA^R$ lines C^R-7, BC^R-2 and EC^R-1 (○) were able to adsorb NK cells more effectively than their corresponding wild-type clones (●). The revertant, RC^R-7 (□) was intermediate in its ability to adsorb NK cells. This experiment was performed four times for CHO-WT, C^R-7 and RC^R-7 and twice for BC^R-2 and EC^R-1 mutants and WT lines with similar results.

NK reactivity (lysis and binding), suggesting a similar single mutation in all three lines provided this alteration.

Macrophage Mediated Lysis of the CHO Lines

Like NK cells, activated macrophage have been shown to kill a wide variety of targets and can react with a variety of transformed tumor lines (2-5,41). In general, NK cells and macrophage do not have the same target specificity (2,4,5). However, one group of investigators has shown that both effectors have identical specificities (3). Recently, it was shown that WGA^R cells have an inverse reactivity with both NK and macrophage effectors as compared to their parental wild type (26). We proceeded to test whether the mutation altering NK reactivity in the C^R-7 line has altered its susceptibility to macrophage mediated lysis. In contrast to our observation on the NK sensitivity of these lines, CHO-WT and RC^R-7 lines were sensitive to activated macrophages while the C^R-7 cells were resistant (Figure 5). Results are shown for incubations of 48 h, since only minimal kill was observed following a 24 h incubation period (results not shown). Macrophages incubated in ConA stimulated rat spleen cell supernatants (as a source of MAF) were equally effective in activating macrophage lysis as were macrophage stimulated with MAF derived from PPD stimulated cells, while unstimulated macrophages were non-lytic. ConA induced MAF supernatants might contain residual ConA (even after absorption with Sephadex) and enhance lysis by cross-linking the target cell to the effector. This, however, was unlikely to be the case since PPD induced MAF was an equally efficient macrophage activator (Figure 5, panels B and C). The murine strain of origin of the macrophage (CBA or DBA/2) also did not influence the observed rate of lysis. These results indicate that the mutation rendering CHO cells resistant to ConA while

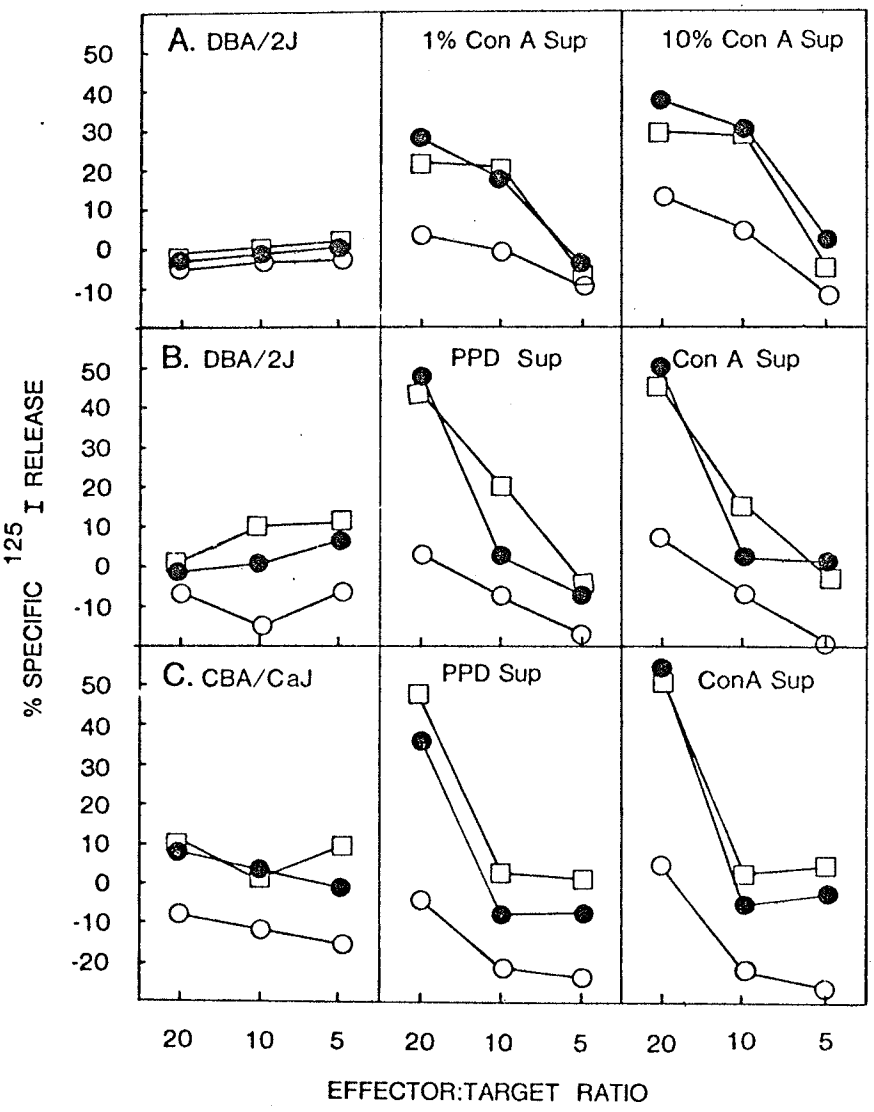

Figure 5. Macrophage lysis of the CHO lines. Normal and activated (ConA and PPD Sup) bone marrow derived macrophages were incubated with ^{125}IUdR labelled C^{R}-7 (○), RC^{R}-7 (□) and CHO-WT (●) cells for 48 h as outlined in methods. This experiment was performed by Dr. K. C. Lee.

increasing NK reactivity, resulted in an inverse or decreased susceptibility of the cell lines to be killed by activated macrophages. Since the mutation affected NK binding/recognition of the tumor cells, we next proceeded to determine if the decrease in macrophage reactivity of the C^R-7 mutant was due to a defect in binding to macrophage effectors.

Binding of Macrophage to CHO lines

Macrophages require physical contact (i.e. binding) with their targets before effecting lysis (42). In examining the binding of radiolabelled macrophage to the CHO lines, it was shown that both non-activated (Figure 6, panel A) and activated (Figure 6, panel B) macrophage more effectively bound to the CHO-WT and RC^R-7 than to the C^R-7 following 30 minutes of incubation. These results suggested that the reduced lytic reactivity (Figure 5) of the C^R-7 line may have been due to a reduced macrophage binding potential of these cells.

Effect of Tunicamycin on NK binding to CHO lines

The antibiotic tunicamycin can block the synthesis of GLcNAc-P-P-dolichol from dolichol-P and UDP-GLcNAc, which is the first step in asparagine-linked oligosaccharide biosynthesis (rev. in 43) and thus can inhibit the glycosylation of glycoproteins (43,44). To test the hypothesis that asparagine-linked oligosaccharides on CHO cell surface membranes are involved in recognition/binding by NK cells, we subjected the CHO cells to various concentrations of tunicamycin and repeated the binding studies. In addition to inhibiting glycosylation, tunicamycin (at high doses or for long periods) can also impair DNA and protein synthesis

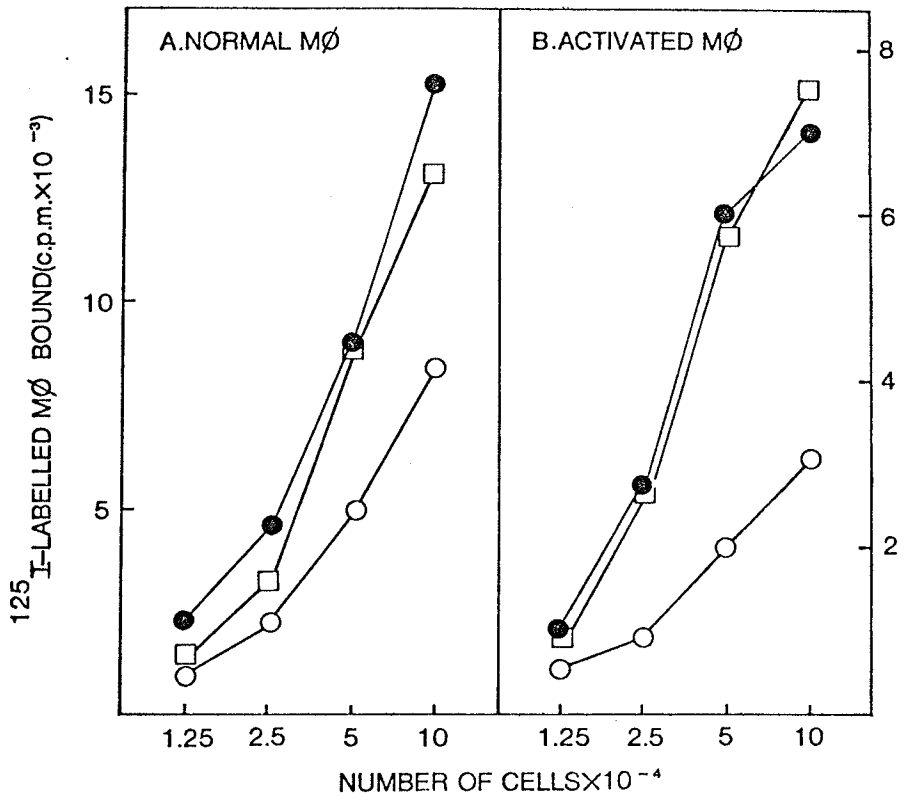

Figure 6. Binding of macrophage to CHO lines. ^{125}IUdR labelled, activated bone marrow derived macrophages were incubated with monolayer cultures of the C^R-7 (○), RC^R-7 (□) and CHO-WT (●) cells for 30 minutes and the amount of bound cells was determined. This experiment was performed by Dr. K. C. Lee.

(33, rev. 4). We had to initially test various doses of tunicamycin to minimize these secondary effects. As shown in Table 1, incubation of C^R-7 cells for 20 h in 0.25-0.5 μg/ml tunicamycin could effectively inhibit glycosylation (^{3}H-mannose incorporation) (26.5-45.7% of the control) while inhibition of both protein synthesis (^{3}H-leucine incorporation) and DNA synthesis (^{3}H-thymidine incorporation) was minimally affected. Higher doses of tunicamycin (1.0 μg/ml) resulted in marked inhibition of protein synthesis. We then tested whether a 20 h preincubation of tunicamycin (0.25-0.5 μg/ml) with the CHO-WT and C^R-7 mutant could effectively inhibit NK binding to the lines. NK cells were incubated with control and tunicamycin treated cells and then the non-adherent cells were tested for NK lytic activity. As shown in Table 2, cells obtained from the tunicamycin treated targets had more lytic activity, suggesting that they less effectively bound NK cells. The effect of tunicamycin on NK lysis of the C^R-7 line could not be determined due to the high levels of spontaneous release of ^{51}Cr by the treated cells (results not shown).

NK Lysis of the ConA^R L6 Myoblast and BW5147.3 Lymphoma

The ConA^R CHO cell lines are thought to have a defect associated with an inability to glucosylate oligo-lipid which can be reversed by the addition of dolichol-P (36). Two other ConA^R mutants, BW5147.3 lymphoma and L6 myoblasts, on the other hand, have a different mutation in that their ability to synthesize dolichol-P-mannose is impaired (mannose transferase activity) and this defect cannot be corrected by the addition of free dolichol-P (45,46). We tested whether the second mutation, which also renders cells resistant to the cytotoxic action of ConA, could alter

Table 1. The effect of tunicamycin on carbohydrate, protein and DNA synthesis in the C^R-7.

Expt.	Tunicamycin Concentration (μg/ml)	Isotope	% of Control*
1	0.25	^{3}H-Mannose	45.7
	0.50	^{3}H-Mannose	26.5
	1.00	^{3}H-Mannose	15.5
	0.25	^{3}H-Thymidine	100.0
	0.50	^{3}H-Thymidine	97.7
	1.00	^{3}H-Thymidine	100.0
	0.25	^{3}H-Leucine	100.0
	0.50	^{3}H-Leucine	79.1
	1.00	^{3}H-Leucine	66.8
2	0.50	^{3}H-Mannose	31.7
	0.50	^{3}H-Thymidine	100.0
	0.50	^{3}H-Leucine	100.0

* All results were expressed as cpm/mg cellular protein before calculation of inhibition of isotope incorporation.

Table 2. The effect of tunicamycin treatment of CHO-WT and C^R-7 on NK binding.

Expt.	Cell Line	Tunicamycin (μg/ml)	No. Plated x 10^6	Final No. x 10^6	% Specific Lysis*	% Absorption
1	--	--	--	--	26.7	--
	CHO-WT	--	1.0	3.5	18.7	30.0
	CHO-WT	--	1.5	4.9	19.9	25.5
	CHO-WT	0.25	2.0	2.4	33.7	0.0
	CHO-WT	0.50	2.0	2.7	30.6	0.0
	C^R-7	--	1.0	2.7	13.9	47.9
	C^R-7	--	1.5	3.5	13.2	50.6
	C^R-7	0.25	2.0	2.1	23.8	11.9
	C^R-7	0.50	2.0	2.1	23.1	13.5
2	--	--	--	--	37.5	--
	C^R-7	--	1.0	1.3	7.3	80.5
	C^R-7	0.50	1.0	1.0	39.8	0.0
3	--	--	--	--	22.6	--
	C^R-7	--	2.0	3.8	5.8	74.3
	C^R-7	0.50	2.0	4.6	11.1	50.9

*C^R-7 line used as target

reactivity with NK cells. As shown in Figure 7, the ConA^R BW5147.3 lymphoma was not significantly altered in NK lysis by both normal and poly I:poly C activated NK cells as compared to its parental wild type. As shown in Figure 8 the ConA^R L6 myoblasts also did not show any major increases in NK reactivity as compared to its parental wild type. NK binding studies, as measured by cold target inhibition with the ConA^R BW5147.3 lymphoma or by NK adherence and depletion with the ConA^R L6 myoblasts also showed no preferential binding as compared to their parental wild type lines (results not shown). The difference in the oligosaccharide defects resulting from different mutations in these ConA^R lines may therefore be a determining factor in their behavior in the NK assays.

DISCUSSION

In this study we have shown that a mutation which renders CHO cells resistant to the toxic action of the lectin ConA has both altered NK and macrophage reactivity. While the mutation resulted in an increase in NK binding and lysis (27) the susceptibility to macrophage mediated lysis was suppressed.

We believe that the reactivity of NK cells or macrophage with these cell lines may involve an important carbohydrate determinant on the tumor cells. The mutation was previously shown to alter cell surface glycoproteins as detected by galactose-oxidase (^{3}H) borohydride technique and lactoperoxidase catalyzed iodination of cell surface polypeptides (30). In this study, the mutation also altered the binding of carbohydrate reactive plant lectins (ConA, WGA and PNA) and a carbohydrate reactive monoclonal antibody (49H.8) to their cell surfaces. Most lectin resistant variants have structurally altered carbohydrate determinants on their cell

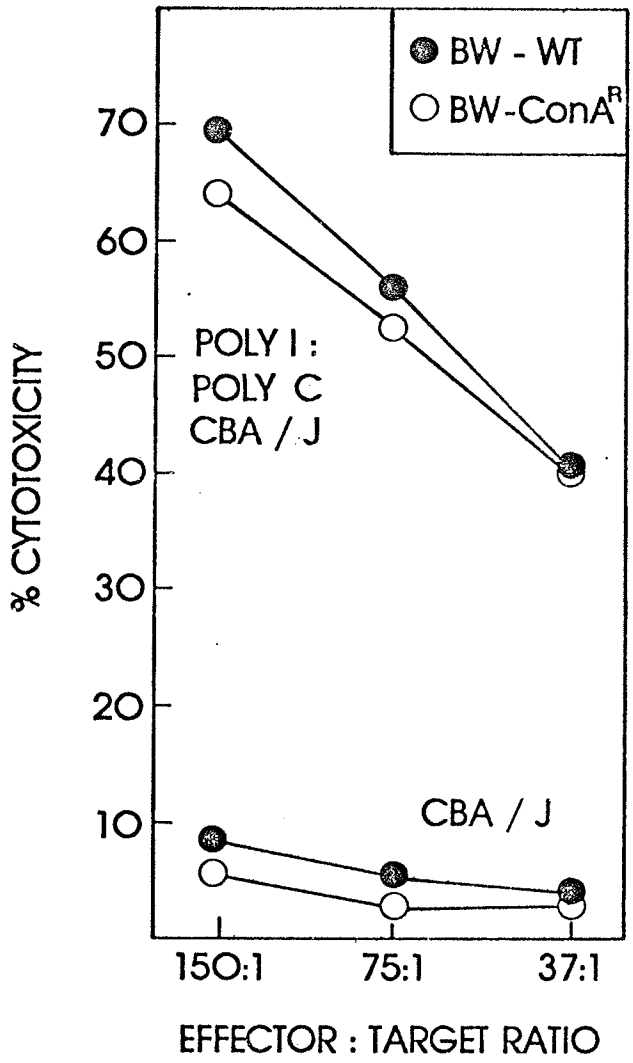

Figure 7. NK cell mediated lysis of the ConA^R BW5147.3 lymphoma. The ConA^R BW5147.3 lymphoma (○) was compared to the parental BW5147.3 wild type (●) for sensitivity to normal or poly I:poly C stimulated CBA/J NK cells for 6 h in a ^{51}Cr release assay.

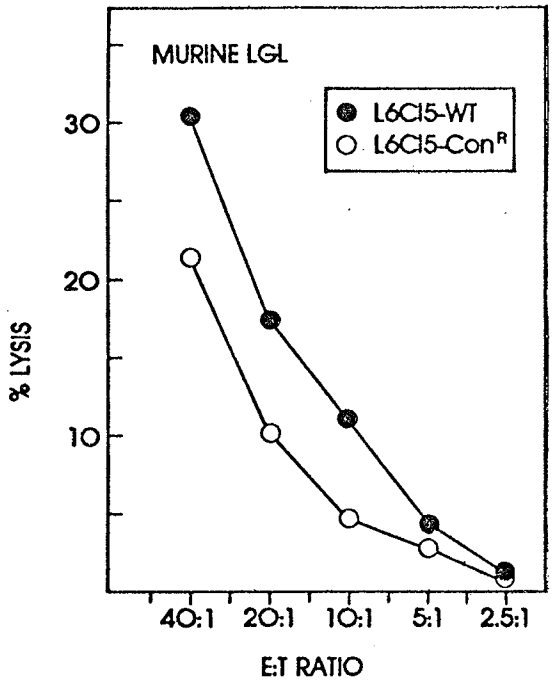

Figure 8. NK mediated lysis of the $ConA^R$ L6 rat myoblast line. NK cells from poly I:poly C stimulated CBA/J mice were purified by nylon wool column (1 h, 37°C) followed by centrifugation (500 x g) on Percoll (Pharmacia) density gradients removing and combining the large granular lymphocyte (LGL) fractions. Adherent L6C15 WT (●) and L6C15 $ConA^R$ (○) cells were prelabelled with ^{51}Cr prior to incubation with purified LGL. Following an 18 h incubation, 100 μl of supernatant was removed and the pellets were dissolved in 2N NaOH. Both were counted in a gamma counter and percent specific lysis was determined.

surface and bind less of the selecting lectin (rev. in 47). In this study it was shown by flow cytometry using FITC-labelled lectins that the $ConA^R$ C^R-7 line binds less ConA than the CHO-WT, which confirms earlier results using radiolabelled lectin (28) and suggests that the mutant has less exposed cell surface mannose determinants. The C^R-7 cells also bound less FITC-WGA, which supports a finding by Stanley et al (48) who showed that $ConA^R$ CHO lines are also mildly resistant to PHA, WGA and LCA. Complementation analysis indicates there can be up to seven distinct groups of lectin resistant mutants, of which the $ConA^R$ phenotype comprises two distinct groups (37). The C^R-7 variants, while binding less ConA and WGA, could bind more PNA, a lectin specific for terminal galactose residues. These results are interesting in that it has been previously shown that terminal galactose residues are involved in binding/recognition of both neuraminidase treated RBC by Kupffer cells (17) and of highly metastatic (but not non-metastatic) tumors to hepatocytes (49). It has also been shown that neuraminidase treatment of tumor cells which exposes galactose determinants can partially increase their NK susceptibility (50). Our results also support the hypothesis that the increased NK reactivity of the C^R-7 mutant may involve galactose determinants in recognition and the hypothesis that the masking of these sites by sialic acid could account for the NK^R phenotype. Whether NK cells have a lectin-like receptor for terminal galactose determinants is unknown and this hypothesis will be explored in future investigations. It should also be mentioned that neuraminidase treated RBC can effectively bind Kupffer cells but not hepatocytes (21), indicating a certain degree of specificity in each system.

The antibiotic tunicamycin, which can block glycosylation of asparagine-linked glycoproteins, appeared to reverse the enhanced NK

binding to the C^R-7 mutant. This would support our hypothesis that an N-linked carbohydrate determinant is important for NK recognition. In addition, tunicamycin can have other effects such as inhibiting DNA and protein synthesis, inhibition of protein secretion and increasing protein degradation (rev. 43,44). We attempted to minimize some of these secondary effects by using concentrations of tunicamycin which did not affect DNA or protein synthesis. We cannot, yet, rule out the possibility that tunicamycin has altered the expression of the NK target structure by its transport and/or positioning into the cell membrane. In contrast, Vose et al (24) were unable to detect an alteration in NK sensitivity of K562 following tunicamycin treatment. This result may either reflect the variation in dominant target structures or tumor cells used in each study, or differences in the detection by the NK subpopulations used in each study. It is well known that NK cells are heterogeneous (51,52), so that a carbohydrate reactive subpopulation may be responsible for the observations made in this study. Others have published data similar to those obtained in this study, finding that tunicamycin treated tumor cells were less NK reactive (53). These authors detected alterations in the synthesis of both gangliosides and asparagine-linked glycoproteins and noted that the tumor underwent differentiation during the drug treatment. THe effect of tunicamycin on gangliosides and differentiation has also been reported by other investigators (54,55). It is well known that NK cells may recognize targets only at certain stages of differentiation (56) and asialated gangliosides have been implicated in NK recognition (57,58). Agents which cause differentiation of tumors (e. g. sodium butyrate, haemin, TPA), have been shown to either decrease (56) or increase (59) NK reactivity. In these studies it was not known whether the carbohydrate expression was altered in the differentiated cells and whether this could

account for the altered NK reactivity. Only in the study of Werkmeister et al (50), where K562 were differentiated with butyrate and made NK resistant, was an associated increase in neuraminidase releasable sialic acid demonstrated on glycoproteins and glycolipids. In our study, whether tunicamycin was altering NK susceptibility by its direct effect on carbohydrate expression or its indirect effect of causing differentiation (and altering carbohydrate determinants) is presently unknown.

It has been previously shown that monosaccharides can inhibit NK lysis (15,16) and macrophage lysis (4,5) and binding (19) to tumor targets. Sugars that block NK lysis are not the same as those affecting macrophage kill, and inhibition patterns are dependent on the tumor target (4,5). We have been able to demonstrate, as summarized in Table 3, that out of a variety of simple monosaccharides, only D-mannose could reduce macrophage lysis, while most were capable of inhibiting NK mediated lysis of the CHO lines. As previously shown with other tumor lines, fucoidan was also able to stimulate macrophage binding to the CHO lines (34). We were unable to reduce NK or macrophage adsorption to C^R-7 or CHO monolayers using the same monosaccharides. These results do not agree with Weir (19), who was able to inhibit the binding of tumor cells to mouse macrophage with simple monosaccharides. The reason for these discrepancies in results are presently unknown, but may reflect the tumors used in each study or the assay systems used to measure binding. For example, in our study bone marrow derived macrophage were used as compared to peritoneal macrophage used by Weir (19) to study tumor binding.

In this study we have shown that three independently selected $ConA^R$ CHO lines (C^R-7, BC^R-2 and EC^R-1) all have increased NK reactivity as compared to their parental wild-type. This suggests that a similar

Table 3. Summary of the effect of carbohydrates and carbohydrate polymers on NK and macrophage binding/lysis of CHO lines.

	Macrophage*		NK Cells	
	Lysis	Binding	Lysis	Binding
Mannose	inhibition	no change	inhibition	no change
GlcNAc	no change	no change	inhibition	no change
Galactose	no change	no change	inhibition	no change
Mannan	no change	no change	not tested	not tested
Fucoidan	no change	stimulation	inhibition	no change

* Experiments performed by Dr. K. C. Lee.

mutation may be was rendering all these cell lines $ConA^R$ and also increasing their reactivity with NK cells.

As also shown in this study, all mutations rendering all tumor cells $ConA^R$ do not necessarily enhance NK lysis or adhesion. The mutation rendering the BW5147.3 lymphoma or L6 rat myoblasts $ConA^R$ did not alter their reactivity to NK cells. There is biochemical evidence that the mutation in the CHO lines is different from that of the BW5147.3 and L6 lines. The $ConA^R$ CHO lines are thought to have a defect associated with an inability to glucosylate oligosaccharide-lipid and can be reversed by addition of dolichol-phosphate (36). The $ConA^R$ BW5147.3 and L6, on the other hand, have a different mutation in that their ability to synthesize dolichol-phosphate mannose is impaired (defect in mannose transferase activity) and this defect cannot be corrected or reversed by the addition of dolichol-phosphate (45,46). The differences in the oligosaccharide defects (and resulting carbohydrate cell surface expression) in these various $ConA^R$ lines may therefore be a determining factor in their behavior in the NK lysis or binding assays.

Nestel et al (26) have recently shown that the metastatic MDAY tumor which initially is NK^R and macrophage sensitive, when mutagenized and selected for resistance to WGA, become non-metastatic, NK^S and macrophage resistant. The authors also report preliminary evidence that the cell lines have altered N-linked oligosaccharide on their cell surface which may explain the alterations in macrophage/NK susceptibility. The inverse relationship between NK and macrophage sensitivities found with the WGA^R lines (26) is analogous to the results presented in this study using the $ConA^R$ CHO lines. As mentioned earlier, the $ConA^R$ CHO lines are also mildly resistant to WGA, indicating that both lectin resistant

variants used by Nestel and colleagues (26) and in our study may have similar altered lectin accessible carbohydrate determinants. It has been previously shown that alveolar macrophage have a lectin-like receptor which preferentially binds mannose, GlcNAc and glucose neoglycoproteins while it does not bind galactose-terminal glycoproteins (60). On the other hand, terminal galactose neoglycoproteins, but not mannose or GlcNAc containing glycoproteins, bind effectively to hepatic membranes (61, rev. 62). These results indicate that certain cells have specific lectin receptors. The lectin resistant variants which have altered expression (increased or decreased) of certain oligosaccharides may provide the distinguishing features required for recognition by these effector cells.

In conclusion, the inverse relationship between NK and macrophage recognition may indicate that both effectors recognize the same glycoprotein. In this model, the alteration caused by the mutation has changed (or unmasked?) the carbohydrate determinant which enhances reactivity to only one effector while decreasing reactivity to another. However,there may be two independent glycoproteins with altered carbohydrate determinants reacting with each effector independently.

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CHAPTER III. TUMOR ACTIVATED NK CELLS TRIGGER MONOCYTE OXIDATIVE METABOLISM

ABSTRACT

We have examined the hypothesis that tumor cells can stimulate a respiratory burst by human NK cells in vitro as measured by luminol dependent chemiluminescence (CL). Percoll purified NK cells, containing 40% HNK-1$^+$ cells and <1 to 4% esterase positive contaminating monocytes, can generate a strong CL response after stimulation with the NK susceptible K562 tumor but not with the NK resistant P815 tumor cells. Although the response was NK dependent, as shown by depletion with NK directed monoclonal antibodies (HNK-1, OKT-11 and OKM-1), the cell generating the CL response was not the NK cell. On the basis of several independent experimental approaches the CL response always required the presence of monocytes in the NK preparation: a) Treatment with a monocyte specific monoclonal antibody (MO2) and complement completely abolished CL; b) The cells producing the CL response were strongly adherent to nylon wool columns (NWC) and LGL preparations containing <0.1% esterase positive cells were inactive; c) NK cells cultured in IL-2 containing medium and tested over several days did not generate CL and d) Optimal numbers of monocytes (<1 to 2%) added to a non-CL NWC purified NK population restored CL while larger or smaller amounts were ineffective. Neither these procedures nor the addition of superoxide dismutase (which completely blocked CL) had any effect on NK lytic activity. We subsequently demonstrated that a factor present in supernatants obtained from NK/K562 incubations, but not from NK or tumor cells alone could stimulate monocyte CL. We therefore propose that the CL response measured in NK enriched Percoll fractions originated from contaminating monocytes which were triggered by factor(s) released from tumor-activated NK cells, and that superoxide anion was not required for NK lysis.

INTRODUCTION

NK cells are a heterogeneous population of cells with phenotypic markers associated with both a T-cell lineage and a monocyte or macrophage lineage and the morphology of a large granular lymphocyte (1,2,3,4). Whether NK cells have a T cell or myeloid origin is controversial and some have speculated that LGL may in fact represent a separate cell lineage (1). Until recently, the ability to generate superoxide anion during an oxidative respiratory burst was thought to occur exclusively in myeloid lineage cells (polymorphs, monocytes and macrophages)(5,6). However, Roder and colleagues (7,8,9,10) found that highly purified NK cells were capable of generating superoxide anion as measured by chemiluminescence (CL) and cytochrome c reduction, following triggering by NK sensitive but not NK resistant tumors. These authors have also shown that the enzyme superoxide dismutase (SOD) can substantially inhibit both superoxide anion generation and NK cytolysis. This would suggest that oxygen radicals may be involved in NK-mediated tumor lysis, a mechanism of target destruction utilized by macrophage and polymorphs (11,12).

The ability of NK cells to generate superoxide anion and its involvement in NK cytolysis has not been substantiated by others (13,14, 15). Patients with chronic granulomatous disease (CGD) who exhibit an impaired phagocytic oxidative metabolism have normal NK lytic function (13,16), an observation that prompted Kay and colleagues (13) to claim that NK cells neither generate CL nor are oxygen intermediates involved in NK mediated cytolysis.

In this study, we present evidence which provides a partial solution to this controversy. We will show that while NK cells themselves cannot generate superoxide anion, they rapidly release factor(s) which can trigger a respiratory burst in monocytes.

MATERIALS AND METHODS

Cell lines - The K562 (NK sensitive) cell line is a erythroleukemia isolated from the pleural effusion of a patient with chronic myelogenous leukemia in terminal blast crisis (17). The K562 line was shown to be free of mycoplasma contamination by repeated plating on PPLO agar and by direct DNA staining using the fluorochrome Hoechst 33258. The P815 (NK resistant) cell line is a methylcholanthrene induced mastocytoma from DBA/2 mice. Cells were cultured in RPMI 1640 (Gibco) supplemented with heat inactivated fetal calf serum (Gibco), penicillin and streptomycin. Cultures were incubated at 37°C with humidified 5% CO_2.

Purification of LGL - Buffy coats obtained from blood donors to the Canadian Red Cross or whole blood from normal volunteers were applied to Ficoll (Pharmacia, density 1.077) and centrifuged at 500 x g for 30 minutes. The mononuclear band was removed and washed in heparin containing medium (10% fetal calf serum in RPMI 1640) and placed either on autologous plasma coated tissue culture petri plates (60 x 15 mm) or, in some experiments, on nylon wool columns (NWC) for 1 h at 37°C to remove adherent monocytes (18). The nonadherent population was then applied to Percoll (Pharmacia) density gradients (10^8 cells per 10 ml gradient) as described by Timonen and Saksela (19). Discontinuous Percoll was made iso-osmolar by adjusting with 10 x phosphate buffered saline and then dilutions were prepared with medium. The density of each fraction increased from top to bottom by 2.5% Percoll increments (Fraction 1 = 40%, P = pellet). Bands and pellet were removed and washed three times prior to each assay. The LGL enriched band, Fraction 2, always contained <1 to 4% monocytes and often <0.5% contamination

when using petri plates for monocyte absorption and <0.1% when using NWC as determined by nonspecific esterase staining (32).

PCM Production and LGL cultures - IL-2 containing culture supernatants were generated by stimulating pooled human tonsil lymphocytes (10^7/ml) with 0.1% V/V PHA-P (Difco) for 24 hours in RPMI containing 1% FCS. The resulting PHA conditioned medium (PCM) was claified by centrifugation, frozen and filter sterilized before use. For culturing, LGL were incubated in 10% PCM with 10% fetal calf serum in RPMl 1640 at 37°C with 5% CO_2.

Chemiluminescence Assay - Chemiluminescence was measured as outlined by Roder et al (7). Briefly, purified LGL (2×10^6) were placed in 0.9 ml of 10% luminol (Sigma) saturated fetal calf serum in RPMl 1640 in glass scintillation vials. Following 30 min of dark adaptation at ambient (20°) temperature, 10^7 K562 in 0.1 ml of medium were added, mixed and immediately counted. CL was measured at 6 second intervals at ambient (20°) temperature in a liquid scintillation spectrophotometer (Beckman LS 9000) in the out of coincidence mode for a duration of 5 to 10 minutes. The values indicated are the mean counts per minute (cpm) above stable background at 30 second intervals.

NK lysis assay - This assay has been previously described in detail (20). Briefly, the NK sensitive K562 was labelled with 100 μCi of ^{51}Cr for 30-45 min at 37°C, washed three times and incubated with purified LGL (200μl final volume) at 10:1, 5:1 and 2.5:1, effector:target ratios in V bottom microtitre plates. Following 4 h at

37°C the plates were centrifuged and 100 μl of supernatant was removed and counted on a gamma counter. Spontaneous ^{51}Cr release was always <10% and usually 5% following 4 h of incubation.

Monocyte Preparation - Mononuclear cells from Ficoll purification of LGL were applied to autologous plasma coated tissue culture plates for 1 h at 37°C with 5% CO_2. Non-adherent cells (LGL containing) were removed and the plates were washed twice with 37°C medium, discarding the non-adherent cells. The plates were then incubated at 4°C for 15-30 minutes. The strongly adherent cells were then gently removed with a rubber policeman, washed and then kept on ice until further use. Staining with non-specific esterase stain indicated >80-90% esterase positive cells.

Monoclonal antibody depletion - Purified LGL (10^7) were incubated for 45 minutes at 4°C with pre-determined concentrations of monoclonal antibodies. OKT-11 (Ortho) was used at $^1/100$ dilution, OKM-1 (Ortho) at $^1/20$ dilution, HNK-1 (Becton Dickenson) at 5 $1/10^6$ cells, MO2 (kindly donated by Dr. J. Roder, Queen's University, Kingston, Canada) at $^1/100$ dilution. Cells were then washed and incubated with 200 μl ($^1/5$ dilution) of pre-absorbed Cedarlane Low-tox rabbit complement at 37°C for 45 minutes. Cells were then washed twice, counted and re-adjusted to 2 x 10^6 per vial for chemiluminescence assays. An aliquot of cells was also simultaneously taken for an assay of NK lytic activity. Control cells were incubated at 4°C as in the experimental antibody group and also received preabsorbed Cedarlane Low-tox complement.

Immunofluorescent staining with HNK-1 and FACS sorting LGL fractions were tested for HNK-1 bearing cells by immunofluorescence using monoclonal anti-HNK-1 and a second FITC-labelled goat anti-mouse IgM $F(ab')_2$ (TAGO, $^1/20$ dilution). Each antibody was incubated with cells in a 100 μl volume for 30-45 minutes at 4°C on ice. Cells were washed and the proportion of fluorescent cells was determined by counting 200 cells under a U.V. microscope. HNK-1 positive and negative cells were also sorted on a Coulter Epics IV Fluorescent activated cell sorter. Cells were placed in RPMI 1640 containing 10% FCS and 10 mM HEPES buffer without NaN_3 and kept on ice throughout the procedure. Appropriate gates were established and the FACS was set at sorting 300 HNK-1^+ cells per second. After 5h 2.8 x 10^6 HNK-1^+ and 10.2 x 10^6 HNK-1^- cells were obtained.

RESULTS

Distribution of NK and CL activity following Percoll density gradients. Peripheral blood lymphocytes were isolated from buffy coats and fractionated on discontinuous Percoll gradients as outlined in Methods. In these experiments, monocytes were removed by adherence on autologous plasma coated petri plates. As shown in Figure 1a, the highest NK lytic activity was obtained from fraction 2 which coincided with the peak of HNK-1^+ monoclonal antibody staining cells (40%). This fraction usually contained <1 to 4% monocytes as determined by non-specific esterase staining. Cells were also tested simultaneously for their ability to generate CL by stimulation with K562 tumor following the

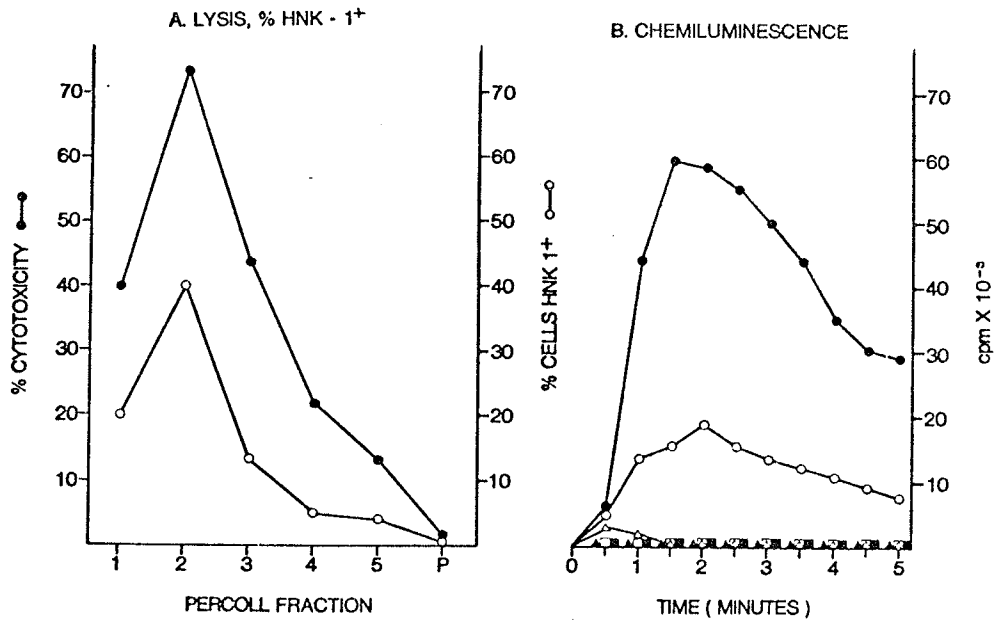

Figure 1

Distribution of NK cells and K562-induced chemiluminescence activity in Percoll density gradient fractions. A. Distribution of NK lytic activity (●) at an effector to target ratio of 10:1. Cell fractions were also tested for HNK-1 bearing cells (○) by immunofluorescence as outlined in methods. B. Luminoldependent chemiluminescence activity in Percoll Fractions. The values shown are the mean cpm above stable background following stimulation with K562 tumor cells. Percoll fractions: (△) fraction 1, (●) fraction 2, (○) fraction 3, (▲) fraction 4, (□) fraction 5, (■) Pellet. This experiment was performed twice with similar results.

procedure described by Roder et al (7) and outlined in the Methods. As shown in Figure 1b, fraction 2 LGL produced most CL activity while lower responses were found in fractions 3 and 1. Fractions 4, 5 and the cell pellet were inactive. Following this observation, that peak CL activity corresponded to fractions containing the most NK cells, we further examined the dependence of the response on this cell population.

Abrogation of CL response by NK and monocyte reactive monoclonal antibodies.

The ability of a Percoll fraction 2 LGL to generate CL could be inhibited by pre-treatment with HNK-1 monoclonal antibody and complement similar to the report by Roder et al (7) (Figure 2a and b). Two other NK reactive monoclonals, OKT-11 and OKM-1, were also effective in abrogating both the CL and the corresponding NK lytic activity. This experiment then, suggested that the CL response of Percoll fractionated LGL was dependent on the presence of cells bearing three NK membrane markers. CL of control cells treated either with antibody (HNK-1) or complement alone were unaffected.

To rule out the possibility that non-specific interactions (i.e., activated complement components) were inhibiting CL during the monoclonal antibody depletions, we examined HNK-1 positive and negative cells obtained by sorting Percoll fraction 2 LGL on the fluorescence activated cell sorter. FACS sorted HNK-1 negative cells showed a significantly diminished CL response when compared to cells handled in an identical manner except for the sorting step (Figure 3). We were, however, unable to obtain sufficient numbers of HNK-1 positive cells by this method to make a direct comparison. In this experiment, untreated Fraction 2 cells were also tested for their ability to generate

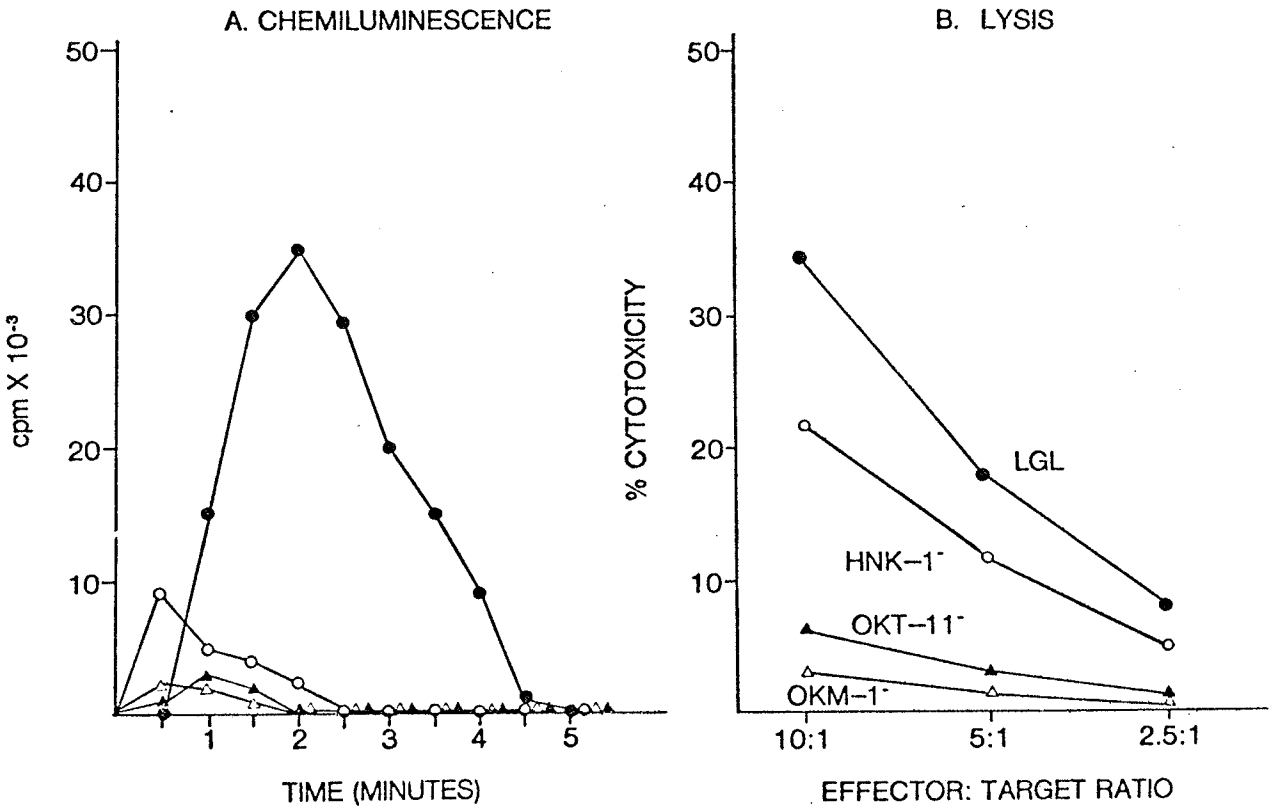

Figure 2

Abrogation of chemiluminescence and NK lytic activity by depletion with NK reactive monoclonal antibodies and complement. Aliquots of 10^7 LGL from fraction 2 of a Percoll gradient were treated with monoclonals and complement. The remaining cells were tested for chemiluminescence and NK activity against K562. Fractions: (●) control LGL with complement, (○) HNK-1 treated with complement, (△) OKM-1 treated with complement and (▲) OKT-11 treated with complement. This experiment was performed twice with similar results.

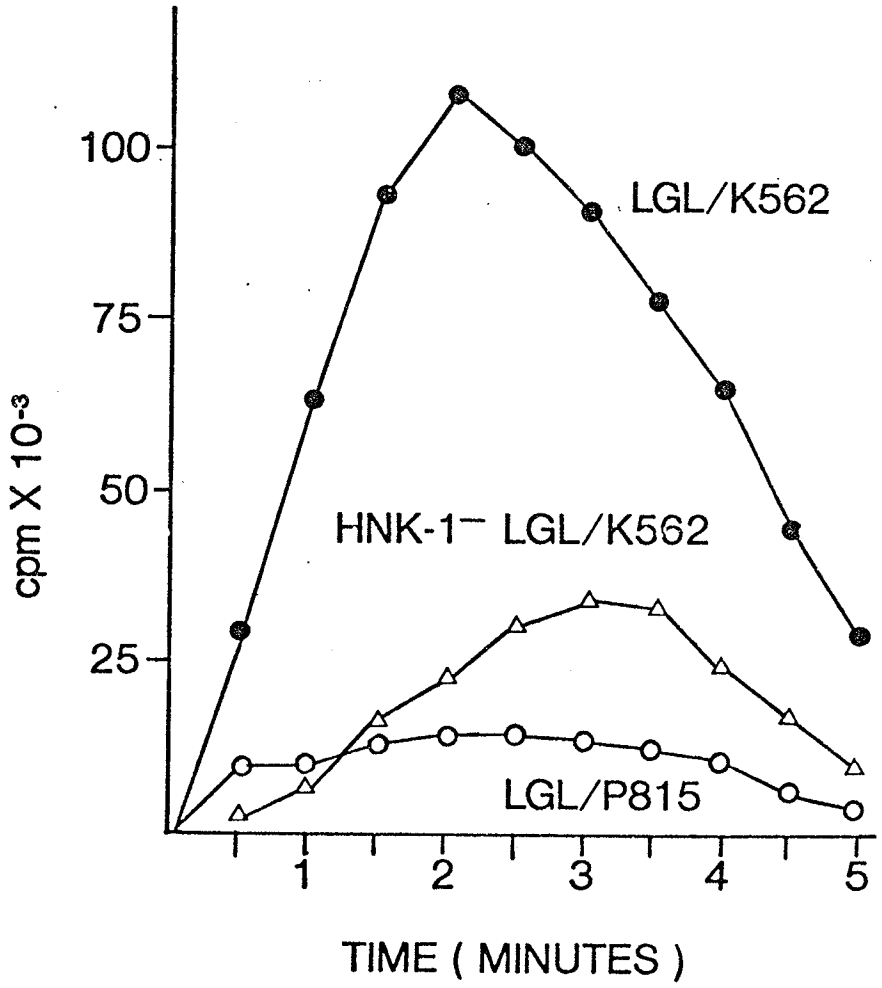

Figure 3

Chemiluminescence of LGL depleted of HNK-1 cells by FACS sorting. Chemiluminescence of HNK-1⁻ sorted cells (△) was compared to control LGL stimulated with either K562 (●) or NK resistant P815 (○).

CL following exposure to NK resistant (NK^R) tumor lines. The NK^S K562 tumor produced a strong CL response when compared to either the NK^R P815 tumor (Figure 3) or L5178Y (data not shown). These results, then, confirm the observations of Roder et al (7), that the cells generating CL were triggered by NK^S and not NK^R tumors and coincided with high NK lytic and HNK-1^+ staining activity.

At this point we examined the possibility that the small number of contaminating monocytes in our LGL preparations were generating the CL response by treating Percoll fractionated NK cells with the monocyte specific monoclonal MO2 antibody and complement. In this experiment, both the MO2 and HNK-1 monoclonal antibodies could effectively remove the CL response (Figure 4a), while only the HNK-1 and not the MO2 antibody and complement treatment could abrogate NK lytic activity (Figure 4b).

Removal of CL responsive cells by nylon wool adherence.

Since the LGL CL response could be eliminated by MO2 monoclonal antibody, it was likely that the residual monocytes in our LGL preparation were the active population. However, NK depletion also reduced CL and it was unclear whether purified NK cells were also responsive. As shown in Figure 5a, if monocyte contamination was reduced to 1-2% by adherence to autologous plasma coated petri plates for 1 h at 37°C, the cells recovered after Percoll density gradients could generate a strong CL response. However, when the mononuclear band was applied to a nylon wool column (NWC) for 1 h at 37°C the nonadherent cells, now contained <0.1% nonspecific esterase positive cells,

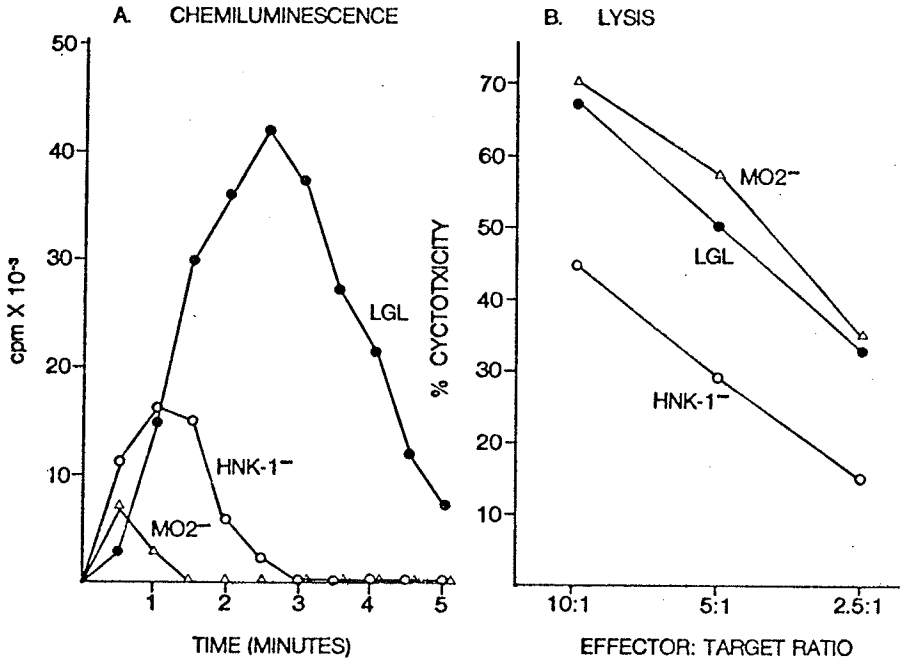

Figure 4

Abrogation of K562 induced CL of LGL with MO2 monoclonal antibody and complement. A. Both MO2 (△) and HNK-1 (○) abolished the chemiluminescence response when compared to control cells treated with complement (●). B. Reduction of lytic activity was observed only after HNK-1 antibody and complement (○). NK lysis was unaffected by MO2 antibody (△) despite complete suppression of the chemiluminescence response. Control cells (●) were treated with complement alone. This experiment was performed twice with similar results.

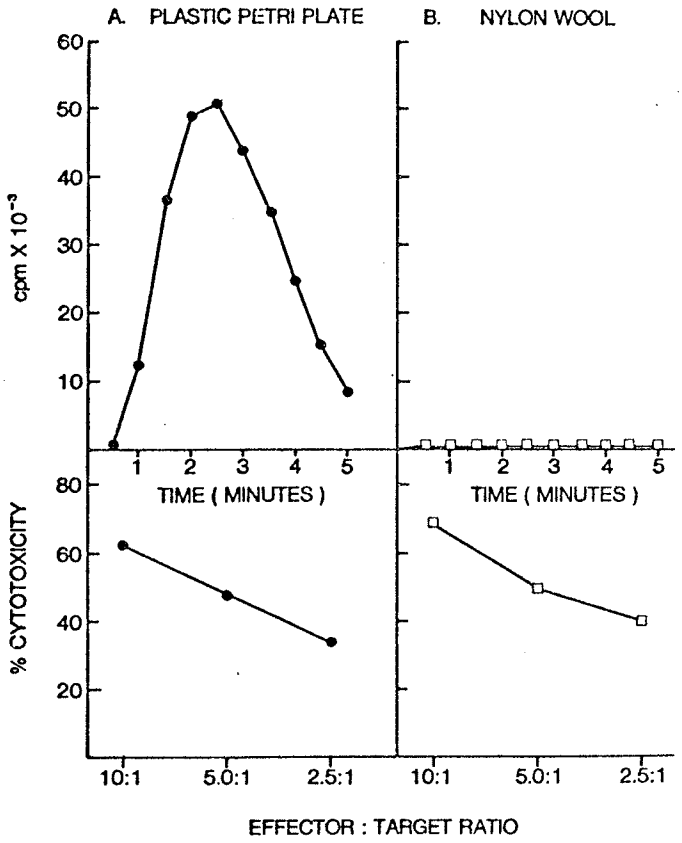

Figure 5

Effect of stringent removal of adherent cells on LGL chemiluminescence and NK lytic activity. A. Mononuclear cells from Ficoll-Hypaque were placed on autologous plasma coated petri plates for 1h at 37°C. Non-adherent cells were removed and fractionated on Percoll gradients. The LGL prepared in this way usually contained <1 to 4% esterase positive contaminating monocytes. These cells were tested for chemiluminescence (upper panel) and NK lytic activity (bottom panel)(●). B. Mononuclear cells were applied to a nylon wool column and incubated for 1h at 37°C. Non-adherent cells were fractionated on Percoll gradients and contained <0.1% esterase positive contaminating monocytes. These LGL were tested for chemiluminescence and NK lytic activity (○) as in A. This experiment was performed three times with similar results.

and following Percoll density centrifugation, did not produce a respiratory burst (Figure 5b). Both procedures yielded close to the same amount of NK lytic activity. This result, then, indicated that the generation of CL was dependent on an adherent, $MO2^+$ cell, a phenotype generally associated with monocytes and macrophages rather than NK cells. It was also clear that depletion of CL responsiveness did not alter NK lytic activity.

PCM cultured NK cells lack CL response.

Purified NK cells can be propagated in IL-2 containing medium for several months (21). Cells maintained in such medium for longer periods of time develop T cell membrane antigens and, while having also maintained some NK phenotypic markers, they represent a subpopulation of the total NK population which have been termed lymphokine activated killer (LAK) cells (22). As shown in Figure 6, when NK cells are placed into medium containing 10% PCM for 24 h at 37°C with 5% CO_2 the CL response is decreased dramatically. This procedure, however, had no effect on NK lytic activity. By day 2, a time period which is insufficient for the differentiation of LAK cells, the CL response is abolished completely (data not shown). Cells tested over several time periods (up to three weeks) in PCM, while maintaining strong lytic activity did not exhibit any detectable CL following stimulation with K562. These results indicate that the population of cells generating CL cannot be cultured in PCM while the NK lytic population remains unchanged. This experiment clearly dissociates lytic activity and CL responsiveness in both NK cells and lymphokine activated killer cells.

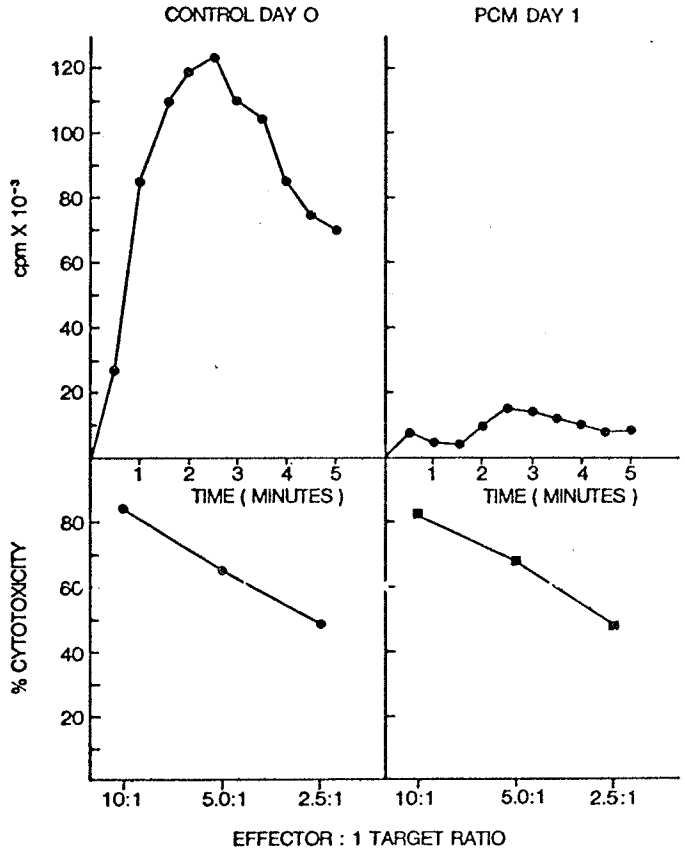

Figure 6

Chemiluminescence and NK lytic activity of freshly isolated and PCM cultured LGL. Control (Day 0) or PCM cultured (Day 1) LGL prepared from plastic non-adherent cells were tested for chemiluminescence as shown in upper panels and for NK lytic activity as shown in lower panels. Experiments performed on cells cultured in PCM for 4, 23 and 25 days (not shown) were also inactive in K562 mediated chemiluminescence despite high levels of lytic activity.

Monocyte reconstitution of CL in LGL preparations.

The possibility that NK cells and monocytes interact in some manner to produce CL was suggested by the requirement of both an $MO2^+$, adherent cell and an HNK-1^+, $OKT11^+$, non-adherent LGL for the response. To test this hypothesis we prepared LGL by NWC. To this non-CL population was added various concentrations (equivalent to 0.1, 1.0, 2.0 and 10.0% contamination) of adherent monocytes recovered from petri dishes. As illustrated in Figure 7, a substantial CL burst was observed in the presence of K562 using 2% purified monocytes while 1 and 0.1% gave a smaller but detectable response. A similar response was observed with the NK^s T cell line MOLT-4 (data not shown). NWC purified LGL or monocytes by themselves were not stimulated by the K562. The monocytes added to the non-CL cells were prepared as outlined in Methods and contained >80-90% esterase positive cells. These experiments were totally dependent on the concentration of monocytes when tested on numerous occasions. If the monocyte concentration was 10%, or less than 0.1%, there was no response to tumor in the presence or absence of NK cells (Figure 7). Monocyte concentrations which gave optimal responses were usually 1 to 2%. Higher monocyte concentrations presumably inhibited CL, an observation that has been previously made in other systems where monocytes can be immunosuppressive (23) and is supported by the findings of Seaman et al (24) who demonstrated that H_2O_2 production by monocytes can effectively suppress NK lytic activity.

It is worth noting that the monocytes used in these experiments were prepared from a strongly adherent population which was recovered by incubation at 4°C followed by mechanical detachment using a rubber

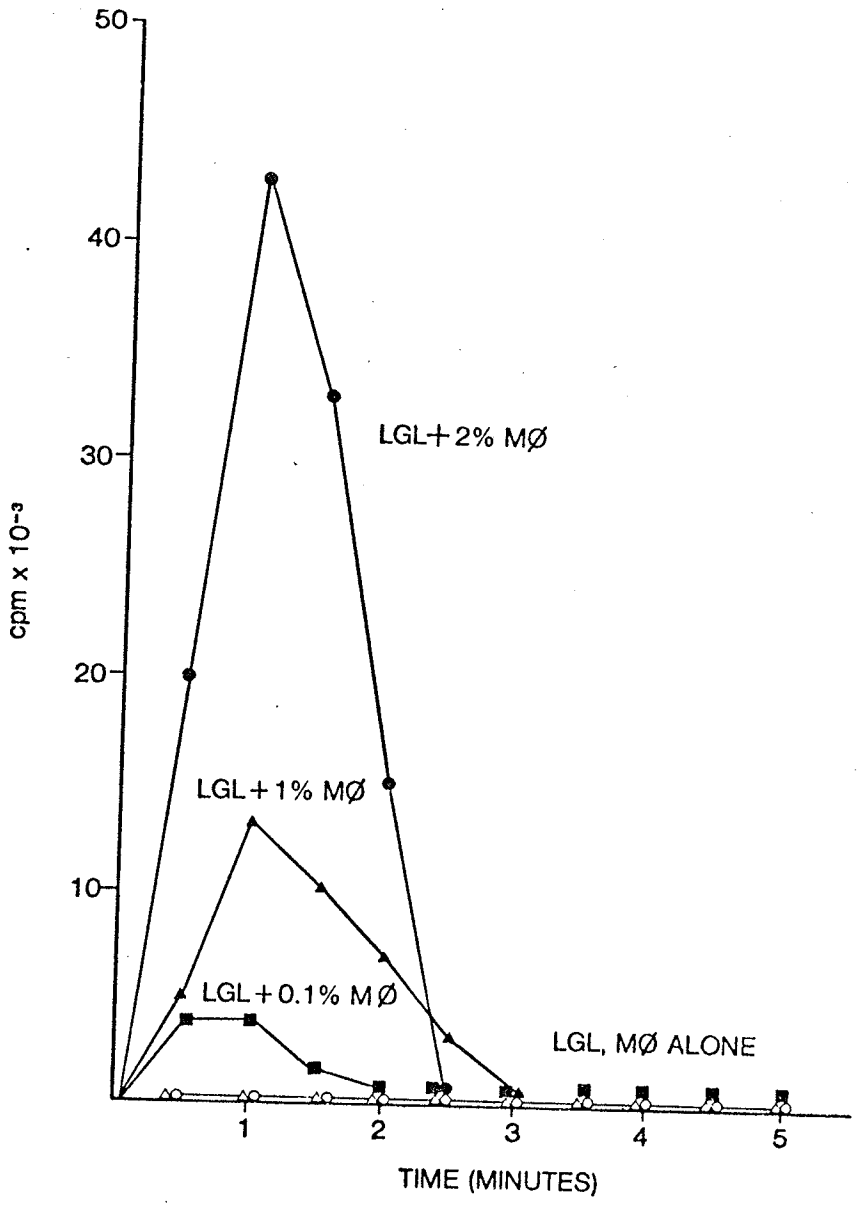

Figure 7

Monocyte reconstitution of chemiluminescence by LGL. Monocytes were prepared by adherence to plastic then recovered and added to the NWC purified LGL preparation. K562 mediated chemiluminescence was restored using 2% (●) and 1% monocyte (▲) contamination, while monocyte concentrations of 0.1% (■), 10% (not shown), monocytes alone (10, 2 and 1%)(△) and LGL alone (○) were unable to generate chemiluminescence following stimulation with K562. This experiment was performed three times with similar results.

policeman. When monocytes were recovered by trypsinization (8), they were not able to restore the CL response. These trypsinized monocytes were also less responsive to other stimuli (e.g. formyl-methionyl-leucyl-phenylalanine) indicating that this methodology was not appropriate for preparing monocytes.

Effect of soluble NK released factor(s) on generating monocyte CL.

From the above studies, it was evident that although the cell generating CL was most probably a monocyte on the basis of the requirement for an $MO2^+$, adherent cell, the presence of cells bearing NK membrane antigens was also necessary. We then tested the hypothesis that NWC purified NK cells (non-CL, monocyte depleted) after being triggered by tumor could release a factor(s) which would, in turn, stimulate a respiratory burst by monocytes. This was exactly what we observed when examining supernatants obtained from the incubation of K562 tumor (5×10^7/ml) and NK cells (10^7/ml) at 37°C for 5-10 min. After centrifugation (200x g) and filtration (0.2μ) 200μl was added to 1×10^6 monocytes and the CL response was recorded (Figure 8). Control supernatants from NK cells or tumor cells incubated without tumor could not trigger monocyte CL, and those obtained from the incubation of NK^R L5178Y and P815 lines with NK cells gave a poorer response than the NK^S K562 (data not shown).

DISCUSSION

In this report we have re-investigated the controversial observation that NK cells can generate a respiratory burst. The results

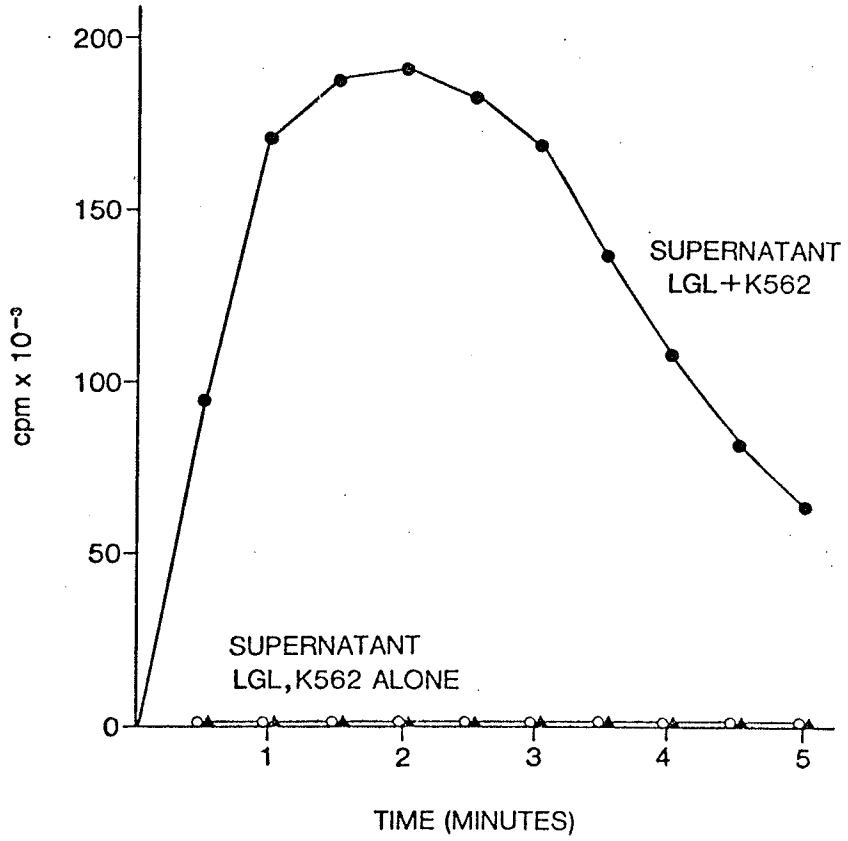

Figure 8

Soluble factors(s) released from LGL during incubation with K562 stimulate monocyte chemiluminescence. Aliquots of supernatants obtained from 5-10 minute incubations of LGL with K562 (●) were able to stimulate chemiluminescence in a purified monocyte preparation (>80-90% esterase positive). Supernatants from LGL (○) or K562 tumor (△) alone were inactive. This experiment was performed twice with similar results.

presented here strongly support the interpretation that the CL detected in Percoll enriched LGL was produced by contaminating monocytes. The CL response was shown to be dependent on a small but critical dose of monocytes in the presence of NK cells as determined by three independent experiments. Firstly, the monocyte reactive monoclonal antibody MO2 and complement, which neither cross-reacts with NK cells (25), nor affects NK lytic activity, completely abolished the CL response. Secondly, when monocytes were more stringently removed by adherence to NWC the CL response was also abrogated. Although most investigators have shown that while NK cells are relatively non-adherent, under certain conditions (e.g. following stimulation by IFN and poly I:poly C) an adherent subpopulation may exist (26). We therefore cannot rule out the possibility that a small nylon wool adherent subpopulation of NK cells was removed by this procedure and that the CL activity resided within this population. Lastly, when purified monocytes are added to a non-CL NK population at 1-2% concentration, one could restore K562 mediated CL activity. These results differ slightly from Roder et al (7) who showed that CL responsive preparations of LGL contained less than 0.1% monocyte contamination. Although we could obtain CL with a 0.1% monocyte concentration, the response was quite small. In our hands, monocytes which were recovered by trypsinization also failed to reconstitute the CL response. This may explain why Helfand et al (8) were unable to observe an increased CL response when 2% purified monocytes prepared by this method were added to their LGL preparations. Initial reports by Roder et al (7) ruled out the possibility of monocyte participation by showing that LGL preparations having increasing monocyte contamination failed to

alter the CL response. This group later suggested in a "Note in Proof" (9) that while monocytes alone cannot generate CL, they can act in synergy with NK cells to generate CL. The experiments in our report do substantiate the involvement of monocytes in the NK CL response. The inability of other investigators (13,14) to observe CL responses in LGL preparations may be due to the requirement of a critical concentration of contaminating monocytes. Extensive purification procedures may remove these monocytes and with them any measurable CL.

Our experiments also substantiate the participation of NK cells in the monocyte CL response. The NK reactive monoclonals (HNK-1 and OKT-11) which do not cross-react with monocytes were able to abolish the CL response. HNK-1 negative cells obtained following FACS sorting also showed a diminished CL response compared to untreated controls. Although the monoclonal OKM-1 was able to abolish the CL response this antibody extensively cross-reacts with both NK cells and monocytes (27). The requirement for NK cells was also evident after the addition of monocytes to NWC purified LGL preparations, where monocytes alone could not be triggered by K562. The additional evidence that cell-free supernatants from NK/tumor interactions caused the rapid release of a soluble factor capable of stimulating monocytes to generate CL supports the idea that monocyte CL required the participation of NK cells. We have also tested the supernatant factor(s) using the monocyte tumor line U937. These PCM differentiated cells have been previously shown to produce a respiratory burst following exposure to a number of non-specific stimuli (28). The supernatants produced from the NK-tumor interaction could also trigger the U937 to generate CL in the absence of a response from control preparations obtained from NK or tumor alone.

Following the interaction with tumors, NK cells can release soluble lymphokines such as interferon, IL-2 and colony stimulating factor (29). Lymphocytes have also been shown to release soluble intermediates (MAF or IFNγ) which can activate monocytes and macrophages, although the activity of these mediators is usually measured over a longer time period. The CL stimulating factors in our studies were produced very rapidly (5-10 mins) and activated monocyte CL within minutes. Another monokine that rapidly stimulates an oxidative burst in neutrophils is leukocyte pyrogen, now known as IL-1 (30). However, whether any lymphokine secreted rapidly from NK cells can activate a rapid CL or oxidative burst in monocytes is unknown. NK cells also have an active phospholipase A2 pathway (31) and most recently, the production of hydroxyl radicals generated through this pathway during the formation of leukotrienes has been demonstrated (15). The characterization of the LGL factors that activate monocytes is presently being undertaken and will be the focus of future investigations. Preliminary evidence indicates that K562 stimulated NK factors also stimulate microbicidal activity in rat alveolar macrophages (Gomez, O'Neill, Pohajdak and Greenberg, unpublished).

The involvement of superoxide anion in the NK lytic mechanism has also been suggested following experiments in which it was found the enzyme superoxide dismutase (SOD) could effectively inhibit NK lysis (7). The inhibition of NK by SOD however, has not been demonstrated by others (15), and in our laboratory, SOD was shown to have minimal effects on NK lysis while completely abolishing CL activity (data not shown). Kay et al (13) reported that patients with chronic granulomatous disease (CGD) whose cells cannot generate superoxide anion have

normal NK lytic levels. In our experiments NWC purified and PCM cultured LGL show little CL activity yet have a completely intact lytic mechanism. These results would strongly argue against the participation of superoxide anion in the NK lytic mechanism.

In conclusion, we have shown that the generation of CL by percoll fractionated LGL is the result of the activation of monocytes by a factor released following NK/K562 interaction and that the ability of NK cells to produce a respiratory burst is not necessary for tumor lysis.

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DISCUSSION AND CONCLUSIONS

A. NK and Macrophage Recognition of a Glycosylation Mutant

The mechanisms involved in recognition of non-self have stimulated the interest of scientists since the dawn of the discovery of the immune system. While a great deal of research has gone into the study of specific receptors and recognition of antigen by antibodies or by T cells, little is known on the less-specific mechanisms utilized by natural killer cells and macrophages.

In this study we have provided a model system to study the recognition system involved in the destruction of tumor cells by two mediators of natural resistance, NK cells and macrophages. Previous results have suggested that a lectin-like recognition system may exist on both NK and macrophages (1-4). According to this hypothesis, NK cells or macrophages can bind to structurally altered oligosaccharides on tumor cells. Earlier results indicated that tumors have altered glycosylation patterns (5,6) as well as lectin binding (rev. 7) and agglutinating properties (8), the necessary alterations required by this hypothesis for NK cell and macrophage interactions.

Most lectin resistant variants have altered cell surface oligosaccharides and usually have less carbohydrate on glycoconjugates that react with the selecting lectin (9). In our study, $ConA^R$ CHO cells bound less FITC-ConA, and radiolabelled ConA (10) and biochemical analyses revealed a decreased incorporation of (^3H) mannose into oligolipid and glycoproteins (11). The ConA CHO lines were shown to have increased susceptibility to NK lysis, probably resulting from their enhanced binding to NK cells. Three independently selected $ConA^R$ CHO

lines all showed this increase in NK reactivity, supporting the suggestion by Wright et al. (48) that a single mutation in oligosaccharide biosynthesis was probably responsible for this NK phenotype. The RC^R-7, a revertant of the C^R-7, was near wild-type, reversing all of the $ConA^R$ pleiotropic features although not quite to the same extent (ConA binding, D_{10} values etc.) (10) as well as NK susceptibility, again providing evidence that the mutation was responsible for the NK phenotype.

The $ConA^R$ mutant C^R-7 grew poorly in nude mice as compared to the CHO-WT or RC^R-7. NK depletion in vivo with an antibody directed against NK cells (asialo GM1) enhanced the growth of the C^R-7 in nude mice, suggesting that NK cells in vivo may be involved in regulating the growth of this tumor.

The mutation rendering the C^R-7 line sensitive to NK cells also inversely decreased its reactivity (binding and killing) to bone marrow derived macrophage. These results would indicate that NK cells and macrophage do not recognize similar epitopes on tumor cells, a result supported by earlier investigators (12,13) in which it was found that tumors that were NK^S were usually macrophage resistant and vice versa. During the course of our investigations, Nestel et al. (14) reported that NK sensitivity of the WGA^R MDAY tumor was inversely related to macrophage susceptibility. The metastatic MDAY tumor, which is NK^R and macrophage sensitive, following mutagenesis and selection in WGA, is WGA^R, non-metastatic, NK^S and macrophage resistant. The authors suggest that NK cells may be the immunoregulatory element in the growth of these cells in vivo, a hypothesis which is similar to the conclusions we derived from our in vivo experiments with the ConA selected lines. We have also shown that the C^R-7 mutant, in addition to binding less ConA, also binds less WGA, supporting the findings of Stanley et al. (15) that

lectin cross-resistance can occur in lectin resistant variants. It is also possible that some of the oligosaccharide structural alterations in the WGA^R cells used by Nestel et al. (14) are similar to those found on the $ConA^R$ CHO cells. Further biochemical analysis will have to be performed before this conclusion can be reached. However, the association of alterations in carbohydrate expression on both lines with alterations in NK and macrophage sensitivity supports the hypothesis that a lectin-like recognition system is utilized by both effectors. It is also evident that the target structures of importance may be different and, possibly, expressed in a mutually exclusive manner.

The inhibitor of N-linked glycosylation, tunicamycin, was able to reverse the NK binding to the CHO lines. This also supports our contention that carbohydrates are important in NK recognition. However, because of the other effects of the antibiotic, caution is needed in drawing this conclusion. Tunicamycin also inhibits membrane protein expression (16) and has been reported to affect cell differentiation (17) and consequently could alter NK susceptibility without directly implicating N-linked sugars as recognition structures. Whether tunicamycin is causing any or all of these effects can only be determined when NK-TS is purified and characterized.

Piontek et al. (18) pointed out that NK recognition may depend more on the tumors adhesive qualities than on a specific recognition system. It is well known that carbohydrate containing proteoglycans (e.g. fibronectin, laminin) are involved in cellular adhesion and can alter the metastatic phenotype of the tumor cell (19). The $ConA^R$ CHO lines used in this study have altered adhesiveness to substratum as shown by cell detachment with trypsin (20). This property was associated in another

study (21) with increased levels of hyaluronic acid, a glycosaminoglycan involved in cell adhesion (22). The WGA^R cells used by Nestel et al. (14) which have altered NK and macrophage susceptibility can bind or attach more efficiently to fibronectin or collagen type IV surfaces but not laminin, as compared to the parental wild-types (23). In this study we have shown that NK cells bind more efficiently to $ConA^R$ CHO lines than to their wild-type clones and it is possible that cell surface carbohydrate expression on the lectin resistant variants could account for this altered adhesive phenomenon. Recently, fibronectin has been shown to both enhance phagocytosis (24) and to promote binding (25) of test particles to macrophages. Proteoglycans involved in cellular adhesion have not been formally tested for their participation in the NK recognition/binding phenomenon and will be the focus of future investigations in this laboratory.

Not all $ConA^R$ cells have increased NK reactivity. As shown in this study, the $ConA^R$ BW5147.3 lymphoma and the L6 rat myoblasts did not show any increases in NK sensitivity as compared to their corresponding wild-types. As discussed earlier, these cells have a different mutation in that their mannose transferase activity is impaired (26,27) while the CHO cells have an altered ability to glucosylate oligolipid (28). One could speculate that the structural alterations in cell surface oligosaccharides are different in these mutants as some evidence suggests (27,49) and that this feature may be the determining reason why only the $ConA^R$ CHO lines have altered NK reactivity.

In conclusion, we have found evidence for the participation of membrane carbohydrates in both NK and macrophage lysis. The use of lectin

resistant variants has provided a model system for the further study of the effects of altered cell surface oligosaccharide expression on NK and macrophage recognition/binding.

B. Tumor Activated NK Cells Trigger Monocyte Oxidative Metabolism

It is well established that cells of the myeloid lineage (polymorphs, macrophages, monocytes) can generate an oxidative or respiratory burst and can utilize the products of this burst (O_2^-, $\cdot OH$, H_2O_2, etc.) as an effective mechanism in the destruction of both bacteria and tumor cells (29-31). Roder and colleagues (32-35) have shown that an LGL population, when triggered by NK^S but not NK^R tumors, can also generate an oxidative burst as measured by chemiluminescence (CL), while others have been unable to detect CL responses in purified LGL populations (36-38).

In our study, we have been able to confirm that NK^S and not NK^R tumors trigger CL in a purified LGL preparation. By using various NK reactive monoclonals (HNK-1, OKT-11, OKM-1) and by FACS sorting (HNK-1), the cell generating CL appeared to be an NK cell. However, using more stringent removal of monocytes by depleting the population with a monocyte reactive monoclonal antibody (MO2) and complement, we were able to show that the cell generating the CL was in fact a monocyte or macrophage. While these results were initially puzzling in that it appeared that both LGL and monocytes were required for an optimal CL response, it was proposed that an interaction between NK cells and tumor was triggering the monocyte and that a possible intermediate (factor?) was involved. In demonstrating that supernatants from LGL/tumor incubations can rapidly (5 - 10 minutes) produce a potent macrophage CL stimulation, we were able to provide evidence for this model.

Purified NK cells when stimulated with either mitogens or lectins can produce and release potent lymphokines and monokines, usually associated with macrophages and T cells, such as IFN, IL-1, IL-2 and CSF (39,40). These results would again suggest that NK cells consist of subpopulations that have properties associated with both lymphoid and myeloid cells. IL-1 can rapidly trigger a CL response in neutrophils (41). One of the major differences between the production of lymphokine by LGL and the CL inducing cytokine identified in our experiments is that lymphokine production usually requires 24 h for synthesis/secretion, while the macrophage stimulatory factor was released within 10 minutes of tumor contact.

During the preparation of this thesis, further experimentation revealed that the LGL/tumor factor(s) can rapidly activate both rat and human aveolar macrophage to kill opsonized S. aureus. This cytokine is also produced by purified LGL that are HNK-1$^+$ but not HNK-1$^-$ after FACS sorting and its release was triggered by NK^S but not NK^R tumors. T cells prepared by E-rosetting (low NK reactivity) were unable to generate the factor(s) while non-E-rosetting populations (high NK activity) could produce active supernatants. The secretion inhibitor monensin, was able to abolish production of the factor while inhibitors of protein synthesis (cycloheximide) or inhibitors of nucleic acid synthesis (actinomycin D) were without effect. On the basis of these results and the kinetics of cytokine production, it is likely that these are preformed molecules that are released by secretion or possibly by degranulation. A major population of NK cells have granules and upon contact with various stimuli (e.g. tumor, Sr^{++}) rapidly degranulate (42,43). We are presently testing the hypothesis that the macrophage activating

factor is present within these granules.

Biochemical characterization indicated that the molecule is trypsin, chymotrypsin and protease sensitive while being RNAase resistant. It is both heat and pH labile, losing reactivity at pH 2.0 and 10.0, 100°C for 5 minutes, while resisting 8°C for 48 h and 56°C for 30 minutes. Preliminary molecular weight determinations indicate a MW in the range of 25,000 $\pm$ 10,000 daltons on Ultrogel AcA44 (Gomez, Pohajdak, O'Neill and Greenberg, manuscript in preparation).

It has been proposed that many macrophage activating factors (MAF) are produced by γ IFN (44,45). NK cells can release IFN after mitogen stimulation (40). In collaboration with Dr. J. Wilkins we have been unable to detect IFN anti-viral activity in an assay which is sensitive to $<$ 1.0 U of IFN. The active supernatants also did not contain IL-2, as measured in an IL-2 dependent proliferation assay. The hormones CSF (500 U/ml), IL-2 (250 U/ml) or IL-1 (100 U/ml) were inactive in our microbial kill assay. It should be mentioned that not all MAFs have anti-viral activity (rev. 46). The identification and association of our factor(s) with other known molecules including MAF is presently under investigation.

In conclusion, we have isolated a novel LGL lymphokine-like factor which can activate macrophages and which may be responsible for the increased CL responses of macrophages noted by us and others (47).

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