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Inhibition of Natural Killer Cell Cytotoxicity by Cell Growth-related Molecules

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Certain MHC class I molecules on target cells are known to inhibit the cytotoxic action of NK cells. By using monoclonal antibody (mAb) Cho-1, we have found inhibitory non-MHC class I cell surface molecules that are noncovalently-associated with 200 kDa and 40 kDa antigens. Poly I-C-induced rat NK cells were not cytotoxic to rat fetus-derived fibroblast WFB cell line. In contrast, NK cells were cytotoxic to H-*ras* oncogene-induced transformants of WFB, W14 and W31. FACS analysis indicated that mAb Cho-1 reacts with WFB, but not with W14 and W31 cells. Thus, this antigen may disappear concomitantly with cell growth and transformation. Cho-1 antigens were also expressed on other NK-resistant lines, such as mouse BALB3T3 fibroblast, EL-4 lymphoma and human fibroblast HEPM. However, they were not expressed on NK-sensitive mouse YAC-1 and H-*ras* transformant (Brash) of BALB3T3 cells. Furthermore, treatment of target cells with IFN-γ clearly induced the cell surface expression of Cho-1 antigens, and conferred a resistance to NK cytolysis on target cells. These data strongly suggest that Cho-I antigen expression may correlate with target cell susceptibility to NK cells. Indeed, treatment of NK-resistant WFB as well as HEPM cells with F(ab')$_2$ fragments of mAb Cho-1 resulted in the acquisition of susceptibility to NK cytolysis. Cho-1 antigens may be novel molecules that regulate the NK resistance of cells.

Key words: NK cell — Inhibitory molecule — MHC class I

NK cells are a distinct subpopulation of lymphoid cells, characterized phenotypically by the cell surface TCR/CD3 receptor complex$^-$, CD16$^+$, CD56$^+$, and Ig$^-$. These cells are lytic to certain neoplastic cells, virally infected cells and bone marrow cells.[1–4] Recent reports have proposed several candidates as NK receptors, which may recognize NK target structures (NKTS) and trigger the signal transduction necessary for NK cytolysis.[5–9] These receptors were considered to be "positive" NK receptors, and were shown to have a structure composed of C-type lectin-like type II transmembrane domain. Previously we demonstrated the existence of a cell-surface NKTS glycoprotein of 86 kDa, whose expression was associated with cell growth and transformation.[1, 10, 11] This molecule might be critical to induce NK cytolysis, and it is of interest to know whether this putative NKTS can bind to positive NK receptors.

Recent studies also clarified that the cytolysis by NK cells may be negatively (protectively) regulated by an important mechanism.[12–14] Loss or reduced expression of MHC class I products may result in the acquisition of susceptibility to NK cells.[15, 16] Conversely, several lines of evidence have indicated that the expression of certain MHC products could inhibit NK function at the target cell level.[17, 18] Furthermore, although the cytotoxicity of NK cells was believed to be non-MHC-restricted, it is suggested that cellular endogenous peptides, which affect the conformational structure of the MHC class I groove, may play a pivotal role in the regulation of NK function.[19, 20] These facts raise the possibility that NK cells may recognize the expression of certain MHC class I products, whereby the cytotoxic function of NK cells is suppressed. Indeed, Ly49 molecule is a receptor for H-2D^d molecules, and is negatively regulated in NK cytolysis.[21, 22]

Thus, it is highly likely that the expression of MHC class I molecules determines, at least to a certain extent, the susceptibility of target cells to NK cytolysis. However, there is also a possibility that regulatory molecules other than MHC class I products are involved in the mechanism of NK cytolysis. In this report, we describe such non-MHC class I molecules, defined by the monoclonal antibody (mAb) Cho-1. This mAb detects noncovalently-associated antigens of 200 kDa and 40 kDa, which are expressed on the cell surface on NK-resistant cells such as fibroblast rat WFB and human HEPM lines. F(ab')$_2$ fragments of mAb Cho-1 as well as anti-MHC class I mAb R48B1 could confer susceptibility to NK cytolysis on these otherwise NK-resistant lines. Our data suggested that the cytolysis by NK cells is negatively regulated not only by the expression of MHC class I products, but also by these additional molecules.

MATERIALS AND METHODS

Animals, cells, procedures for mAb development, FACS analysis, and cytotoxicity assay using NK cells These

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materials and methods have been described in our previous papers.[1,10,23,24] Briefly, W14 and W31 cells are EJ-*ras* oncogene-transformants of the parental WKA rat fetus fibroblast line, WFB. They are independent clones as determined by Southern blot analysis using an EJ-*ras* oncogene probe.[10,24,25] Brash is the EJ-*ras* oncogene-transformant of BALB3T3.[26] The anchorage-independent growth *in vitro* and tumorigenic potential *in vivo*, as well as the determination of oncogene insertion on Southern blot analysis, were also described in our previous papers.[10] NK-sensitive mouse YAC-1, human K562 and NK-resistant mouse EL-4 were also described previously.[1,23] NK-resistant HEPM is a human embryonal palatal fibroblast line, and this was kindly provided by the Japanese Cancer Research Resources Bank. mAbs Cho-1 and Cho-2 were obtained by hybridization of NS-1 myeloma cells[1] and mouse spleen lymphocytes immunized with WFB cells. Poly I-C (Sigma Chemical Co., St. Louis, MO)-induced rat splenic NK cells and human peripheral blood NK cells were obtained according to our previous methods.[1,10,27] It was clearly demonstrated that the majority of poly I-C-induced NK cells were 3.2.3 antigen (NKR-P1)-positive, since the depletion of 3.2.3 antigen-positive cells resulted in the loss of NK activity.[1] The cytotoxicity assays using ^{51}Cr-labeled target cells were usually performed at different effector/target ratios for 6–10 h after mixing effector and target cells.

F(ab′)$_2$ fragment preparation of mAbs and effect of mAbs on susceptibility to NK cells Mouse IgG F(ab′)$_2$ fragments were obtained by pepsin digestion as described previously.[9,28] In this study, we obtained F(ab′)$_2$ fragments of mAbs 109 (IgG1) and R48B1 (IgG1) which recognize NKTS[1,10] and rat MHC class I nonpolymorphic determinant,[29] respectively. We also prepared F(ab′)$_2$ fragments of mAbs Cho-1 (IgM), Cho-2 (IgM) and A15 (IgM). mAb A15 detects 70 kDa heat shock proteins. The pepsin digestion of these mAbs with IgM isotype was done by the method described elsewhere.[30] The fractions containing F(ab′)$_2$ fragments were obtained by Sephadex G-150 gel filtration and checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). These F(ab′)$_2$ preparations reacted similarly to intact mAbs with the cell surface antigens as assessed by FACS, and were used in the cytotoxicity assays of poly I-C-induced NK cells against WFB to assess the involvement of mAb-defined molecules. In this study, target cells were pretreated with these preparations at 4°C for 60 min.

Cell surface radioiodinization, radioimmunoprecipitation, Western blot analysis, and SDS-PAGE The procedures for immunoprecipitation and cell surface iodinization used in this study have been described elsewhere.[10,24] We also used Western blot analysis as described previously.[1,27]

IFN-γ treatment of cells In some experiments, we treated cells with recombinant rat IFN-γ (ICN Biochemicals, Cleveland, OH). WFB, W14 and W31 cells were cultured in the presence of 10 units of this cytokine at 37°C for 24 h, then washed with phosphate-bufferd saline (PBS), and used in the cytotoxicity and FACS analyses.

RESULTS

Susceptibility of cells to rat NK cells We determined the susceptibility of target cells to cytotoxicity by poly I-C-induced rat splenic NK cells. In our previous study,[1] these NKR-P1-positive,[6] indicating that these effector cells were indeed rat NK cells. As shown in Fig. 1, these effector cells lyzed NK-sensitive YAC-1, but not NK-resistant EL-4 cells. Fig. 1 also shows that nontransformed rat fetal fibroblast line WFB was not lyzed, whereas H-*ras* oncogene-transformed lines, W14 and W31, of parental WFB were destroyed by NK cells. BALB3T3 showed only minimal susceptibility, but its H-*ras*-transformed Brash was clearly susceptible to NK cells.

Cell surface expression of Cho-1 antigens To detect cell surface molecules that are expressed on NK-resistant WFB, but not on NK-sensitive W14 and W31 cells, mice were immunized with WFB cells and mAbs Cho-1 and Cho-2 were developed. As shown in Fig. 2, FACS analysis indicated that these mAbs reacted with WFB. However, the antigens defined by these mAbs were not expressed on NK-sensitive W14 and W31 transformants, indicating that the expressions of these antigens are

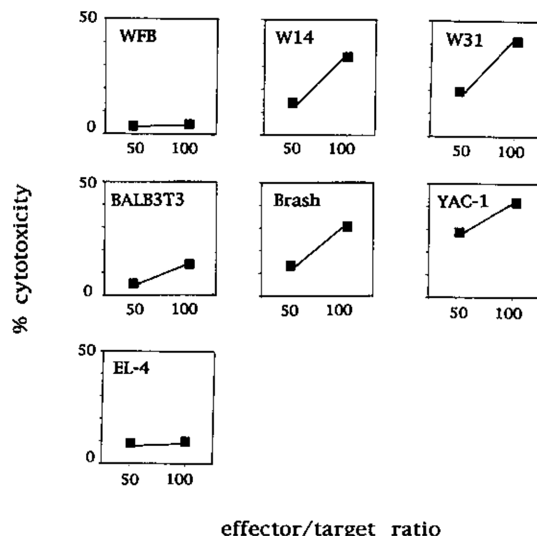

Fig. 1. Susceptibility of cells to rat NK cells. ^{51}Cr-labeled target cells were mixed with poly I-C-induced rat splenic NK cells at effector/target cell ratios of 50 and 100.

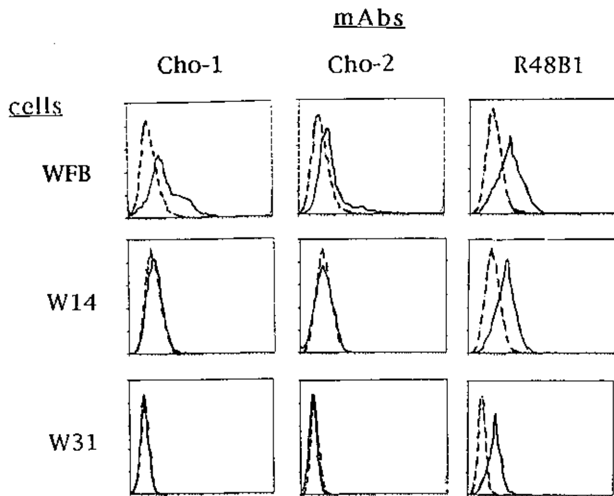

Fig. 2. FACS profiles of the cell surface expression of Cho-1 antigens. Parental WFB and its transformants, W14 and W31 cells, were treated with (——) or without (---) a saturating amount of mAbs Cho-1, Cho-2 or R48B1 at 4°C for 60 min, and were analyzed with FACS.

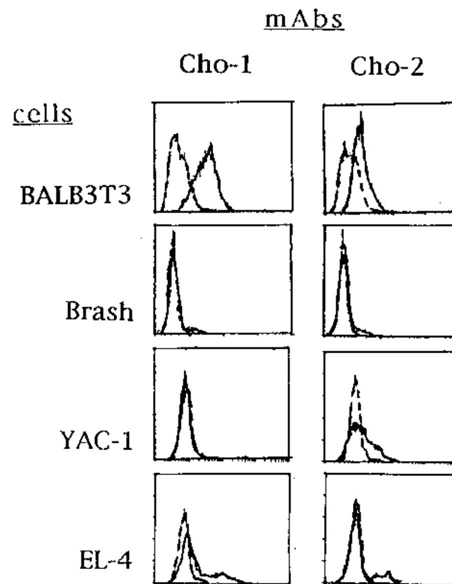

Fig. 3. FACS profiles of the cell surface expression of Cho-1 antigens in mouse cell lines. The cells were treated with (——) or without (---) a saturating amount of mAbs Cho-1 and Cho-2 at 4°C for 60 min, and were analyzed with FACS.

reduced concomitantly with cell growth and transformation. Obviously these antigens were not MHC class I molecules, since the FACS pattern of Cho-1 and Cho-2 mAbs was clearly different from that of R48B1, which detects rat MHC class I antigens.

Since there is a possibility that Cho-1 and Cho-2 antigen expressions may parallel NK-resistancy, we checked several other NK-resistant and NK-sensitive lines. As shown in Fig. 3, Cho-1 antigens were strongly expressed on NK-resistant BALB3T3, but only minimally on EL-4 cells. The expression of Cho-1 antigens was not detected on NK-sensitive Brash and YAC-1 cells. In contrast, Cho-2 antigens were clearly expressed on BALB3T3 and YAC-1 cells, suggesting no clear relationship between Cho-2 expression and NK-resistancy. Among human cells, NK-sensitive K562 did not express Cho-1 antigens, but NK-resistant HEPM expressed Cho-1 antigens (data not shown).

Involvement of Cho-1 antigens in susceptibility to NK cells Since the above data imply that Cho-1 antigen expression may be related to susceptibility to the cytotoxicity of NK cells, we examined whether Cho-1 antigens are indeed involved. To this end, we isolated $F(ab')_2$ fragments of mAbs Cho-1 and Cho-2, R48B1 and #109, and ^{51}Cr-labeled WFB target cells were treated with saturating amounts of these fragments. Then, poly I-C-induced rat splenic NK cells were added to the mixtures. As shown in Fig. 4, $F(ab')_2$ fragments of Cho-1 as well as R48B1 mAbs could confer NK susceptibility upon otherwise NK-resistant WFB cells. WFB cells treated with these preparations acquired NK susceptibility similar to that of NK-sensitive W31 cells. In contrast, $F(ab')_2$ fragments of Cho-2 and #109 mAbs did not influence the susceptibility.

These observations were confirmed by a cold target inhibition experiment. Fig. 5 indicates that the cytotoxicity of NK cells towards W31 was inhibited by the addition of WFB cold target cells treated with $F(ab')_2$ fragments of mAb Cho-1, although this inhibitory potential was not as strong as that of cold W31 cells. Cold WFB cells treated with $F(ab')_2$ fragments of anti-MHC class I R48B1 mAb also inhibited the cytotoxicity towards W31 (data not shown). In contrast, WFB treated with $F(ab')_2$ fragments of mAb Cho-2 or without mAb did not affect the cytotoxicity. Furthermore, we could rule out the possibility that mAb Cho-1 up-regulates the cytotoxicity by binding to NK cells, since NK cells treated with $F(ab')_2$ fragments of this antibody did not show enhanced cytotoxicity towards WFB or W31 (data not shown). These data indicate that not only MHC class I, but also Cho-1 antigens act as protective molecules against NK cytotoxicity.

We also assessed the involvement of Cho-1 antigens in human NK cytotoxicity. A human embryonal palatal fibroblast line, HEPM, expresses Cho-1 antigens. As shown in Table I, this line is relatively resistant to human resting peripheral blood NK cells. However, HEPM cells became NK-sensitive when the cells were treated with a

saturating amount of $F(ab')_2$ fragments of mAb Cho-1, but not with mAbs Cho-2 and A15. These facts suggest that Cho-1 antigens act as protective molecules in human cells as well.

Effect of IFN-γ on Cho-1 antigen expression, and comparison of relative NK protective potential between Cho-1 and MHC class I molecules The expression of certain MHC class I molecules is known to be inhibitory for NK cytotoxicity, and this seems to be closely linked with the effect of IFN-γ. Therefore, it is of interest to determine whether Cho-1 antigen expression and NK susceptibility are influenced by IFN-γ treatment. As shown in Fig. 6A, Cho-1 antigen expression on WFB cells was obviously enhanced by IFN-γ treatment. The enhancing potential of IFN-γ on Cho-1 antigen expression was similar to that on MHC class I antigen expression. We then studied the relative potential of protective action of Cho-1 and MHC class I antigens. WFB cells cultured in the presence or absence of IFN-γ were treated with a saturating amount of $F(ab')_2$ fragments of mAbs Cho-1 and R48B1, and

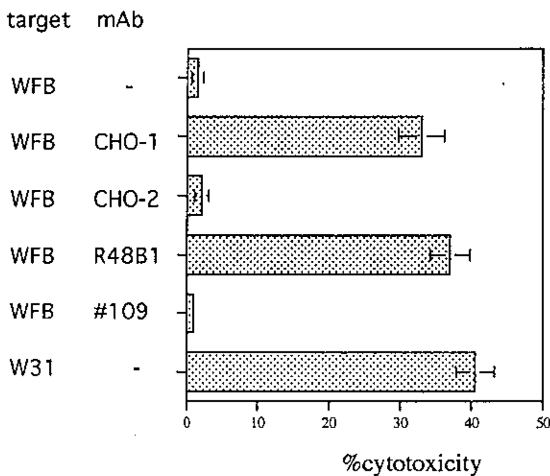

Fig. 4. Involvement of Cho-1 antigens in the cytotoxic action of NK cells. ^{51}Cr-labeled WFB cells were treated with a saturating amount of $F(ab')_2$ fragments of mAbs Cho-1, Cho-2, R48B1 and #109 at 4°C for 60 min, washed with PBS, and mixed with poly I-C-induced rat splenic NK cells at an effector/target cell ratio of 100. The cytotoxic potential of these NK cells against W31 cells was also determined as a positive control. Bars represent mean ± SE.

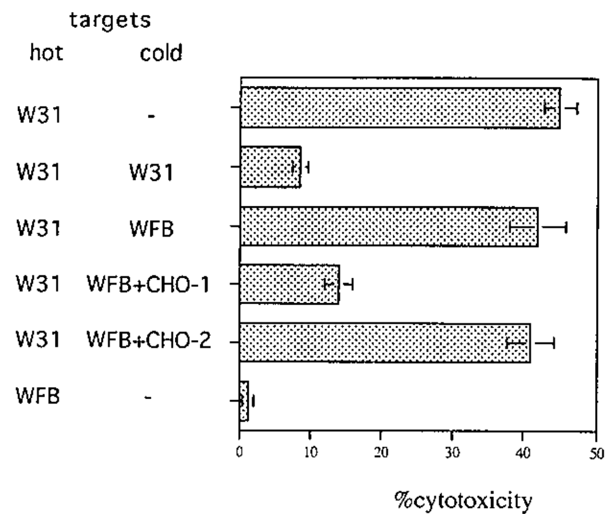

Fig. 5. Cold target inhibition assays for Cho-1 antigen involvement in NK cytotoxicity. ^{51}Cr-labeled (hot) W31 cells were mixed with ^{51}Cr-non-labeled (cold) WFB cells that were pretreated with or without a saturating amount of $F(ab')_2$ fragments of mAbs Cho-1 and Cho-2. Hot and cold target cells were mixed at equal numbers of cells. Cold W31 cells were also used in the experiment. Then, poly I-C-induced rat splenic NK cells were added to the cytotoxicity assays at an effector/hot target cell ratio of 100. Hot W31 and WFB alone were also used as positive and negative controls, respectively, in the experiment. Bars represent mean ± SE.

Table I. Involvement of Cho-1 Antigens in the Cytotoxic Action of Human NK Cells[a]

$F(ab')_2$ mAb treatment	% cytotoxicity against HEPM		K562	
	E/T 25	50	25	50
—	6.0±2.5	7.2±1.8	31.4±5.5	38.5±6.0
Cho-1	19.8±3.6[b]	26.0±5.6[b]	ND[c]	42.2±2.2
Cho-2	ND	8.0±2.5	ND	ND
A15	ND	8.8±2.1	ND	ND

a) ^{51}Cr-labeled HEPM and K562 cells were treated for 60 min at 4°C with or without a saturating amount of $F(ab')_2$ fragments of mAbs Cho-1, Cho-2 and A15, washed with PBS, and used in the cytotoxicity assays with human NK cells at 25 and 50 E/T ratios.

b) $P<0.05$.

c) Not determined.

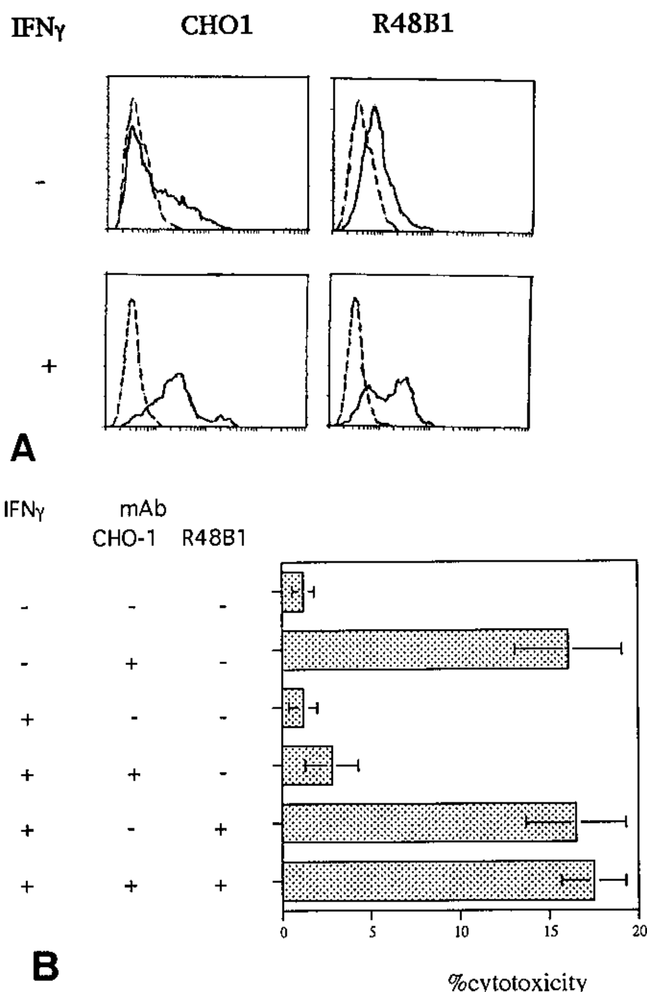

Fig. 6. Effect of IFN-γ on Cho-1 antigen expression, and comparison of their NK protective potential with that of MHC class I molecules. (A) FACS profiles of IFN-γ effect on Cho-1 antigen expression. WFB cells were cultured in the absence (−) or presence (+) of 10 units of rat IFN-γ at 37°C for 24 h, and were assessed with FACS for the antigen expression (——) defined by mAb Cho-1 and anti-rat MHC class I mAb R48B1. (---), FITC-conjugated anti-mouse Ig alone. (B) NK protective potential of Cho-1 and MHC class I antigens. WFB cells as employed in (A) were labeled with ^{51}Cr, and treated with $F(ab')_2$ fragments of mAbs Cho-1 and/or R48B1. Then, poly I-C-induced rat splenic NK cells were added at an effector/target cell ratio of 100 for cytotoxicity assay. Bars represent mean ± SE.

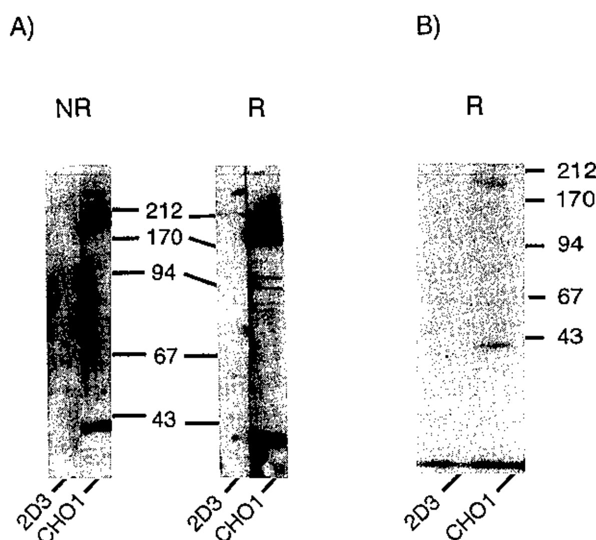

Fig. 7. Immunochemical characteristics of Cho-1 antigens. (A) Western blot analysis of WFB cell lysates with mAb Cho-1 and a control mAb 2D3 (IgM) under nonreducing (NR) and reducing (R) conditions (with 5.0% 2-ME) on 8.0% SDS-PAGE. (B) SDS-PAGE analysis of immunoprecipitates with mAbs Cho-1 or 2D3 and ^{125}I-labeled-WFB cell surface lysates. Immune complexes were subjected to 8.0% SDS-PAGE under reducing conditions.

poly I-C-induced rat NK cells were added. Fig. 6B indicated that once WFB cells were treated with IFN-γ, mAb Cho-1 could not confer NK susceptibility upon them. However, anti-MHC class I R48B1 still could confer NK susceptibility upon otherwise NK-resistant cells. The inductive potential of NK susceptibility with a mixture of $F(ab')_2$ fragments of mAbs Cho-1 and R48B1 was similar to that of R48B1 alone. Collectively, these data suggest that MHC class I molecules are the dominant protective molecule against NK cytotoxicity, and that Cho-1 antigens may play a subsidiary role.

Immunochemical characteristics of Cho-1 antigens In Western blot analysis using WFB cell lysates, mAb Cho-1 detected molecules of approximately 200, 190 and 40 kDa under nonreducing as well as reducing conditions (5% 2-ME) in SDS-PAGE (Fig. 7A). Furthermore, as shown in Fig. 7B, mAb Cho-1 formed immunoprecipitates with WFB cell surface antigens of approximately 200 kDa and 40 kDa under reducing conditions. This was also the case under nonreducing conditions (data not shown).

DISCUSSION

It is known that NK cytotoxicity is inhibited by MHC class I molecule expression and their receptors.[12–14] Several studies have shown that the expression of certain MHC class I haplotype elements reduces susceptibility to NK cells,[15, 16] while expression of the reduced β_2m molecule increases the NK susceptibility.[18] Ly49 is supposed to be a receptor for H-2D^d molecules, and to inhibit the NK cytotoxicity.[21, 22] It is also indicated that the nature

of peptide fragments which bind to MHC class I, and the conformation of the MHC class I molecule may be important for the inhibition of NK cytotoxicity.[19, 20] The substitution of particular amino acid residues can drastically affect the susceptibility to NK cytotoxicity. For example, the alteration of His74 to Asp74 converted HLA-A2 from a susceptible to a resistant class I allele in C1R tumor cells.[31] In addition to these allogeneic systems, it was also demonstrated that certain self MHC class I alleles could have potential effects on susceptibility to NK cytotoxicity.[32–34] However, autologous PHA-stimulated lymphoblasts are usually not lyzed by NK cells. Thus, although it is clear that certain MHC class I molecules are potent negative regulators of NK cytotoxicity, the molecular basis of NK cytotoxicity still remains unclear, and other inhibitory molecules, may be expressed on the target cell surface. In this study, we have attempted to detect such molecules.

In our previous study,[1, 10] WKA rat fetus-derived WFB line showed a resistance to syngeneic NK cells, whereas its H-*ras* oncogene-transformants, W14 and W31, showed high NK susceptibility. In the present study, we found that there is no drastic difference in the expression of MHC class I among these lines. This supports the idea that WFB may express NK regulatory molecules other than MHC class I on the cell surface. We obtained mAbs which react with cell surface molecules expressed on WFB, but not on W14 and W31. These mAb Cho-1-defined molecules may negatively regulate NK cytotoxicity, since $F(ab')_2$ fragments of Cho-1 mAb could confer NK susceptibility on WFB. This was also true for NK-resistant human fibroblast line HEPM. Furthermore, YAC-1, a highly NK-susceptible mouse cell line, lacked the Cho-1 antigen, but NK-resistant EL-4 expressed this antigen on the cell surface.

Several features of Cho-1 antigen, including its immunochemical characteristics, indicated that the Cho-1 molecule is different from mAb R48B1-defined MHC class I molecules. However, these molecules have similar immunobiologic characteristics. Namely, the expression of Cho-1 and MHC class I molecules, like that of many multiple other molecules, was up-regulated by IFN-γ treatment. The expression of both Cho-1 and MHC class I molecules could confer NK-resistancy on the cells. Interestingly, once WFB cells were treated with IFN-γ, mAb Cho-1 could no longer confer NK susceptibility on WFB cells. However, anti-MHC class I mAb R48B1 could still induce susceptibility. Therefore, we speculate that the NK-regulatory potential of Cho-1 molecules is weaker than that of MHC class I. Perhaps Cho-1 acts as a subsidiary NK regulatory element.

Immunohistochemical studies of various rat tissues by using Cho-1 mAb indicated that Cho-1 antigens are mainly distributed on the cell surface and in the extracellular matrix. It has been suggested that certain extracellular matrix proteins inhibit T cell activation.[35] However, mAb Cho-1 did not react with extracellular matrix proteins such as tenascin, fibronectin, laminin and vitronectin in ELISA assay (data not shown).

The fact that Cho-1 expression is down-regulated upon neoplastic cell transformation is reasonable in view of the anti-tumor surveillance role of NK cells. The reduced expression of Cho-1 antigens that accompanies transformation of WFB, as well as BALB3T3 cells, may be related to the enhanced susceptibility to NK cytotoxicity. Namely, Cho-1 molecules may act as a negative (protective) regulator of NK cytotoxicity. In contrast, our previous studies[1, 10] indicated that cell transformation also resulted in the enhanced expression of NKTS, an 86 kDa glycoprotein which acts as a positive (susceptible) NK target molecule. In fact, W14 and W31 transformants of WFB, and Brash transformant of BALB3T3 cells strongly expressed this glycoprotein on the cell surface, whereas parental nontransformed WFB and BALB3T3 cells expressed only a minimal level. The expression of this 86 kDa molecule appeared to be critical in the lethal attack by NK cells.[1, 10, 23] Taken together, these facts imply that transformants, but not normal counterparts, become susceptible to NK cells due to the loss of Cho-1 and the enhanced expression of NKTS. We speculate that the overall susceptibility to NK cells of each target cell may be determined by the relative balance of expression between MHC class I, Cho-1 and 86 kDa molecules.

Although mAb Cho-1 could detect cell surface 200 kDa and 40 kDa molecules, it is currently unknown which molecule is important for the negative-regulatory function of Cho-1 molecules. We are attempting to isolate each of these molecules, and to assess their functional significance. We also do not know how the 190 kDa molecule, which is detected in Western blot analysis, is related to the 200 kDa and 40 kDa antigens. Moreover, it would be interesting to identify the NK receptor which recognizes Cho-1 molecules. Since the molecular nature of Cho-1 seems to be different from that of MHC class I, we speculate that Ly49 homologues are not involved in this recognition. Further analyses may provide us with better information on the exact role of Cho-1 molecules in NK cytotoxicity.

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