ANALYSIS OF THE STRUCTURE AND THE MODULATION OF A SPECIFIC ALLOANTIGEN OF MURINE NATURAL KILLER CELLS

by

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ABSTRACT

Natural killer (NK) cells can kill tumor and virally infected cells in a manner that is only beginning to be characterized at the molecular level. Results reported in the last few years support the concept that NK cells may display two types of receptors; one triggering NK cell lysis upon recognition of target cell carbohydrate structures, and one inhibiting cytotoxicity when engaged by particular target cell MHC class I molecules. Our laboratory produced a mAb (4LO3311) identifying a murine NK cell surface antigen, and provided evidence that this antigen is an activating receptor. In this work, we established that, in fact, mAb 4LO3311 recognizes the NK2.1 antigen, formerly defined by an NZB anti-BALB/c antiserum. We characterized NK2.1 as a probably transmembrane disulfide-linked dimer of highly glycosylated 65 kDa subunits. Cell and tissue distribution of NK2.1 is consistent with this antigen being NK cell specific. Contrary to what we had previously observed, all splenic NK cells are NK2.1$^+$, although a subpopulation expresses only a very low level of NK2.1, undetectable with the flow cytometer we used in earlier experiments. Interleukin-2, which is known to increase NK cell activity, up-regulated NK2.1 expression, suggesting a possible relationship between increased NK2.1 expression and increased NK cell activity. On the other hand, NK2.1 expression appeared to be down-regulated by some host MHC molecules, possibly through interaction with NK2.1. Our results suggest that NK2.1 interacts principally with H-2K^b and probably H-2D^b and D^k haplotypes. Finally, although the gene encoding NK2.1 has not yet been cloned, we have determined that it maps to chromosome 6, in association with the NKR-P1 and Ly-49 gene families in the NK gene complex.

RÉSUMÉ

Les cellules NK ont la capacité de détruire des cellules tumorales ou infectées de virus par un procédé dont les bases moléculaires sont à peine caractérisées. Les résultats publiés au cours des dernières années suggèrent que les cellules NK exprimeraient deux types de récepteurs; un déclenchant l'activité lytique des cellules NK suite à la reconnaissance d'une structure glucidique sur la cellule cible, et un inhibant cette activité lorsqu'il se lie à certaines molécules de classe I du CMH, présents sur la cellule cible. Notre laboratoire a produit un AcM (4LO3311) définissant un antigène de surface des cellules NK murines et a démontré qu'il était possible d'augmenter l'activité lytique des cellules NK via cet antigène. Dans ce travail, nous avons établi que l'AcM 4LO3311 reconnaît en fait l'antigène NK2.1, originalement identifié par un sérum NZB anti-BALB/c. Cet antigène est constitué de deux sous-unités de protéines de 65 kDa, hautement glycosylées, probablement transmembranaires, et reliées par un ou des pont(s) disulfures. La distribution cellulaire et tissulaire du NK2.1 supporte l'hypothèse voulant que cet antigène soit spécifique aux cellules NK. Contrairement à ce que nous avions précédemment observé, toutes les cellules NK spléniques expriment le NK2.1, mais une fraction de ces cellules n'exprime qu'un très faible taux de NK2.1, non détectable avec le cytofluoromètre utilisé lors de nos expérimentations antérieures. L'interleukine-2, connue pour sa capacité à accroître l'activité lytique des cellules NK, augmente aussi l'expression du NK2.1, suggérant un lien possible entre l'expression accrue de cet antigène et l'augmentation de l'activité NK. D'autre part, l'expression du NK2.1 semble modulée à la baisse par la présence de certaines molécules de classe I du CMH pouvant

potentiellement interagir avec le NK2.1. Nos résultats nous portent à croire que le NK2.1 interagirait plus particulièrement avec l'haplotype H-2K^b et probablement aussi avec les haplotypes H-2D^b et H-2D^k. Le gène codant pour le NK2.1 n'a pas encore été cloné, mais il a été localisé sur le chromosome 6, en association avec les familles de gène NKR-P1 et Ly-49, dans le complexe NK.

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LIST OF ABBREVIATIONS

A-LAK	:	Adherent-LAK
ADCC	:	Antibody-dependent cellular cytotoxicity
CD	:	Cluster of differentiation
CRD	:	Carbohydrate recognition domain
FcR	:	Receptor for the Fc portion of immunoglobulin
FITC	:	Fluorescein isothiocyanate
GAMIG	:	Goat anti-mouse immunoglobulin
GPI	:	Glycosyl-phosphatidylinositol
GVHD	:	Graft-versus-host disease
H-2	:	Histocompatibility-2
Hh-1	:	Hemopoietic histocompatibility-1
HLA	:	Human leukocyte antigen
hNKR-P1A	:	Human NKR-P1A
ICAM	:	Intercellular adhesion molecule
IFN	:	Interferon (e.g., IFN-γ)
Ig	:	Immunoglobulin
IL	:	Interleukin (e.g., IL-2)
kDa	:	Kilo dalton
LAK	:	Lymphokine-activated killer
LGL	:	Large granular lymphocyte
mAb(s)	:	Monoclonal antibody(ies)
MFI	:	Mean fluorescence intensity
MHC	:	Major histocompatibility complex
musNKR-P1	:	Mouse NKR-P1
NCAM	:	Neural cell adhesion molecule

NCC	:	Nonspecific cytotoxic cell
NK	:	Natural killer
NKAT	:	Natural killer-associated transcript
NKCF	:	Natural killer cytotoxic factor
NKR	:	Natural killer receptor
NWNA	:	Nylon wool nonadherent
PAGE	:	Polyacrylamide gel electrophoresis
Pgp-1	:	Phagocyte glycoprotein-1
PI-PLC	:	Phosphatidylinositol-specific phospholipase C
PMN	:	Polymorphonuclear (leukocyte)
Poly I:C	:	Polycytidylic acid-polyinosinic acid
Prp	:	Proline-rich protein
RAG	:	Recombination activating gene
RFLP	:	Restriction length polymorphism
RI	:	Recombinant inbred
rNKR-P1	:	Rat NKR-P1
SA-PE	:	Streptavidin-conjugated phycoerythrine
SCID	:	Severe combined immunodeficient
SDS	:	Sodium dodecyl sulfate
TAP	:	Transporter for antigen presentation
TCR	:	T cell receptor
TGF	:	Transforming growth factor
TNF	:	Tumor necrosis factor

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PROLOGUE

This thesis describes the analysis of the structure and modulation of the murine NK2.1 alloantigen, an NK cell activation receptor. I have chosen the option provided in Section 7 of the Guidelines Concerning Thesis Preparation of the Faculty of Graduate Studies and Research of McGill University, which reads as follows:

> "Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly-duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis.
>
> If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory.** The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.
>
> The thesis must still conform to all other requirements of the "Guidelines for Thesis Prepration". **The thesis must include:** A Table of Contents, an abstract in English and in French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. **Under no circumstances can a co-author of any component of such a thesis serve as a examiner for that thesis."**

This provision allows me to include the texts of three manuscripts (chapters 2, 3, and 4) concerning the thesis project. In addition to Dr. Suzanne Lemieux, who supervised all the steps of this work, Mrs. Yvette Lusignan, a senior technician in our laboratory, is the only co-author of two manuscripts (chapter 2 and 4). Mrs. Lusignan produced the NZB anti-BALB/c antiserum (chapter 2), determined the phenotype of (129 x C57BL/6)F_1 x 129 backcross progeny, and helped in the analysis of NK2.1 expression on NK cells from some congenic and congenic recombinant mice (chapter 4).

CHAPTER 1

Introduction

Review of the literature

1.1 - INTRODUCTION

The history of natural killer (NK) cells really began in the mid 70's when two different research groups observed naturally occurring cell-mediated cytotoxicity in lymphoid cell suspensions from a variety of normal inbred mouse strains (Herberman *et al.*, 1975a; Kiessling *et al.*, 1975a). A few years before, it had been reported that blood cells from many normal human adults had cytotoxic reactivity against acute leukemia blast cells, but this reactivity was considered an artifactual phenomenon (Rosenberg *et al.*, 1972). The cells responsible for this spontaneous cytotoxicity, also called killer activity, were not identified but appeared unlikely to be T or B cells since very high reactivity was seen in athymic nude mice (Kiessling *et al.*, 1975b) and in mice deprived of B cells by neonatal treatment with anti-IgM antiserum (Gidlund *et al.*, 1979). Moreover, none of the conventional markers T or B mature lymphocytes were found on the killer cells (Herberman *et al.*, 1975b; Kiessling *et al.*, 1975b). The term natural killer or NK was then proposed (Kiessling *et al.*, 1976) to define the cells responsible for the spontaneous killing of a number of target cells without prior sensitization and restriction by antigens of the major histocompatibility complex (MHC).

Ten years later, the definition of NK cells still remained evasive because cells involved in natural killing were heterogeneous. At the Fifth International Workshop on NK cells in 1988, a general consensus was obtained and NK cells were defined as follows:

> "NK cells are CD3$^-$ T cell receptor (α, β, γ, δ)$^-$ large granular lymphocytes (LGL). They commonly express certain cell surface markers such as CD16 and CD56 in humans and NK1.1/NK2.1 in mice. They mediate cytolytic reactions that do not require expression of class I or class II MHC molecules on the target cells."[1]

In 1995, the biology of NK cells is much better understood due notably to the production, over the years, of monoclonal antibodies (mAbs) reacting with NK cell specific antigens. Hence, the accuracy of the 1988 definition of NK cells is now questionable. Indeed, a number of cell surface receptors almost exclusively expressed by NK cells and involved in positive or negative regulation of the NK cell responsiveness have been identified and a role for MHC class I molecules in NK cell killing has been described. Nevertheless, the structure(s) responsible for the recognition of target cells still remain to be elucidated. The subject of this thesis is the characterization of the structure and the regulation of the NK2.1 alloantigen, one of the few murine NK cell activation receptors that have been identified thus far. Before describing the work that has been done, I will present an overview of NK cell biology with a comprehensive description of the cell surface antigens involved in NK cell functions. Most of the discussion will concern murine NK cells with references to the human and rat systems only when appropriate.

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[1]Fitzgerald-Bocarsly *et al.*, 1988.

1.2 - ONTOGENY AND DISTRIBUTION OF NK CELLS

Whereas commitment to the B or T cell lineage is based on the rearrangement of immunoglobulin (Ig) or T cell receptor (TCR) genes respectively, the exact NK cell lineage still remains obscure. The picture is also complicated by the fact that NK cells share a number of phenotypic and functional characteristics with T cells suggesting a possible common origin for these cell types. Nevertheless, NK cells undoubtedly represent a discrete leukocyte subpopulation possibly constituting a third lineage of lymphoid cells. In this section, I will summarize the actual knowledge on the origin, differentiation and distribution of NK cells and their relationship to the T cell lineage.

1.2.1 - Origin and differentiation of NK cells

Natural killer cells are bone marrow-derived lymphocytes that are also dependent on an intact bone marrow environment for their differentiation. The first suggestion of a bone marrow origin for NK cells came from the demonstration that lethally irradiated mice, reconstituted with bone marrow from donors showing high or low splenic NK cell activity, expressed the splenic NK cell activity of the donor strain (Haller *et al.*, 1977). Moreover, the necessity for functional bone marrow in NK cell development has been demonstrated by experiments with ^{89}Sr-treated mice (Haller and Wigzell, 1977; Kumar *et al.*, 1979; Levy *et al.*, 1981). Although other lymphoid and myeloid cell populations were normally reconstituted in mice treated with ^{89}Sr, a bone-seeking isotope, NK cell activity never recovered. In another system, mice whose bone marrow had been

destroyed by chronic exposure to 17-β-estradiol had apparently normal numbers of splenic NK cells but very low NK activity (Seaman *et al.*, 1979; Hackett *et al.*, 1985). Taken together, these results suggest that an intact bone marrow environment is necessary for the functional maturation of NK cells.

Various cytokines are involved in the development of NK cells from bone marrow precursors, the central one being interleukin-2 (IL-2). Using *in vitro* systems, it has been demonstrated that IL-2 can support the development of functional NK cells from the bone marrow of normal mice (Kalland, 1986a; Koo and Manyak, 1986). Mature NK cells were also generated in IL-2-stimulated cultures of bone marrow cells from mice pretreated with 5-fluorouracil (5-FU), which eliminates the more mature cells while sparing less differentiated precursors (Migliorati *et al.*, 1987a). In addition to IL-2, other cytokines are implicated in the differentiation of NK cells but interestingly, most of them appear to modulate the effect of IL-2 rather than having a direct effect. It is the case for IL-1α and β, tumor necrosis factor α (TNF-α), lymphotoxin or TNF-β, and interferon γ (IFN-γ) which all potentiate the IL-2-dependent *in vitro* differentiation of NK cells from 5-FU resistant bone marrow progenitors (Migliorati *et al.*, 1987a; 1987b; 1988; 1989). The synergistic effect of IL-1α would be due, at least partly, to increased TNF-α and IFN-γ production by undefined accessory cells (Delfino *et al.*, 1991). More recently, it was reported that IL-7, in combination with IL-2 and stem cell factor, could also support differentiation of pluripotent $CD34^+$ human bone marrow progenitors into $CD56^+$ NK cells (Silva *et al.*, 1994). IL-12, which was originally described as a natural killer stimulatory factor (Kobayashi *et*

al., 1989) or cytotoxic lymphocyte maturation factor (Stern *et al.*, 1990), is produced by B cells and macrophages [reviewed in (Brunda, 1994)]. Although this lymphokine does not apparently act on NK cell development, IL-12 is of major importance for NK cell activation. Indeed, IL-12 can enhance NK cell-mediated cytotoxicity and induce IFN-γ production by NK cells (Kobayashi *et al.*, 1989; Wolf *et al.*, 1991; Perussia *et al.*, 1992; Gately *et al.*, 1994). Interestingly, IL-12 can, like IL-2, directly induce the proliferation of NK cells *in vitro* but inhibits the proliferation of NK cells induced by high doses of IL-2 (Perussia *et al.*, 1992).

Some cytokines, such as IL-4, were shown to inhibit or stimulate IL-2-dependent *in vitro* generation of NK cells from bone marrow precursors, depending on the concentration of IL-2 (Migliorati *et al.*, 1991; Ballas and Rasmussen, 1993). Whether a similar situation exists *in vivo* has not been demonstrated. IL-3 also has a dual effect as it can significantly inhibit the *in vivo* and *in vitro* generation of NK cells from bone marrow precursors (Kalland, 1986b), but synergizes with IL-2 to promote the proliferation of $CD3^+$ LGL derived from the bone marrow (Minato *et al.*, 1988). On the other hand, transforming growth factor-β (TGF-β) and granulocyte-macrophage colony-stimulating factor (GM-CSF) significantly inhibit the IL-2-dependent *in vitro* development of NK cells (Migliorati *et al.*, 1989).

It is now known that the third chain of the IL-2R, the γ chain, is also a component of the IL-4, IL-7, IL-13, and IL-15 receptors (Kondo *et al*, 1993; Russell *et al.*, 1993; Noguchi *et al.*, 1993; Zurawski *et al.*, 1993; Giri *et al.*, 1994). This common γ chain, apparently plays a very important

role in the development of lymphoid cells since mice with a targeted deletion of the γ chain have substantially reduced numbers of T and B cells and no detectable NK cells (DiSanto *et al.*, 1995).

Cytokines thus play a key role in the *in situ* differentiation of NK cells within the bone marrow and it is likely that this cell compartment contains all the required factors to sustain normal NK cell development. Supporting this hypothesis, the generation of lymphoid cells with phenotypic and functional characteristics of mature NK cells has been reported in murine long-term bone marrow cultures, in the absence of exogenous growth factors (Pollack *et al.*, 1992). Under those conditions however, the maturation of NK cell precursors appears to require direct contact with bone marrow stroma since NK1.1$^-$Thy-1$^-$ bone marrow cells generated lytic NK1.1$^+$ cells when cultured directly on irradiated stromal cell layer but not when they were separated from the stroma by a 0.45-μm microporous membrane (Tsuji and Pollack, 1995).

1.2.2 - The NK cell lineage

Some investigators have suggested the assignment of NK cells to the myeloid (Dorshkind *et al.*, 1985) or the monocytic lineage (Lohmann-Matthes *et al.*, 1979) while others suggested that NK cells represent a discrete subset of lymphocytes (Hackett *et al.*, 1986a). It has also been documented that NK cells develop normally in severe combined immunodeficient (SCID) mice (Dorshkind *et al.*, 1985) and SCID patients (Peter *et al.*, 1983). Moreover, NK cells undergo normal phenotypic and functional development in the absence of a functional thymus (Hackett *et*

al., 1986b) as well as in recombinase-activating gene (RAG)-deficient mice (Shinkai *et al.*, 1992). In fact, NK cells neither rearrange TCR or Ig genes nor express the products of these genes at the cell surface (Lanier *et al.*, 1986a; 1986b; Tutt *et al.*, 1986; Biron *et al.*, 1987). These observations strongly suggest that NK cell receptors are different from B and T cell receptors and may not require gene rearrangement for expression and function. Nevertheless, NK cells share a number of phenotypic and functional properties with T cells (Trinchieri, 1989). However, at least one case has been reported where a subject having normal T and B cells had no NK cells (Biron *et al.*, 1989). NK cells and T cells thus probably belong to related lineages having somewhat divergent differentiation pathways.

Consistent with this hypothesis, it has been reported that fetal thymocytes isolated from mouse embryos at 14.5 days of gestation, most of which are $CD4^-CD8^-TCR^-CD16^+NK1.1^-$ differentiated into $TCR\alpha\beta^+CD16^-$ T cells expressing either CD4 or CD8 if transplanted in a thymic microenvironment, whereas they selectively generated $CD4^-CD8^-TCR^-CD16^+NK1.1^+$ cells when injected intravenously (i.v.) to irradiated recipient (Rodewald *et al.*, 1992). When cultured in the presence of IL-2, $CD4^-CD8^-TCR^-CD16^+$ thymocytes differentiated into cells having phenotypic and functional characteristics of NK cells. Brooks *et al.* (1993) also showed that immature thymocytes from C57BL/6 14-day embryonic mice have a strong and sustained proliferation in response to IL-2 when pretreated with PMA and IL-4 for 24 h. The cell lines obtained in this manner lacked lineage-specific markers of mature T cells, B cells, and myeloid cells, but

expressed NK1.1 and asialo GM1. They also efficiently killed NK-sensitive targets.

The current knowledge on the developmental relationship between human NK and T cells has recently been reviewed (Spits *et al.*, 1995). It is suggested that a common T/NK progenitor cell exists, just upstream of commited T- and NK- precursors, but downstream from a common lymphoid progenitor cell. Supporting this hypothesis, Hori *et al.* (1992) have isolated two apparently identical $CD7^{+}CD2^{low}CD3^{-}CD4^{-}CD8^{-}CD5^{-}CD6^{-}CD11b^{+}CD16^{-}CD56^{-}$ clones from human fetal liver that express cytoplasmic CD3-δ (cCD3-δ) and cCD3-ε, have not rearranged the genes of the TCRαβ or γδ, and show no NK cell activity. These cell lines proliferate in response to IL-2, IL-4, and IL-7 but not to IL-3. However, these clones never differentiated into mature NK or T cells. It has been suggested that these clones might represent a common T and NK precursor present in the fetal liver. The emergence of T, B, and NK cells from a common lymphoid precursor is further substantiated by the observation that mice functionally-deficient for the transcription factor Ikaros lack T, B, and NK cells (Georgopoulos *et al.*, 1994).

1.2.3 - Organ and tissue distribution of NK cells

Following their maturation, some NK cells randomly leave the bone marrow for the periphery (Miller, 1982) where they accumulate, mainly in the spleen and peripheral blood (Dorshkind *et al.*, 1985). The life span of mature splenic NK cells was evaluated to range from a few days to a couple of months (Miller, 1982; Pollack and Rosse, 1987). In the mouse,

NK cells or NK-like activity have also been observed in the intestinal epithelium and lamina propria (Tagliabue *et al.*, 1982; Carman *et al.*, 1986; Tutt *et al.*, 1986). According to Alberti *et al.* (1985), the intraepithelial LGLs have the same phenotype as splenic and peripheral NK cells. On the other hand, Mowat *et al.*, (1983) have reported that NK cells of the gut differ from splenic NK cells in that a longer incubation period is required to detect their *in vitro* cytotoxic activity. Therefore, the presence of NK cells in the gastro-intestinal tract is still a controversial issue and the NK or NK-like cells that may be present in that organ are likely to be at least functionally distinct from peripheral NK cells. These results suggest that NK cells may have adapted to efficiently defend various tissues.

The presence of NK cells in the thymus also remains a controversial issue, especially in light of the putative related lineage of T and NK cells. Indeed, in C57BL/6 mice, ~10% of double negative (DN) thymocytes express the NK1.1 antigen (Ballas and Rasmussen, 1990a). However, these cells are not lytic for YAC-1 target cells and dimly express CD3ε with 60% expressing TCR-Vβ8. The NK1.1$^+$ DN thymocytes are thus both functionally and phenotypically distinct from splenic NK cells and could represent thymic NK/T bipotential progenitor cells. Nevertheless, NK cell activity against YAC-1 target cells has been reported in the thymus of C.B-17 SCID mice (Garni-Wagner *et al.*, 1990). The cells responsible for that activity were asialo GM1$^+$IL2Rα$^-$J11d$^-$CD3$^-$CD4$^-$CD8$^-$. On the basis of this phenotype, these cells were identified as NK cells. The lymph nodes, liver, and lung also display low levels of NK cell activity (Herberman *et al.*, 1975a; Lohmann-Matthes *et al.*, 1979; Itoh *et al.*, 1982;

Peter *et al.*, 1983; Stein-Streilein *et al.*, 1983; Holt *et al.*, 1985; Dorshkind *et al.*, 1985; Foure *et al.*, 1990). Finally, a number of murine decidual cells in the first trimester of pregnancy express NK1.1 and asialo GM1 (Croy *et al.*, 1985; Linnemayer and Pollack, 1991).

1.3 - FUNCTIONAL RELEVANCE OF NK CELLS

Depending on their localization, NK cells contribute to different aspects of immunity through direct killing of target cells. Alternatively, NK cells can be triggered via low affinity receptors for IgG (FcγRIII or CD16) to kill IgG-coated target cells by antibody-dependent cellular cytotoxicity (ADCC) (Perussia *et al.*, 1989). Moreover, it has been reported that human NK cells activated through CD16 or with IL-2 can produce IFN-γ, TNF, GM-CSF, CSF-1, M-CSF, and IL-3 (Anegon *et al.*, 1988; Cuturi *et al.*, 1989). Neither G-CSF, IL-1α nor IL-1β were however detected under similar stimulatory conditions. Interleukin-12 was also shown to stimulate, either alone or in synergy with IL-2, the production of IFN-γ by human NK cells (Chan *et al.*, 1991). Through cellular cytotoxicity or cytokine secretion, NK cells contribute to the limitation of tumor cell growth, play an important role in the resistance to certain microbial infections, and participate in the regulation of hematopoiesis. Unfortunately, their killing ability toward "non-self" or "altered-self" cells make NK cells partly responsible for bone marrow graft rejection and graft-versus-host disease (GVHD). Finally, NK cells are also involved in maternal-foetal interactions and in regulation of Ig production by B cells.

1.3.1 - Immune surveillance against cancer

As mentioned above, NK cells were initially defined by their *in vitro* ability to kill tumor cells (Herberman *et al.*, 1975a; Kiessling *et al.*, 1975a). That property suggested that NK cells might also kill tumor cells *in vivo* and therefore, be involved in immune surveillance against cancer. In support of this hypothesis, a direct correlation between the level of NK activity and resistance to transplanted syngeneic tumors was reported soon thereafter (Haller *et al.*, 1977; Talmadge *et al.*, 1980). It was also shown that the increased susceptibility of cyclophosphamide-treated mice to transplanted malignant cells could be reversed by the adoptive transfer of NK clones or NK-enriched suspensions (Warner and Dennert, 1982). The involvement of T cells in that process was excluded since neither cytotoxic T cell clones nor T cells could confer tumor resistance under identical conditions. In addition, specific depletion of NK cells with anti-NK1.1 mAb *in vivo* also impaired host defence against malignancy, without affecting other cellular immune functions (Seaman *et al.*, 1987). Similarly, NK cell deficiency in humans suffering from the Chediak-Higashi syndrome and in mice carrying the *beige* (bg) mutation correlates with a higher susceptibility to lymphoproliferative diseases (Roder *et al.*, 1979; Abo *et al.*, 1982). In agreement with these observations, it was reported that the incidence of spontaneous tumors was slightly higher in bg/bg homozygous mice than in +/bg heterozygous mice (Haliotis *et al.*, 1985). Taken together, these observations support a role for NK cells in surveillance against spontaneously arising tumors.

There is also experimental evidence that NK cells contribute to limit the spread of metastatic cells. This includes the observation of increased incidence of experimental pulmonary metastases in cyclophosphamide-treated mice which is prevented by adoptive transfer of purified NK cells (Hanna and Burton, 1981). In addition, anti-asialo GM1-treated mice showed a depressed NK activity and exhibited increased formation of lung and liver metastases after i.v. injection of B16 melanoma or Lewis lung carcinoma (Wiltrout *et al.*, 1985a). On the other hand, injection with the pyran copolymer maleic anhydride divinyl ether, a synthetic biological response modifier, resulted in increased NK activity in the spleen, the lung, and the liver and significantly reduced metastatic spread in the lung and liver (Wiltrout *et al.*, 1985a).

1.3.2 - Resistance to microbial infections

It has been documented that NK cells are implicated in early resistance to certain microbial infections (Fitzgerald and Lopez, 1986). Of the antimicrobial functions of NK cells, the antiviral activity is certainly the most convincingly documented, especially in the case of murine herpesvirus (HSV-1), cytomegalovirus (MCMV), and Theiler's virus infections (Welsh, 1986; Fitzgerald *et al.*, 1985; Paya *et al.*, 1989). Indeed, it has been reported that mice having low NK cell activity are more susceptible to herpes simplex and MCMV infections. Furthermore, adoptive transfer of bone marrow cells from a MCMV-resistant to a lethally-irradiated susceptible mouse confers resistance to a subsequent viral infection (Bukowski *et al.*, 1985). Similarly, adoptive transfer of cloned NK cells, but not T cells, to suckling mice 1 day before injecting

them with MCMV also confers resistance to infection (Bukowski *et al.*, 1985). Moreover, *in vivo* depletion of NK cells by inoculation of anti-asialo GM1 antiserum or anti-NK1.1 mAb was shown to increase the susceptibility of treated mice to MCMV and also Theiler's virus infections (Bukowski *et al.*, 1984; Paya *et al.*, 1989; Welsh *et al.*, 1990).

In humans, there is a convincing piece of direct evidence for the crucial role of NK cells in protection against herpesvirus infections. Biron *et al.* (1989) have reported the case of a teenage girl having a complete and apparently isolated deficiency of NK cells. That girl experienced repeated viral infections, including primary disseminated varicella and cytomegalovirus pneumonia as well as severe primary cutaneous herpes simplex infections. Neither $CD16^+$ nor $CD56^+$ cells were present in the peripheral blood lymphocytes (PBL) of this patient and no NK activity was detectable, even after *in vitro* stimulation with IL-2 or IFN-γ. Nevertheless, neutrophil, T cell, and B cell functions were apparently normal. In fact, with antiviral therapy and supportive care, the patient eventually recovered from each infection with normal T-cell and B-cell responses. From that case report, it clearly appears that NK cells act as a first line of defense in order to limit virus dissemination during the early phases of infection, whereas the more delayed specific responses mediated by T cells and antibodies are responsible for the ultimate control of the viral infection.

NK cells can also control infections by other pathogens, including *Listeria monocytogenes* (LM) (Dunn and North, 1991), *Salmonella typhimurium* (Ramarathinam *et al.*, 1993), the fungus *Cryptococcus neoformans*

(Lipscomb *et al.*, 1987; Hidore and Murphy, 1989), and the protozoa *Tripanosoma cruzi* (Hatcher and Kuhn, 1982) and *Toxoplasma gondii* (Hauser and Tsai, 1986). Interestingly, NK cells apparently do not act directly on the bacteria but rather cooperate with macrophages, via the secretion of cytokines. The best evidence for that cooperation comes from LM infection in the SCID mouse. In that infection, LM-infected macrophages release TNF-α, IL-1, and IL-12 (Bancroft *et al.*, 1989; Rogers *et al.*, 1992; Tripp *et al.*, 1993). Lymphokine-activated NK cells in turn produce IFN-γ (Bancroft *et al.*, 1991; Dunn and North, 1991). Indeed, it has been shown that a subcutaneous inoculation of a sublethal number of LM resulted in the early appearance of IFN-γ-producing $NK1.1^+asialoGM1^+Thy\text{-}1^+CD4^-CD8^-$ cells in draining lymph nodes (Dunn and North, 1991). Early elimination of these cells with anti-NK1.1 mAb or treatment with anti-IFN mAb resulted in severe exacerbation of infection, confirming the importance of NK cells and IFN-γ. The IFN-γ secreted by NK cells then directly stimulates macrophages to release bactericidal nitric oxide (Beckerman *et al.*, 1993). This cytokine cascade is counterbalanced by IL-10 which inhibits TNF-α and IL-12 production (Tripp *et al.*, 1993). This pathway is not unique to listeriosis and has been observed following infection with different bacteria and protozoa (Bancroft, 1993; Appelberg *et al.*, 1994), including *Toxoplasma* (Johnson *et al.*, 1993).

On the other hand, destruction of the fungus *C. neoformans* appears to proceed through direct cell-mediated killing somewhat different from tumor killing in that the attachment of NK cells to the fungus is relatively slow (2 h) as compared to the attachment of NK cells to tumor cells (20

min.). Moreover, NK cells are attached less intimely to *C. neoformans* than to tumor target cells (Hidore and Murphy, 1989; Murphy *et al.*, 1991). Nevertheless, rat NK cells can bind to the fungal pathogen *C. neoformans* and inhibit its growth through the release of their cytolysin-containing cytoplasmic granules (Hidore *et al.*, 1990).

1.3.3 - Regulation of hematopoiesis

The regulation of hematopoiesis by NK cells is supported by the observation that purified IL-2-activated NK cells of SCID mice are able to support the growth of hematopoietic cells *in vitro* without addition of exogenous hematopoietic growth factors (Murphy *et al.*, 1992a). NK cells not only positively regulate hematopoiesis but can also inhibit hematopoietic colony formation. Indeed, Murphy *et al.*, (1992a) also reported that NK cells could inhibit hematopoietic proliferation when syngeneic bone marrow cells and cytokines necessary to support optimal growth (IL-2, IL-3 and GM-CSF) were added. Since anti-IFN-γ antibodies partially reversed the observed inhibition, it appears that IFN-γ mediates or at least contributes to the inhibitory effect of NK cells. Therefore, NK cells may support the growth of hematopoietic cells in limiting conditions yet inhibit hematopoiesis under optimal conditions, thus keeping a balanced hematopoiesis. A similar dual effect of NK cells on hematopoiesis was also observed in humans. When incubated for several hours with allogeneic or autologous bone marrow cells, purified NK cells exhibit a colony-inhibiting activity that synergize with IFN-γ to suppress granulocytic-monocytic colony-forming units (CFU-GM) and to support

the development of erythroid burst-forming units (Degliantoni *et al.*, 1985a; 1985b; Pistoia *et al.*, 1985).

1.3.4 - Other NK-related functions

Bone marrow transplantation provides curative treatment for some patients with hematopoietic malignancies. The contribution of NK cells in maintaining homeostasis, particularly at the hematopoietic level, unfortunately brings major problems after a bone marrow transplantation. Indeed, patients deprived of marrow cells by intensive chemotherapy or radiotherapy and reconstituted with compatible allogeneic bone marrow may reject the bone marrow cell graft or develop GVHD.

The involvement of NK cells in rejection of bone marrow cell grafts (Yu *et al*, 1992) and in GVHD (Ghayur *et al.*, 1987; 1988; MacDonald and Gartner, 1991; 1992) has been well documented in the mouse model. Even before the formal identification of NK cells, Bennett (1973) had shown that ^{89}Sr-treated recipient mice failed to reject marrow allografts. The involvement of NK cells as the principal effectors of bone marrow graft rejection was further confirmed following the observation that rejection was abrogated after NK cell depletion by treatment of recipient mice with anti-asialo GM1 antiserum (Okumura *et al.*, 1982) and more specifically with anti-NK1.1 mAb (Lotzova *et al.*, 1983). Rejection of bone marrow cell grafts can also occur through hybrid resistance, a phenomenon by which lethally irradiated F_1 hybrid mice reject parental hematopoietic cell grafts, even though other parental grafts are tolerated [reviewed in (Bennett, 1987)]. This phenomenon is controlled by a recessively inherited

minor histocompatibility locus named Hemopoietic histocompatibility-1 (Hh-1) located within the Histocompatibility-2 (H-2) complex, between the H-2S and H-2D regions (Cudkowicz and Stimpfling, 1964; Bennett, 1972). Interestingly, a particular NK cell subpopulation is responsible for the rejection of Hh-1^d but not Hh-1^b bone marrow grafts (Sentman *et al.*, 1989a). It is unknown whether other NK cell subpopulations can mediate rejection of bone marrow cells expressing different Hh-1 alleles.

Warner and Dennert (1982) have challenged the exclusive role of NK cells ($NK1.1^+CD3^-$) in acute rejection of marrow allografts. In fact, they isolated a T-cell clone with NK-like activity which was able to transfer allogeneic resistance. Moreover, using antibody plus complement-mediated depletion of spleen cells, Yankelevich *et al.* (1989) have shown that the effector cell capable of transferring allogeneic as well as hybrid resistance, was not only $NK1.1^+$ and asialo $GM1^+$, but also $CD3^+CD4^-CD8^-$ and $TCR\alpha\beta^+$. It was thus concluded that $NK1.1^+$ T cells, rather than classical NK cells were responsible for bone marrow cell grafts rejection.

The participation of NK cells in GVHD was suggested by the observation that bone marrow cells from normal but not NK-deficient bg/bg mice were able to induce severe GVHD with histopathological lesions and profound B and T cell suppression in either normal or bg/bg recipients (Ghayur *et al.*, 1987). Moreover, it was verified that the cells mediating the GVHD reaction were of donor origin since donor-derived $Thy\text{-}1^+$ asialo$GM1^+$ $Mac\text{-}1^+$ $Lyt\text{-}1^-$ $Lyt\text{-}2^-$ Ia^- LGLs were found in the site of tissue injury in mice with acute GVHD (Ferrara *et al.*, 1989) and cells mediating NK or NK-like activity in F_1-hybrid mice with acute GVHD were also of

donor origin (MacDonald and Gartner, 1991). A convincing demonstration of the active participation of donor-derived NK cells in GVHD was afforded by the observation that selective elimination of asialo GM-1$^+$ or NK1.1$^+$ cells from transplanted bone marrow cell suspensions prevented the development of lethal GVHD (Ghayur *et al.*, 1988; MacDonald and Gartner, 1992).

Although these results identified NK cells as potential effectors of GVHD, evidence also exists that donor-type activated NK cells can suppress the generation of GVHD and promote marrow engraftment during allogeneic bone marrow transplantation (Murphy *et al.*, 1992b). Therefore, the exact role of NK cells in the pathology of GVHD remains to be elucidated. It is conceivable that the dual effect of NK cells on hematopoiesis may be responsible for either occurrence of GVHD or improved engraftment of bone marrow cells, depending on the activation stage of the donor NK cells and the bone marrow environment of the recipient.

The presence of NK cells in the uterus during pregnancy is consistent with a role for NK cells in regulating the relationship between the mother and the foetus. Lytic NK cells are unlikely to be necessary for successful embryonic development since lytic NK cells are absent from the decidua of bg mice although pregnancy progresses normally (Croy *et al.*, 1985). A correlation between NK cell activity and the spontaneous abortion rate has moreover been reported in the murine model (Gendron and Baines, 1988). Furthermore, the abortion rate was observed to increase after treatment of mice with polyinosinic:polycytidylic (poly I:C) acid, an agent that induces NK cell activity (Gidlund *et al.*, 1978), and to decrease after anti-asialo

GM1 antiserum treatment (De Fougerolles and Baines, 1987). NK cells may also contribute to support the development of the foetus by their ability to secrete IFN-γ, which has been shown to promote placental growth (Athanassakis *et al.*, 1987). Alternatively, decidual NK cells may contribute to suppress the immune response of the mother against the embryo. Indeed, decidual cells distinct from peripheral NK cells, in that they lack expression of asialo GM-1 (Slapsys *et al.*, 1986), have been shown to suppress NK and CTL lytic activities as well as ADCC (Kolb *et al.*, 1984; Clark and Chaouat, 1986).

NK cells also contribute to down-regulate the production of Igs by B cells. This function of NK cells was suggested by experiments in which depletion of NK1.1$^+$ cells from C57BL/6 mice before or at the time of immunization resulted in an increased frequency of plasma cells in the spleen (Robles and Pollack, 1986). Conversely, *in vivo* activation of NK cells by poly(I:C) resulted in inhibition of B cell responses (Khater *et al.*, 1986). Interestingly, regulation of the B cell response by NK cells appears to be mediated by IFN-γ. Indeed, using an *in vitro* system, activated B cells were shown to stimulate IL-2-activated NK cells to secrete IFN-γ (Michael *et al.*, 1989) which in turn inhibits B cell proliferation and differentiation (Michael *et al.*, 1991).

1.4 - Phenotypic Properties of Murine NK Cells

When they were first described, NK cells were qualified as "null cells" since no characteristic T-cell and B-cell antigens were found at their surface. Indeed, as mentioned in section 1.2.1, NK cells do not express

TCR heterodimers ($\alpha\beta$ or $\gamma\delta$) or surface Igs (Lanier *et al.*, 1986a; 1986b; Tutt *et al.*, 1986; Biron *et al.*, 1987). Nevertheless, NK cells share some cell surface antigens with T cells and cells of the monocyte/macrophage lineage and express a number of adhesion molecules and cytokine receptors as well. A summary of the common surface antigens expressed by either fresh or IL-2-activated murine NK cells is presented in Table I. Most of these antigens are not expressed by the total NK cell population but rather define subpopulations of variable size. In addition to the antigens listed in Table I, ubiquitous cell surface antigens (e.g. MHC class I) are of course also expressed by NK cells.

1.4.1 - Murine NK cell "specific" antigens

Over the years, most likely because of the development of anti-NK mAbs, a number of antigens principally expressed by NK cells have been described in mice, rats, and humans. Because most of them have not yet been found on other cell types, they are still considered NK cell specific.

The production of a (C3H x BALB/c)F_1 anti-CE antiserum allowed to identify the first NK cell alloantigen which was called NK1.1 (Glimcher *et al.*, 1977). This anti-NK1.1 antiserum was then used by different groups of investigators to define a cell population that had lytic activity against the Moloney virus-induced T cell lymphoma YAC-1 target cell (Glimcher *et al.*, 1977; Pollack *et al.*, 1979; Koo *et al.*, 1980). By flow cytometry analysis, it was further established that all of the NK cell activity present in C57BL/6 spleen cell suspensions was contained in the NK1.1$^+$ population (Tam *et al.*, 1980). A few years later, Koo and Peppard (1984)

Table I. Cell surface antigens expressed by murine natural killer cells.

Antigen[a]	Other name(s)	Reference(s)
NK1.1	-	Glimcher *et al.*, 1977
NK2.1	-	Burton and Winn, 1981
	-	Pollack and Emmons, 1982
LGL-1	Ly-49G	Mason *et al.*, 1988
5E6	SW5E6, Ly-49C	Sentman *et al.*, 1989a
3A4	SW3A4	Sentman *et al.*, 1989b
4LO439	-	Lemieux *et al.*, 1991
4LO3311	-	Lemieux *et al.*, 1991
2B4	SW2B4	Sentman *et al.*, 1989b
Asialo GM1	-	Kasai *et al.*, 1980
Ly-10	-	Koo *et al.*, 1982
Ly-11	-	Meruelo *et al.*, 1980
Ly-49A	YE1/48, YE1/32	Chan and Takei, 1986
	A1	Nagasawa *et al.*, 1987
MEL-14	Ly-22	Ballas and Rasmussen, 1990a
FcεRI-γ	γ	Lanier *et al.*, 1991a
CD2	-	Nakamura *et al.*, 1990
CD3-ζ	ζ	Lanier *et al.*, 1989a
CD11a/CD18	LFA-1, Ly-15	Nishimura and Itoh, 1988
CD11b/CD18	Mac-1, Ly-40	Holmberg and Ault, 1984
CD11c/CD18	p150/95	Lanier *et al.*, 1985
CD16	FcγRIII	Perussia *et al.*, 1989
CD28		Nandi *et al.*, 1994
CD44	Pgp-1, Ly-24	Ballas and Rasmussen, 1990b
CD45	T200, Ly-5	Pollack *et al.*, 1979
CD56	NCAM	Lanier *et al.*, 1989b
CDw90	Thy-1	Koo *et al.*, 1980

[a] Antigens in bold print are considered NK cell-specific. Underlined antigens have been described in humans. Their presence on murine NK cells is suspected but has not been clearly established.

were successful in selecting a hybridoma producing an anti-NK1.1 mAb designated PK136.

The NK1.1 antigen has been characterized as a disulfide-linked dimer of 39 kDa type II glycoproteins (Sentman *et al.*, 1989b; Ryan *et al.*, 1992) and is considered by many as the most specific serologic determinant of mouse NK cells. However, the NK cell specificity of NK1.1 is questionable since recent improvements in flow cytometry and cell isolation techniques have revealed the existence of NK1.1+TCRαβ+ within bone marrow cells (Sykes, 1990), spleen cells, and thymocytes (Koyasu, 1994). Whether these NK1.1+TCRαβ+ cells should be considered NK1.1+ T cells or TCRαβ+ NK cells remains however debatable.

After the identification of the NK1.1 antigen, several groups of investigators endeavored to produce anti-NK mAbs or antisera in order to define new NK cell antigens. The strain distribution of the NK1.1 antigen and the other known murine NK cell-"specific" antigens is presented in Table II.

Burton and Winn (1981) produced a mouse alloantiserum by immunizing CE mice with spleen cells from CBA mice. The detected alloantigen was expressed by NK cells and was tentatively designated NK1.2 since it was believed to detect an allelic form of NK1.1. Another group produced an NZB anti-BALB/c antiserum reacting with another NK cell alloantigen they named NK2.1 (Pollack and Emmons, 1982). As the strain distribution of the antigens detected by the CE anti-CBA and NZB anti-BALB/c antisera were almost similar, the two antisera were further considered to con-

Table II. Strain distribution of murine NK cell-"specific" antigens[a].

Strain	NK1.1[#†]	NK2.1[*◊]	LGL-1[¤]	5E6[¶]	3A4[†∞]	4LO439[‡]	4LO3311[‡]
129	-	-	+	-	-	-	-
A	-	+	?	?	-	-	+
AKR	-	+	+	?	-	-	+
BALB/c	-	+	+	+	-	-	+
C3H	-	+	+	+	-	-	+
C57BL/6	+	+	+	+	+	+	+
C57BL/10	+	?	?	?	?	+	+
C57BR	-	-	?	?	-	-	-
C57L	-	-	?	?	?	-	-
C58	+/-	-	?	?	-	-	-
CBA	-	+	?	?	-	-	+
CE	+	-	?	?	+	-	-
DBA/1	-	+	?	?	-	-	+
DBA/2	-	+	+	?	-	-	+
LP	-	+	?	?	?	-	+
MA/My	+	-	?	?	+	-	-
NZB	+	-	?	+	+	-	-
NZW	?	?	?	?	?	-	-
RIIIS	?	?	?	?	?	-	-
SJL	+/-	-	+	?	-	-	-
SM	-	-	?	?	-	-	-
ST	+	?	?	?	?	-	+

[a]The strain distribution of the listed antigens was compiled from Koo and Peppard (1984)[#], Pollack and Emmons (1982)[*], Burton *et al.* (1988)[◊], Mason *et al.* (1988)[¤], Sentman *et al.* (1989a[†]; 1989b[¶]; 1989c[∞]), and Lemieux *et al.* (1991)[‡]. Question marks indicate that expression of this antigen in the indicated mouse strain is unknown.

contain anti-NK2.1 antibodies (Burton *et al.*, 1988). Considering that NK1.1 and NK2.1 antigens are both expressed by C57BL/6 NK cells, it is excluded that they are allelic. Following a study with (C3H x BALB/c)F_1 anti-CE (anti-NK1.1) and NZB anti-BALB/c (anti-NK2.1) antisera, it was suggested that NK1.1$^+$ and NK2.1$^+$ cells of the C57BL/6 mouse might contain different functional NK cell populations but that all NK1.1$^+$ cells express NK2.1 and *vice versa* (Emmons and Pollack, 1985). At that time, prior to the beginning of the present study, no anti-NK2.1 mAb had been produced and for that reason, little information was available on the NK2.1 alloantigen.

The LGL-1 murine NK cell antigen was identified with the 4D11 rat anti-mouse liver-derived LGL (Mason *et al.*, 1988). The initial analysis of LGL-1 revealed that this antigen was a single protein of 87 kDa (Mason *et al.*, 1988). However, further studies revealed that LGL-1 is in fact expressed as a homodimer of disulfide-linked of 40 kDa *N*-glycosylated subunits (Mason *et al.*, 1994a). The cells stained with mAb 4D11 have the characteristic morphology of large granular lymphocytes and represent a cell population of variable size in all strains tested including 129, C57BL/6, BALB/c, AKR, DBA/2, C3H, and SJL (Mason *et al.*, 1988). In the C57BL/6 mouse, LGL-1$^+$ cells account for 50% of the NK1.1$^+$ population (Mason *et al.*, 1990). However, most of lymphokine-activated killer (LAK) activity as well as ADCC is apparently derived from the NK1.1$^+$LGL-1$^-$ population (Mason *et al.*, 1990).

The 5E6 antigen is a disulfide-linked homodimer of 54 kDa subunits expressed by ~50% of fresh and IL-2-propagated NK1.1$^+$ cells of the

C57BL/6 mouse (Sentman *et al.*, 1989a). The 5E6 antigen is also expressed by a fraction of BALB/c, C3H, and NZB NK cells. In three-color flow cytometry analysis using YE1/48 (anti-Ly-49A, see below), 5E6, and PK136 mAbs, Brennan *et al.* (1994) showed that NK1.1$^+$ cells of the C57BL/6 mouse are either Ly-49A$^-$5E6$^-$ (50.8%), Ly-49A$^-$5E6$^+$ (25.3%), Ly-49A$^+$5E6$^-$ (18.7%), or Ly-49A$^+$5E6$^+$ (5.2%). In these analyses, some NK1.1$^-$CD3$^+$ as well as NK1.1$^-$CD3$^-$ cells expressing Ly-49A or 5E6 were also detected. These results contributed to further support the concept of the heterogeneity of NK cells which dates back to the early studies of murine NK cells (Stutman *et al.*, 1978; Kumar *et al.*, 1979; Lust *et al.*, 1981; Minato *et al.*, 1981; Burton *et al.*, 1981; Emmons and Pollack, 1985).

In an attempt to produce mAbs directed against pan NK cell antigens, Sentman *et al.* (1989b) obtained another series of mAbs named 2B4, 3A4, and 4B12. The strain distribution of the antigens detected by these three new mAbs was similar to that of the NK1.1 antigen. While the 4B12 mAb was shown to bind a unique epitope of the NK1.1 molecule, the 2B4 and 3A4 mAbs apparently detected new molecules expressed by all NK cells of the C57BL/6 mouse. Even though the 2B4 antigen is expressed by all NK cells of selected strains, it cannot really be considered NK cell-specific since it is also expressed by a significant number of non-MHC-restricted T cells cultured with high doses of IL-2 (Garni-Wagner *et al.*, 1993). The nature of the 3A4 antigen is still undefined but the 2B4 antigen has been characterized as a monomeric 66 kDa protein homologous to members of the immunoglobulin superfamily (Garni-Wagner *et al.*, 1993; Mathew *et al.*, 1993).

Monoclonal antibodies directed against murine NK cell-specific alloantigens were also produced by immunization of 129 mice with NK-enriched spleen cells from C57BL/6 mice (Lemieux *et al.*, 1991, see Appendix I). The resulting 4LO439 and 4LO3311 mAbs reacted with fresh and IL-2-activated NK cells but not with T or B cells nor with activated macrophages. The strain distribution of the antigen detected by the 4LO3311 mAb was similar to that of the NK2.1 antigen, suggesting that the 4LO3311 mAb might be reacting with NK2.1. However, such an identity of specificity remained to be confirmed. The 4LO3311 mAb was also used to further define the heterogeneity of murine NK cells in NK1.1$^+$ as well as NK1.1$^-$ inbred mouse strains and their progeny (Morelli *et al.*, 1992). The 4LO3311 mAb was shown to react with a subpopulation of NK cells rather than with all NK cells and the frequency of 4LO3311$^+$ cells as well as the density of the corresponding antigen on NK cells were variable from one strain to another. As for the 4LO439 mAb, it appeared to identify a new NK cell alloantigen expressed by a subpopulation of NK cells present exclusively in C57BL mice. The structure and the functional relevance of the molecules detected by these two mAbs were yet to be elucidated at the time I began my Ph. D. studies.

1.4.2 - Cell surface antigens shared by NK cells and other leukocytes

Of the non-exclusive NK antigens, the Ly-49A antigen is undoubtedly the one that has attracted the most interest in recent studies on the functions of murine NK cells. Two groups, working independently, have produced YE1/32, YE1/48 (Chan and Takei, 1986), and A1 (Nagasawa *et al.*, 1987)

mAbs against antigens expressed by rare T cell tumors of C57BL/6 origin. The two molecules identified have been characterized as disulfide-linked dimers of ~45 kDa subunits. Further studies revealed that all these mAbs actually recognized the same molecule (Chan and Takei, 1989; Yokoyama *et al.*, 1989) now known as Ly-49A (Wong *et al.*, 1991).

Using either the YE1/48 or A1 mAb, the Ly-49A antigen was not found on normal T and B cells (Nagasawa *et al.*, 1987; Chan and Takei, 1989; Yokoyama *et al.*, 1990) but was detected at high levels on EL-4 and MBL-2 murine T lymphoma cell lines, on which it had first been described (Chan and Takei, 1986). Increasing interest for Ly-49A came after the observation that this antigen was expressed by 15-20% of splenic NK cells (Yokoyama *et al.*, 1990). Using a fourth anti-Ly-49A mAb (JR9-318), Ly-49A antigen was also found on a TCR$\alpha\beta^+$ subpopulation of intestinal intraepithelial lymphocytes and on a small population of cells in the adult thymus (Roland and Cazenave, 1992). Therefore, contrary to the observations reported with YE1/48, YE1/32, and A1 mAbs, the Ly-49A antigen may be expressed by some normal T cell subpopulations. The functional relevance of Ly-49A on NK cells has been extensively studied in the recent years and will be presented in section 1.5.4 and in chapter 4.

The glycosphingolipid asialo GM1 is one of the first antigens to have been identified on murine NK cells. Its expression on the vast majority of NK cells was shown by complement-dependent depletion of splenic NK cell activity after treatment of spleen cells with a rabbit anti-asialo GM1 antiserum, raised against mouse brain tissue (Kasai *et al.*, 1980). Subsequently, it was reported that i.v. injection of anti-asialo GM1

antiserum into BALB/c nude mice completely abrogated NK cell activity (Kasai *et al.*, 1981). However, the expression of asialo GM1 is not restricted to NK cells. Indeed, cytotoxic T lymphocytes (CTL) generated in mixed lymphocyte cultures (Suttles *et al.*, 1986; Ting *et al.*, 1986) and tumoricidal activated macrophages (Wiltrout *et al.*, 1985b) may also express this glycolipid. The anti-asialo GM1 antiserum nevertheless represented a valuable tool to conduct *in vitro* and *in vivo* studies of NK cells before more specific anti-NK mAbs were available.

Receptors for the Fc fragment of IgG have also been extensively studied, especially because of their role in ADCC. The presence of FcγRIII on most murine NK cells was shown by Perussia *et al.* (1989) using the 2.4G2 mAb, which identifies FcγRII (CD32) and FcγRIIα, later renamed FcγRIII (Ravetch and Kinet, 1991). However, Northern blot analyses revealed that only transcripts for the FcγRIII were present in NK cells (Perussia *et al.* (1989). The FcγRIII expressed by murine NK cells is a 40-60 kDa trans-membrane protein, member of the Ig superfamily (Fridman *et al.*, 1992).

Human NK cells express FcγRIIIA (CD16) as a non-covalently associated multimeric complex with the ζ chain of the CD3 complex (CD3-ζ) or the γ chain of the FcεRI (FcεRI-γ) in either ζ:ζ, ζ:γ, or γ:γ disulfide-linked dimers (Lanier *et al.*, 1989a; Lanier *et al.*, 1991a; Anderson *et al.*, 1990; Vivier *et al.*, 1991a). Although it is believed that mouse CD16 is similarly associated to ζ:ζ, ζ:γ, or γ:γ dimers, the presence of ζ on murine NK cells has never been documented. However, the γ-chain transcripts have been found in murine NK cells, although they do not express FcεRI, suggesting a physiological role for γ in murine FcγRIII expression, function, or both

(Ravetch and Kinet, 1991). In fact, cotransfection of FcγRIII and γ genes results in increased and stabilized expression of FcγRIII on COS cells (Ra *et al.*, 1989; Kurosaki and Ravetch, 1989).

1.4.3 - The NK gene complex

The existence of a gene cluster encoding NK cell surface proteins was initially suggested by the identification, first in the mouse and then in the human, of a series of closely related genes expressed in NK cells.

The Ly-49 gene has been cloned and the deduced amino acid sequence indicates that this antigen is a type II integral membrane protein (extracellular carboxy terminus), highly homologous to members of the C-type lectin supergene family (Chan and Takei, 1989; Yokoyama *et al.*, 1989). This family includes the low affinity receptor for IgE (CD23), the asialoglycoprotein receptor, the selectins MEL 14 and CD62, and the very early activation molecule CD69 (Drickamer and Taylor, 1993). Although these proteins are only weakly homologous to each other, they all have a carbohydrate recognition domain (CRD) and six conserved cysteine residues in their extracellular portion. Genomic Southern blot analyses of C57BL/6, BALB/c, and C3H mice with Ly-49 cDNA have indicated that other genes homologous to Ly-49 may exist in the mouse genome (Chan and Takei, 1989). Indeed, at least eight cDNA clones, highly homologous but not identical to Ly-49 have been isolated, thus confirming the existence of a Ly-49 multigene family within the superfamily of C-type animal lectins (Wong *et al.*, 1991; Smith *et al.*, 1994; Brennan *et al.*, 1994). In addition, some cDNA clones appear to represent deletional variants of one

of the Ly-49 genes (Smith *et al.*, 1994). Because of the presence of multiple genes in this family, the antigen detected by YE1/48 and A1 mAbs was later renamed Ly-49A and the others Ly-49B through Ly-49H (Wong *et al.*, 1991; Smith *et al.*, 1994; Brennan *et al.*, 1994). The molecules encoded by Ly-49B through Ly-49H have not been identified, with the exception of Ly-49C which encodes the 5E6 molecule (Stoneman *et al.*, 1993; Brennan *et al.*, 1994), and Ly-49G which encodes LGL-1 (Mason *et al.*, 1994b).

Analyses of restriction fragment length polymorphism (RFLP) from inbred and AKXL (AKR x C57L), BXH (C57BL/6 x C3H), and BXD (C57BL/6 x DBA/2) recombinant inbred (RI) mouse strains indicated that the Ly-49 multigene family maps to the distal segment of mouse chromosome 6, in close association with the proline-rich protein (Prp) locus (Yokoyama *et al.*, 1990). One year earlier, analysis of NK1.1 expression in BXD RI mouse strains had revealed that the yet undefined gene encoding NK1.1 maps to the same distal part of mouse chromosome 6 (Sentman *et al.*, 1989b), subsequently named the NK gene complex (Yokoyama *et al.*, 1991).

In the meantime, the NKR-P1 surface protein was identified on all rat NK and LAK cells, and most polymorphonuclear leukocytes (PMN) by the 3.2.3 mAb (Chambers *et al.*, 1989). It is noteworthy that NKR-P1 was subsequently found to be expressed at very low levels on a subset of splenic CD5$^+$ cells (van den Brink *et al.*, 1990; Chambers *et al.*, 1992; Kaufman *et al.*, 1993a) and on small subsets of splenic rat TCR$\alpha\beta^+$ or TCR$\alpha\beta^-$ T cells, the latter presumably expressing TCR$\gamma\delta$ (Brissette-

Storkus *et al.*, 1994). Therefore, rat NKR-P1 (rNKR-P1) should not really be considered as NK cell-specific, even though its functional relevance for NK cells (discussed in section 1.5.2) has been documented (Chambers *et al.*, 1989; Ryan *et al.*, 1991).

The rNKR-P1 has a structure similar to that of most mouse NK cell antigens (NK1.1, Ly-49A, 5E6, and LGL-1), in that it is expressed on NK cells as a disulfide-linked homodimer of ~60 kDa (Chambers *et al.*, 1989). The gene encoding rNKR-P1 has been cloned and the predicted amino acid sequence indicates that this antigen is a type II integral membrane protein (Giorda *et al.*, 1990). The extra-cytoplasmic domain also shows features of a C-type animal lectin. Although Southern blot analyses of rat genomic DNA suggested a single gene for rNKR-P1 (Giorda *et al.*, 1990), Yokoyama and Seaman (1993) have reported an unpublished observation by J. C. Ryan that at least four cross-hybridizing NKR-P1 cDNAs with related but distinct sequences are present in rat NK cells.

Using the rNKR-P1 cDNA as a probe, Giorda and Trucco (1991) isolated three related but distinct mouse NKR-P1 cDNAs initially identified 2, 34, and 40. In accordance with their Genbank designations, these cDNA were later renamed musNKR-P1A, musNKR-P1B, and musNKR-P1C (Yokoyama and Seaman, 1993). At nearly the same time, two other mouse NKR-P1 cDNA were cloned by PCR amplification using oligonucleotide primers derived from the published rNKR-P1 cDNA sequence (Yokoyama *et al.*, 1991; Ryan *et al.*, 1992). These clones were in fact identical to those isolated by Giorda and Trucco. Of most interest, analysis of RFLP variants from inbred mouse strains as well as BXD and AKXL RI strains

indicated that mouse NKR-P1 genes are genetically linked to the Prp and Ly-49 loci in the NK gene complex (Yokoyama *et al.*, 1991). Furthermore, the strain distribution pattern of mouse NKR-P1 and Ly-49 identified one recombinant among the AKXL RI strains, indicating that the mouse NKR-P1 gene family is distinct from the Ly-49 gene family.

The musNKR-P1A, B, and C molecules are highly homologous with each other (74-87% amino acid identity) and with rNKR-P1 (61-74% amino acid identity), but weakly homologous (27% amino acid identity) with Ly-49 (Giorda and Trucco, 1991; Yokoyama *et al.*, 1991). However, the 3' untranslated region of rNKR-P1 is more closely related to musNKR-P1A than to musNKR-P1B or C. It is thus believed that the musNKR-P1A would be the mouse homologue of rNKR-P1. Interestingly, a strain-specific expression of the three mouse NKR-P1 genes was observed and correlated with the expression of NK1.1 (Giorda *et al.*, 1992). On the basis of nucleotide sequence analysis of the promoter region of the three NKR-P1 genes, it was suggested that differences in expression of the different genes probably do not result from alteration in the upstream regions of these genes, but may rather be caused by the expression of strain-specific transacting factors, which remain to be identified.

Structural as well as functional similarities between NK1.1 and rNKR-P1 (see below) prompted the hypothesis that NK1.1 might be encoded by a member of the mouse NKR-P1 gene family. Although initial efforts to detect NK1.1 expression at the cell surface of COS cells transfected with musNKR-P1 cDNAs were unsuccessful (Yokoyama *et al.*, 1991; Giorda *et al.*, 1992), expression of mNKR-P1.9, a cDNA identical to musNKR-P1C,

in *Sf*9 cells using the baculovirus expression system resulted in specific expression of the NK1.1 antigen (Ryan *et al.*, 1992). This clearly identified NK1.1 as a member of the NKR-P1 receptor-like protein family.

The products of seven out of the eleven members of the Ly-49 and NKR-P1 gene families have thus not yet been identified. The presence of their corresponding mRNA transcripts nevertheless suggests that they are expressed by NK cells. Other type II integral membrane glycoproteins belonging to the C-type lectin supergene family and encoded by genes located in or near the NK gene complex are also expressed by NK cells. One exemple is the CD69 molecule (Lanier *et al.*, 1988; Karlhofer and Yokoyama, 1991). Another is a family of related cDNA clones, designated NKG2-A, B, C, and D, isolated from a human NK cell clone library (Houchin *et al.*, 1991). The products of these genes have not yet been identified but the putative proteins display only very limited amino acid sequence homology with NKR-P1 and Ly-49 and would likely not represent their human homologues. By Southern blot analysis of a series of human/hamster hybrid somatic cell lines, the NKG2 multigene family has been mapped to the human chromosome 12 in association with the Prp gene (Yabe *et al.*, 1993). Inasmuch as Prp lies in a linkage group conserved between human chromosome 12p and the distal segment of mouse chromosome 6 (Searle *et al.*, 1987), where the NKR-P1 and Ly-49 gene families are located, that segment of the human chromosome 12p could contain the human NK gene complex. Consistent with this hypothesis, the human homologue to mouse and rat NKR-P1 (hNKR-P1A) has recently been isolated and the gene mapped to chromosome 12p (Lanier *et al.*, 1994). The hNKR-P1A molecule shows 46% homology

with mouse and rat NKR-P1 at the amino acid level and has the structural features of a C-type lectin-like disulfide-linked homodimer, but differs from mouse and rat NKR-P1 in its cell distribution. In fact, whereas all rat NK cells express NKR-P1 and all NK cells from the C57BL/6 mouse express musNKR-P1C, hNKR-P1A is expressed only by a subpopulation of NK cells. Moreover, although rodent T cells only infrequently express NKR-P1, ~25% of human T cells, including both $CD4^+$ and $CD8^+$ T cells, express hNKR-P1A. Functionally, hNKR-P1A also appears to be different from rodent NKR-P1. This aspect will be discussed in section 1.5.

As mentioned earlier, the CD69 antigen belongs to the C-type lectin family (Ziegler *et al.*, 1993; Lopez-Cabrera *et al.*, 1993; Hamann *et al.*, 1993). Although the physiological function of CD69 is unclear, it has been reported that cross-linking of CD69 on human T cells with antibodies in the presence of a second signal such as phorbol ester results in proliferation involving the induction of the IL-2 and IL-2Rα genes (Cebrian *et al.*, 1988; Testi *et al.*, 1989; Nakamura *et al.*, 1989). This result thus suggests that CD69 may be an activation receptor. Therefore, CD69 is not only structurally but also functionally related to the NKR-P1 family. Human and mouse CD69 genes have in fact been mapped to their respective NK gene complexes (Schnittger *et al.*, 1993; Lopez-Cabrera *et al.*, 1993; Ziegler *et al.*, 1994).

In the rat, no formal identification of an NK gene complex has been reported. Nevertheless, taking into account that a synteny group is conserved on rat chromosome 4, mouse chromosome 6, and human chro-

mosome 12p (Szpirer *et al.*, 1991), it is conceivable that a rat NK gene complex may exist and map to chromosome 4, near the Prp gene.

1.5 - MOLECULAR STRUCTURES INVOLVED IN NK CELL KILLING

As described in section 1.3, NK cells have the capacity to recognize and kill tumor cells, virus-infected cells, as well as normal cells. Even though the NK cell-mediated cytotoxic mechanism consists of several steps, this process can be separated into two major phases: the binding and the killing phases (Herberman *et al.*, 1986). In the binding phase, a number of receptor-ligand interactions instruct NK cells to kill or to spare the cells they interact with. Some receptors trigger NK cell lysis while others inhibit the killing process. Present knowledge on the events occurring at the binding stage will be described in detail in the following sections.

In the killing phase, a lethal hit is delivered to the target cell. This phase has been dissected into a sequence of discrete stages (Herberman *et al.*, 1986; Trinchieri, 1989). Briefly, the NK cell azurophilic granules first become concentrated in the part of the effector cell that is in close contact with a target cell. Then, the granule-packaging Golgi apparatus is directed toward the contact site and a number of proteins of the cytoskeleton are reoriented toward the target cell soon after binding (Kupfer and Dennert, 1984; Kupfer *et al.*,1985; Dennert *et al.*, 1985).

These events are followed by exocytosis of cytoplasmic granules and the release of a complex of cytotoxic proteins called NK cell cytotoxic factor or NKCF (Wright and Bonavida, 1982; 1983a). The granules contain the

killer cell's lethal products including trypsin-like serine proteases known as granzymes A to G (Jenne and Tschopp, 1988; Jenne *et al.*, 1989), a pore-forming protein or perforin (Young and Cohn, 1987), and a protein called fragmentin which induces DNA fragmentation (Shi *et al.*, 1992). The perforin has significant homology with the C9 component of complement and polymerizes in the plasma membrane of the target cell to form tube-like pores (Lichttenheld *et al.*, 1988). Recently, the key role of perforin in NK cell killing of tumor and virus-infected cells was re-evaluated in perforin-deficient mice (Kägi *et al.*, 1994). These mice have normal numbers of NK cells which, however, do not lyse virus-infected or tumor target cells *in vitro*. Moreover, perforin-deficient mice infected i.v. with a low dose of lymphocytic choriomeningitis virus (LCMV) still had high titers of virus in the spleen and in the liver by the time LCMV-infected normal and heterozygous mice had completely eliminated the virus. Following the membrane damage caused by perforin, the DNA of the target cell undergoes rapid breakdown into oligonucleosomal fragments and the cell dies with morphological changes consistent with apoptosis (Duke *et al.*, 1983; 1986). A recently identified protein called fragmentin, contained in NK cell granules, capable of causing DNA fragmentation in YAC-1 targets and producing severe chromatin condensation within 1 h, was proposed to induce the NK cell-mediated apoptosis (Shi *et al.*, 1992). The composition of NKCF is still poorly defined with the exception of TNF which has been identified as one of the components (Wright and Bonavida, 1983b; Degliantoni *et al.*, 1985b). After the lethal hit delivery, the cell conjugate breaks up and the NK cell initiates a new killing cycle.

1.5.1 - The NK cell receptor

Whereas the recognition structures or receptors of T and B cells are well characterized, the nature of the NK cell receptor (NKR) remains elusive despite the sustained efforts of many laboratories. In fact, accumulated data would suggest that NK cells do not express a unique receptor like TCR for T cells and Ig for B cells, but rather an array of several molecules involved in recognition and triggering of NK cell-mediated activity. Nevertheless, Harris *et al.* (1991) did identify a conserved molecule involved in recognition and killing of NK target cells and proposed it as a candidate NK cell antigen receptor. This molecule was first identified with a mAb (5C6) directed against nonspecific cytotoxic cells (NCC) of teleost fish, these cells being analogous to mammalian NK cells (Evans *et al.*, 1988). Subsequently, the antigen identified by the 5C6 mAb was also detected on human (Harris *et al.*, 1991) and rat NK cells (Jaso-Friedmann *et al.*, 1992). Biochemical analysis revealed that the molecule expressed by human and rat NK cells was a complex of non-covalently associated 38 and 42 kDa proteins, distinct from the TCR (Harris *et al.*, 1991; Jaso-Friedmann *et al.*, 1992). In fish, rat, and man, the 5C6 mAb specifically binds to and inhibits the lytic activity of NK cells but has no effect on ADCC, indicating that it identifies a functionally relevant molecule involved in the first phase of cytolysis i.e. target cell recognition and binding. Moreover, binding of the 5C6 mAb to NWNA rat spleen cells results in a rapid increase in intracellular calcium concentration ($[Ca^{2+}]_i$), suggesting that it can also trigger the activation of NK cells (Jaso-Friedmann *et al.*, 1992).

Another putative NK cell receptor and its ligand were described in humans (Frey *et al.*, 1991; Bino *et al.*, 1992). In an attempt to define the structures involved in tumor cell recognition by human NK cells, Bino *et al.*, (1992) produced a mAb (mAb 36) against K562 tumor cell membrane glycoproteins, which inhibits LGL binding and lysis of NK-susceptible targets. Subsequently, an anti-idiotypic (anti-Id) antiserum was produced against mAb 36 and was found to bind to human $CD3^-$LGLs, but not to other resting cells including $CD3^+$ T cells, macrophages, neutrophils, and B cells, suggesting that the molecule reacting with the anti-Id was NK-specific (Frey *et al.*, 1991). The anti-Id antiserum was shown to inhibit the LGL-mediated binding and lysis of K562 targets. However, the same effectors retained the ability to mediate ADCC. Moreover, the antigen identified with the anti-Id appeared to be a functional receptor since anti-Id antibodies mediated redirected lysis of FcR^+ NK-resistant Raji targets and $F(ab')_2$ fragments of the anti-Id triggered the release of serine esterases and IFN-γ. The target cell antigen was not extensively characterized but tunicamycin treatment of target cells revealed that it was a carbohydrate-associated molecule.

Initial biochemical and functional analyses revealed that the majority of the anti-Id reactivity was directed against three proteins of 80, 110, and 150 kDa, the last one being the functionally relevant molecule (Frey *et al.*, 1991). Using an anti-Id antiserum, the genes encoding the human 150 kDa protein and its murine homologue were cloned revealing >80% homology based on their predicted amino acid sequences (Anderson *et al.*, 1993). The sequences further suggested that the human and mouse molecules, named NK-tumor recognition 1 (NK-TR1), had an amino terminus

domain highly homologous to cyclophilin. Further analyses of the recombinant protein showed that NK-TR1 possesses both peptidyl-propyl *cis-trans* isomerase and chaperone-like functions (Rinfret *et al.*, 1994). NK-TR1 is encoded by a single copy gene mapping to human chromosome 3 (3p21-3p23) and mouse chromosome 9 (Young *et al.*, 1993). Two sites of alternative splicing are present in the 5' region of the NK-TR1 mRNA, resulting in the production of immature protein (Rinfret and Anderson, 1993). Interestingly, the production of mRNA encoding mature NK-TR1 protein is increased following activation of NK cells with IL-2. The requirement of NK-TR1 for NK activity was recently studied in antisense NK-TR1 transfectants in the rat LGL cell line RNK-16 (Giardina *et al.*, 1995). Transfectants with low levels of NK-TR1 expression showed drastically reduced levels of cytotoxicity against NK-susceptible targets and vaccinia virus-infected cell lines. However, lectin-dependent cytotoxicity, reverse ADCC through NKR-P1, and ADCC were similar in high and low NK-TR1 expressing clones, indicating that the lytic machinery was intact. From these experiments, it was concluded that NK-TR1 is an essential element in a signaling pathway leading to killing of tumor or virally-infected cells.

The relevance of NK-TR1 in tumor killing was also suggested by an *in vivo* study. Indeed, individuals susceptible to or clinically manifesting von Hippel-Lindau (VHL) disease, characterized by a high incidence of certain tumors, have a genetic defect at the 3p25-3p27 region of chromosome 3, i.e. just telomeric to the NK-TR1 gene (Hosoe *et al.*, 1990; Glenn *et al.*, 1990). Interestingly, individuals affected by VHL disease were generally

shown to have low expression of NK-TR1 correlating with a lack of NK cell activity (Ortaldo *et al.*, 1992).

1.5.2 - Activation molecules

The 5C6 and NK-TR1 antigens can both be considered activation molecules but are unique in that antibodies directed against them also block the binding of NK cells to target cells. The activation molecules described in the present section are also NK cell receptors but appear to act at a post-binding step exclusively. Some of the criteria defining activation receptors include increased NK cell cytotoxicity, redirected lysis of FcR^+ target cells, secretion of cytokines, granule exocytosis, tyrosine phosphorylation, phosphoinositide turnover, and increased $[Ca^{2+}]_i$.

Although most of the functional studies of CD16 have been done in humans and although CD16 is not restricted to NK cells, this antigen remains the best characterized of the triggering molecules expressed by NK cells. As presented in section 1.3, NK cells have the capacity to kill IgG-coated target cells by a process called ADCC. This ability suggested that FcγRIII, or CD16, had the capacity to trigger NK cell lysis (Perussia *et al.*, 1989). Indeed, antibodies to human CD16 mediate redirected lysis of otherwise resistant FcR^+ target cells (van de Griend *et al.*, 1987). Moreover, cross-linking of CD16 with specific mAb induces various activation processes including the expression of IL-2Rα, the activation of protein kinase C, secretion of IFN-γ and TNF-α, as well as a rapid increase in $[Ca^{2+}]_i$ and the turnover of phosphoinositides (Anegon *et al.*, 1988; Cassatella *et al.*, 1989; O'Shea *et al.*, 1991). NK cell activation

induced by anti-CD16 mAb results in phosphorylation of the associated ζ chain, suggesting that this chain actually transduces the activation signal (Vivier *et al.*, 1991b; O'Shea *et al.*, 1991). Interestingly, NK cell-mediated lysis of target cells uncoated with antibodies does not result in phosphorylation of the ζ chain, suggesting that human NK cells express a CD16-independent activation pathway in which ζ is not involved.

The CD16:ζ:γ complex mentioned in section 1.4.2 has been shown to also include the CD2 molecule (Vivier *et al.*, 1991c), the physiological ligand of LFA-3 (CD58) (Springer *et al.*, 1987). Interestingly, mAbs directed against certain epitopes of CD2 but not others were able to increase NK cell activity (Siliciano *et al.*, 1985). The functional relevance of CD2 was also studied in the mouse. In that system, only a marginal inhibition of YAC-1 cell lysis by NK and LAK cells was observed when CD2 was blocked with specific mAbs, suggesting that CD2 provides only a minor contribution to murine NK and LAK cell activities (Nakamura *et al.*, 1990). Nevertheless, transfection of wild-type murine CD2 cDNA in a $CD2^{-}CD3^{-}$ NK-like LGL clone led to CD2-dependent binding and killing of P815 target cells, while the expression of CD2 with a truncated cytoplasmic domain led to binding but not to killing of target cells (Nakamura *et al.*, 1991). Hence, the CD2 molecule would appear to contribute to the transduction of activation signals in NK cells although other receptors are likely involved.

Even though rNKR-P1 has been detected on PMN and some T cells, this molecule was the first rodent NK-associated cell surface antigen shown to trigger NK cell lysis. The 3.2.3 anti-rNKR-P1 mAb was indeed shown to

enhance the cytolytic activity of NK and A-LAK cells against FcγR+ but not FcγR- target cells (Chambers *et al.*, 1989). In addition, triggering of A-LAK cells through the rNKR-P1 molecule resulted in the release of *N*-α-benzyloxycarbonyl-L-lysine thiobenzyl (BLT)-esterases contained in the cytoplasmic granules. Further studies also indicated that $F(ab')_2$ fragments of anti-rNKR-P1 mAb or the 3.2.3 producing hybridoma stimulated phosphoinositide turnover and a rise in $[Ca^{2+}]_i$ in the rat NK cell line RNK-16 (Ryan *et al.*, 1991). All these observations are consistent with the NKR-P1 being an activation receptor on NK cells. The ability of rNKR-P1 to trigger NK cell lysis was recently confirmed using rNKR-P1-deficient RNK-16 cells (Ryan *et al.*, 1995). Although rNKR-P1-deficient RNK-16 cells killed some tumor targets, including YAC-1, they were selectively unable to kill IC-21 macrophage, B-16 melanoma, and C1418 lymphoma targets. Re-expression of rNKR-P1 however restored lysis of IC-21 targets and transmembrane signaling in response to IC-21. Even though intracellular signals were observed in response to B-16 and C1418 targets, these cell lines remained resistant, suggesting here again that other NK cell molecules are required for the efficient killing of certain tumor targets and supporting the idea that there are not one but many NK cell receptors involved in natural killing.

It was demonstrated recently that the Ca^{2+}-dependent CRD of rNKR-P1 can bind a diversity of oligosaccharide ligands, mainly gangliosides and glycoaminoglycans, some of which are present on NK-susceptible tumor target cells (Bezouska *et al.*, 1994). The saccharide ligands were shown to compete with soluble recombinant rNKR-P1 for binding to YAC-1 cells. They also inhibited the lysis of YAC-1 cells by fresh NK cells and by

RNK-16 cells. Moreover, incubation of RNK-16 cells with liposome-incorporated but not isolated ligands elicited phosphoinositide turnover and a rise in cytoplasmic Ca^{2+} to levels comparable with those observed after cross-linking of NKR-P1 with antibodies. This confirmed that cross-linking of rNKR-P1 molecules at the cell surface was required to transduce the activation signal. Most interestingly, a clinical relevance for this finding is provided by the observation that NK-resistant P815 target cells were killed almost as efficiently as YAC-1 targets by fresh NK cells following a preincubation of the target cells with saccharide ligand-containing liposomes. Although rNKR-P1 can bind carbohydrates, it may also recognize a peptide epitope. A similar situation has been reported for the low-affinity receptor for IgE (CD23), another member of the C-type lectin family, which can bind glycosylated epitopes of IgE (Richards and Katz, 1990; Delespesse *et al.*, 1992) but can also bind IgE in a carbohydrate-independent manner (Verselli *et al.*, 1989).

Activation of NK cell lysis through the NK1.1 molecule, the murine homologue of rNKR-P1, has also been described (Karlhofer and Yokoyama, 1991). Indeed, redirected lysis of the FcR^+ Daudi target cells, but not of YAC-1 or other FcR^- tumor cells, by fresh and IL-2-activated NK cells from C57BL/6 mice is obtained in the presence of PK136 anti-NK1.1 mAb. Since no lysis of Daudi target cells was observed when the Fc portion of PK136 was blocked with protein A, the lysis of Daudi cells appears to require the Fc portion of the PK136 mAb. Garni-Wagner *et al.* (1993) have observed that PK136 mAb can induce lysis not only of FcR^+ target cells but also of YAC-1 (FcR^-) cells, suggesting that the PK136 mAb can directly activate NK cell lysis. Nevertheless, Fab fragments of

PK136 failed to enhance the lysis of YAC-1 cells indicating that the Fc portion of the mAb was a prerequisite. Overall, the activation mechanism of NK cell lysis through the NK1.1 antigen has yet to be clarified. It has been suggested that under certain conditions, NK1.1 could be cross-linked when the Fab portion of PK136 mAb is bound to an NK1.1 molecule of one effector cell and its Fc portion is engaged by an FcγR of an adjacent NK cell (Garni-Wagner *et al.*, 1993). This hypothesis remains however to be verified.

The 2B4 antigen has also been described as a function related receptor on murine NK cells (Garni-Wagner *et al.*, 1993). In the presence of anti-2B4 mAb, the lytic activity of IL-2-activated but not freshly isolated murine NK cells against FcR^+ and FcR^- target cells was significantly enhanced. Contrary to the observation made with NK1.1, anti-2B4 Fab fragments were as efficient as intact mAb in increasing lysis of YAC-1 cells, clearly indicating that the augmentation of NK cell killing through the 2B4 is FcR-independent. Supporting the involvement of 2B4 in NK cell activation, anti-2B4 mAb was also shown to stimulate IFN-γ secretion and granule exocytosis by IL-2-activated NK cells. Although no consensus sequence for tyrosine phosphorylation is contained in the 2B4 sequence, signal transduction could occur via serine, threonine, or tyrosine phosphorylation of the 2B4 protein since a number of these residues are present in its cytoplasmic domain (Mathew *et al.*, 1993).

Finally, NK cell antigen detected by the 4LO3311 mAb, namely NK2.1, was shown to trigger cytotoxic activity of both fresh and IL-2-activated NK cells (Morelli and Lemieux, 1993). Cells expressing the 4LO3311

antigen were found to be significantly more lytic against YAC-1 targets than 4LO3311$^-$ cells. This observation suggested that the 4LO3311 antigen could be involved in the triggering of NK cell killing. Consistent with this hypothesis, immobilized 4LO3311 mAb induced granule exocytosis by LAK cells and binding of soluble mAb selectively enhanced the lysis of FcγR$^+$ and FcγR$^-$ NK-sensitive target cells. Activation of NK cells with 4LO3311 mAb was confirmed to be Fc-independent as F(ab')$_2$ and Fab fragments of 4LO3311 mAb were as efficient as intact mAb in enhancing NK cell-mediated cytolytic activity. However, killing of NK-resistant P815 target cells was not induced by 4LO3311 mAb. Considering that the resistance of the P815 target to NK cell killing is due to its inability to form conjugates with fresh NK cells (Roder and Kiessling, 1978), activation through the 4LO3311 antigen would appear to take place at a post-binding step.

1.5.3 - MHC class I molecules

Since its formal identification, NK cell activity has been considered to be MHC-unrestricted because allogeneic as well as xenogeneic cells and MHC-deficient cells were killed by NK cells. However, accumulating data indicate that expression of MHC class I molecules dictates the sensitivity of a cell to NK lysis. In the human and the mouse, the susceptibility of target cells to NK cell lysis has been inversely correlated with the level of MHC class I expression (Trinchieri, 1994; Kärre, 1995). Because of the considerable amount of information on this topic, only the most striking results and the most recent findings will be discussed.

One of the first suggestions that MHC class I expression plays a role in resistance to NK cell lysis came with the observation that the murine prototype NK-sensitive YAC-1 cell line became resistant to NK cell lysis when grown *in vivo*, but returned to its sensitive status when recultured *in vitro*, concomitant with a decreased MHC class I expression (Becker *et al.*, 1979). It was later reported that certain MHC antigen-deficient lymphomas were rejected *in vivo* more readily than MHC antigen-expressing lymphomas (Kärre *et al.*, 1986). In addition, hemopoietic cells from MHC class I-deficient mice were rejected by lethally irradiated, wild type mice unless the recipients were depleted of NK cells (Bix *et al.*, 1991). It was also reported that resistance to NK cell-mediated lysis can be restored by reconstitution of MHC class I expression through transfection of the gene encoding β_2-microglobulin (Quillet *et al.*, 1988; Ljunggren *et al.*, 1990; Sturmhöfel and Hämmerling, 1990; Maio *et al.*, 1991). In another system, Glas *et al.* (1995) recently reported that rejection of certain H-2^b tumor cell lines by SCID mice was abrogated by introduction of H-$2D^d$ but not H-$2K^d$ or L^d into these allogeneic tumors. The observed rejection of tumor cells was dependent on NK cells since all tumor cells grew progressively when inoculated into SCID mice depleted of asialo $GM1^+$ cells, regardless of MHC class I expression.

The correlation between reduced MHC class I expression and NK sensitivity appears not to be universally applicable. Indeed, transfection of the NK susceptible H-$2K^b$ loss variant of the murine BW7756 hepatoma (Hepa-1) with the H-$2K^b$ gene had no effect on its susceptibility to NK cell killing (Nishimura *et al.*, 1988). In addition, two murine lymphoma cell lines (ASL1 and ASL1w), both expressing high levels of H-2K and H-2D

proteins, differed greatly in their susceptibility to NK cell activity (Chervenak and Wolcott, 1988).

Similar observations were made in the human system where the NK susceptibility of three series of cloned cell lines was shown to vary inversely with target cell class I human leukocyte antigen (HLA) expression (Storkus *et al.*, 1987). Moreover, transfection of MHC class I genes into a series of NK susceptible class I-deficient cell lines confered resistance to NK cell-mediated lysis (Storkus *et al.*, 1989a; Shimizu and DeMars, 1989). However, other studies indicated to the contrary that susceptibility of target cells to NK cell activity was independent of the level of HLA-A, -B, or -C expression by target cells (Leiden *et al.*, 1989; Peña *et al.*, 1990).

The role of MHC class I expression in NK cell lysis thus appears controversial. Nevertheless, it is now generally agreed that expression of MHC class I molecules or at least certain alleles on target cells has an inhibitory effect on NK cell lysis, possibly by overruling triggering signals. More precisely, it has been reported that MHC class I expression on target cells can deliver an inhibitory signal to NK cells without blocking target recognition (Kaufman *et al.*, 1993b) and that the resistance to NK cell-mediated cytotoxicity in the human and the mouse was conferred by the $\alpha 1/\alpha 2$ domains of MHC class I molecules, in or near the peptide-binding cleft (Storkus *et al.*, 1989b; Karlhofer *et al.*, 1992; Kane, 1994; Sentman *et al.*, 1994). In the human, critical amino acid residues in the peptide-binding groove have been identified at position 80 of HLA-B molecules (Moretta *et al.*, 1994; Litwin *et al.*, 1994; Cella *et al.*, 1994) and

at positions 77 and 80 of HLA-C molecules (Ciccone *et al.*, 1992b; Colonna *et al.*, 1993a; 1993b).

If the peptide groove of MHC class I molecules is implicated in the protection against NK cell cytotoxicity, one could expect that the peptide carried by these molecules might be involved. The importance of the peptide was very recently studied in the murine and the human systems using the transporter for antigen presentation (TAP)-deficient mouse cell line RMA-S (Correa and Raulet, 1995; Malnati *et al.*, 1995). The TAP molecules are responsible for importing peptides from the cytoplasm to the endoplasmic reticulum where they associate with MHC class I molecules. In TAP-deficient cells, some empty class I molecules will nonetheless reach the cell surface but in a state which is highly unstable and will thus rapidly dissociate at 37°C resulting in sensitivity to NK cell lysis (Townsend *et al.*, 1989; Franksson *et al.*, 1993). However, when the cells are cultured at 26°C, empty MHC class I molecules maintain their integrity and can be stabilized by loading with exogenous peptides (Ljunggren *et al.*, 1990; Rock *et al.*, 1991). Correa and Raulet (1995) reported that, in the mouse, inhibition of NK cell activity occurred with almost all the H-2D^d-binding peptides they tested, suggesting that NK cells recognize class I-peptide complexes largely independently of peptide composition. On the other hand, Malnati *et al.* (1995) reported that only specific self peptides that bind to the HLA-B27 MHC class I molecule confer protection from NK cell lysis. It thus appears unclear whether the MHC molecule itself or an epitope formed by the MHC molecule and the peptide confers protection. Alternatively, it has been proposed that protective self peptides promote interaction of MHC class I with a

particular NK cell subset (Kärre, 1995). This hypothesis would explain why cells expressing a normal level of MHC class I molecules but loaded with foreign peptides, like virally-infected cells, are efficiently killed by NK cells.

1.5.4 - Inhibitory molecules

Two models have been proposed to explain the mechanism by which expression of MHC class I molecules may mediate protection against NK cell cytotoxicity (Ljunggren and Kärre, 1990). The "target interference" or "masking" model postulates that interaction of MHC class I molecules with an undefined target structure would prevent recognition by NK cells and thus, killing. This model implies that a target structure should be capable of triggering NK cell lysis and be accessible to interference by MHC class I molecules. Such a target structure has not as yet been identified. In the "effector inhibition" or "negative signaling" model, the initial triggering of NK cell activity by broadly distributed target structures would be overruled by an inhibitory signal from a specific receptor on NK cells upon recognition of particular MHC class I alleles on the target cell. More experimental results agree with the second model and inhibitory receptors have been identified and characterized. According to the "effector inhibition" model, a summary of the possible situations occurring when an NK cell encounters a putative target cell is illustrated in Figure 1.

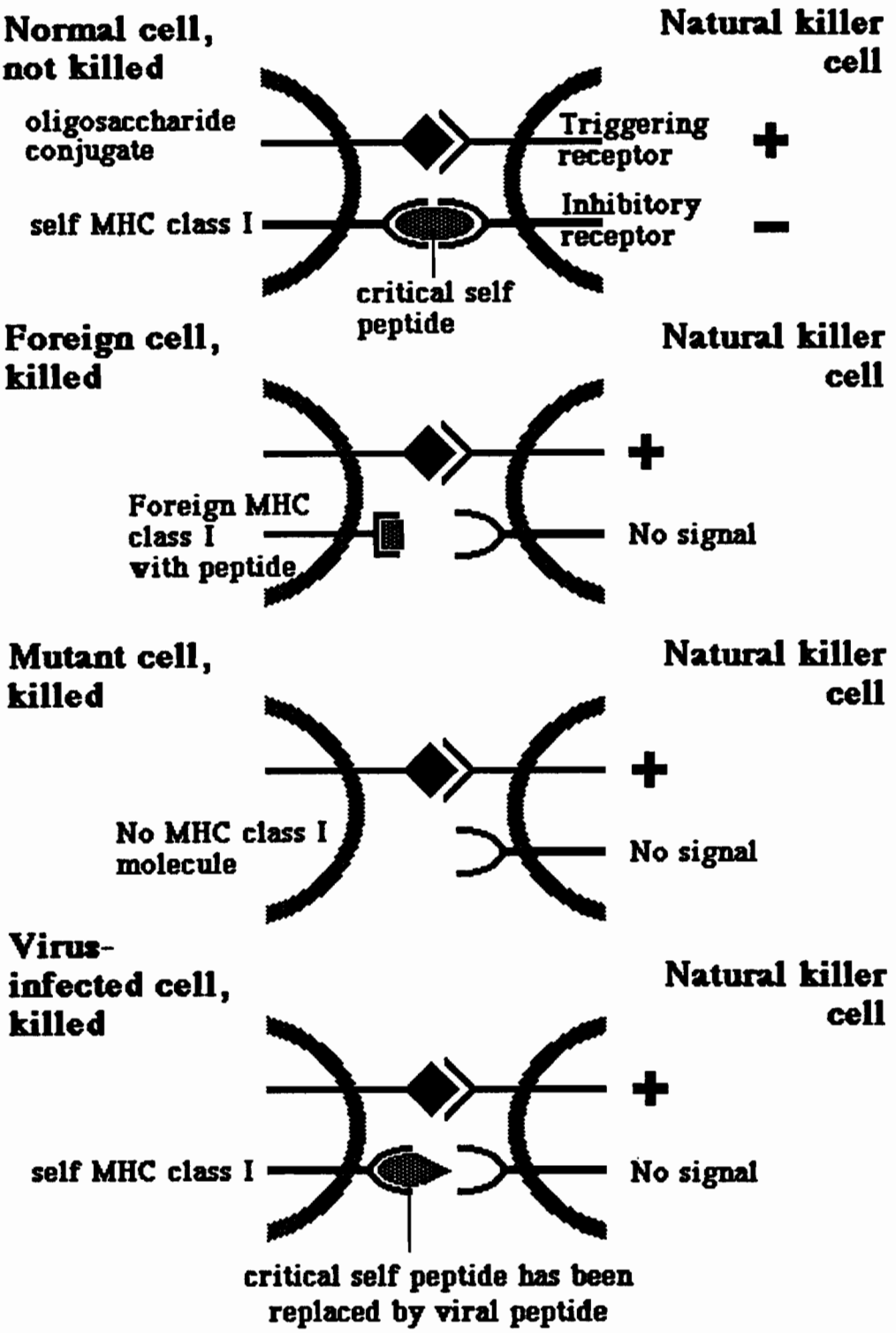

Figure 1. How natural killer cells selectively eliminate nonself cells (adapted from Kärre, 1994).

1.5.4.1 - Mouse inhibitory receptors

Ly-49A$^+$ and Ly-49A$^-$ NK cells are identical with respect to their phenotype (NK1.1$^+$, FcγRIII$^+$, asialo GM1$^+$) and their capacity to kill YAC-1 targets. However, whereas Ly-49A$^+$ cells were unable to kill most H-2^d and H-2^k target cells, these target cells were efficiently killed by Ly-49A$^-$ cells (Karlhofer *et al.*, 1992). It has also been demonstrated that NK-susceptible target cells became resistant to lysis by Ly-49A$^+$ cells after transfection with cDNA encoding H-2D^d but not H-2K^d or H-2L^d and that this resistance was also extended to other killing mechanisms including ADCC and redirected lysis. In fact, it appears that all types of NK cell-mediated lysis of lymphoblasts, of tumor cells, and of almost any target by ADCC can be inhibited by appropriate class I expression on the target cell (Correa *et al.*, 1994). Direct binding of Ly-49A to H-2D^d and D^k, but not H-2K^b, K^d, K^k, or D^b was shown using the EL4 lymphoma cell line, which expresses Ly-49A, and H-2 molecules immobilized on plastic (Kane, 1994). Ly-49A appears to engage the α1/α2 domains of H-2D^d since antibodies to Ly-49A or to α1/α2 but not α3 domains of H-2D^d inhibit binding of Ly-49A$^+$ cells to isolated H-2D^d molecules (Kane, 1994) and restore killing of target cells expressing this MHC class I molecule (Karlhofer *et al.*, 1992). The interaction between Ly-49A and H-2D^d or D^k also appears to affect the NK cell repertoire (Karlhofer *et al.*, 1994; Olsson *et al.*, 1995). To avoid redundancy, I will not describe here this aspect of the interaction between Ly-49A and H-2D^d and D^k since it will be discussed in detail in chapter 4.

Although definitive proof is still missing, binding of Ly-49A to H-2D^d could involve the carbohydrates of MHC class I molecules. Indeed, as for the rNKR-P1, the CRD of the Ly-49A molecule appears to be functional since treatment of H-2D^d target cells with tunicamycin restored their susceptibility to Ly-49A$^+$ NK cell lysis (Daniels *et al.*, 1994). The anionic polysaccharide fucoidan, which is composed of fucose residues, some of which are sulfated (Patankar *et al.*, 1993), was shown to bind specifically to CHO cells transfected with the Ly-49A cDNA in a calcium-dependent manner (Daniels *et al.*, 1994). This binding was moreover inhibited by sulfated glucose and galactose.

Apart from Ly-49A, no other inhibitory receptor has been identified thus far in the murine model but the 5E6 molecule, encoded by the Ly-49C gene is a logical candidate. In fact, COS cells transfected with Ly-49C cDNA can bind H-2^d, H-2^k, H-2^b, and H-2^s cell lines, and antibodies to either H-2^s or Ly-49C molecules inhibit this binding, suggesting that the Ly-49C molecule might, as does Ly-49A, react with MHC class I molecules (Brennan *et al.*, 1994). Although the ligand for Ly-49C remains to be determined, recent studies by Brennan *et al.* (1995) indicate that Ly-49C mediates carbohydrate recognition. More precisely, the sulfated glycans fucoidan, λ-carrageenan, and dextran sulfate were found to inhibit adhesion of H-2^s cells and binding of 5E6 mAb to Ly-49C-tansfected COS cells.

1.5.4.2 - Human inhibitory receptors

In the human, the proposed NK cell inhibitory receptors are designated p58, Kp43 (CD94), and NKB1. Two distinct members of the p58 molecular family have been identified with GL183 (Moretta *et al.*, 1990a) and EB6 or HP3E4 (Moretta *et al.*, 1990b; Melero *et al.*, 1994) mAbs. GL183 and EB6 molecules are expressed on subpopulations of $CD16^+CD56^+$ cells either as a single chain 58 kDa protein or more frequently as a 58 kDa protein noncovalently associated with a 55 kDa polypeptide. On the basis of GL183 and EB6 expression, four different NK cell subsets have been identified (Moretta *et al.*, 1990b), suggesting that NK cells are equipped with clonally distributed receptors. The Kp43 antigen is somewhat different. It is a 70 kDa disulfide-linked dimer expressed by a variable proportion of $CD16^+$ NK cells, a subset of $TCR\gamma\delta^+$ T cells, and 10% of the $CD8^+CD56^+TCR\alpha\beta^+$ T cell clones that have been tested (Aramburu *et al.*, 1990). Upon stimulation with IL-2, essentially all NK cells will express Kp43 albeit at varying levels. By flow cytometry analysis, three distinct $Kp43^+$ groups of clones have been identified among GL183/EB6 phenotypic groups from a single individual namely $GL183^-EB6^-$, $GL183^+EB6^+$, and $GL183^-EB6^+$ clones with low to high levels of Kp43 expression (Moretta *et al.*, 1994). Finally, the NKB1 receptor has recently been identified as a 70 kDa glycoprotein expressed by a subset of NK cells and NK cell clones (Litwin *et al.*, 1994).

The availability of NK cell clones in the human system have allowed the identification of distinct groups of alloreactive clones. Such clones, which can be derived from a single individual, have been divided into at least

five groups on the basis of their patterns of cytolytic activity against a panel of allogeneic phycohemaglutinin (PHA) blasts (Ciccone *et al.*, 1992a) or their recognition of MHC class I molecules (Colonna *et al.*, 1993a; 1993b; Cella *et al.*, 1994). The expression of given HLA-B and HLA-C alleles have been associated with protection from NK cell lysis by distinct subsets of NK clones (Ciccone *et al.*, 1992b; Moretta *et al.*, 1993; 1994; Gumperz *et al.*, 1995). More precisely, the ability to discriminate between two groups of HLA-C alleles, represented by Cw1, Cw3, Cw7, Cw11, and by Cw2, Cw4, Cw6, have been attributed to GL183$^+$EB6$^+$ and GL183$^-$ EB6$^+$ NK clones, respectively (Moretta *et al.*, 1990a; 1990b). The reactivity to HLA-B7 and Bw4 alleles have more recently been attributed to NK clones expressing Kp43 and NKB1, respectively (Moretta *et al.*, 1993; Gumperz *et al.*, 1995). Even though expression of certain HLA-A alleles have been associated with protection from killing by some NK clones, the NK cell receptor mediating that protection has not as yet been identified (Storkus *et al.*, 1991). The NK cell repertoire is further enhanced by the existence of clones expressing various combinations of the p58 receptors with Kp43 as previously discussed. Interestingly, co-expression of the two p58 receptors on some NK clones appears to result in their inhability to kill all normal HLA-C$^+$ target cells (Vitale *et al.*, 1995). Overall, the expression of the various combinations of the different inhibitory receptors probably account for the NK cell repertoire. This repertoire, however, is not believed to be as extended as the T- or B-cell repertoire, given that a sizeable fraction of NK cells in a given individual are alloreactive NK clones.

Recently, four NK-specific cDNAs, designated NK-associated transcripts NKAT1, NKAT2, NKAT3, and NKAT4 have been cloned from human NK cells (Colonna and Samaridis, 1995). The predicted proteins encoded by these genes are members of the Ig supergene family. Most interestingly, the NKAT1 gene was shown to encode the EB6/HP3E4 molecule. The NKAT2 gene was expressed in NK clones with different HLA-C specificities and was proposed to encode the p58 receptor detected by the GL183 mAb. This has, however, not been clearly demonstrated. Finally, either NKAT3 or NKAT4 or both genes were expressed in NK clones that were inhibited by Bw4 alleles, suggesting that one or both of these genes might code for NKB1.

The characterization of p58 and Kp43 molecules suggested that they were involved in the activation of NK cell lysis. Indeed, stimulation of NK cells with anti-GL183 mAb plus PMA resulted in TNF-α secretion and cross-linking of GL183 with specific mAb induced $[Ca^{2+}]_i$ increase in GL183$^+$ clones (Moretta *et al.*, 1990a). Intact GL183 mAb or its F(ab')$_2$ fragment and EB6 mAb also increased the cytolytic activity of NK cell clones toward different human tumor target cell lines (Moretta *et al.*, 1990a; 1990b). Similarly, it was reported that incubation of IL-2-activated NK cells with either anti-Kp43 mAb or its F(ab')$_2$ fragment induced significant lysis of normally resistant autologous and allogeneic T cell blasts (Aramburu *et al.*, 1991) and that TNF-α production was also triggered through the Kp43 molecule (Aramburu *et al.*, 1993). It thus appears possible that NK receptors can transduce inhibitory or activation signals depending on their affinity for MHC class I, and possibly critical peptides, as well as on other undefined physiological conditions.

Unlike its mouse and rat homologues, hNKR-P1A was not able to mediate redirected lysis of FcR+ target cells by freshly isolated peripheral blood NK cells (Lanier *et al.*, 1994). Moreover, although the anti-hNKR-P1A mAb did not affect the NK killing of human K562 target cells, intact anti-hNKR-P1A mAb but not its $F(ab')_2$ fragment inhibited the spontaneous killing of murine P815 targets by certain human NK cell clones. A possible explanation could be that when hNKR-P1A molecules are sufficiently cross-linked by anti-hNKR-P1A mAb, a negative signal is transmitted into the NK cell. Hence, hNKR-P1A would have opposite signaling functions compared to its rat and mouse homologues. Alternatively, hNKR-P1A may not represent the human homologue of rodent NKR-P1 but may rather be a member of the Ly-49 family or may be related to the p58 or Kp43 molecules. One could thus hypothesize that hNKR-P1A should bind to an HLA class I molecule. However, when hNKR-P1A+ and hNKR-P1A- NK cell clones were analyzed for their ability to recognize HLA class I-deficient EBV-LCL cell lines, transfected with 19 different HLA-A, -B, or -C alleles, no correlation between hNKR-P1A expression and HLA class I recognition was observed. Further studies are thus required to clarify the exact role of hNKR-P1A and its relationship with activation and inhibitory receptors identified thus far.

Although the signals transmitted by most of the activation receptors have been described, the intracellular events following crosslinking of the inhibitory receptors still remain undefined. Association of p58 molecules with ζ:ζ, ζ:γ, or γ:γ as well as with the signal transducing $p56^{lck}$ molecule have been reported but, under experimental conditions where signaling through CD16 resulted in tyrosine phosphorylation of the CD16-associated

ζ chain, no phosphorylation of the p58-associated ζ chain has been observed following activation through p58 receptors (Bottino *et al.*, 1994). Engagement of the MHC class I specific receptor may block this activation cascade, but this would have to be downstream of early activation events since the increased $[Ca^{2+}]_i$ and phosphatidylinositol turnover, are not inhibited by MHC class I recognition (Kaufman *et al.*, 1993b).

1.5.5 - Accessory molecules

In addition to the receptors that activate or inhibit NK cell lysis, a number of accessory molecules appear to be involved in NK cell-mediated cytotoxicity (Hersey and Bolhuis, 1987). I will limit the discussion to three of particular interest, CD45, CD56, and the β2 integrins.

Alternative splicing of a single CD45 gene defines a series of mouse cell surface glycoproteins, ranging in relative molecular mass from 180 000 to 220 000, expressed exclusively on hematopoietic cell lineages (Scheid and Triaglia, 1979; Tung *et al.*, 1981; Saga *et al.*, 1988). Murine NK cells express the smallest isoform of CD45 also known as Ly-5 or T200 (Kasai *et al.*, 1979). The involvement of CD45 in NK cell lysis was first suggested by the ability of anti-Ly-5 antibodies to block the binding of NK cells to YAC-1 target cells (Kasai *et al.*, 1979). More recently, the critical role of CD45 in regulating the signaling responses of rat NK cells was demonstrated using a $CD45^-$ mutant of the rat NK cell line RNK-16 (Bell *et al.*, 1993). These mutant cells were unable to kill tumor targets and cross-linking of NKR-P1 or CD2 with specific mAbs did not induce tyrosine phosphorylation and increased $[Ca^{2+}]_i$ in these mutants as in wild-type

RNK-16. It is unclear how the CD45 molecule participates in NK cell-mediated activity, but the presence of two tandem domains with tyrosine phosphatase activity in the cytoplasmic region of CD45 (Tonks *et al.* 1988; Charbonneau *et al.*, 1988) suggests that dephosphorylation of a yet undefined protein is required to remove an inhibitory signal. Such a regulatory mechanism has been observed in T- and B-cell responses (Koretzky *et al.*, 1991; Justement *et al.*, 1991).

The CD56 antigen, defined by the anti-Leu-19 and NKH-1 mAbs, is a 200 to 220 kDa transmembrane isoform of the neural cell adhesion molecule (NCAM, Griffin *et al.*, 1983; Hercend *et al.*, 1985; Lanier *et al.*, 1989b). This antigen is a member of the immunoglobulin supergene family (Cunningham *et al.*, 1987) and is expressed by virtually all human NK cells and by a subset of T cells and IL-2-activated thymocytes mediating MHC-unrestricted cytotoxicity (Lanier *et al.*, 1987). Even though most human NK cells are defined as $CD3^{-}CD16^{+}CD56^{+}$, CD56 is far from being restricted to NK cells since it is expressed by several cell types in hematopoietic, muscle, and neural tissues (McClain and Edelman, 1982; Lanier *et al.*, 1987). The NCAM expressed by neural and muscle tissues is involved in homotypic adhesive interactions (Edelman, 1986). Similarly, the CD56 antigen expressed by human NK cells can also function as an adhesion molecule in NK-target interactions (Nitta *et al.*, 1989). However, using a panel of $CD56^{-}$ mutant and $CD56^{+}$ transfectant target cells, it was found that expression of CD56 on these targets did not affect their susceptibility to fresh or IL-2-activated NK cell-mediated cytotoxicity, suggesting that the homophilic CD56 interaction does not have a major role in NK cell cytotoxicity (Lanier *et al.*, 1991b).

The β2 integrins share a common β chain (CD18) noncovalently associated with one of the three α subunits CD11a (LFA-1), CD11b (Mac-1), or CD11c (p150/95) to form adhesion complexes (Arnaout, 1990). Although NK cells express the three CD11/CD18 integrins, neither CD11b/CD18 nor CD11c/CD18 are directly involved in the binding of NK cells to target cells. However, interaction of CD11a/CD18 with intercellular adhesion molecule-1 (ICAM-1 or CD54) or ICAM-2 appears to form an important adhesion complex in NK cell-mediated lysis (Timonen *et al.*, 1988). Indeed, a strong correlation has been observed between the level of LFA-1 expression and NK cell activity (Nishimura and Itoh, 1988). In humans, the role of CD11a/CD18 and ICAM-1 was further demonstrated by inhibition of the attachment of NK cells to susceptible targets and of NK cell-mediated activity by anti-CD11a mAbs (Schmidt *et al.*, 1985; Timonen *et al.*, 1988) or soluble forms of ICAM-1 (Becker *et al.*, 1991). More recently, Aramburu *et al.*, (1993) reported that the secretion of TNF-α induced by triggering human LAK cells with anti-Kp43 mAb was inhibited by anti-CD11a, anti-CD18, and anti-ICAM-1 mAbs.

1.6 - OBJECTIVES OF THE RESEARCH PROJECT

Natural killer cells are undoubtedly important elements of the immune system, particularly in the non-specific first line of defense preceding the specific T- and B-cell responses, and also contribute to preserve the general homeostasis of the body. The different NK cell-mediated activities are regulated through a number of cell surface receptors involved in the binding of NK cells to their target cells and in the triggering or inhibition of NK cell-mediated functions.

When this study was initiated, most of the rodent and human function-associated NK cell antigens had only recently been identified and some had been partly characterized. In the mouse, the NK1.1 and NK2.1 antigens, which had not yet been either structurally or functionally characterized, were considered to be the most characteristic NK cell markers. Immunization of 129/Sv ($NK1.1^-NK2.1^-$) mice with NK-enriched spleen cells from C57BL/6 ($NK1.1^+NK2.1^+$) mice had allowed us to produce anti-NK mAbs (Lemieux *et al.*, 1991, Appendix I). One of them, the 4LO3311 mAb, reacted with an antigen specifically expressed by a subpopulation of splenic NK cells having the same strain distribution as the NK2.1 antigen, suggesting that the 4LO3311 mAb might be reacting with the NK2.1 antigen. The antigen detected by the 4LO3311 mAb is of particular interest based on its broad distribution among the most commonly used laboratory mouse strains as compared with other murine NK cell antigens, particularly NK1.1, and its implication in activation of the NK cell activity. Therefore, in an attempt to define this antigen, its expression in NK cell subpopulations, and its relationship to other function-associated NK cell antigens, the following objectives were set:

- to biochemically characterize the alloantigen identified with the 4LO3311 mAb
- to verify the putative identity between the NK2.1 alloantigen and the molecule detected by the 4LO3311 mAb
- to investigate the regulation of the 4LO3311-reactive alloantigen expression
- to map the gene encoding the protein recognized by the 4LO3311 mAb.

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CHAPTER 2

"The murine NK2.1 antigen: A 130 kDa glycoprotein dimer expressed by a natural killer cell subset of the spleen, thymus, and lymph nodes"

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FOREWORD

A number of antigens principally expressed by murine NK cells and having distinct strain distributions have been described in chapter 1. Of these antigens, NK1.1 is considered by many as a pan-NK cell marker. Even though this antigen has a narrow strain distribution, the production of the PK136 anti-NK1.1 mAb was a major breakthrough in the study of murine NK cells. Indeed, this mAb has been very useful in studying the heterogeneity and biological relevance of murine NK cells.

The NK2.1 antigen, which was originally identified by an NZB anti-BALB/c antiserum, has a broader strain distribution than NK1.1. It is indeed expressed in most strains of mice commonly used in research. As mentioned in the first chapter, our laboratory had recently derived anti-NK mAb producing hybridomas from 129 anti-C57BL/6 immune spleen cells. Interestingly, the strain distribution of the antigen detected by one of these mAbs, the 4LO3311 mAb, was similar to that of the NK2.1 antigen. Although the selection of these anti-NK mAb-producing hybridomas and the formal characterization of their strain and cell distributions were not part of my research project, I significantly contributed to these experiments which constituted the basis of my Ph. D. thesis. Since the 4LO3311 mAb has been extensively used for the experiments described in this thesis, I have appended a reprint of the original paper, in which I was a co-author, describing the production and initial characterization of this mAb (Appendix I).

At the beginning of this study, the nature and function of NK1.1 and NK2.1 were unknown. In this chapter, the results obtained pertaining to the first two objectives of my research project are presented. We determined some of the biochemical properties of the antigen detected by the 4LO3311 mAb. The NK1.1 antigen was concurrently characterized from NK-enriched spleen cell suspensions. Most importantly, we provided evidence that the 4LO3311 mAb reacted with the NK2.1 antigen. Finally, we extended the cell and tissue distribution of the NK2.1 antigen by showing that in addition to being expressed by a population of splenic NK cells, the NK2.1 antigen was present on minor subpopulations of the thymus and lymph nodes. However, unlike the rat NKR-P1, neither NK1.1 nor NK2.1 were found on PMN leukocytes.

ABSTRACT

Murine natural killer (NK) cells express a few antigens not found on other leukocyte subsets. The NK1.1 antigen, that is present in only a few mouse strains, has been extensively characterized whereas our knowledge of the NK2.1 antigen, which is more commonly expressed, remains, as yet limited. Our laboratory has previously reported the production of a mAb (4LO3311) recognizing a murine NK cell-specific molecule with a similar strain distribution as the NK2.1 antigen formerly defined with an NZB anti-BALB/c antiserum. In this study, we demonstrate by sequential immunoprecipitation that 4LO3311 represents the first NK2.1 antigen-specific mAb. This reagent was used to immunoprecipitate the NK2.1 antigen from ^{125}I-labeled lysates of fresh NK-enriched spleen cells. SDS-PAGE analyses revealed that the NK2.1 antigen is expressed at the cell surface as a *N*-glycosylated disulfide-linked protein dimer with ~65 kDa subunits. The NK2.1 antigen is likely to be anchored in the plasma membrane by a peptide moiety since its expression on NK cells was not affected by treatment with phosphatidylinositol-specific phospholipase C. In addition to being present on a splenic NK cell subset, the NK2.1 antigen is shown to be expressed by a small number of $CD4^-CD8^-$ thymocytes and by a subset of $CD4^-CD8^-IgG^-$ lymph node cells. Finally, it is shown here that unlike the NKR-P1, the rat homologue of the murine NK1.1 antigen, neither the NK2.1 nor the NK1.1 antigen is expressed by polymorphonuclear leukocytes.

INTRODUCTION

Natural killer (NK) cells constitute a leukocyte subset distinct from mature T and B lymphocytes and from cells of the myeloid lineage [reviewed in (Trinchieri, 1989)]. Although they share several surface antigens with other cell types, NK cells also express specific molecules considered to be NK cell markers. Several of the NK antigens of rodent NK cells have been recently characterized from IL-2-propagated NK cells or NK cell lines. The rat NKR-P1 antigen is a disulfide-linked protein dimer composed of 30 kDa subunits (Chambers *et al.*, 1989). Giorda *et al.* (1990) cloned the gene encoding the NKR-P1 molecule and reported a significant amino acid homology with C-type lectins. Corresponding mouse NKR-P1 genes have also recently been cloned (Giorda and Trucco, 1991; Yokoyama *et al.*, 1991). These genes map to a region of the chromosome 6 identified as the NK complex (Yokoyama *et al.*, 1991). This region also includes a multigene family encoding Ly-49 (Yokoyama *et al.*, 1990), a murine antigen having similar biochemical properties with the rat NKR-P1 antigen (Chan and Takei, 1986; Nagasawa *et al.*, 1987). The Ly-49 antigen is expressed by a minor subset of NK cells (Yokoyama *et al.*, 1990).

Although the NK1.1 antigen, detected by the PK136 mAb, is expressed by most NK cells of C57BL/6 mice, this antigen cannot be considered as a pan-NK cell marker since only a few mouse strains are NK1.1$^+$ (Koo and Peppard, 1984; Sentman *et al.*, 1989a). Furthermore, our laboratory has recently established that the NK1.1 antigen is not expressed by all NK cells in NZB mice (Morelli *et al.*, 1992). The NK1.1 antigen has been detected on a subclone of the CTLL-2 cell line from which it was first partially

characterized as a 39 kDa molecule by SDS-PAGE analysis under reducing conditions (Sentman *et al.*, 1989b). The NK1.1 antigen immunoprecipitated from C57BL/6 IL-2-stimulated spleen cells was recently confirmed to be a glycosylated disulfide-linked homodimer and, most importantly, was identified as the mouse homologue of the rat NKR-P1 antigen (Ryan *et al.*, 1992).

The NK2.1 antigen, identified with an NZB anti-BALB/c antiserum, is an NK cell-specific marker expressed in most mouse strains commonly used in laboratory research (Pollack and Emmons, 1982). Surprisingly, even though this antigen was identified several years ago, its molecular structure remains undefined, most likely because of the unavailability of a specific mAb.

We have recently produced a mAb (4LO3311) recognizing a molecule specifically expressed by a subset of murine NK cells (Lemieux *et al.*, 1991; Morelli *et al.*, 1992). Given the similar strain distribution of the antigen identified by this mAb to the one detected by the NZB anti-BALB/c antiserum, we hypothesized that the two reagents may well detect the same molecule. In the present study, we demonstrate by sequential immunoprecipitation that the 4LO3311 mAb does indeed recognize the NK2.1 antigen on a splenic NK cell subset. Importantly, the NK2.1 and the NK1.1 antigens were immunoprecipitated and biochemically characterized from fresh NK-enriched spleen cells rather than IL-2-propagated NK cells or NK cell lines. We also show that the NK2.1 antigen is expressed by a minor population of peripheral and mesenteric lymph node cells depleted of T and IgG^+ cells and by a subset of $CD4^-CD8^-$ thymocytes. Unlike the

rat NKR-P1, neither the NK2.1 nor the NK1.1 antigen is expressed by PMN leukocytes. The putative relationship between the NK2.1 antigen and the members of the NKR-P1 family is discussed.

MATERIALS AND METHODS

Mice

BALB/cAnN, C57BL/6N, and C3H/HeN mice were obtained from Charles River Canada Inc. (St. Constant, Québec) and NZB/BINJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice of either sex were used at 6-10 weeks of age.

Monoclonal antibodies and anti-NK2.1 antiserum

The production of the 4LO3311 (IgG_3) mAb, which reacts with NK cells of selected mouse strains, has been previously described (Lemieux *et al.*, 1991). The PK136 (IgG_{2a}) anti-NK1.1 mAb-secreting hybridoma was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Affinity-purified 4LO3311 and PK136 mAbs were conjugated to biotin or FITC as described (Lemieux *et al.*, 1991). An unrelated mouse IgG_3 mAb produced in our laboratory and an IgG_{2a} mAb kindly provided by Dr. P. J. Talbot (Institut Armand-Frappier) were used as isotype controls. The hybridoma producing a rat anti-mouse CD4 (IgG_{2b}) mAb (clone MT4) was a gracious gift of Dr. F. J. Dumont (Merck, Sharp, and Dohme Research Laboratories, Rahway, NJ) and the rat anti-mouse CD8 (IgG_{2a}) mAb-secreting hybridoma (clone 53-6.72) was purchased from ATCC. FITC-conjugated rat anti-mouse Thy-1.2 (IgG_{2b}) mAb was obtained from Becton Dickinson (Mountain View, CA). The anti-Ly-5.2

(IgG_{2a}) mAb was purchased from Cedarlane Laboratories Ltd (Hornby, Ontario) and conjugated to FITC.

An NZB anti-BALB/c (anti-NK2.1) antiserum was produced by the method described by Pollack and Emmons (1982) modified as follows: 10-week-old NZB male mice were injected i.p. with 6 x 10^5 BALB/c NK-enriched spleen cells (see below) and boosted twice i.v. with 2 x 10^6 cells at biweekly intervals. Immunized mice were bled and their sera tested individually on syngeneic spleen cells by complement-dependent cytotoxicity to ensure the absence of autoantibodies. To detect anti-NK antibodies, sera were tested on C3H spleen cells by complement-dependent NK-inhibition assay (Lemieux *et al.*, 1991) using a nonimmune NZB serum and the 4LO3311 mAb as negative and positive controls, respectively. NK-reacting antisera devoid of detectable autoantibodies were pooled for subsequent analyses.

Preparation of NK-enriched spleen cell suspensions

Splenic NK cells were enriched as previously described (Lemieux *et al.*, 1991). Briefly, spleen cells from normal mice were filtered twice through nylon wool columns and depleted of $CD4^+$ and $CD8^+$ cells using rat anti-mouse CD4 and rat anti-mouse CD8 mAbs, and sheep anti-rat IgG-coated magnetic beads (Dynal Inc., Great Neck, NY). NK-enriched cells were washed twice in PBS before they were used for flow cytometry analyses or immunoprecipitations.

Cell staining and flow cytometry analysis

Cells were incubated with biotin- or FITC-conjugated mAbs as described (Lemieux *et al.*, 1991). Binding of biotinylated-4LO3311 mAb was detected by phycoerythrin-labeled streptavidin (SA-PE) (Becton Dickinson). For the detection of NK2.1+ cells with the NZB anti-BALB/c antiserum, nylon wool nonadherent (NWNA) spleen cells were incubated with the antiserum and then with FITC-conjugated goat anti-mouse IgG (GAMIG, Jackson Immunoresearch Laboratories Inc., West Grove, PA). Samples were analyzed on an EPICS C flow cytometer (Coulter Electronics, Hialeah, FL) equipped with a 5-W argon laser. The percentage of positive cells was obtained after background fluorescence, corresponding to unlabeled cells or cells incubated with SA-PE or GAMIG-FITC alone, was subtracted from experimental samples using the Immuno program from the Easy 2 software (Coulter Electronics).

Surface protein iodination, immunoprecipitation, and SDS-PAGE analysis

Cell surface proteins of NK-enriched spleen cells were labeled with $Na^{125}I$ (ICN Biomedical, Mississauga, Ontario), using the Enzymobead radioiodination reagent (Bio-Rad Laboratories Canada, Mississauga, Ontario), according to the manufacturer's directions. Labeled cells were washed four times in PBS and proteins were solubilized in lysis buffer (50 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl, 2 m*M* PMSF, 0.5% Nonidet P-40, and 1% BSA, all from Sigma Chemical Co., St. Louis, MO) for 60 min on ice before removal of nuclei by centrifugation at 12 000 x *g* for 10 min. Nonspecific binding components were removed from the cell lysates by three cycles of incubation with Protein G-Sepharose (Pharmacia Fine Chemicals, Baie d'Urfé, Québec) for 1 h at 4°C under rotation followed

by centrifugation at 2 000 x *g* for 2 min. Aliquots of the clarified radioiodinated cell extracts were incubated with mAbs or antisera for 1 h at 4°C before adding Protein G-Sepharose for an overnight incubation at 4°C under rotation. Immunoprecipitates were washed four times in lysis buffer without BSA and eluted from Protein G-Sepharose by boiling for 5 min in 125 m*M* Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, supplemented with 5% 2-mercaptoethanol for the reduced samples. Proteins were separated by electrophoresis on 10 % polyacrylamide gels. Slab gels were fixed in 50% methanol, dried, and exposed overnight to Kodak XAR-2 films (Picker International Canada, Ville St. Laurent, Québec) at - 70°C.

Removal of N-linked carbohydrates

The immunoprecipitates were boiled for 5 min in 50 m*M* Tris-HCl, pH 8.5, 1.25% Nonidet P-40, 5% 2-mercaptoethanol, 0.05% sodium azide, 50 m*M* EDTA and 0.1% SDS. *N*-linked carbohydrates were digested by incubation for 20 h at 37°C in the presence of 5-10 U/ml *N*-Glycosidase F (from *Flavobacterium meningosepticum*, Boehringer Mannheim Canada, Laval, Québec). The reaction was stopped by boiling samples for 5 min before SDS-PAGE analysis.

Treatment of cells with phosphatidylinositol-specific phospholipase C (PI-PLC)

NK-enriched spleen cells (5 x 10^6) from BALB/c mice were incubated with 250 mU of PI-PLC from *Bacillus cereus* (Boehringer Mannheim Canada) in 500 µl of PBS containing 10 ng/ml of BSA for 1 h at 37°C (Seaman *et al.*, 1991). The effect of PI-PLC on the expression of the

NK2.1 antigen, and of the Thy-1.2 antigen as a control was monitored by flow cytometry.

Preparation of unfractionated and NK-enriched leukocyte suspensions from different sources

Thymi and lymph nodes (mesenteric or peripheral) obtained from normal BALB/c or C57BL/6 mice were rinced and trimmed to remove contaminating blood or fat. Single cell suspensions were then stained with labeled antibodies and analyzed by flow cytometry. When specified, cell suspensions were depleted of $CD4^+$ and $CD8^+$ cells using the protocol described for spleen cells and of IgG^+ cells using sheep anti-mouse IgG-coated magnetic beads (Dynal Inc.) before staining. PMN leukocytes were obtained from BALB/c or C57BL/6 mice injected i.p. with 2 ml of 12.5% sodium caseinate (Sigma Chemical Co.) in isotonic saline solution, pH 7.2 (Colepicolo *et al.*, 1990). Mice were sacrificed 18 h after injection and the peritoneal cells were harvested by washing the peritoneal cavity with 10 ml of PBS. After centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals), pelleted cells, which were 90% PMN leukocytes, were collected.

RESULTS

Biochemical analysis of the antigen detected by the 4LO3311 mAb and of the NK1.1 antigen extracted from fresh NK-enriched spleen cells

Variations in the frequency of $4LO3311^+$ cells as well as in the density of the 4LO3311 antigen at the cell surface were previously detected among 11 strains reacting with the 4LO3311 mAb (Morelli *et al.*, 1992). BALB/c

(4LO3311$^+$NK1.1$^-$) mice were selected for immunoprecipitating the antigen detected by the 4LO3311 mAb because their NK cells express this antigen at the highest density. About 40 to 50% of BALB/c NK-enriched spleen cells were stained with the 4LO3311 mAb with a mean fluorescence channel of 150 (Fig. 1A). Likewise, NZB (4LO3311$^-$NK1.1$^+$) mice were considered a good source of NK1.1$^+$ cells for comparatively immunoprecipitating the NK1.1 antigen from fresh NK-enriched spleen cells. Indeed, even though the NK1.1 antigen was dimly expressed by NK cells of NZB mice (mean fluorescence channel of 50), 60 to 75% of the cells recovered in the NWNA, CD4$^-$CD8$^-$ spleen cell fraction were NK1.1$^+$ (Fig. 1B).

SDS-PAGE analysis under nonreducing conditions revealed that the 4LO3311 mAb precipitates a protein of ~130 kDa (Fig. 2A). No equivalent material was seen after incubation of the same extract with an isotype control mAb. Under reducing conditions, the protein immunoprecipitated with the 4LO3311 mAb appeared as a broad band of ~65 kDa yielding a band of ~32 kDa after removal of *N*-linked carbohydrates (Fig. 2B). No difference in the biochemical properties of the antigen immunoprecipitated by the 4LO3311 mAb was detected when NK-enriched cell lysates from C3H (4LO3311$^+$NK1.1$^-$) and C57BL/6 (4LO3311$^+$NK1.1$^+$) mice were used (data not shown). Concurrently, the NK1.1 antigen precipitated from NZB NK-enriched cell extracts with the PK136 mAb appeared to be composed of two disulfide-linked ~39 kDa subunits. When *N*-linked sugars were removed, the molecular weight of the NK1.1 protein dropped to ~27 kDa. The NK1.1 antigen immunoprecipitated from fresh splenic NK cells of NZB mice would therefore be similar to the one characterized from a subclone of the

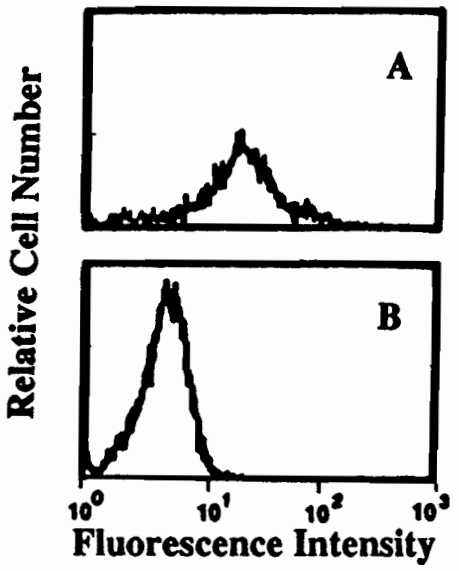

Fig. 1. Frequency of 4LO3311$^+$ and NK1.1$^+$ cells in NK-enriched spleen cell suspensions. NWNA, CD4$^-$ CD8$^-$ spleen cells from BALB/c mice (A) were incubated with biotin-conjugated 4LO3311 mAb followed by SA-PE and those from NZB mice (B) were stained with FITC-labeled PK136 anti-NK1.1 mAb. Positive cell frequency was calculated as described under Materials and Methods.

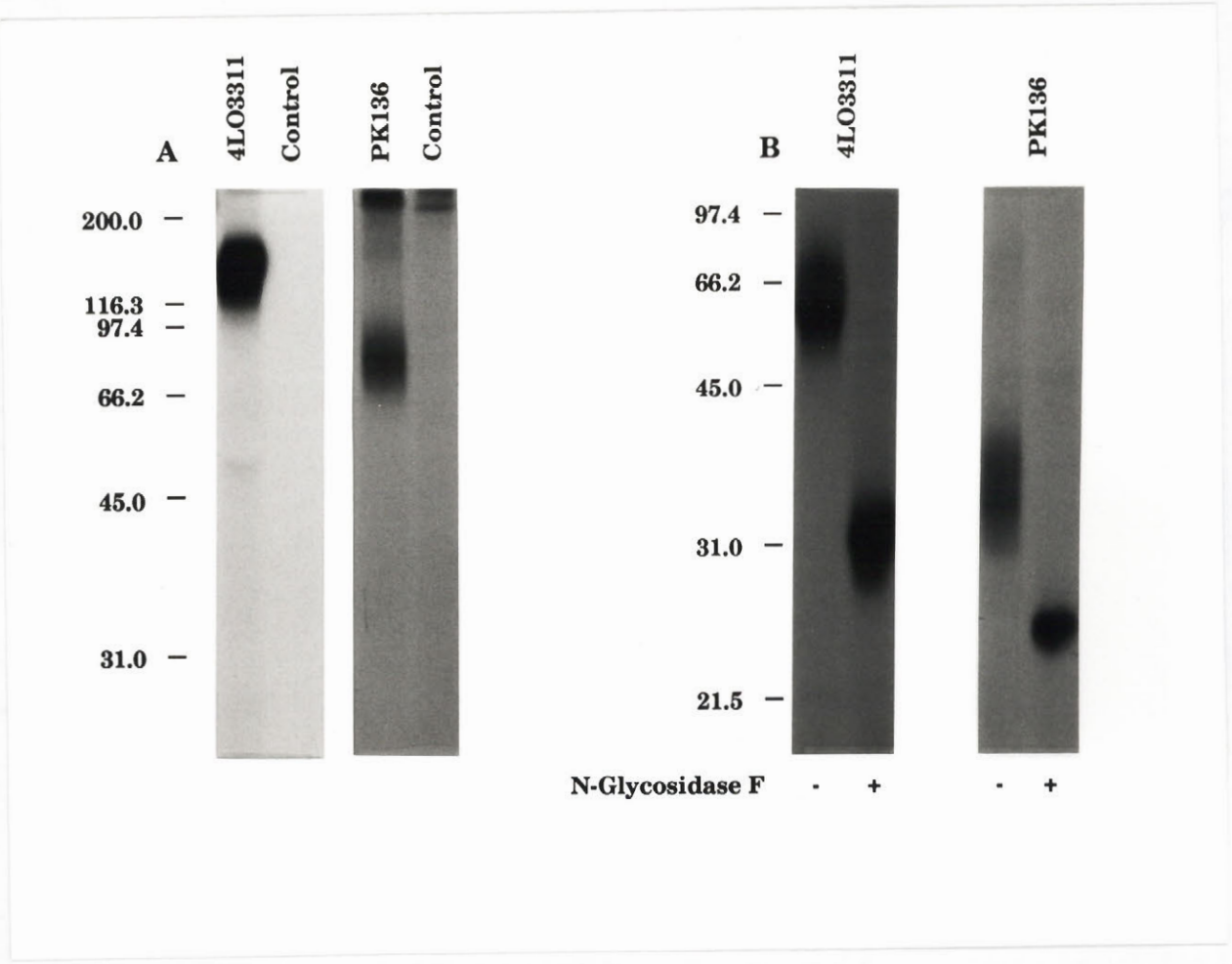

Fig. 2. SDS-PAGE analysis of NK-specific antigens. Radioiodinated lysates of BALB/c (left lanes) or NZB (right lanes) NK-enriched spleen cells were respectively immunoprecipitated with the 4LO3311, the PK136 anti-NK1.1, or corresponding isotype control mAbs. Samples were run on 10% polyacrylamide gels under nonreducing (A) or reducing (B) conditions. In panel B, immunoprecipitates were either untreated (-) or treated (+) with N-Glycosydase F before SDS-PAGE analysis under reducing conditions.

CTLL-2 cell line (Sentman *et al.*, 1989b) and from C57BL/6 IL-2-propagated NK cells (Ryan *et al.*, 1992). No protein was precipitated from BALB/c NK-enriched cell lysates with the PK136 anti-NK1.1 mAb and from NZB NK-enriched cell extracts with the 4LO3311 mAb (data not shown).

Identity of the antigen detected by the 4LO3311 mAb

To confirm our hypothesis that the 4LO3311 mAb might actually detect the NK2.1 antigen, an NZB anti-BALB/c (anti-NK2.1) antiserum was produced and used for flow cytometry analysis and sequential immuno-precipitation with the 4LO3311 mAb. We first observed by flow cytometry analysis that the NZB anti-BALB/c antiserum and the 4LO3311 mAb detected the same frequency of positive cells (~6%) in BALB/c NWNA spleen cell suspensions (data not shown). When BALB/c NWNA spleen cells were incubated with the NZB anti-BALB/c antiserum before staining with biotin-conjugated 4LO3311 mAb and SA-PE, positive cells were no longer detected, suggesting that the two reagents reacted either with the same antigen or with closely associated molecules (data not shown). In addition, the ~130 kDa protein immunoprecipitated by the 4LO3311 mAb was not present in radioiodinated cell lysates that have been pre-cleared with an NZB anti-BALB/c antiserum (Fig. 3A). Conversely, no material was precipitated by an NZB anti-BALB/c antiserum when NK-enriched cell lysates were pre-cleared with the 4LO3311 mAb (Fig. 3B). Pre-clearing with nonimmune NZB serum or isotype control mAb was ineffective (Fig. 3A and 3B). These results confirmed that the 4LO3311 mAb detects the NK-2.1 antigen that had not yet been identified by any other mAb.

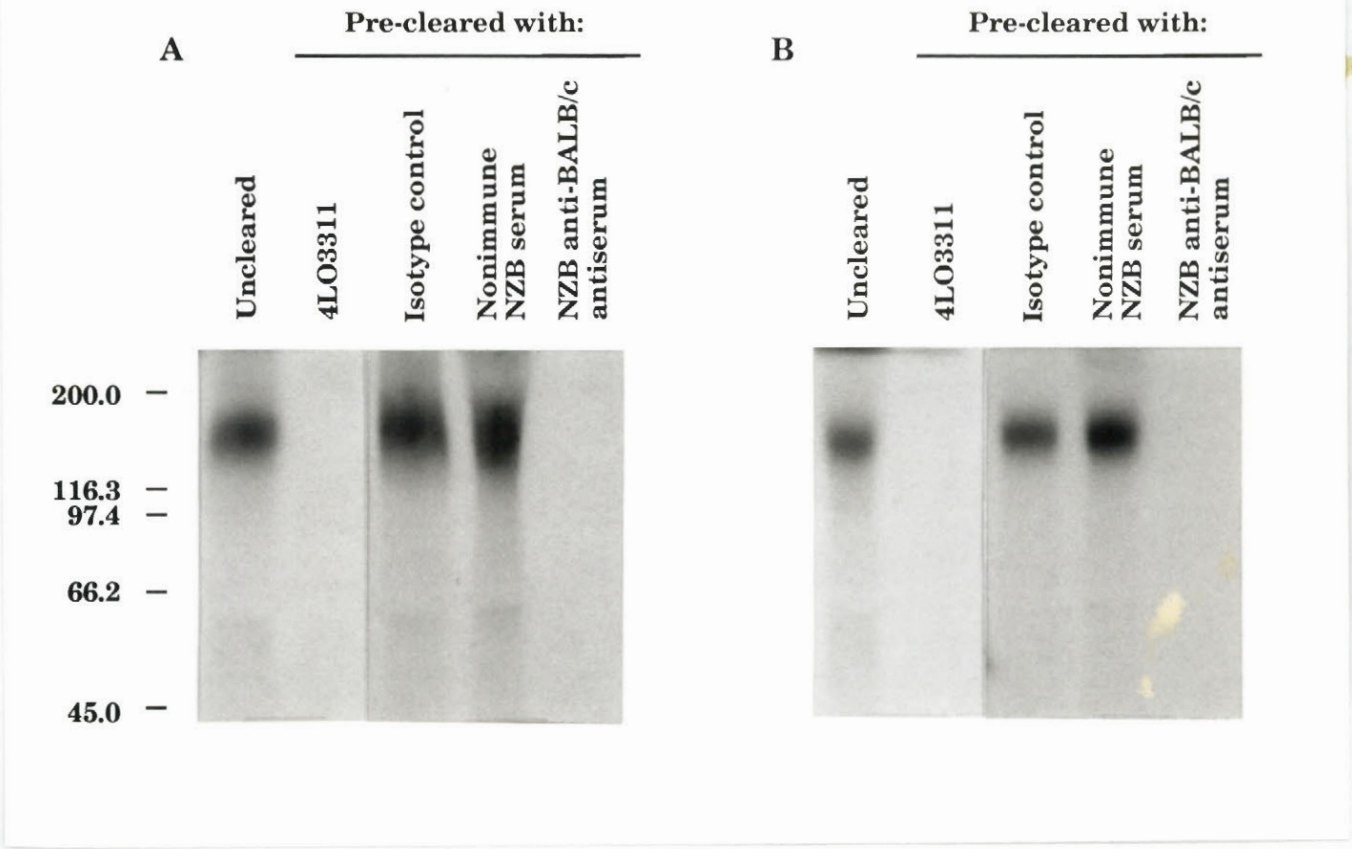

Fig. 3. Sequential immunoprecipitation of the antigen detected by the 4LO3311 mAb and an NZB anti-BALB/c (anti-NK2.1) antiserum. ^{125}I-labeled lysates of BALB/c NK-enriched spleen cells were immunoprecipitated with the 4LO3311 mAb (A) or an NZB anti-BALB/c antiserum (B) before and after three cycles of pre-clearing with either reagent or negative control mAb or serum. Samples were analyzed by SDS-PAGE under nonreducing conditions.

Sensitivity of the NK2.1 antigen to PI-PLC

In order to determine whether the NK2.1 antigen is attached to the cell surface by a glycosyl-phosphatidylinositol (GPI)-anchor, the expression of the NK2.1 antigen was assessed after treatment of NK-enriched spleen cells with PI-PLC. The expression of the GPI-anchored Thy-1.2 antigen (Tung *et al.*, 1986) was concurrently measured to ensure the efficacy of the enzyme treatment. Under conditions where the expression of the Thy-1.2 antigen on NWNA spleen cells (Fig. 4B) was reduced by 70-80% after incubation with PI-PLC (Fig. 4D), the expression of the NK2.1 antigen on NK-enriched spleen cells (Fig. 4A) was not modified (Fig. 4C).

Cell and tissue distribution of the NK2.1 antigen

The NK2.1 antigen has so far only been found on splenic NK cells (Lemieux *et al.*, 1991). Herein, we extended our analysis of the expression of the NK2.1 antigen to other tissues and cells. In addition to being expressed by a subset of splenic NK cells, the NK2.1 antigen was found on ~10% of $CD4^-CD8^-$ thymocytes as well as on ~10% of T and IgG^+ cell-depleted peripheral and mesenteric lymph node cells but was undetectable on unfractionated thymocytes and on total lymph node cells (Table 1). Like it was previously observed for fresh NK cells (Lemieux *et al.*, 1991), the NK2.1 antigen was present on only a portion (60%) of IL-2-propagated BALB/c NK-enriched spleen cells (Table 1). This result is in agreement with our previous conclusion that the NK2.1 antigen defines a particular NK cell subset rather than all NK cells (Morelli *et al.*, 1992).

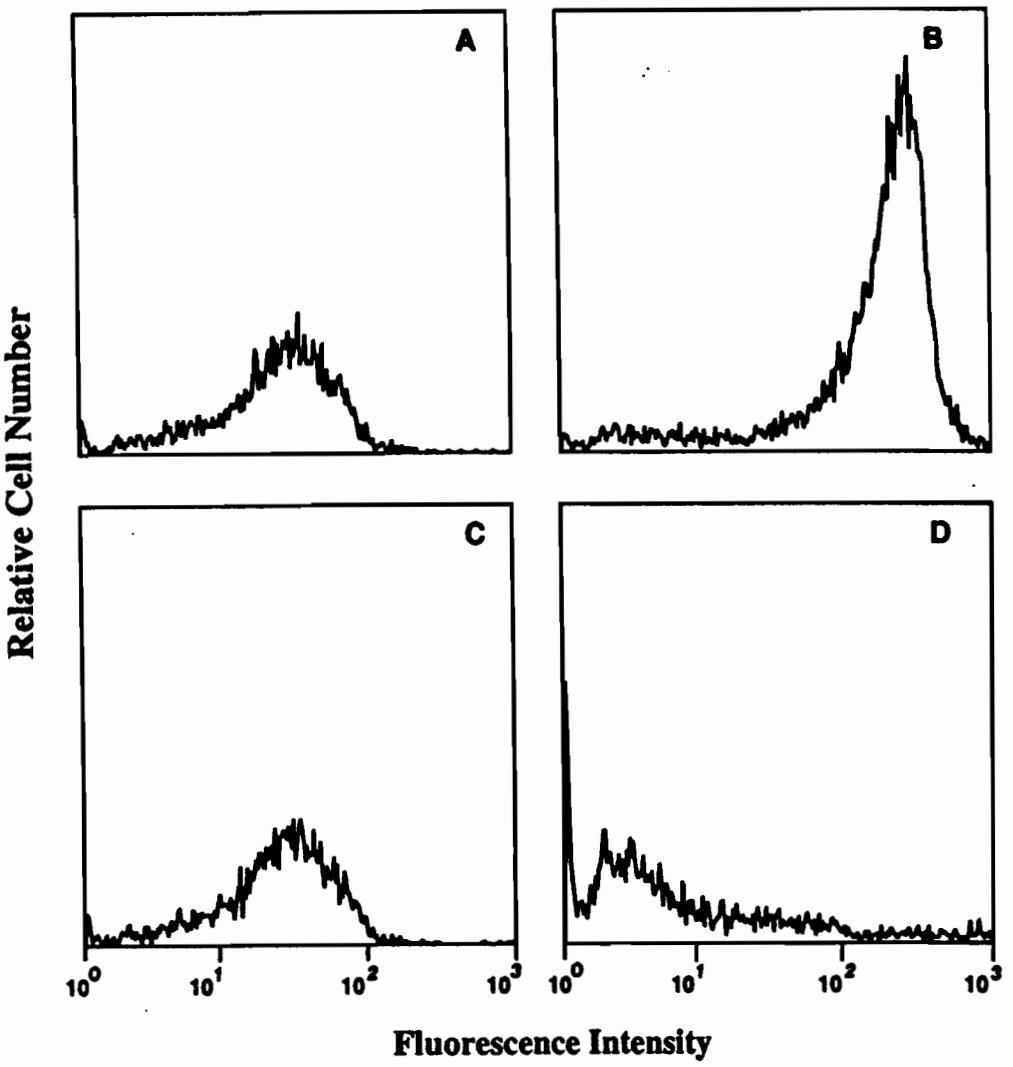

Fig. 4. Effect of PI-PLC treatment on the cell surface expression of the NK2.1 antigen. BALB/c NK-enriched spleen cells (A, C) were either untreated (A) or treated (C) with PI-PLC before they were stained with biotin-conjugated 4LO3311 anti-NK2.1 mAb and SA-PE and analyzed by flow cytometry. Expression of the GPI-anchored Thy-1.2 antigen on untreated (B) or enzyme-treated (D) BALB/c NWNA spleen cells was concurrently evaluated for controlling the efficacy of the enzyme treatment.

Table I. Expression of the NK2.1 antigen by cells of different lymphoid tissues of BALB/c mice

Cells	Percentage of positive cells[a]
Spleen cells	2
NWNA spleen cells	6
NK-enriched spleen cells	50
IL-2-stimulated NK-enriched spleen cells[b]	60
Thymocytes	<1
$CD4^-CD8^-$ thymocytes	10
Peripheral lymph node cells	<1
$CD4^-CD8^-IgG^-$ peripheral lymph node cells	10
Mesenteric lymph node cells	<1
$CD4^-CD8^-IgG^-$ mesenteric lymph node cells	10

[a]The frequency of $NK2.1^+$ cells in cell suspensions was determined by flow cytometry as described under Materials and Methods.

[b]NK-enriched spleen cells were cultured for five days in the presence of mrIL-2 (500 U/ml, Boehringer Mannheim Canada).

The NK2.1 antigen shares common properties not only with the NK1.1 antigen but also with the rat NKR-P1 antigen which, in addition to being expressed by NK cells and lymphokine-activated killer (LAK) cells, is detected on PMN leukocytes, although at a much lower density (Chambers *et al.*, 1989). Given the recently demonstrated identity between the NK1.1 and the corresponding mouse NKR-P1 antigen (Ryan *et al.*, 1992), it was of interest to determine whether the NK1.1 and the NK2.1 antigens are also expressed by PMN leukocytes. As illustrated in Fig. 5A, the 4LO3311 anti-NK2.1 mAb did not bind to PMN leukocytes of BALB/c mice. No reactivity with the 4LO3311 mAb was detected either when PMN leukocytes were obtained from C57BL/6 mice (Fig. 5B). A positive reaction was obtained when C57BL/6 PMN leukocytes were incubated with the PK136 anti-NK1.1 mAb (Fig. 5C) but the reactivity was no longer detected when PMN leukocytes were incubated with an aggregated control IgG_{2a} mAb before staining with FITC-labeled PK136 mAb (Fig. 5D). Under similar blocking conditions, the reactivity of PMN leukocytes with another IgG_{2a} mAb detecting the Ly-5 pan-leukocyte antigen (Holmes and Morse, 1988) remained unaffected (Fig. 5E and 5F).

DISCUSSION

We have previously established that the 4LO3311 mAb detects, on murine NK cells, a polymorphic antigen having a strain distribution similar to that of the NK2.1 antigen (Lemieux *et al.*, 1991) formerly defined by the NZB anti-BALB/c antiserum (Pollack and Emmons, 1982). However, after this antiserum was produced, it was reported that most NK cells express a particular type of low affinity Fc receptors for IgG, the FcγRIIα (Perussia

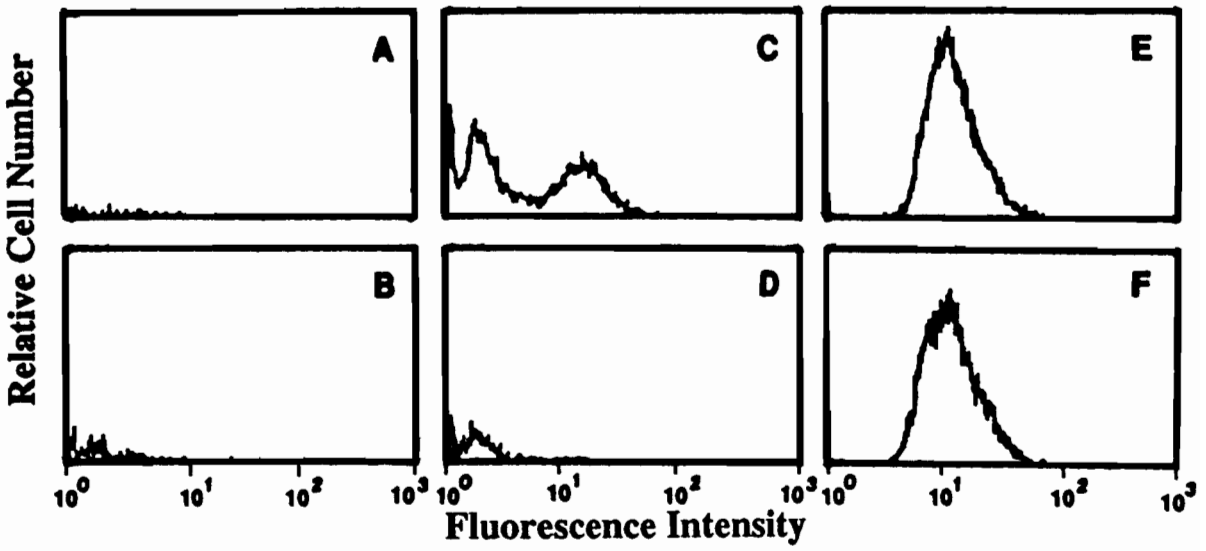

Fig. 5. Reactivity of PMN leukocytes with anti-NK mAbs. Peritoneal PMN leukocytes were elicited in BALB/c (A) or C57BL/6 (B-F) mice with sodium caseinate as described under Materials and Methods. PMN leukocytes were stained with FITC-labeled 4LO3311 anti-NK2.1 mAb (A and B), FITC-labeled PK136 anti-NK1.1 mAb (C and D) or FITC-labeled anti-Ly-5.2 mAb (E and F) as a control. In two cases (D and F), the cells were incubated with unlabeled aggregates of an irrelevant IgG_{2a} mAb before staining. The binding of specific mAbs to PMN leukocytes was assessed by flow cytometry.

et al., 1989), later renamed FcγRIIIα (Ravetch and Kinet, 1991), and the Pgp-1 marker (Ballas and Rasmussen, 1990a). These molecules are polymorphic antigens respectively encoded by genes at the *Ly-17* (Hibbs *et al.*, 1985; Holmes *et al.*, 1985) and *Ly-24* loci (Morse *et al.*, 1987). NZB and BALB/c mice express different alleles at these loci (Morse *et al.*, 1987). Therefore, the NZB anti-BALB/c antiserum originally produced may have contained NK-reactive antibodies of different specificities and it was not excluded that the 4LO3311 mAb could have been directed against one of these molecules. We demonstrate here that the 4LO3311 mAb and an NZB anti-BALB/c antiserum recognize a single ~130 kDa antigen clearly distinct from Pgp-1 and FcγRIIIα, which are 80-95 kDa and 40-60 kDa glycoproteins, respectively (Hughes and August, 1982; Ravetch and Kinet, 1991). We conclude that the 4LO3311 mAb does recognize the NK2.1 antigen. We also show that this molecule is a novel dimeric cell surface glycoprotein expressed by a subset of NK cells concentrated in the spleen of mice but also representing a minor population of double negative thymocytes and of $CD4^-CD8^-IgG^-$ lymph node cells.

Given its apparent molecular weight and its peculiar strain distribution, the NK2.1 antigen is clearly distinct with respect to other murine NK cell-specific alloantigens characterized thus far. However, the NK2.1 antigen shares common features with most of them. For instance, the NK2.1 antigen is expressed at the NK cell surface as a disulfide linked dimer of *N*-glycosylated proteins most likely to be anchored in the plasma membrane by a peptide moiety. The murine Ly-49 and NK1.1 antigens (Chan and Takei, 1986; Nagasawa *et al.*, 1987; Chan and Takei, 1989; Yokoyama *et al.*, 1989; Ryan *et al.*, 1992) as well as the rat NKR-P1

antigen (Chambers *et al.*, 1989; Giorda *et al.*, 1990) have such biochemical properties. Although the NK2.1 antigen is a much larger molecule (130 kDa) than the NK1.1 (78 kDa), the Ly-49 (88 kDa) and the rat NKR-P1 (60 kDa) antigens, it is noticeable that they all appear to be composed of subunits of almost the same size (25-32 kDa) in their deglycosylated form, although we do not know whether the NK2.1 antigen is a homo- or heterodimer. The 5E6 antigen is a less documented molecule expressed by a subset of murine NK cells (Sentman *et al.*, 1989c). Interestingly, the 5E6 antigen was characterized as a dimeric protein of the same molecular weight as the Ly-49. However, these two antigens are expressed in C57BL/6 mice by NK1.1$^+$ cell subsets of different sizes (Sentman *et al.*, 1989c; Yokoyama *et al.*, 1990). The degree of glycosylation of the 5E6 antigen and how it is attached to the cell membrane have not yet been reported.

Concerning the role played by NK-specific antigens in NK cell functions, it has been established that the rat NKR-P1 and its NK1.1 murine homologue are triggering molecules on NK cells (Chambers *et al.*, 1989; Karlhofer and Yokoyama, 1991). Indeed, crosslinking of these antigens with their specific mAbs can trigger redirected lysis of FcγR-bearing target cells. Redirected lysis of P815 FcR$^+$ cells has also been obtained with the 5E6 mAb but the observation that 5E6$^+$ cells are specifically reactive with the determinant 2 of Hh-1^d and Hh-1^f antigens raised the issue that the 5E6 antigen may act as a specific receptor for this determinant and in this way may be involved in the rejection of incompatible bone marrow cell grafts rather than being an accessory molecule in the elimination of tumor targets (Sentman *et al.*, 1989c). In

contrast, anti-Ly-49 mAb does not induce lysis of Daudi FcR$^+$ cells (Karlhofer *et al.*, 1992), which are susceptible to anti-NK1.1 antibody-induced lysis (Karlhofer and Yokoyama, 1991). Alternatively, a role for Ly-49 as an inhibitory receptor in the recognition of targets expressing the H-2D^d MHC class I alloantigen has recently been proposed (Karlhofer *et al.*, 1992). We have evidence that the 4LO3311 anti-NK2.1 mAb can trigger the cytolytic activity of NK cells (Morelli and Lemieux, unpublished results). Therefore, considering the structural and functional properties of the NK2.1 antigen, it is tempting to speculate that this antigen may well be encoded by a gene belonging or linked to the NKR-P1 multigene family. It is however unlikely that the NK2.1 gene would be one of the three NKR-P1 genes cloned by Giorda and Trucco (1991). Indeed, even though NKR-P1 genes highly homologous to those of C57BL/6 mice were identified in BALB/c mice by PCR amplification of total RNA isolated from adherent LAK (A-LAK) cells, NKR-P1 RNA transcripts were not detected by Northern hybridization even after BALB/c A-LAK cells are cultured for 7 or 14 days with IL-2 (Giorda *et al.*, 1992). As we have established by flow cytometry analysis that the NK2.1 antigen is on the contrary expressed at a very high level by BALB/c A-LAK cells, this molecule is apparently encoded by another gene. The putative relationship of the NK2.1 antigen to the NKR-P1 family will be resolved only when the cloning and sequencing of the NK2.1 gene, now ongoing in our laboratory, will be completed.

For a long time, the thymus was considered to be free of NK cells. However, after enriching NK cells, Ballas and Rasmussen (1990b) recently identified a subset of NK1.1$^+$ cells among CD4$^-$CD8$^-$ thymocytes of

C57BL/6 mice. Using a similar procedure, we were able to detect an NK2.1$^+$ cell population of the same size (~10%) in double negative thymocyte suspensions of BALB/c mice. At this stage, we cannot determine whether these NK2.1$^+$ cells represent only a subset or all thymic NK cells. As Ballas and Rasmussen (1990b) have established that thymic NK cells are devoid of cytolytic activity, there is, for the moment, no way to determine whether the NK2.1$^-$ NK cell subset detected in the spleen is also present in the thymus. Appropriate cell selection and availability of specific anti-NK mAbs having allowed us and others to detect NK cells in the thymus, we anticipated that low frequency of NK cells could well be found in other lymphoid tissues. It was indeed the case for BALB/c lymph nodes in which we identified a small number of NK2.1$^+$ cells. This observation is consistent with the natural cytotoxic reactivity against RBL-5 tumor cells that was observed several years ago in peripheral and mesenteric lymph nodes of BALB/c nude mice (Herberman *et al.*, 1975).

Unlike the rat NKR-P1, neither the NK1.1 nor the NK2.1 antigen is expressed by murine PMN leukocytes. Even though the PK136 anti-NK1.1 mAb bound to these cells, the inhibition of the reaction with aggregated isotype control mAb indicates that the anti-NK1.1 mAb likely reacted with PMN leukocytes through their FcγR. The absence of nonspecific binding of the 4LO3311 anti-NK2.1 (IgG_3) mAb to PMN leukocytes is probably due to the low affinity of the FcγRII and FcγRIIIα expressed by these cells for IgG_3 (Ravetch and Kinet, 1991). To our knowledge, the occurrence of specific receptors for IgG_3 on PMN leukocytes has not been reported.

Since the NK2.1 antigen shares common properties with the murine NK1.1 and the rat NKR-P1 antigens, it will now be of major interest to establish whether these dimeric glycoproteins all react with similar structures on NK cell targets. To the best of our knowledge, none of the natural ligand of these NK cell triggering molecules has as yet been identified.

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CHAPTER 3

"Modulation of the NK2.1 expression on splenic natural killer cells by interleukin-2"

Gosselin, P. and Lemieux, S.

(Unpublished results)

FOREWORD

In the previous chapter, we demonstrated that the 4LO3311 mAb recognized the NK2.1 molecule which we characterized as a highly glycosylated disulfide linked protein dimer of 130 kDa. This molecule is probably a transmembrane protein as its expression is unaffected by a PI-PLC treatment which sheds GPI-anchored proteins from the cell surface.

Our laboratory further demonstrated that intact anti-NK2.1 mAb as well as its $F(ab')_2$ and Fab fragments had the capacity to trigger the lytic activity of fresh and IL-2-stimulated NK cells towards susceptible target cells, suggesting that NK2.1 may function as an activation antigen. Interestingly, $NK2.1^+$ NK-enriched spleen cells were shown to be significantly more lytic than their negative counterpart. Since the lytic activity of NK cells is markedly enhanced after stimulation with IL-2, and since, as reported in the previous chapter, the percentage of $NK2.1^+$ cells is increased when NK-enriched spleen cell suspensions are cultured for 5 days in the presence of IL-2, it was of interest to further investigate the effect of IL-2 on $NK2.1^+$ cells.

This chapter includes a series of unpublished observations showing that NK2.1 expression is enhanced by IL-2 and suggesting that this lymphokine has the capacity to induce expression of NK2.1 on some $NK2.1^-$ cells. These observations, together with the aforementioned higher lytic capacity of $NK2.1^+$ cells, are consistent with a role for the NK2.1 molecule in natural killing.

ABSTRACT

The NK2.1 Ag is a triggering molecule found on 40-50% of fresh BALB/c NK-enriched spleen cells, most of which (>90%) express asialo GM1. When these cells are cultured in the presence of IL-2, the frequency of NK2.1$^+$ cells increases to 60%, suggesting a preferential growth of NK2.1$^+$ cells or an induced expression of NK2.1 on some NK2.1$^-$ cells. In the present study, we demonstrated that the increased frequency of NK2.1$^+$ cells did not result from a preferential growth of NK2.1$^+$ cells since sorted NK2.1$^+$ and NK2.1$^-$ cells proliferated at the same rate when stimulated with IL-2. This result also indicated that apart from their requirement for exogenous IL-2, each cell population was self-sufficient for growth. The increased frequency of NK2.1$^+$ cells resulted rather from an IL-2-induced expression of NK2.1 on some NK2.1$^-$ cells. Indeed, whereas the frequency of NK2.1$^+$ cells among fresh NK-enriched cells from NK2.1-depleted mice was negligible, 25-30% NK2.1$^+$ cells were found after 3 days of culture with IL-2. In addition, we found that IL-2 up-regulated the expression level of the NK2.1 antigen on positive cells, an effect potentiated by the calcium ionophore A23187. The chronology of the IL-2-induced events was as follows: 1) increased surface expression of NK2.1 after 4 h, 2) increased NK2.1$^+$ cell frequency after 24 h, and 3) active cell proliferation detectable on day 2. We thus conclude that, as for many other functionally relevant NK cell surface molecules, the expression of NK2.1 can be up-regulated by IL-2, a cytokine that can also drive the maturation of some NK2.1$^-$ cells into NK2.1$^+$ cells.

INTRODUCTION

NK cells play an important role in the limitation of growth and metastatic spread of a variety of tumors, and in resistance to certain microbial infections, without requiring prior sensitization or stimulation (Trinchieri, 1989). All resting NK cells constitutively express the β-chain of the IL-2R (Phillips *et al.*, 1989). When cultured in high concentrations of IL-2, NK cells become activated and proliferate without requirement for accessory cells or cofactors (Trinchieri *et al.*, 1984; Phillips and Lanier, 1987). Compared to resting NK cells, these lymphokine-activated killer (LAK) cells display a greater cytotoxic potential towards a wider variety of target cells (Grimm *et al.*, 1982). LAK cells also acquire surface antigens commonly associated with activated T cells such as CD25, the α chain of the IL-2R (Siegel *et al.*, 1987) and the very early activation molecule, CD69 (Lanier *et al.*, 1988; Karlhofer and Yokoyama, 1991). Expression of CD69 on NK cells is rapidly induced, within 4 h of culture with IL-2, and parallels the IL-2-induced cytotoxicity against NK-resistant solid tumor target cells (Lanier *et al.*, 1988). In the rat, IL-2 also induces the expression of a glycosyl-phosphatidylinositol (GPI)-anchored protein called gp42 on all LAK cells within 6 to 8 days of culture (Imboden *et al.*, 1989; Seaman *et al.*, 1991). Importantly, gp42 appears to deliver intracellular signals in the rat NK cell line RNK-16, suggesting that it may function as an activation receptor (Seaman *et al.*, 1991).

The murine NK2.1 antigen is a heavily glycosylated cell surface protein dimer of 130 kDa, expressed on a subpopulation of NK cells from the spleen, thymus, and lymph nodes from several mouse strains (Lemieux *et*

al., 1991; Gosselin *et al.*, 1993). Although NK2.1$^+$ and NK2.1$^-$ NK-enriched spleen cell fractions can both kill YAC-1 targets, NK2.1$^+$ cells are significantly more lytic (Morelli and Lemieux, 1993), raising the possibility that the NK2.1 antigen is involved in triggering NK cell lysis. In support of this hypothesis, our laboratory has demonstrated that triggering of NK cells through the NK2.1 molecule induces granule exocytosis by LAK cells and increases lysis of susceptible tumor targets by fresh or IL-2-activated NK cells (Morelli and Lemieux, 1993).

We have previously reported an increased frequency of NK2.1$^+$ cells in LAK cell suspensions generated from BALB/c NK-enriched spleen cells (Gosselin *et al.*, 1993). In the present study, we demonstrate that this effect resulted from an induced expression of NK2.1 on some NK2.1$^-$ cells. In addition, we show that the level of NK2.1 expression was rapidly increased following IL-2 stimulation and that a rise in intracellular calcium concentration ($[Ca^{2+}]_i$) potentiates this up-regulation.

MATERIALS AND METHODS

Mice

BALB/cAnN mice of either sex were purchased from Charles River Canada Inc. (St-Constant, Québec) and used at 6-10 weeks of age.

Medium and reagents

All cultures were performed in complete medium consisting of RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10% fetal calf serum (all from GIBCO

Laboratories, Burlington, Ontario), 25 mM Hepes buffer and 5 x 10^{-5} M 2-ME (Sigma Chemical Co., St Louis, MO). Mouse recombinant IL-2 (mrIL-2, Boehringer Mannheim Canada, Laval, Québec) was always included at 500 U/ml. Calcium ionophore A23187 was purchased from Sigma Chemical Co. and used at a final concentration of 1 μM.

Monoclonal antibodies

The 4LO3311 anti-NK2.1 mAb (mouse IgG3) was produced in our laboratory and conjugated to FITC as previously described (Lemieux *et al.*, 1991). An irrelevant FITC-conjugated mouse IgG3 mAb was purchased from Pharmingen (San Diego, CA).

Flow cytometry analyses and cell sorting

BALB/c splenic NK cells were enriched by selective elimination of nylon wool adherent, $CD4^+$, and $CD8^+$ cells and labeled with FITC-conjugated 4LO3311 anti-NK2.1 mAb or IgG3 control mAb as described (Gosselin *et al.* 1993). Samples were analyzed on an Epics C flow cytometer and the percentage of $NK2.1^+$ cells was obtained after fluorescence of cells incubated with FITC-conjugated control mAb was subtracted from experimental samples using the Immuno program from the Easy 2 data analysis software (Coulter Electronics). For some experiments, $NK2.1^+$ and $NK2.1^-$ NK-enriched spleen cells were isolated by sorting fresh BALB/c NK-enriched spleen cells, stained with FITC-conjugated 4LO3311 mAb, on the EPICS C flow cytometer.

NK cell cultures

NK-enriched spleen cells were diluted in 10 ml of complete medium, at a concentration of 1 x 10^6 cells/ml, and incubated in 25-cm^2 tissue culture flasks (Corning Works, Corning, NY) at 37°C, in a 5% CO_2 humidified atmosphere. When the incubation period exceeded 96 h, IL-2-propagated cells were divided and expanded as long as needed in fresh complete medium changed every 48 h.

Proliferation assays

A constant number (5 x 10^4) of unfractionated NK-enriched spleen cells or NK2.1$^+$ and NK2.1$^-$ cells were seeded in 200 µl of complete medium in flat-bottomed 96-well tissue culture microplates and incubated for up to 5 days under the aforementioned conditions. Cultures were pulsed with 1 µCi of ^{3}H-thymidine (CN Biomedical, Mississauga, Ontario) 4 h before the end of the incubation period and cell proliferation was assessed by measuring the ^{3}H-thymidine uptake with a liquid scintillation counter.

In vivo *depletion of NK2.1$^+$ cells*

Mice were inoculated once i.v. with 100 µl of 4LO3311 ascitic fluid diluted in 100 µl of PBS, 48 h before harvesting the spleen. Control mice were given PBS only. The efficacy of depletion was monitored by flow cytometry using FITC-conjugated anti-NK2.1 mAb.

RESULTS AND DISCUSSION

We have previously reported that whereas ~50% of fresh BALB/c NK-enriched spleen cells express the NK2.1 antigen, the frequency of NK2.1+

cells reaches ~60% after 5 days of culture in the presence of IL-2 (Gosselin *et al.*, 1993). From this observation, we hypothesized that this increased percentage of NK2.1+ cells may result from a preferential growth of NK2.1+ cells or from the acquisition of the NK2.1 antigen by some NK2.1- cells. In order to verify the first hypothesis, the relative growth rates of NK2.1+ and NK2.1- cell populations were compared by measuring the ^{3}H-thymidine uptake in sorted cells cultured for 5 days in the presence of IL-2. The sorted cell populations were always >95% pure as assessed by flow cytometry analysis (data not shown). In two separate experiments, ^{3}H-thymidine uptakes in NK2.1+ and NK2.1- cell populations were similar (Table I), indicating that both cell populations were proliferating at the same rate. This result also indicated that apart from their requirement for exogenous IL-2, NK2.1 positive and negative cells were independent of each other for growth. These observations thus ruled out a possible overgrowth of NK2.1+ cells, as well as a selective death of NK2.1- cells.

To investigate whether the increased frequency of NK2.1+ cells in LAK cell suspensions might result from an induced expression of NK2.1 on some NK2.1- cells, NK-enriched spleen cells from 4LO3311 mAb-treated BALB/c mice were cultured in the presence of IL-2. The number of NK2.1+ cells in these cell suspensions was negligible compared to those harvested from PBS-treated control mice, confirming the efficacy of the mAb-mediated depletion (Fig. 1). However, after 1 day of culture with IL-2, 17% of the cells expressed NK2.1, although at a lower level than NK-enriched spleen cells isolated from PBS-treated mice, incubated under the same conditions. After 3 days of culture, 28% of NK-enriched spleen

Table I. IL-2-induced proliferation of sorted populations of NK-enriched spleen cells.

	^{3}H-thymidine uptake (cpm ± SD)	
Cells	Experiment 1	Experiment 2
Unsorted	138 135 ± 20 530	76 420 ± 3 405
NK2.1$^+$	135 485 ± 18 660	67 612 ± 9 852
NK2.1$^-$	168 070 ± 14 532	60 785 ± 6 275

NK-enriched spleen cells were either unsorted or sorted on the basis of NK2.1 expression and cultured in 96-well microplates at 5 x 10^4 cells/well in 200 µl of complete medium. On day 5, cultures were pulsed for 4 h with ^{3}H-thymidine (5µCi/ml). Results are expressed as the mean ± SD of quadruplicate samples.

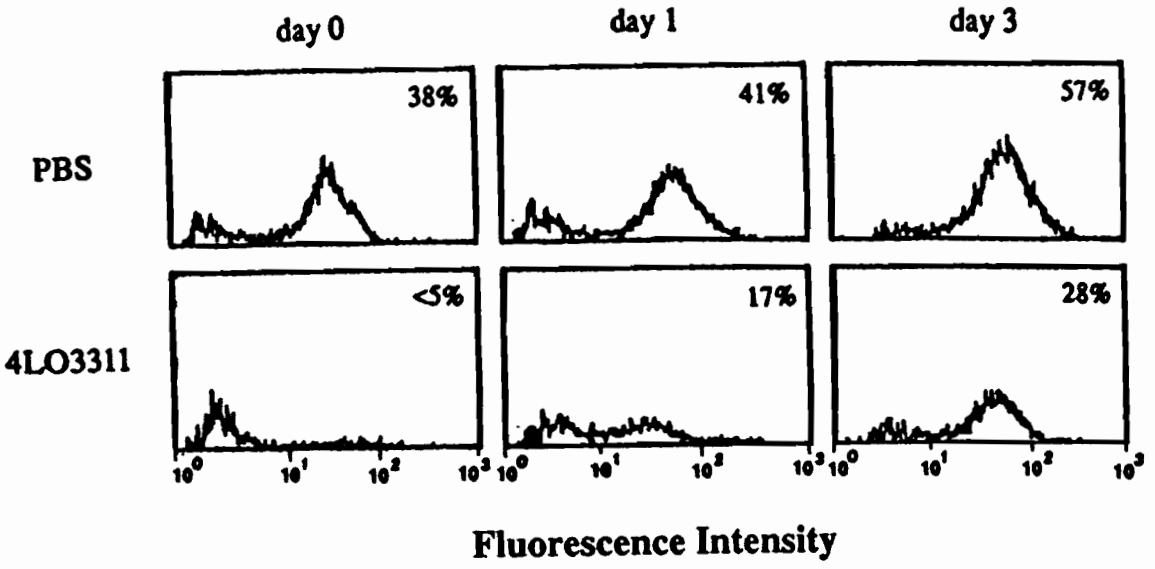


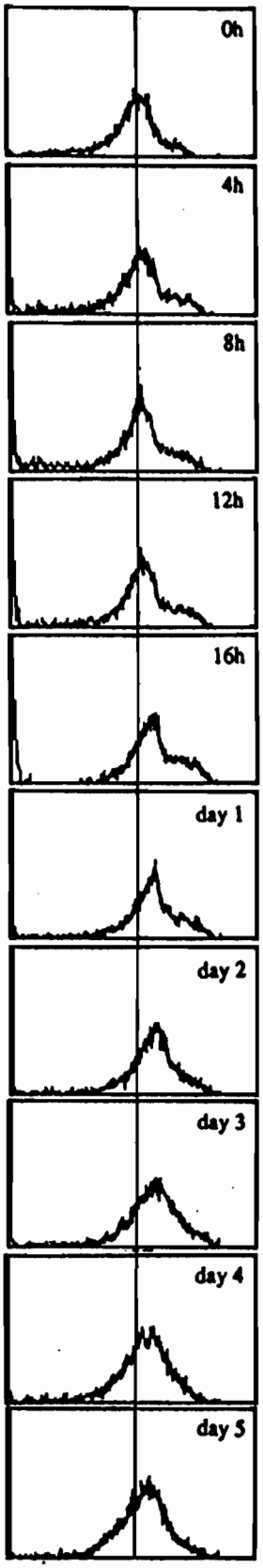
Figure 1. IL-2-induced expression of the NK2.1 antigen on NK2.1$^-$ cells. Mice were inoculated i.v. with 200 µl of PBS or 4LO3311 ascitic fluid 48 h before NK-enriched spleen cells were prepared and cultured with mrIL-2 (500 U/ml). At the indicated time points, cells were collected, stained with FITC-labeled 4LO3311 mAb and analyzed by flow cytometry. Results illustrated are representative of 3 different experiments.

cells from NK2.1-depleted mice expressed NK2.1 at a level similar to that of cells from PBS-treated mice. As expected from our previous observations (Gosselin *et al.*, 1993), the frequency of NK2.1$^+$ cells in LAK cell suspensions generated from PBS-treated control mice gradually increased from day 0 to day 3. These results thus strongly suggested that IL-2 can drive the maturation of some NK2.1$^-$ cells into NK2.1$^+$ cells.

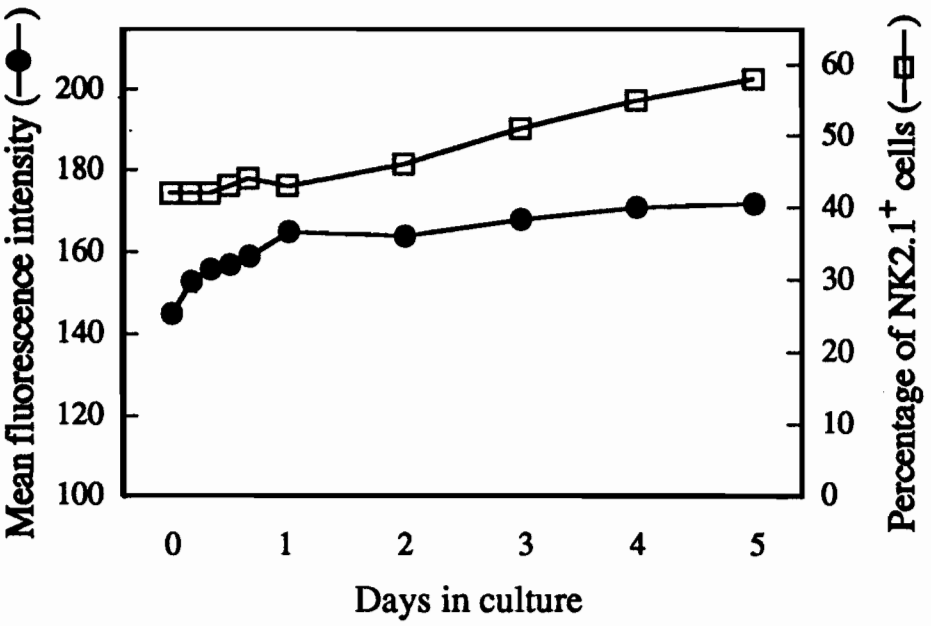
To more precisely characterize the effect of IL-2 on NK2.1 expression, the kinetics of the IL-2-induced expression of NK2.1 was studied. BALB/c NK-enriched spleen cells were cultured in the presence of 500 U/ml of IL-2 and the cell surface expression of NK2.1 was monitored by flow cytometry at different culture time points. Interestingly, a progressive increase in the NK2.1 cell surface expression was detected during the first 24 h of culture as shown by an increase in the mean fluorescence intensity (MFI) channel (Fig. 2A and 2B). Almost all the increased expression occurred within the first 24 h and the high NK2.1 expression level was maintained throughout the IL-2 stimulation period. Actually, the IL-2-induced up-regulation of NK2.1 expression was detectable as soon as 4 h after initiation of stimulation. An increase in the frequency of NK2.1$^+$ cells was detected only after 2 days of culture, and progressed slowly throughout the culture period (Fig. 2B). There were no further changes either in the frequency of NK2.1$^+$ cells or in the level of NK2.1 expression when cells were cultured for more than 5 days (data not shown).

Figure 2. Kinetics of the IL-2-induced up-regulation of NK2.1 antigen expression. NK-enriched spleen cells were grown in complete medium over a 5 day period. At indicated time points, cells were harvested and stained with FITC-labeled 4LO3311 mAb. The MFI channel and the frequency of NK2.1$^+$ cells were monitored by flow cytometry (A and B). Cell yields (expressed as % of cells added at onset) were calculated and proliferation rates of 5 x 10^4 cells (plated in triplicates on day 0) were measured after a 4 h pulse with ^{3}H-thymidine (C). Results illustrated are representative of 3 different experiments.

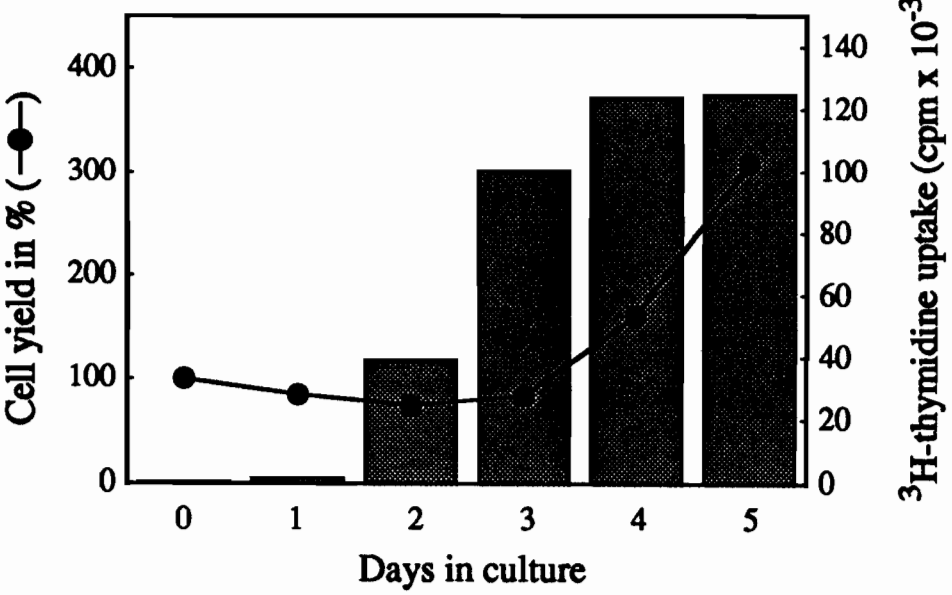
-A-


0h
4h
8h
12h
16h
day 1
day 2
day 3
day 4
day 5

-B-


Mean fluorescence intensity (—●—)
200
180
160
140
120
100
60
50
40
30
20
10
0
Percentage of NK2.1+ cells (—□—)
0
1
2
3
4
5
Days in culture

-C-


Cell yield in % (—●—)
400
300
200
100
0
140
120
100
80
60
40
20
0
3H-thymidine uptake (cpm x 10-3)
0
1
2
3
4
5
Days in culture

The relationship between the IL-2-induced proliferation of NK cells and the increased NK2.1 expression was studied by measuring the ^{3}H-thymidine incorporation during the 5 days of stimulation. The incorporation of ^{3}H-thymidine was detectable from day 2 but an increase in cell yield became measurable only on day 4 (Fig. 2C), thus after the increase in the frequency of NK2.1^{+} cells (Fig. 2B). Similar results were obtained with IL-2 at 100 rather than 500 U/ml, although cell proliferation was lower (data not shown). Altogether, these results clearly illustrate the IL-2-mediated up-regulation of NK2.1 expression, and support our hypothesis that IL-2 can induce expression of NK2.1 on some NK2.1^{-} cells. NK-enriched spleen cells were never cultured for more than 7 to 10 days as cell death became prohibitive at longer incubation periods. The inability of murine NK cells to maintain a sustained *in vitro* proliferation, despite a vigorous initial response to IL-2, has also been reported by others (Karlhofer *et al.*, 1995).

In the human, the IL-2-induced proliferation of CD56^{low} human NK cells was shown to be potentiated by a brief co-stimulation with calcium ionophore, apparently by increasing the IL-2Rα expression level (Robertson *et al.*, 1993). It was thus of interest to investigate the effect of a rise in $[Ca^{2+}]_i$ on NK2.1 expression. When added on day 0, the calcium ionophore A23187 blocked NK cell proliferation and cells rapidly entered a senescent phase and died within 24 h (data not shown). However, when it was added for 18 h, after an initial culture period of 48 h, the calcium ionophore A23187 up-regulated the level of NK2.1 expression, 15-20 MFI channels beyond the IL-2-induced expression, without affecting the percentage of NK2.1^{+} cells (Table II). Interestingly, the up-regulation of

Table II. Effect of the calcium ionophore A23187 on NK2.1 antigen expression.[a]

Exp.	IL-2 stimulation	A23187	Percentage of NK2.1 positive cells	MFI channel
1	-	-	50	145
	+	-	59	166
	+	+	57	184
2	-	-	45	150
	+	-	56	163
	+	+	51	180
3	-	-	42	112
	+	-	57	141
	+	+	61	161

[a] NK-enriched spleen cells were either unstimulated or cultured for 3 days in the presence of IL-2. The calcium ionophore A23187 was added at a final concentration of 1 μM 18 h before the end of the incubation period. The percentage of NK2.1$^+$ cells and the MFI channel were determined by flow cytometry analysis of fresh or stimulated cells, stained with FITC-labeled 4LO3311 mAb.

NK2.1 expression by the calcium ionophore was detectable whether NK cells were cultured for only 4 h or for up to 4 days before adding A23187 (data not shown).

Results presented herein indicate that the IL-2-mediated effects observed in NK-enriched spleen cell cultures occurred chronologically. In the first 24 h, IL-2 induced an up-regulation of NK2.1 expression on NK2.1$^+$ cells. From day 2, there was a recruitment of new NK2.1$^+$ cells from the negative pool. Even though DNA synthesis was detectable from day 2, an increase in the cell yield was seen only on day 4, resulting from equivalent proliferation of NK2.1$^+$ and NK2.1$^-$ cell populations. Interestingly, although NK2.1 expression reached a plateau when NK cells were stimulated with IL-2 only, the density of NK2.1 molecules on NK cells can be further augmented by an additional signal such as an increase in $[Ca^{2+}]_i$.

Using purified human NK cells, it has been observed that the IL-2-induced augmentation of NK cell-mediated cytotoxicity can be detected within 4 h, with a peak at 18 h, without requiring cell division (Lanier *et al.*, 1985; Phillips and Lanier, 1987). In addition, the IL-2-induced expression of CD69 occurs within 4 h of stimulation, thus before cell proliferation (Lanier *et al.*, 1988). The IL-2-induced modulation of NK2.1 reported herein thus resembles that of the activation receptor CD69, although only a fraction of murine NK cells apparently acquire NK2.1 expression. Our data suggest that the IL-2-induced up-regulation of NK2.1 expression is a calcium-dependent early activation event. Since we have previously reported that the NK2.1 antigen is an activation receptor (Morelli and Lemieux, 1993), enhanced NK2.1 expression may contribute to the

increased NK cell-mediated activity of LAK cells. Our observations also raise the possibility that NK2.1$^+$ cells are more activated or mature cells than NK2.1$^-$ cells, although further investigations are needed to confirm this hypothesis.

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CHAPTER 4

"The activation receptor NK2.1 is encoded by a gene linked to the NK gene complex and its expression is regulated by H-2- and non-H-2-dependent factors"

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(Submitted for publication in the Journal of Immunology)

FOREWORD

In the mouse, two gene families encoding activation or inhibition receptors are located in the NK gene complex mapping on the distal segment of chromosome 6. These genes, known as NKR-P1 and Ly-49, encode type II integral membrane disulfide-linked glycoproteins. NK2.1 thus shares some structural properties with NKR-P1 and Ly-49 gene products, exemplified in the C57BL/6 mouse by NK1.1 and Ly-49A, respectively. These similarities raise the possibility that NK2.1 may be encoded by either an NKR-P1 or Ly-49 gene, or by a distinct but related gene. It is precisely the case for CD69, an activation antigen expressed by IL-2-activated NK cells and encoded by a gene distinct from NKR-P1 and Ly-49 but mapping to the NK gene complex.

Our previous investigations suggested that NK2.1 shares another property with members of the Ly-49 family. Indeed, like Ly-49A, Ly-49C (5E6), and Ly-49G (LGL-1), NK2.1 appears to define an NK cell subpopulation rather than being expressed by all NK cells. As presented in the previous chapter, the number of NK2.1$^+$ cells, as well as the level of NK2.1 expression, can be increased by IL-2. We reported previously that IL-2-activated NK-enriched spleen cells expressing NK2.1 are more lytic towards YAC-1 cells than the cell population on which the NK2.1 molecule was not detected. Altogether, these findings support a role for NK2.1 in natural killing.

Our laboratory has also reported strain variations in the frequency of NK2.1$^+$ cells and in the density of NK2.1 expression. These observations

suggested that in addition to IL-2, undefined factors, possibly related to the H-2 haplotype, can regulate NK2.1 expression. In agreement with this hypothesis, although in another system, Karlhofer *et al.* (1994) reported that the cell surface expression of Ly-49A was modulated by host MHC class I molecules.

In this chapter, we analyzed the phenotype of NK-enriched spleen cells from (C57BL/6 x 129)F_1 x 129 backcross progeny to show that the NK2.1 gene is genetically linked to the NK1.1 and Ly-49C genes. By using an Epics XL flow cytometer, which can discriminate slight variations in fluorescence intensities, and inbred, MHC-congenic, and -congenic recombinant mice, we studied the possible relationship between NK2.1 expression and H-2 haplotypes. The sensitivity of the Epics XL apparatus has first allowed us to observe that, contrary to what we previously thought, the NK2.1 antigen is expressed by almost all splenic NK cells though at least two cell populations of different expression levels are defined. Most interestingly, we also found that H-2- and non-H-2-dependent factors control the *in vivo* expression of NK2.1 at the surface of splenic NK cells.

ABSTRACT

A dual receptor system composed of activation and inhibitory receptors apparently controls NK cell-mediated lysis. In the C57BL/6 mouse, the NK1.1 molecule, which is present on all NK cells, acts as an activation receptor whereas Ly-49A, expressed only on an NK cell subpopulation, can inhibit NK cell lysis of most target cells expressing H-2D^d or D^k molecules. Both types of receptors are disulfide-linked protein dimers belonging to related families of type II integral membrane proteins, homologous to C-type animal lectins, and encoded by genes located in a region of chromosome 6 named the NK gene complex. We previously reported that NK2.1 is a disulfide-linked glycoprotein dimer with a broad strain distribution which may function as an activation receptor. We demonstrate herein that the gene encoding NK2.1 is closely linked to the NK1.1 (NKR-P1C) and 5E6 (Ly-49C) genes. We further show that the 4LO3311 anti-NK2.1 mAb reacts with most nylon wool nonadherent, CD4$^-$CD8$^-$ spleen cells from NK2.1$^+$ mouse strains, defining two to three distinct subpopulations varying in NK2.1 expression level. Flow cytometry analyses of NK-enriched spleen cells from a number of inbred, congenic, and congenic recombinant strains revealed that like Ly-49A, NK2.1 expression is modulated by H-2-dependent factors although the participation of non-H-2-dependent factors is also suspected. Our data suggest that NK2.1 may interact with host MHC molecules, and raise the possibility that H-2K^b, D^k, and D^b molecules are NK2.1 ligands.

INTRODUCTION

NK cells can kill tumor cells and virus-infected cells without need for prior sensitization and without requiring target cell expression of MHC molecules (Trinchieri, 1989). Instead, it is of general agreement that MHC class I molecules expressed by target cells may interrupt the lytic process of NK cells, following their interaction with specific inhibitory receptors (Yokoyama, 1995). Ly-49A, which selectively binds H-2D^d and D^k (Karlhofer *et al.*, 1992; Kane, 1994), is so far the only molecule known to have such inhibitory properties in the mouse. However, accumulating data indicate that other inhibitory receptors yet to be identified must be present on murine NK cells (Carlow *et al.*, 1990; Roth *et al.*, 1994).

Ly-49A belongs to a family of type II integral membrane proteins homologous to C-type animal lectins (Nagasawa *et al.*, 1987; Chan and Takei, 1989; Yokoyama *et al.*, 1989). The other Ly-49 gene products are still unknown with the exception of the 5E6 and LGL-1 molecules, encoded by Ly-49C and Ly-49G2 genes, respectively (Stoneman *et al.*, 1993; Mason *et al.*, 1994; Brennan *et al.*, 1994). Whereas the biological function of LGL-1 remains obscure, the 5E6 molecule was shown to be expressed on a subpopulation of NK cells responsible for the rejection of H-2^d/Hh-1^d but not H-2^b/Hh-1^b bone marrow allografts by irradiated recipients (Sentman *et al.*, 1989a). More recently, it was reported that 5E6 may function as a receptor for MHC class I molecules of diverse haplotypes (Brennan *et al.*, 1994), raising the possibility that interaction of 5E6 with particular MHC molecules might inhibit NK cell lysis of particular targets. On the other hand, the 5E6 molecule might be an

activation receptor as anti-5E6 mAb can induce redirected lysis of FcγR+ target cells (Sentman *et al.*, 1991), a property shared by some members of another family of NK receptors, the NKR-P1 family.

NK1.1, the mouse homologue to rat NKR-P1, is encoded by the musNKR-P1C gene (Ryan *et al.*, 1992), which belongs to a family of clustered genes distinct from Ly-49 but also encoding lectin-like proteins (Giorda and Trucco, 1991; Yokoyama *et al.*, 1991). Ly-49 and musNKR-P1 are linked gene families mapping to the distal segment of mouse chromosome 6 named the NK gene complex (Giorda and Trucco, 1991; Yokoyama *et al.*, 1990; 1991; Wong *et al.*, 1991; Smith *et al.*, 1994). Antibodies to rat and mouse NKR-P1 can trigger NK cell lysis of FcγR+ NK resistant target cells (Chambers *et al.*, 1989; Karlhofer and Yokoyama, 1991). Anti-rat NKR-P1 mAb can also induce granule exocytosis, phosphoinositide turnover, and a rise in intracellular calcium in LAK cells or the RNK-16 cell line (Chambers *et al.*, 1989; Ryan *et al.*, 1991). Therefore, NKR-P1 molecules likely play a prominent role in natural killing.

Using the A1 anti-Ly-49A mAb for flow cytometry analysis of NK cells from a series of congenic and transgenic mice expressing different H-2 alleles on C57BL/10 (B10) background, Karlhofer *et al.* (1994) recently reported that Ly-49A was down-regulated on NK cells expressing D^d or D^k molecules. In contrast, NK1.1 expression was apparently not influenced by the host MHC haplotype. Using A1 and JR9-318 mAbs, which detect different epitopes of Ly-49A (Roland and Cazenave, 1992), Olsson *et al.* (1995) confirmed the host MHC class I-dependent down-regulation of Ly-49A expression in D8 (C57BL/6 expressing the H-$2D^d$

transgene) mice. However, Brennan *et al.* (1994) found no MHC-related change in NK cell expression of Ly-49A in B10 congenic mice, when YE1/48 and YE1/32 mAbs, also specific for Ly-49A, were used for flow cytometry analyses. These findings argue against a down-regulation of the Ly-49A expression. They would rather suggest that epitopes recognized by A1 and JR9-318 mAbs are rendered cryptic following interaction of Ly-49A with membrane-bound H-2D^d or D^k host molecules.

Previous studies from our laboratory have shown that contrary to NK1.1 (Hackett *et al.*, 1986), NK2.1 is expressed by a subpopulation rather than all NK cells (Lemieux *et al.*, 1991; Morelli *et al.*, 1992; Gosselin *et al.*, 1993; Morelli and Lemieux, 1993). We also reported that the percentage of NK2.1$^+$ cells in nylon wool nonadherent (NWNA) spleen cells as well as the level of NK2.1 expression are variable among positive mouse strains (Morelli *et al.*, 1992). We characterized the NK2.1 antigen as a highly glycosylated disulfide-linked protein dimer of 65 kDa subunits (Gosselin *et al.*, 1993). We further showed that immobilized anti-NK2.1 mAb induced granule exocytosis from IL-2-activated cells and that soluble mAb as well as its F(ab')$_2$ and Fab fragments increased lysis of NK susceptible target cells by resting or IL-2-activated cells (Morelli and Lemieux, 1993). These results are consistent with NK2.1 being a relevant molecule in natural killing.

We report herein that the NK2.1 gene is genetically linked to the NKR-P1 and Ly-49 genes. We further demonstrate that as observed for Ly-49A, NK2.1 expression is influenced by the host MHC haplotype.

MATERIALS AND METHODS

Mice

AKR/N, BALB/cAnN, C57BL/6N, C3H/HeN and DBA/2N mice were purchased from Charles River Canada Inc. (St-Constant, Québec). 129/J, A/J, C57BL/10SnJ, CBA/J, LP/J, B10.A/SgSnJ, B10.A(2R)/SgSnJ, B10.A(5R)/SgSnJ, B10.BR/SgSnJ, B10.D2/nSnJ, B10.D2(R103)/EgDvEgJ, B10.D2(R107)/EgDvEgJ, C.B10/LiMcdJ, C.C3/LiMcdJ and NZB/BINJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME).

Monoclonal antibodies

The 4LO3311 anti-NK2.1 mAb (mouse IgG3) was generated in our laboratory (Lemieux *et al.*, 1991). The hybridoma PK136 producing anti-NK1.1 mAb (mouse IgG2a) (Koo and Peppard, 1984) was purchased from ATCC (Rockville, MD). These mAbs were purified by affinity chromatography and biotinylated using standard methods. Biotinylated 5E6 mAb (anti-Ly-49C, mouse IgG2a) (Sentman *et al.*, 1989a) was obtained from Pharmingen (San Diego, CA). Phycoerythrin-labeled A1 mAb (anti-Ly-49A, mouse IgG2a) (Nagasawa *et al.*, 1987) was kindly provided by Dr. K. P. Kane (University of Alberta, Edmonton, Alberta, Canada). Irrelevant biotinylated mouse IgG3 mAb, purchased from Pharmingen (San Diego, CA), and mouse IgG2a mAb, generously provided by Dr. P. J. Talbot (Institut Armand-Frappier), were used as isotype controls.

Enrichment of splenic NK cells

Splenic NK cells were enriched by selective depletion of T lymphocytes from NWNA cells using anti-CD4 and anti-CD8 rat mAbs and sheep anti-rat IgG-coated magnetic beads (Dynal Inc., Great Neck, NY) as previously described (Gosselin *et al.*, 1993). We reported elsewhere that more than 90% of these cells react with anti-asialo GM1 antiserum (Morelli and Lemieux, 1993).

Cell staining and flow cytometry analysis

NK-enriched spleen cells were incubated for 30 min on ice with optimal concentrations of phycoerythrin-labeled A1 mAb or biotinylated mAbs. Binding of biotinylated mAbs was detected with phycoerythrin-labeled streptavidin (SA-PE, Becton Dickinson, Mountain View, CA). Control samples were incubated with biotinylated isotype control mAb and SA-PE. In the phenotypic study of (129 x C57BL/6)F_1 x 129 backcross animals, 10 000 cells/sample from individual mice were analyzed on a Coulter Epics C flow cytometer (Coulter Electronics, Hialeah, FL). The analysis of NK2.1 expression in inbred, congenic, and congenic recombinant mice was performed with a Coulter Epics XL-MCL. Data collected correspond to 10 000 cells/sample.

RESULTS

Linkage of the NK2.1 gene to those of the NK gene complex

To investigate whether the NK2.1 gene might be associated with those of the NK gene complex, the expression of NK1.1 and NK2.1 on NK-enriched spleen cells from 80 individual mice of the (129 x C57BL/6)F_1 x

129 backcross progeny was determined by flow cytometry analysis. This strain combination was suitable for a linkage study since 129 mice are NK1.1$^-$NK2.1$^-$ whereas both antigens are expressed in C57BL/6 (Lemieux *et al.*, 1991; Pollack and Emmons, 1982). Because only a low number of dimly fluorescent cells were detected after incubation of C57BL/6 NWNA spleen cells with 4LO3311 anti-NK2.1 mAb (Morelli *et al.*, 1992), the backcross analysis was performed on NK-enriched spleen cells in order to get more reliable results. Our data showed that NK1.1 and NK2.1 genes never segregated in backcrossed mice (Table I), strongly suggesting that the two genes are closely linked. The expression of the 5E6 molecule, which is also present in C57BL/6 but not in 129 mice (Sentman *et al.*, 1989b), was concurrently followed. We observed that all of the 36 backcrossed mice expressing the NK2.1 antigen also expressed the 5E6 molecule and no 5E6$^+$NK2.1$^-$ mouse was found (data not shown). In agreement with the results obtained in the backcross analysis, a separate study from our laboratory revealed that none of the recombinant inbred mice of the 129XB6 series, kindly provided by Dr. J.-L. Guenet (Institut Pasteur, Paris, France), were NK1.1 or NK2.1 single positive (unpublished observations). It thus appears that the NK2.1 gene maps to chromosome 6 either near or within the NK gene complex, in association with the NKR-P1 and Ly-49 gene families.

H-2- and non-H-2-dependent variations in NK2.1 expression among inbred mouse strains

In earlier studies based on complement-dependent NK cytotoxicity inhibition assays with 4LO3311 mAb as well as mAb-mediated *in vivo* depletion of NK2.1$^+$ cells, we reported that NK2.1 identifies a subpopulation rather

Table I. Expression of NK1.1 and NK2.1 antigens in the (129 x C57BL/6)F_1 x 129 backcross progeny[a]

	NK1.1$^+$	NK1.1$^-$	Total
NK2.1$^+$	36	0	36
NK2.1$^-$	0	44	44
Total	36	44	80

[a] Expression of NK1.1 and NK2.1 was determined by flow cytometry analysis of NK-enriched spleen cells from individual animals as described in *Materials and Methods*. Results shown correspond to the number of mice expressing a given phenotype.

than all NK cells (Morelli *et al.*, 1992). This conclusion was further supported by flow cytometry analysis of BALB/c NK-enriched spleen cells since only 40-50% of these cells were stained with 4LO3311 biotinylated mAb and SA-PE (Gosselin *et al.*, 1993). Further analysis of NK2.1 expression using a flow cytometer with a lower detection threshold and a 4- rather than 3-decade log amplifier, revealed that most BALB/c NK-enriched cells that were previously considered negative express low levels of the NK2.1 antigen (Fig. 1). This low reactivity is not due to non-specific adsorption of the mAb since no similar staining was seen with NK-enriched cells from 129 and NZB ($NK2.1^-$) mouse strains. The $NK2.1^{low}$ population was present in all $NK2.1^+$ strains tested and showed limited variation from one strain to another in its mean fluorescence intensity (MFI) (Table II).

The $NK2.1^{high}$ cell population, on the other hand, showed variability both in the percentage of positive cells and in MFI being low in C57BL/6 and C57BL/10 ($H\text{-}2^b$) mice, intermediate in AKR, C3H, and CBA ($H\text{-}2^k$) mice, and high in BALB/c and DBA/2 ($H\text{-}2^d$) mice (Fig. 1 and Table II). These findings confirm and extend our previous observations with NWNA spleen cells (Morelli *et al.*, 1992). Interestingly, $NK2.1^{high}$ cells of A/J ($H\text{-}2^a$) mice, which express $H\text{-}2K^k$ and D^d molecules, showed a MFI in the range of $H\text{-}2^d$ mice. With three clearly distinct cell populations and an $NK2.1^{high}$ cell population with a much higher MFI than other $H\text{-}2^b$ strains, LP mice showed a unique phenotype compared to all other mouse strains. Altogether, these results indicate that H-2-linked factors may influence NK2.1 expression and also strongly suggest that non-H-2-dependent factors are involved.

Figure 1. NK2.1 expression on NK-enriched spleen cells from different inbred mouse strains. NWNA $CD4^{-}CD8^{-}$ spleen cells were stained with biotinylated anti-NK2.1 mAb (dark lines) or isotype control mAb (dotted lines) and SA-PE and then analyzed by flow cytometry. The log fluorescence intensity is presented on the x axis while the y axis represent the cell count. The histograms are representative of three to five experiments.

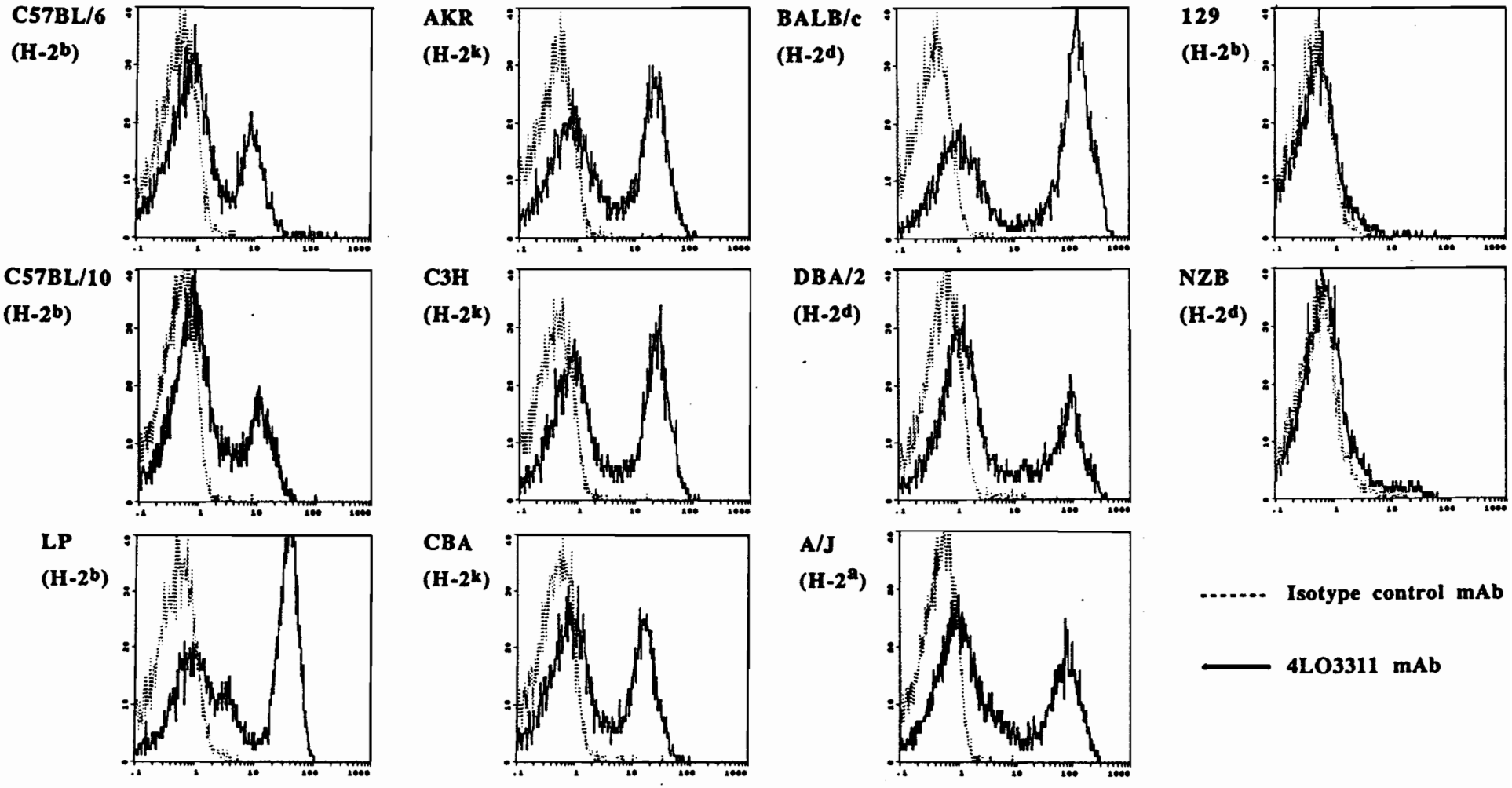

C57BL/6
(H-2b)
AKR
(H-2k)
BALB/c
(H-2d)
129
(H-2b)
C57BL/10
(H-2b)
C3H
(H-2k)
DBA/2
(H-2d)
NZB
(H-2d)
LP
(H-2b)
CBA
(H-2k)
A/J
(H-2a)
Isotype control mAb
4LO3311 mAb

Table II. Expression of NK2.1 on NK-enriched spleen cells from various inbred mouse strains[a]

Strain	H-2 haplotype	Percent ± SD of NK2.1^{low}	Percent ± SD of NK2.1^{high}	MFI ± SD for NK2.1^{low}	MFI ± SD for NK2.1^{high}
C57BL/6	b	57 ± 4	28 ± 2	0.7 ± 0.1	10 ± 1
C57BL/10	b	67 ± 2	25 ± 6	0.8 ± 0.1	11 ± 1
LP[b]	b	51 ± 8	33 ± 8	0.5 ± 0.1	27 ± 4
AKR	k	49 ± 6	40 ± 5	0.8 ± 0.1	20 ± 2
C3H	k	51 ± 2	39 ± 3	0.8 ± 0.1	24 ± 3
CBA	k	56 ± 2	33 ± 2	0.8 ± 0.1	17 ± 2
A	a	59 ± 4	33 ± 2	1.0 ± 0.2	78 ± 10
BALB/c	d	43 ± 4	52 ± 5	1.1 ± 0.2	115 ± 15
DBA/2	d	63 ± 3	28 ± 1	1.1 ± 0.2	66 ± 8
129	b	<1	<1	N.A.[c]	N.A.
NZB	d	<1	<1	N.A.	N.A.

[a] The expression of NK2.1 on NK-enriched spleen cells from 3 to 5 individual mice of each strain was analyzed by flow cytometry as described in *Materials and Methods*.

[b] In this mouse strain, an NK2.1^{int} cell populations representing 13 ± 2 % of the cells with a MFI of 4 ± 1 was also clearly detectable in every mouse tested.

[c] Not applicable

Variations in NK2.1 expression in H-2-congenic mice

To better evaluate the modulation of NK2.1 by H-2 haplotypes, we carried a series of flow cytometry analyses of NK2.1 expression on NK-enriched spleen cells from selected MHC-congenic strains. Since C57BL/10 and BALB/c mice showed respectively the NK2.1^{high} cell populations with the lowest and highest MFI, we reasoned that if NK2.1 expression is indeed H-2-dependent, the NK2.1 phenotype of congenic mice would tend towards the phenotype of the mouse strain contributing the H-2 allele, and thus vary in opposite directions in B10 and BALB/c congenic mice expressing a given haplotype. With MHC-congenic strains on B10 background, the MFI of NK2.1^{high} cells was enhanced in B10.D2 (H-2^{d}) mice but remained unchanged in B10.BR mice (H-2^{k}) (Fig. 2). No significant variations in the percentage and MFI of the NK2.1^{low} cell population and in the frequency of NK2.1^{high} cells were detected. The staining of NK1.1^{+} cells by PK136 mAb remained unaffected in B10 congenic strains expressing H-2^{d} or H-2^{k} haplotypes whereas the Ly-49A expression detected with the A1 mAb was significantly reduced (Fig. 2), thus confirming the data reported by Karlhofer *et al.* (1994).

As expected, when similar analyses were done with MHC-congenic strains on the BALB/c background, a down-regulation of NK2.1 expression on NK2.1^{high} cells was observed in C.C3 (H-2^{k}) and C.B10 (H-2^{b}) mice (Fig. 3), while no significant change in the percentage of the NK2.1^{high} cell population was detected. Together, these results indicate that the H-2 haplotype or, alternatively, H-2-associated genes, regulate NK2.1 expression.

Figure 2. Phenotypic analysis of NK-enriched spleen cells from B10 congenic lines. NK-enriched spleen cells were stained with PE-labeled anti-Ly-49A mAb, biotinylated anti-NK2.1 or anti-NK1.1 mAb and SA-PE (dark lines) or with isotype control mAb and SA-PE (dotted lines) and analysed by flow cytometry. The percentage of NK2.1^{high}, NK1.1^{+} or Ly49A^{+} cells and the corresponding MFI are shown in upper right corners (% cells/MFI).

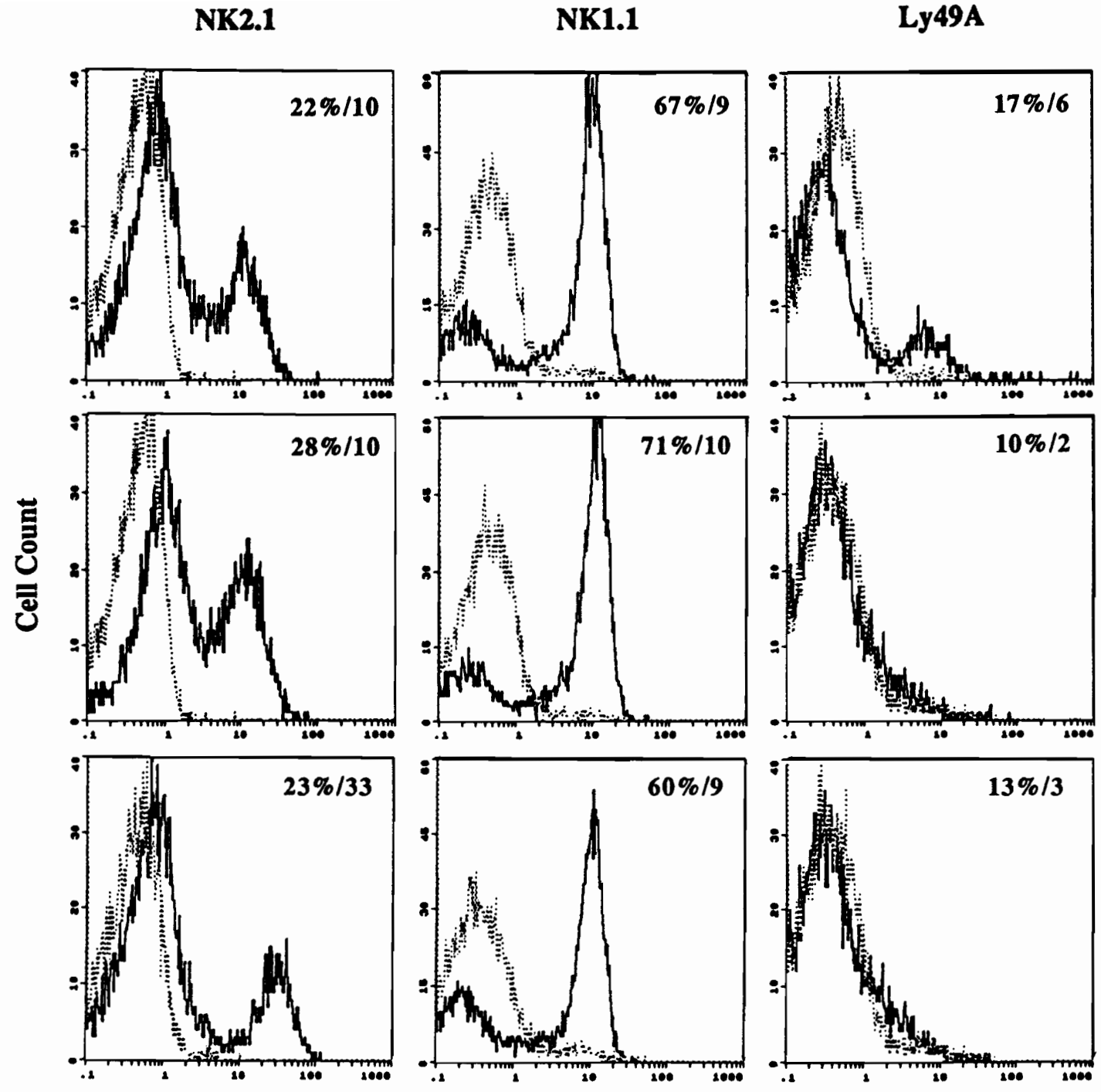
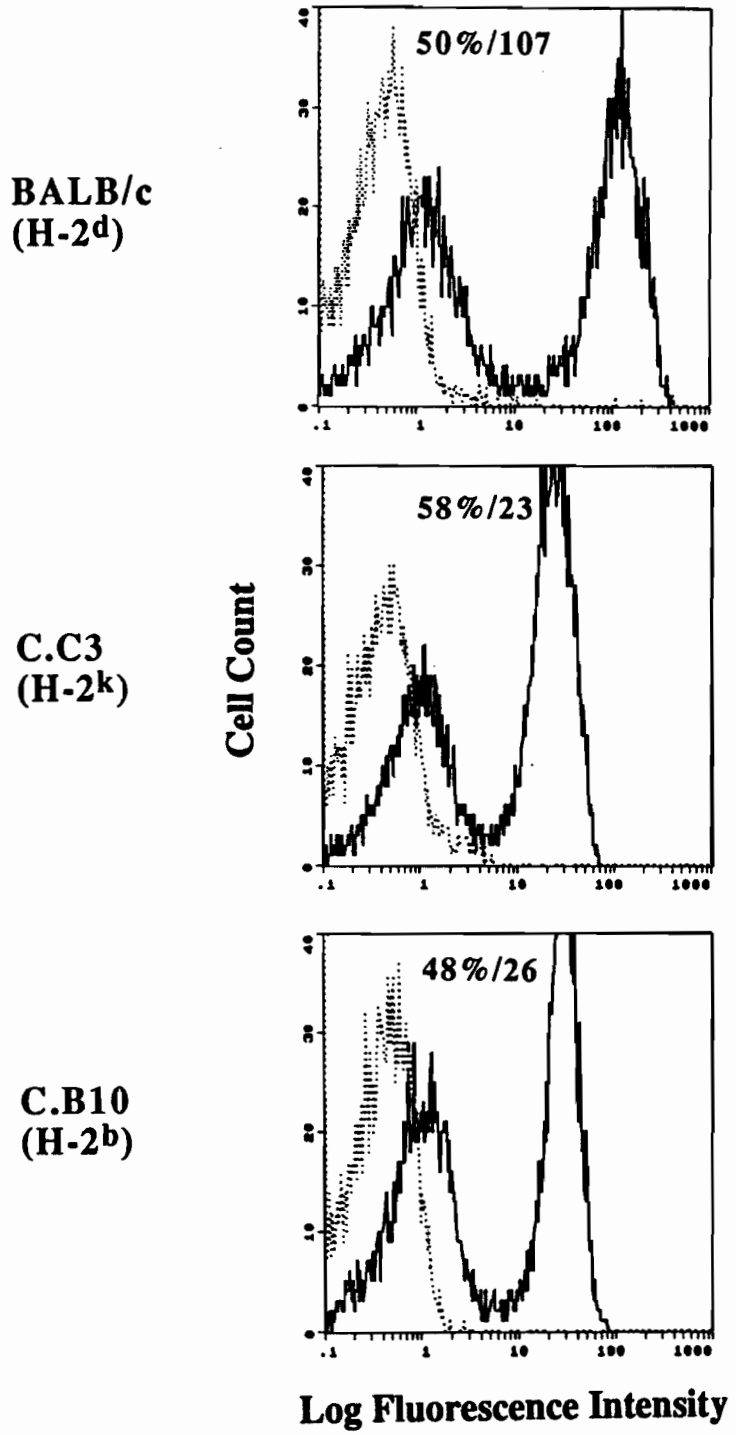

NK2.1
NK1.1
Ly49A
C57BL/10
(H-2b)
B10.BR
(H-2k)
B10.D2
(H-2d)
22%/10
67%/9
17%/6
28%/10
71%/10
10%/2
23%/33
60%/9
13%/3
Cell Count
Log Fluorescence Intensity

Figure 3. NK2.1 expression on NK-enriched spleen cells from H-2 congenic mice on the BALB/c background. NK-enriched spleen cells were stained with biotinylated anti-NK2.1 mAb (dark lines) or isotype control mAb (dotted lines) and SA-PE before being analysed by flow cytometry. The histograms are representative of three to five experiments. The percentage of NK2.1^{high} and the corresponding MFI are shown (% cells/MFI).


BALB/c
(H-2d)
C.C3
(H-2k)
C.B10
(H-2b)
50%/107
58%/23
48%/26
Cell Count
Log Fluorescence Intensity

NK2.1 expression is mainly modulated by a genetic factor centromeric to the H-2D locus

To clarify further the contribution of H-2 loci or H-2-associated genes in regulating NK2.1 expression, a series of B10 congenic strains carrying a chromosome 17 differential segment of variable length inherited from DBA/2 or A mice were used. As illustrated in Fig. 4, the differential segment inherited from the donor strain in B10.D2 and B10.A mice includes additional loci telomeric to H-2D and centromeric to H-2K (Klein *et al.*, 1982; Vincek *et al.*, 1989; 1990). In the B10.D2(R103) strain, only a region of undetermined length centromeric to H-2D derives from DBA/2 whereas the H-2D locus is inherited from the B10 inbred partner (Fig. 4). In this line, the MFI of NK2.1^{high} cells was enhanced, but at a slightly lower level than in B10.D2 mice (Table III). In B10.D2(R107), the differential segment inherited from the DBA/2 donor strain includes H-2D and a long region telomeric to that locus, whereas the H-2K, H-2A, and H-2E loci are inherited from the B10 inbred partner (Fig. 4). Interestingly, in that congenic recombinant strain, the MFI of NK2.1^{high} cells remained at the B10 strain level.

A similar modulation of NK2.1 expression was observed with B10 congenic strains carrying a differential segment of variable length from A mouse origin (Fig. 4 and Table III). Indeed, in B10.A(2R) mice, in which the differential segment covers a region of undetermined length centromeric to the H-2D locus, the MFI of NK2.1^{high} cells was enhanced but not as much as in B10.A. In contrast, in B10.A(5R) mice in which the differential segment covers the H-2D locus and a region telomeric to H-2D, the level of NK2.1 expression on NK2.1^{high} cells was not significantly

Figure 4. The minimal length of the chromosome 17 differential segment inherited in B10 congenic and congenic recombinant mice. The open bar indicates the minimal length of the differential segment and includes the name of the donor strain or question marks when the origin of that particular segment is unknown. The solid line indicates that this portion of the chromosome is of B10 origin. The centromere of chromosome 17 is located on the left side. The localization of selected loci and the length of differential segments were compiled from Klein *et al.*, 1982; Vincek *et al.*, 1989; 1990.

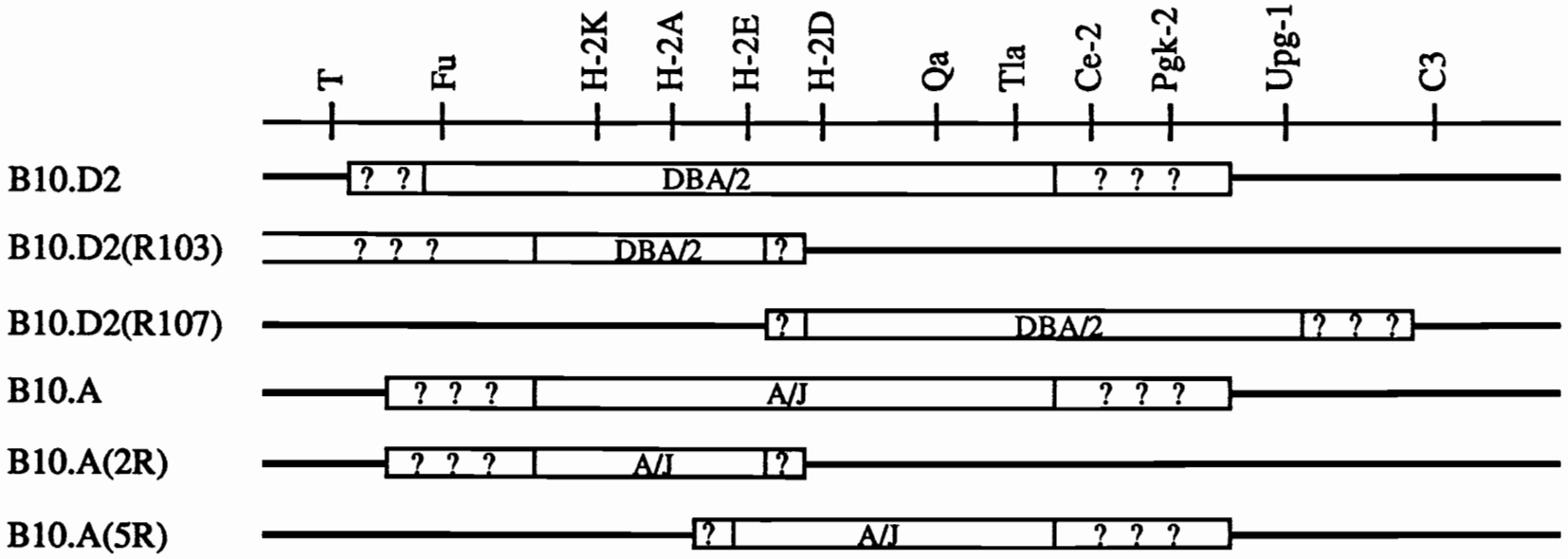

T
Fu
H-2K
H-2A
H-2E
H-2D
Qa
Tla
Ce-2
Pgk-2
Upg-1
C3
B10.D2
? ?
DBA/2
? ? ?
B10.D2(R103)
? ? ?
DBA/2
?
B10.D2(R107)
?
DBA/2
? ? ?
B10.A
? ? ?
A/J
? ? ?
B10.A(2R)
? ? ?
A/J
?
B10.A(5R)
?
A/J
? ? ?

Table III. Expression of NK2.1 on NK-enriched spleen cells from MHC congenic and congenic recombinant mice[a]

Strain	H-2 haplotype	H-2 alleles[b] for K	A	E	D	%NK2.1^{high} ± SD	MFI ± SD
C57BL/10	b	b	b	b	b	28 ± 2	10 ± 1
B10.D2	d	d	d	d	d	24 ± 2	38 ± 3
B10.D2(R103)	g3	d	d	d / b		22 ± 9	24 ± 7
B10.D2(R107)	i7	b	b	b / d		24 ± 8	9 ± 3
B10.A	a	k	k	k	d	28 ± 5	29 ± 6
B10.A(2R)	h2	k	k	k / b		29 ± 5	19 ± 2
B10.A(5R)	i5	b	b / k		d	28 ± 2	13 ± 3

[a] See legend to Table II.

[b] For each strain, the haplotype of each MHC subregion is shown with a slash indicating the site of recombination when applicable.

changed compared to the inbred partner. Although the possibility remains that NK2.1 expression may be influenced by H-2-linked genes rather than by H-2 loci, it is clear that the main genetic element down-regulating NK2.1 expression is centromeric to the H-2D locus. Since the lowest level of NK2.1 expression was observed in H-2^b mice, the b haplotype at the H-2K, H-2A, or H-2E loci are the prime candidates. The lower levels of NK2.1 expression in B10.BR (H-$2K^k$, H-$2D^k$) and B10.A(2R) (H-$2K^k$, H-$2D^b$) compared to B10.A (H-$2K^k$, H-$2D^d$) suggest that the k and b haplotypes at the H-2D locus may also contribute to down-regulating NK2.1 expression.

DISCUSSION

The majority of NK cell-associated molecules playing a role in activation or inhibition of NK cell functions are type II integral membrane disulfide-linked dimeric proteins having significant homology with the C-type lectin protein family (Yokoyama, 1995). In the mouse, most genes encoding NK-specific molecules are located in the NK gene complex of chromosome 6 (Yokoyama *et al.*, 1989; 1991; Wong *et al.*, 1991; Ryan *et al.*, 1992), a region which also includes the very early activation antigen CD69 another C-type lectin homologue (Ziegler *et al.*, 1994) which is not NK-specific but apparently serves as a triggering receptor for IL-2 activated NK cells (Testi *et al.*, 1994). It is of interest that the NK2.1 gene, which encodes a disulfide-linked glycoprotein dimer (Gosselin *et al.*, 1993) through which NK cell lytic properties can be triggered (Morelli and Lemieux, 1993), is linked to, or is even located in, the NK gene complex. Although the characterization is preliminary, the structural similarities between NK2.1

and proteins encoded by the NKR-P1 and Ly-49 genes, raise the possibility that NK2.1 may be a member of one of these families. Cloning of a cDNA encoding the NK2.1 protein, now ongoing in our laboratory, will clarify this issue. NK1.1, Ly-49A, 5E6, or LGL-1 are as yet the only known protein products encoded by members of the NKR-P1 and Ly-49 gene families. It is excluded that NK2.1 is an allelic form of one of these molecules since they are all co-expressed with NK2.1 in C57BL/6 mice (Mason *et al.*, 1988; Sentman *et al.*, 1989b; Yokoyama *et al.*, 1990; Lemieux *et al.*, 1991). The partial amino acid sequencing of the amino terminus of NK2.1, purified by affinity chromotography, revealed no homology with any of the predicted amino acid sequences corresponding to Ly-49 genes (unpublished observation), which share 80 to 90% of their amino acid residues in this region (Sentman *et al.*, 1991; Smith *et al.*, 1994). However, the amino terminus of the predicted amino acid sequence of NK1.1 (Ryan *et al.*, 1992) shares 40% homology with the sequence we obtained for NK2.1, supporting the possibility that NK2.1 may be a member of the NKR-P1 family.

We previously reported that BALB/c NWNA spleen cells, depleted of $CD4^+$ and $CD8^+$ cells, contained $NK2.1^+$ and $NK2.1^-$ cell populations (Gosselin *et al.*, 1993). We observed now with more sensitive flow cytometry that most of the NK-enriched spleen cells from all NK2.1-expressing strains tested were stained by the 4LO3311 mAb, defining $NK2.1^{low}$, $NK2.1^{high}$, and, in one case, $NK2.1^{int}$ populations. The biological relevance of these cell populations is yet to be elucidated. One possibility is that they may represent cells at different stages of activation or differentiation. This would agree with our observation that the

frequency of BALB/c NK2.1^{high} cells as well as the level of NK2.1 expression is up-regulated by IL-2 (Gosselin *et al.*, 1993 and unpublished data). Alternatively, cell populations expressing different levels of NK2.1 may represent distinct groups of clones as for Kp43^{low}, Kp43^{int}, and Kp43^{high} human NK clones that were isolated from a single donor (Moretta *et al.*, 1994). Interestingly, Kp43 (CD94) molecules act as inhibitory receptors only in clones with bright fluorescence, suggesting that the number of interactions between Kp43 molecules and their HLA ligands may be critical for the induction of the negative signal necessary to protect target cells from lysis. Functional differences between Ly-49A^{low} and Ly-49A^{high} cells were also reported recently (Olsson *et al.*, 1995). Previous observations from our laboratory support a similar relationship between function and NK2.1 expression level. We have indeed reported that NK2.1^{high} IL-2-stimulated cells were several times more lytic towards YAC-1 targets than the other NK-enriched cell population now characterized as NK2.1^{low} (Morelli and Lemieux, 1993).

Similar to our detection of NK cell populations expressing different levels of NK2.1 in mice of a given strain, variations in Ly-49A expression were demonstrated in a study with the YE1/48 mAb which allows the detection of Ly-49A^{high} and Ly-49A^{low} cell populations in C57BL/6 mice (Brennan *et al.*, 1994). Ly-49A^{high} cells could be further subdivided into 4 populations on the basis of NK1.1 and 5E6 expression and 5E6^{+} cells not expressing Ly-49A could also be subdivided into NK1.1^{+} and NK1.1^{-} populations. These findings, together with data reported herein, illustrate the complex pattern of heterogeneity within the NK cell population in the mouse, especially in C57BL which express all the activation and inhibitory

receptors identified thus far in that species. It can be anticipated that the target cell specificity and the functional properties of each NK cell population depend, as for human NK cell clones, on both the level of expression and the particular array of activation and inhibitory receptors present at the cell surface. It may however be difficult to address this question, given the difficulty in deriving murine NK cell clones.

By comparing NK2.1 expression in several inbred mouse strains, we observed that high, intermediate, and low NK2.1 expression levels of the NK2.1^{high} population correlated with the d/a, k and b H-2 haplotype, respectively. This observation raised the possibility that NK2.1 expression may be regulated by H-2 or H-2-linked genes in a way similar to the down-regulation of Ly-49A, recently reported in B10 congenic mice expressing H-2D^{d} or D^{k} MHC class I molecules (Karlhofer *et al.*, 1994). The enhanced NK2.1 expression in B10.D2 (H-2^{d}) compared to B10 (H-2^{b}) and B10.BR (H-2^{k}) and, conversely, the reduced NK2.1 expression in C.B10 (H-2^{b}) and C.C3 (H-2^{k}) compared to the BALB/c (H-2^{d}) inbred partner strongly support this hypothesis. It is thus tempting to speculate that as for Ly-49A, some MHC class I alleles may be ligands for NK2.1. Should down-regulation of NK2.1 expression in H-2 congenic mice be due to interaction of NK2.1 with host MHC class I molecules, H-2D^{d} which specifically binds Ly-49A (Karlhofer *et al.*, 1992; Kane, 1994) is unlikely to be involved since the highest expression of NK2.1 is found in inbred and congenic mouse strains expressing H-2D^{d}. Likewise, H-2K^{d} and K^{k} molecules are unlikely ligands of NK2.1 considering the enhanced MFI of NK2.1^{high} cells in B10.D2 (H-2K^{d}, H-2D^{d}) and B10.A (H-2K^{k}, H-2D^{d}) mice. On the other hand, the low MFI of NK2.1^{high} cells in B10 (H-2K^{b},

H-2D^b), B10.D2(R107) (H-2K^b, H-2D^d), and B10.A(5R) (H-2K^b, H-2D^d) mice would be consistent with H-2K^b interacting with NK2.1. This interpretation is also supported by the reduced NK2.1 expression in C.B10 (H-2K^b, H-2D^b) mice compared to the BALB/c inbred partner. The low NK2.1 expression in B10.BR (H-2K^k, H-2D^k) mice compared to B10.A (H-2K^k, H-2D^d) may indicate that NK2.1 can also interact with the H-2D^k molecule. This would also explain the reduced expression of NK2.1 in C.C3 mice. Similarly, since the MFI of NK2.1^{high} cells in B10.D2(R103) (H-2K^d, H-2D^b) and B10.A(2R) (H-2K^k, H-2D^b) is slightly lower than in B10.D2 (H-2K^d, H-2D^d) and B10.A (H-2K^k, H-2D^d), respectively, it cannot be excluded that NK2.1 interacts with H-2D^b molecules.

Unlike Ly-49A, which is recognized by several mAbs binding to different epitopes (Chan and Takei, 1986; Nagasawa *et al.*, 1987; Olsson *et al.*, 1995), the 4LO3311 mAb was the only anti-NK2.1 mAb available for this study. For this reason, it has not been possible to evaluate whether the apparent down-regulation of NK2.1 expression in mice expressing particular H-2 haplotypes was due to masking of the 4LO3311-reactive epitopes with host MHC molecules or, alternatively to reduction in the number of NK2.1 molecules expressed at the NK cell surface. Having recently selected hamster-mouse hybridomas secreting anti-NK2 mAbs with different epitope specificities (Paré and Lemieux, manuscript in preparation), we now have the appropriate tools to directly address this question.

The putative interaction of NK2.1 with the H-2K^b, D^k and D^b alleles would relate NK2.1 to Ly-49A, Kp43, p58 and NKB1 which were all

shown to bind particular MHC class I molecules (Moretta *et al.*, 1990a; 1990b; 1994; Karlhofer *et al.*, 1992; Litwin *et al.*, 1994). This raises the possibility that, in addition to being involved in activating lysis of susceptible tumor target cells (Morelli and Lemieux, 1993), NK2.1 may also act as an inhibitory receptor upon interaction with appropriate MHC molecules. There is at least one example of such a dual function for a human NK cell receptor. Indeed, in addition to its capacity to deliver an inhibitory signal to NK cells, Kp43 apparently also has the capacity to trigger NK cell lysis (Aramburu *et al.*, 1991) and to up-regulate TNF-α production (Aramburu *et al.*, 1993).

Two of the three class I molecules that may possibly interact with NK2.1 are different from those interacting with Ly-49A thus giving support to a specific target cell repertoire for murine NK cell populations. If as postulated NK2.1 is a receptor for H-2K^b, it would explain the reduced susceptibility of H-2K^b transfected YAC-1 cells to lysis by CBA/J, C57BL/6 and A/J mice (Carlow *et al.*, 1990) which all express NK2.1 (Lemieux *et al.*, 1991). However, other yet unknown receptors with different specificities should also exist since we have found no evidence that NK2.1 interacts with H-2K^d molecules which were recently shown to have the capacity to inhibit the cytotoxicity of DBA/2 and C57BL/6 NK cells in a Ly-49A-independent fashion (Roth *et al.*, 1994).

Although observations reported in this study are consistent with particular MHC class I molecules interacting with NK2.1, the possibility remains that the down-regulation of NK2.1 expression is rather regulated by MHC class II genes or even by H-2-linked genes. The use of congenic strains indeed

has its limitations since congenic mice generally differ from their inbred partners at hundreds of genes inherited from the donor strain, the exact length of the differential segment often being unknown (Klein *et al.*, 1982). Our data at least clearly indicates that the main H-2-associated element down-regulating NK2.1 expression is telomeric to the T locus but centromeric to H-2D. In an extensive evaluation of the role of MHC molecules in NK-cell mediated rejection of allogeneic, semi-syngeneic, and MHC-matched bone marrow grafts, Öhlén *et al.* (1995) found no evidence for a role of MHC class II molecules in this system. On the other hand, HLA class II-deficient tumor cells were reported to have an increased susceptibility to NK cell lysis, compared to the parent cell lines (Lobo and Patel, 1994). Whether MHC class II molecules interact with NK2.1 and thus contribute to the down-regulation of its NK cell surface expression will need further investigation.

It is noteworthy that although NK2.1 expression in B10.D2 mice is enhanced compared to B10 mice, it does not reach the expression level observed in the donor DBA/2 mice. Similarly, the introduction of a B10 differential segment including the H-2 complex into BALB/c mice did not reduce NK2.1 expression to the level found in B10 mice, thus indicating that non-H-2-dependent factors may also contribute to the regulation of NK2.1 expression. Differences in the MFI of NK2.1^{high} cells in BALB/c and DBA/2 mice, which both express the H-2^{d} haplotype, also support a role for non-H-2-dependent factors. We found, in LP mice, one exception to the low NK2.1 expression in mice expressing this haplotype. This is another indication that non-H-2-dependent factor(s) are involved in modulating NK2.1 expression. Putative regulatory elements acting on the

transcription of the NK2.1 gene or modulating NK2.1 mRNA or protein synthesis or stability are possible mediators of these differences. The occurrence of NK2.1^{high} cells with high fluorescence intensity in LP mice certainly challenges our hypothesis that H-2K^b and D^b alleles bind NK2.1. It may suggest that under certain circumstances, interaction of NK2.1 with host MHC molecules may be overridden by other as yet unidentified signals. A similar paradoxical situation is illustrated by the absence of binding of Ly-49A$^+$ cells to YAC-1 cells although these target cells express their main ligand H-2D^d (Karlhofer *et al.*, 1992; Brennan *et al.*, 1994).

Our results showing that NK2.1 expression is influenced by the H-2 haplotype raise the possibility that the NK2.1 molecule, which has already been shown to have activation properties, interacts with MHC class I molecules as do the NK cell inhibitory receptors. Further studies are however required to determine whether the down-regulation of NK2.1 expression seen in H-2-congenic mice is due to such an interaction with MHC class I molecules and/or whether other H-2 or H-2-linked gene products (also) contribute to this regulation.

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CHAPTER 5

General discussion and conclusions

5.1 - THE NK2.1 MOLECULE IN NK CELL BIOLOGY

When I started this research project, it was of general agreement that murine NK cells normally express NK1.1 and/or NK2.1 alloantigens. Formerly identified with alloantisera, these two molecules were detected in tissues where spontaneous NK cell activity was found. The selection of the PK136 anti-NK1.1 mAb-secreting hybridoma (Koo and Peppard, 1984) has been determinant for further characterization of the biological role of NK cells in the murine model, especially through the selective depletion of NK1.1 cells. The use of the PK136 mAb has however been limited since only a few strains (C57BL, NZB, CE, MA/My, and SJL) express the NK1.1 molecule.

Our laboratory had selected two anti-NK mAb-producing hybridomas with different specificity (Lemieux *et al.*, 1991), one of which reacting with a molecule of similar strain distribution as NK2.1, originally identified by an NZB anti-BALB/c antiserum (Pollack and Emmons, 1982). In chapter 2, I clearly established that one of our anti-NK mAbs, the 4LO3311 mAb, is specific for the NK2.1 antigen. Our initial characterization of the NK2.1$^+$ cell population by complement-dependent NK cell inhibition assay or by flow cytometry analysis suggested that the NK2.1 molecule was found on a subpopulation rather than all NK cells (Lemieux *et al.*, 1991; Morelli *et al.*, 1992). Results presented in chapters 2 and 3 are consistent with this hypothesis. From recent flow cytometry analyses, it however appears that the NK-enriched spleen cell population not reacting with the 4LO3311 mAb in our previous studies actually expresses a low level of NK2.1 (chapter 4). In addition, we found that expression of NK2.1 is

down-regulated in mice expressing H-2^b and H-2^k haplotypes (chapter 4) and that H-2-independent factors also appear to control the expression level of NK2.1. As discussed in chapter 4, variations in the level of NK2.1 expression may be related to regulatory elements acting on the transcription of the NK2.1 gene or on the stability of NK2.1 mRNA or protein. Analysis of the genomic structure of C57BL/6 and BALB/c NKR-P1 genes has suggested that differences in their level of expression may be caused by strain-specific transacting factors (Giorda *et al.*, 1992). It is thus quite possible that a similar situation occurs for NK2.1. Spleen cell subpopulations expressing low and high level of another NK cell receptor, Ly-49A, have also been reported (Brennan *et al.*, 1994) and Ly-49A expression may be down-regulated by H-2^d haplotype (Karlhofer *et al.*, 1994). A functional relevance for a low expression level of Ly-49A was recently proposed (Olsson, *et al.*, 1995). While Ly-49A$^+$ cells of D8 mice, which express a low level of Ly-49A, have failed to kill BALB/c Con A blasts expressing a high level of H-$2D^d$, they have efficiently killed SP2/0 tumor cells, which express H-$2D^d$ at a low level. These two target cells were however protected from killing by Ly-49A$^+$ cells from the C57BL/6 mouse which express a higher level of Ly-49A. Olsson and colleagues consequently formulated what they have called the "receptor-calibration" hypothesis, whereby a low density of an inhibitory receptor on the effector cell allows higher sensitivity for detection of reduced expression of its MHC class I ligand on a potential target cell. This theory has still to be verified.

Since our laboratory has established that NK cell lysis can be triggered through the NK2.1 molecule (Morelli and Lemieux, 1993), we speculated

that NK2.1^{low} (formerly NK2.1^{-}) and NK2.1^{high} cells may have different properties. In agreement with this hypothesis, we have already reported that NK2.1^{high} IL-2-stimulated cells are several times more lytic than the NK2.1^{low} counterpart while both cell populations share the same overall phenotype (Morelli and Lemieux, 1993). In the present study, I determined that even though NK2.1^{low} and NK2.1^{high} cell populations proliferated equally well when cultured in the presence of IL-2, this cytokine enhanced the NK2.1 expression on NK2.1^{high} cells and recruited NK2.1^{high} cells from the NK2.1$^{low/-}$ cell population (chapter 3). Considering the chronology of the increased NK2.1 expression and the induction of cell proliferation, these IL-2-mediated effects likely follow different pathways.

In addition to being expressed at a high level in NWNA, CD4^{-}CD8^{-} spleen cell suspensions, the NK2.1 antigen has been detected on a low number of CD4^{-}CD8^{-}IgG^{-} lymph node cells and on a small population of double negative thymocytes. These observations raise important questions regarding the ontogeny of NK2.1^{+} cells and the biological functions they may have in these organs. NK cell activity in the lymph nodes has been known for many years (Herberman *et al.*, 1975), but to the best of my knowledge, cells expressing NK cell antigens have never been described. Since, as mentioned in section 1.3.4, NK cells contribute to the regulation of immunoglobulin production by B cells, it is possible that NK2.1^{+} cells carry out this function in the lymph nodes. The presence of NK2.1^{+} cells in the thymus is of major interest considering the developmental relationship between NK cells and T cells. It however remains unclear whether NK2.1^{+}CD4^{-}CD8^{-} thymocytes are "classical" NK cells, or immature T

cells expressing a molecule generally found on peripheral NK cells. Since fetal thymocytes from C57BL/6 mice were shown to differentiate into mature NK1.1$^-$ T cells when transplanted in a thymic microenvironment but into NK1.1$^+$ NK cells when injected i.v. to irradiated recipient (Rodewald *et al.*, 1992) or cultured with IL-2 (Brooks *et al.*, 1993), one can hypothesize that NK2.1$^+$ cells found in the thymus of adult mice are rather mature NK cells. In the spleen, the NK2.1$^+$ cells express neither CD4, CD8, TCR$\alpha\beta$, or $\gamma\delta$ (Morelli and Lemieux, 1993), but most of them express a low level of CD3ε (unpublished observation). It however remains to be established whether NK2.1$^+$CD4$^-$CD8$^-$ thymocytes express CD3 and/or TCR ($\alpha\beta$ or $\gamma\delta$). It has been reported that the NK1.1$^+$ cells which were found in the thymus were unable to kill YAC-1 cells (Ballas and Rasmussen, 1990). However, NK cell activity against YAC-1 cells was observed with thymocyte suspensions from NK1.1$^-$ SCID mice (Garni-Wagner *et al.*, 1990). The possibility that NK2.1$^+$ cells, which are present in the thymus of SCID mice (S. Shibata, NIH, Shinjku-ku, Tokyo, Japan, personal communication), are more differentiated cells with lytic activity towards YAC-1 targets may reconcile these apparently contradictory findings. This is however yet to be determined as is the overall phenotype of thymus and lymph node NK2.1$^+$ cells and their capacity to secrete IFN-γ, TNF, and other lymphokines. A role for NK2.1$^+$ cells in regulating intrathymic T cell development has also to be considered.

Most of the activation or inhibitory receptors involved in regulating NK cell lysis share a common dimeric structure including a CRD in their extracellular domain, and are encoded by clustered genes located on mouse chromosome 6 named the NK gene complex. I characterized the NK2.1

molecule as a disulfide-linked dimer of 65 kDa subunits (chapter 2), and showed that the gene encoding NK2.1 is linked to those of the NK gene complex (chapter 4). These observations raise the possibility that the NK2.1 molecule may be encoded by a member of the Ly-49 or NKR-P1 gene families. Eight members of the Ly-49 gene family have been described and named Ly-49A through Ly-49H (Wong *et al.*, 1991; Smith *et al.*, 1994; Brennan *et al.*, 1994) but only Ly-49A, C, and G encode known proteins i.e. Ly-49A, 5E6, and LGL-1, respectively (Wong *et al.*, 1991; Stoneman *et al.*, 1993; Brennan *et al.*, 1994; Mason *et al.*, 1994). Ly-49A is an inhibitory receptor (Karlhofer *et al.*, 1992) and the 5E6, encoded by the Ly-49C gene, may function as an activation receptor (Sentman *et al.*, 1991). These findings indicate that all of the Ly-49 gene products do not necessarily have similar functions. Therefore, it cannot be excluded that the NK2.1 antigen may be encoded by a gene of the Ly-49 gene family. One way to address this question would be to verify whether COS cells transfected with each of the Ly-49 genes will be recognized by the 4LO3311 mAb. Ly-49C was in fact identified as a gene encoding the 5E6 molecule using this system (Brennan *et al.*, 1994). However, unpublished observations I made during my Ph. D. studies suggest that the NK2.1 antigen is not encoded by a Ly-49 gene. I have indeed purified a small amount of NK2.1 proteins by affinity chromatography and obtained a probable partial amino acid sequence of the NK2.1 amino terminus with the assistance of the Laboratory of Protein and Peptide Sequencing from the Biotechnology Research Institute at Montreal. There is only one amino acid shared between the sequence obtained for NK2.1 and members of the Ly-49 family (Figure 1). It is noteworthy that members of the Ly-49 gene

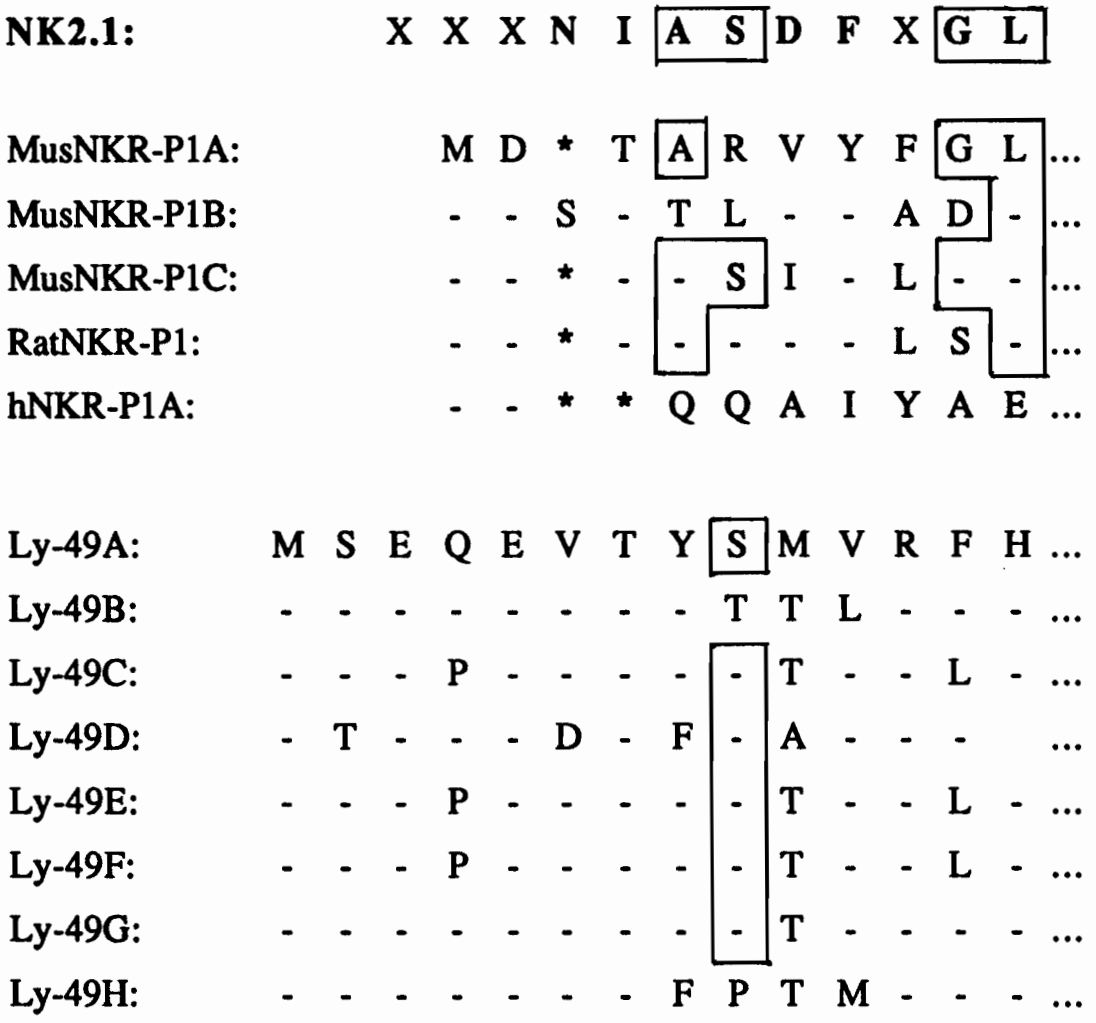

Figure 1. Comparison of the amino acid sequence at the NH_2-terminus of NK2.1 with the corresponding predicted sequences of mouse, rat, and human NKR-P1 and with members of the Ly-49 family. The NK2.1 amino acid sequence was obtained by sequencing the NH_2-terminus of affinity purified NK2.1 proteins. Dashes (-) represent amino acid identity to musNKR-P1A or Ly-49A, stars (*), gaps; and boxes, identity to NK2.1.

families share up to 91% identity in their predicted amino acid sequences (Smith *et al.*, 1994; Brennan *et al.*, 1994).

However, the sequence of NK2.1 is more homologous to that of NKR-P1 sequences, particularly NKR-P1C (NK1.1). These results would suggest that NK2.1 is not related to Ly-49 but rather to NKR-P1. Since, as discussed in chapter 2, none of the three known NKR-P1 genes is likely to encode NK2.1, this molecule may be encoded by a new member of the NKR-P1 family or by a gene possibly belonging to a new gene family. A definite answer to this hypothesis will be obtained only with the cloning of the NK2.1 gene. The complete sequencing of the NK2.1 molecule will also indicate whether NK2.1 is a type II protein with a CRD. If so, the functional properties of the CRD will have to be investigated.

I have performed a series of experiments in order to clone the NK2.1 gene using the expression cloning method described by Aruffo and Seed (1987). This method has been used by many laboratories to clone cell surface molecules identified by specific mAbs including the rat and human NKR-P1 antigens (Giorda *et al.*, 1990; Lanier *et al.*, 1994). I constructed a cDNA library into the expression vector pcDNAIneo with mRNA purified from BALB/c LAK cells. An A-LAK cDNA library from SCID (NK2.1$^+$) mice and from which the genes encoding the 5E6 and LGL-1 antigens have been originally cloned (Stoneman *et al.*, 1993; Mason *et al.*, 1994) was kindly provided by Dr. V. Kumar (Dallas, TX) and concurrently used with our cDNA library. COS-7 cells, transiently transfected with each cDNA library using the DEAE-dextran method for the first round and protoplast fusion for subsequent rounds, were screened for NK2.1 expression using

4LO3311 (IgG3) mAb and sheep anti-mouse IgG3 coated magnetic beads (Dynal). After 4 rounds of transfection and selection, an increased frequency of selected cells from ~2 to ~10% was obtained with each cDNA library. In each case, 48 cDNA clones were digested with appropriate restriction enzymes. Clones from the BALB/c cDNA library contained no inserts but plasmids from the SCID cDNA library contained inserts of different sizes ranging from ~1000 to ~3000 base pairs. Plasmids having inserts of similar sizes were grouped and a representative member of each group was transfected into COS-7 cells using the DEAE-dextran method or Lipofectin (Bethesda Research Laboratories). Transfected cells were cultured for 3 days, stained with biotinylated 4LO3311 or isotype control mAb and SA-PE, and analyzed by flow cytometry. Unfortunately, expression of NK2.1 was not detected on COS-7 cells transfected either ways with any of the clones. Since the 4LO3311 mAb is still able to immunoprecipitate the NK2.1 antigen in its deglycosylated form (unpublished observation), this mAb apparently detects an unglycosylated epitope of NK2.1. Therefore, the inability of the 4LO3311 mAb to bind transfected COS cells unlikely results from variations in the glycosylation of NK2.1 molecules expressed by COS cells. However, the 4LO3311 mAb might detect a conformational epitope since native but not denaturated NK2.1 proteins react with the 4LO3311 mAb in dot blot experiments (unpublished observation). It is also possible that, for still unknown reasons, these transfections did not allow NK2.1 expression, even if a gene encoding NK2.1 was present. A somewhat similar situation has been reported with NK1.1 where Giorda and Trucco (1992) had not been able to detect NK1.1 on COS-7 cells transfected with any of the musNKR-P1 genes. On the other hand, Ryan *et al.* (1992) were able to detect NK1.1 at

the surface of *Sf*9 cells infected with recombinant musNKR-P1C/baculovirus. The baculovirus expression system may thus be useful in identifying a cDNA clone encoding NK2.1. Alternatively, a degenerate oligonucleotide probe could be designed from the partial amino acid sequence of NK2.1 and used to screen a cDNA library by Southern blot hybridization. This probe would however be highly degenerated due to the 4 possible codons for the alanine and glycine residues and the 6 codons corresponding to leucine and serine residues.

One of the most challenging theories on the regulation of NK cell activity is that NK cell lysis is triggered through activation receptors engaged by oligosaccharide ligands on target cells, and that this activation can be over-ruled by interaction of inhibitory receptors with selected MHC class I molecules on target cells (see Figure 1 in chapter 1). Reduction of MHC class I expression (e.g., on tumor cells) or expression of foreign MHC class I alleles or peptides (e.g., on transplanted allogeneic cells or virally infected cells) prevents the triggering of the inhibitory signal, thus leading to lysis of the "non-self" or "altered-self" cell. This means that under normal conditions, NK cells can be constantly activated through activation receptors, like NK2.1, but are maintained in a repressed state through inhibitory receptors like Ly-49A. Such a situation is unlikely to be energy efficient for NK cells and would thus appear physiologically improbable. Nevertheless, a similar situation apparently occurs with cytotoxic T cells activated by bacterial superantigens. Indeed, Phillips *et al.* (1995) have reported that many cytotoxic human CD4+CD3+TCR$\alpha\beta$+ and CD8+CD3+TCR$\alpha\beta$+ T cell clones expressed NKB1 and possibly other inhibitory receptors which recognize polymorphic MHC class I molecules.

Engagement of these receptors by appropriate HLA class I would inhibit cytotoxic T cells from killing superantigen-coated target cells they would otherwise kill. Hence, it appears that there may be a modulation in T cell killing similar to that of NK cell killing. One possible interpretation of these results is that there may be a balance in positive and negative signalling (Barinaga, 1995). A foreign MHC-peptide complex that tightly binds to a TCR might send an activating signal that would be more powerful than the signal delivered by the inhibitory receptor, thus leading to killing of the target cell. On the other hand, an MHC-peptide complex which does not fit perfectly in the TCR might send weaker activating signals that would be overwhelmed by the inhibitory signal. That kind of "fine-tuning" might thus also occur in NK cell lysis and would explain the necessity for positive and negative signalling. Another level of "fine-tuning" might involve the recognition of the peptide associated with the MHC class I molecule by activation or inhibitory NK cell receptors. It is however still unclear whether the MHC molecule itself or the MHC-peptide complex confers protection from NK cell killing. Indeed, as presented in section 1.5.3, murine Ly-49A$^+$ cells apparently recognize MHC class I-peptide complexes largely independently of the peptide composition (Correa and Raulet, 1995) while different human NK cell clones are only inhibited when specific peptides are associated with MHC class I molecules (Malnati *et al.*, 1995). Similar experiments with other NK cell receptors or NK cell clones and peptide-MHC complexes are required to clarify this issue. In addition, if activation receptors also bind MHC class I, as suggested by our results (chapter 4), the involvement of the peptide contained in the peptide groove of MHC class I molecules in activation of NK cell killing will have to be investigated.

One can also wonder whether activation receptors really exist or if activation simply results from the absence of inhibition. A recent study by Correa *et al.* (1994) suggests that lysis of a target cell by NK cells must be triggered by any one of a set of distinct target cell ligands, but that all of these signals can be overruled by MHC class I-mediated inhibition. In the mouse, the only known inhibitory receptor is Ly-49A, a molecule expressed by a small subpopulation (15-20%) of C57BL NK cells (Yokoyama *et al.*, 1990). Identification of other murine inhibitory receptors as well as further studies of the intracellular events following interactions of an inhibitory receptor with an MHC class I molecule on the target cell is required to clarify this interesting and evolving subject.

5.2 - CONCLUSIONS

Initial investigations on NK cells have suggested that these cells were nonspecifically killing different target cells. Today, it is of general agreement that like T cells, NK cells represent an important group of lymphocytes involved in immunosurveillance. Indeed, tumor cells or virally infected cells which could avoid T cell lysis by reducing or altering expression of MHC class I molecules will be killed by NK cells. Results presented herein, together with previous observations from our laboratory, indicate that the NK2.1 antigen is a receptor playing a key role in the NK cell-mediated immunosurveillance. Cloning of the NK2.1 gene should now clarify the relationship between NK2.1 and activation as well as inhibitory receptors. Future studies should also establish whether MHC class I molecules, particularly H-2K^b, D^b, and D^k, are natural ligands of NK2.1, and elucidate intracellular events following the interaction between

NK2.1 and its ligand. An improved knowledge of the structure and the regulation of the NK2.1 antigen as well as its functional relevance in NK cell functions undoubtedly contributes to deepen our understanding of the biology of NK cells.

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5.4 - CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

Chapter 2:

- By sequential immunoprecipitation using the 4LO3311 mAb and an NZB anti-BALB/c (anti-NK2.1) antiserum, I determined that the 4LO3311 mAb is reacting with NK2.1.

- SDS-PAGE analyses under nonreducing and reducing conditions allowed me to show that NK2.1 is expressed at the NK cell surface as a disulfide-linked dimer of 65 kDa protein subunits with abundant *N*-linked sugars. On the basis of its resistance to PI-PLC treatment, the NK2.1 antigen is likely to be a transmembrane protein.

- By using flow cytometry analysis, I demonstrated that a fraction of double negative thymocytes and $CD4^-CD8^-IgG^-$ lymph node cells express NK2.1 and that the percentage of $NK2.1^+$ cells in NK-enriched spleen cell suspensions is increased from ~50% to ~60% after a short-term culture in the presence of IL-2.

Chapter 3:

- Further analysis of the effect of IL-2 on NK2.1 expression allowed me to show that the increased frequency of $NK2.1^+$ cells following IL-2 stimulation resulted from an induced expression of NK2.1 on some $NK2.1^-$ cells.

- In a kinetic study, I showed that IL-2 increases surface expression of NK2.1 after only 4 h of stimulation, increases NK2.1+ cell frequency after 24 h, and induces cell proliferation after 3 days.

Chapter 4:

- I demonstrated that the NK2.1 gene is linked to the NKR-P1 and Ly-49 multigene families of the NK gene complex on the distal segment of chromosome 6.

- I determined that most NK-enriched spleen cells of commonly used mouse strains express the NK2.1 antigen defining two to three distinct subpopulations varying in NK2.1 expression level.

- By studying NK2.1 expression on NK-enriched spleen cells from inbred, congenic, and congenic recombinant mouse strains, I demonstrated that expression of the NK2.1 antigen is regulated by H-2-dependent factors, likely to be H-2K^b, D^k, and D^b molecules, and by non-H-2-dependent factors yet to be identified.

APPENDIX I

CELLULAR IMMUNOLOGY 134, 191-204 (1991)

Identification of Murine Natural Killer Cell Subsets with Monoclonal Antibodies Derived from 129 Anti-C57BL/6 Immune Spleen Cells[1]

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Two hybridomas producing monoclonal antibodies reactive with natural killer cells were selected after fusion of 129 anti-C57BL/6 immune spleen cells with P3X63-Ag8.653 myeloma cells. Treatment of normal or stimulated cells with the 4LO3311 or the 4LO439 mAb and rabbit complement inhibited natural killer and antibody-dependent cellular cytotoxicities, whereas cell lysis mediated by natural cytotoxic cells, cytotoxic T lymphocytes, or activated macrophages was unaffected. Lymphokine-activated killer activity was reduced after complement-mediated treatment of interleukin-2-stimulated spleen cells with the 4LO3311 mAb but not after treatment with the 4LO439 mAb. Similar treatment of spleen cells with either mAb had no effect on the mitogen-induced proliferation of T and B lymphocytes and did not alter the frequency of antibody plaque-forming cells in immune spleen cell suspensions. The 4LO3311 and 4LO439 mAbs thus appear to be specific for NK cells and their progeny. Flow cytometry analysis confirmed that 4LO3311$^+$ and 4LO439$^+$ cells are phenotypically identical to NK-1.1$^+$ cells. The epitope recognized by the 4LO3311 mAb has the same strain distribution as the NK-2.1 alloantigen previously detected with NZB anti-BALB/c antiserum, whereas the 4LO439 mAb appears to identify a new NK cell marker exclusively expressed in mice of C57BL lineage. The relationship of the molecules detected with either the 4LO3311 or the 4LO439 mAb to polymorphic antigens of the Ly series is discussed.

INTRODUCTION

Most mouse strains express either NK-1.1 or NK-2.1 Natural killer (NK)[3]-specific antigens that were originally detected with (C3H × BALB/c)F_1 anti-CE (1) and either CE anti-CBA (2) or NZB anti-BALB/c (3) antisera, respectively. As both NK-1.1 and NK-2.1 antigens have been detected in C57BL mice, that they are antithetical molecules encoded by a single gene locus is excluded (3, 4). Koo and Peppard (5) first succeeded in producing an anti-NK-1.1 monoclonal antibody (mAb)-designated PK136. All the

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[3] Abbreviations used: ADCC, antibody-dependent cellular cytotoxicity; Con A, concanavalin A; CTL, cytotoxic T lymphocyte; E:T, effector:target; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GAMIG, goat anti-mouse IgG; GARIG, goat anti-rabbit IgG; LAK, lymphokine-activated killer; LPS, lipopolysaccharide; mAb(s), monoclonal antibody(ies); NC, natural cytotoxic; NK, natural killer; NWNA, nylon wool nonadherent; poly(I:C), polyinosinic acid–polycytidylic acid.

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splenic NK cell activity against YAC-1 target cells being included in sorted NK-1.1$^+$ cells (6), this mAb has been extremely useful in establishing, on the basis of other cell surface criteria or specific functional properties, that NK-1.1$^+$ cells constitute a heterogeneous population. Indeed, only one-half of NK-1.1$^+$ cells express Thy-1 antigen (6). Likewise, another murine NK-specific mAb (SW5E6) produced against interleukin-2-propagated (Il-2) NK-1.1$^+$ cells was shown to bind to ~50% of sorted NK-1.1$^+$ cells (7). Interestingly, treatment of irradiated recipients with SW5E6 antibodies abolished their capacity to reject *Hh-1*^{d}- but not *Hh-1*^{b}-incompatible bone marrow cell grafts (7). A rat mAb (4D11) reacting with LGL-1, a nonpolymorphic antigen of murine NK cells, has also been produced (8). It was further established that NK-1.1$^+$LGL-1$^+$ cells constitute a particular subset of NK cells (~50% of NK-1.1$^+$ cells) which have strong lytic activity against YAC-1 target cells but which are not lymphokine-activated killer (LAK) precursor or effector cells (9). Recently, Ballas and Rasmussen (10) showed that 40 to 60% of splenic NK-1.1$^+$ cells express MEL 14, the murine homing receptor. They also identified, among CD4$^-$CD8$^-$ thymocytes, an NK-1.1$^+$MEL 14$^-$ cell subset which was phenotypically distinct from splenic NK-1.1$^+$ cells.

Most studies of NK-1.1$^+$ cell heterogeneity have been done with C57BL/6 mice. This is not surprising since all other commonly used strains do not express the NK-1.1 alloantigen. Monoclonal antibodies recognizing NK-2.1, a specificity more commonly distributed (2, 3), would be desirable to allow comparable studies in NK-1.1$^-$ strains. The production of such a reagent has not yet been achieved. In the present paper we report the production and characterization of two new murine anti-NK mAbs. According to the cell specificity and strain distribution of the corresponding alloantigens, the reactivity of the 4LO3311 mAb is consistent with its recognizing NK-2.1, whereas the 4LO439 mAb identifies a new NK-specific marker exclusively expressed in C57BL strains.

MATERIALS AND METHODS

Mice. C57BL/6N, DBA/2N, BALB/cAnN, and C3H/HeN mice of both sexes were purchased from Charles River Canada Inc. (St.-Constant, Quebec). All other mouse strains were obtained from the Jackson Laboratory (Bar Harbor, ME).

Medium. Tumor cell cultures and assays were done in RPMI 1640 medium supplemented with 2 m*M* L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, (GIBCO Laboratories, Burlington, Ontario), and 25 m*M* Hepes buffer (Sigma Chemical Co., St. Louis, MO). The medium was also supplemented with fetal bovine serum (FBS, GIBCO Laboratories), at final concentrations adapted for each cell culture or assay.

Tumor cell lines. YAC-1, P815, RDM4, and WEHI-164 cells used as target cells in cytotoxicity assays were maintained in RPMI 1640 medium supplemented with 10% FBS. C57BL/6-derived EL-4 thymoma and AKR-derived BW5147 thymoma tumor cell lines used in cell specificity analyses were maintained under the same conditions. CTLL-2 cells were propagated in the presence of IL-2.

Stimulation of NK cell activity. Mice with low spontaneous NK cell activity were inoculated i.p. with 100 μg polyinosinic acid–polycytidilic acid (poly(I:C), Terochem Laboratories Ltd., Mississauga, Ontario) 24 hr before their spleen cells were used in NK cell inhibition assays.

Immunization and fusion for mAb production. C57BL/6 spleen cells were enriched for NK cells by the sequential elimination of non-NK cells according to a method we

have recently developed (11). Briefly, macrophages are eliminated first by adherence to plastic, after which T cells are excluded from multicellular aggregates formed by agglutination of the other cells with wheat germ lectin (Sigma Chemical Co.). After dissociation of the aggregates with *N*-acetyl-D-glucosamine (Sigma Chemical Co.) and elimination of erythrocytes by osmotic shock, NK cells are separated from the remaining cells, mostly B cells, by filtration through a nylon wool column. With this procedure, almost all the input NK cell activity, enriched 10- to 30-fold, is recovered in the final fraction. 129/SvJ mice were primed i.p. with $1-5 \times 10^6$ C57BL/6 NK-enriched spleen cells and they were reimmunized i.v. 3 weeks later with 2×10^6 cells. Immune spleen cells, collected 3 days after boost, were fused with nonsecreting P3X63-Ag8.653 myeloma cells (653) in the presence of PEG1000 (Sigma Chemical Co.) as previously described (12).

Screening of hybridoma supernatants. Hybridomas were screened for the production of anti-NK antibodies by testing the complement-dependent capacity of growth positive culture supernatants to inhibit the lysis of YAC-1 target cells by C57BL/6 (NK-1.1$^+$NK-2.1$^+$) splenic NK cells. Culture supernatants of 653 myeloma cells were used as negative controls in these NK inhibition assays. Hybridomas of interest were cloned two times by limiting dilution and each clone was expanded to produce a single large pool of supernatant for the analysis of the antibody specificity. Isotypes of anti-NK mAbs were determined by double-immunodiffusion on 10× concentrated hybridoma supernatants using rabbit monospecific anti-mouse immunoglobulins (Miles Laboratories, Inc., Elkhart, IN).

Cell cytotoxicity assays. NK, Antibody-dependent (ADCC), LAK, and CTL cytotoxicities were measured in standard 4- to 6-hr ^{51}Cr-release assays as described previously (11), incubation being prolonged to 18–24 hr for natural cytotoxic (NC) cell and macrophage cytotoxicity assays. Spleen cells from normal or poly(I:C)-stimulated mice were used as effector cells in NK, NC, and ADCC assays, target cells being YAC-1, WEHI-164, and antibody-coated P815 cells, respectively. The antiserum used for coating target cells for the ADCC assays was prepared in C57BL/6 mice. Heat-inactivated antiserum was incubated with radiolabeled P815 target cells for 60 min at 37°C, before effector cells were added. LAK cells were generated in bulk culture by incubating 5×10^7 spleen cells in 10 ml of medium supplemented with 10% FBS, 5×10^{-5} *M* 2-mercaptoethanol (Sigma Chemical Co.), and 200 U/ml of recombinant IL-2 (Boehringer Mannheim Canada Ltd., Dorval, Quebec) for 5 days at 37°C in a humidified 5% CO_2 atmosphere. Viable cultured cells were isolated by centrifugation on Lympholyte M (Cedarlane Laboratories Ltd., Hornby, Ontario) and then used in a cytotoxicity assay against NK-resistant P815 target cells. Spleen cells from either C3H (*H-2*^{k}) mice immunized with P815 (*H-2*^{d}) cells or C57BL/6 (*H-2*^{b}) mice immunized with RDM4 (*H-2*^{b}) cells were used as effector cells for CTL cytotoxicity assays. Effector cells for macrophage-mediated cytotoxicity were thioglycolate-elicited peritoneal exudate cells harvested from C3H or C57BL/6 mice. Plastic adherent cells were incubated for 24 hr with 200 μl of lymphokine-rich supernatant collected from concanavalin A (Con A)-stimulated spleen cell cultures before their cytotoxic activity was measured by addition of P815 target cells.

Inhibition of cell-mediated cytotoxicity by antibody plus complement. Effector cells were incubated 30 min at 4°C with hybridoma supernatants and then 45 min at 37°C with diluted rabbit complement (Pel-Freez Biologicals Inc., Rogers, AR). Cytotoxic activity of treated cells, washed twice and resuspended to the original volume, was then compared with that of untreated cells tested at different effector:target (E:T)

ratios. The inhibiting capacity of the mAb was calculated from the determination of the number of untreated effector cells necessary to produce lysis equivalent to the residual cytotoxic activity of treated cells. A complement control or an isotype antibody control of unrelated specificity was included in every experiment. Anti-NK-1.1 mAb produced by the PK136 hybridoma (American Type Culture Collection, Rockville, MA), anti-Ly-2 mAb (Cedarlane Laboratories), anti-H-2K^k mAb (Becton Dickinson, Mountain View, CA), anti-H-2^b antiserum (B10.A anti-B10 antiserum obtained from Dr. E. Skamene, McGill Centre for Host Resistance, Montreal, Quebec), and anti-Ia mAbs (Cedarlane Laboratories) were used as positive controls in NK, CTL, NC, and macrophage cytotoxicity assays, respectively.

Flow cytometry analysis. For single-color analysis, nylon wool nonadherent (NWNA) spleen cells were stained with optimal concentrations of 4LO3311, 4LO439, or PK136 (anti-NK-1.1) mAb and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (GAMIG) specific for the γ chain (Jackson Immunoresearch Laboratories Inc., West Grove, PA). Control samples were incubated with isotype control mAb and FITC–GAMIG or with FITC–GAMIG alone. For two-color analysis, cells were further depleted of CD8$^+$ and/or CD4$^+$ cell subsets by 30 min incubation on ice with rat anti-Ly-2(CD8) mAb (clone 53-6.72, ATCC) or rat anti-L3T4(CD4) mAb (clone MT4, kindly provided by Dr. F. J. Dumont, Merck, Sharp, and Dohme Research Laboratories, Rahway, NJ) or both sequentially, followed by the elimination of antibody-coated cells with sheep anti-rat IgG-coated magnetic beads (Dynabeads, Dynal Inc., Great Neck, NY) using a ratio of five beads/cell. Purified 4LO3311, 4LO439, and PK136 mAbs extracted from ascitic fluid by affinity chromatography on GammaBind G PrePack cartridges (Genex Corp., Gaithersburg, MD) were conjugated to biotin and/or FITC according to standard procedures. The following reagents were also used in the study: phycoerythrin-conjugated anti-L3T4, biotin-conjugated anti-Ly-2, biotin-conjugated anti-Thy-1, and phycoerythrin-labeled streptavidin for detection (all from Becton Dickinson), rabbit anti-asialo GM1 antiserum (Wako Chemicals USA Inc., Richmond, VA), and FITC- or biotin-conjugated goat anti-rabbit IgG (GARIG, Jackson Immunoresearch Laboratories Inc.). Analysis of 10,000 cells/sample was performed on an EPICS C cytofluorograph (Coulter Electronics, Hialeah, FL) equipped with a 5-W argon laser. The fluorescence intensity measured was expressed on a logarithmic scale. In the two-color analysis, gates dividing the four quadrants were set on the basis of control samples which consisted either of unlabeled cells for autofluorescence or cells incubated with phycoerythrin-labeled streptavidin or with FITC- or biotin-conjugated GARIG for background staining. Samples incubated with each mAb alone were also run for comparison.

RESULTS

Selection of Strains for the Production and Detection of Murine Anti-NK mAbs

In addition to NK-1.1 and NK-2.1 molecules, Thy-1 and several other non-NK-specific alloantigens, mostly belonging to the Qa (Qa-2, Qa-4, and Qa-5) and Ly (Ly-1, Ly-5, Ly-6, Ly-10, and Ly-11) series, are expressed on some or all murine NK cells (reviewed in (4, 13)). H-2-compatible C57BL/6 and 129 strains share identical alleles at all of these loci (14–16). In addition, C57BL/6 NK cells express both NK-1.1 and NK-2.1 alloantigens, whereas 129 cells express neither (3, 5). This strain combination thus offered a theoretical opportunity to produce monoclonal antibodies against both murine NK cell markers in a single fusion. 129/SvJ mice from the Jackson Laboratory

colony were used for immunization because they are the only 129 mice not producing IgM anti-IgG autoantibodies that could impair the detection of anti-NK antibodies (17).

The lymphocyte function-associated antigen-1 (LFA-1) (18), the receptor for the Fc portion of IgG2b/IgG1 immunoglobulins (FcγRII) (19, 20), and the phagocyte glycoprotein-1 (Pgp-1) (21) have been also shown to be expressed on most NK cells. As for the other aforementioned non-NK-specific alloantigens, these three molecules are polymorphic antigens which are encoded by genes at *Ly-15* (22), *Ly-17* (formerly *Ly-m20*) (23–25), and *Ly-24* (14) loci, respectively. 129 and C57BL/6 share the same Ly-15 and Ly-24 specificities but they express different alleles at the Ly-17 locus (14). Serologically detected Ly-17 polymorphism was recently shown to correlate with two amino acid substitutions at positions 116 and 161 of the extracellular portion of FcγRIIβ1 (26). Even though the FcγRIIα is encoded by a different gene, its external portion has 95% homology with FcγRIIβ1 and FcγRIIβ2, which are otherwise identical in their external domains (27–29). It is still unknown whether Ly-17 polymorphism is also detectable on FcγRIIα, which would be the case if the separation of α and β FcγRII genes occurred after Ly-17-related nucleotide substitutions arose. It was recently reported that only the FcγRIIα transcripts are present in C57BL/6 NK-1.1$^+$ cells (20). As Ly-17 polymorphism of FcγRIIα has not been ruled out and since 129 and C57BL/6 mice express different Ly-17 specificities, hybridomas secreting anti-Ly-17.2 antibodies could be produced and the reaction of their supernatants could be wrongfully interpreted as being NK specific, unless a careful selection of strains were carried out for testing of the mAbs. Therefore, mouse strains were classified according to their NK cell phenotype and their Ly-17 specificity in order to identify the most appropriate strains to screen hybridoma supernatants for the presence of anti-NK-1.1 and anti-NK-2.1 antibodies (Table 1).

Only four of the strains listed meet the requirements for this purpose, as they express the 129 allele at the Ly-17 locus and only one of the two NK-specific alloantigens. NZB (NK-1.1$^+$NK-2.1$^-$) and C3H (NK-1.1$^-$NK-2.1$^+$) mice were selected for antibody screening because they also display a high spontaneous NK cell activity.

Characterization of Hybridomas Secreting Anti-NK Antibodies of Different Specificities

Hybridoma supernatants from wells containing growing cell colonies were first tested for their complement-dependent capacity to inhibit NK cell activity mediated by

TABLE 1

Classification of Mouse Strains According to NK Cell Phenotype and Ly-17 Specificity[a]

Ly-17 specificity	NK cell phenotype			
	NK-1.1$^+$NK-2.1$^+$	NK-1.1$^+$NK-2.1$^-$	NK-1.1$^-$NK-2.1$^+$	NK-1.1$^-$NK-2.1$^-$
Ly-17.1	—	CE, NZB	A/J, C3H/He	SJL, SM, 129
Ly-17.2	C57BL/6	MA/My	BALB/c, CBA/J, DBA/1, DBA/2	C57BR, C58

[a] The strain distribution of the Ly-17 (formerly Ly-m20) alloantigen was compiled from Ref. (23) and the NK cell phenotypes from flow cytometry analysis with the PK136 (anti-NK-1.1) mAb (7) and from complement-dependent NK inhibition assays or flow cytometry analyses with NZB anti-BALB/c (anti-NK-2.1) antiserum (3).

C57BL/6 (NK-1.1$^+$NK-2.1$^+$) spleen cells. Positive hybridomas were cloned and subcloned by limiting dilution and their supernatants were then assayed for the complement-dependent capacity to inhibit NZB (NK-1.1$^+$NK-2.1$^-$) and C3H (NK-1.1$^-$NK-2.1$^+$) splenic NK cell activity. Two hybridomas producing anti-NK mAbs of different specificities were selected in this way (Table 2).

As the 4LO3311 mAb inhibited C57BL/6 and C3H NK cell activity but had no effect on NZB NK cells, that it detects the NK-1.1 alloantigen is excluded, but it could recognize the NK-2.1 alloantigen. On the other hand, although the 4LO439 mAb strongly inhibited C57BL/6 NK cell activity, it had no significant effect on spleen cells expressing the NK-1.1 or the NK-2.1 specificity alone. Neither the 4LO3311 nor the 4LO439 mAb had any NK-inhibiting capacity in the absence of complement, indicating that the alloantigens they recognize are not involved in the binding of NK cells to YAC-1 target cells. Immunodiffusion analysis revealed that the mAb present in 4LO3311 and 4LO439 hybridoma supernatants are both of IgG3 isotype with *k* light chains.

Cell Specificity of the 4LO3311 and 4LO439 mAbs

To ascertain the NK cell specificity of the two mAbs produced, their capacity to inhibit several other specific and nonspecific immune cell functions was tested. Cells collected from C3H and C57BL/6 were used to characterize the 4LO3311 and 4LO439 mAbs, respectively. Treatment of C3H spleen cells with the 4LO3311 mAb and complement inhibited both NK cell activity (Fig. 1a) and ADCC equally well, (Fig. 1b), but only slightly depressed LAK cytotoxicity (Fig. 1c). This finding is consistent with the known heterogeneity of the LAK cell population (30). As expected, the 4LO3311 mAb and complement treatment was totally inefficient in inhibiting tumor cell lysis

TABLE 2

Identification of Hybridomas Secreting Anti-NK mAb of Different Specificities[a]

Spleen cell source	Percentage lysis of untreated cells			Residual lysis after incubation with mAb + C		
				4LO3311	4LO439	Control[b]
	150:1	75:1	37:1	150:1	150:1	150:1
C57BL/6	25	14	5	17 (38)[c]	14 (50)	26 (0)
	32	24	13	25 (40)	20 (60)	32 (0)
	39	26	19	32 (31)	25 (59)	42 (0)
NZB	39	27	14	38 (3)	40 (0)	38 (3)
	25	18	12	25 (0)	27 (0)	27 (0)
	30	21	16	31 (0)	30 (0)	31 (0)
C3H	34	26	11	18 (65)	33 (11)	35 (0)
	29	23	11	13 (71)	28 (6)	28 (6)
	32	19	10	17 (56)	31 (3)	31 (3)

[a] Results shown for each strain were obtained in three different experiments.

[b] Supernatant from 653 myeloma cell culture.

[c] Percentage suppression calculated as described under Materials and Methods. Under similar conditions, C57BL/6 and NZB NK cell activities were respectively inhibited by 69 ± 6% and 34 ± 1% with the PK136 anti-NK-1.1 hybridoma supernatant, whereas C3H NK cell activity was unaffected.

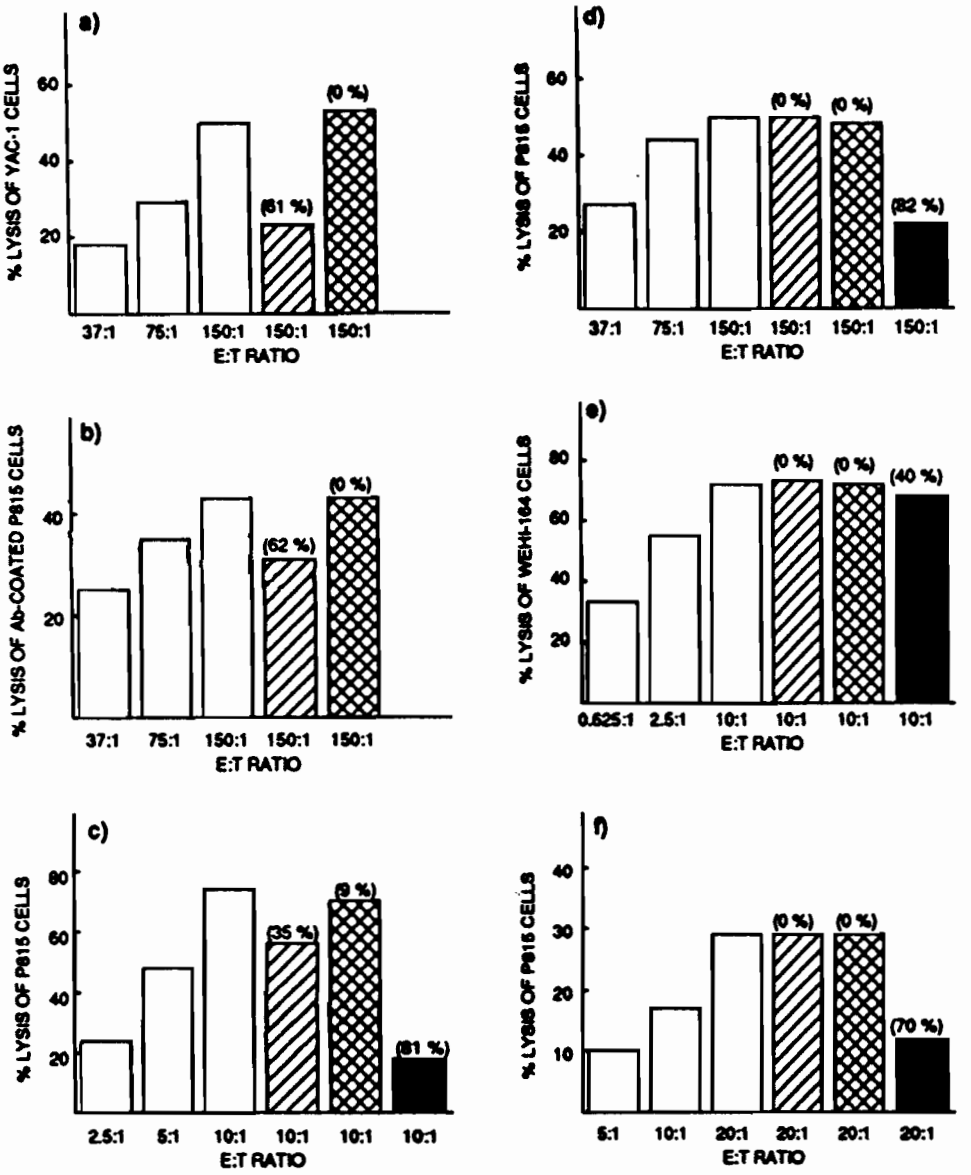

FIG. 1. Effect of the 4LO3311 mAb plus complement treatment on the cytotoxic activity of different C3H cell subsets. Normal spleen cells (a, b, e), rIL-2-stimulated spleen cells (c), alloimmune spleen cells (d), or activated macrophages (f) were incubated with medium alone (open bars), with the 4LO3311 mAb (hatched bars), with the isotype control mAb (cross-hatched bars), or with the positive control mAbs (solid bars) and complement before they were tested with appropriate target cells for NK (a), antibody-dependent (b), LAK (c), CTL (d), NC (e), and macrophage (f) cytotoxicity. The percentage of suppression indicated in parenthesis was calculated in reference to control cells incubated in medium alone. The positive control mAbs in CTL, NC, and macrophage cytotoxicity assays are identified under Materials and Methods.

by alloimmune CTL (Fig. 1d), NC cells (Fig. 1e), and activated macrophages (Fig. 1f). Similar results were obtained after treatment of C57BL/6 cells with the 4LO439 mAb in the presence of complement except that LAK activity was not at all depressed by the treatment (Fig. 2). Con A- and LPS-induced lymphoproliferation, as well as an-

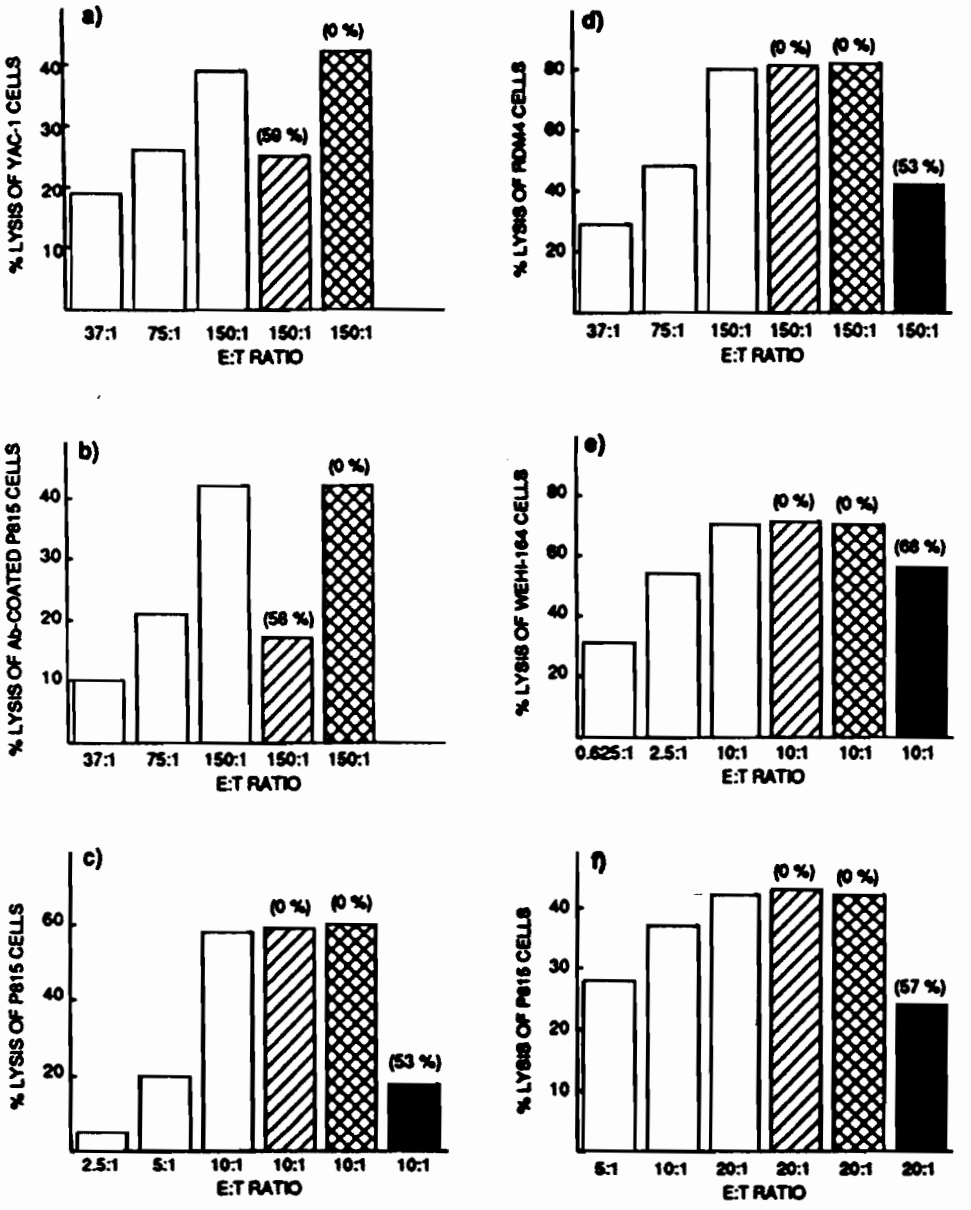

FIG. 2. Effect of the 4LO439 mAb plus complement treatment on the cytotoxic activity of different C57BL/6 cell subsets. See legend for Fig. 1 except that hatched bars represent cells treated with the 4LO439 mAb and complement.

tibody plaque-forming cell frequency, was also unaffected by treatment of normal or immune spleen cells with either mAb and complement (data not shown). Therefore, the alloantigens detected by the 4LO3311 and 4LO439 mAbs are most likely specific for NK cells and their progeny, as they are not expressed on NC cells, resting or activated T and B lymphocytes, and activated macrophages.

Flow Cytometry Analysis of Cells Recognized by the 4LO3311 and 4LO439 mAbs

In order to confirm the phenotype of cells reactive with the 4LO3311 and 4LO439 mAbs, one- and two-color immunofluorescence analyses were performed. PK136 anti-NK-1.1 mAb was used as a control.

To begin, C3H, C57BL/6, and NZB NWNA spleen cells were studied after incubation with each mAb and FITC–GAMIG. C3H and C57BL/6 but not NZB spleen cells were stained with the 4LO3311 mAb and neither NZB nor C3H spleen cells reacted with the 4LO439 mAb, thus corroborating the results obtained in complement-dependent NK inhibition assays (Fig. 3 and Table 2). A smaller number of less bright cells was detected with the 4LO3311 mAb in C57BL/6 (2.8%) than in C3H (12.6%) spleen cell suspensions, an observation which also correlates with the capacity of the mAb to inhibit NK cell lysis. Although C57BL/6 spleen cells were stained with the three mAbs, distinctive profiles were obtained with each mAb not only in regard to the number of positive cells but also in terms of fluorescence intensity. The negativity of NZB (NK-1.1$^+$NK-2.1$^-$) and C3H (NK-1.1$^-$NK-2.1$^+$) spleen cells with the 4LO439 mAb rules out the possibility that it detects the NK-1.1 or the NK-2.1 molecules or an associated antigen. Therefore, this 4LO439 mAb could recognize either a non-NK-specific alloantigen not yet reported as being present on the NK cell surface or a previously undescribed NK-specific alloantigen as well. In another series of experiments, neither the 4LO3311 nor the 4LO439 mAb stained YAC-1, EL-4, RDM4, BW5147, or CTLL-2 lymphoid cell lines (data not shown).

In order to determine if 4LO3311$^+$ and 4LO439$^+$ cells coexpress T and NK cell markers, two-color immunofluorescence studies were also performed. Coexpression of L3T4 and Ly-2 alloantigens was studied on NWNA cells, further enriched for NK cells by the elimination of either the Ly-2$^+$ or the L3T4$^+$ cell subset. Coexpression of Thy-1, asialo GM1, and NK-1.1 antigens was studied on NWNA cells depleted of both Ly-2$^+$ and L3T4$^+$ cell subsets. The frequency of NK cells in T-cell-depleted

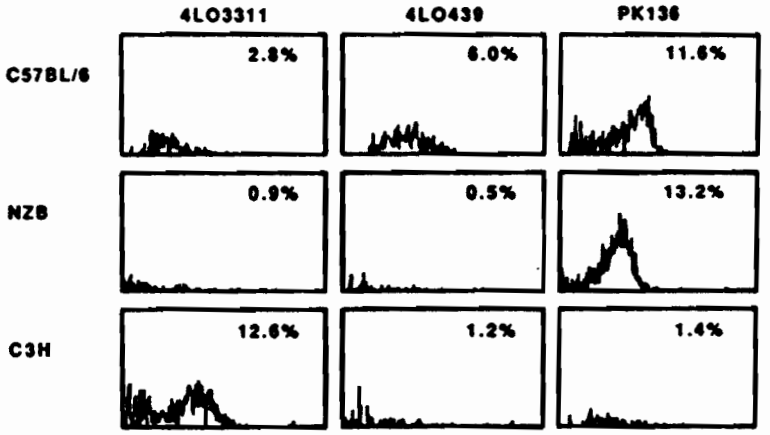

FIG. 3. Indirect immunofluorescence analysis of spleen cells with different anti-NK mAb. Representative histograms corresponding to the staining of NWNA spleen cells with the 4LO3311, 4LO439, and PK136 (anti-NK-1.1) mAbs and FITC–GAMIG are illustrated. Numbers in upper-right corners correspond to the percentage of positive cells remaining after background staining was subtracted using Easy2 IMMUNO data analysis software (Coulter Electronics). At least three similar analyses have been done with spleen cells of each strain. The *x* and *y* axes represent the fluorescence intensity and cell number, respectively.

suspensions was measured by direct immunofluorescence with FITC- or biotin-conjugated anti-NK mAbs, the elimination of T-cell subsets being controlled with phycoerythrin-conjugated anti-L3T4 mAb and with biotin-conjugated anti-Ly-2 mAb and phycoerythrin-conjugated streptavidin. A typical NK cell enrichment is illustrated in Fig. 4. The PK136$^+$ and 4LO439$^+$ cells were enriched 4.6- and 4.2-fold, respectively, in C57BL/6 spleen cell suspensions depleted of both T cell subsets (lower panel). The enrichment of C3H 4LO3311$^+$ cells following elimination of L3T4$^+$ and Ly-2$^+$ T cells was of the same magnitude (data not shown). The results reported in Tables 3, 4, and 5 indicate that C57BL/6 4LO439$^+$ cells and C3H 4LO3311$^+$ cells otherwise have the same phenotype as C57BL/6 NK-1.1$^+$ cells. Cells coexpressing either 4LO3311, 4LO439, or NK-1.1 and L3T4 were negligible (Tables 3 and 4). 4LO3311$^+$ cells were also Ly-2$^-$ (Table 4), but about 30% of NK-1.1$^+$ and 40% of 4LO439$^+$ cells were found to express a low amount of Ly-2 in L3T4-depleted cell suspensions (Table 3). The phenotype of murine NK cells would thus be similar to human NK cells which do not express CD4, but do express CD8 antigen in a proportion of 30–50% (13). As was previously reported for NK-1.1$^+$ cells (6), some but not all 4LO3311$^+$ and 4LO439$^+$ cells express Thy-1 antigen (Tables 3 and 4). With regard to NK cell markers, the majority of 4LO3311$^+$ and 4LO439$^+$ cells express asialo GM1 as well as NK-1.1 antigen (Table 5). Most 4LO3311$^+$ and 4LO439$^+$ cells in C57BL/6 mice belong to different cell subsets, since only 42% of 4LO3311$^+$ cells are included in the 4LO439$^+$ cell subset.

Strain Distribution of Alloantigens Recognized by the 4LO3311 and 4LO439 mAbs

The strain distribution pattern of the alloantigens detected with the 4LO3311 and 4LO439 mAbs was determined in complement-dependent NK-inhibition assays using

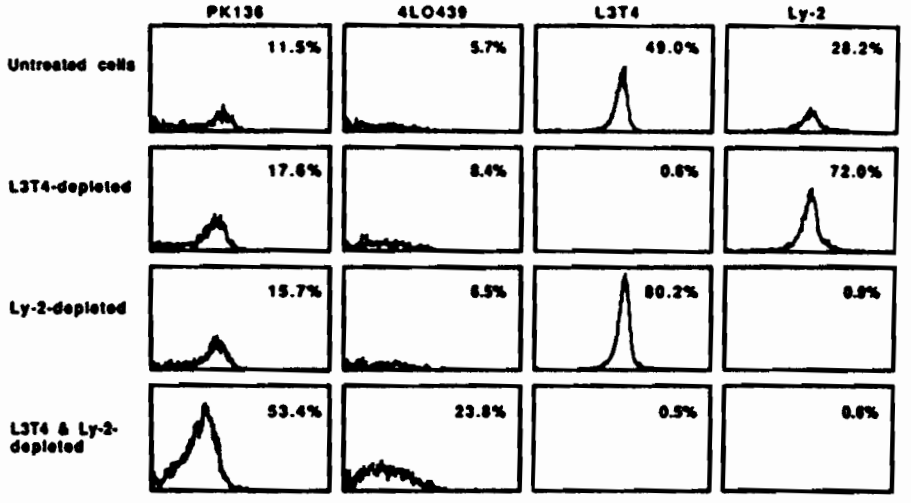

FIG. 4. Frequency of PK136(NK-1.1)$^+$ and 4LO439$^+$ cells in NWNA and L3T4- and/or Ly-2-depleted spleen cell suspensions. Cells prepared as described under Materials and Methods were incubated with FITC-labeled PK136, FITC-labeled 4LO439, phycoerythrin-labeled anti-L3T4, or biotin-labeled anti-Ly-2 and phycoerythrin–streptavidin. Each histogram illustrates the residual frequency of positive cells after background staining was substracted using IMMUNO Easy2 data analysis software. This is a representative experiment of four identical analyses. The *x* and *y* axes represent the fluorescence intensity and cell number, respectively.

TABLE 3

Expression of T Lymphocyte Alloantigens on 4LO439$^+$ and PK136$^+$ Cells[a]

Percentage Ly-2-depleted cells expressing			Percentage L3T4-depleted cells expressing			Percentage Ly-2- and L3T4-depleted cells expressing		
L3T4 only	4LO439 only	4LO439 and L3T4	Ly-2 only	4LO439 only	4LO439 and Ly-2	Thy-1 only	4LO439 only	4LO439 and Thy-1
64 ± 6	3 ± 1	1 ± 1	58 ± 18	4 ± 2	3 ± 1	33 ± 11	4 ± 1	7 ± 3
L3T4 only	PK136 only	PK136 and L3T4	Ly-2 only	PK136 only	PK136 and Ly-2	Thy-1 only	PK136 only	PK136 and Thy-1
70 ± 14	9 ± 3	1 ± 1	55 ± 10	10 ± 2	4 ± 1	22 ± 10	18 ± 9	16 ± 5

[a] C57BL/6 NWNA cells depleted of L3T4 and/or Ly-2 T cells were incubated with FITC-conjugated 4LO439 or PK136 mAb and either phycoerythrin-conjugated anti-L3T4, biotin-conjugated anti-Ly-2, or anti-Thy-1 and phycoerythrin-conjugated streptavidin. Results correspond to percentage of positive cells (mean ± S.D. from three experiments) showing red fluorescence, green fluorescence, or both, above background staining.

a panel of 22 inbred mouse strains (Table 6). Half of the strains tested express the alloantigen recognized by the 4LO3311 mAb and this strain distribution pattern correlates with the one reported for the alloantigen detected with NZB anti-BALB/c (anti-NK-2.1) antiserum (3). DBA/1 spleen cells which were positive only in flow cytometry studies with this antiserum (3) were unquestionably reactive in NK cell inhibition assays with 4LO3311 mAb. To our surprise, the specificity detected with the 4LO439 mAb was expressed only in C57BL/6 and C57BL/10 mice. The identification of this new C57BL strain-restricted murine NK cell marker thus emphasizes the uniqueness of these strains in regard to NK cell phenotype as these mice are the only ones which express both the NK-1.1 and the NK-2.1 alloantigens.

DISCUSSION

Here we report the production and the characterization of two anti-NK mAbs, derived from 129/SvJ mice immunized with C57BL/6 NK-enriched spleen cells. In

TABLE 4

Expression of T Lymphocyte Alloantigens on 4LO3311$^+$ Cells[a]

Percentage Ly-2-depleted cells expressing			Percentage L3T4-depleted cells expressing			Percentage Ly-2- and L3T4-depleted cells expressing		
L3T4 only	4LO3311 only	4LO3311 and L3T4	Ly-2 only	4LO3311 only	4LO3311 and Ly-2	Thy-1 only	4LO3311 only	4LO3311 and Thy-1
66 ± 2	4 ± 1	0 ± 1	45 ± 3	8 ± 2	0 ± 1	44 ± 8	9 ± 4	8 ± 3

[a] C3H NWNA spleen cells depleted of L3T4 and/or Ly-2 T cell subsets as described under Materials and Methods were incubated with biotin-conjugated 4LO3311 mAb, phycoerythrin-conjugated streptavidin, and FITC-conjugated anti-L3T4, anti-Ly-2, or anti-Thy-1 mAb. Results correspond to percentage of positive cells (mean ± S.D. from three experiments) showing green fluorescence, red fluorescence, or both, above background staining.

TABLE 5

Expression of NK Cell Markers on 4LO3311$^+$ and 4LO439$^+$ Cells[a]

Cells tested	Percentage of cells coexpressing		
	Asialo GM1	PK136 (NK-1.1)	4LO439
4LO3311$^+$	88 ± 11[b]	76 ± 6	42 ± 12
4LO439$^+$	97 ± 4	78 ± 1	—

[a] C57BL/6 NWNA spleen cells depleted of L3T4$^+$ and Ly-2$^+$ cells were used for these experiments, except for expression of asialo GM1 on 4LO3311$^+$ cells, which was determined with C3H spleen cells.

[b] Mean ± S.D. (from three experiments).

view of the cell specificity and the strain distribution of the detected molecules, the 4LO3311 mAb could putatively recognize the NK-2.1 antigen previously characterized with NZB anti-BALB/c and CE anti-CBA antisera (2, 3), whereas the 4LO439 mAb binds to a new specificity exclusively expressed in mice of C57BL strains.

The strain distribution pattern of the alloantigen recognized by the 4LO3311 mAb corresponds to that determined for NK-2.1 with NZB anti-BALB/c antiserum. Although this strongly suggests that the 4LO3311 mAb recognizes the NK-2.1 specificity, this finding alone is as yet insufficient to allow this conclusion. By employing either CE anti-CBA or NZB anti-BALB/c antisera for typing, the NK-2.1 alloantigen was previously shown to be coexpressed on all NK-1.1$^+$ cells of C57BL/6 mice (6, 31). The cell population detected in C57BL/6 spleen cell suspensions with the 4LO3311 mAb is much smaller than that stained with the PK136 (anti-NK-1.1) mAb. This is not due to low avidity of the mAb, as the number of 4LO3311$^+$ cells and their fluorescence intensity in C3H are comparable to those observed in C57BL/6 and NZB spleen cell suspensions with the PK136 mAb. The differences compared to previous reports in regard to the percentage of C57BL/6 NK cells reacting with the 4LO3311 mAb (putatively anti-NK-2.1) may however be due to the presence of NK-reactive antibodies other than anti-NK-2.1 in NZB anti-BALB/c and CE anti-CBA/J antisera. NZB and BALB/c mice actually express different Ly-17 and Ly-24 specificities and CE and CBA/J have different alleles at *Ly-10*, *Ly-17*, and *Ly-24* loci which identify polymorphism of alloantigens expressed on most NK cells.

TABLE 6

Strain Distribution of NK-Specific Alloantigens Detected with the 4LO3311 and 4LO439 mAbs[a]

Strain	4LO3311	4LO439
A/J, AKR/J, BALB/cAnN, C3H/He, CBA/J, DBA/1J, DBA/2N, LP/J, ST/bJ	+	−
C57BL/6N, C57BL/10SnJ	+	+
C57BR/cdJ, C57L/J, C58/J, CE/J, MA/MyJ, NZB/BINJ, NZW/LacJ, RIIIS/J, SM/J, SJL/J, 129/SvJ	−	−

[a] Determined in complement-dependent NK inhibition assays. At least three mice of each strain were individually tested. Mice with low spontaneous NK cell activity were stimulated with 100 μg poly(I:C) 24 h before assay.

No mice other than those of C57BL strains, among 22 inbred strains tested, were found to express the molecule detected with the 4LO439 mAb. The reactivity of three other anti-NK mAbs, SW3A4, SW4B12, and SW2B4, recently derived from 129/J mice, immunized with C57BL/6-purified and IL-2-propagated NK-1.1$^+$ cells, was also shown to be limited to C57BL/6 and B10.A mice (32). On the basis of the strain distribution of the detected epitopes and their cosegregation in BXD recombinant inbred mice, the SW3A4, SW4B12, and PK136 mAbs all likely detect the NK-1.1 alloantigen. Although B10.A spleen cells also react with the 4LO439 mAb (data not shown), this reagent clearly detects a different molecule as the treatment of NZB, CE, and MA/My (NK-1.1$^+$) spleen cells with the mAb and complement does not alter their NK cell activity, nor can the 4LO439 mAb stain NZB NWNA spleen cells. It is also unlikely that the 4LO439 and SW2B4 mAbs detect the same antigen, as all NK cell activity was found in the SW2B4$^+$ sorted cell fraction (29), whereas different numbers of C57BL/6 cells were stained with the PK136 and 4LO439 mAbs.

The 4LO3311 and 4LO439 mAbs most probably detect subsets of NK cells. However, that either one could be equivalent to the SW5E6 anti-NK mAb, a reagent which recognizes a subset of NK cells present not only in C57BL mouse strains but also in NZB mice is excluded (7). Because of its restricted strain distribution pattern, that the antigen detected with the 4LO439 mAb could be LGL-1 is also excluded (8). On the other hand, it remains possible that the 4D11 anti-LGL-1 mAb, which identifies a subset of NK-1.1$^+$ cells in C57BL/6 mice, could detect an invariant epitope of the molecule identified with the 4LO3311 mAb. Nine of the 10 strains reported to react with the 4D11 mAb are indeed NK-2.1$^+$ strains whose splenic NK cell activity is otherwise inhibited by treatment with the 4LO3311 mAb in the presence of complement. SJL was the only NK-2.1$^-$4LO3311$^-$ strain tested with the 4D11 mAb and the frequency of 4D11$^+$ spleen cells in these mice was shown to be very low in comparison with that in all other tested strains. This is likely to be due to the low frequency of NK cells in SJL mice, which are characterized, not only by a low level of spontaneous NK cell activity, but also by low responsiveness to interferon and interferon inducers (33). However, to establish whether the 4D11 and 4LO3311 mAbs detect different antigens or the same molecule, it would be necessary to know if the LGL-1 antigen is also expressed in NZB, CE, and MA/My mice, which all belong to NK-1.1$^+$NK-2.1$^-$ strains, which are unreactive with the 4LO3311 mAb.

Over 50 cell surface molecules have been identified thus far on murine leucocytes (14). Given that the expression of most of these antigens on NK cells has never been tested for, it could therefore be possible that the molecules detected with either the 4LO3311 or the 4LO439 mAb are identical to one of the Ly specificities. However, most are excluded as possible candidates as they are either nonpolymorphic molecules or alloantigens expressed on resting or activated T and/or B lymphocytes (34). In fact, incompatibility between 129 and C57BL/6 mice has been identified at only eight of the *Ly* loci controlling polymorphic antigens (14). Strain distributions of these alloantigens rule out the possibility that any of them would be identical to the molecule detected with the 4LO3311 or the 4LO439 mAb, which therefore are considered as NK cell specific. In regard to the heterogeneity of NK cell functions and the fine specificity of the anti-NK mAbs described here, these new reagents should thus be valuable in determining whether 4LO3311$^+$ and 4LO439$^+$ NK cells are involved in a defined NK function.

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