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Characterization of a novel function-associated molecule (FAM) on murine natural killer cells

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The University of Arizona, 1994
CHARACTERIZATION OF A NOVEL FUNCTION-ASSOCIATED MOLECULE (FAM) ON MURINE NATURAL KILLER CELLS

by

Reuben Kapur

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
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1994
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Reuben Kapur entitled CHARACTERIZATION OF A NOVEL FUNCTION-ASSOCIATED MOLECULE (FAM) ON MURINE NATURAL KILLER CELLS. and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of DOCTOR OF PHILOSOPHY.

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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STATEMENT BY AUTHOR

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SIGNED: Reuben Kapur
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ABSTRACT

True antigen receptors on murine natural killer (NK) cells have not been identified. In this study, using a novel monoclonal antibody (mAb) directed against a 43 kDa function-associated molecule (FAM) specific to murine NK cells, a "receptor-like" structure has been characterized. It was observed by two-color flow cytometric analysis that the anti-FAM mAb specifically binds to a subpopulation of nylon wool nonadherent (NWNA) lymphocytes (19-20%). The expression of FAM was restricted to NK cells that expressed the NK1.1 antigen. Analysis of FAM expression in various lymphoid tissues revealed that splenocytes expressed the greatest numbers of mAb(+) cells. Lymphokine activated killer (LAK) cells and adherent lymphokine activated killer (ALAK) cells expressed higher levels of FAM. It was demonstrated that the anti-FAM mAb inhibited the lysis and the conjugate formation (i.e. recognition) of target cells by NK cells. Stimulation of NK cells with anti-FAM mAb resulted in enhanced cytotoxicity as well as lymphokine production by NK cells. Redirected lysis of the anti-FAM mAb bearing hybridoma indicated that this structure is an activating (i.e., signal-transducing) molecule. Modulation of FAM from the surface of NK cells resulted in a significant loss of cytotoxicity as well as conjugate formation.

A partial amino acid sequence of the anti-FAM mAb recognized molecule from rat NK cells was found to be closely related to an intermediate filament, vimentin. Anti-vimentin mAb reacted with NK cells, as shown by flow cytometry, and further, inhibited the cytotoxicity and the conjugate formation between NK cells and target cells. Moreover, northern blot analysis and polymerase chain reaction (PCR) analysis revealed no differences in the size of the mRNA between NK cells that express this molecule and cells that are negative for the expression of this
molecule. Treatment of NK cells with anti-sense oligonucleotides against the vimentin gene inhibited the expression and the lytic activity of NK cells against target cells.

Taken as a whole, these results demonstrated that the "vimentin-like" NK cell surface molecule FAM may play an important role in the recognition, signal-transduction, and lytic mechanism of murine NK cells.

The ligand for FAM on fish and human NK target cells has been shown to be a 43 kDa molecule. An anti-idiotypic (id) mAb against the NK target cell antigen-specific mAb (18C2) inhibits the cytotoxicity of rat NK cells by binding to their membrane. It has been shown that the anti-id mAb recognizes a vimentin-like FAM on the membrane of NK cells. This study describes that the ligand for FAM is also present on murine transformed cell lines. The mAb 18C2 recognized a 43 kDa molecule on murine NK target cells and inhibited the lysis and conjugate formation (i.e. recognition) of target cells by NK cells. Further, the role of MHC class I antigens in NK cell cytotoxicity was examined. It was determined that target cells that express low levels of MHC class I antigens in association with the novel target cell antigen were more sensitive to NK cell lysis.
CHAPTER 1

INTRODUCTION

Natural killer (NK) cells constitute a minor subpopulation of lymphocytes that are characterized in vitro as being non-adherent, non-phagocytic, display a large, granular morphology and are found in unstimulated peripheral lymphoid tissues and blood (1-3). NK cells play a major role in resistance to growth and metastatic spread of tumors (4,5). In addition, NK cells play a role in eliminating certain viral and bacterial infections (6-8). NK cells display antibody dependent cell mediated cytotoxicity (ADCC) (1-3), regulate B cell immunoglobulin synthesis (1-3), and mediate the phenomenon of "hybrid resistance" in bone marrow transplantation (9). However, controversy still exists with regard to NK cell lineage with respect to other immune cells (10) and to the relationship of NK cells to other effector cells such as lymphokine activated killer (LAK) cells and adherent lymphokine activated killer (ALAK) cells (11).

One of the major functions of NK cells is the destruction of malignant and virally-infected cells. It is generally accepted that NK cells lyse their targets in the following sequential manner: recognition and conjugation of the NK cell to the target cell (12); triggering or activation of the NK cells (12,13); release of a cytotoxic substance (14,15); binding of the cytotoxic substance to the target cell; and membrane damage, or DNA fragmentation, resulting in target cell death (16). However, one of the controversial questions in NK cell biology is by what structure(s) is NK cell antigen recognition and lysis mediated? T cells recognize products of the MHC (17) expressed on antigen presenting cells. However, it was not until the last few years that the T cell antigen receptor (TCR) was discovered,
isolated and characterized (18). Although freshly isolated NK cells express the delta and the zeta chains of the CD3/T cell receptor (TCR) complex (19,20), NK cells do not however, display other components of the complex, do not express mRNA for mature T cell receptor chains, and do not rearrange TCR genes (21).

The identification of the TCR and its ligand (MHC/peptide antigen) was facilitated by the use of mAbs which recognized these structures and inhibited various T cell functions. In terms of NK cell biology however, mAb inhibition of function has been less frequently documented, both in terms of mAbs directed against target cells and against the effector cells.

The structures involved in NK cell recognition of target cells and the subsequent triggering of the effector cell have not yet been determined. Although several candidates have been considered, no definite NK antigen receptors have been identified. It is generally believed that NK cell cytotoxicity is mediated in part by several cell interaction/ adhesion molecules such as CD2 and LFA-1 which interact with ligands such as LFA-3 and ICAM-1 on target cells (22,23). Though none of these molecules are specific to NK cells, mAbs against these molecules have been shown to partially inhibit NK cell lysis, although their relationship to an NK antigen receptor is not known (24-26).

The nature of the target cell antigen that is recognized by NK cells is not known. It has been shown that on certain target cell types the levels of MHC class I antigen expression inversely correlates with target cell susceptibility to NK cell lysis (27-31). These results have been explained by utilizing the "missing self" hypothesis, which suggests that NK cells recognize and lyse target cells that lack the expression or express low levels of MHC class I molecules. It also has been postulated that MHC class I expression may mask the target cell antigen responsible for recognition
by NK cells (32), or that recognition of certain MHC class I molecules may deliver a negative signal to NK cells (32).

Therefore, in an effort to identify an "antigen-receptor" on NK cells and the antigen that is recognized on target cells, mAbs were raised against both NK cells and NK target cells. Using these mAbs a novel function-associated molecule (FAM) with "receptor-like" characteristics on NK cells and, a novel target cell antigen on NK target cells have been characterized at the cellular as well as at the molecular level.
RESEARCH AIMS

The principle aim of this research was to characterize a function-associated molecule (FAM) on murine natural killer (NK) cells at the cellular and molecular level, and to characterize a novel murine NK-target cell antigen using monoclonal antibodies (mAbs). The specific goals were:

(1) to identify if various effector cell populations express FAM, using an anti-FAM mAb;

(2) to determine the biological effects of the anti-FAM mAb on various effector cell populations;

(3) to determine if FAM is a signal-transducing molecule;

(4) to characterize FAM at the molecular level;

(5) to identify murine NK target cells that express a target cell antigen recognized by the anti-target cell mAb; and

(6) to determine the biological effects of the anti-target cell mAb.
Literature Review

Natural killer (NK) cells have been defined as large granular lymphocytes (LGL) that do not express the CD3 antigen or any of the known T cell receptor (TCR) chains, but that do express CD16 and CD56 surface markers in humans and NK1.1/NK2.1 in mice. Also, NK cells mediate cytolytic reactions even in the absence of class I or class II major histocompatibility complex (MHC) expression on the target cells (33).

Certain T lymphocytes, particularly upon activation, may display cytolytic activity that resembles that of NK cells. These lymphocytes are more appropriately described as displaying "NK-like" activity or "non-MHC" restricted cytotoxicity (33). Lymphokine-activated killer (LAK) cells are NK cells that have been cultured in the presence of high doses of IL-2 (34).

It is generally believed that NK cells develop in the bone marrow. The transplantable NK precursor cell has been analyzed in detail by bone marrow transplantation in anti-asialo GM1 antibody-injected mice and by detection of NK activity as ability of the animals to clear i.v.-injected, radio-labeled YAC-1 tumor cells from the lung (35-37). Differentiation of NK cell precursors in vivo requires an intact marrow microenviornment, as shown by the failure of estradiol-treated mice to sustain NK cell differentiation (37). Bone marrow cells from severe combined immunodeficient (scid) mice contain a normal frequency of NK precursors, indicating that the NK precursors cells are distinct from those of T and B lymphocytes (36,37). More recently it has been demonstrated that mature, functional NK cells, identified as membrane (m)CD3-CD7+CD45+CD56+ lymphocytes, are abundantly present in human fetal liver and spleen as early as six weeks of gestation (38). The majority of these fetal NK cells express CD3 epsilon
and delta proteins in the cytoplasm but not on the cell surface. Fetal NK cell clones stably express cytoplasmic CD3 epsilon-gamma and CD3 epsilon-delta complexes, and the zeta chain. Fetal NK cells can mediate non-MHC-restricted cytotoxicity against NK-sensitive tumors, function in antibody-dependent cellular cytotoxicity (ADCC) assays, respond to IL-2, and produce interferon-gamma and tumor necrosis factor-alpha after stimulation in vitro (38). While TCR gene rearrangement clearly discriminates between T and NK cell types, T cells and NK cells are remarkably similar with respect to expression of other membrane receptors and immune effector functions. NK cells develop normally in athymic nude mice, demonstrating the thymus-independent maturation of this lineage (39). NK cells are however, present at a very low frequency in both the human (40) and the mouse (41) thymus. Thymic NK cells express the prototypic phenotype: mCD3-CD16+ in humans and mCD3-NK1.1+ in appropriate mouse strains. It is unclear whether these thymic NK cells are resident cells or are mature, recirculating lymphocytes. Recently, it was reported that the majority of thymocytes isolated from fetal mice of 14.5 days gestation express CD16 (42). When CD16+ fetal thymocytes were transplanted by intravenous injection into recipient mice and the splenocytes of the recipients grown in vitro with IL-2, NK1.1+ NK cells of donor origin were detected in these cultures (42). Adoptive transplantation of the CD16+ fetal thymocytes by intrathymic injection resulted in the generation of CD4+ and CD8+ thymocytes. These results indicate that either NK cells and T cells arise from a common CD16+ progenitor present in day 14.5 fetal thymus, or that within the CD16+ fetal thymic population two different cell types exist, a transplantable T-cell progenitor and a low frequency of mature NK cells that expand when cultured with IL-2. These studies are consistent with, but do not prove, the existence of a common T cell-NK cell progenitor.
NK cells have also been implicated in regulation of the immune system. There is considerable evidence that NK cells regulate B cell functions in that depletion of NK cells either in vivo or in vitro results in alterations in levels of antibody production (43-45). It has been shown that co-culture of purified B and NK cells results in measurable alterations in the responses of both cell types which are dependent on the activation status of the B lymphocytes. Low density B lymphocytes (presumably in vivo pre-activated) can enhance the production of gamma-IFN by NK cells (46), which in turn induces these B cells to switch to IgG2a production after mitogenic stimulation (47). It has also been demonstrated that NK cells can also produce other unknown soluble factors which induce B lymphocytes to proliferate and secrete IgM (48).

NK cells also play a major role in the regulation of hematopoiesis. NK cells mediate hybrid resistance of F1 mice to parental bone marrow transplantation (49,50). Parental hematopoietic or lymphoid grafts do not survive in lethally irradiated F1 hybrids, even though these animals are universal recipients of grafts of other types of parental tissue (49). The genetic control of hybrid resistance contrasts with the classical transplantation studies which show that the graft compatibility rests predominantly on multiple genetic determinants of cellular antigens inherited codominantly: the histocompatibility (H) antigens. The F1 hybrid anti-parent reaction has been explained by assuming the existence of a class of noncodominant genes, designated Hh for hematopoietic histocompatibility, with tissue distribution restricted to hematopoietc cells (49). Hybrid resistance is reduced in NK-deficient beige mice (50), and the ability to reject bone marrow (B.M) in a genetically restricted way is adoptively transferred by clones with NK cell activity (51). It has also been shown that NK cells inhibit both autologous and allogeneic bone marrow colony forming units (CFU) (52-55). NK cells require several hours of contact with
the bone marrow cells before plating in semisolid media, in order to mediate maximum inhibition (54). B.M-derived CFU-granulocyte/macrophage (GM) and CFU-erythrocyte (E) are maximally inhibited by NK cells (56-58). In addition, studies have shown that peripheral blood- derived CFU-GM are not inhibited, but rather stimulated by NK cells (59,60).

NK cells also play an important role in the early stages of viral infections. During viral infections, both NK and T cell populations are activated and undergo proliferation. The kinetics of the responses and the factors that regulate them however, differ. Using the model of lymphocytic-choriomeningitis virus (LCMV) infection (61-63), it has been shown that NK cell activation, blastogenesis, and proliferation peak around day 3 post-infection (63) and that the responding cell population has a CD3-NK1.1+ surface phenotype (63-65). At later times, e.g., day 7 post-infection, the decline in NK cell proliferation and function is accompanied by T cell proliferation and an increase in LCMV-specific T cell activation (63,66,67). Cytomegalovirus (MCMV) infection of the mouse is presently the system with the most convincing evidence that NK cells play a role in resistance to virus infection in vivo. This was shown by performing experiments in different mouse strains, by altering NK cell activity in vivo with stimulators and inhibitors, including treatment with anti-asialo-GM1 antiserum, and by adoptive transfer experiments (68-71). Injection of anti-asialo-GM1 antiserum up to the third day of infection increases the virus titer up to 1000-fold (70,71).

Although NK cells were identified on the basis of the naturally occurring cytotoxic activity, this cell type exerts additional functions, as described above. The most striking characteristic of NK cells is that resting circulating NK cells, present at all times in all healthy individuals, are "natural" functionally active cells. That is, NK cells are able to lyse a cell within minutes when confronted for the first time with
the appropriate target structure. There is considerable variability in the sensitivity of different cell types to NK-mediated cytotoxicity. The recognition structure on NK cells has not been identified, although it is believed that more than one structure is involved and that different target cells might be recognized through different structures.

The prototypic target cell lines, K562 for human and YAC-1 for mouse and rat NK cells, are among the most sensitive cell lines to NK lysis in each system. Several different factors play a role in determining cell lysis. The ability of a cell line to bind to NK cells is necessary but not sufficient to render it sensitive to lysis (72). In order to activate the NK cell cytotoxic mechanism, a structure on the target cells, possibly distinct from the one responsible for cell binding, must trigger the effector cells (72,73). When target cells are bound to NK cells and the lytic mechanism is activated, lysis of target cells still depends on the intrinsic sensitivity of the target cells to the lytic mechanism. Certain types of target cells activate the cytotoxic ability of NK cells and therefore appear sensitive to NK cells (74-77).

Several antigens present on target cell membranes have been proposed as possible NK cell target structures. The data are conflicting and evidence in favor of the role of a single molecule has not been confirmed. It is possible that NK cells recognize different molecules that either play a primary role as target molecules responsible for conjugate formation and/or triggering, or that exert an accessory but not essential role in increasing the binding affinity between NK and target cells. Initially, the transferrin receptor (TfR) was implicated as a possible target antigen on the basis of cytotoxicity inhibition by anti-TfR antibodies, and a correlation between TfR expression on target cells and sensitivity to NK cells (78-80). However, in several other studies (81-83) these results could not be reproduced, indicating that the role, if any, of the TfR as target cell antigen was not unique. Expression of
the CD15 (3-fucosyl-N-acetyl-lactosamine hapten) antigen on cell lines has been correlated with binding to and lysis by NK cells (84). This finding, together with the ability of anti-CD15 antibody to inhibit NK cell-mediated lysis (85), suggested a role for the CD15 in NK cell killing. Other investigators (86) have identified the 140 kDa heterodimer detected by antibody 4F2 as the target cell structure recognized by several non-MHC-restricted cytotoxic cell clones that express an identical TCR idiotype and recognize target cells through the TCR in an antigen-specific fashion. However, no role of the 4F2 antigen was found in the killing mediated by human peripheral blood CD3-, TCR- NK cells (87,88). Several reports have described the ability of simple or complex sugars to inhibit NK cell-mediated lysis (89,90). Inhibition was observed only at high sugar concentrations. Although it is known that sugars act at a post-binding stage to inhibit lysis, the mechanism of this inhibition remains unclear (90,91). The ability of certain glycopeptides from target cell membranes to inhibit NK cell-mediated lysis suggests the requirement for target cell expression of certain carbohydrate structures (92), though these results are controversial.

More recently, many experimental observations have implicated an inverse correlation between expression of class I MHC antigens on target cells and sensitivity to NK lysis. Differentiation of normal thymocytes results in increased H-2 expression and decreased sensitivity to lysis (93-95). In the YAC-1 and other cell lines, low expression of class I antigens correlates with high sensitivity to NK cells and limited growth potential in vivo, whereas variants with high MHC class I expression are resistant to NK cells and are highly metastatic in vivo (96-100). However, other studies failed to demonstrate an absolute correlation between class I MHC expression and sensitivity to NK lysis (101-103). Overall, studies suggest that in some cases class I MHC antigen expression prevents the triggering of NK cells
and the formation of NK-target cell conjugates (100). However, in other cases this is not true, possibly because other structures are present on the target cell membrane and are recognized by NK cells. Recently, the idea that MHC class I expression protects targets against NK lysis (96,104,105) has been confirmed by gene transfection experiments (28,29,106). Transfection of human leukocyte antigen (HLA) class I genes into an HLA class I-negative mutant cell line restored the NK-resistant phenotype. However, while HLA-A3, Aw68, B7, B27 and Bw58 protected against NK lysis, HLA-A2 did not (31). It was demonstrated that the inability of HLA-A2 to protect stems from the presence of a histidine residue at position 74 in the alpha-1 domain of the molecule (107). Site-directed mutagenesis of His 74 to Asp 74 rendered HLA-A2 protective from NK lysis (107).

To further determine the role of MHC class I molecules, addition of synthetic viral peptides to target cell cultures has been shown to increase their susceptibility to NK lys:is (108), suggesting that viral peptides can displace a protective self peptide to render the cells susceptible to NK lysis. Addition of certain synthetic self peptides also increases NK susceptibility (109), indicating protection is conferred by a particular self peptide or a group of self peptides, that have to compete for presentation with other, non-protecting self peptides. Also, using RMA-S cells that cannot load class I MHC molecules with peptide (108), and therefore lack MHC class I cell surface expression and are prone to NK lysis, addition of exogenous peptide restores MHC class I molecule expression, but does not convert the cells to the NK-resistant phenotype (109). This result is probably due to the fact that only specific peptides can protect against NK lysis.

Further evidence also demonstrates the involvement of MHC molecules in NK cell recognition. Studies in humans indicate that NK cells kill allogeneic cells selectively. The first evidence of specific NK cell-mediated reactions in humans was
obtained by co-culturing T cell-depleted populations with irradiated allogeneic mononuclear cells, in a mixed lymphocyte reaction (MLR) (110). The resulting MLR-activated CD3-16+ NK cells lysed target cells derived from the stimulating donor. These MLR-activated NK cells failed to lyse autologous cells. Culture of peripheral blood CD3-16+ NK cells with irradiated autologous cells did not produce effector cells capable of mediating lysis of autologous or allogenic cells. These results indicated that appropriate alloreactive precursors can be induced selectively in allogeneic, but not in autologous MLR. Also, clonal analysis indicated that individual NK cells are heterogeneous, since only a proportion of clones could lyse normal target cells derived from any given allogeneic donor. It has also been demonstrated that different NK cells isolated from a given individual could recognize different allogeneic target cells. Studies have shown that distinct groups of alloreactive clones exist within an individual (111). Clones belonging to each of these groups displayed a homogeneous pattern of cytolytic activity against a panel of allogenic PHA blasts.

The idea that NK cell recognition of MHC-mismatched cells might depend on incomplete expression of self molecules has now been confirmed in a variety of mouse and human target cell systems, including the use of MHC heavy chain and beta2M gene-transfections for restoration of MHC class I expression (103,112-115). Studies with transgenic mice provided the first evidence that MHC class I genes regulate the NK cell repertoire of transplant recipients (116), as well as the NK susceptibility of grafted cells (117). Introduction of an H-2D<sup>d</sup> transgene into B6 (H-2<sup>b</sup>) mice enabled the NK cells to reject lymphoma (116) and bone marrow (117) grafts that lacked the H-2D<sup>d</sup> gene but were otherwise syngeneic to the host. Recently, evidence that loss of MHC class I expression leads to NK sensitivity, at least in some types of normal cells, has become available. Bone marrow cells from
beta2M gene "knockout" mice are rejected by normal beta2M-expressing mice, and NK cells from the latter kill beta2M-deficient blasts in vitro (118). Interestingly, NK cells of the beta2M-deficient mice themselves were not capable of attacking blasts or bone marrow cells that lacked beta2M-expression, suggesting some type of tolerance towards autologous cells had occurred.

With the exception of the CD16 molecule involved in ADCC, there is no conclusive information on the type of receptor used by NK cells for target cell recognition and killing. It was hypothesized that, at least in some experimental systems, NK cell activity depended on the presence of natural cytophilic antibodies bound in vivo to CD16 that was directed against target cell surface antigens (119-121). Although this mechanism may play a role in some systems, it is clearly not a general mechanism for NK cell-mediated cytotoxicity, as shown by several lines of evidence. One, anti-IgG or anti-CD16 antibodies inhibit ADCC but not NK killing (122-127). Two, phorbol diesters induce down-modulation of CD16 and inhibition of ADCC but not of NK killing (128). And three, NK cells from scid mice and from SCID patients are cytotoxic both in vitro and in vivo even though the animals and the patients do not produce IgG.

The surface molecules of the CD11/CD18 family appear to play important functional roles in NK killing. Patients with a severe deficiency of the common CD18 chain, are deficient in NK cell activity (129,130). Antibodies to the CD18 chain efficiently block NK cell-mediated cytotoxicity by preventing binding of NK cells to target cells (131,132). Patients with selective deficiency in LFA-1 expression are also deficient in NK cell cytotoxicity (130). A series of antibodies directed against various epitopes of the LFA-1 molecule inhibit both NK cell-mediated cytotoxicity and CTL-mediated cytotoxicity. These antibodies inhibited lysis at the effector cell level by preventing NK-target cell conjugate formation (133-135).
CD11a (LFA-1) is therefore an important adhesion molecule in the interaction of cytotoxic cells, both NK and CTL, with target cells. Differential requirements for CD11a molecules in the binding of different target cells on different clones suggest that either CD11a is one of the several different receptors that NK cells can use for binding, or that it has an accessory function that is essential only when other receptors responsible for target cell are not of sufficient affinity. No evidence has been obtained that CD11a is a functional receptor capable of signal transduction and triggering of NK cells.

Antibodies against non-lineage-restricted epitopes of the CD45 molecule inhibit NK cell-mediated cytotoxicity but not CTL-mediated lysis, acting at the effector cell level at a post-binding stage (134). Thus, the CD45 molecule appears to interact with surface molecules on at least some target cell types. Some of these anti-CD45 antibodies block lysis of a wide range of target cells (134). A rabbit anti-idiotypic antiserum generated against an antibody designated 9.1C3 was shown to be highly reactive with K562 cells, precipitating two molecules of 94 and 79 kDa, and inhibited NK-mediated lysis of K562 cells at a post-binding stage (136).

Laminin and its receptors represent another adherence system that plays a role in NK cell killing (137). Rodent and human NK cells, more than other lymphocyte types, express laminin-like structures recognized by anti-laminin antibodies (138-140). Anti-laminin antibodies block cytotoxicity at a post-binding stage, without inhibiting NK-target cell interactions (139,140). Expression of laminin-like molecules on the NK cell surface increases upon stimulation with IL-2 (140). Thus, it is possible that a laminin-like molecule on the NK cell acts to trigger the lytic mechanism by binding to the target cells through laminin receptors (140).

The CD2 antigen expresses at least three distinct epitopes. CD2 is an antigen-independent pathway of T cell activation, and treatment of T cells with anti-
CD2 antibodies induce expression of IL-2R, secretion of IL-2, and proliferation of T cells (141-144). Treatment of peripheral blood NK cells with anti-CD2 mAb does not induce cell proliferation (144), probably due to a lack of IL-2 production (145). Anti-CD2 antibodies stimulate the expression of the IL-2 receptor in NK cell clones (146) and increase the cytotoxic activity of both fresh NK cells (147) and NK cell clones (148,149), and anti-CD2 mAb treated NK cells show increased adhesion to the target cells (149).

Anti-idiotypic antibodies directed against an antibody specific for a glycoprotein of human K562 target cells react with an 80 kDa molecule on NK cells, block target binding and lysis by NK cells, and enhance cytotoxicity and induce IFN production (150).

Of the different molecules for which a role has been proposed in NK-target cell binding or in post-binding events during NK cell-mediated cytotoxicity, only CD16 and CD2 have been shown to act directly in signal transduction and activation of the cytotoxic mechanisms (149,151). Currently, it is believed that neither CD16 nor CD2 appear to be required for non-MHC-restricted cytotoxicity, as cytotoxic lymphocytes that lack either molecule can still mediate natural killing (152-154).

Recently, other cell surface molecules with unknown ligands have been described on NK cells that can also activate cytotoxicity. NKRPI was identified on rat NK cells and an antibody against this molecule can induce redirected lysis of FcR+ target cells, as well as mediate other activation and signal transduction events (154). The cloned cDNA for NKRPI encodes a putative type II integral membrane protein with 22% homology with members of the C-type lectin superfamily (156). The NK1.1 antigen expressed by murine NK cells can initiate many of the same activities observed with NKRPI stimulation, and is the murine equivalent of NKRPI (155). In humans, a family of related cDNAs (NKG2) has been identified
with expression restricted to NK cells and coding for putative polypeptides displaying homology with NKRPI (157). Additional molecules, such as pNKR1 (158), the molecule reacting with the mAb PP35 (159), also appear to be activating structures on NK cells. Two additional, novel signal transducing molecules on human NK cells have recently been reported. p38 is expressed on virtually all human NK cells and subpopulations of T cells. Perturbation of p38 with anti-p38 antibody stimulated NK cell-mediated redirected cytotoxicity and production of IFN. The kp43 mAb recognizes a human NK cell-associated molecule that has been shown to induce significant non-MHC-restricted lysis of relatively resistant autologous and allogenic T cell blasts (160). Additionally, a novel NK associated molecule 2B4 present on murine NK cells and cultured T cells that mediate ("NK-like") non-MHC-restricted killing has been reported (161). Antibodies to 2B4 antigen can activate non-MHC-restricted killing and also transduce other signals resulting in IFN secretion and granule exocytosis (161).

Recently, human NK cells have been shown to display a clonally distributed ability to recognize MHC class I alleles. Five distinct groups of alloreactive clones, each displaying a different specificity have been identified (111). Whereas specific recognition of MHC molecules by alloreactive cytotoxic T lymphocytes leads to triggering of their cytolytic function, in the case of NK lymphocytes a given HLA allele was found to confer specific protection from lysis by a group of NK clones displaying a defined specificity (162,163). It has been shown that a clonally distributed receptor on NK cells specifically involved in MHC class I recognition exists (163,164). The NK-specific p58 molecule family (165) has been shown to be a possible NK receptor for MHC. Single members of this family have been identified by the GL183 (163) and EB6 (165) mAbs, and were found to be confined to subsets of human NK cells. It has also been shown that mAb-mediated masking of p58
molecules leads to restoration of lysis of MHC class I protected target cells by NK clones recognizing defined specificites.

Exactly how NK cells recognize allogeneic or MHC-deficient cells is not understood. Two models originally proposed to explain recognition of MHC class-I-deficient cells (163) are applicable to human alloreactive NK cell clones. These models can be described as "effector inhibition" and "target interference". According to the "effector inhibition" model, appropriate MHC class I molecules deliver an inactivating signal which blocks the NK cells ability to mediate cytolysis. This may follow NK cell-target cell interaction mediated by adhesion/activation molecules and their ligands. The lack of this negative signal results in target cell lysis. According to this model, the NK cell receptor recognizes MHC class I molecules. The "target interference" model suggests that the appropriate MHC class I molecules probably mask a putative self epitope which represents the actual molecular target for NK cells, thus protecting cells from lysis. The presence of an inappropriate HLA allele, as in some allogeneic cells, or the lack of expression of the appropriate allele, as in some virus-infected or tumor cells, renders the NK cell target structure available for binding by NK cell receptors. According to this model, the NK cell receptor recognizes a non-MHC self epitope and the interaction between ligand and the receptor results in NK cell activation, leading to triggering of the lytic machinery and target cell lysis.
Included in chapter 2 is a summary of four papers entitled "Evolutionary Conservation of a Human Function-Associated Molecule on Murine Natural Killer Cells: Expression and Function" which was accepted for publication in the Scandinavian Journal of Immunology, "Involvement of a Novel Function-Associated Molecule (FAM) on Murine Natural Killer Cells in Signal-Transduction" which was submitted for publication to the Scandinavian Journal of Immunology, "Molecular Characterization of a Novel Function-Associated Molecule (FAM) Murine Natural Killer Cells Using an Antisense Oligonucleotide Approach" which was prepared for submission to Journal of Experimental Medicine and "Reduced Levels of Major Histocompatibility Complex Class I Antigen In Association with Expression of A Novel Target Cell Antigen Renders Murine Target Cells Susceptible to NK Cell Lysis" which was submitted for publication to Immunology Letters. The manuscripts for these papers can be found in the Appendices A, B, C, and D respectively.

The first paper describes the expression and biological function of FAM on various NK cell populations. The second paper describes the role of FAM in murine NK cell signal-transduction. The third paper describes the characterization of FAM on NK cells at the molecular level. Finally, the fourth paper describes the characterization of a novel murine NK target cell antigen. My contributions to these papers include the experimental design as well as all the data that has been presented in each one of these papers.
CHAPTER 2

PRESENT STUDY

The methods, results, and conclusions of this study can be found in four papers which are appended to this dissertation. The following is a summary of the most important findings of these papers. The first paper, "Evolutionary Conservation of a Human Function-Associated Molecule on Murine Natural Killer Cells: Expression and Function", demonstrates the presence of FAM on the majority of fresh murine NK cells, 5 day LAK cells, ALAK cells and SCID NK cells. FAM expressing NK cells can also be detected in other lymphoid organs. The expression of FAM is restricted to NK1.1 expressing NK cells, which have been implicated in mediating NK cell cytotoxicity. It was demonstrated that anti-FAM mAb inhibited up to 100% of the cytotoxicity of target cells by each one of these effector cell populations. Conjugate assays demonstrated that FAM was involved in recognition, as up to 80% inhibition in conjugate formation between NK cells and target cells was observed. The functional role of FAM in other species has been previously demonstrated (166-168). The above findings define the role of FAM in cytotoxicity of murine NK cells.

The second paper, "Involvement of a Novel Function-Associated Molecule (FAM) on Murine Natural Killer Cells In Signal-Transduction" presents evidence that FAM on fresh NK cells, 5 day LAK cells and ALAK cells is also a signal-transducing molecule. As shown, anti-FAM mAb stimulation of each of the effector cell populations results in enhanced cytotoxicity as well as lymphokine production. Redirected lysis of the anti-FAM mAb expressing hybridoma and inhibition of target cell lysis upon pretreating effector cells with the soluble anti-FAM mAb,
confirms that this structure is an activating molecule. Down-modulation of FAM resulted in an 80% decrease in cytotoxicity and up to an 70% decrease in conjugate formation. One can conclude from these findings that FAM plays an important role in recognition, signal-transduction and the lytic mechanisms of fresh NK cells, LAK cells and ALAK cells.

The third paper, "Molecular Characterization of a novel Function-Associated Molecule (FAM) on Murine Natural Killer Cells Using an Antisense Oligonucleotide Approach", characterizes FAM at the molecular level. It has been demonstrated that a partial amino acid sequence of FAM is 100% identical to the core region of an intermediate filament, vimentin (169). Cross-reactive determinants have been shown to exist between purified FAM and vimentin by western blot analysis using anti-FAM and anti-vimentin mAbs (170). In this paper it is demonstrated that FAM on murine NK cells is also related to vimentin at the molecular level. Northern blot analysis using a full length vimentin cDNA probe, demonstrated no differences in the size of the mRNA between FAM(+) and FAM(-) cells. Polymerase chain reaction (PCR) experiments using primers specific for the head, core and tail regions of the vimentin gene also revealed no differences in the size of the PCR products between these two cell types. To further determine the relatedness of FAM and vimentin at the RNA level, FAM(+) cells were treated with antisense oligonucleotides directed against the head and the core region of the vimentin gene. This treatment inhibited the expression of FAM and also inhibited the lytic activity of NK cells against target cells. Moreover, it was demonstrated that an anti-vimentin mAb reacts with NK cells and inhibits the cytotoxicity and conjugate formation between NK cells and target cells.

The fourth paper, "Reduced Levels of Major Histocompatibility Complex Class I Antigen In Association with Expression of A Novel Target Cell Antigen
Renders Murine Target Cells Susceptible to NK Cell Lysis", characterizes a novel murine NK target cell molecule. Characterization of this molecule on human NK target cells has been previously reported (170). In this paper it is demonstrated that the anti-target cell mAb also recognizes a similar structure on murine NK sensitive (YAC-1) as well as resistant target cells (P815 and EL-4), by flow cytometry. Functional studies demonstrated that the anti-target cell mAb inhibits the lysis and conjugate formation of both NK sensitive and resistant target cells, by fresh NK cells, 5 day LAK cells and ALAK cells. Further, the role of MHC class I antigens in NK cell cytotoxicity was examined. It was observed that the target cells most sensitive to NK cell lysis express low levels of MHC class I antigens in association with the novel target cell antigen. In conclusion, each of the effector cell populations recognize a common novel target cell antigen, but target cells that express low levels of MHC class I antigens are most susceptible to lysis by NK cells.
APPENDIX A
Evolutionary Conservation of a Human Function-Associated Molecule on Murine Natural Killer Cells: Expression and Function

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ABSTRACT

Using a novel anti-natural killer (NK) cell monoclonal antibody (mAb), we have recently identified an evolutionarily conserved function-associated molecule (FAM) present on fish, rat and human NK cells. This molecule is involved in NK cell function as anti-FAM mAbs inhibit cytotoxicity, stimulate lymphokine secretion and inhibit conjugate formation between effector cells and target cells. We now have examined murine NK cells for the presence of this structure. It was observed by two-color flow cytometric analysis that the anti-FAM mAb 5C6 specifically bound to a subpopulation of nylon wool nonadherent splenic lymphocytes (19-20%). The expression of the FAM molecule was restricted to NK cells that expressed the NK1.1 antigen. Neither T cells, B cells, nor macrophages reacted with the anti-FAM mAb. Analysis of FAM expression in various lymphoid tissues revealed that splenocytes expressed the greatest numbers of mAb (+) cells. Generation of lymphokine activated killer (LAK) cells and adherent lymphokine activated killer (ALAK) cells resulted in higher levels of FAM expression. The anti-FAM mAb 5C6 also detected the presence of FAM on fresh SCID NK cells. It was demonstrated that the anti-FAM mAb 5C6 inhibited the lysis of target cells by endogenous NK cells, activated NK cells, 5d LAK cells, ALAK cells and SCID NK cells. Moreover, conjugate assays demonstrated this molecule to be involved in recognition between NK cells and target cells.

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INTRODUCTION

Natural killer (NK) cells constitute a small population of lymphocytes that display a large, granular morphology and are found in unstimulated peripheral lymphoid tissues and blood [1-3]. NK cells exhibit "natural" cytolytic activity against certain tumor cells and virally infected cells in a non-major histocompatibility (MHC) restricted manner [1-3]. NK cells display antibody dependent cell mediated cytotoxicity [1-3], appear to regulate B cell immunoglobulin synthesis [1-3], and mediate the phenomenon of "hybrid resistance" in bone marrow transplantation [4]. Although freshly isolated NK cells express the delta and the zeta chains of the CD3/T cell receptor (TCR) complex [5,6], they do not display other components of the complex, do not express mRNA for mature T cell receptor chains, and do not rearrange TCR genes [7]. After activation in vitro with lymphokines, such as interleukin-2 (IL-2), CD3(-) NK cells can be induced to lyse a broader panel of target cells than freshly isolated NK cells [8,9]. The first step in the lytic mechanism of NK cells is the binding to susceptible target cells by postulated receptors which recognize specific target cell structures [7]. Although it is now clear that physical contact is required for target cell killing and that various adhesion molecules participate in this contact (e.g., LFA-1, CD2 etc), little is known regarding the identity of the NK cell recognition apparatus necessary for conjugate formation between effector and target cells.

We have previously described the mammalian NK cell equivalent in teleost fish, referred to as nonspecific cytotoxic cells (NCC) [10]. NCC share many characteristics with mammalian NK cells such as the requirement for cell-cell contact for lysis, similar Mg$^{2+}$ and Ca$^{2+}$ concentrations required for binding and killing, and the lysis of a variety of transformed (human and murine) cell lines [11-13]. Recently, we have reported on the production and characterization of mAbs
made against fish NCC [10]. These mAbs bind to NCC and block the cytotoxicity of these cells at the conjugate formation stage. Immunoprecipitation and immunoblotting was used to demonstrate that this function-associated molecule found on NCC was a 43 kD protein. A similar molecule was immunoprecipitated from human NK cells using the mAb 5C6 [14].

In the present study we have analyzed the effects of the anti-function associated molecule mAb 5C6 on various NK cell populations from murine lymphoid tissues. We report that the anti-FAM mAb 5C6 binds to fresh and cultured murine NK cells and that anti-FAM mAb 5C6 inhibits murine NK cell activity by preventing the formation of conjugates with YAC-1 target cells.
MATERIALS AND METHODS

MICE: C57BL/6 mice were obtained from SASCO (Omaha, Nebraska) and kept in a pathogen free animal facility at our institution. SCID mice were obtained from University animal care, University of Arizona. Mice were used in experiments at 6 to 10 wks of age.

REAGENTS AND CELL LINES: Human recombinant interleukin-2 (IL-2) was the kind gift of Dr. Emmanuel Akporiaye (Department of Microbiology & Immunology, University of Arizona) and Amgen (Thousand Oaks, CA). The mAbs Thy1.2, Lyt2 and L3T4 were obtained from Becton Dickinson (Mountain View, CA); mAbs NK1.1, Lyt2-PE, L3T4-PE and Thy1.2-PE were obtained from Pharmingen (San Diego, CA). All mAbs were used as suggested by the manufacturer. Fluorescein conjugated goat anti-mouse IgG, IgM was used as a secondary antibody (Olympus Corp, Lake Success, NY). Cell cultures were maintained in RPMI 1640 (Flow Laboratories, Rockville, MD) supplemented with amino acids and antibiotics, and containing 10% fetal bovine serum (Hy-Clone, Logan, UT). This media will hereafter be referred to as RPMI-10%.

DERIVATION OF MONOCLONAL ANTIBODY: The anti-NCC mAb 5C6 was derived as described [10]. Briefly, mice were immunized with purified fish NCC (from Ictalurus punctatus), spleen cells were fused with the P3-X63-Ag8.653 myeloma, and wells positive for growth were screened by ELISA and flow cytometry with NCC. All of the anti-NCC mAbs selected for use in the experiments were of IgM isotype.
ISOLATION OF EFFECTOR CELLS: Spleen, lymph nodes and thymus were aseptically removed and single cell suspensions were prepared in RPMI-10%. Mononuclear cells were obtained after centrifugation through lympholyte-M (Accurate, Westbury, NY) cell separation medium (1700 rpm, 30 minutes, room temperature). In some experiments, spleen mononuclear leukocytes were passed over nylon wool columns to remove macrophages and B cells. Briefly, $10^8$ spleen cells in 2 ml of RPMI-10%, were added to a 20 cc syringe containing 1 gm of sterile nylon wool. The cells were incubated for 45 minutes at 37°C and the nylon wool was gently washed with 50 ml of RPMI-10%. The nonadherent cells were collected, washed, and used in the assays as fresh endogenous NK cells. This method consistently reduced the percentage of B cells and macrophages in the spleen preparations to less than 2%. Activated NK cells were obtained by culturing nylon wool nonadherent (NWNA) cells in RPMI-10% medium containing 50 U/ml rIL-2 for 12 hrs. LAK cells were obtained by culturing fresh NWNA cells in complete medium supplemented with 250 U/ml rIL-2 at $2 \times 10^6$ cells per ml for a period of at least 5 days. Adherent lymphokine activated killer (ALAK) cells were derived by a procedure described earlier [15]. Briefly, fresh NWNA cells were cultured in complete medium supplemented with 1000 U/ml rIL-2 at $2 \times 10^6$ cells per ml for a period of 48 hrs at 37°C, after which tissue culture flasks were rinsed three times with prewarmed RPMI 1640 to remove all nonadherent cells. The remaining adherent cells were expanded for another 6 to 8 days with 1000 U/ml of rIL-2 and medium changed as necessary. The adherent cells were removed with Ca$^{2+}$ and Mg$^{2+}$ free PBS containing 0.02% EDTA. The cells were washed in complete medium before use in the functional assays.
FLOW CYTOMETRIC ANALYSIS: Cell surface phenotypes and mAb binding were analyzed by one and two-color flow cytometry with the use of a Becton Dickinson FACStar Plus flow cytometer. For one color flow cytometry, controls consisted of fluorescein conjugated goat anti-mouse IgG, IgM and avidin-PE antibodies. For the two-color flow cytometric analysis, cells were first stained with anti-FAM mAb and an anti-IgG, IgM-FITC second antibody (green fluorescence). The cells were then stained with a directly PE-conjugated lymphocyte subset mAb (e.g., L3T4-PE). The cells were then analyzed for simultaneous red and green fluorescence. Viable cells were gated by a combination of forward light scatter and size. A minimum of 10,000 cells were analyzed for each histogram and the data are presented on a log scale.

CYTOTOXICITY ASSAYS: Effector cells were tested for cytolytic activity in a standard microcytotoxicity assay [16]. Percent specific lysis was calculated according to the formula:

\[
\%\text{SPECIFIC LYSIS} = \frac{\text{TEST cpm} - \text{CONT cpm}}{\text{MAX cpm} - \text{CONT cpm}} \times 100
\]

Lytic unit at 20% lysis (LU\textsubscript{20}) for 1 X 10\textsuperscript{6} effector cells is defined as the number of cells required to cause 20% lysis of target cells. It is calculated by computer-assisted regression analysis as described by Pross et al [17]. All effector to target (E/T) ratios were performed in triplicate. Spontaneous \textsuperscript{51}Cr-release under any of the conditions never exceeded 10%.

In experiments in which the effector cells were tested with the mAb, the effector cells were placed in the wells of the microtiter plates and incubated with the mAb for 1 hr at 4\textdegree C before the addition of the target cells to the wells. In other
experiments the effector cells were preincubated with the mAb at 4C for 1 hr in conical tubes, cells were washed and then used in cytotoxicity assays. The percent inhibition of cytotoxicity was based on comparisons of the LU$_{20}$ and was calculated according to the formula:

\[
\frac{\text{TEST LU}_{20}}{\text{CONT LU}_{20}}
\]

\[
\% \text{ INHIBITION} = 1 - \frac{\text{TEST LU}_{20}}{\text{CONT LU}_{20}} \times 100
\]

CONJUGATE ASSAYS: The effects of the mAb on the ability of the various effector cell populations to form conjugates with YAC-1 tumor cells was examined as described previously [18]. Briefly, equal numbers of effector cells and target cells (0.5 X 10$^6$ each) were mixed in 1 ml of RPMI-10%, the mixture was centrifuged for 2 minutes at 50 x g and incubated for 15 minutes at room temperature. At this time cells were vigorously pipetted (four to five times) to disrupt spontaneous conjugates, an aliquot was placed on a hemocytometer and conjugates were enumerated.
RESULTS

TISSUE DISTRIBUTION OF THE EPITOPE RECOGNIZED BY ANTI-FAM MAB 5C6: The cellular distribution of the determinant recognized by the anti-FAM mAb 5C6 in fresh murine tissues was analyzed by flow cytometry. The FAM expression in the C57BL/6 strain of mice was compared to other murine T cell markers such as CD3 and Thy1.2 (Table 1). The mAb 5C6 bound to approximately 20% of NWNA fresh spleen cells, 9% of thymocytes and 9% of lymph node cells. In comparison to mAb 5C6, mAb NK.1.1 (a pan NK cell marker) stained approximately 28% of fresh NWNA splenic lymphocytes, 11% of thymocytes and 9% of lymph node cells. 75% of the spleen cells stained positive with the mAb against the CD3 molecule, while 29% of the thymocytes and 73% of lymph node cells reacted positively with this mAb. 85% of the spleen cells, approximately 100% of thymocytes and 77% of the lymph node cells stained positive with the anti-Thy1.2 mAb. No reactivity was observed of mAb 5C6 with B cells or macrophages. Thus, anti-FAM mAb 5C6 reactivity was similar to anti-NK1.1 binding but distinct from T cell staining.

COMPARISON OF FAM EXPRESSION ON ENDOGENOUS NK CELLS, ACTIVATED NK CELLS, 5 DAY LAK CELLS, ALAK CELLS AND SCID NK CELLS: To examine the fine specificity of the mAb 5C6 for murine NK cells, two-color flow cytometric analysis was performed using various NK effector cell populations. NWNA cells were stained with mAb 5C6 in combination with mAbs that recognized T cell specific markers (i.e., CD4 and CD8). As shown in Figure 1A and 1B, approximately 20% of the NWNA cells stained positive for the mAb 5C6. The mAb NK1.1 bound approximately 30% of NWNA cells (Figure 1C and 1D). As shown in Figure 1A-1D, helper T cells as well as cytotoxic T cells as identified by
mAbs L3T4 and Lyt2 respectively, demonstrated no reactivity with either of the two mAbs (i.e., NK1.1 and/or 5C6). Furthermore, neither B cells nor monocytes (as identified by mAb B220 and MAC-1, respectively) reacted with the mAbs (data not shown). To further confirm the fine specificity of the mAb, splenocytes from T and B cell deficient SCID mice were analyzed by two-color flow cytometry using mAb 5C6 and Thy1.2. The marker recognized by the mAb Thy1.2 is found on the majority of T cells as well as on NK cells. As shown in Figure 2B approximately 84% of SCID splenocytes stained positive for mAb 5C6. Approximately 40% of the splenocytes stained for mAb 5C6 as well as Thy1.2. Approximately 44% of the splenocytes were 5C6(+) but Thy1.2(-). An increase in the percentage of 5C6(+) cells was due to the elimination of myeloid cells from SCID mice spleens after centrifugation through lympholyte-M cell separation medium. To determine if the expression of the FAM was restricted to NK cells that expressed the NK1.1 antigen, a pan NK cell marker, LAK cells were stained with mAbs NK1.1 and 5C6. It was observed that all the FAM expressing NK cells also expressed the NK1.1 antigen (31% in Figure 2A).

An increased expression of the FAM was observed on 5 day LAK cells (30%)(Figure 3B) and ALAK cells (50%)(Figure 3C). When NWNA cells were cultured for 5 days in IL-2 and subsequently stained with mAb 5C6 and Thy1.2 (Figure 3B), most of the cells that stained positive for mAb 5C6 were also positive for mAb Thy1.2 (20%). A small percentage of NK cells stained positive with mAb 5C6, but negative with mAb Thy1.2 (13%). When 5 day LAK cells were stained with mAb NK1.1 and Thy1.2 (Figure 3A), approximately 20% of the LAK cells stained positive for mAb NK1.1. Approximately 30% of LAK cells stained positive for both mAb NK1.1 as well as mAb Thy1.2. The majority of the LAK cells stained positive with mAb Thy1.2, but negative with mAb 5C6 (57%). Finally, the
expression of FAM was significantly higher on cells that were enriched for NK cells and cultured for 10 to 12 days in IL-2, i.e. ALAK cells (51% in Figure 3C).

THE ANTI-FAM MAB 5C6 INHIBITS THE CYTOTOXICITY OF MURINE ENDOGENOUS NK CELLS, ACTIVATED NK CELLS, 5D LAK CELLS, ALAK CELLS AND SCID NK CELLS: Previously, we have shown that the mAb 5C6 inhibited the cytolytic activity of fish NCC [10] and human NK cells [14]. Having observed that this mAb bound specifically to murine NK cell populations (i.e., endogenous NK cells, activated NK cells, 5d LAK cells, ALAK cells and SCID NK cells) it was of interest to determine what effects this mAb might have on murine NK cell lysis. It was observed that mAb 5C6 inhibited the lysis of YAC-1 target cells by endogenous NK cells, activated NK cells, 5d LAK cells, ALAK cells and SCID NK cells (Table 2). Nearly one hundred percent inhibition of lysis was observed with the anti-FAM mAb 5C6 treated activated NK cells, 5d LAK cells, ALAK cells and SCID NK cells. When mAb 5C6 treated endogenous NK cells were used as a source of effector cells, 70% inhibition of lysis was observed (Table 2).

As a control, the mAb Thy1.2 was used in the cytotoxicity assays. Although this mAb bound to the effector cells, it only significantly inhibited the lysis of YAC-1 target cells by endogenous NK cells and not by the other effector cell populations (Table 2). Among activated NK cells, 5 day LAK cells, ALAK cells and SCID NK cells, the inhibition of cytotoxicity was consistently higher with the mAb 5C6. Thus, the mAb Thy1.2 served as a control for possible nonspecific effects and indicated that the mAb 5C6 specifically inhibited NK cell cytotoxicity. Upon target cell incubation with mAb 5C6 alone, the mAb failed to inhibit target cell lysis, indicating that the inhibitory effects of the mAb were due to binding of the mAb to effector cells and not to the target cells (data not shown).
THE ANTI-FAM MAB 5C6 BLOCKS RECOGNITION OF THE TARGET CELLS BY EFFECTOR CELLS: In an effort to determine the mechanism for the inhibitory effects of the mAb, single cell conjugate assays were conducted with each of the previously used effector cell populations. As shown in Table 3, mAb 5C6 was able to inhibit conjugate formation of the effector cells with the YAC-1 tumor cells. Inhibition of target cell binding was observed with endogenous NK cells, activated NK cells, 5 day LAK cells and ALAK cells. The percent inhibition of conjugate formation observed with endogenous NK cells, activated NK cells, 5d LAK cells and ALAK cells as a source of effector cells was 75%, 70%, 68%, and 79% respectively. Further, the control anti-effector cell mAb Thy1.2 had minimal effect on conjugate formation by these effector cell populations.
DISCUSSION

In an effort to elucidate the recognition mechanism of NK cells, mAbs were derived against teleost fish NCC [10], a supposed evolutionary precursor to mammalian NK cells. NCC share many characteristics with NK cells, among which is the ability to bind to and lyse a variety of human and murine transformed cells [10]. This property of NCC seemed to indicate that NCC and NK cells share similar recognition structures. We previously have described the production and characterization of the anti-NCC mAb 5C6 [10]. This mAb binds to a structure on NCC which is involved in the formation of conjugates between effector cells and target cells. Similar results were obtained with human NK cells [14].

The purpose of the present investigation was to determine the cellular distribution of the FAM in murine tissues and to examine the effects of the anti-NCC mAb on murine NK cell function. Our data showed that mAb 5C6 was specific for murine NK cells; that mAb 5C6 inhibited NK cell-mediated cytotoxicity and therefore recognized a function-associated molecule on murine NK cells; and that these function-associated molecules were involved in the formation of target cell/effector cell conjugates which leads to the expression/triggering of NK cell lysis.

Flow cytometric analyses of murine spleen cells indicated that anti-FAM mAb 5C6 specifically bound to murine NK cells. The specificity of the mAb 5C6 for NK cells was also confirmed by staining SCID spleen cells with the mAb 5C6 (Figure 2B). SCID mice are deficient in both T and B cell populations and only have NK cells as part of their lymphocyte repertoire. The fine specificity of the mAb 5C6 for FAM on NK cells was further confirmed by performing a two-color flow cytometry analysis using mAbs NK1.1 and 5C6. It was observed that the expression of FAM was restricted to NK cells that expressed the NK1.1 antigen (Figure 2A).
An enrichment of murine NK cells by ALAK treatment resulted in an approximately 12 fold increase in cytotoxic activity, which correlated with the increased percent of mAb 5C6 positive cells obtained by flow cytometry. The mAb 5C6 specifically bound to 20% of NWNA spleen cells and approximately 30% of LAK and 50% of ALAK cells, indicating an increase in mAb 5C6 binding as NK cells were enriched. Our data also suggest that an increase in FAM expression occurs upon culturing endogenous NK cells in the presence of high doses of IL-2 (LAK cells) which correlates with an increase in the cytotoxic activity of these cells. It is therefore reasonable to assume that NK cells that express FAM are also responsible for mediating target cell lysis. To further confirm the role played by FAM on NK cells in mediating cytotoxicity, incubation of various effector cell populations with the anti-FAM mAb resulted in nearly complete inhibition of target cell lysis. These results suggest that the FAM plays a major role in NK cell cytotoxicity.

The mAb NK1.1 (a pan NK cell marker) consistently recognized a higher percentage of NK cells from the spleen as compared to the mAb 5C6. The percentage of NK cells recognized by the mAb NK1.1 from the thymus and lymph nodes were comparable to the percentage of cells that stained positive with the mAb 5C6 from the same lymphoid organs. From our data it appeared that the mAbs recognized overlapping sets of NK cells. It is evident that the mAb 5C6 recognizes only a subpopulation of NK cells, and that these cells are probably responsible for mediating NK cytotoxicity.

Binding of the mAb 5C6 to murine NK cells resulted in the loss of cytotoxic activity by preventing the formation of conjugates with target cells. While both mAb 5C6 and Thy1.2 bound to NK cells, only the anti-FAM mAb produced a dose-dependent inhibition of murine NK cell cytotoxicity against YAC-1 target cells (data
not shown). The fact that mAb Thy1.2 bound to the murine NK cells but did not inhibit their cytotoxic activity (with the exception of endogenous NK cells) indicated that the inhibition observed with mAb 5C6 was not due to steric hindrance. The inhibition of target cell lysis observed with mAb 5C6 was due to the binding of the mAb to an effector cell structure, as mAb preincubation of target cells followed by washing prior to the assay did not have an effect on the levels of cytotoxicity (data not shown). Some inhibition in cytotoxicity of YAC-1 target cells was observed upon incubating effector cell populations with the mAb Thy1.2. It was therefore possible that the Thy1.2 molecule may play a minor role in NK mediated killing.

The mechanism of mAb 5C6 inhibition of cytotoxicity was studied by using single cell binding assays. It was found that mAb 5C6 inhibited cytotoxicity by preventing the formation of NK cell/YAC-1 target cell conjugates. The percentage of conjugate forming cells was correlated with the cytotoxic ability of effector cells. The percent inhibition observed in single cell conjugate assays using the mAb 5C6 was less than what we observed in microcytotoxicity assays. The discrepancy in these results may be attributed to the differences in the nature of the above assays. Microcytotoxicity assays are performed to measure the ability of the effector cells to lyse target cells. Conjugate assays are performed to assess the ability of the effector cells to form conjugates with (i.e. recognition of) the target cells.

Our data thus indicate that the cell surface structure recognized by mAb 5C6 is a function-associated molecule specifically present on murine NK cells and is involved in the early stages of the effector cell/target cell interaction. NK cell recognition proteins are distinct from the TCR molecules necessary for cytotoxic T cell mediated recognition. Other investigators have implicated laminin-like proteins in NK cell function. These proteins act at a later stage in the lytic cycle in that anti-laminin antibodies do not affect conjugate formation although they block
cytotoxicity [19]. Recently, some other molecules have been implicated to be functionally relevant to NK cell cytotoxicity. A disulfide-linked cell surface antigen, NKR-P1, is expressed by all NK cells and LAK cells [20]. NKR-P1 bears significant homology (22% identity) with the C-type lectin supergene family, which includes the mouse NK cell Ly-49 Ag [21] and NK1.1 Ag [22]. The NK1.1 molecule is present on all fresh and IL-2 propagated NK cells [23-25]. Although NK1.1 is often used to identify murine NK cells, its function is unknown at this point. A recent study suggests that it may serve as an activation molecule for NK cells [26]. Because the mAb 5C6 defined molecules are necessary for conjugate formation, their function in NK cell cytotoxicity would occur in the early stages of the lytic cycle. Previous biochemical characterization has indicated that FAM is distinct from each of these molecules. Thus, mAb 5C6 identifies a novel molecule involved in NK cell function.

The molecule recognized by the mAb 5C6 is present on teleost fish NCC, human NK cells and murine NK cells. It is possible that the molecules in fish, man and mouse are not identical, but that the portion of the molecule recognized by the mAb 5C6 and that is involved in NK cell function is very conserved antigenically.

In conclusion, we have identified a novel function-associated molecule on murine NK cells. This molecule is specifically present only on a subpopulation of NK cells and is involved in mediating NK cell functions, as shown by the ability of the mAb against this molecule to inhibit both conjugate formation as well as cytotoxicity of NK-sensitive target cells.

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REFERENCES


### TABLE 1

TISSUE DISTRIBUTION OF THE NK CELL FUNCTION-ASSOCIATED MOLECULE

<table>
<thead>
<tr>
<th>MAB</th>
<th>SPLEEN</th>
<th>THYMUS</th>
<th>LYMPH NODE</th>
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<tbody>
<tr>
<td>NK1.1</td>
<td>28.2%</td>
<td>10.7%</td>
<td>8.7%</td>
</tr>
<tr>
<td>5C6</td>
<td>20.2%</td>
<td>9.3%</td>
<td>9.5%</td>
</tr>
<tr>
<td>CD3</td>
<td>75.0%</td>
<td>29.0%</td>
<td>73.0%</td>
</tr>
<tr>
<td>THY1.2</td>
<td>85.0%</td>
<td>99.0%</td>
<td>77.4%</td>
</tr>
</tbody>
</table>

Data is presented as the percent specific binding of cells labeled by each mAb as detected by flow cytometric analysis. Background values have been subtracted. Spleen cells were passed over nylon wool to eliminate B cells and macrophages prior to analysis. 10 ug of mAb 5C6 was used in each experiment. 10 ug of each of the other mAbs were used. Each experiment was performed three times.
### TABLE 2

**COMPARISON OF CYTOTOXIC ACTIVITY OF VARIOUS NK CELL POPULATIONS**

<table>
<thead>
<tr>
<th>Effector Cells</th>
<th>Treatment</th>
<th>LU$_{20}/10^6$</th>
<th>Percent Inhibition</th>
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<tr>
<td>Endogenous NK cells</td>
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<td>10</td>
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<td></td>
<td>5C6</td>
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<td>70</td>
</tr>
<tr>
<td></td>
<td>Thy 1.2</td>
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<td>50</td>
</tr>
<tr>
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<tr>
<td></td>
<td>5C6</td>
<td>1</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>Thy 1.2</td>
<td>634</td>
<td>0</td>
</tr>
<tr>
<td>5 Day LAK cells</td>
<td>Media</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>5C6</td>
<td>3</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>Thy 1.2</td>
<td>228</td>
<td>24</td>
</tr>
<tr>
<td>ALAK cells</td>
<td>Media</td>
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<td></td>
</tr>
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<td></td>
<td>5C6</td>
<td>2</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>Thy 1.2</td>
<td>171</td>
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</tr>
<tr>
<td>SCID NK cells</td>
<td>Media</td>
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</tr>
<tr>
<td></td>
<td>5C6</td>
<td>3</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Thy 1.2</td>
<td>150</td>
<td>18</td>
</tr>
</tbody>
</table>

Effector cell populations were derived as described and tested for lytic activity in the presence or absence of the indicated mAbs. YAC cells were used as targets. 10 µg of mAb 5C6 and 10 µg of mAb Thy1.2 was used in each experiment. Each experiment was performed in triplicate.
<table>
<thead>
<tr>
<th>POPULATION</th>
<th>% CONJUGATES</th>
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<tr>
<td></td>
<td>CONTROL 5C6</td>
</tr>
<tr>
<td>Endogenous NK cells</td>
<td>40 10 35</td>
</tr>
<tr>
<td>Activated NK cells</td>
<td>53 28 48</td>
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<tr>
<td>5d LAK cells</td>
<td>52 19 53</td>
</tr>
<tr>
<td>ALAK cells</td>
<td>55 12 45</td>
</tr>
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</table>

Effector cell populations were derived as described. Conjugate assays were performed using YAC-1 target cells as described in Materials and Methods. 10 ug of mAb 5C6 and 10 ug of mAb Thy1.2 was used in each experiment. Each experiment was repeated three times.
FIGURE LEGENDS

FIGURE 1: Binding specificity of the anti-FAM mAb as determined by flow cytometric analysis. NWNA cells were isolated as described and stained with mAb 5C6 and FITC conjugated anti-IgM (green fluorescence, x-axis) antibody, followed by direct staining with PE (phycoerythrin)-conjugated mAb (red fluorescence, y-axis). (A) NWNA cells stained with mAb 5C6-FITC and L3T4-PE. (B) NWNA cells stained with mAb 5C6-FITC and Lyt2-PE. (C) NWNA cells stained with mAb NK1.1-FITC and L3T4-PE. (D) NWNA cells stained with mAb NK1.1-FITC and Lyt2-PE.

FIGURE 2: Flow cytometric analysis of mAb 5C6 binding to murine LAK cells and SCID splenocytes. (A) 5d LAK cells stained with mAb NK1.1-FITC (green fluorescence, x-axis) and 5C6-PE (red fluorescence, y-axis). (B) SCID splenocytes stained with mAb 5C6-FITC (green fluorescence, x-axis) and Thy1.2-PE (red fluorescence, y-axis).

FIGURE 3: Flow cytometric analysis of mAb 5C6 binding to murine LAK cells and ALAK cells. (A) 5d LAK cells stained with mAb NK1.1-FITC (green fluorescence, x-axis) and Thy1.2-PE (red fluorescence, y-axis). (B) 5d LAK cells stained with mAb 5C6-FITC (green fluorescence, y-axis) and Thy1.2-PE (red fluorescence, y-axis). (C) ALAK cells stained with mAb 5C6-FITC (green fluorescence, y-axis).
Figure 1.
Figure 2.
Figure 3.
APPENDIX B
Involvement of A Novel Function-Associated Molecule (FAM) on Murine Natural Killer Cells In Signal Transduction

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ABSTRACT

In this study we have identified a novel signal-transducing molecule present on the majority of murine natural killer (NK) cells. We have previously shown that this molecule is expressed on 20% of fresh nylon wool nonadherent (NWNA) lymphocytes, 30% of lymphokine activated killer (LAK) cells and 50% of adherent lymphokine activated killer (ALAK) cells. This molecule, designated FAM for function-associated molecule, is also involved in recognition between NK cells and target cells, as anti-FAM mAb inhibits both the cytotoxicity and conjugate formation between NK cells and target cells. We now show that the stimulation of NK cells with anti-FAM mAb results in enhanced cytotoxicity as well as lymphokine production by NK cells. Further, redirected lysis of the anti-FAM mAb bearing hybridoma further confirms that this structure is an activating (i.e., signal-transducing) molecule. Modulation of FAM from the surface of NK cells results in a significant loss of cytotoxicity and conjugate formation. Together these results suggest that FAM may play an important role in the recognition, signal-transduction, and lytic mechanisms of murine NK cells, LAK cells, and ALAK cells.

Dr David T. Harris, Department of Microbiology and Immunology, Bldg#90, University of Arizona, Tucson, AZ, 85721, USA
INTRODUCTION

Natural killer (NK) cells exhibit "natural" cytolytic activity against virus-infected cells and certain tumor cells in a non-major histocompatibility complex (MHC) restricted fashion [1]. NK cells are usually defined as CD16^+ 56^+ 3^- large granular lymphocytes (LGL) in humans [2], and by the expression of the surface marker NK1.1 in the mouse. NK cells are present and functional in nude as well as in severe combined immunodeficiency (SCID) mice [3].

Although, NK cell antigen receptors have not been defined, several molecules have been described that may be involved in non-MHC-restricted killing. Molecules such as LFA-1, CD2, and CD45 on NK cells, upon interaction with their respective ligands on target cells result in NK cell activation and target cell death [4]. Such molecules are not only present on NK cells, but are also expressed on other lymphocytes that do not mediate non-MHC restricted cytotoxicity. Recently, several NK cell-specific molecules have been described that appear to be involved in signal-transduction. These include four novel molecules present on human NK cells [5-8], NKR-P1 on rat NK cells [9], and NK1.1 and 2B4 expressed on murine NK cells [10,11]. It is clear that the above molecules play an important role in non-MHC-restricted killing. However, it is not certain whether these molecules are involved in the cytotoxicity of fresh NK cells, IL-2 cultured NK cells, lymphokine activated killer (LAK) cells or adherent lymphokine activated killer (ALAK) cells, or if any of these molecules are antigen receptors.

Previously, using a novel monoclonal antibody (mAb) against the function-associated molecule (FAM), we identified an evolutionarily conserved molecule on fish [12], rat [13], human [5], and mouse NK cells [14]. On murine NK cells, the expression of FAM is restricted to NK cells that express the NK1.1 antigen [14]. Activation of NK cells to become LAK and ALAK cells results in higher levels of
FAM expression [14]. We have also demonstrated that anti-FAM mAb inhibits the cytotoxicity and conjugate formation of target cells by fresh NK cells, LAK cells and ALAK cells [14]. Since FAM is involved in recognition, a function normally associated with a "receptor-like" molecule, an attempt was made to determine if FAM can also function as a signal-transducing molecule.

In the present study, we demonstrate that FAM on endogenous NK cells, 5 day LAK cells and ALAK cells functions as a signal-transducing/activation molecule. Stimulation of these cells with anti-FAM mAb results in enhanced cytotoxicity and lymphokine (TNF-alpha) production by NK cells. Redirected lysis of the anti-FAM mAb bearing hybridoma also suggests FAM to be involved in signal-transduction. Down-modulation of FAM from the surface of NK cells results in decreased cytotoxicity and conjugate formation by various NK cell populations. These findings suggest that FAM may play an important role in target cell recognition, cell-mediated cytotoxicity and signal-transduction.
MATERIALS AND METHODS

MICE: C57BL/6 mice were obtained from SASCO (Omaha, Nebraska) and kept in a pathogen free animal facility at our institution. Mice were used in experiments at 6 to 10 wks of age.

REAGENTS AND CELL LINES: Human recombinant interleukin-2 (IL-2) was the kind gift of Dr. Emmanuel Akporiaye (Department of Microbiology & Immunology, University of Arizona) and Amgen (Thousand Oaks, CA). Cell cultures were maintained in RPMI 1640 (Flow Laboratories, Rockville, MD) supplemented with amino acids and antibiotics, and containing 10% fetal bovine serum (Hy-Clone, Logan, UT). This media will hereafter be referred to as RPMI-10%.

DERIVATION OF MONOCLONAL ANTIBODY: The anti-FAM mAb 5C6 was derived as described [12]. Briefly, mice were immunized with purified fish nonspecific cytotoxic (NCC) cells (from Ictalurus punctatus), spleen cells were fused with the P3-X63-Ag8.653 myeloma, and wells positive for growth were screened by ELISA and flow cytometry with NCC. The anti-FAM mAb selected for use in the experiments was of the IgM isotype.

ISOLATION OF EFFECTOR CELLS: Spleens were aseptically removed and single cell suspensions were prepared in RPMI-10%. Mononuclear cells were obtained after centrifugation through lympholyte-M (Accurate, Westbury, NY) cell separation medium (250 x g, 30 minutes, room temperature). In some experiments,
spleen mononuclear leukocytes were passed over nylon wool columns to remove macrophages and B cells. Briefly, $10^8$ spleen cells in 2 mls of RPMI-10%, were added to a 20 cc syringe containing 1 gm of sterile nylon wool. The cells were incubated for 45 minutes at 37C and the nylon wool was gently washed with 50 ml of RPMI-10%. The nylon wool nonadherent (NWNA) cells were collected, washed, and used in assays as a source of fresh endogenous NK cells. This method consistently reduced the percentage of B cells and macrophages in the spleen preparations to less than 2%. Lymphokine activated killer (LAK) cells were obtained by culturing fresh NWNA cells in complete medium supplemented with 250 U/ml rIL-2 at 2 X $10^6$ cells per ml for a period of at least 5 days. Adherent lymphokine activated killer (ALAK) cells were derived by a procedure described earlier [15]. Briefly, fresh NWNA cells were cultured in complete medium supplemented with 1000 U/ml rIL-2 at 2 X $10^6$ cells per ml for a period of 48 hrs at 37C, after which tissue culture flasks were rinsed three times with prewarmed RPMI 1640 to remove all nonadherent cells. The remaining adherent cells were expanded for another 6 to 8 days with 1000 U/ml of rIL-2 and medium changed as necessary. The adherent cells were removed with Ca$^{2+}$ and Mg$^{2+}$ free PBS containing 0.02% EDTA. The cells were washed in complete medium before use in functional assays.

**CYTOTOXICITY ASSAYS:** Effector cells were tested for cytolytic activity in a standard microcytotoxicity assay [16]. Lytic units at the 20% lysis level (LU$_{20}$) for $1 \times 10^6$ effector cells was calculated by computer-assisted regression analysis as described by Pross et al [17]. All effector to target (E/T) ratios were performed in triplicate. Spontaneous $^{51}$Cr - release under any of the conditions never exceeded 10%.
In experiments in which the effector cells were tested with the mAb, the effector cells were plated in the wells of the microtiter plates and incubated with the mAb for 1 hr at 4C before the addition of the target cells to the wells. In other experiments the effector cells were preincubated with the mAb at 4C for 1 hr in conical tubes, cells were washed and then used in cytotoxicity assays. The percent inhibition of cytotoxicity was based on comparisons of the LU_{20}.

CONJUGATE ASSAYS: The effects of the mAb on the ability of the various effector cell populations to form conjugates with YAC-1 tumor cells was examined as described previously [18]. Briefly, equal numbers of effector cells and target cells (0.5 X 10^6 each) were mixed in 1 ml of RPMI-10%, the mixture was centrifuged for 2 minutes at 50 x g and incubated for 15 minutes at room temperature. At this time cells were vigorously pipetted (four to five times) to disrupt spontaneous conjugates, an aliquot was placed on a hemocytometer and conjugates were enumerated.

NK CELL STIMULATION WITH ANTI-FAM MAB: Various effector cell populations were treated with the anti-FAM mAb for a period of 4 hrs at a concentration of 10 ugs per 1 X 10^6 effector cells. The treated cells were repeatedly washed with 37C media to remove any cell surface bound anti-FAM mAb. To measure lymphokine production by NK cells upon anti-FAM mAb treatment, fresh NK cells were treated with anti-FAM mAb for a period of 24 hrs with 10 ugs/ml of anti-FAM mAb. Cells were washed with 37 C media, and total cellular RNA was extracted [19]. Briefly, washed cells were resuspended in isotonic TEN buffer (0.15 M NaCl, 10 mM Tris-HCl, pH 7.8, 1 mM EDTA). The RNase inhibitor, vanadyl ribonucleoside complex was added to a final concentration of 5 mM followed by 10% NP40 detergent to lyse the cells. The samples were centrifuged for 10 minutes
at 1500 g. The supernatant were added to 2X extraction buffer (0.35 M NaCl, 20 mM Tris-HCl, pH 7.8, 2 mM EDTA) containing 20% (w/v) sodium dodecyl sulfate (SDS). The supernatants were extracted three times with equal volumes of phenol/chloroform. 1 ug of total RNA was used for constructing first strand cDNA [20]. For preparing a 30 ul cDNA reaction mixture 3 ul of mouse Moloney leukemia virus reverse transcriptase (MMLV-RT) (200 U/ml), 1 ul of RNasin (40 U/ml), 6 ul of 5X MMLV-RT buffer, 3 ul of oligo d(T) (0.5 mg/ul), 3 ul of acetylated BSA (1 mg/ml) and 1.5 ul of dNTP mix (10 mM each) and 8 ul of DepH20 were added together. The samples were incubated at 65 C for 5 minutes to denature the RNA and then quenched on ice. This RT mix was then added to each sample and incubated at 42 C for 1 hr. Polymerase chain reaction (PCR) was performed on each of these samples using primers specific for TNF-alpha (Clontech, Palo Alto, CA). The samples were amplified for 30 cycles, consisting of 94 C for 1 minute, 60 C for 2 minute, and 72 C for 3 minutes.

MODULATION OF FAM WITH ANTI-FAM MAB: Down-modulation of FAM from the surface of NK cells was achieved by treating various effector cell populations with 10 ug/ml of anti-FAM mAb for 30 minutes at 4 C, cells were subsequently cross-linked with a goat anti-mouse IgM mAb and incubated for 18 hrs at 37 C. Cells were washed repeatedly with 37 C media to remove any cell surface bound mAb. Flow cytometric analysis was performed to determine the loss of FAM expression from the surface of NK cells.

FLOW CYTOMETRIC ANALYSIS: Cell surface phenotype and mAb binding were analyzed by flow cytometry with the use of a Becton Dickson FACStar Plus flow cytometer. Controls consisted of fluorescein conjugated goat anti-mouse
IgM antibody. Viable cells were gated by a combination of forward light scatter and size. A minimum of 10,000 cells were analyzed for each histogram and the data are presented on a log scale.
RESULTS

ANTI-FAM MAB TREATMENT ENHANCES THE LYtic ACTIVITY OF FRESH NK CELLS, 5 DAY LAK CELLS AND ALAK CELLS. Studies with T cells have demonstrated that activation by specific ligands can be mimicked with mAb against the ligand's receptor. To test the hypothesis that stimulation of NK cells with anti-FAM mAb results in NK cell activation, various effector cell populations were treated with anti-FAM mAb at a concentration of 10 ug/ml per 1 x 10^6 effector cells, after which cells were washed repeatedly with 37 C media to remove any residual mAb that might be bound to the cell surface. As shown in Figure 1, anti-FAM mAb treatment of various effector cell populations resulted in a 2-5-fold increase in cytotoxicity against YAC-1 target cells. An increase in the cytotoxicity displayed by endogenous NK cells (from 4 LU_20 to 21 LU_20), by 5 day LAK cells (from 53 LU_20 to 114 LU_20), and by ALAK cells (from 115 LU_20 to 214 LU_20) was observed. We have previously demonstrated [14] that the isotype control mAb Thy1.2 binds to various effector cell populations, but does not inhibit the cytotoxicity or conjugate formation between the effector and target cell populations. To examine if the isotype control Thy1.2 mAb may result in activation of NK cells, various effector cell populations were treated with mAb Thy1.2 as described in Materials and Methods. MAb Thy1.2 treated cells exhibited marginal, if any, enhancement of NK cell cytotoxicity (Figure 1).

STIMULATION OF NK CELL LYMPHOKINE PRODUCTION BY ANTI-FAM MAB. It has been reported that stimulation of NK cells with tumor cells results in the secretion of various lymphokines [21], most probably via antigen receptor interactions. Since anti-FAM mAb treatment of effector cells results in
enhanced cytotoxicity of NK target cells, an attempt was made to determine if anti-FAM mAb treatment of NK cells also results in lymphokine production. It has been previously reported that IL-2 activated NK cells make a variety of cytokines (including IFN-gamma and TNF-alpha), as compared to fresh NK cells (22). In an effort to specifically determine if anti-FAM mAb stimulation of fresh NK cells results in TNF-alpha production, fresh NK cells were stimulated with anti-FAM mAb for a period of 24 hrs. Total RNA was extracted from both mAb treated and untreated cells. cDNA was synthesized and PCR analysis was performed using primers specific for TNF-alpha. As shown in Figure 2, a PCR product corresponding to the size of TNF-alpha was observed in the anti-FAM mAb-treated cells.

**Redirected Killing of Anti-FAM MAB Expressing Hybridoma Cells by Endogenous NK Cells, 5 Day LAK Cells and ALAK Cell Populations.** It has been demonstrated [23] that mAbs directed against the T cell antigen receptor (TCR) are able to mediate redirected lysis of normally unrecognized target cells. To examine if FAM on murine NK cells can mediate redirected lysis, the anti-FAM mAb expressing hybridoma was used as a target cell in cytolytic assays. The P3 myeloma used for the hybridoma fusions served as a control target cell. Each of the effector cell populations tested were efficient at lysing the anti-FAM mAb bearing hybridoma (Table 1), but were inefficient at killing the P3 myeloma (Table 1). Moreover, up to 94% inhibition of the cytotoxicity against the anti-FAM mAb bearing hybridoma was observed upon pretreating the effector cells with the anti-FAM mAb (Table 1), demonstrating the specificity of the lytic assay.
MODULATION OF FAM EXPRESSION WITH ANTI-FAM MAB. To further determine the role of FAM in NK cell recognition and cytotoxicity, an attempt was made to down-modulate the expression of FAM from the surface of NK cells. As shown in Figure 3A, 30% of untreated 5 day LAK cells stained positive with anti-FAM mAb. In comparison, 2% of anti-FAM mAb modulated cells stained positive with the anti-FAM mAb (Figure 3B). Similar results were observed with other effector cell populations (data not shown). Previously we have shown the involvement of FAM in both NK cell recognition and cytotoxicity [14]. To further examine the role of FAM in NK cell cytotoxicity, anti-FAM mAb modulated fresh NK cells, 5 day LAK cells, and ALAK cells were examined for their ability to lyse YAC-1 target cells. An average of 80% decrease in the cytotoxicity of NK target cells was observed. As shown in Figure 4, a decrease in $LU_{20}$ from 20 to 4, 80 to 20 and 140 to 30 was observed with fresh NK cells, 5 day LAK cells, and ALAK cells, respectively. To determine if the decreased cytotoxicity by various effector cell populations was due to the lack of conjugate formation (i.e., recognition) between effector cells and the target cells, single cell conjugate assays were performed between FAM modulated NK cells and target cells. As shown in Table 2, up to an 70% decrease in conjugate formation was observed between FAM modulated NK cells and YAC-1 target cells.
DISCUSSION

The cell surface molecule NK1.1 is considered the most specific marker for murine NK cells [24]. Moreover, all murine NK cell cytotoxicity is contained within the NK1.1(+) cell population [25]. However, a true NK cell antigen receptor has not been identified at the present time. Using a mAb (5C6), we have recently identified a novel function-associated molecule (FAM) on murine NK cells [14]. FAM is expressed by 20% of fresh NWNA cells, 30% of 5 day LAK cells, and 50% of ALAK cells [14]. We have also demonstrated that all FAM(+) NK cells also express the NK1.1 antigen [14]. The shared expression of FAM by fresh NK cells, 5 day LAK cells, and ALAK cells and the ability of anti-FAM mAb to inhibit the cytotoxicity of NK target cells, suggests FAM to be involved in a common recognition mechanism utilized by each of these effector cell populations.

We now demonstrate that FAM on murine NK cells is a signal-transducing molecule. Stimulation of NK cells with anti-NK1.1 [10], anti-2B4 [11] and anti-p38 [7] mAbs have been reported to enhance NK cell cytotoxicity. To determine if anti-FAM mAb treatment of NK effector cell populations also results in enhanced cytotoxicity, fresh NK cells, 5 day LAK cells and ALAK cell populations were treated with the anti-FAM mAb. An approximate 2-5-fold increase in the cytotoxicity of the effector cell populations against YAC-1 target cells was observed (as compared to untreated effector cell populations). This increase in cytotoxicity suggests that FAM plays an important role in NK cell activation. In comparison, treatment with an isotype control mAb Thy1.2 which binds to each of the effector cell populations but does not block function resulted in only a marginal increase in the lysis of target cells. These findings suggest that the mAb Thy1.2 recognized molecule on NK cells does not play a significant role in NK cell signal-transduction/activation.
NK cells are potent producers of lymphokines, and this function can be induced by many stimuli that also trigger lysis. Stimulation with the anti-FAM mAb resulted in the production of TNF-alpha by fresh NK cells, similar to C1.7 mAb [7] and anti-2B4 mAb [11] induced lymphokine production. Further, the ability of cytotoxic cells to specifically lyse hybridoma cells that produce antibodies against a triggering molecule on the effector cells has been used to determine the ability of a molecule to transduce signals into the cell (i.e., redirected lysis) [23]. Normally insensitive target cells are rendered susceptible to lysis by bearing an anti-receptor mAb on its surface. This process bypasses the normal receptor-antigen recognition step while triggering the antigen-bearing effector cell. Our results demonstrate that each of the effector cell populations were efficient at lysing the anti-FAM mAb expressing hybridoma cell, suggesting a role of FAM in signal-transduction/cellular activation.

Other molecules in humans, mice and rats capable of mediating "NK-like" non-MHC restricted cytotoxicity have been implicated in NK cell activation [7-11,26,27]. A mAb to CD7 on NK cells induces calcium mobilization, secretion of IFN-gamma, proliferation and increased cytolytic activity. However, the CD7 molecule does not transduce a lytic signal, as it neither mediates redirected killing of FcR+ murine mastocytoma P815 nor triggers lysis of a hybridoma expressing the antibody in a membrane bound form [26]. In contrast FAM on human [5] and murine NK cells mediates the redirected lysis of the FAM expressing hybridoma, and is therefore directly involved in transducing lytic signals. Further, this specific function can be blocked upon pretreating NK cells with soluble anti-FAM mAb. A significant increase in the lytic activity of resting NK cells, but not of IL-2-activated NK cells has been reported by anti-CD7 mAb stimulation [26]. In comparison, anti-CD16 mAb treatment of NK cells does not result in enhanced cytotoxicity, but does
result in calcium mobilization [27]. A mAb directed against the surface dimer Kp43 also enhances the cytotoxicity of IL-2-activated NK cells [8]. In the mouse model, anti-2B4 and anti-NK1.1 mAbs have been shown to enhance NK cell cytotoxicity [10,11]. The anti-2B4 mAb specifically augments the cytotoxicity of IL-2 cultured NK cells, but has no effect on fresh NK cells [11]. The role of the anti-NK1.1 mAb is controversial, as it is not clear if anti-NK1.1 mAb treatment results in the activation of fresh NK cells or IL-2 cultured NK cells. In comparison, our results demonstrate that the anti-FAM mAb enhances the lytic activity of fresh NK cells, 5 day LAK cells, and ALAK cells. To our knowledge, FAM is the first molecule that has been implicated in playing a role in signal-transduction in each of these effector cell populations. Therefore, NK cell signaling induced upon anti-FAM mAb binding is different from that obtained with mAbs detecting NK1.1, 2B4, and the human CD7 antigen.

To further analyze the role of FAM in NK cell cytotoxicity, an attempt was made to down-modulate FAM from the surface of the various effector cell populations. As shown by flow cytometry, up to an 100% decrease in the expression of FAM was observed. Down-modulation of FAM also resulted in a 80% decrease in cytotoxicity and a 71% decrease in conjugate formation by various effector cell populations. These results correlate with the findings obtained upon treating effector cells with the anti-FAM mAb [14]. Absolute inhibition in the cytotoxicity of target cells was not achieved upon modulating FAM from the surface of NK cells. It is possible that either the modulated effector cells still express low levels of FAM that were not detected by flow cytometry and/or the loss of FAM results in the upregulation of other molecules on NK cells that are involved in NK cell cytotoxicity. One can hypothesize that each of these distinct effector cell populations utilize similar molecules in mediating NK-like cytotoxicity.
In conclusion, FAM is an evolutionarily conserved molecule which is expressed on cell surface of fish NCC [12], human NK cells [5], rat NK cells [13], and mouse NK cells [14], and is also involved in signal-transduction. The enhanced cytotoxicity, lymphokine production, susceptibility to lysis of anti-FAM mAb secreting hybridoma cells, and the specific inhibition of killing by soluble anti-FAM mAb are consistent with the participation of FAM on fresh NK cells, 5 day LAK cells, and ALAK cells in the killing process. In addition, modulation of FAM from NK cell surface results in the inhibition of cytotoxicity and conjugate formation by NK cells. To our knowledge, our results are the first demonstration that the cytotoxic activity of fresh NK cells, 5 day LAK cells and ALAK cells can be triggered by a common anti-FAM mAb recognizing molecule. Overall, these results indicate that FAM is intimately involved in murine NK cell recognition and function.

ACKNOWLEDGMENTS

We thank Miss Barb Carolus for assisting us with flow cytometry. This work was performed as partial fulfillment for the doctoral degree of Reuben Kapur.
REFERENCES


**TABLE 1**

ANTI-FAM MAB MEDIATED REDIRECTED LYSIS BY MURINE NK CELLS.

<table>
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<th>Effector Cells</th>
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<th>Percent Inhibition</th>
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Effector cells were derived as described and tested for lytic activity in the presence or absence of anti-FAM mAb. The anti-FAM mAb producing hybridoma cells (5C6(H)) and the P3 myeloma fusion partner were used as targets. 10 μg of anti-FAM mAb was used where indicated. Each experiment was performed in triplicate.
TABLE 2
DOWN-MODULATION OF FAM RESULTS IN DECREASED CONJUGATE FORMATION.

<table>
<thead>
<tr>
<th>POPULATION</th>
<th>FAM(+)</th>
<th>FAM(-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh NK cells</td>
<td>53</td>
<td>21</td>
</tr>
<tr>
<td>5 day LAK cells</td>
<td>56</td>
<td>20</td>
</tr>
<tr>
<td>ALAK cells</td>
<td>60</td>
<td>17</td>
</tr>
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Effector cell populations were purified as described in Materials and Methods. The expression of FAM was modulated as described in Materials and Methods. Conjugate assays were performed using YAC-1 target cells. Each experiment was repeated three times.
FIGURE LEGENDS

FIGURE 1: Increased lytic activity by anti-FAM mAb treated NK cells. Various effector cell populations were treated with anti-FAM mAb and anti-Thy1.2 mAb as described in Materials and Methods. Treated effector cells were tested for lytic activity against YAC-1 targets. Each experiment was performed in triplicate.

FIGURE 2: Lymphokine production by fresh NK cells. Fresh NK cells were stimulated in vitro for 24 hrs as described in the Materials and Methods. RT-PCR was performed to analyze the expression of mRNA for TNF-alpha. Lane 1: positive control for TNF-alpha. Lane 2: Unstimulated NK cells. Lane 3: Anti-FAM mAb stimulated NK cells.

FIGURE 3: Anti-FAM mAb mediated down-modulation of FAM. The expression of FAM on 5 day LAK cells was compared between anti-FAM mAb treated and untreated 5 day LAK cells. Flow cytometry was performed as described in Materials and Methods. Treated and untreated LAK cells were stained with anti-FAM mAb. An anti-IgM antibody was used as a control.

FIGURE 4: Decreased lytic activity of FAM(-) NK cells. The expression of FAM on various effector cell populations was down-modulated as described in Materials and Methods. FAM(+) and FAM(-) cells were tested for lytic activity against YAC-1 targets. Each experiment was performed in triplicate.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Molecular Characterization of a Novel Function-Associated Molecule (FAM) on Murine Natural Killer Cells Using an Antisense Oligonucleotide Approach

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ABSTRACT

We have previously described a novel function-associated molecule (FAM) present on fish nonspecific cytotoxic cells (NCC), as well as human, rat and murine NK cells. FAM is involved in NK cell recognition and cytotoxicity, as anti-FAM mAb inhibits both the recognition and cytotoxicity of target cells by various effector cell populations. Moreover, FAM also acts as a signal-transducing molecule on NK cells, as anti-FAM mAb stimulates lymphokine production as well as the redirected lysis of the anti-FAM mAb expressing hybridoma. A partial amino-acid sequence of the FAM isolated from rat NK cells has been found to be closely related to an intermediate filament, vimentin. We now demonstrate that FAM on murine NK cells is molecularly related to vimentin. Anti-vimentin mAb reacts with NK cells, as shown by flow cytometry, and further, inhibits cytotoxicity and conjugate formation between murine NK cells and target cells. Moreover, northern blot analysis using a full length vimentin cDNA probe revealed no differences in mRNA transcripts between FAM(+) NK cells and FAM(-) BALB/C 3T3 fibroblasts (both are identical to that of the classical vimentin mRNA). Further, polymerase chain reaction (PCR) experiments performed with FAM(+) NK cells and FAM(-) BALB/C 3T3 fibroblasts, using primers specific for the head, core and the tail region of the vimentin gene, revealed no differences in the size of the PCR products. Finally, FAM(+) NK cells treated with anti-sense oligonucleotides directed against the head and the core region of the vimentin gene inhibited the expression of FAM on FAM(+) NK cells and also inhibited the lytic activity of NK cells against target cells.
INTRODUCTION

Natural killer (NK) cells are a subpopulation of lymphocytes characterized as being nonadherent, nonphagocytic and able to mediate non-major histocompatibility complex (MHC) restricted cytotoxicity (1,2). NK cells do not express cell surface TCR molecules or CD3 proteins (3-5). However, NK cells do express the CD3 zeta chain (6) in association with FcRIII for IgG (7). NK cells play a major role in resistance to growth and metastatic spread of a wide variety of tumors (1,2), and display resistance to certain viral and bacterial infections (8-10). NK cells have been shown to release cytokines, proliferate and mediate enhanced cytotoxicity in response to interleukin-2 (IL-2). These lymphokine activated killer (LAK) cells (11,12) are morphologically and antigenically identical to NK cells (13,14). Recently, a simple technique for the isolation of large numbers of highly purified, activated NK cells from splenocytes by selective adhesion to plastic has been developed (15). These adherent lymphokine activated killer (ALAK) cells also are morphologically and antigenically similar to NK cells. It is generally accepted that NK cells lyse their targets in a sequential manner: recognition and conjugation of the NK cell to the target cell (16); triggering of the NK cell (16,17); release of a cytotoxic substance (18,19); binding of the cytotoxic substance to the target cell; and membrane damage, or DNA fragmentation, resulting in target cell death (20).

The membrane structures involved in NK cell recognition of target cells and their subsequent triggering have not yet been determined. Although several candidates have been identified, no definite NK recognition receptors have yet been identified. It is generally believed that NK cell cytotoxicity is mediated in part by several cell interaction/adhesion molecules such as CD2 and LFA-1 which interact with ligands such as LFA-3 and ICAM-1 on target cells (21,22). It is also possible
that there are specific receptors on NK cells that recognize specific antigens on target cells. Such molecules in humans, rats and mice have been reported (23-30).

In earlier reports we have shown that nonspecific cytotoxic cells (NCC) from fish are capable of lysing a wide variety of both human and murine transformed cell lines (31). We hypothesized that NCC recognize a wide variety of transformed cell lines through a conserved receptor-like molecule. In an effort to determine the nature of these molecule, monoclonal antibodies (mAbs) were made against fish NCC. One such mAb (5C6) recognizes a function-associated molecule (FAM) on fish NCC (31), as well as human (23), rat (32) and murine NK cells (33). FAM is involved in NK cell recognition, cytotoxicity and signal-transduction. Anti-FAM mAb blocks the recognition and the cytotoxicity of target cells by NK cells. Moreover, it stimulates lymphokine production and redirected lysis of anti-FAM mAb expressing hybridoma cells. A partial amino-acid sequence of the FAM isolated from rat NK cells has been found to be closely related to an intermediate filament, vimentin (34). We now demonstrate that anti-vimentin mAb binds to murine NK cells and inhibits the cytotoxicity and conjugate formation between NK cells and target cells. Northern blot and polymerase chain reaction (PCR) analyses suggest that the mRNA encoding for FAM is similar to that of vimentin, if not identical. Finally, antisense oligonucleotides directed against the vimentin gene inhibit FAM expression and NK cell cytotoxicity.
MATERIALS AND METHODS

MICE. C57BL/6 mice were obtained from SASCO (Omaha, Nebraska) and kept in a pathogen free animal facility at our institution. SCID mice were obtained from University animal care, University of Arizona. Mice were used in experiments at 6 to 10 wks of age.

REAGENTS AND CELL LINES. Human recombinant interleukin-2 (IL-2) was the kind gift of Dr. Emmanuel Akporiyae (Department of Microbiology & Immunology, University of Arizona) and Amgen (Thousand Oaks, CA). The mAb Thy1.2-PE was obtained from Pharmingen (San Diego, CA) and used as suggested by the manufacturer. Monoclonal antibody 13.2 (IgM), anti-human foreskin fibroblast vimentin (V5255), was purchased from Sigma. Fluorescein conjugated goat anti-mouse IgM was used as a secondary antibody (Olympus Corp, Lake Success, NY).

DERIVATION OF ANTI-FAM MAB. The anti-FAM mAb 5C6 was derived as described (31). Briefly, mice were immunized with purified fish NCC (from Ictalurus punctatus), spleens were fused with the P3-X63-Ag8.653 myeloma, and wells positive for growth were screened by ELISA and flow cytometry with NCC. The anti-FAM mAb selected for use in the experiments was of the IgM isotype.

ISOLATION OF EFFECTOR CELLS. Spleens were aseptically removed and single cell suspensions were prepared in RPMI-10%. Mononuclear cells were obtained after centrifugation through lympholyte-M (Accurate, Westbury, NY) cell separation medium (200 x g, 30 minutes, room temperature). In some experiments,
spleen mononuclear leukocytes were passed over nylon wool columns to remove macrophages and B cells. Briefly, $10^8$ spleen cells in 2 ml of RPMI-10%, were added to a 20 cc syringe containing 1 gm of sterile nylon wool. The cells were incubated for 45 minutes at 37°C and the nylon wool was gently washed with 50 ml of RPMI-10%. The nylon wool nonadherent (NWNA) cells were collected, washed, and used in the assays as fresh endogenous NK cells. This method consistently reduced the percentage of B cells and macrophages in the spleen preparations to less than 2%. Lymphokine activated killer (LAK) cells were obtained by culturing fresh NWNA cells in complete medium supplemented with 250 U/ml rIL-2 at 2 X 10^6 cells per ml for a period of at least 5 days. Adherent lymphokine activated killer (ALAK) cells were derived by a procedure described earlier (35). Briefly, fresh NWNA cells were cultured in complete medium supplemented with 1000 U/ml rIL-2 at 2 X 10^6 cells per ml for a period of 48 hrs at 37°C, after which tissue culture flasks were rinsed three times with 37°C RPMI 1640 to remove all nonadherent cells. The remaining adherent cells were expanded for another 6 to 8 days with 1000 U/ml of rIL-2 and medium changed as necessary. The adherent cells were removed with Ca^{2+} and Mg^{2+} free PBS containing 0.02% EDTA. The cells were washed in complete medium before use in the functional assays.

FLOW CYTOMETRIC ANALYSIS. Cell surface phenotypes and mAb binding were analyzed by one color and two-color flow cytometry with the use of a Becton Dickinson FACStar plus flow cytometer. For one color flow cytometry, controls consisted of fluorescein conjugated goat anti-mouse IgM antibody. For the two-color flow cytometric analysis, cells were first stained with anti-FAM mAb and an anti-IgM-FITC second antibody (green fluorescence). The cells were then stained with a directly PE-conjugated Thy1.2 mAb. The cells were then analyzed for simultaneous
red and green fluorescence. Viable cells were gated by a combination of forward light scatter and size. A minimum of 10,000 cells were analyzed for each histogram and the data are presented on a log scale.

**CYTOTOXICITY ASSAYS.** Effector cells were tested for cytolytic activity in a standard microcytotoxicity assay (36). Lytic units at the 20% lysis level ($LU_{20}$) for $1 \times 10^6$ effector cells was calculated by computer-assisted regression analysis as described by Pross et al (37). All effector to target (E/T) ratios were performed in triplicate. Spontaneous $^{51}$Cr-release under any of the conditions never exceeded 10%.

In experiments in which the effector cells were tested with the mAb, the effector cells were placed in the wells of the microtiter plates and incubated with the mAb for 1 hr at 4C before the addition of the target cells to the wells. In other experiments the effector cells were preincubated with the mAb at 4C for 1 hr in conical tubes, cells were washed and then used in cytotoxicity assays. The percent inhibition of cytotoxicity was based on comparisons of the $LU_{20}$.

**CONJUGATE ASSAYS.** The effects of the mAb on the ability of the various effector cell populations to form conjugates with YAC-1 tumor cells was examined as described previously (38). Briefly, equal numbers of effector cells and target cells ($0.5 \times 10^6$ each) were mixed in 1 ml of RPMI-10%, the mixture was centrifuged for 2 minutes at $50 \times g$ and incubated for 15 minutes at room temperature. At this time cells were vigorously pipetted (four to five times) to disrupt spontaneous conjugates, an aliquot was placed on a hemocytometer and conjugates were enumerated.
NORTHERN BLOT ANALYSIS. Total cellular RNA was extracted as previously described (39). Briefly, washed cells were resuspended in isotonic buffer TEN (0.15 M NaCl, 10 mM Tris-HCl, pH 7.8, 1 mM EDTA). The RNase inhibitor, vanadyl ribonucleoside complex was added to final concentration of 5 mM followed by 10% NP-40 detergent to lyse the cells. The samples were centrifuged for 10 minutes at 400 g. The supernatant were added to 2X extraction buffer (0.35 M NaCl, 20 mM Tris-HCl, pH 7.8, 2 mM EDTA) containing 20% (w/v) sodium dodecyl sulfate (SDS). The supernatants were extracted three times with equal volumes of phenol/chloroform. Northern analysis was performed as described (40). Briefly, 25 ug of total RNA was electrophoresed through 1% agarose/formaldehyde gels, transferred to nylon membranes, UV-cross-linked at 0.3 joules/cm² using a Bioslinker (Bios Corp., New Haven, CT), and probed with a ³²P-labeled 1 Kb cDNA vimentin probe (Oncor, Gaithersburg, MD). Hybridizations and washings were carried out as suggested by the manufacturer.

POLYMERASE CHAIN REACTION (PCR) ANALYSIS. Total cellular RNA was isolated as described above. cDNA was synthesized from total RNA using the uni-amp plus kit (Clontech, Palo Alto, CA). Briefly, 1 ug of total RNA was used for constructing first strand cDNA. For preparing a 30 ul cDNA reaction mixture, 3 ul of mouse Moloney leukemia virus reverse transcriptase (MMLV-RT) (200 U/ml), 1 ul of RNasin (40 U/ml), 6 ul of 5X MMLV-RT buffer, 3 ul of oligo d(T) (0.5 mg/ul), 3 ul of acetylated BSA (1 mg/ml) and 1.5 ul of dNTP mix (10 mM each). The samples were incubated at 65 C for 5 minutes to denature the RNA and then quenched on ice. This RT mix was then added to each sample and incubated at 42 C for 1 hr. PCR was performed on each of these samples using primers specific for the head, core and the tail region of the vimentin gene. Briefly, seven different primers
against different regions of the vimentin gene were synthesized. Primer 1 corresponds to the head region of the vimentin gene (5'-CAA / CCG / GAG / CTA / TGT / GAC / CAC / -3'), primer 2 corresponds to the region approximately 40 nucleotides up stream of primer 1 (5'-TGT / CTA / CCA / GGT / CTG / TGT / CCT / -3'), primer 3 corresponds to the head region of the vimentin gene, approximately 200 nucleotides downstream of primer 2 (3'-CTC / TTC / CAT / CTT / GAC / GTC / CTC / -5'), primer 4 corresponds to the conserved core region of the gene, and is approximately 350 nucleotides downstream of the primer 3 (3'- GAC / GTG / CTA / CTT / CTC / TAG / GTC / -5'), primer 5 also corresponds to the conserved core region of the vimentin gene, and is approximately 110 nucleotides downstream of the primer 4 (3'- TTC / TTG / GAG / GTC / CTC / CGG / CTC / -5'), primer 6 also corresponds to the conserved core region of the vimentin gene (5'- AAG / AAC / CTC / CAG / GAG / GCC / GAG / -3'), and primer 7 corresponds to the tail region of the vimentin gene (3'- GAG / TCG / TAG / TGC / TAC / TGG / AAC / -5'). The samples were amplified for 30 cycles, consisting of 94 C for 1 minute, 60 C for 2 minutes, and 72 C for 3 minutes.

SENSE AND ANTI-SENSE OLIGONUCLEOTIDE TREATMENT. Sense and anti-sense treatment of FAM(+) positive NK cells was performed as described earlier (41). Briefly, 21 mer sense and anti-sense oligonucleotides were generated to the core and the head region of the vimentin gene (Macromolecular structures facility, University of Arizona). Sense oligonucleotides used in experiments were of the following sequence: 5'- TGT / CTA / CCA / GGT / CTG / TGT / CCT / -3' (against the head region) and 5'- CTG / CAC / GAT / GAA / GAG / ATC / CAG / -3' (against the core region). Anti-sense oligonucleotides were of the following sequence: 3'- ACA / GAT / GGT / CCA / GAC / ACA / GGA / -5' (against the head
region) and 3'-GAC /GTG /CTA /CTT /CTC /TAG /GTC /-5' (against the core region). FAM(+) 5 day LAK and ALAK cell populations were treated with either sense or anti-sense oligonucleotides for a period of 24 hrs at a final concentration of 50 uM in 96 well plates. The cells then were washed and used either for flow cytometric analysis and cytotoxicity assays.
RESULTS

ANTI-VIMENTIN MAB BINDS TO SCID NK CELLS. We have previously shown that a partial amino acid sequence of the rat FAM is closely related to an intermediate filament, vimentin (34). In addition, anti-vimentin mAb reacts with rat NK cells (34). It was of interest to determine if anti-vimentin mAb also reacted with FAM expressing murine NK cells. As shown in Figure 1, the anti-FAM mAb binds to approximately 50% of SCID NK cells. In comparison, an anti-vimentin mAb reacts with approximately 25% of SCID NK cells (Figure 1). Further, the mAb fluorescence intensity was higher on cells stained with anti-FAM mAb, as compared to anti-vimentin mAb (Figure 1).

ANTI-VIMENTIN MAB INHIBITS THE CYTOTOXICITY OF TARGET CELLS BY SCID NK CELLS. Previously we have demonstrated that the anti-FAM mAb inhibits the cytotoxicity of target cells by NK cells (23,31-33). Since anti-vimentin mAb also reacts with NK cells, an attempt was made to determine if antivimentin mAb also would inhibit the lysis of target cells. As shown in Figure 2, antivimentin mAb treatment of NK cells resulted in 63% inhibition in the killing of target cells. In comparison, 93% inhibition in the cytotoxicity of target cells was observed upon treating NK cells with the anti-FAM mAb. As a control mAb, anti-Thy1.2 was used. This mAb binds to NK cells (33), but does not inhibit the cytotoxicity of NK target cells (Figure 2). Each of the mAbs used in the experiments were of the IgM isotype. In addition, we have shown that the FAM on NK cells is involved in conjugate formation (i.e., recognition) between NK cells and target cells (23,31-33). Pretreatment of NK cells with anti-FAM mAb and subsequently mixing with target cells inhibits the ability of these cells to form conjugates. To determine if
anti-vimentin mAb would also inhibit conjugate formation (i.e., recognition), single-cell conjugate assays were performed. As shown in Table 1, upon treating NK cells with anti-vimentin mAb 50% inhibition in conjugate formation was observed between NK cells and target cells. Approximately 75% inhibition in conjugate formation was observed when anti-FAM mAb treated NK cells were used in conjugate assays. Similar to previous results (33), anti-Thy1.2 mAb did not have any effect on conjugate formation (Table 1).

MOLECULAR CHARACTERIZATION OF THE THE VIMENTIN-LIKE FAM. We have previously shown that a partial amino acid sequence of FAM isolated from rat NK cells was identical to the core region of vimentin (34). A full length vimentin cDNA probe was used to determine if the mRNA that encodes FAM and vimentin were identical. Total RNA was extracted from cells that express FAM (i.e. SCID NK cells, fresh NK cells from C57BL/6 mice and ALAK cells) and cells that are negative for FAM expression (i.e. EL-4, P815 and 3T3 fibroblasts). As shown in Figure 3, upon hybridizing the blot with a full length vimentin cDNA probe only one mRNA transcript was detected in each of the lanes. The size of the mRNA was approximately 2 Kb, which corresponded to the mRNA size that encodes for the classical vimentin protein. To determine if subtle differences exist in the mRNA from FAM(+) and FAM(-) cells, PCR analysis was performed with ALAK cells and BALB/C 3T3 fibroblast cells. Primers were synthesized to different regions of the vimentin gene (i.e. head, core, and tail). PCR was performed on cDNA from both FAM expressing ALAK cells (lanes 1-5) and FAM(-) BALB/C 3T3 fibroblasts (lanes 1'-5'), using the following primer combinations: 1/7 (head/tail), 1/4 (head/core), 2/5 (head/core), 6/7 (core/tail), and 2/3 (head/head), respectively (Figure 4). PCR fragments of the expected sizes were obtained upon priming cDNA
from FAM(-), vimentin (+) 3T3 fibroblasts. A 1300 bp fragment was observed when the primer combination 1/7 was used. 631 bp, 843 bp, 532 bp, and 300 bp fragments were obtained upon using primer combinations 1/4, 2/5, 6/7 and 2/3, respectively (lanes 1'-5'). PCR fragments of identical sizes were obtained from FAM(+) cells (lanes 1-5). These results demonstrated that the mRNA that encodes vimentin and FAM are either identical or related, to the extent that PCR cannot detect subtle differences in the size of the two mRNA.

To determine if vimentin anti-sense oligonucleotide treatment of FAM(+) NK cells would modulate the expression of FAM on NK cells, FAM(+) NK cells were treated with sense and anti-sense oligonucleotides synthesized against the head and the core regions of the vimentin gene. As shown in Figure 5A and 5B, sense oligonucleotide treated FAM(+) 5 day LAK and ALAK cells expressed normal levels of FAM (i.e. 30% and 50% respectively). In comparison, a 90% decrease in the expression of FAM was observed with 5 day LAK and ALAK cells upon a 24 hr antisense oligonucleotide treatment (Figure 5A and 5B). Modulation of FAM due to antisense oligonucleotide treatment of FAM(+) 5 day LAK and ALAK cells resulted in an 85% and a 70% decrease in the cytotoxicity of NK target cells respectively (i.e. 90 to 10 LU's for 5 day LAK cells and 250 to 80 LU's for ALAK cells)(Figure 6). In comparison, cells that were treated with the sense oligonucleotides demonstrated normal levels of target cell lysis (i.e. 95 LU's for 5 day LAK cells and 238 LU's for ALAK cells (Figure 6).
DISCUSSION

The FAM is a novel evolutionarily conserved cell surface protein on fish NCC (31), human NK cells (23), rat NK cells (32), and mouse NK cells (33), as demonstrated by flow cytometric analysis of NK cells, and by 125I surface labeling and immunoprecipitation experiments (23). In addition, anti-FAM mAb inhibits cytotoxicity and conjugate formation by NK cells. FAM has also been shown to be a signal-transducing molecule, as anti-FAM mAb treatment of NK cells stimulates lymphokine production, enhances NK cell cytotoxicity, and mediates redirected lysis (23). A partial amino acid sequence of FAM isolated from rat NK cells has been shown to be highly related to the core region of the intermediate filament (IF), vimentin (34). We now demonstrate that murine NK cell FAM is molecularly related to vimentin.

IF are composed of distinct intermediate filament proteins (IFP) that are encoded by a multigene family, the members of which are regulated developmentally in a tissue specific fashion (42,43). The expression of IFP is predominantly restricted to cells of mesenchymal origin, undifferentiated cells, and cancer cells. Moreover, the expression of vimentin is growth regulated (44). Vimentin is expressed in all stages of B cell development, but is lost during terminal differentiation of B cells into plasma cells (45). Previous studies have shown that vimentin is induced in normal resting murine T lymphocytes during the Go to S phase transition by mitogens and growth factors, including ConA (46), polyamines (46), and IL-2 (47). These studies suggest vimentin to be a growth responsive gene in T cells.

The function of vimentin during growth and differentiation is not clear (48). Vimentin appears to link the nucleus to the plasma membrane (49), and becomes
phosphorylated during mitotic reorganization (50). Based on these findings it can be postulated that vimentin plays an important role in signal-transduction and intracellular transport processes. Although the function of vimentin in lymphocytes is not understood, vimentin cocaps with lymphocyte antigen receptors (51) and undergoes extensive cytoplasmic reorganization in B cells after antigen receptor cross-linking (52).

Similar to FAM, vimentin also is highly evolutionarily conserved in its structure, cellular localization, and amino acid sequence (particularly in the core region) (53). Vimentin along with other IFs has a conserved central alpha-helical rod domain of 311-314 amino acids, which is flanked by end domains of variable size and chemical character (53). Differences in the sizes and properties of IF subunits are due almost entirely to the variability in portions of the end domains, and the basis for the structural uniformity of diverse IF appears to reside in the conserved structure of the rod domains (53).

The presence of vimentin either in the cytoplasm or on the cell surface of NK cells has never been reported. However, cross-reactive determinants have been shown to exist between Thy1 on lymphocytes and vimentin (54). Vimentin also shares determinants with CD23 (low affinity IgE receptor) on B cells, as anti-CD23 mAb cross reacts with vimentin (55). A mAb to an unidentified 43 kd cell surface protein on a human leukemia cell line also cross reacts with vimentin (56). In the present study we demonstrate that an anti-vimentin mAb binds to a cell surface protein on murine NK cells, and inhibits cytotoxicity and conjugate formation between NK cells and target cells. The percentage of NK cells reacting with the anti-vimentin mAb was less than that observed with the anti-FAM mAb. Further, the ability of anti-vimentin mAb to inhibit NK cell cytotoxicity and conjugate formation also was less than that observed with anti-FAM mAb. Previously, we have
demonstrated that shared epitopes exist on FAM and vimentin, which are recognized by anti-FAM and anti-vimentin mAbs (34), although the molecular weights of these cross reactive proteins (vimentin (57 kDa) and FAM (50-53 kDa)) are different. It is conceivable that the epitope recognized by anti-vimentin mAb on FAM is not directly involved in target cell antigen recognition. This could explain why the anti-vimentin mAb only partially inhibits the cytotoxicity and conjugate formation between NK cells and target cells. Alternatively, the affinity of the anti-vimentin mAb for the epitope on FAM may not be as high as that of the anti-FAM mAb, which may result in only a partial inhibition in the cytotoxicity and conjugate formation between NK cells and target cells.

Previously, we have shown that a partial amino acid sequence of FAM was identical to the core region of vimentin (34). To examine the relationship between FAM and vimentin at the molecular level, northern blot analysis were performed on FAM(+) and FAM(-) cells. An attempt was made to determine if the size of the mRNA that encodes these two proteins was identical. Using a full length vimentin cDNA probe, only a single mRNA transcript corresponding to the size of the classical vimentin was detected. Since we were unable to detect any other bands on the northern blot, we believe that the mRNA encoding FAM and vimentin are either identical or highly related. To further examine for differences in the mRNA that encode for FAM and vimentin, PCR analysis was performed on cDNA derived from FAM(+) and FAM(-) cells. In particular, we looked for subtle differences in the size of PCR products between the two cell types that may have arisen as a result of alternate splicing of a single mRNA. Primers made against different regions of the vimentin gene were used to amplify various regions of the cDNA from FAM(+) and FAM(-) cells. No differences in the sizes of the PCR products were observed between the two cell types. These results suggest that the mRNA that encodes FAM
is identical to the mRNA that encodes vimentin. Finally, to examine the functional relationship of FAM and vimentin, and to determine whether the mRNA encoding these two proteins are identical, an antisense oligonucleotide approach was utilized. This strategy has been used successfully in the past to study specific gene function of perforin (41) and c-myc (57). Upon treating FAM(+) cells with sense and antisense oligonucleotides made against the core (i.e. conserved) and the head (variable) region of the vimentin gene, we observed a 93% and a 90% loss in the expression of FAM on 5 day LAK and ALAK cells upon antisense treatment. The decrease in the expression of FAM on 5 day LAK and ALAK cells also resulted in a decrease in the cytotoxicity of YAC-1 target cells. As shown in Figure 6, a complete inhibition in the cytotoxicity of target cells was not achieved upon treating FAM(+) cells with antisense oligonucleotides. It is possible that the loss of FAM results in the utilization or up-regulation of other NK cell function-associated molecules, which may play a role in NK cell cytotoxicity. These findings further strengthen the importance of FAM in NK cell cytotoxicity. Based on the partial amino acid sequence, cell surface staining, northern blot analysis, PCR analysis, and the antisense oligonucleotide treatment, we firmly believe that FAM on murine NK cells is highly related to vimentin, if not identical. The mechanism by which "vimentin-like" FAM is expressed on the surface of NK cells is not clear. It is possible that a small undetected transmembrane domain (exon) might be added to FAM, which allows it to anchor in the plasma membrane and function as a receptor-like molecule. It is also conceivable that post-translational processing of vimentin in NK cells in some fashion allows the "vimentin-like" FAM to be transported to the cell surface. It should be noted that the molecular weight of FAM is slightly smaller than the classical vimentin. Studies are being conducted to determine exactly how "vimentin-like" FAM gets expressed on the surface of NK cells.
In conclusion, experiments conducted at both the cellular as well as at the molecular level demonstrate FAM to be highly related to (if not identical with) an IF, vimentin. FAM, like vimentin, is a highly evolutionarily conserved molecule and plays an important role in NK cell cytotoxicity. Further experiments are being conducted to determine how the vimentin related FAM is expressed on the surface of NK cells and functions as a potential receptor-like molecule.

ACKNOWLEDGMENTS

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REFERENCES


### TABLE 1

Inhibiton of NK Cell Conjugate Formation by an-anti Vimentin mAb.

<table>
<thead>
<tr>
<th>% Conjugates</th>
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<tbody>
<tr>
<td>Cells</td>
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<tr>
<td>scid NK cells</td>
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</tbody>
</table>

The effector cell population was purified as described. Conjugate assays were performed using YAC-1 target cells as described in Materials and Methods. 10 ug of mAb 5C6, Thy1.2 and anti-vimentin were used in each experiment. Each experiment was repeated three times.
FIGURE LEGENDS

FIGURE 1: Binding specificity of the anti-FAM and anti-vimentin mAbs as determined by flow cytometric analysis. SCID NK cells were isolated as described and stained with mAb 5C6-FITC or anti-vimentin mAb-FITC.

FIGURE 2: Effect of anti-vimentin mAb on NK cell cytotoxicity. Cytotoxicity assays were performed as described in Materials and Methods. SCID NK cells were incubated either with anti-FAM mAb, anti-vimentin mAb or anti-Thy1.2 mAb for 1 hr. After incubation, the cells were washed and assayed for the ability to lyse $^{51}$Cr-labeled YAC-1 target cells in a 4-hr assay. Results are expressed as LU$_{20}$.

FIGURE 3. Northern blot analysis of FAM transcripts. Total RNA (20 ug) from FAM expressing cells, and cells negative for FAM expression were electrophoresed in a formaldehyde agarose gel, blotted, and hybridized with a $^{32}$P-labeled full-length vimentin cDNA probe. Lanes 1-3 contain RNA from FAM(-) cells (EL-4, P815 and 3T3, respectively). Lanes 4-6 contain RNA from FAM(+) ALAK cells, SCID NK cells and fresh NK cells, respectively.

FIGURE 4. PCR analysis of FAM(+) ALAK cells and FAM(-) 3T3 fibroblasts. The following primer combinations were used on cDNA from FAM(+) cells. Lane 1,1/7 (head/tail); 2,1/4 (head/core); 3,2/5 (head/core); 4,6/7 (core/tail); and 5,2/3 (head/head), respectively. cDNA from 3T3 fibroblasts was amplified using identical primer combinations (Lanes 1'-5').
FIGURE 5. Modulation of FAM expression by anti-sense oligonucleotide treatment. (A) Sense and anti-sense oligonucleotide treated 5 day LAK cells were stained with anti-FAM mAb-FITC. (B) Sense and anti-sense oligonucleotide treated ALAK cells were stained with anti-FAM mAb-FITC.

FIGURE 6. Modulation of FAM by anti-sense oligonucleotide treatment results in decreased NK cell cytotoxicity. 5 day LAK cells and ALAK cells were treated with either sense oligonucleotides or anti-sense oligonucleotides for a period of 24 hrs, cells were washed, and assayed for lysis of YAC-1 in a Cr51-release assay. Results are expressed in LU20.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5A.
Figure 5B.
Figure 6.
APPENDIX D
Reduced Levels of Major Histocompatibility Complex Class I Antigen In Association with Expression of A Novel Target Cell Antigen Renders Murine Target Cells Susceptible to NK Cell Lysis

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SUMMARY

We have previously characterized a novel monoclonal antibody (mAb), termed 18C2, which binds to and inhibits the lysis of target cells by human natural killer (NK) cells. We now show that the anti-target cell mAb 18C2 also recognizes a similar structure on the murine NK sensitive target cell YAC-1, as well as on NK resistant target cells P815 and EL-4, as observed by flow cytometry. Functional studies demonstrated that the mAb 18C2 inhibited the lysis of both NK sensitive YAC-1 target cells, as well as NK resistant target cell lines P815 and EL-4 by freshly-isolated nylon wool nonadherent (NWNA) NK cells, 5 day lymphokine activated killer (LAK) cells and adherent lymphokine activated killer (ALAK) cells. The inhibitory activity of the mAb 18C2 occurred at the target cell level only. Single cell conjugate assays demonstrated that the structure recognized by the mAb 18C2 was involved in recognition between NK cells and NK target cells, as the mAb inhibited conjugate formation between a variety of effector cells and various target cell lines tested. Further, the role of major histocompatibility complex (MHC) class I antigens in NK cell cytotoxicity was examined. We observed that target cells expressing low levels of MHC class I antigens in association with the novel target cell antigen were more sensitive to NK cell lysis, as compared to cells that co-express high levels of the MHC class I antigen and the target cell antigen. Further, the presence of this antigen across different species suggests this target cell antigen/structure to be highly evolutionarily conserved.
INTRODUCTION

Natural killer (NK) cells exhibit "natural" cytolytic activity against virus infected cells and certain tumor cells in a non-major histocompatibility (MHC) restricted fashion (1). Although NK cell-mediated cytotoxicity is not MHC restricted, lysis is influenced by MHC antigen expression. It has been shown that on certain target cell types the level of MHC class I expression inversely correlates with target cell susceptibility to NK cell lysis (2-6). An attempt has been made to explain the above results utilizing the missing self hypothesis, which suggests that NK cells recognize and lyse target cells that lack the expression of or express low levels of MHC class I molecules (7). Alternative mechanisms have been postulated to explain the effects of MHC class I on NK cell activity. MHC class I expression may mask the target cell antigen responsible for recognition by NK cells, or recognition of certain class I molecules may deliver a negative signal to NK cells. Recent studies (8) support the negative signal hypothesis. For example Ly-49, a murine NK cell surface molecule, appears to deliver a negative signal to NK cells upon recognition of MHC class I molecules on target cells (9). Molecules like SW5E6 (10-12) and GL183/EB6 (13,14) also seem to interact with MHC class I molecules. In contrast, recent findings (15) suggest that MHC class I molecules do not play an important role in NK cell cytotoxicity. Transfection of MHC class I alleles that confer resistance to NK cell killing into the NK sensitive target cell K562 does not render them resistant to NK cell lysis, suggesting that molecules other than MHC class I might play an important role in NK cell cytotoxicity. In all probability NK cell cytotoxicity can also occur through MHC independent mechanisms (16-19).

Previously, it was observed that fish nonspecific cytotoxic cells (NCC) recognize and lyse a variety of both human and murine transformed cells (20-24).
We hypothesized that NCC recognize a conserved antigen on the surface of both human and murine transformed cell lines. In an effort to determine the nature of this target cell antigen, mAbs were made against a human NK target cell line (25). A novel mAb (18C2) was isolated and characterized (24). This mAb not only reacts with a novel antigen on human NK target cells, but also inhibits its lysis by NCC (25). In addition, this mAb also recognizes a similar molecule on the surface of a common fish protozoan parasite *Tetrahymena pyriformis* (24), a target normally killed by NCC.

We now demonstrate that the mAb 18C2 also recognizes a similar structure on both NK sensitive and NK resistant murine target cells. Upon incubation of the target cells with the mAb a dramatic reduction in the cytotoxicity of both NK sensitive as well as NK resistant target cells was observed using various effector cell populations. These effects were exerted at the recognition (i.e., conjugate formation) stage as demonstrated by single cell conjugate assays. Further, the role of MHC class I antigens in NK cell cytotoxicity was examined. Target cells that co-expressed low levels of MHC class I antigens in association with the target cell antigen were more sensitive to NK cell lysis than target cells that co-expressed higher levels of MHC class I antigen and the target cell antigen, with MHC class I antigens seeming to play a dominant role. Our findings thus suggest that co-expression of the target cell antigen and low levels of MHC class I antigen expression determine target cell sensitivity to murine NK cell lysis.
METHODS

Mice: C57BL/6 mice were obtained from SASCO (Omaha, Nebraska) and kept in a pathogen free animal facility at our institution. Mice were used in experiments at 6 to 10 wk of age.

Reagents and cell lines: Human recombinant interleukin-2 was the kind gift of Dr. Emmanuel Akporiaye (Department of Microbiology and Immunology, University of Arizona) and Amgen (Thousand Oaks, CA). The mAb B220 was purchased from Becton Dickinson (Mountain Veiw, CA) and was used as suggested by the manufacturer. Fluorescein conjugated goat anti-mouse IgG, IgM was used as a secondary antibody (Olympus Corp., Lake Success, NY). Monoclonal antibodies against different major histocompatibility complex (H-2) haplotypes were obtained from ATCC and grown in our laboratory. Supernatants from these hybridomas were used for staining the target cells.

Derivation of the anti-target cell monoclonal antibody: The anti-target cell mAb was derived as described (24). Briefly, mice were immunized with NC-37 tumor cells and spleen cells fused with the murine P3-X63-AG8.653 myeloma. Wells positive for growth were screened by ELISA and flow cytometry. The mAb 18C2 selected for use in the experiments was of the IgM isotype.

Isolation of effector cells: Spleens were aseptically removed and single cell suspensions were prepared in RPMI 1640 with 10% FBS. The mononuclear cells were obtained after centrifugation through lympholyte-M (Accurate, Westbury, NY) cell separation medium (250 x g, 30 minutes, room temperature). In some
experiments, spleen mononuclear leukocytes were passed over nylon wool columns to remove macrophages and B cells. Briefly, $10^8$ spleen cells in 2 ml of RPMI 1640, 10% FBS, were added to a 20 cc syringe containing 1 gm of sterile nylon wool. The cells were incubated for 45 minutes at 37 C and the nylon wool was gently washed with 50 ml of RPMI, 10% FBS. The nylon wool nonadherent (NWNA) cells were collected, washed, and used as a source of fresh NK cells. This method consistently reduced the percentage of B cells in the spleen preparations to less than 2%. 5d lymphokine activated killer (LAK) cells were obtained by culturing fresh NWNA cells in complete medium supplemented with 250 U/ml rIL-2 at $2 \times 10^6$ cells per ml for a period of at least 5 days. Adherent lymphokine activated killer (ALAK) cells were obtained by culturing fresh NWNA cells in complete medium supplemented with 1000 U/ml rIL-2 at $2 \times 10^6$ cells per ml for a period of 48 hrs at 37 C, after which tissue culture flasks were rinsed three times with prewarmed RPMI 1640 to remove all nonadherent cells. The remaining adherent cells were expanded for another 6 to 8 days with rIL-2 and medium changed as necessary. The adherent cells were removed with Ca$^{2+}$/Mg$^{2+}$ free PBS containing 0.02% EDTA. The cells were washed in complete medium before use in the functional assays.

Flow cytometric analysis: Cell surface phenotypes and mAb binding were analyzed by flow cytometry with the use of a Becton Dickinson FACStar Plus flow cytometer. Controls consisted of fluorescein conjugated goat-anti-mouse IgG, IgM antibody. Viable cells were gated by a combination of forward light scatter and size. A minimum of 10,000 cells were analyzed for each histogram and the data are presented on a log scale.
Cytotoxicity assays: Effector cells were tested for cytolytic activity in a standard microcytotoxicity assay (26). Lytic units at the 20% lysis level (LU20) for 1 X 10^6 effector cells was calculated by computer-assisted regression analysis as described by Pross et al (27). All effector to target (E/T) ratios were performed in triplicate. Spontaneous ⁵¹Cr-release under any of the conditions never exceeded 10%.

In experiments in which the target cells were tested with the mAb, the target cells were placed in the wells of the microtiter plates and incubated with the mAb at 4 C for 1 hr, cells were washed and used in cytotoxicity assays. The percent inhibition was based on comparisons of the LU20.

Conjugate assays: The effects of the mAb on the ability of the various effector cell populations to form conjugates with various tumor cell lines was examined as described previously (28). Briefly, equal numbers of effector cells and target cells (0.5 X 10^6 of each) were mixed in 1 ml of RPMI-10%, the mixture was centrifuged for 2 minutes at 50 x g and incubated for 15 minutes at room temperature. At this time the cells were vigorously pipetted (four to five times) to disrupt spontaneous conjugates, an aliquot was placed on a hemocytometer and conjugates were enumerated. In some cases target cells were incubated with the mAb 18C2 for 30 minutes at 4 C prior to being added to effector cells.
RESULTS

The anti-target cell mAb 18C2 inhibits the lysis of both sensitive and resistant target cell lines by various murine NK effector cell populations. Previously, we have shown that the mAb 18C2 inhibits the cytolytic activity of human NK cells (25) and fish NCC (24). It was of interest to determine what effects this mAb might have on the ability of murine NK cells to lyse target cells. It was found that mAb 18C2 inhibited the lysis of both NK sensitive (Table 1) as well as NK resistant (Table 2) target cells by fresh NK cells, 5d LAK cells and ALAK cells. Using the prototypical NK sensitive target cell YAC-1, up to 83% inhibition in cytotoxicity (a decrease in the cytotoxicity from 30 to 5 LU20) was observed when fresh NK cells were used as a source of effector cells (Table 1). An irrelevant mAb (B220) of the same IgM isotype as the mAb 18C2 which also binds to YAC-1 tumor cells (data not shown) had no effect on the lysis of YAC-1 target cells (Table 1). Inhibition of YAC-1 target cell lysis by fresh NK cells was greater as compared to 5 day LAK cells or ALAK cells (83% vs 68% and 53%, respectively) (Table 1). Pretreatment of the effector cells with the mAb had no effect on lysis (data not shown), implying that the inhibitory effects were located at the target cell level. When the NK resistant target cells P815 and EL-4 were used, similar results were obtained. As shown in Table 2, the mAb 18C2 inhibited the lytic activity of all three effector cell populations against both P815 and EL-4 target cells (68% and 61% inhibition of lysis for fresh NK cells; 45% and 30% inhibition of lysis for 5 day LAK cells; 50% and 39% inhibition of lysis for ALAK cells, respectively). The IL-2 cultured 5 day LAK cells and ALAK cells were more resistant to inhibition by the mAb 18C2 as compared to activated NK cells.
The anti-target cell mAb 18C2 blocks recognition of target cells by NK effector cells. To determine at what stage of the lytic cycle mAb 18C2 exerted its effects, single cell conjugate assays were conducted with each of the effector cell populations. Treatment of the NK sensitive target cell YAC-1 with the mAb 18C2 resulted in up to 70% inhibition of conjugate formation (Table 3). The noninhibitory mAb B220 had no significant effect on conjugate formation (Table 3). The percent inhibition of conjugate formation observed with fresh NK cells, 5d LAK cells and ALAK cells was 70%, 64% and 46%, respectively (Table 3). This data correlated with the results obtained with inhibition of cytotoxicity of YAC-1 target cells using the same effector cell populations. Finally, similar results were obtained when the NK resistant targets P815 and EL-4 were tested for conjugate formation with the various effector cell populations (Table 4). Treatment of P815 and EL-4 target cells with mAb 18C2 resulted in an approximate 60%, 52%, and 43% inhibition in the conjugate formation with fresh NK cells, 5 day LAK cells, and ALAK cells, respectively.

Flow cytometric analysis of mAb 18C2 binding to various NK target cell lines. Previously, we have shown that the anti-target cell mAb 18C2 bound to human NK sensitive as well as NK resistant tumor cell lines (25). Flow cytometry was utilized to examine mAb 18C2 binding to murine NK sensitive and NK resistant tumor cell lines. As shown in Figure 1A, mAb 18C2 stained the NK sensitive target cell line YAC-1. The expression of this target cell antigen/structure was observed to be expressed at higher levels on the NK resistant target cell line P815 (Figure 1C). In comparison EL-4, another NK resistant target cell line, also expressed low levels of the target cell antigen (Figure 1B). The anti-target cell mAb was shown to be specific for an antigen/structure on murine target cell lines as it did not react with
any of the effector cell populations (data not shown). In an effort to determine if murine target cell sensitivity to NK cell lysis was moderated by differential expression of MHC class I antigens on the target cells, the expression of MHC class I antigens on the NK target cells was examined by flow cytometry. As shown in Figure 1 (panels A, B, and C), the NK resistant target cells P815 and EL-4 expressed significantly higher levels of MHC class I molecules as compared to the NK sensitive target cell YAC-1.
DISCUSSION

Previously, we have shown that NCC are capable of lysing a wide variety of transformed cells of both human and mouse origin (20-24). In order to elucidate the mechanism of interaction between NCC and target cells, mAbs were raised against the NCC-sensitive NC-37 target cell (24). A mAb termed 18C2 was isolated and characterized. This mAb not only bound to NC-37 target cells but also recognized a similar structure on a fish protozoan parasite, Tetrahymena pyriformis. Further, the mAb 18C2 was able to inhibit the cytolysis of NC-37 target cells by fish NCC (24). The inhibition of cytotoxicity occurred as a result of lack of recognition (i.e., block in conjugate formation) between fish NCC and NC-37 target cells as shown by single cell conjugate assays (24). Similar results have been published using human NK sensitive and resistant target cell lines (25).

The purpose of the present investigation was to determine if the anti-target cell mAb 18C2 also recognized a similar structure on murine NK sensitive and resistant target cells, and to determine if this mAb was capable of inhibiting the lysis of target cells by various effector cell populations. Our data showed that the mAb 18C2 bound to approximately 40% of YAC-1 target cells, 80% of P815 target cells and 16% of EL-4 target cells (Figure 1). Further, the mAb 18C2 was specific for the target antigen/structure, as it did not bind to any of the effector cell populations. In addition, the mAb 18C2 inhibited the cytotoxicity of both the NK sensitive target cell YAC-1, as well as the NK resistant target cells P815 and EL-4 by each of the effector cell populations. In general, the inhibition in cytotoxicity was much greater against NK sensitive target cells as compared to NK resistant target cells. Up to 83% inhibition in cytotoxicity was achieved using YAC-1 target cells (Table 1), as compared to 68% inhibition in cytotoxicity of P815 target cells (Table 2). Our data
demonstrates that the inhibition in lysis of target cells was greater when fresh NK cells were used as effector cells in comparison to 5 day LAK cells or ALAK cells. It is possible that upon long term culture of NK cells in the presence of rIL-2, that the effector cells are able to recognize and bind to additional target antigens/structures other than the one recognized by mAb 18C2. It is clear from our data that all three of the effector cell populations are capable of recognizing a common target antigen/structure on each of the target cells tested. It is also probable that NK cells recognize more than one antigen structure on target cells, as the mAb 18C2 only partially inhibits the cytotoxicity of NK target cells (39-83% inhibition of killing). Among the various target cells tested, mAb 18C2 mediated inhibition of YAC-1 tumor cells was the highest. It is conceivable that the target/antigen structure recognized by mAb 18C2 on YAC-1 tumor cells plays a dominant role in NK-target cell interaction. In comparison, tumor cells such as P815 and EL-4 may possess additional recognition structures that may play an equally important role in NK-target cell interaction. The inhibition of target cell lysis observed with mAb 18C2 was due to binding of the mAb to a target cell antigen/structure, as mAb preincubation of various effector cells followed by washing prior to the assay did not have an effect on the observed levels of cytotoxicity (data not shown).

In order to determine at what stage of the lytic cycle mAb 18C2 was exerting its effect (i.e., early or later stages of the lytic cycle), single cell conjugate assays were performed. It was observed that the mAb 18C2 inhibited the cytotoxicity of target cells by preventing the formation of conjugates (i.e., recognition) between various effector cell populations and both NK sensitive and resistant target cells. An average of 60% inhibition in conjugate formation was observed between the various effector cell populations and YAC-1 target cells. An average of 48% and 34% inhibition in conjugate formation was observed between the various effector cells
and P815 and EL-4 target cells, respectively. In general, the percent inhibition in conjugate formation was significantly higher for the NK sensitive target cell YAC-1, as compared to NK resistant target cell lines (Table 4). The percent inhibition observed in single cell conjugate assays using the mAb 18C2 correlated with the observed inhibition of cytotoxicity using the same mAb. Our data thus suggests that the cell surface structure recognized by mAb 18C2 is present not only on NK sensitive target cells YAC-1, but also on NK resistant target cells P815 and EL-4, and is probably necessary for the early stages of the effector/target cell interactions.

Interestingly, the expression of the target antigen/structure on the NK resistant P815 target cells was much higher as compared to other target cells (i.e., YAC-1 and EL-4). In spite of a greater expression of the target antigen/structure on P815 cells, these target cells were not killed as well as YAC-1 target cells. It has previously been shown that certain tumor cells that express very high levels of certain class I molecules are resistant to NK cell killing (2-6). In an effort to determine the role of MHC class I antigens in murine NK cell lysis, the expression of the MHC class I molecules in conjunction with the novel target cell antigen was examined. As shown (Figure 1A), NK target cells that expressed low levels of MHC class I antigens in conjunction with the target cell antigen were generally more susceptible to NK cell lysis (Table 1). In comparison, the NK resistant targets P815 and EL-4 (Figure 1B and 1C) expressed significantly higher levels of MHC class I antigens and were more resistant to NK cell lysis (Table 2). These results are similar to recently published data (29) on various human target cells that co-express MHC class I antigens and the target cell molecule. It is possible that high levels of MHC class I antigens on NK resistant target cells in some fashion interacts with the novel target cell antigen and alters its recognition by NK cells. This altered form of recognition would render target cells resistant to NK cell lysis. Target cells that
express low levels of the MHC class I antigens are probably not effective at altering
the recognition between NK cells and target cells, and therefore are more
susceptible to NK cell lysis. Our data suggests that the co-expression of the MHC
class I antigen expression and the target cell antigen determines how susceptible a
target cell is to NK cell lysis, with MHC class I antigen expression playing the
dominant role.

In conclusion, the molecule recognized by the mAb 18C2 is present on
human target cells, on the fish protozoan parasite *Tetrahymena pyriformis*, and on
murine target cells. The presence of this antigen across different species implies that
the molecule has been conserved through evolution. This evolutionary conservation
may imply that there have been specific selective pressures throughout evolution to
maintain this molecule. We postulate that in order to observe detectable levels of
cytotoxicity of transformed cells or virally infected cells, target cells should express a
certain threshold number of these molecules as well as low levels of expression of
MHC class I molecules in order to be recognized and lysed by NK cells.

ACKNOWLEDGMENTS

We thank Miss Barb Carolus for assisting us with flow cytometry. This work was
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REFERENCES


FIGURE LEGEND

FIGURE 1. Flow cytometry profiles of murine NK target cells labeled with mAbs versus the novel target cell antigen and H-2 class I molecules. Fluorescein-conjugated goat-anti-mouse-IgG, IgM was used as a control antibody. (A) YAC-1 target cells stained with mAb 18C2 and anti-H-2K$^k$D$^d$ mAb. (B) EL-4 target cells stained with mAb 18C2 and anti-H-2K$^b$D$^b$ mAb. (C) P815 target cells stained with mAb 18C2 and anti-H-2K$^d$D$^d$ mAb.
TABLE 1
The Anti-Target Cell Mab 18C2 Inhibits the Lysis of Yac-1 Target Cells by Various Murine NK Effector Cell Populations

<table>
<thead>
<tr>
<th>Effector cells</th>
<th>Treatment</th>
<th>LU$_{20}/10^6$</th>
<th>Percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh NK cells</td>
<td>Media</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>18C2</td>
<td>5</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>B220</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>5d LAK cells</td>
<td>Media</td>
<td>76</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>18C2</td>
<td>24</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>B220</td>
<td>79</td>
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<tr>
<td>ALAK cells</td>
<td>Media</td>
<td>83</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>18C2</td>
<td>39</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>B220</td>
<td>87</td>
<td>0</td>
</tr>
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</table>

Fresh NK cells, 5d LAK cells and ALAK cells were derived as described and tested for lytic activity in the presence or absence of the indicated mAbs. YAC-1 cells were used as targets. 10 ug of mAb 18C2 and 10 ug of mAb B220 were used in each experiment. Each experiment was performed in triplicate.
**TABLE 2**

The Anti-Target Cell Mab 18C2 Inhibits the Cytotoxicity of NK Resistant Target Cells by Various Murine NK Effector Cell Populations

<table>
<thead>
<tr>
<th>Effector Cells</th>
<th>Treatment</th>
<th>LU$_{20}/10^6$</th>
<th>%Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P815</td>
<td>EL-4</td>
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<tr>
<td>Fresh NK cells</td>
<td>Media</td>
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<td>18</td>
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<tr>
<td>NK cells</td>
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</tr>
<tr>
<td></td>
<td>B220</td>
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<td>5d LAK cells</td>
<td>Media</td>
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<td></td>
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<tr>
<td></td>
<td>B220</td>
<td>53</td>
<td>49</td>
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</table>

Fresh NK cells, 5 day LAK cells and ALAK cells were derived as described and tested for lytic activity in the presence or absence of the indicated mAbs. P815 and EL-4 tumor cells were used as targets. 10 ugs of mAb 18C2 and 10 ug of mAb B220 were used in each experiment. Each experiment was performed in triplicate.
TABLE 3
Effect of Mab 18C2 Treatment on Murine NK Effector Cell Conjugate Formation with Yac-1 Target Cells

<table>
<thead>
<tr>
<th>Population</th>
<th>% Conjugates</th>
<th>Percent Inhibition by mAb 18C2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>18C2</td>
</tr>
<tr>
<td>Fresh NK cells</td>
<td>60</td>
<td>18</td>
</tr>
<tr>
<td>5d LAK cells</td>
<td>58</td>
<td>21</td>
</tr>
<tr>
<td>ALAK cells</td>
<td>54</td>
<td>29</td>
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</table>

Effector cell populations were derived as described. Conjugate assays were performed using YAC-1 target cells. 10 ug of mAb 18C2 and 10 ug of mAb B220 were used in each experiment. Each experiment was repeated three times.
TABLE 4

The Effect of Mab 18C2 Treatment on Murine NK Effector Cell Conjugate Formation with P815 and EL-4 Target Cells

<table>
<thead>
<tr>
<th>Population</th>
<th>P815</th>
<th>EL-4</th>
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<tbody>
<tr>
<td></td>
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<td>Fresh NK cells</td>
<td>79</td>
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<tr>
<td>5 day LAK cells</td>
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<td>30</td>
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<tr>
<td>ALAK cells</td>
<td>72</td>
<td>40</td>
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Effector cell populations were derived as described. Conjugate assays were performed using P815 and EL-4 target cells. 10 ug of mAb 18C2 was used in each of this experiment. Each experiment was repeated three times.
Figure 1.
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VERIFICATION OF REVIEW
BY THE INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

PHS Assurance No. A-3248-01 - USDA No. 86-3
IACUC Control # 93-037

Title: GVL and GVHD Activities of Cord Blood

Principal Investigator: David T. Harris, PhD

Department: Microbiology

Submission Date: February 15, 1993

Agency: American Cancer Society

The University of Arizona Institutional Animal Care and Use Committee reviews all sections of proposals relating to animal care and use. The above-named proposal:

[ ] Has been reviewed and approval withheld.

[XX] Has been reviewed and approved by IACUC on April 22, 1993

Revisions (if any), are listed below:
NONE

Approval valid through April 21, 1996

Michael A. Cusanovich, Ph.D.
Vice President for Research

Date: April 23, 1993
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