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SURFACE Ly-5 GLYCOPROTEIN IN MURINE NATURAL KILLER (NK) CELL DEVELOPMENT, TARGET BINDING AND CYTOTOXICITY: FUNCTIONAL STUDIES AND PRELIMINARY BIOCHEMICAL CHARACTERIZATION
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By

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ABSTRACT

SURFACE Ly-5 GLYCOPROTEIN IN MURINE NATURAL KILLER (NK) CELL DEVELOPMENT, TARGET BINDING AND CYTOTOXICITY: FUNCTIONAL STUDIES AND PRELIMINARY BIOCHEMICAL CHARACTERIZATION

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The role of surface Ly-5 glycoprotein expression in the binding and lysis of susceptible tumor targets by natural killer cells was studied in murine splenocytes reacted with anti-Ly-5 serum. Conjugate assays demonstrated that abrogation of tumor cell lysis by anti-Ly-5 serum involved the inhibition of NK cell binding to susceptible YAC-1 targets. Treatment of cell sorter purified Ly-5- cells with interferon converted 22% of the cells to a Ly-5+ phenotype and induced NK activity against YAC-1 targets. Sorted Ly-5- cells cultured three weeks in interleukin and interferon containing medium (CM) acquired substantial surface Ly-5 with concomitant high levels of NK activity that remained susceptible to inhibition by anti-Ly-5 serum. To gain further insight into the physical properties of Ly-5 glycoprotein as a candidate surface molecule for NK cell interaction with target cells,

preliminary biochemical characterization of Ly-5 on the plasma membrane of murine NK cell-enriched splenocytes was performed. Radioiodination of surface proteins followed by immunoprecipitation of Ly-5 and polyacrylamide gel electrophoresis (SDS-PAGE) revealed a relative molecular mass (Mr) of 205,000 which was indistinguishable from that of macrophage-associated Ly-5. While trypsinization of viable cells did not diminish the amount of immunofluorescently detectable surface Ly-5, SDS-PAGE analysis of the radioiodinated glycoprotein revealed that it had been cleaved to a peptide with an approximate Mr of 125,000 which resisted further trypsinization. The peptide remained closely associated with the plasma membrane, did not exist as a disulfide-linked heterodimer and was released only after detergent solubilization of membrane proteins. The Mr of the immunoprecipitable fragments generated from trypsinized viable cells and NP-40 solubilized membrane proteins were identical, suggesting that the fragment lacked the intracytoplasmic peptide sequence of the intact Ly-5 glycoprotein. The data presented suggest that surface Ly-5 glycoprotein expression is important for binding of NK cells to YAC-1 targets, that Ly-5- precursors of NK cells are present in murine splenic tissues and can be induced by CM to become highly active NK cells with increased surface Ly-5 expression. The persistent susceptibility of these cells to inhibition of cytotoxic activity by anti-Ly-5 serum and

biochemical characteristics consistent with those of a putative NK cell receptor provide additional evidence of an important role for the Ly-5 glycoprotein in the NK cell cytolytic mechanism.

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Finally, to my wife Barbara, for her inestimable importance to my life and work, shall go my unending gratitiude.

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LIST OF ABBREVIATIONS

ADCC	antibody dependent cellular cytotoxicity
AK	activated killer, anomalous killer
avidin-FITC	fluoresceinated avidin
B6	C57BL/6J
biotin/avidin-FITC	sequential reaction with biotinylated anti-mouse IgG and fluoresceinated avidin
BSA	bovine serum albumin
C	complement
°C	degrees Centigrade
CM	conditioned medium
ConA	concanavalin A
cpm	counts per minute
CT	chymotrypsin
CTL	cytotoxic T lymphocytes
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
FBS	fetal bovine serum
FcR	receptors for the Fc portion of Ig
FcγR	receptors for the Fc portion of IgG
GPS	guinea pig serum

HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
Ia	immune response region associated
IFN	interferon
Ig	immunoglobulin
IL-2	interleukin-2
IL-3	interleukin-3
K	killer
KCIL	killer cell independent lysis
KD	kilodaltons
LDCC	lectin dependent cellular cytotoxicity
LGL	large granular lymphocytes
LPO	lactoperoxidase
M	molar
mAb	monoclonal antibody
2-ME	2-mercaptoethanol
MHC	major histocompatability complex
MLC	mixed lymphocyte culture
MLC-AK	mixed lymphocyte culture-activated killer
Mr	relative molecular mass
NC	natural cytotoxic
NK	natural killer
NKCF	natural killer cytotoxic factor
NP-40	Nonidet-P40
NWNA	nylon wool nonadherent
P	papain
PBL	peripheral blood lymphocytes

PBS	phosphate buffered saline
PHA	phytohemagglutinin
PMSF	phenylmethylsulfonylfluoride
SAC	Staphlococcus aureus cells
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacryl-amide gel electrophoresis
sIg	surface immunoglobulin
T	trypsin
TBC	tumor binding cells
TCGF	T cell growth factor, interleukin-2
TEMED	N,N,N',N'-tetramethylethlyene-diamine
UF	unfractionated
WGA	wheat germ agglutinin

INTRODUCTION

The experiments described in this dissertation have been devised to identify and characterize developmental, biochemical and functional aspects of a relatively recently discovered cytotoxic effector cell, the natural killer (NK) cell. First identified in the early 1970's (Rosenberg, et al, 1972; Kiessling, et al, 1975a), NK cells have been implicated in various aspects of host defense mechanisms against cancer (Herberman, 1981), microbial infections (Hatcher and Kuhn, 1982; Murphy and McDaniel, 1982) and in graft rejection (Kiessling, et al, 1977). Despite the considerable efforts of numerous investigators exploring the nature and function of NK cells, relatively little is known about the progenitors of NK cells, the cell lineage to which NK cells belong, mechanisms leading to their functional maturation and cell surface molecules that are important in one or more steps of the cytolytic reaction sequence.

The approach taken in the experiments described in the first article was to identify, on the basis of inhibition by specific antibody, a candidate surface molecule on natural killer cells whose expression is important to NK cell-mediated cytotoxicity against

malignant cells. A molecule identified in this manner and studied for its role in NK cell development and function was the Ly-5 glycoprotein (also called T200 glycoprotein or leukocyte common antigen). Ly-5 comprises a molecularly heterogeneous group of glycoproteins found on cells of lymphoid, myeloid and monocytoid origin (Scheid and Triglia, 1979).

Once identified as a protein that may participate in the NK cell-mediated lysis of tumor cells, Ly-5 was further examined to determine whether it mediates any of the steps of the cytolytic mechanism, namely, target cell binding, programming for lysis or killer cell-independent lysis (Hiserodt, et al, 1982). The results from conjugate assays indicated that Ly-5 glycoprotein acts as a receptor on NK cells that mediates binding to the YAC-1 lymphoma target cell.

In order to identify precursors of NK cells and those processes leading to their functional maturation to cytotoxic effector cells, it was postulated that the surface Ly-5 glycoprotein which appeared to be important for NK activity might be lacking from the surface of pre-NK cells. This hypothesis was tested by fluorescence-activated cell sorting of murine splenocyte suspensions into highly purified Ly-5+ and Ly-5- populations. Sorted cells were tested for NK activity before and after treatment with agents that have been shown to augment NK activity, such as interferon (IFN)

and interleukin-2 (IL-2; Brooks, 1983). Observed differences in NK activity were correlated with changes in surface Ly-5 glycoprotein expression occuring under the same conditions. Additional aliquots of sorted cells were placed into three week in vitro culture in medium containing IFN and IL-2 and compared for Ly-5 expression and NK activity. The results of these experiments indicated that Ly-5- pre-NK cells are present in murine splenic tissues and that IFN- and IL-2-containing preparations induce conversion of NK-inactive Ly-5- pre-NK cells to potently cytolytic NK cells bearing very high surface levels of Ly-5 glycoprotein. Even at a low effector:target ratio, the cytotoxic activity of in vitro cultured NK cells greatly exceeded that of freshly isolated NK cells. This activity was significantly inhibited by anti-Ly-5 serum, further supporting a role for the Ly-5 glycoprotein in the cytolytic process.

Having established evidence for the importance of Ly-5 in murine NK cell development, target cell binding and cytotoxicity, it became of interest to biochemically characterize the glycoprotein on NK cells. The molecular weight heterogeneity of Ly-5 observed between purified and cloned populations of T cells, B cells and monocytes (Trowbridge, et al, 1975; Tung, et al, 1981), raised the question of whether the molecular weight form of Ly-5 on NK cells is similar to that found on other lymphoid or myeloid populations or if instead it comprises a unique

form of the glycoprotein. Experiments described in the second article were designed to identify the apparent molecular weight of Ly-5 glycoprotein obtained from NK cell-enriched populations in comparison to that of whole spleen cells, thymocytes and lymph node cells. The results indicated that NK cell-derived Ly-5 is more similar in migration on SDS-PAGE to T cells and macrophages than B cells, in agreement with current theories on the cell lineage of NK cells (Habu and Okumura, 1982).

Studies of the various facilitators and inhibitors of NK cell function have led to a hypothetical model of the NK cell lytic mechanism (Hiserodt, et al, 1983), which ascribes certain properties to the putative NK receptor for target cell interaction. While this model is more fully described in the following sections, one such property would be the susceptibility of the receptor to cleavage by trypsin-like proteases in a manner that does not completely degrade the fragment generated, allowing it to maintain its interaction with the target cell and participate in subsequent steps of the lytic cycle. In support of this model and the possibility that Ly-5 is a NK cell receptor, the results to be described indicate that trypsin treatment of NK cell-enriched splenocytes generates a fragment of Ly-5 that resists further degradation by trypsin and retains its ability to bind anti-Ly-5 mAb. When viewed with data presented on the role of Ly-5 in target cell binding and cytotoxicity, the

results further implicate Ly-5 as a NK cell receptor, in that: 1. Ly-5 mediates binding of NK cells to YAC-1 tumor cells; 2. Ly-5 has an external protease-sensitive site that is cleaved by gentle trypsinization; 3. the remainder of the external domain fragment generated by gentle trypsinization resists further degradation by high concentrations of trypsin, and; 4. the trypsin-generated fragment maintains its ability to bind to anti-Ly-5 mAb, a likely requirement for continued binding to the target cell for the programming and killer cell-indepedent lysis steps of NK cell-mediated cytolysis.

While additional activities of the Ly-5 fragment need to be demonstrated to confirm its importance as a NK receptor, the results described in this dissertation strongly suggest an important and fundamental role for Ly-5 glycoprotein in the natural killer cell system.

LITERATURE REVIEW

Natural killer (NK) cells are lymphocytes characterized in part by their ability to bind and lyse certain neoplastic cells without prior sensitization. Their discovery came in the process of investigating mechanisms of cell-mediated cytotoxic reactivity against tumors, when Rosenberg, et al, (1972) found that lymphocytes from normal individuals who were free of cancer or known exposure to cancer patients had cytotoxic activity against leukemic cells. Other studies (McCoy, et al, 1973; Oldham and Herberman, 1973; Takasugi, et al, 1973; Rosenberg, et al, 1974) showed similar reactivity of lymphocytes from healthy individuals against tumor-derived cell lines. Natural cell-mediated cytotoxicity was subsequently demonstrated to occur in normal rats (Nunn, et al, 1973) and mice (Kiessling et al, 1975a).

Splenic natural killer cell activity in mice and rats is an age-dependent phenomenon, appearing at approximately three weeks of age, peaking at five to eight weeks and declining to negligible levels by the age of twelve weeks (Nunn, et al, 1973; Kiessling, et al, 1975b). However, when peripheral blood lymphocytes (PBL) are examined,

murine NK cell development more closely resembles that seen in humans, with significant and stable circulating levels of NK activity maintained for most of the adult life of the mouse (Herberman and Holden, 1978; Lanza and Djeu, 1982).

Natural killer cells are nonphagocytic, nonadherent cells that comprise 1-5% of splenocytes and peripheral blood lymphocytes (Kiessling, et al, 1975b; Herberman, et al, 1975). Their activity is highest in blood and spleen, lower in lymph nodes, lungs, gut-associated lymphoid tissue and bone marrow, and generally absent from thymus (Reynolds, et al, 1981). Using the bone seeking isotope, 89-Sr, or by inducing osteopetrosis with estradiol, NK cell differentiation has been shown to be marrow-dependent (Haller, et al, 1977; Seaman, et al, 1979; Lust, et al, 1981, Kumar, et al, 1982). In humans, NK cells have a characteristic morphology on Giemsa staining, with a relatively high cytoplasmic-to-nuclear ratio, kidney-shaped nuucleus, pale cytoplasm and azurophilic granules which has led to their description as large granular lymphocytes (LGL) (Timonen, et al, 1979). Although somewhat smaller, murine NK cells also appear to possess the same LGL morphology and preferentially sediment at 55-60% interfaces on Percoll discontinuous density gradient centrifugation (Tagliabue, et al, 1982).

Many of the current theories on the nature, function and cellular origin of natural killer cells have resulted

from the characterization of surface antigens on these cytotoxic effectors. Like most murine cells of lymphopoietic or myelopoietic origin, NK cells express the high molecular weight glycoprotein, Ly-5 (Pollack, et al, 1979; Scheid and Triglia, 1979; Cantor, 1979). Approximately 50% of NK cells bear low levels of Thy-1, which is characteristic of T lymphocytes (Herberman, et al, 1978). Thymocytes and NK cells are surface Ly-11+, while mature T cells and cytotoxic T lymphocytes are not (Meruelo, et al, 1980). In common with macrophages is the expression on NK cells of MAC-1 and Mph-1.2 antigens (Springer, et al, 1978; Archer and Davies, 1974), as well as asialo GM1 (Kasai, et al, 1980; Okumura, 1982). While essentially all human NK cells highly express receptors for the Fc portion of IgG (FcγR), only a few receptors are identified on murine NK cells and receptors for complement are lacking (Herberman, et al, 1975; Herberman, et al, 1977). Murine NK cells bear Qa-4 and Qa-5 on their cell surfaces and selectively express the NK-1 antigen, but lack the B cell antigens IgM and Lyb-2 (Koo, et al, 1980; Glimcher, et al, 1977). Taken together, the data from comparative surface phenotyping does not allow a designation of cell lineage to NK cells.

In order to further characterize natural killer cells, describe their function and gain insight into their cell lineage, it is convenient to compare NK-mediated cytotoxicity with other forms of cellular cytotoxicity.

Natural cytotoxic (NC) cells share with NK cells the ability to lyse certain tumor cells without prior sensitization, but their cytotoxicity is directed toward adherent tumor cell lines derived from solid tumors, while NK cells are preferentially cytotoxic to lymphoid cell lines (Stutman, et al, 1981). The strain distribution of NK and NC activities differ as well, with certain strains of mice having high NC and low NK activity, and vice versa (Stutman, et al, 1978). Stutman, et al (1978), also showed that murine splenic NC activity appears earlier than NK activity, and unlike the latter persists at high levels in aged animals. The kinetics of the cytotoxic response was also different in the two cell types, with significant NK activity appearing within 4 hours, while NC activity required a minimum of 12-18 hours to be observable (Paige, et al, 1978). Differential sensitivity to blocking by simple sugars has also been observed, with NC activity blocked by the addition of D-mannose, while NK activity is inhibited by a variety of simple sugars (Stutman, et al, 1980). Characterization of cell surface phenotype by treatment with antibody and complement has shown that murine NK cells express asialo GM1 (Young, et al, 1980), Qa5 (Pollack, et al, 1979), Ly-11 (Meruelo, et al, 1980) and NK-1 antigens (Burton, 1980), whereas NC cells appear to express only low levels of asialo GM1 but none of the other surface antigens (Lattime, et al, 1981). Finally, Djeu, et al (1983), have

shown that interleukin-3 (IL-3) allows the selective growth of NC but not NK cells or their precursors, while Henney, et al (1981), demonstrated that interleukin-2 (IL-2) augments NK activity and supports the long-term culture of NK cells in vitro. It is of note that in vitro NK activity declines rapidly at 37 ℃ in the absence of IL-2 or other stimulatory substances, while NC activity is stable under these conditions (Herberman, et al, 1975; Djeu, et al, 1983).

Unlike the many properties that serve to distinguish NK cells from NC cells, killer (K) cells mediating antibody-dependent cellular cytotoxicity (ADCC) show great similarity to NK cells. Ojo and Wigzell (1978), showed that murine NK activity and ADCC directed against certain targets varied in parallel with age, organ distribution and genotype. In addition, both activities were labile in vitro at 37 ℃, were augmented by Corynebacterium parvum injection and blocked by cold target inhibition with either target type. Analysis of human K cells (Perussia, et al, 1979), indicated that these cells express the same cell surface phenotype and large granular lymphocyte (LGL) morphology as NK cells. It appears reasonable to conclude on the basis of available data that NK and K cell activities are separate functions of a single effector cell.

The phenomenon of lectin-dependent cellular cytotoxicity (LDCC) is also differentiable from classical

NK activity and other forms of cellular cytotoxicity. Bonavida and Bradley (1976), showed in mice that lymphocytes must be presensitized in order to generate LDCC activity. Sensitized CTL can be induced by addition of ConA to exert nonspecific spontaneous lytic activity against many cells, including some NK-susceptible targets (Bradley and Bonavida, 1981). When these studies were extended to include spontaneous LDCC in nonstimutated lymphocytes from high and low NK-active mouse strains (Bonavida, 1982), it was observed that in mice with low NK activity, cytotoxicity against NK-susceptible but not NK-resistant targets was facilitated by the addition of ConA, but not by other lectins capable of stimulating CTL-LDCC, such as phytohemagglutinin (PHA) or wheat germ agglutinin (WGA). This activity has been designated NK-LDCC and was abolished by alpha-methyl-D-mannoside, a ConA inhibitor. No significant enhancement of cytotoxicity was observed in lymphocytes from high NK-active strains. The effector cells in NK-LDCC differed from the classical NK effector cell in surface phenotype, being eliminated by treatment with anti-Thy-1.2 and complement (C) but not by anti-asialo GM1 + C. While these data do not allow a precise understanding of the relationship of NK activity to LDCC, the inverse relationship observed among mouse strains between NK activity and NK-LDCC may eventually provide insight into the processes leading to the development of functional natural killer cells.

A comparison of NK cells and cytotoxic T lymphocytes (CTL) reveals many differences and some similarities. Unlike CTL, NK cells appear to lack any major histocompatability restriction for cytotoxic activity (Herberman and Ortaldo, 1981; Stutman, et al, 1978), evidence of immunologic memory (Dennert, 1980), or the need for priming to induce a cytotoxic response (Herberman and Holden, 1978). Characterization of cell surface phenotype by abrogation of activity following reaction with specific antibody and complement suggests that murine NK cells but not CTL express Ly-11.2, Qa5 and asialo GM1, while CTL but not NK cells express Lyt-2 (Meruelo, et al, 1980, Minato, et al, 1981; Kasai, et al, 1980). While both effector cell types express the hematopoietic differentiation antigen Ly-5/T200, only NK cell activity is blocked by anti-T200 or anti-Ly-5 in the absence of complement (Seaman, et al, 1981; Kasai, et al, 1979). Conversely, anti-RAT* sera appears to block both the binding and post-binding lethal hit stage of CTL-mediated lysis without affecting ADCC or NK activity (Hiserodt and Bonavida,1981). Roder, et al (1979), isolated cell surface glycoproteins from NK-susceptible targets that specifically blocked binding of NK cells but not CTL to their respective targets, suggesting that different receptors are involved in the two cytolytic processes. The effect of interferon treatment on target cells differs as well, resulting in increased sensitivity of

CTL-susceptible targets but decreased sensitivity of NK-susceptible targets to the respective forms of cytotoxicity (Welsh, et al, 1981).

Under certain circumstances, it appears possible to show both NK and CTL activities in a single cloned lymphocyte population. Brooks (1983), generated two allospecific CTL clones in medium containing 2% ConA-induced spleen cell supernatant, which has been termed conditioned medium (CM). One clone, AIG7, demonstated H-2b specificity and lysed only the H-2b-bearing target EL4 under the conditions described above. However, after one week culture in 40% CM, AIG7 cells lysed not only EL4 cells but also the NK-susceptible targets YAC-1 and 27v-IC2 (L5178Y-lymphoma), while having no activity against the NK-resistant targets P815 (H-2d) and av (L5178Y-lymphoma). This effect was reversible by returning the cultures to 2% CM. Partially purified interferon (IFN) and interleukin-2 (IL-2) preparations individually supported the augmented response, and their effects were synergistic when used in combination, while addition of ConA to 2% CM did not promote NK activity. Similar effects were seen with the other CTL clone studied, suggesting that at least in some circumstances a single cell can give rise to both NK and CTL activities, that CTL are not necessarily terminally differentiated cells and that IFN and IL-2 are mediators of this phenomenon.

Determining whether NK and CTL activities result from different cell types or alternate functions of a single effector cell was further complicated by the finding that mitogen- or allogeneic cell-stimulated lymphocytes express not only CTL but also NK and ADCC activities (MacDonald and Bonnard, 1975; Ortaldo, et al, 1977). In addition, the cells responsible for the NK-like activity in these cultures have been shown to bear high surface levels of Thy-1 (Dennert, et al, 1981; Brooks, et al, 1982), while only about 50% of unstimulated splenic NK cells express even low levels of Thy-1 (Herberman, et al, 1978). The cytotoxic activities observed in such cultures led to additional cell designations, such as activated killer (AK), mixed lymphocyte culture-activated killer (MLC-AK), anomolous killer (AK), NK-like effector, NK-T and lymphokine-activated killer cells (reviewed by Timonen, 1983). Seeley, et al (1979), have identified several similarities between NK and MLC-AK cells, including low affinity receptors for sheep red blood cells, similar ranges of target cell sensitivity, cross competition between sensitive targets and the lack of an amnestic response or immunologic memory. However, numerous studies exist to suggest significant differences between the various activities. In human mixed lymphocyte cultures (MLC), Zarling and Bach (1982) have shown that most if not all of the cells responsible for CTL activity are OKT3+ and OKT8+ while NK-like cells are OKT3- and OKT8-. Abo

and Balch (1982) found evidence that the cell surface phenotoype and kinetics of response differ between NK and MLC-AK cells, with human NK cells expressing the HNK-1 antigen and having spontaneous activity while MLC-AK cells lacked this surface marker and required 7-day stimulation with alloantigen or mitogen to acquire cytotoxic activity. MLC-AK cells also appear to lack cell surface receptors for the Fc portion of immunoglobulin (FcR), that are characteristic of human NK cells (Seeley, et al, 1979). In addition, certain target recognition structures appear to be unique to NK cells (Takasugi, et al, 1977).

While several features serve to contrast NK and MLC-AK cells, the latter differ in several respects from allospecific CTL as well. Cytotoxic T lymphocytes display classic memory responses whereas activated killer cells do not; their respective targets also fail to cross compete in cold target inhibition studies (Seeley, et al, 1979). Seeley and Golub (1978), studied the kinetics of development of the two responses during <u>in vitro</u> culture and found that peak activity of MLC-AK cells occured at 4-5 days and returned to baseline by day 9, while CTL activity peaked at 6-7 days and remained active for months. Further understanding of the role of the various cytotoxic effector cells to one another is expected as additional clones of cells posssessing one or more of these cytotoxic activities become available for study.

Studies into the mechanism of natural killer cell-mediated cytotoxicity and its regulation have shown that cytotoxicity results from a multiple-step process. Roder and Kiessling (1978), identified a subpopulation of nylon wool nonadherent murine splenocytes that bound specifically to NK-sensitive target cells, suggesting that target cell binding was an important step in the cytolytic process. They went on to show that: binding preceeded lysis and was inhibited by EDTA; the tumor-binding cells (TBC) resembled small lymphocytes with membrane specialization in areas of tumor cell contact; metabolic inhibitors and serine protease inhibitors blocked lysis without affecting target cell binding and interferon-inducing agents augmented lysis without increasing the number of TBC (Roder, et al, 1978). Data from other laboratories has confirmed that cytotoxicity requires binding between NK effectors and target cells, is temperature-independent and Mg++-dependent (Carpen, et al, 1981; Quan, et al, 1982; Hiserodt, et al, 1982).

The Ca++ pulse method, in which effector-target cell conjugates are held in medium with Mg++/EGTA and CaCl2 subsequently added, served to establish that binding alone is not sufficient for activation of the lytic mechanism in NK cell-mediated cytolysis and that activation and programming for lysis is Ca++-dependent and requires energy (Hiserodt, et al, 1982; Quan, et al, 1982). Saksela, et al (1982), employed fluorochrome-coupled

lectins that bind preferentially to the golgi apparatus of fixed and detergent-solubilized cells to show that programming for lysis is associated morphologically with movement of the golgi apparatus toward the target cell. They also observed that the resultant lytic phase of NK-target interaction was inhibited by monensin, a carboxylic ionophore that specifically blocks vesicular traffic of golgi derived vacuoles to the cell surface (Tartakoff, 1981), in support of the stimulus secretion model of NK cytolysis proposed by Roder, et al (1980).

After Ca++-dependent programming, the addition of EDTA to NK-target conjugates followed by dispersion into dextran containing medium was shown to dissociate all conjugates, but not interfere with target cell lysis as determined by 51-Cr release, thereby confirming the existence of a killer cell-independent lysis (KCIL) step in the overall cytotoxic process (Hiserodt, et al, 1982). KCIL was found to be Ca++ and Mg++-independent but protease and antiprotease sensitive (Hiserodt, et al, 1983; Hudig, et al, 1981). After detaching from the KCIL-programmed target, the NK cell is apparently refractory to further cell binding or lysis for a period of time, in which it is said to be recycling (Timonen, T., et al, 1982; Ullberg and Jondal, 1981). The existence of KCIL and evidence for a stimulus secretion model of NK cytolysis supported a search for soluble mediators of the cytotoxic process. Using effector cells incubated with

PHA or ConA in the bottom of a miniature Marbrook chamber, Wright and Bonavida (1981), demonstrated that the effector cells released soluble mediators which traversed a 0.2 micron Nucleopore membrane barrier to specifically lyse NK-sensitive target cells cultured in the top of the chamber. They also showed that the mediators, termed natural killer cell-derived cytotoxic factors (NKCF), were released from spleen cells but not thymocytes, had a molecular weight greater than 12,000 daltons, were stable at -20 °C, inactivated by heating to 100°C for 15 minutes, and inhibited in their activity by the addition of alpha-methyl-D-mannoside, D-galactose, or N-acetyl-D-galactosamine, which distinguishes them from lymphotoxin activity against the same targets (Toth, and Granger, 1979). However, unlike the rapid kinetics of cellular NK activity in vitro, NKCF-induced cytotoxicity required incubation periods of 40 hours to cause significant target cell lysis as measured by trypan blue exclusion (Wright and Bonavida, 1981; Farram and Targan, 1983), suggesting that additional effector cell-associated components may be necessary for full expression of cytotoxic activity.

The finding that proteases inhibited both target cell binding and lysis while antiproteases blocked only lysis (Roder, et al, 1978), implicated a protein with protease activity in the lytic step of NK-mediated cytolysis. Hudig, et al (1981), demonstrated that

alpha-1-antichymotrypsin is a potent inhibitor of NK activity and similar results were shown for alpha-2-macroglobulin, a potent inhibitor of many endopeptidases including chymotrypsin (Gravagna, et al, 1982; Laurell and Jeppson, 1975). In detailed studies of protease inhibition of the KCIL stage of human NK cytolysis, Hiserodt, et al (1983a, 1983b), found that trypsin (T), chymotrypsin (CT), and papain (P) inhibited lysis well beyond the Ca++-dependent programming step. However, in an NKCF assay only T and CT but not P inhibited cytolysis. Preincubation of target cells with T, CT or P failed to diminish their ability to absorb NKCF or to serve as cold target competitors in an NKCF assay, but abolished their ability to serve as targets in a standard NK assay due to decreased binding by effector cells. Pretreatment of effector cells with T and CT but not P blocked their abiltiy to mediate NK cytolysis. The authors concluded that the receptor on target cells for binding to the effector was T-, CT- and P-sensitive, the receptor for NKCF was protease-resistant and that both NKCF and the NK receptor for binding to target antigen were T- and CT-sensitive but P-resistant (Hiserodt, et al, 1983b). Taken together with data from other studies, the authors proposed a multistage hypothetical model for human natural killing, in which: 1. Mg++-dependent binding of NK cell surface receptor to target cell antigen occurs, followed by; 2. Ca++-dependent binding to an

extracellular site analogous to that seen in CTL cytolytic mechanisms (Martz, et al, 1982): 3. enzymatic cleavage of a component of the NK surface receptor (lytic focusing fragment), which remains attached to the target cell and has otherwise cryptic high avidity subunits for NKCF that are exposed in the process of cleavage; 4. intracellular flux of Ca++ through calcium channels to activate the more protracted phase of Ca++-dependent programming. These channels can be blocked by the calcium channel blocking agent verapamil (Hiserodt, et al, 1982a); 5. secretion of NKCF; 6. focusing and polymerization of NKCF by the target cell-bound lytic focusing fragment, possibly through disulfide bond formation, since 2-mercaptoethanol additon after the Ca++-dependent step inhibits cytotoxicity (Hiserodt, et al, 1982b), and; 7. interaction of the assembled lytic complex with the target cell membrane in a manner analogous to the membrane attack complex of the complement system, leading to colloid osmotic lysis. This hypothetical model serves as a framework for the inquiry into potential mediators of the various stages of the cytolytic process as well as testing of the hypothesis itself.

Evidence has accumulated in recent years to suggest a role for natural killer cells in host defense mechanisms against cancer (Herberman and Holden, 1978a; Hanna and Fidler, 1980; Talmadge, et al, 1980; Warner and Dennert, 1982), viral, fungal and parasitic infections (Welsh,

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1979; Murphy and McDaniel, 1982; Hatcher and Kuhn, 1982), in graft rejection (Kiessling, et al, 1977; Datta, et al, 1979; Warner and Dennert, 1982), and even in the regulation of normal hematopoietic cell differentiation (Abruzzo and Rowley, 1983). This broad spectrum of activity has also raised interest in the possible immunotherapeutic use of NK cells in the treatment of disease. Of particular interest in this regard is the identification of physiologic and pharmacologic regulators of NK cytolytic activity, as well as the development of functional NK cells from precursor cells.

Interferon has important but mixed effects on natural killer cell activity against susceptible tumor targets. Interferon rapidly boosts NK activity (Roder, et al, 1978; Herberman, et al, 1980; Ortaldo, et al, 1982), apparently by increasing the number of TBC that lyse their targets (one way of defining an NK precursor cell), accelerating lysis and recycling capacity but not by increasing the number of effectors that bind target cells (Roder, et al, 1978; Ullberg and Jondal, 1981; Targan and Stebbing, 1982). One exception to this is the ability of interferon to increase the number of TBC against anchorage-dependent tumor and nontumor targets in humans (Timonen, et al, 1982). In addition to their ability to respond to interferon with increased cytolytic activity, NK cells were shown to be capable of producing interferon in response to target cell contact in a positive feedback mechanism

(Timonen, et al, 1980). While interferon is known to have direct inhibitory effects on tumor cells (Gresser, et al, 1970), it also seems capable of protecting certain normal and malignant cells from NK-mediated cytolysis when such cells are incubated with interferon prior to contact with NK cells (Welsh, et al, 1981), although such treatment increases their susceptibility to CTL-mediated cytotoxicity. Trinchieri, et al (1981), showed that normal cells were more rapidly and effectively protected from NK cytolysis by interferon than tumor cells, suggesting a mechanism for the selective destruction of tumor cells in vivo.

Interleukin-2 (IL-2, T cell growth factor, TCGF), is an potent augmentor of NK activity and is the only growth factor detected to date that is capable of maintaining the long-term proliferation of cytolytically active NK cells (Henney, et al, 1981). In fact, NK cultures and clones have been generated and evaluated using IL-2 and interferon-containing conditioned medium (CM) supernatants obtained from splenocyte cultures stimulated with the mitogenic lectins PHA or ConA (Nabel, et al, 1981a and 1981b; Brooks, et al, 1982). IL-2 binding studies of NK cells cultured in the presence or absence of interferon has led Kuribayashi, et al (1981), to suggest that a synergistic effect of the two lymphokines may occur, with interferon causing an increase in the number or affinity of IL-2 receptors on NK cells as part of the augmentation

process.

Studies investigating the cell surface phenotype of cells responding to interferon and IL-2 with increased cytotoxicity has led to the description of subsets of NK cells (Minato, et al, 1981; Minato and Bloom, 1982; Burton, et al, 1982). Based on residual cytotoxic activity of murine spleen and marrow cells following treatment with specific antibody and complement, as well as the response to IL-2 and interferon preparations, Minato, et al (1981), identified four phenotypically different cytotoxic cell populations: 1. NK-I cells, prominent in spleen, Thy-1-, Qa5+, Ly-5+, augmented by interferon; 2. NK-T cells, prominent in spleen, Thy-1+, Qa5+, Ly-5+, augmented by interferon and IL-2; 3. NK-M cells, restricted to marrow, Thy-1-, Qa5-, Lyt-2-, Ly-5+, not modulated by IFN or IL-2, and; 4. TK cells, in spleen, Thy-1+, Qa5-, Lyt-2+, Ly-5+, augmented by IL-2 but not IFN, lyse CTL-sensitive target but not NK-sensitive target. These results and others employing complement-mediated lysis to determine cell surface phenotype must be interpreted with caution, since surface marker expression does not always indicate susceptibility to antibody + C (Woody, et al, 1977; Dennert, et al, 1980). Even so, the probable existence of heterogeneity within the natural killer cell system and the identification of relevant surface antigens has served to guide the study of NK cell development and lineage.

Despite the abundance of literature regarding the nature of natural killer cells, their function and relationship to other immunologic effectors, relatively little is currently known about precursor or stem cells that give rise to NK cells. One approach to this problem involves the examination of cell surface antigens expressed on differentiated cells but lacking on their immature counterparts. In addition, if such a surface antigen is also implicated in an important aspect of cellular function, it represents a unique opportunity to explore the developmental biology and biochemistry of functional cellular maturation. Such may be the case with the murine lymphopoietic and myelopoietic cell surface differentiation glycoprotein, Ly-5. Several studies have shown that polyvalent anti-Ly-5 sera block NK activity, even in the absence of complement (Kasai, et al, 1979; Pollack, et al, 1979; Minato, et al, 1980), suggesting that the Ly-5 surface molecule may directly participate in one or more steps of NK-mediated cytolysis. After reports showed that Ly-5 and T200 antigens are expressed on identical murine cell surface proteins (Siadak and Nowinski, 1980), and that Ly-5 is polymorphic (Komuro, et al, 1975; Omary, et al, 1980), while T200 is not (Trowbridge, et al, 1975; Trowbridge and Mazauskas, 1976), Seaman, et al (1981), found that anti-T200 monoclonal antibody (mAb) blocked NK cytolysis without affecting CTL activity against the same target. In

addition, they observed that pretreatment of YAC-1 target cells with anti-T200 did not alter their susceptibility to NK cytotoxicity even though by immunofluorescence these targets express T200, and that anti-T200 mAb must be continuously present in the NK cell assay for inhibitory activity to be observed. This requirement may help to explain the variable effects of anti-Ly-5 sera on NK activity observed in some experiments (Minato, et al, 1980). In addition, some evidence exists to suggest that different subsets of NK cells may vary in their susceptibility to inhibition by anti-Ly-5 sera, with NK-I cells blocked more effectively than NK-M cells (Minato, et al, 1981).

Direct antibody binding studies to determine the distribution of Ly-5/T200 on hematopoietic cells have revealed that Ly-5+ cells include all known sets of T cells, prothymocytes, myeloid cells, monocytes, macrophages, natural killer cells and subsets of B cells, while Ly-5- cells include erythrocytes, proerythroblasts, hepatocytes and kidney cells (Komuro, et al, 1975; Scheid and Triglia, 1979). Susceptibility to lysis by anti-Ly-5 + C has also been used to estimate the percentage of lymphocytes that express surface Ly-5 and determine their biological function (Minato, et al, 1980, but several studies have indicated that surface Ly-5 positivity does not necessarily correlate with the ability to be lysed with antibody and complement. Minato, et al (1980), found

that anti-Ly-5 sera + C lysed 30-40% of CBA/J and 7% of BALB/c nude mouse splenocytes and abrogated NK activity, leading them to conclude that such were the percentages of Ly-5+ lymphocytes in the respective mouse strains and that all mature Ly-5+ cells were lysed by this technique. Following a three hour treatment with an interferon-containing preparation, they identified recovery of NK activity and additional cells susceptible to lysis by anti-Ly-5 sera + C, from which they concluded that Ly-5- precursors of NK cells exist in murine spleen, and that these cells are induced by interferon to convert to Ly-5+ cells with natural killer cell function. However, using a second step antibody (anti-rat Ig), Seaman, et al (1981), showed that anti-T200 mAb lysed 82% of C57BL/6 splenocytes, suggesting that additional Ly-5+ cells may have been present in CBA/J and BALB/c nude spleens that resisted complement-mediated lysis. Further supportive evidence came from Dennert, et al (1980), who found that in the absence of a second step antibody, anti-T200 mAb lysed more than 95% of C57BL/6 thymocytes, but only 40% of spleen cells, 20-30% of bone marrow cells and 5-10% of BALB/c nude spleen cells, in close aggreement with the results of Minato, et al (1980). However, the former investigators also looked at the effect of anti-T200 + C on CFU-S and prothymocytes and found that neither were diminished by this treatment. In addition, they subjected nonerythrocytic thymic, splenic and bone

marrow cells to quantatative analytical cytofluorimetry and found that 78% of splenocytes, 91% of thymocytes and 81% of bone marrow cells were Ly-5+, verifying that a large percentage of Ly-5+ cells exist in bone marrow and spleen that resist lysis with anti-Ly-5 and complement (Dennert, et al, 1980). From these results and the evaluation of CTL activity, helper-T cell function, mixed lymphocyte reactivity, T and B cell mitogenesis and T cell-independent B cell responsiveness, the authors also demonstrated that anti-T200 mAb is selectively cytotoxic for thymocytes and mature thymus-dependent T lymphocytes. Based on the above information, it appears unsettled as to whether splenic Ly-5- precursor cells actually convert to Ly-5+ NK effector cells as proposed by Minato, et al (1980), since it may be equally possible that Ly-5+ NK cells could have been blocked in their cytolytic capacity by anti-Ly-5 sera but were resistant to complement-mediated lysis, following which the cells regenerated additional cell surface Ly-5 or other relevant molecules in the presence of interferon or other lymphokine activity to regain cytolytic function. In addition, the incremental increase in cells susceptible to lysis by anti-Ly-5 + C after interferon treatment may have represented a cellular maturation event akin to that of prothymocytes to thymocytes and been unrelated to new NK effectors generated from precursors. In order better understand NK cell development and its regulation,

additional information is needed regarding the role of Ly-5 in the mechanism of natural killer cell-mediated cytotoxicity and alternate approaches developed to determine the cell surface phenotype of precursors and effectors.

Before proceeding to describe the biochemistry and heterogeneity of the Ly-5 system, it is useful to establish the relevance of this system to human natural killer cells. Omary, et al (1980), identified the human homologue of murine T200 glycoprotein and documented the following similarities between the two systems: 1. the peptide structure of the glycoprotein is highly conserved between the two species, a property shared by only a few other cell surface proteins such as Ia, Ig, Thy-1 and MHC antigens; 2. the molecule is broadly distributed within the hematopoietic system but not detectable on nonhematopoietic cells, and; 3. structural differences between the forms of the glycoprotein are found on T and B lymphoblastoid cell lines. Sparrow and McKenzie (1983), showed that anti-human T200 mAb specifically blocks binding and NK-mediated lysis of K562 target cells without affecting CTL responses. Targan and Newman (1983), presented evidence to suggest that T200 may also participate in the early part of the Ca++-dependent programming step of NK lysis, in that 13.1 mAb, which recognizes an epitope on T200, blocks lysis if added prior to Ca++ and does so without altering conjugate formation.

Together, these data strongly suggest an important and fundamental role for Ly-5/T200 in the natural killer cell system and establish the murine system as a reasonable model for exploring the nature and function of the Ly-5 glycoprotein.

Any attempt to ascribe biological significance to the Ly-5 glycoprotein must contend with the fact that the expression of Ly-5 is not unique to NK cells. Early evidence that differences in Ly-5 may exist among various lymphoid cell populations came from Trowbridge, et al (1975), who found that the glycoprotein migrates on sodium docecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with a relative molecular mass (Mr) of 170,000-190,000 for thymocytes and T cells, while B cells express a 220,000 form. Similar Mr were estimated by Dunlap, et al (1978). Michaelson, et al (1979), found three peaks for splenocytes, at 175, 185 and 220 kilodaltons (KD), while thymocytes had a 175 KD peak which was broad, typical of heavily glycosylated proteins. Coffman and Weissman (1981), developed a monoclonal antibody that reacted specifically with B cells, precipitating the 220,000 dalton form of T200, which they designated B220. Tung, et al (1981), studied the Ly-5 expression of 7 cloned cell lines and found that each expressed only one of three forms: T cells, 200,000; macrophages, 205,000 and; B cells, 220,000. Cleveland peptide maps for the three molecular weight forms were

identical, suggesting that differences in the molecular weights of the various forms of Ly-5 may reside in the carbohydrate portion of the glycoprotein. SDS-PAGE analysis of Lyt-2,3+ CTL clones revealed additional apparent forms of Ly-5 with Mr of 210,000 and 215,000 (Tung, et al, 1983). The relative molecular mass of Ly-5 that is expressed on NK cells may provide insight into their cell lineage but has yet to be characterized.

Trowbridge, et al (1977), identified T200 as a cell surface protein that is partially degraded by gentle trypsinization. Saturating binding experiments suggest that murine splenocytes express approximately 20,000 T200 molecules per cell, while thymocytes express slightly more, marrow cells slightly less and certain lymphomas up to 5 times as much of this glycoprotein (Trowbridge, 1978). Omary and Trowbridge (1980), further characterized T200 as a phosphorylated transmembrane glycoprotein with a relatively protease-resistant domain exposed on the cell surface and containing the antigenic determinant recognized by anti-T200 mAb. Trypsinization of radioiodinated, Nonidet-P40 (NP-40) solubilized cell surface proteins from a murine T cell lymphoma line (BW5147), showed that the protease-resistant fragment migrated with a Mr of 100,000 on SDS-PAGE, contained all of the sites labeled by lactoperoxidase (LPO)-catalyzed radioiodination of viable cells and most if not all of the mannose-containing oligosaccharide units of the T200

glycoprotein. The remainder of the protein was extensively degraded by trypsin and contained phosphoserine residues that could be labeled with 32-PO4 in vivo. The existence of a protease-resistant outer domain in a molecule which has been shown to be important in NK-mediated cytolysis, a process that involves proteases, makes Ly-5/T200 a candidate molecule for facilitating the binding and/or lytic stages of natural killer cell cytotoxic reactivity against susceptible tumor cells.

Watson, et al (1981), looked at intracytoplasmic forms of Ly-5/T200 in murine T and B cells and found that EL-4 cells (a T cell leukemia) produced a 170 KD precursor that reacted with anti-Ly-5 sera and was not expressed on the cell surface, while the corresponding precursor form in LPS blasts (B cells) had a molecular weight of 190 KD. Precursors were shown to have higher 3H-mannose:3H-glucosamine ratios than their immediate products and lacked the 32-PO4 incorporation characteristic of the mature forms of the glycoprotein.

Investigation of the oligosaccharide portion of human Ly-5/T200 glycoprotein (also known as human leukocyte common antigen), has revealed that it labels with 3H-mannose, 3H-galactose and 3H-fucose (van Eijk and Muhlradt, 1981), carries O- and N-glycosidically linked, sialated carbohydrate chains and that the O-linked chains of B cells but not T cells are highly susceptible to degradation by endo-beta-galactosidase (Childs, et al,

1983). Using neuraminidase treatment of human B and T cell lines, Morishima, et al (1982), concluded on the basis of mobility changes on SDS-PAGE that the lower molecular weight form of Ly-5/T200 seen in T cell lines had higher sialic acid content than the higher molecular weight form in B cell lines and are therefore more highly glycosylated or have greater chain branching. The oligosaccharide structure of murine Ly-5 glycoprotein is as yet undetermined.

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SURFACE Ly-5 GLYCOPROTEIN IN MURINE NATURAL KILLER (NK) CELL DEVELOPMENT, TARGET BINDING AND CYTOTOXICITY*

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[1]Abbreviations: avidin-FITC, fluoresceinated avidin; B6, C57BL/6 mice; biotin/avidin-FITC, sequential reaction with biotinylated anti-mouse IgG and fluoresceinated avidin; C, complement; CM, conditioned medium; ConA, concanavalin A; CTL, cytotoxic T lymphocytes; FBS, fetal bovine serum; GPS, guinea pig serum; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; mAb, monoclonal antibody; NK, natural killer; PBS, phosphate buffered saline; sIg, surface immunoglobulin; TBC, tumor binding cells.

ABSTRACT

The role of surface Ly-5 glycoprotein expression in the binding and lysis of susceptible tumor targets by natural killer cells was studied using nonphagocytic, nylon wool nonadherent, T cell-depleted splenocytes from 6-8 week old C57BL/6 mice which were reacted with anti-Ly-5 serum in the presence or absence of a source of complement. A conjugate assay was used to demonstrate that abrogation of tumor cell lysis by anti-Ly-5 serum involved the inhibition of NK cell binding to susceptible YAC-1 targets. Indirect immunofluorescent labeling and cell sorting were used in the physical separation of $Ly\text{-}5^+$ and $Ly\text{-}5^-$ cells to examine the effect of interferon and interleukin preparations on Ly-5 expression and NK activity. Three hour treatment of sorted $Ly\text{-}5^-$ cells with murine $\alpha+\beta$ interferon resulted in conversion of 22% of the cells to an $Ly\text{-}5^+$ phenotype, as well as a significant increase in the percent specific lysis of NK-susceptible YAC-1 targets when compared to freshly sorted $Ly\text{-}5^-$ cells (29.5 ± 1.9 vs 2.6 ± 4.0; $p < .001$). In vitro proliferation of sorted $Ly\text{-}5^-$ cells was induced by three week culture in 40% supernatant from ConA stimulated BALB/c splenocytes (CM), followed by repeat analysis of Ly-5 expression and cytotoxic activity. Cell sorter purified $Ly\text{-}5^-$ cells cultured in CM acquired substantial surface Ly-5 with concomitant high levels of NK activity that remained susceptible to inhibition by anti-Ly-5 serum. The data presented suggest that surface Ly-5 glycoprotein expression is important for binding of NK cells to YAC-1 targets. In addition, $Ly\text{-}5^-$ precursors of NK cells are present in murine splenic tissues and can be induced by CM to become highly active NK cells with increased surface Ly-5 expression. The persistent susceptibility of these cells to inhibition of cytotoxic activity by

anti-Ly-5 serum provides additional evidence of an important role for the Ly-5 glycoprotein in the natural killer cell cytolytic mechanism against certain targets.

Introduction

The Ly-5 glycoprotein is a transmembrane alloantigen expressed on the surface of most cells of lymphopoietic or myelopoietic origin in mice (1,2) and appears to be a homologue of the leukocyte common antigen (LCA) in humans (3,4). This cell surface glycoprotein has been identified on cytotoxic T lymphocytes (CTL) and natural killer (NK) cells (5,6), yet only NK cell-mediated cytotoxicity was shown to be inhibited by treatment with specific antiserum in the absence of complement (7,8). This property indicated that fundamental differences exist in the two forms of cytotoxicity and that the Ly-5 glycoprotein may function in one or more steps of the NK cell lytic mechanism (i.e., binding, programming and killer cell-independent lysis) against certain targets. Additionally, the consistent expression of the Ly-5 glycoprotein on functionally mature NK cells and its apparent importance in the lytic process make more likely the possibility that cytolytically inactive NK precursor cells exist which lack significant surface Ly-5 glycoprotein expression ($Ly\text{-}5^-$).

The isolation and purification of viable $Ly\text{-}5^-$ cells allows for the analysis of regulatory factors that induce the proliferation and functional maturation of pre-NK cells as well as determining the relationship between Ly-5 expression and cytotoxic capability. Of the available methods for enriching lymphoid cell suspensions in $Ly\text{-}5^-$ cells, the use of anti-Ly-5 serum and complement (9) appears to be unsatisfactory since Ly-5 expression correlates poorly with complement-

mediated cytotoxic susceptibility and T cells are selectively eliminated by this procedure (10). Viable NK cells could persist in such preparations but not be identifiable in a standard NK assay due to the inhibitory effect of anti-Ly-5 serum. Similarly, recovery of NK activity in such preparations upon treatment with interferon or interleukins could be due either to recruitment of pre-NK cells or recovery of antibody-bound NK cells or both.

Using in vitro culture and functional analysis of cell sorter purified Ly-5^- and Ly-5^+ murine splenocytes, we report here that Ly-5^- precursors of NK cells are present and can be induced by interferon and interleukin preparations to become highly active NK effector cells. Cytotoxic activity is accompanied by increased surface expression of Ly-5 glycoprotein. The ability of anti-Ly-5 to inhibit cytotoxicity suggests that the Ly-5 glycoprotein participates in the cytolytic process. We further report that the Ly-5 glycoprotein is a mediator of murine natural killer cell binding to YAC-1 tumor cells.

Materials and Methods

Mice. C57BL/6J, BALB/cJ, A.SW/SnJ and SJL/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in our facility. Some of the C57BL/6J mice were also used as breeders. Animals used in these experiments were five to eight weeks of age.

Target Cells. The NK-susceptible YAC-1 lymphoma of A/Sn mice and the NK-resistant P815 mastocytoma of DBA/2 mice were generous gifts from Dr. G. Cudkowicz, and were grown in RPMI-1640 culture medium (Gibco Laboratories, Grand Island, NY), supplemented with 15 mM HEPES (Sigma Chemical Co., St. Louis, MO), penicillin/streptomycin (Gibco) and 10% heat-inactivated (56°C x 30 min) fetal bovine serum (Microbiological

Associates, Walkersville, MD). Cells were maintained in a 37°C, 5% CO_2, humidified incubator.

Preparation of NK Effector Cells. C57BL/6J spleen cell suspensions were aseptically prepared in RPMI-1640 medium with 10% fetal bovine serum (FBS) as described previously (11). Lysis of erythrocytes was achieved by 10 second exposure of spleen cell suspensions to double distilled water followed by a return to isotonicity with concentrated phosphate buffered saline (PBS). Adherent and phagocytic cells were removed by 45 minute incubation with pre-washed carbonyl iron (50 mg/1 x 10^8 cells, 37°C, 5% CO_2) followed by application of a bar magnet to the side of the tube and aspiration of nonphagocytic cells. Alternatively, adherence to plastic surfaces (75 cm^2 polystyrene tissue culture flasks, Corning Glass Works, Corning, NY) was employed and non-adherent cells collected. Nonadherent, nonphagocytic cells were depleted of B cells by passage through nylon wool columns (Fenwal Laboratories, Deerfield, IL) containing 0.6 gm nylon fiber loosely packed into 10 ml plastic syringes (Becton, Dickinson and Co., Rutherford, NJ) that were pre-incubated with medium and FBS. Four ml containing 3 x 10^7-5 x 10^7 cells were loaded into the columns and incubated 60 min in a 37°C, 5% CO_2 environment, after which nylon wool nonadherent cells were collected from the bottom of the column by the dropwise addition of 25 ml medium and FBS to the top. Nylon wool nonadherent splenocytes were treated with monoclonal anti-Thy 1.2 (New England Nuclear, Boston, MA), and complement (guinea pig serum, GPS, Gibco) for 60 min at 37°C, 5% CO_2, to eliminate T cells. Cellular viability was determined by trypan blue exclusion. For some experiments, nylon wool column separation and anti-Thy 1.2 + GPS treatment was

replaced by discontinuous density gradient centrifugation of splenocytes in Percoll (Sigma), as described previously (12). Briefly, 100% Percoll (nine parts Percoll, one part 10X PBS) was diluted in medium to make final concentrations of 30, 40, 50, 60 and 70% (v/v), and gently layered in order of decreasing density in conical 15 ml plastic tubes. 1×10^8 plastic nonadherent cells were layered on top and centrifuged at 375 x G for 30 minutes at room temperature. By this procedure, NK effectors were concentrated in a band at the interface between the 50% and 60% Percoll layers. Cells were collected, washed in medium and kept on ice until use.

Natural Killer Cell Cytotoxicity Assay. YAC-1 and P815 tumor cells were labeled with ^{51}Cr, washed three times and placed into separate soft, V-shaped plastic microtiter wells at 4×10^4 cells/well. Specified numbers of NK cell-enriched spleen cells or sorted and *in vitro* cultured splenocytes were added to the microtiter wells and the plates centrifuged at 100 rpm x 60 seconds. Certain wells were pre-treated with monospecific or monoclonal (New England Nuclear) anti-Ly 5.1, at dilutions of 1:10 and 1:1000, respectively. After incubation for 4-6 hours on a rotating platform (40 rpm) at 37°C, 5% CO_2, the plates were centrifuged at 400 rpm x 10 min and half of the supernatant was removed and counted in a gamma counter. The wells were individually cut and placed into tubes containing their respective supernatants and total counts measured. Percent ^{51}Cr release and specific lysis were calculated by the following formulas:

$$\% \ ^{51}Cr \text{ release} = \frac{2 \times \frac{1}{2} \text{ supernatant cpm}}{\text{total cpm}} \times 100\%$$

$$\% \text{ specific lysis} = \frac{\% \text{ release in test} - \% \text{ spontaneous release}}{\% \text{ maximal release} - \% \text{ spontaneous release}} \times 100\%$$

where the addition of medium and FBS was used to determine spontaneous release and 3% perchloric acid was used to achieve maximal release.

Target Cell Binding Assay. 1×10^5 NK effector cells in plastic round bottom tubes were mixed with an equal number of YAC-1 or P815 target cells in 0.2 ml culture medium. Cell mixtures were incubated at 37°C x 8 minutes, centrifuged at 200 x G, 5 min and reacted for 30 min on ice. Cells were gently aspirated using a pasteur pipette and examined microscopically. The percentage of single effector (small) cells bound to single target (large) cells was determined visually after counting 100 effector cells. Each sample was performed in triplicate.

Fluorescent Labeling, Cytofluorimetric Analysis and Cell Sorting. NK effector cells were adjusted to 1.2×10^7 cells/ml in PBS and incubated for 30 minutes with an equal volume of murine monospecific or monoclonal anti-Ly-5 at final dilutions of 1:10 and 1:1000, respectively. Cells were washed, centrifuged and reacted sequentially on ice with biotinylated anti-mouse IgG and fluoresceinated avidin (avidin-FITC, Vector Laboratories, Burlingame, CA), at dilutions of 1:80 and 1:100, respectively. Cells treated with fluoresceinated avidin with and without biotinylated anti-mouse IgG served as controls for the determination of specific fluorescence. Cells were analyzed cytofluorimetrically in an Orthocytofluorograf 50H cell sorter/2150 comuputer system, using narrow angle helium-neon laser scatter and right angle argon laser fluorescence parameters. Cells were sorted into $Ly\text{-}5^+$ and $Ly\text{-}5^-$ populations and collected aseptically into RPMI-1640 medium and 50% FBS. Sorted cells were washed, centrifuged and resuspended in

culture medium for subsequent treatment and analysis. Viability of sorted cells as determined by trypan blue exclusion always exceeded 80%.

Interferon Treatment and Analysis of Sorted Ly-5$^-$ Splenocytes. Sorted Ly-5$^-$ cells were incubated 3 hours with 5000 units of murine interferon (Lee Biomolecular, San Diego, CA), in culture medium or in medium alone at 37°C. Cells were then assessed cytofluorimetrically for acquired expression of Ly-5 using anti-Ly-5 IgG, biotinylated anti-mouse IgG and avidin-FITC as described above. A comparison of the natural killer cell activities of unfractionated and sorted Ly5$^+$ and Ly5$^-$ C57BL/6 splenocytes was made after incubation of effector cells in the presence or absence of murine interferon. The ^{51}Cr release assay was performed as outlined above, except that 5 x 10^3 target cells/well were used, with an effector:target ratio of 12.5.

In Vitro Culture of Sorted Ly-5$^-$ and Ly-5$^+$ Splenocytes. Conditioned medium (CM) was used to support the *in vitro* culture of sorted Ly-5$^-$ and Ly-5$^+$ splenocytes and was generated by 48 hour culture of BALB/c spleen cells (5 x 10^6/ml) with 10 g/ml concanavalin A (ConA, Sigma) in culture medium. Residual ConA was removed by the addition of 1 g/100 ml Sephadex G-50 (Pharmacia, Piscataway, NJ). The supernatant was passed through 0.2 micron Nalgene filters and tested for its ability to support the *in vitro* proliferation of C57BL/6J spleen cells. The CM used in these experiments was composed of 40% ConA BALB/c splenocyte culture supernatant and 60% RPMI-1640 medium containing 10% FBS.

Production of Anti-Ly-5 Serum. Six-week old SJL/J female mice were injected intraperitoneally with 1 x 10^8 spleen cells and thymocytes from female A.SW/SnJ mice every 2 weeks (13). Animals were aseptically bled from the tail vein on days 7 and 10 after the fourth and subsequent

injections. Sera were pooled and absorbed with SJL/J spleen and thymic cells (one part packed SJL/J cells, four parts sera, v/v). Monoclonal anti-Ly-5.1 IgG_{2a} was purchased from New England Nuclear (Lot FPB 217).

Statistical Analysis. Data were analyzed using the two-tailed Student's t test to determine the significance of differences between experimental groups.

Results

Inhibition of NK Activity by Anti-Ly-5 Serum. Splenic NK effector cells were isolated from 5-8 week old C57BL/6J mice and incubated with monospecific anti-Ly-5 serum in microtiter wells for 60 min at 37°C in the presence or absence of endogenous or exogenous sources of complement. ^{51}Cr labeled YAC-1 target cells were then added and the incubation was continued an additional 4 hours. The specific lysis of NK-susceptible YAC-1 target cells was significantly diminished by anti-Ly-5 serum, regardless of the presence or absence of a source of complement (Table I). Heat inactivation of the serum prior to its use ruled out an endogenous murine source of complement in the abrogation of NK activity. Furthermore, the high cellular viability of each treatment group indicated that NK cells were relatively resistant to complement mediated lysis, in that splenic T cells were effectively lysed by anti-Ly-5 + C (10, personal observations).

Inhibition of Target Cell Binding by Anti-Ly-5 Serum. To test the hypothesis that anti-Ly-5 serum interferes with the target binding step of NK-mediated cytolysis, discontinuous density gradient centrifugation was used to obtain splenocyte populations enriched in or depleted of NK effectors. Anti-Ly-5 serum, normal mouse serum or an equal volume of medium was added to the cell suspensions for 30 min, followed by removal

TABLE I

Effect of Anti-Ly-5 Serum on NK Cell-Mediated Cytotoxicity against YAC-1 Target Cells[a]

Effector cell treatment[b]	% Specific lysis[c]	Average % reduction in lysis
Medium alone	45.1 ± 4.8	
Complement (C) control	41.8 ± 6.1	7.3
Anti-Ly-5 serum alone	15.1 ± 0.6	66.5*[e]
Heat-inactivated anti-Ly-5 serum[d]	5.9 ± 3.3	86.9*
Anti-Ly-5 serum + C	5.6 ± 2.2	87.6*
Heat-inactivated anti-Ly-5 serum + C	2.3 ± 1.1	94.9**

[a]Effector:target ratio = 25.

[b]Cell viability in all treatment groups exceeded 95%, as measured by trypan blue exclusion.

[c]Mean ± S.D. of triplicate samples (σ_{n-1}).

[d]Serum heated to 56°C x 30 min to inactivate endogenous complement before use.

[e]* $p < .001$, ** $p < .0001$.

of excess antibody and the addition of YAC-1 or P815 tumor cells in a target cell binding assay (Table II). Pretreatment of NK effectors with anti-Ly-5 serum substantially blocked binding to NK-susceptible YAC-1 targets (decreasing the number of conjugates/100 lymphocytes from 17 to 5), while normal mouse serum as a source of immunoglobulin had no effect. The NK-resistant P815 target was not bound by effector cells despite the absence of antiserum. In a parallel experiment, treatment of YAC-1 tumor cells with anti-Ly-5 serum prior to the target cell binding assay resulted in an increase in conjugate formation ($35.7 \pm 7.4\%$), indicating that an interaction of antiserum with target cells was not the cause of decreased binding by NK effectors.

Cytofluorimetric Analysis of Ly-5 Expression on NK Effector Cells and Separation of Ly-5$^-$ and Ly-5$^+$ Cells by Cell Sorting. NK effector cells underwent indirect immunofluorescent labeling with murine monoclonal anti-Ly-5.1 IgG (anti-Ly-5 mAb), biotinylated anti-mouse IgG and fluoresceinated avidin. Cells labeled with avidin-FITC alone or in combination with biotinylated anti-mouse IgG (i.e., biotin/avidin-FITC) served as controls to determine background fluorescence and contaminating surface immunoglobulin positive (sIg$^+$) B cells, respectively. To verify adequate removal of splenic B cells, which are labeled by the indirect immunofluorescence technique independent of their surface Ly-5 expression, unfractionated and nylon wool nonadherent splenocytes were compared for percent specific fluorescence after biotin/avidin-FITC treatment (Figure 1). Nylon wool column separation was shown to remove more than 90% of the sIg$^+$ cells, leaving only 3% nonspecific fluorescence in the anti-Ly-5 mAb treated suspension. Figure 2 shows the gating procedure used to define and sort Ly-5$^-$ and

TABLE II

Inhibitory Effect of Anti-Ly-5 Serum on Target Cell Binding by NK Cells

		Conjugates per 100 lymphocytes[a]		
Target cell	% Percoll fraction	NK cells	NK cells + normal serum	NK cells + anti-Ly-5
YAC-1	60[c]	17.0 ± 3.6	17.3 ± 4.0	5.7 ± 1.5*[b]
P815	60	0.3 ± 0.6	ND[d] ND	
YAC-1	70[e]	4.3 ± 1.5	2.0 ± 1.0	3.0 ± 1.7
P815	70	0.3 ± 0.6	ND ND	

[a]Values represent mean ± S.D.of triplicate samples blinded to observer (σ_{n-1}).

[b]$p < .005$.

[c]60% Percoll fraction (enriched in NK cells).

[d]ND = not done.

[e]70% Percoll fraction (depleted of NK cells).

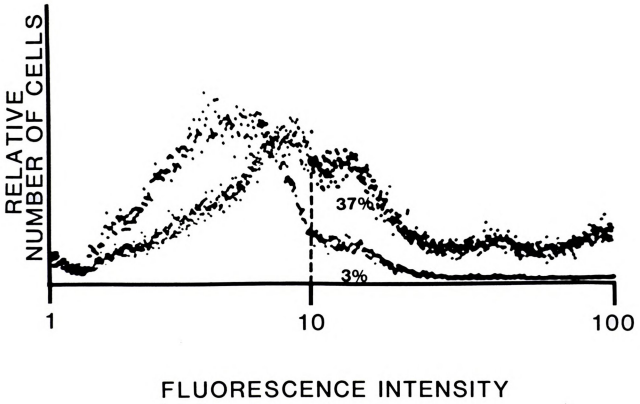

Fig. 1. Fluorescence histograms of unfractionated (UF) and nylon wool nonadherent (NWNA), C57BL/6J splenocytes labeled with biotinylated anti-mouse IgG and fluoresceinated avidin. Relative cell number is plotted on the ordinate and fluorescence intensity on the abscissa. The dashed line (- - - - -) signifies the 99th percentile for autofluorescence of unlabeled spleen cells. The percentage of total cells exceeding this level of fluorescence in each of the treated groups is listed beneath their respective histograms. Nylon wool column separation substantially eliminates nonspecific immunofluorescence that is unrelated to specific surface marker expression in this system.

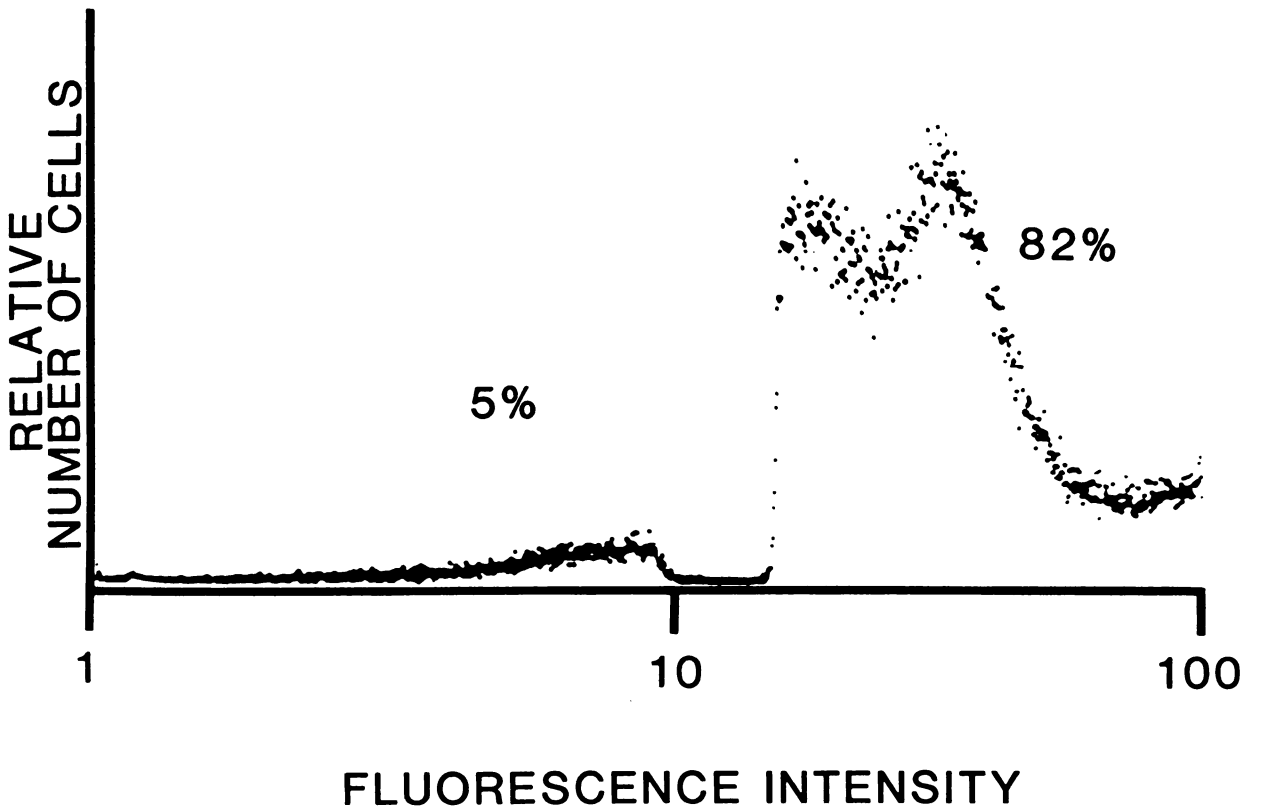

Fig. 2. Fluorescence histogram of viable NWNA, anti-Thy 1 + GPS treated C57BL/6J splenocytes treated with anti-Ly-5 mAb + biotin/avidin-FITC. The percentages shown indicate those cells having defined $Ly\text{-}5^-$ and $Ly\text{-}5^+$ phenotypes. The baseline region between the $Ly\text{-}5^-$ and $Ly\text{-}5^+$ cell populations indicates the gating procedure used by the cell sorter to discriminate fluorescent from non-fluorescent cells.

Ly-5$^+$ cells. The vast majority of nonphagocytic, nylon wool non-adherent, T cell-depleted splenocytes expressed relatively high amounts of surface Ly-5 glycoprotein. Ly-5$^-$ cells were purified by cell sorting and cytofluorimetric analysis of sorted cells indicated successful separation, with 4% positive cells in the Ly-5$^-$ fraction (Figure 3A) and 5% negative cells in the Ly-5$^+$ fraction (Figure 3B).

Effect of Interferon Treatment on Sorted Ly-5$^-$ Cells. Sorted Ly-5$^-$ cells were incubated for 3 hours at 37°C, 5% CO_2, in the presence of murine interferon (α + β, 5000 units) or in medium alone. Cell suspensions were then examined cytofluorimetrically for increased surface expression of Ly-5 glycoprotein (Figure 3). When compared with sorted Ly-5$^-$ and Ly-5$^+$ cells (Figure 3A, 3B) both the medium control and interferon treated cells showed slight increases in fluorescence when labeled with biotinylated anti-mouse IgG + avidin-FITC (Figure 3C, 3E). However, only the interferon treated cells demonstrated a significant increase in specific surface Ly-5 expression under the same conditions, with 22% of the cells converting to a Ly-5$^+$ phenotype (Figure 3D, 3F).

To define the relationship between interferon-induced increases in surface Ly-5 expression and acquired NK activity among sorted Ly-5$^-$ cells, unsorted and cell sorter purified whole B6 splenocytes were compared in their ability to lyse NK-susceptible YAC-1 targets (Table III). Ly-5$^-$ sorted cells lacked cytotoxic activity immediately after sorting (2.6 $\pm$ 4.0% specific lysis), but when cultured in medium containing interferon under conditions shown to increase surface Ly-5 expression, they acquired substantial NK activity, even at a relatively low effector:target ratio of 12.5 (29.5 $\pm$ 1.9% specific lysis; $p < .001$). The degree of activity exceeded that generated by the incubation of

Fig. 3. Two-dimensional cytograms (narrow angle scatter versus right angle fluorescence) of sorted Ly-5$^-$ C57BL/6J splenocytes before and after 3 hour incubation with 5000 units murine interferon (α + β) or medium control. Each group was then labeled with anti-Ly-5 mAb (1:1000) + biotin/avidin-FITC and compared with a biotin/avidin-FITC control. A. Sorted Ly-5$^-$ cells prior to incubation with interferon or medium. B. Sorted Ly-5$^+$ cells indicating the degree of fluorescence characteristic of Ly-5$^+$ cells. C. Sorted Ly-5$^-$ cells incubated with medium alone x 3 hr, biotin/avidin-FITC control. D. Sorted Ly-5$^-$ cells incubated with medium alone x 3 hr, labeled with anti-Ly-5 + biotin/avidin-FITC. E. Sorted Ly-5$^-$ cells incubated with interferon x 3 hr, biotin/avidin-FITC control. F. Sorted Ly-5$^-$ cells incubated with interferon x 3 hr, labeled with anti-Ly-5 + biotin/avidin-FITC. Note that an additional 22% of the cells (43.6-21.2), have converted to an Ly-5 phenotype when treated with the interferon preparation.

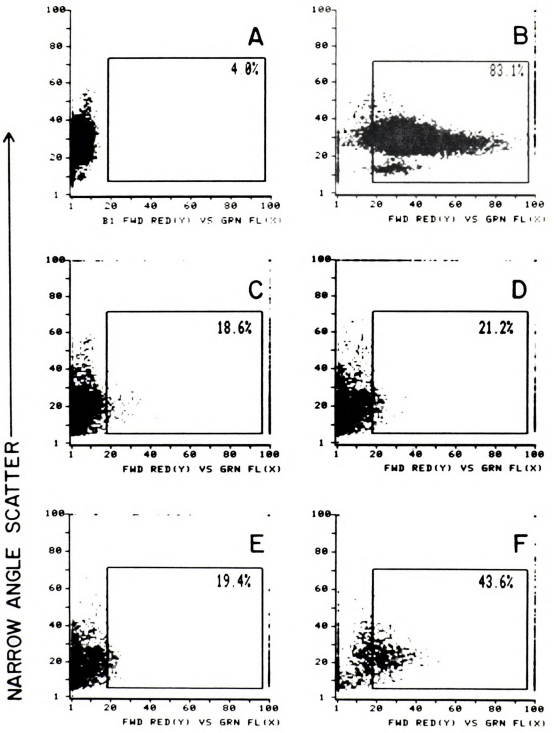
A
4.0%
B
83.1%
C
18.6%
D
21.2%
E
19.4%
F
43.6%
FWD RED(Y) VS GRN FL(X)
NARROW ANGLE SCATTER
FLUORESCENCE INTENSITY

Figure 3

Table III

Effect of Interferon Treatment on NK Activity of Sorted Ly-5$^+$ and Ly-5$^-$ C57BL/6 Splenocytes[a]

	% Specific Lysis of YAC-1 Targets[b,c]		
	Medium x	Medium + Interferon (5000 u/ml)	
Splenocytes	0 Hours[d]	3 Hours	x 3 Hours
Whole spleen	1.7 ± 6.4	9.0 ± 0.3	15.7 ± 3.8
Sorted Ly-5$^+$	11.1 ± 5.9	8.5 ± 1.9	11.3 ± 3.8
Sorted Ly-5$^-$	2.6 ± 4.0	19.7 ± 6.0	29.5 ± 1.9[*e]

[a]Whole B6 splenocytes labeled with anti-Ly-5.1 + biotinlyated anti-mouse IgG + avidin-FITC as described in Materials and Methods, sorted into fluorescent (Ly-5$^+$) and nonfluorescent (Ly-5$^-$) populations.

[b]4 hour ^{51}Cr-release assay, 5 x 10^3 YAC-1 targets/well, effector:target ratio = 12.5.

[c]Mean ± S.D. of triplicate samples (σ_{n-1}).

[d]Assay of cells immediately after sorting; sorted cell populations >95% pure for defined cell population.

[e]*$p < .001$.

whole B6 splenocytes with interferon (15.7 ± 3.8%), or of interferon treated Ly-5$^+$ sorted cells from whole spleen suspensions (11.3 ± 2.5%).

Effect of In Vitro CM Culture of Sorted Ly-5$^-$ and Ly-5$^+$ Cells on Surface Ly-5 Glycoprotein Expression and NK Activity. Sorted Ly-5$^-$ and Ly-5$^+$ cells were placed into culture for a period of 21 days with CM to support the proliferation of cells necessary for subsequent cytofluorimetric analysis of Ly-5 expression and NK activity. In both the Ly-5$^-$ and Ly-5$^+$ cell cultures, CM induced the conversion of almost all of the cells to an Ly-5$^+$ phenotype (Figure 4), with an average level of fluorescence at least 10 times greater than freshly isolated NK effector cells (Figure 2). CM cultured Ly-5$^-$ cells were heterogeneous in their Ly-5 expression, with two distinct levels of fluorescence identified (Figure 4A). Sorted Ly-5$^+$ cells appeared more uniform in their high level of surface Ly-5 expression after culture in CM, with a single peak level of fluorescence (Figure 4B).

To identify a possible association between the conversion of Ly-5$^-$ cells to Ly-5$^+$ cells, the development of NK activity and the role of the Ly-5 glycoprotein in the cytotoxic process, sorted and CM cultured cells were used as effectors in a standard NK assay (Table IV). Anti-Ly-5 mAb was added to some of the wells to examine its effect on cytotoxicity. An effector to target ratio of 2 was used, a ratio that is usually insufficient to allow the demonstration of NK activity in a standard 4 hour ^{51}Cr release assay (14). After showing that spent media from the CM cultures were without cytotoxic activity, it was clear that both of the sorted cell populations that were cultured in CM developed potent and selective cytotoxic activity against the NK-susceptible YAC-1 target. In addition, the cytotoxic process was significantly diminished

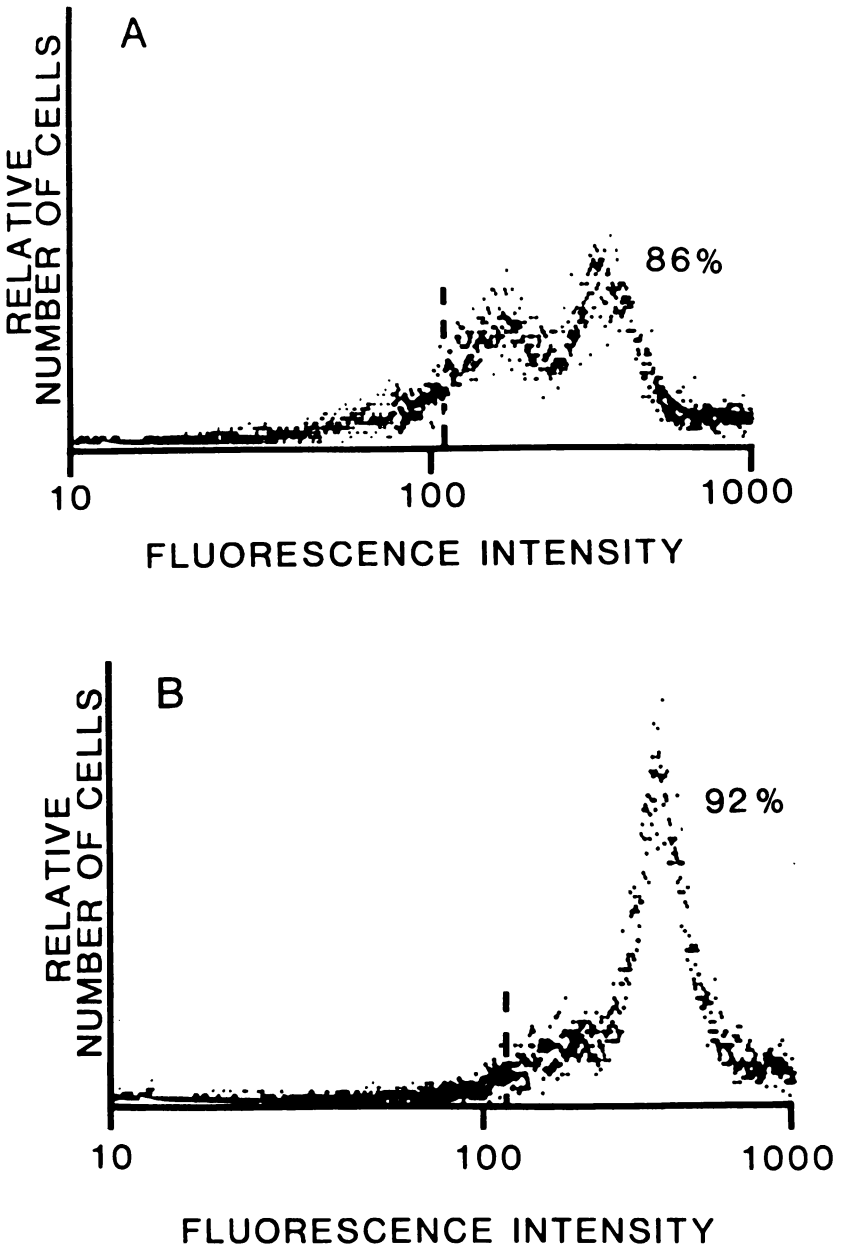

Fig. 4. Fluorescence histograms of sorted Ly-5$^-$ and Ly-5$^+$ C57BL/6 splenocytes after 3 week culture in conditioned medium (CM). Note that the relative fluorescence intensity scale is 10 times higher than for cells analyzed prior to culture in CM. Cells were labeled with anti-Ly-5 + biotin/avidin-FITC in a manner identical to that for cells studied prior to culture in CM. The dashed line (- - - - -) represents the 85th percentile for fluorescence intensity of the biotin/avidin-FITC controls. A. Sorted Ly-5$^-$ cells cultured in CM, showing conversion to a highly Ly-5$^+$ phenotype that is heterogenous in nature, with two populations of cells bearing different amounts of Ly-5 discernible. B. Sorted Ly-5$^+$ cells cultured in CM, showing a relatively homogenous population of cells expressing high amounts of Ly-5.

TABLE IV

Increased NK Activity of Sorted C57BL/6 Spleen Cells Cultured 3 Weeks *in vitro* in Conditioned Medium and Inhibition by Anti-Ly-5 mAb

Medium or cell Population	Antibody Treatment	% Specific lysis[a] YAC-1	P815
Spent medium from CM cultured Ly-5$^-$ sorted cells	none	-2.1 ± 2.1	1.6 ± 1.4
Spent medium from CM cultured Ly-5$^+$ sorted cells	none	-1.6 ± 0.9	-0.8 ± 1.5
Sorted Ly-5$^+$ cells	none	85.7 ± 2.5	28.0 ± 2.4
	anti-Ly-5	53.1 ± 2.5*[b]	31.3 ± 2.1
Sorted Ly-5$^-$ cells	none	84.6 ± 5.2	22.7 ± 3.2
	anti-Ly-5	63.0 ± 1.1*	10.9 ± 1.4

[a]Effector to target ratio = 2.

[b]* $p < .001$.

but not eliminated by the addition of anti-Ly-5 mAb in the absence of complement. Some cytotoxic activity was also observed against the usually NK-resistant P815 target. This process was not affected by the addition of anti-Ly-5 mAb to sorted Ly-5$^+$ CM cultured cells, but an inhibitory influence against sorted Ly-5$^-$ CM cultured effectors was suggested.

Discussion

In these studies, we have investigated the hypothesis that functional maturation of natural killer cells is accompanied by increased surface expression of Ly-5 glycoprotein. We also have identified a mechanism by which antiserum directed against Ly-5 glycoprotein inhibits NK-mediated cytolysis of YAC-1 tumor cells. From these data, we conclude that Ly-5$^-$ NK precursors exist in murine splenic tissues and can be induced by conditioned medium to proliferate and differentiate to become potent NK effector cells. As determined by cytofluorometric analysis, these cytotoxic effector cells also express substantially greater amounts of surface Ly-5 glycoprotein than freshly isolated NK cells. In addition, the surface Ly-5 glycoprotein expressed on sorted and CM cultured effectors appears to be important for cellular function, in that specific antiserum diminished cytotoxicity against the NK-susceptible YAC-1 target. The lack of complete abrogation of NK activity was likely due to the polyclonal nature and heterogeneity of the cultured cells, with certain effector subsets highly inhibited by anti-Ly-5 serum while other subsets were unaffected (13). While steric hindrance could be invoked as a nonspecific means of inhibiting NK cell-mediated cytotoxicity, this seems unlikely since Thy-1 glycoprotein is also highly expressed on CM cultured NK effectors (15,16), yet anti-

Thy-1 mAb does not inhibit cytotoxicity (our unpublished observations). It is also unlikely that cytotoxicity was due to a form of nonspecific CTL activity, since lysis was inhibited by anti-Ly-5 serum, which has been shown to specifically inhibit lysis by NK cells but not CTL (7). This is also supported by the finding that CTL-susceptible P815 targets were comparatively resistant to lysis (Table IV).

Previous studies of natural killer cell precursors in humans have generally shown that pre-NK cells bind to target cells as well as NK effectors (17,18), and that interferon augments NK activity without increasing the number of tumor binding cells (TBC; 19,20). Our findings in mice on the importance of Ly-5 glycoprotein in YAC-1 target cell binding and the ability of interferon to induce surface Ly-5 expression among Ly-5$^-$ cells suggest that nonbinding pre-NK cells are also present. Such cells may be difficult to detect in standard assays in which TBC are assessed individually, since Ly-5$^-$ pre-NK cells appear to comprise at most only a few percent of typical NK-enriched splenocyte preparations.

In one other study of pre-NK cells, Minato, et al. (9), used anti-Ly-5 serum and complement to detect and deplete Ly-5$^+$ murine splenocytes. They observed that 30-40% of CBA/J and 7% of BALB/c nude mouse splenocytes were susceptible to lysis by anti-Ly-5 + C, with remaining viable cells having no NK activity. Interferon treatment was associated with a return of NK activity and additional cells susceptible to lysis by anti-Ly-5 + C. From these results, they concluded that Ly-5$^-$ pre-NK cells were present in murine spleen and were stimulated by interferon to convert to Ly-5$^+$ functional NK cells. However, using a second step antibody, Seaman, et al. (7), showed that anti-T200 mAb, which also

specifically binds to the Ly-5 glycoprotein (21), lysed 82% of C57BL/6 splenocytes, suggesting that additional Ly-5^+ cells may have been present in CBA/J and BALB/c nude spleens that resisted complement-mediated lysis. Further evidence in support of this possibility was obtained by Dennert et al. (10), who found that in the absence of a second step antibody, anti-T200 mAb lysed more than 95% of C57BL/6 thymocytes, but only 40% of spleen cells, 20-30% of bone marrow cells and 5-10% of BALB/c nude spleen cells. By analytical cytofluorimetry of nonerythrocytic cells, they found that 78% of splenocytes, 91% of thymocytes and 81% of bone marrow cells were Ly-5^+, confirming that a large percentage of Ly-5^+ cells exist in bone marrow that resist lysis with anti-Ly-5 + C. In addition, using tests of B and T cell function they were able to establish that anti-T200 mAb is selectively cytotoxic for thymocytes and mature T cells. Hence treatment with anti-Ly-5 + C followed by the finding of abrogation of NK activity cannot be taken as presumptive evidence that mature NK cells have been eliminated, since, i) many viable Ly-5^+ cells remain in treated spleen suspensions; ii) Ly-5^+ NK cells resist lysis with anti-Ly-5 + C (Table I) and iii) NK cells treated with anti-Ly-5 are inhibited in their cytotoxic activity without being lysed or removed from the cell suspension. Restoration of NK activity under such conditions by the addition of interferon could result either from recruitment of Ly-5^- pre-NK cells or regeneration of lytic function among Ly-5^+ NK cells.

The use of a cell sorter to obtain Ly-5^- cells and examine their ability to convert to Ly-5^+ NK effectors allows more precise assessment of this process. While some of the effects seen in CM cultured cells could have resulted from proliferation of the few contaminating Ly-5^+

cells in the sorted Ly-5$^-$ preparation; no reliable method exists for the removal of all contaminating cells. This is especially true in view of the resistance of Ly-5$^+$ NK cells to lysis by anti-Ly-5 + C. The probable significance of contaminating Ly-5$^+$ cells is lessened by our observation that interferon treatment induced significant conversion of Ly-5$^-$ cells to a Ly-5$^+$ phenotype without inducing proliferation (Figure 3). The presence of Ly-5$^-$ NK precursors in murine spleen is further supported by the results listed in Table III, which show that the conversion to a Ly-5$^+$ phenotype is accompained by the acquisition of substantial NK activity.

The observation that anti-Ly-5 serum blocks binding of NK cells to YAC-1 targets suggests that the Ly-5 glycoprotein is involved in the binding step of NK mediated cytolysis and is, therefore, a NK cell receptor. It is of current interest in this laboratory to determine whether Ly-5 also participates in subsequent steps of the NK-mediated cytolytic mechanism, namely, programming for lysis and killer cell-independent lysis (22). In a detailed hypothetical model supported by some experimental evidence, Hiserodt et al. (23), characterized the likely properties of the NK cell receptor as, i) binding to the target cell in a Mg^{++} dependent fashion, ii) possessing an external site for Ca^{++} binding, iii) being susceptible to proteolytic cleavage yet retaining its ability to interact functionally with the target cell, and, iv) having the ability to focus secreted NK cytotoxic factors to generate a lytic complex capable of irreversibly damaging the target cell membrane. The identification of Ly-5 glycoprotein as a mediator of target cell binding will allow further testing of this hypothetical model. Targan and Newman (24), provided recent evidence in the human NK

system that the Ly-5/T200 glycoprotein may participate in the early part of the Ca^{++}-dependent programming step of NK cell-mediated cytolysis in that 13.1 mAb, which recognizes an epitope of T200, blocks lysis if added prior to Ca^{++} and does so without altering conjugate formation. A different anti-human T200 monoclonal antibody, F2.5, was recently shown to reduce the capacity of human NK cells to bind to K562 target cells (25), in agreement with our results in the murine system. Together, these data strongly suggest an important and fundamental role for Ly-5/T200 in the natural killer cell system.

Any attempt to ascribe biological significance to the Ly-5 glycoprotein must contend with the fact that its expression is not unique to NK cells. Ly-5 is present on most cells of myelopoietic and lymphopoietic origin (2). However, heterogeneity has been observed in the molecular weight of the glycoprotein among T cells, B cells and monocytes (26,27) despite apparent identity in peptide maps (27). While the molecular weight of Ly-5 expressed on NK cells has not been established, the glycoprotein may be sufficiently different in its glycosylation to give it unique properties.

The clinical implications of the data presented here relate to recent evidence of the importance of NK cells in the immunosurveillance of cancer (reviewed in 28) in graft rejection (29) and in graft-versus-host (GVH) disease (30). Understanding the mechanism of NK receptor interaction with target cells and its precise role in the lytic process should lead to advances in the clinical immunotherapy of certain neoplasms. In addition, the isolation and purification of NK cells or their precursors followed by <u>in vitro</u> CM culture to induce proliferation and functional augmentation may represent an autologous means of

improving host defense mechaniams against cancer (31). The finding of an antibody that blocks NK cell function without altering cell viability may provide an additional strategy for the prevention of graft rejection and GVH disease in transplant recipients.

Acknowledgements:

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PRELIMINARY BIOCHEMICAL CHARACTERIZATION OF SURFACE Ly-5 GLYCOPROTEIN ON MURINE NATURAL KILLER CELLS*

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Running Head: Characterization of NK Cell Ly-5 Glycoprotein

Footnotes:

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[1]Abbreviations: avidin-FITC, fluorescein conjugated avidin; B6, C57BL/6J; BSA, bovine serum albumin; C, complement; °C, degrees centigrade; ConA, concanavalin A; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; IgG, immunoglobulin G; KCIL, killer cell-independent lysis; KD, kilodaltons; LGL, large granular lymphocytes; M, molar; mAb, monoclonal antibody; M_r, relative molecular mass; 2-ME, 2-mercaptoethanol; NK, natural killer; NKCF, natural killer cytotoxic factor; NP-40, Nonidet-P40; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonylfluoride; SAC, Staphylococcus aureus cells; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TEMED, N,N,N',N'-tetramethylethylenediamine.

[2]Zaroukian, M. H., W. J. Esselman and H. C. Miller. 1984. Surface Ly-5 glycoprotein in murine natural killer (NK) cell development, target binding and cytotoxicity. Submitted for publication.

Abstract

We previously reported the ability of monoclonal anti-Ly-5 IgG to block effector cell participation in the target binding step of murine natural killer (NK) cell-mediated cytotoxicity against YAC-1 lymphoma. In order to gain further insight into the physical properties of Ly-5 glycoprotein as a candidate surface molecule for NK cell interaction with target cells, the present study provides a preliminary biochemical characterization of Ly-5 on the plasma membrane of murine NK cell-enriched splenocytes. Nonadherent, T cell-depleted C57BL/6J splenocyte suspensions were enriched for NK cells by discontinuous Percoll density gradient centrifugation. Lactoperoxidase-catalyzed radioiodination of surface proteins on these intact cells followed by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed a relative molecular mass of 205,000 for Ly-5 glycoprotein, with an electrophoretic mobility indistinguishable from that of macrophage-associated Ly-5. While trypsin treatment of viable effector cells did not diminish the amount of immunofluorescently detectable surface Ly-5, SDS-PAGE analysis of the radioiodinated glycoprotein revealed that it had been cleaved to a peptide with an approximate M_r of 125,000 which resisted further trypsinization. The 125 KD fragment remained closely associated with the plasma membrane and could not be released by treatment of viable trypsinized cells with 1 M NaCl, 1% 2-mercaptoethanol or simple sugars known to interfere with NK cell-mediated cytotoxicity, appearing in the soluble phase only after detergent treatment of cells.

Reducing conditions revealed no evidence that the trypsin generated Ly-5 fragment existed as a disulfide-linked heterodimer. The apparent M_r of the immunoprecipitable fragments generated in trypsinized viable cells and NP-40 solubilized membrane proteins were identical, suggesting that the fragment does not contain the intracytoplasmic peptide sequence of the intact Ly-5 glycoprotein. The forces holding the Ly-5 fragment to the plasma membrane after trypsinization remain obscure.

Introduction

Natural killer (NK) cells have been implicated in various aspects of host defense mechanisms against cancer (1), microbial infections (2), in graft rejection (3) and in the regulation of normal hematopoiesis (4). The multiple activities of these cytotoxic effectors has prompted numerous investigations into the mechanism of NK cell-mediated cytolysis (5-8) and molecules important in the lytic process. Such efforts have led to the recognition that NK cell-mediated cytolysis occurs in several steps: i) Mg^{++}-dependent target cell binding, ii) Ca^{++} -dependent programming of the NK effector for target cell lysis, iii) stimulus secretion of NK cytotoxic factor (NKCF), iv) focusing of NKCF on the target cell as a part of the killer cell-independent lysis (KCIL) step, and v) colloid osmotic lysis of the target cell.

Based on experiments examining the effect of proteases and protease inhibitors on NK activity, Hiserodt et al. (8), proposed a model for NK cell-mediated lysis in which the properties of a putative NK receptor

were defined. According the this model, a surface molecule on NK cells that participates in target cell lysis could be expected to have the following characteristics: i) a transmembrane structure, with an external domain and a cytoplasmic component, ii) the ability to mediate binding of the NK cell to its target in a Mg^{++}-dependent manner, iii) a binding site for Ca^{++}, iv) susceptibility to cleavage of the external domain by trypsin-like proteases in a manner that otherwise preserves its structural integrity, allowing continued interaction with the target cell during subsequent stages of the lytic mechanism, v) NKCF binding sites, and vi) the ability to integrate into a membrane attack complex that particpiates in tumor cell lysis.

Attempts to identify candidate surface molecules on NK cells whose expression is important in NK cell-mediated cytotoxicity against tumor cells have generally involved the use of antibodies that inhibit NK activity at the effector cell level. One group of surface molecules identified in this manner as important in NK cell-mediated cytotoxicity in both murine and human NK cell systems is the Ly-5/T200 glycoprotein complex. Several studies have shown that antibodies directed against Ly-5 and T200 glycoproteins block NK activity at the effector cell level, even in the absence of complement (9-11). Ly-5 and T200 have since been shown to be distinct antigenic determinants on identical cell surface proteins (12). While molecular weight heterogeneity has been observed for Ly-5 among T cells, B cells and macrophages (13,14), the

molecular weight form of Ly-5 expressed on NK cells and its relationship to cells of known hematopoietic lineage has yet to be characterized.

Using lactoperoxidase-catalyzed radioiodination, immunoprecipitation and SDS-polyacrylamide gel electrophoretic analysis of cell surface proteins from NK cell-enriched murine splenocyte populations, we report here that NK cell-derived Ly-5 glycoprotein migrates on SDS-PAGE with a relative molecular mass (M_r) of 205,000. When compared to the migration pattern of Ly-5 glycoproteins from a mixture of splenic T cells, B cells and macrophages, NK cell-derived Ly-5 migrates in a manner indistinguishable from that of macrophages. We further report that trypsin treatment of radiolabeled NK cell-enriched suspensions generates a fragment with a M_r of 125,000 that resists further trypsinization and retains the ability to bind anti-Ly-5 mAb. Combined with recent observations that Ly-5 mediates murine NK cell binding to YAC-1 target cells and is expressed in increased amounts during differentiation of pre-NK cells to NK effectors, the results reported here suggest an important and fundamental role for Ly-5 glycoprotein in the natural killer cell system.

Materials and Methods

Cells and cell culture. Six to eight week old female C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) for use in these experiments and served as donors of splenocytes and thymocytes. Respective cell suspensions were aseptically prepared as described pre-

viously (15), in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) containing L-glutamine and supplemented with 15 mM HEPES (Sigma Chemical Co., St. Louis, MO), penicillin-streptomycin (100 u/ml and 100 μg/ml, respectively) and 10% heat-inactivated (56°C x 30 min) fetal bovine serum (Microbiological Associates, Walkersville, MD). In some experiments, splenocytes were propagated by in vitro culture for 1 week in 40% conditioned medium which was generated by 48 hour culture of BALB/c spleen cells (5 x 10^6/ml) with 10 μg/ml concanavalin A (ConA, Sigma) in culture medium as previously described (16).

Splenic NK cell isolation. Splenocyte suspensions were depleted of adherent and phagocytic cells by 45 minute incubation (37°C, 5% CO_2) in Corning 75 cm^2 polystyrene tissue culture flasks (2 x 10^7 cells/flask in 10 ml; Corning Glass Works, Corning, NY). Nonadherent cells were adjusted to 1.2 x 10^7/ml and incubated with an equal volume of monoclonal anti-Thy-1.2 (1:500, New England Nuclear, Boston, MA) and complement (guinea pig serum, 1:5; Gibco) for 60 min at 37°C, 5% CO_2, in order to eliminate T cells. Cells were then adjusted to 1 x 10^8/ml and subjected to discontinuous density gradient centrifugation in Percoll (Sigma), as described previously (17). Briefly, 100% Percoll (nine parts Percoll, one part 10X PBS), was diluted in RPMI-1640 medium without FBS to make final concentrations of 30, 40, 50, 60 and 70% (v/v), and gently layered in order of decreasing density in conical 15 ml plastic tubes. 1 x 10^8 plastic nonadherent, T cell-depleted splenocytes were layered on top and centrifuged at 375 x G for 30 minutes at room

temperature. By this procedure, NK effectors were concentrated in a band at the interface between the 50% and 60% Percoll layers. Cells were collected, washed in medium and kept on ice until use. Enrichment of NK cell activity was verified in a standard 4 hour ^{51}Cr release assay as previously described.[2]

Fluorescent labeling and cytofluorimetric analysis. In order to determine the effect of trypsin treatment on surface Ly-5 expression, NK cell-enriched B6 splenocyte suspensions underwent indirect immunofluorescent labeling as previously described[2]. Briefly, nonphagocytic, nylon wool nonadherent, anti-Thy-1 + C resistant splenocytes were adjusted to 1.2×10^7 cells/ml in PBS and reacted on ice for 30 min with an equal volume of monoclonal anti-Ly-5.1 (1:500 in PBS + 5% BSA; New England Nuclear, Boston, MA). Cells were washed, centrifuged and reacted sequentially on ice with biotinylated anti-mouse IgG and fluoresceinated avidin (avidin-FITC, Vector Laboratories, Burlingame, CA), at dilutions of 1:40 and 1:500, respectively. Some cells were treated only with biotinylated anti-mouse IgG and avidin-FITC to serve as controls for the determination of specific fluorescence. Cells were analyzed cytofluorimetrically in an Orthocytofluorograf 50H cell sorter using narrow angle helium-neon laser scatter and right angle argon laser fluorescence parameters.

Radiolabeling of cell surface proteins. Lactoperoxidase-catalyzed radioiodination of intact viable cells was performed essentially as previously described (18). Briefly, 1×10^8 cells were suspended in 150$_\mu$ l

of PBS to which 100 μl of lactoperoxidase (1 mg/ml in 0.2 M PBS, Sigma Chemical Co.) was added, along with 20 μl of 2 M PO_4 (pH 7.3), 2 mCi carrier-free Na ^{125}I (Amersham Corporation, Arlington Heights, IL, 16.3 mCi/μg iodine), and 20 μl of 0.06% H_2O_2. After 15 min at 30°C, the reaction was terminated by adding 1.5 ml KI-PBS (5 mg/ml KI). Cells were centrifuged at 1000 rpm x 10 min, washed in KI-PBS, repelleted and either resuspended in PBS for trypsin treatment or saved for immediate detergent solubilization of membrane proteins.

Trypsinization of cell surface proteins. Following radioiodination, some cells were adjusted to 2 x 10^7/ml in PBS and subjected to proteolytic treatment with trypsin (1 mg/ml in PBS, 25 μl/10^7 cells; Worthington Biochemical Corp., Freehold, NJ) for up to 20 minutes at room temperature. The reaction was terminated by the addition of Trasylol (250 KIU/10^7 cells; Bayer Werk Elberfeld, Bayer, Germany) and PMSF (0.1 M in acetone, 5 μl/10^7 cells; Sigma). Cells were centrifuged at 1000 rpm x 10 min followed by separation of supernatant and pellet.

Detergent solubilization of radioiodinated cell surface proteins. Radioiodinated pelleted cells were suspended in 1 ml 1% NP-40 (Particle Data Laboratory, Elmhurst, IL) in PBS containing 100 KIU/ml Trasylol protease inhibitor (FBA Pharmaceuticals, New York, NY), 0.02 mg/ml ovomucoid trypsin inhibitor (Sigma), and 0.02% NaN_3. After 30 min on ice, suspensions were placed in 3 ml conical glass centrifuge tubes and centrifuged at 0°C, 10,000 rpm x 20 min. The supernate containing NP-40 solubilized radiolabeled surface proteins was then added to 3 ml Na_2SO_4-

saturated ethanol and kept at -20°C overnight. The cold ethanol precipitate was collected by centrifugation at 13,000 rpm x 20 min and the supernatant discarded. The precipitate was solubilized in 50 µl 1% NP-40, sonicated and centrifuged (Airfuge, Beckman Instruments, Inc., Palo Alto, CA) at 100,000 G x 10 min. A second extraction using an additional 25 µl NP-40 was performed and pooled with the first solubilization to improve yield. Samples were counted in a gamma counter and stored at -20°C for subsequent immunoprecipitation.

Immunoprecipitation of Ly-5 glycoprotein. NP-40 solubilized proteins were added to the pellet from 100 µl of 10% Staphlacoccus aureus cells (Pansorbin, Calbiochem, La Jolla, CA), to remove any proteins capable of binding nonspecifically to SAC and immunoglobulins binding specifically to the protein A component. After 15 min on ice, the suspensions were airfuged at 15 psi x 10 min and the supernatants retained. To each supernatant was added 20 µl of monoclonal anti-Ly-5 (1:100 in PBS with 5% BSA, New England Nuclear, Boston, MA) and the mixture kept on ice for 30 minutes, then added to SAC, mixed and reacted on ice an additional 15 minutes. The antigen-antibody-SAC complexes were collected by centrifugation, washed, and the radioiodinated Ly-5 glycoprotein liberated from the complexes by reaction with 2.3% SDS (Bio-Rad) sample buffer containing 5% 2-mercaptoethanol (Sigma), 0.0625 M Tris-Cl (Sigma) and 10% glycerol for 30 minutes at room temperature. Supernatants were recovered after airfuging at 15 psi x 10 min and boiled at

100°C x 2 min. Radioactivity was determined in a gamma counter and samples saved for SDS-polyacrylamide gel electrophoresis.

Use of simple sugars, salt solutions and disulfide inhibitors. In order to investigate possible mechanisms to explain the observation that Ly-5 glycoprotein moieties remained associated with the cell surface despite proteolytic treatment, intact viable trypsinized cells were reacted with agents known to disrupt cell surface molecular interaction or covalent peptide linkages involving disulfide bonds. Simple sugar inhibitors of NK cell activity were used individually at concentrations of 5 mM and included mannose-6-phosphate, N-acetyl glucosamine, D-mannose and L-fucose. Radioiodinated cells were trypsinized in the presence of one of these sugars and Ly-5 immunoprecipitates from pellet and supernatant compared for their content of Ly-5 glycoprotein or an immunoprecipitable degradation product. Other aliquots of cells were treated with either 1 M NaCl or 0.1% 2-ME after trypsin treatment of cells to determine whether either of these conditions resulted in the release of immunoprecipitable material from the cell surface.

Comparison of the apparent molecular weight of the Ly-5 fragments generated by trypsinization of intact cells and NP-40 solubilized cell surface proteins. To explore the possibility that the Ly-5 fragment generated by trypsin treatment of intact cells contained the trans-membrane and intracytoplasmic domains of the Ly-5 glycoprotein, at least a portion of which has been shown to be sensitive to trypsinization (20), viable radioiodinated 60% Percoll-fractionated splenocytes were

trypsinized as described above either before or after solubilization of membrane proteins in 1% NP-40. Trasylol and PMSF were added to trypsinized intact cells to halt proteolysis before the addition of NP-40. An additional aliquot of cells underwent trypsinization followed by the addition of NP-40 but a delay in the addition of Trasylol and PMSF for an additional 20 minutes. Following cold ethanol precipitation and solubilization in 1% NP-40, Ly-5 immunoprecipitates were collected and analyzed by SDS-PAGE as described below.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Ly-5 immunoprecipitates and whole cell surface radiolabeled protein samples in SDS buffer were electrophoresed on 1.5 mm thick 3-10% polyacrylamide (Bio-Rad) linear gradient gels which contained 0.1% SDS (w/v), 5% glycerol, 25% running gel buffer (1.5 M Tris-Cl, pH 8.8, Sigma), 0.05% ammonium persulfate (Fisher Scientific, Fair Lawn, NJ) and 0.033% TEMED (Aldrich Chemical Co, Milwaukee, WI). A linear gradient generating apparatus (MRA, Clearwater, FL) was employed for this purpose. Gels were allowed to polymerize under a layer of overlay buffer (0.375 M Tris-Cl, pH 8.8, 0.1% SDS), for 4 hours, after which a 2.5% acrylamide stacking gel was applied to the top of the linear gradient gel and allowed to polymerize x 30 min. The stacking gel components differed from the running gel only in percent acrylamide and the substitution of 25% stacking gel buffer (0.5 M Tris-Cl, pH 6.8) for running gel buffer. Tank buffer consisted of 0.025 M Tris-Cl, 0.192 M glycine and 0.1% SDS (pH 8.3). Bromphenyl blue served as the dye front. A Hoefer vertical

slab gel apparatus (Hoefer Scientific Instruments, San Francisco, CA) was used for electrophoresis, which was performed under 40 mA constant current conditions until the dye front reached the distal end of the gel. High molecular weight protein standards (BioRad Laboratories, Richmond, CA) were included in several lanes to allow precise molecular weight comparisons. After electrophoresis, gels were stained overnight in Coomassie blue solution (0.125% Coomassie blue, 50% methanol, 10% acetic acid) followed by sequential destaining (50% methanol, 10% acetic acid and 5% methanol, 7% acetic acid solution, respectively) and drying x 60 min on a Hoefer slab gel dryer.

Autoradiography. Dried gels were placed on clean glass plates and autoradiography performed at -70°C using Kodak XAR-X-ray film with Cronex intensifying screens (DuPont Inc., Wilmington, DE).

Results

Enrichment of NK cell activity by discontinuous density gradient centrifugation. As large granular lymphocytes (LGL) with a relatively large cytoplasmic-to-nuclear volume ratio (19), NK cells can be physically separated from other lymphocytes by centrifugation through a discontinuous density gradient of Percoll. In the present study, gradients were established in 10% increments from 30% to 70%. NK cell activity was found to be highest in those cells concentrated in a band at the interface between 50% and 60% Percoll (Table I). No significant NK activity was observed in the other bands. As a result, cells from

Table I

Enrichment of Natural Killer Cell Activity by Percoll Discontinuous Density Gradient Centrifugation of Plastic-nonadherent, T Cell-depleted Splenocyte Suspensions.

	% Specific lysis[a]	
Percoll fraction[b]	YAC-1	P815
control[c]	4.3 ± 0.7	1.3 ± 0.5
50%	5.9 ± 0.6	7.5 ± 2.3
60%	44.1 ± 1.3*[d]	2.0 ± 1.6
70%	11.5 ± 2.2	2.1 ± 0.6

[a]Values represent mean ± S.D. of triplicate samples. YAC-1 is an NK-sensitive lymphoma of A/Sn mice. P815 is a NK-resistant mastocytoma of DBA/2 mice. Effector:target ratio = 25.

[b]Indicates the percent Percoll layer that prevented further sedimentation of the cell band collected at its upper interface.

[c]Cells tested for NK activity prior to fractionation through Percoll gradients.

[d]* $p < .001$.

the 60% Percoll band were used for biochemical characterization of Ly-5 glycoprotein.

Persistence of immunofluorescently detectable Ly-5 on NK cells after trypsinization. It has been established that antisera to Ly-5 glycoprotein inhibit NK cell-mediated cytotoxicity (9-11). In order to determine whether the loss of NK activity observed following the treatment of NK effectors with trypsin (5) is associated with the loss of immunologically detectable surface Ly-5, NK cell-enriched B6 splenocyte suspensions were trypsinized (1 mg/ml x 20 min), followed by indirect immunofluorescent labeling with murine monoclonal anti-Ly-5, biotinylated anti-mouse IgG and avidin-FITC. Controls consisted of non-trypsinized cells labeled as just described or using only the biotinylated antibody and avidin-FITC. Figure 1A shows the specific Ly-5 immunofluorescence profile of NK-enriched B6 splenocytes before trypsin proteolysis of surface proteins. Trypsin treatment did not decrease the amount of immunofluorescently detectable surface Ly-5 glycoprotein on these effector cells (Figure 1B). The fluorescense was not the result of nonspecific adherence of biotinylated antibody to trypsinized cells in that cells treated only with biotinylated antibody and avidin-FITC were no more fluorescent than unlabeled cells (data not shown).

Molecular weight of Ly-5 glycoprotein on NK cells. SDS-PAGE evidence of molecular weight heterogeneity has been observed for Ly-5 glycoproteins isolated from cells of different hematopoietic lineage

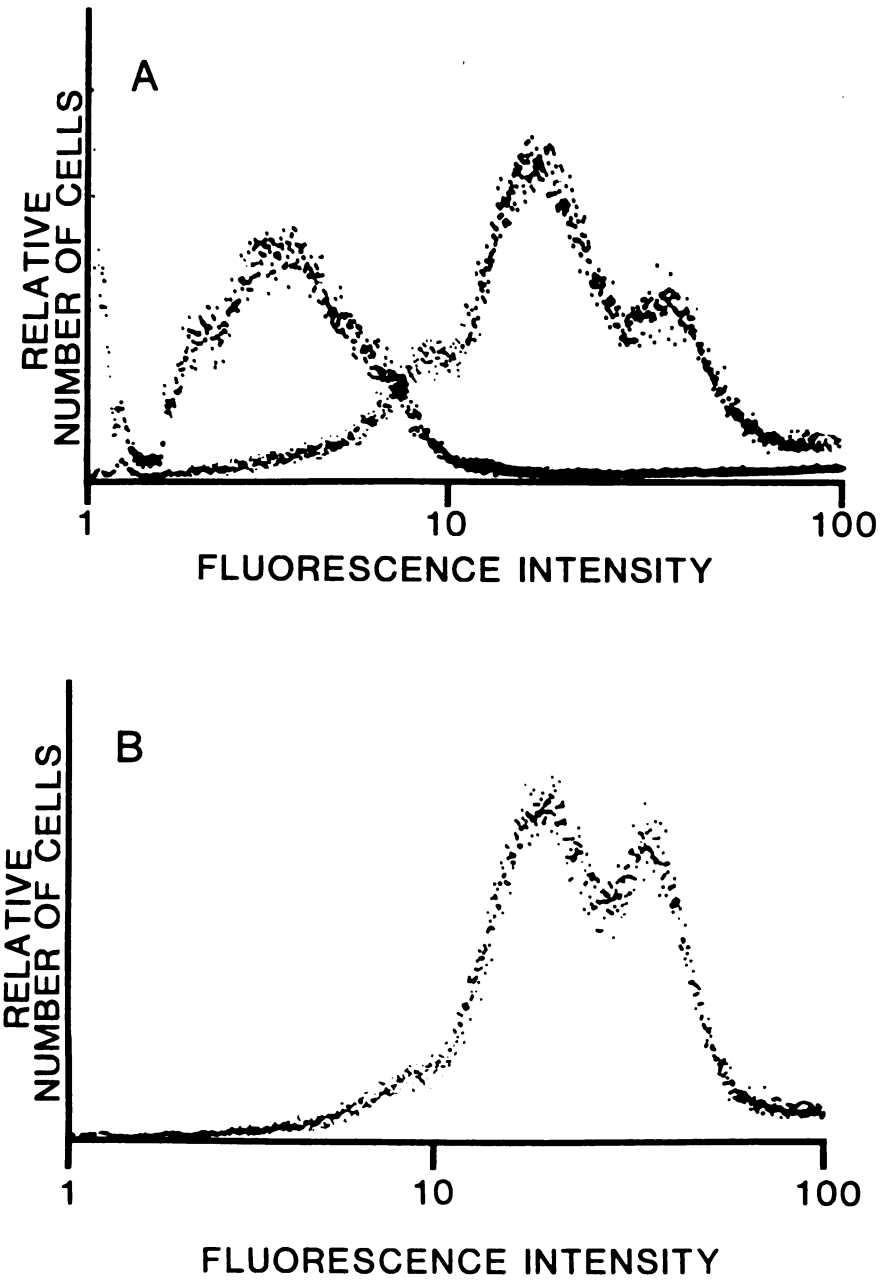

Figure 1. Fluorescence histograms of control and trypsinized NK cell-enriched B6 splenocyte suspensions labeled with murine monoclonal anti-Ly-5, biotinylated anti-mouse IgG and fluoresceinated avidin. Relative cell number is plotted on the ordinate and fluorescence intensity on the abscissa.

A. Specific fluorescence of untrypsinized splenocytes labeled as above in comparison to control cells labeled only with biotinylated anti-mouse IgG and avidin-FITC. 82% of cells labeled with anti-Ly-5 mAb express levels of fluorescence greater than the 99th percentile for fluorescence of control cells. B. Specific Ly-5 immunofluorescence profile of the same cells following tretment with 1 mg/ml trypsin x 20 min at 25°C. No diminution in immunofluorescently detectable surface Ly-5 is observed following trypsinization.

(13,14), resulting in estimates of 185-200 KD for T cells, 205 KD for macrophages and, 220 KD for B cells. The relative rarity of NK cells in murine spleen does not allow molecular weight determination or characterization of Ly-5 on these effectors without the removal of other contaminating cells. In order to determine the apparent molecular weight of NK cell surface Ly-5 glycoprotein and compare it with that of cells of known lineage, macrophage and T cell-depleted 60% Percoll fractionated B6 splenocytes underwent lactoperoxidase-catalyzed radioiodination of surface proteins followed by immunoprecipitation with monoclonal anti-Ly-5 and SDS-PAGE analysis. When compared to an immunoprecipitate of radioiodinated whole B6 splenocytes, Ly-5 glycoprotein derived from the NK cell-enriched Percoll fraction migrated as a broad band with an apparent M_r of approximately 205,000, essentially equivalent to that of macrophages and intermediate between that characteristic of B cells and T cells (Figure 2).

Effect of trypsinization on NK cell surface Ly-5 glycoprotein. Trypsin treatment of intact and detergent solubilized membrane proteins of murine lymphocytes has been shown to result in the generation of a peptide with an approximate M_r of 100,000 that is immunoprecipitable with antisera to the T200 antigenic determinant (20). In that cell sorter analysis of trypsinized NK cell-enriched splenocytes revealed no diminution in the amount of immunofluorescently detectable surface Ly-5 glycoprotein (Figure 1), experiments were directed to determine whether NK cell surface Ly-5 is cleaved into a peptide of similar molecular

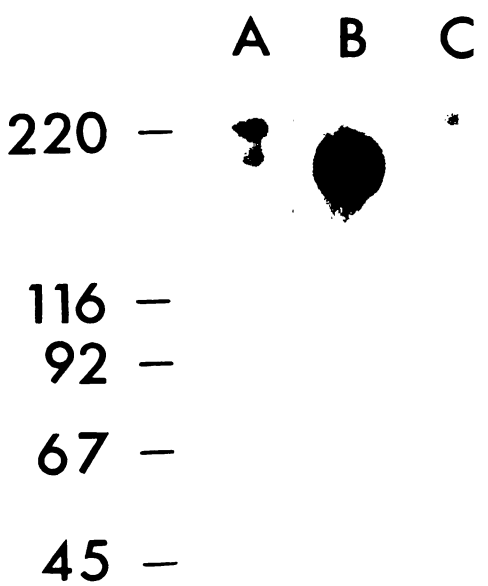

Figure 2. Autoradiographic analysis of SDS-polyacrylamide gel electrophoresis of Ly-5 immunoprecipitates from lactoperoxidase-catalyzed surface radioiodination of unfractionated and NK cell-enriched splenocyte suspensions. Lanes A and C show the labeled species precipitated by anti-Ly-5 mAb treatment of NP-40 solubilized whole spleen suspensions cultured 1 week in vitro in the presence of 40% conditioned medium as described in Materials and Methods. Lane B shows the labeled species precipitated by anti-Ly-5 mAb treatment of NP-40 solubilized 60% Percoll-fractionated B6 splenocytes.

weight and, if so, whether trypsinization releases significant amounts of the peptide from the cell surface into the medium. Percoll-fractionated NK-enriched splenocytes were radioiodinated and trypsinized as described in Materials and Methods. Ly-5 immunoprecipitates of proteins from viable pelleted cells and the trypsin supernatant were generated and electrophoresed on SDS-polyacrylamide gels. Figure 3 shows that trypsinization of these cells generates a fragment of Ly-5 which migrates as a broad band on SDS-PAGE with an approximate M_r of 125,000. Comparison of immunoprecipitates from viable trypsinized cells and the trypsin supernatant indicates that essentially all of the 125 KD Ly-5 fragment remains associated with the plasma membrane. That the Ly-5 glycoprotein is highly susceptible to proteolytic cleavage to a 125 KD fragment (21), is further suggested by the observation that even without deliberate trypsinization, failure to include antiproteases during detergent solubilization of membranes results in the conversion of significant amounts of the Ly-5 glycoprotein to the 125 KD fragments (Figure 3, lane C). Once generated, the 125 KD fragment resists further degradation by trypsin, even with high concentrations and prolonged incubation (Figure 4, lane C).

Inability of simple sugars, high salt or 2-mercaptoethanol to release Ly-5 fragment from trypsinized cells. It was possible that certain covalent or noncovalent interactions were responsible for the persistant association of the 125 KD Ly-5 fragment with the cell surface after trypsinization. The potential mechanisms investigated in the pre-

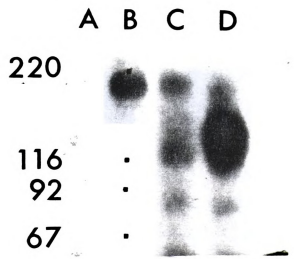

Figure 3. Effect of trypsinization of surface radioiodinated 60% Percoll-fractionated B6 splenocytes. Autoradiograph of SDS-polyacrylamide gel of Ly-5 immunoprecipitates under conditions of deliberate protease inhibitor addition, trypsin treatment or absence of either agent. Lane A shows the Ly-5 immunoprecipitate of the supernatant medium in which intact 60% Percoll-fractionated cells were trypsinized, showing a lack of release of detectable immunoprecipitable Ly-5 from the plasma membrane. Lane B is an Ly-5 immunoprecipitate of 60% Percoll-fractionated splenocytes solubilized in NP-40 in the presence of the protease inhibitors Trasylol and trypsin ovomucoid inhibitor. Lane C represents the immunoprecipitate pattern obtained when radioiodinated cell surface proteins from B6 thymocytes were solubilized in NP-40 without protease inhibitors. Partial digestion to an immunoprecipitable fragment with an approximate molecular weight of 125 KD is apparent. Lane D is from an Ly-5 immunoprecipitate of cell surface proteins on viable 60% Percoll fractionated cells treated with trypsin. The vast majority of the Ly-5/T200 glycoprotein is digested to a fragment with an apparent molecular weight of 125 KD that remains associated with the cell surface.

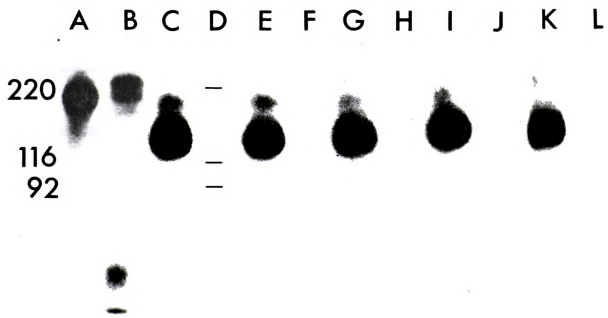

Figure 4. Inability of simple sugars to release Ly-5 fragment from trypsinized cells. Autoradiograph of Ly-5 immunoprecipitates from trypsinized 60% Percoll-fractionated cells treated with various simple sugars at a concentration of 5 mM x 30 min. Immunoprecipitates of treated pelleted cells and the respective supernatants were compared for their relative content of the Ly-5 fragment. Lane A represents immunoprecipitated Ly-5 from radiolabeled 60% Percoll-fractionated cells prior to trypsin treatment. Lane B shows the Ly-5 immunoprecipitate of whole B6 splenocytes cultured 1 week in vitro in 40% conditioned medium. Lanes C through L are Ly-5 immunoprecipitates of radioiodinated 60% Percoll fractionated cells subjected to trypsinization followed by simple sugar addition: C, PBS control, pellet; D, PBS control, supernatant; E, mannose-6-phosphate, pellet; F, mannose-6-phosphate, supernatnant; G, N-acetyl-D-glucosamine, pellet; H, N-acetyl-D-glucosamine, supernatant; I, D-mannose, pellet; J, D-mannose, supernatant; K, L-fucose, pellet; L, L-fucose, supernatant. None of the simple sugars tested released significant amounts of the 125 KD fragment from the cell into the supernatant.

sent study included, i) binding of carbohydrate portions of the glycoprotein to lectin-like receptors on the cell surface, ii) ionic or hydrogen bonding forces disruptible by solutions of high ionic strength, and iii) interchain disulfide bonding of the 125 KD fragment to another portion of the originally intact Ly-5 glycoprotein that anchors the protein to the plasma membrane, i.e., conversion of an intrachain disulfide bond to an interchain one by proteolytic cleavage of the peptide between the cystine residues. To investigate these possibilities, aliquots of Percoll-fractionated trypsinized viable cells were reacted individually with several carbohydrates known to interfere with NK activity (D-mannose, mannose-6-phosphate, L-fucose and N-acetyl-D-glucosamine), 1 M NaCl or 0.1% 2-mercaptoethanol. Cells were centrifuged and immunoprecipitates of pelleted cells and supernatants after treatment compared for relative content of the 125 KD Ly-5 fragment (Figures 4,5). With the possible exception of 2-ME treatment (Figure 5, lane G) no significant release of the 125 KD fragment was seen with any of these treatments. Since the ability of 2-ME to induce release of the fragment from the cell surface even at concentrations up to 1% was only slight and not generally reproducible (data not shown), isolation and SDS-PAGE analysis was performed under reducing and nonreducing conditions to directly examine whether the trypsinized Ly-5 fragment on intact cells exists as a disulfide-linked heterodimer. Under nonreducing conditions (Figure 6, lane B), the Ly-5 fragment displayed slightly greater mobility on SDS-PAGE than when electrophoresis was performed in the

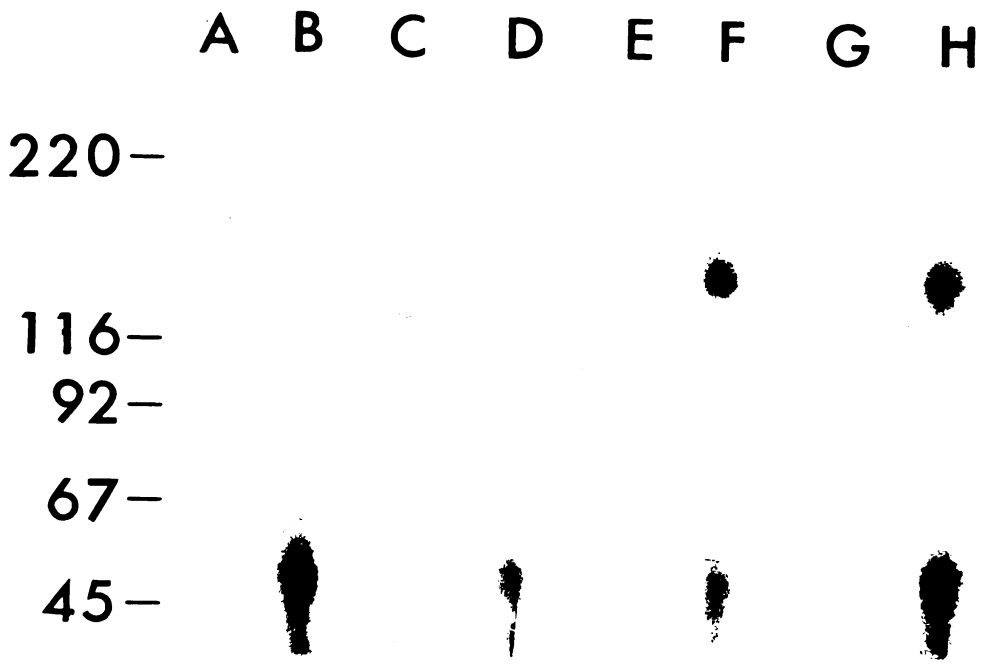

Figure 5. SDS-polyacrylamide gel autoradiograph of Ly-5 immunoprecipitates from trypsinized 60% Percoll-fractionated splenocytes and trypsin supernatants. Trypsinized viable cells were pelleted and resuspended in a solution of 1 M NaCl or in PBS containing 0.1% 2-mercaptoethanol at 25°C x 30 min followed by centrifugation, separation of supernatant and pellet and comparison of the amount of immunoprecipitable Ly-5 glycoprotein from each fraction. Lane A is a control supernatant from trypsinized cells. Lane B is an immunoprecipitate from non-trypsinized cells. Lane C is from pelleted trypsinized cells in which the Ly-5 immunoprecipitate was solubilized in SDS sample buffer lacking 2-ME. Lane D is the same immunoprecipitate as C, except that the precipitate was solubilized in SDS sample buffer containing 2-ME. Lane E is the immunoprecipitable fraction of supernatant from pelleted trypsinized cells suspended in 1 M NaCl, while lane F is the corresponding cell-associated immunoprecipitate after NaCl treatment. Lane G represents solubilized Ly-5 after treatment of pelleted cells with 0.1% 2-ME in PBS, with lane H indicating the immunoprecipitable Ly-5 from the cell pellet after incubation with 2-ME.

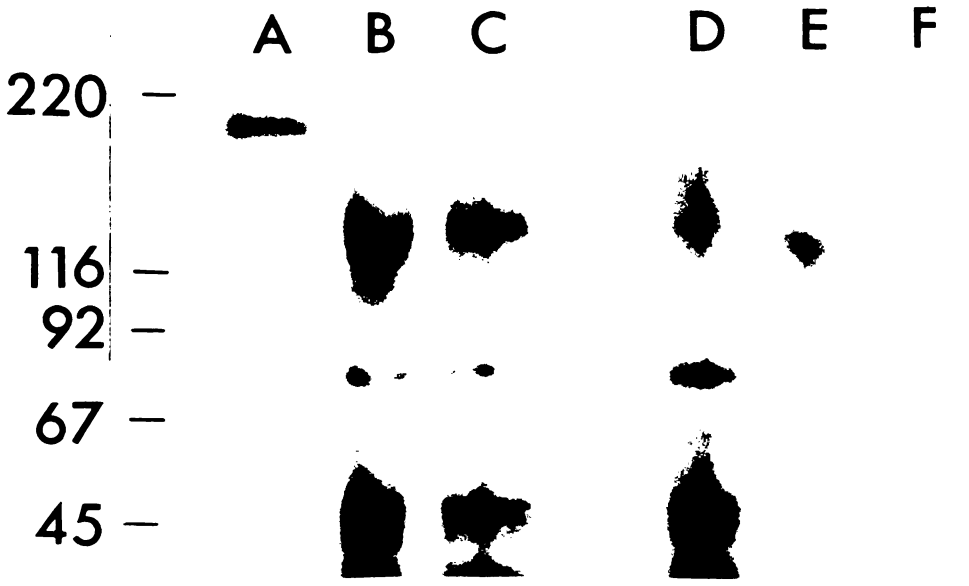

Figure 6. Evidence that the 125 KD Ly-5 fragment does not exist as a disulfide-linked heterodimer. Trypsinized 60% Percoll-fractionated cells were trypsinized and Ly-5 immunoprecipitates solubilized in SDS sample buffer containing or lacking 2-mercaptoethanol. For designated lanes, preparative SDS-polyacrylamide gel electrophoresis was performed on an immunoprecipitate solubilized in SDS sample buffer without 2-ME. The gel was cut into 1 mm slices and the 125 KD fragment identified by counting slices in a gamma counter. Gel slices containing the 125 KD fragment were eluted into SDS sample buffer without 2-ME and divided into aliquots. To one sample was added 2-ME to a final concentration of 5%. Samples were heated to 100°C x 2 min and subjected to SDS-PAGE analysis. Lane A shows a standard Ly-5 immunoprecipitate. Lane B is an Ly-5 immunoprecipitate of trypsinized cells in SDS sample buffer without 2-ME. Lane C is an aliquot of the sample used in lane B to which 2-ME is added to a final concentration of 5% (v/v). Lane D is an immunoprecipitate from trypsinized cells directly solubilized in SDS sample buffer containing 2-ME. Lane E shows the electrophoretic mobility of the eluted 125 KD fragment in SDS sample buffer without 2-ME. Lane F is an aliquot of the same material in Lane E to which 2-ME is added to a final concentration of 5% (v/v).

presence of 2-ME (Figure 6, lane C), indicating that it did not exist as a heterodimer linked by a disulfide bond. This was further confirmed by elution of the 125 KD fragment from the nonreducing gel and repeat SDS-polyacrylamide gel electrophoresis in the presence or absence of 2-ME (Figure 6, lanes E and F).

Trypsinization of Ly-5 from intact cells or NP-40 solubilized membrane proteins yields fragments of identical apparent molecular mass. It has been established that Ly-5 possesses an intracytoplasmic domain which can be labeled by $^{32}PO_4$ and is extensively degraded by trypsin (20). It was of interest to determine whether the somewhat higher relative molecular mass and persistent membrane association of the Ly-5 fragment observed in this study was due to the inclusion of the intramembranous and cytoplasmic domains of the glycoprotein in fragments generated by trypsinization of intact cells. If true, the enzymatic digestion of these domains would be expected to occur only in NP-40 solubilized cells and result in a lower apparent M_r for the Ly-5 fragment. However, SDS-PAGE analysis of Ly-5 immunoprecipitates from intact cells and NP-40 solubilized membrane proteins subjected to trypsinization (Figure 7, lanes B and C, respectively), revealed that the Ly-5 fragments generated had apparently identical M_r, suggesting that trypsin generation of the fragment occurs entirely in the external domain.

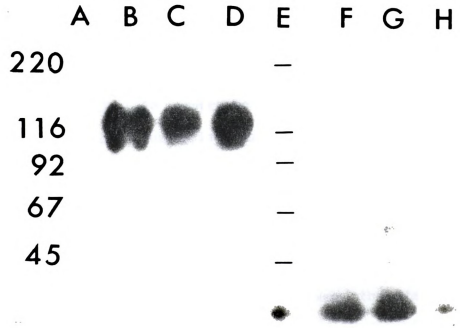

Figure 7. Apparent molecular weight identity of the 125 KD Ly-5 fragment generated by trypsinization of intact cells and NP-40 solubilized cell surface proteins. Surface radioiodinated, 60% Percoll-fractionated splenocytes were trypsinized before and/or after solubilization in 1% NP-40 as described below. Ly-5 immunoprecipitates and immunoprecipitable SAC supernatants underwent SDS-polyacrylamide gel electrophoresis and autoradiography. Lane A is a standard Ly-5 immunoprecipitate. Lane B represents the Ly-5 fragment from cell surface proteins solubilized in NP-40 prior to trypsinization. Lane C is the Ly-5 fragment generated by trypsin treatment of intact cells followed by the addition of Trasylol and PMSF before NP-40 solubilization. Lane D shows the Ly-5 fragment from intact cells treated with trypsin which were then solubilized in NP-40 with a delay in the addition of trypsin inhibitors for an additional 20 min. Lanes E, F, G and H are the nonimmunoprecipitable SAC supernatants from the starting material used in immunoprecipitation of samples used in Lanes A, B, C and D, respectively.

Discussion

In a previous study[2], we employed specific antiserum and conjugate assays to show that Ly-5 glycoprotein is a mediator of murine natural killer cell binding to YAC-1 target cells. Using fluorescence-activated cell sorting, we further demonstrated that within murine splenic tissues are Ly-5 pre-NK cells which can be induced by interferon and interleukin containing preparations to convert to $Ly\text{-}5^+$ NK effectors. The acquired NK activity remained susceptible to inhibition by anti-Ly-5 mAb, suggesting an important role for Ly-5/T200 glycoprotein in the natural killer cell cytotoxic mechanism against YAC-1 lymphoma targets. This is further supported by studies in the human natural killer cell system, where an antibody (13.1) to the human homologue of murine Ly-5/T200 was shown to block the NK lytic mechanism between the stages of target binding and Ca^{++}-dependent programming (22). Thus, separate determinants on a single glycoprotein have been implicated in successive stages of NK cell-mediated lysis, suggesting that Ly-5/T200 glycoprotein acts as a NK cell receptor mediating the lytic reaction sequence.

The experiments reported herein revealed several biochemical features of the Ly-5 glycoprotein isolated from murine splenocyte suspensions enriched in NK cells and allowed comparison with available data from other studies regarding its disposition on the plasma membrane of other lymphocytes. A determination of the consistency of these biochemical properties with a current model of the mechanism of NK cell-mediated lysis (8) was also possible.

Based on SDS-PAGE analysis, an apparent approximate relative molecular mass of 205,000 was assigned to NK cell-associated Ly-5 glycoprotein. Its mobility when compared to whole splenocytes (Figure 2), and apparent M_r were indistinguishable from that of Ly-5 glycoprotein isolated from macrophages (14). However, on some gels (Figure 3) the glycoprotein appeared to have a mobility similar to that of thymocytes. With its high degree of glycosylation (23), the broad bands observed on SDS-PAGE and the relatively small differences observed between macrophages and T cells (14), an unequivocal association of murine NK cell surface Ly-5 glycoprotein with a particular cell lineage is not possible. Such uncertainty is consistent with the results of previous studies in which NK cells were shown to have cell surface phenotypes and cellular functions resembling both macrophages and T cells (24-28).

The observation that trypsin treatment of intact cells results in a immunoprecipitable fragment of Ly-5 with approximate M_r of 125,000 that resists further trypsinization is similar to the results of Trowbridge et al. (21), who identified a fragment of T200 on thymocytes with an apparent M_r of 100,000. Omary and Trowbridge (20), further characterized T200 from NP-40 solubilized membrane extracts of the murine lymphoma cell line BW5147 by surface radioiodination, metabolic labeling with $^{32}PO_4$, ^{3}H-mannose or ^{35}S-methionine and analysis of the effect of proteolysis on the glycoprotein. In surface radioiodinated cells, approximately 80% of the original T200 associated cpm was found in the 100 KD fragment generated by trypsin treatment. The fragment

also possessed most of the mannose-containing oligosaccharide portions of the glycoprotein. Analysis of isolated membrane preparations labeled by lactoperoxidase-catalyzed iodination revealed that the remainder of the molecule included a cytoplasmic domain which was extensively degraded by trypsin and contained phosphoserine residues that in viable cells could be labeled metabolically with $^{32}PO_4$. Among the conclusions and implications of the data was that Ly-5/T200 had an external domain containing most of the mannose-containing oligosaccharides and all of the amino acid residues labeled by radioiodination of viable cells, suggesting that the 100 KD fragment comprised most or all of the external domain of T200 and that the trypsin-sensitive peptide sequence was near the site of insertion of the glycoprotein into the plasma membrane. In the absence of additional associations with the membrane or other membrane-bound molecules, the 100 KD fragment would be expected to become detached from the surface of trypsinized viable cells and appear in the surrounding medium. Our experimental observations that the 125 KD Ly-5 fragment remained firmly bound to the plasma membrane after trypsinization of viable cells until solubilized by detergent suggested that the 125 KD fragment may contain the intramembranous and cytoplasmic portions of the glycoprotein. Additionally, the data suggested that the trypsin-sensitive site on the external domain of Ly-5/T200 may reside closer to the distal end of the molecule than the membrane insertion site. This would be expected to create an additional peptide fragment of Ly-5 with an expected M_r of approximately 75,000 but

which may not be detectable in these experiments due to further extensive degradation by trypsin, relative lack of amino acid residues susceptible or accessible to labeling by lactoperoxidase-catalyzed iodination or absence of antigenic determinants for Ly-5 or T200 antisera necessary for immunoprecipitation and isolation of the peptide. This possibility was supported by a recent study in humans (29), in which a monoclonal antibody (9.1C3) that blocks NK cell activity was shown to precipitate 66 KD and 77 KD proteins associated with the T200 glycoprotein complex. Whether these peptides represent proteolytic degradation products of Ly-5/T200 has not been established. In some of the gels included in the current study (Figures 3, 6 and 7) a band was visualized at an apparent M_r of 85,000 KD but was considered to be a contaminating molecule, in that it was present in great abundance in nonimmunoprecipitates of both trypsinized and nontrypsinized samples, was not concentrated in Ly-5 immunoprecipitates or substantially depleted from samples in which Ly-5 had been removed by immunoprecipitation (data not shown). The possibility that the 85 KD band represented a portion of the external domain of the glycoprotein which lacks a Ly-5 antigenic determinant could not be excluded.

The 125,000 M_r of the trypsinized Ly-5 fragments observed in the present experiments was somewhat greater than the 100,000 estimate reported by others (20,21). While procedural differences and broad banding pattern of the glycoprotein may have accounted for the observed differences, it was possible that trypsinization of Ly-5 on intact cells

generated a larger peptide containing transmembrane and intracytoplasmic amino acid sequences not present in the 100,000 fragment generated by trypsinization of NP-40 solubilized Ly-5. The demonstrated susceptibility of the cytoplasmic portion of Ly-5/T200 to extensive degradation by trypsin (20) is in support of this postulate. However, direct molecular weight comparison of the immunoprecipitable Ly-5 fragments generated by trypsin treatment of intact versus NP-40 solubilized cells and repeat trypsinization of the NP-40 solubilized fragment generated from intact cells failed to provide evidence for this, although the high degree of glycosylation and potentially small amount of intracytoplasmic peptide cleaved may have obscured evidence of differences. Repeat analysis after cytoplasmic domain labeling with $^{32}PO_4$ should more definitively answer this question and is currently under investigation.

Recent studies have focused on Ly-5 as a possible NK cell receptor participating in the mechanism leading to tumor cell lysis. Antisera reacting with different antigenic determinants associated with the glycoprotein have been shown to block target cell binding[2], events linking target binding to Ca^{++}-dependent programming (22) and post-activation stages of NK cell-mediated lysis (29,30). Ly-5 has also been shown to possess a transmembrane structure with a intracytoplasmic component (20), which has been postulated as a likely characteristic of an NK cell receptor (8). We have observed here that Ly-5 glycoprotein from NK-enriched splenocyte suspensions is highly susceptible to trypsinization, generating a fragment that resists further proteolytic

degradation and retains structural integrity sufficient to bind anti-Ly-5 mAb. While our previous data[2] suggest that the Ly-5 determinant is important in NK cell binding to a lymphoma target, it appears unlikely that the Ly-5 fragment is directly involved in the killer cell-independent lysis step of NK cell-mediated cytotoxicity in that it is not released from the plasma membrane by trypsinization. Although the ability of membrane-bound Ly-5 fragment to mediate continued binding to target cells has not been established, it is likely that any in vivo target cell binding mediated by the membrane-bound fragment would be disrupted by endogenous proteases responsible for its generation. Such proteolysis would be expected to lead to cleavage of receptors on the target cell, which has been shown to block their susceptibility to binding and lysis by NK effectors (5,7,8). The possibility that proteolysis of Ly-5 glycoprotein or a closely associated molecule liberates a peptide from the cell surface capable of participating in the lethal hit stage of NK cell-mediated lysis is suggested by recent reports (29,30), but requires further investigation.

In attributing unique biological properties to NK cell-associated Ly-5 glycoprotein, one must address the fact that the expression of Ly-5 is not unique to these effector cells. The molecular weight heterogeneity observed for Ly-5/T200 (13,14,31) suggests structural variations among cells of different hematopoietic lineage that may alter the function of the glycoprotein. An example of documented differences in the structure of the glycoprotein was provided by Coffman and Weisman

(32), who developed a monoclonal antibody that reacted specifically with the 220,000 dalton form of T200 that is present on B cells, which they designated B220. Other studies have provided additional evidence of differences between T and B cells in the protein structure of Ly-5 and its intracytoplasmic precursors (33,34). Variation in the oligosaccharide components of Ly-5 among different lymphocyte populations as a contributing factor for Ly-5 heterogeneity awaits forther study.

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SUMMARY AND CONCLUSIONS

In these studies, inquiry was made into fundamental aspects of murine natural killer cell development and function. Using specific antiserum to inhibit NK cell function, Ly-5/T200 glycoprotein was identified as a candidate cell surface molecule important in NK cell-mediated cytotoxicity. Monoclonal anti-Ly-5 was used to probe the stage in the lytic mechanism in which the Ly-5 determinant participates. The results of target cell binding assays indicated that the Ly-5 determinant or a closely related structure is involved in the Mg++-dependent binding stage of the NK cell lytic mechanism against YAC-1 lymphoma target cells. The importance of Ly-5 to NK cell function was further supported by the finding that cell sorter purified splenocytes lacking significant amounts of surface Ly-5 glycoprotein were without NK activity. One possible mechanism by which the endogenous immunomodulators interferon and interleukin-2 augment natural killer cell activity was identified, in that medium containing these agents was observed to increase the surface Ly-5 expression and NK cell activity of sorted Ly-5- cells. Prolonged *in vitro* culture in medium containing IFN and IL-2 was

associated with at least a tenfold increase in surface Ly-5 expression and potent NK cell activity that remained significantly inhibitable by anti-Ly-5 mAb.

Once evidence for the importance of Ly-5 in murine NK cell development, target binding and cytotoxicity was established, it was of interest to gain insight into the biochemistry of the glycoprotein on NK cells to allow comparison with other hematopoietic cells. The relative molecular mass of the glycoprotein obtained from NK cell-enriched spleen cell suspensions was 205,000 and was indistinguishable from the Mr obtained for macrophages. The results from proteolysis experiments indicated that trypsinization of intact cells did not alter the amount of immunofluorescently detectable Ly-5 on the plasma membrane. Immunoprecipitation and SDS-polyacryalamide gel electrophoretic analysis of surface radioiodinated cells revealed that the glycoprotein was cleaved to a 125 KD fragment that remained bound to the plasma membrane and was not liberated into the soluble phase by treatment with simple sugars, high salt or disulfide inhibitors. The fragment was resistant to further degradation by trypsin and retained its ability to bind anti-Ly-5 mAb. At least some of these properties are consistant with current models of the properties of a putative NK cell receptor.

Taken together, the findings described in this dissertation, along with the results of others in the human and murine natual killer cell systems, suggest an

important and fundamental role for Ly-5/T200 in the NK cell-mediated cytotoxic mechanism against tumor cells.