Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 2361–2364, April 1989
Immunology

Reversal of natural killing susceptibility in target cells expressing transfected class I HLA genes

(natural killing/target structure/class I antigens)

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Communicated by Bernard Amos, January 3, 1989

ABSTRACT **A number of studies have suggested that resistance of target cells to natural killing (NK) may be correlated with their level of expression of major histocompatibility complex (MHC) class I antigens. To examine this hypothesis directly, a NK-sensitive class I-deficient human B-cell line was transfected with MHC class I genes. The expression of transfected HLA, but not H-2, class I gene products resulted in loss of susceptibility to human NK-mediated conjugation and cytolysis. Furthermore, this protection did not extend to cytotoxicity mediated by interleukin 2-stimulated human NK effector cells.**

Natural killer (NK) cells are defined operationally by their potent endogenous and major histocompatibility complex (MHC)-unrestricted cytolytic activity against selected target cells (1). As yet, the recognition elements involved in NK-mediated cytolysis remain poorly defined. At the target cell level, elucidation of the NK ligand(s) has been complicated by recently discovered cell adhesion molecules such as the LFA-1 family of ligands (I-CAM and others; ref. 2) and LFA-3 (2). Monoclonal antibodies directed against these molecules are capable of partially inhibiting NK-mediated conjugation and cytolysis (2). These accessory molecules do not appear to represent NK ligands themselves but rather appear to strengthen effector–target cell conjugates after the initial cognate interaction (2).

Further difficulty in determining the NK ligand(s) may reside in the potential masking effects of target cell MHC class I antigens. While target cell MHC class I antigens may not act as restricting elements for NK cells (in contrast to cytotoxic T lymphocytes; CTLs), recent reports suggest an inverse correlation between the level of class I antigen expression by target cells and their sensitivity to NK cytotoxicity *in vitro* (3–6) and *in vivo* (6, 7). This is consistent with a putative role for NK cells *in vivo*; i.e., to monitor and regulate low class I-expressing tumor cells and metastases (1, 7, 8) that are refractory to CTL lysis (6, 7).

To examine this phenomenon in a controlled and quantitative fashion, we have transfected human and murine MHC class I genes into an HLA-A,-B null B-cell line that is sensitive to human NK-mediated conjugation and cytolysis. We report that target cell surface expression of the exogenous HLA, but not H-2, class I antigens is sufficient to impart resistance to human NK and that this resistance varies quantitatively with the level of HLA class I antigens expressed by the target cell.

MATERIAL AND METHODS

Cells and Media. All cell lines were cultured in Iscove's modified Dulbecco's medium (IDMEM) supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine (all reagents from GIBCO) at 37°C in 5% CO_2/95% air. The B-lymphoblastoid cell line (B-LCL) C1R was derived from the HMy.2 B-LCL (9) by γ-irradiation and selected for class I loss by antibody and complement as described (10). C1R expresses no HLA-A or -B locus gene products. The CEM.NK^R T-LCL is an immunoselected NK-resistant variant of the CEM T-LCL (11).

Effector cells were purified from the peripheral blood of healthy human donors as described (3). NK preparations were >60% Leu 19-positive, >85% OKM1-positive, >60% Leu 11b-positive, <1% MO2-positive, <5% OKT3-positive, and <15% B1-positive. In some experiments, these purified NK preparations were activated with recombinant interleukin 2 (rIL-2) (5 units/ml; AMGEN, Thousand Oaks, CA) for 24 hr, at 37°C, in 5% CO_2/95% air. CTLs (anti-Bw58) were generated in one-way mixed lymphocyte cultures in which the responder and stimulator cells differed in Bw58 expression. These CTLs lysed the HLA-Bw58 transfectants specifically and were only weakly reactive (i.e., background LAK activity) with C1R, irrelevant class I transfectants, and K562 (data not shown).

DNA. Class I genomic clones included HLA-A3 (p1-1.1-3, the gift of E. P. Cowan, National Institutes of Health), HLA-Bw58 (pBw58, the gift of J. Ways and P. Parham, Stanford University Medical Center; ref. 12), HLA-B7 (pJY150, the gift of V. Engelhard, University of Virginia Medical School; ref. 13), H-2D^p (pRM15, the gift of J. A. Frelinger, University of North Carolina; ref. 14), and H-2K^b (C-15-1, the gift of B. Wallace, City of Hope Medical Center, Duarte, CA; ref. 15). The ouabain-resistance gene pSV2α1 (rat α1 ouabain-resistance gene cDNA in pSV2neo; ref. 16) was the gift of R. Levenson (Yale University, School of Medicine).

Transfection. Transfection was performed by electroporation using a Bio-Rad gene pulser and capacitance extender (Bio-Rad). C1R cells (10^7 cells per 500 μl of serum-free IDMEM) were mixed with 20 μg of *Eco*RI linearized class I and 2 μg of *Pvu* I linearized pSV2α1 DNA. Two transfection conditions were used: (*i*) 250 V, 500 μF; and (*ii*) 210 V, 960 μF. These conditions resulted in 50–90% cell death. After transfection, cells were incubated at 4°C for 10 min followed by transfer into 10 ml of IDMEM containing 10% fetal bovine serum and incubation for 24 hr at 37°C in 5% CO_2/95% air. Cells were transferred into culture medium containing 2 μM ouabain (Sigma) and plated into 96-well flat-bottomed culture plates at 10^4 cells per well. These wells also contained a confluent monolayer of γ-irradiated feeder cells (NIH 3T3.PMV.Bgl.neo.PAP, the gift of E. Linney, Duke Univer-

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Abbreviations: NK, natural killer (killing); MHC, major histocompatibility complex; CTL, cytotoxic T lymphocyte; LCL, lymphoblastoid cell line; rIL-2, recombinant interleukin 2; MFC, mean fluorescence channel.

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sity Medical Center). Proliferating transfectants were isolated 3–5 weeks after drug selection and were maintained in ouabain-containing medium as bulk cultures.

Immunofluorescence. C1R and the ouabain-resistant transfectants were examined for class I expression by indirect immunofluorescence (3) using the W6/32 monomorphic monoclonal antibody. W6/32 reacts with both human and murine class I molecules when they are associated with human β_2-microglobulin (17). Monoclonal antibodies directed against polymorphic determinants of class I antigens included GAP.A3 (anti-HLA-A3; ref. 18), BB7.1 (anti-HLA-B7; ref. 19), 7-16-10 (anti-H-2D^p; ref. 20), 28-8-6S (anti-H-2K^b; ref. 21). The 7-16-10 and 28-8-6S antibodies were kindly provided by J. A. Frelinger (University of North Carolina). The 4E antibody (anti-HLA-B locus, the gift of S. Young Yang, Sloan–Kettering Memorial Cancer Center; ref. 22) was used to confirm HLA-Bw58 expression. Fluorescein-labeled F(ab')$_2$ goat anti-mouse IgG plus IgM was obtained from Jackson ImmunoResearch and used at a dilution of 1:50. Results for immunofluorescence are reported as mean fluorescence channel (MFC) values in arbitrary units. Standard deviation values for the MFC values reported in this paper ranged from 6% to 11%.

Conjugate, Cold-Target, and Cytolytic Assays. Single-cell binding and cold-target inhibition assays were performed as described (3, 11). NK, CTL, and LAK cytolytic assays were evaluated by standard 4-hr ^{51}Cr-release assays as described (3).

RESULTS

Transfected Class I Genes Protect Target Cells from NK. The class I antigen-deficient mutant human B-cell line HMy2.C1R (C1R) was previously shown to exhibit enhanced NK susceptibility relative to its class I-positive parent (3). C1R clones transfected with class I genomic clones (HLA-A3, -B7, Bw58, or H-2D^p, -K^b) were screened for expression of the appropriate class I gene products by immunofluorescence using monomorphic and polymorphic monoclonal antibodies and by sensitivity to alloantigen-specific CTL lines. Transfectants expressing the class I gene were further examined for sensitivity to either endogenous or IL-2-activated NK effector cells.

In those cases in which multiple transfectants were isolated expressing a common class I antigen, the level of class I expression was found to vary over a broad range (Table 1). Two-dimensional gel analysis indicated that the class I molecules were biochemically indistinguishable from the corresponding class I molecules expressed endogenously in continuously grown cell lines (data not shown). When the sensitivity of these transfectants to highly purified endogenous NK effector cells was plotted as a function of class I expression, several points became evident (Fig. 1). First, each of the HLA class I gene products was capable of conferring a degree of protection against NK-mediated cytolysis to the C1R transfectants. Second, this degree of protection was dependent on the level of cell-surface class I antigen expression. Third, expression of transfected murine H-2D^p and -K^b class I genes failed to confer any protection against cytotoxicity mediated by human NK cells.

Table 1. Parameters of C1R transfectants

Target/ transfectant	Class I mAb reactivity (MFC)		Allogeneic CTL, % lysis (E/T = 3:1)	NK conjugation frequency, %
	W6/32	Polymorphic		
C1R-control	23	<10	9 ± 1	40 ± 2
C1R.Bw58.18	58	52	37 ± 2	36 ± 4
C1R.Bw58.20	393	345	43 ± 2	32 ± 4
C1R.Bw58.8	485	437	41 ± 3	24 ± 2
C1R.Bw58.5	506	465	42 ± 3	24 ± 5
C1R.Bw58.12	556	505	44 ± 3	18 ± 4
C1R.Bw58.2	795	730	44 ± 5	12 ± 2
C1R.A3.1	105	59	NT	34 ± 2
C1R.A3.3	218	169	NT	15 ± 3
C1R.B7.1	892	618	NT	27 ± 4
C1R.D^p.10	109	44	NT	36 ± 5
C1R.D^p.2	209	115	NT	40 ± 4
C1R.D^p.16	505	451	NT	40 ± 3
C1R.K^b.5	570	680	NT	38 ± 2
C1R.K^b.7	700	780	NT	41 ± 3

NT, not tested; mAb, monoclonal antibody; E/T, effector/target cell ratio.

Protection Is Reflected in Reduced Effector–Target Cell Conjugation. To examine the level at which NK sensitivity is affected by this class I protective mechanism, we performed single cell-binding and cold-target inhibition assays (Table 1; Fig. 2). In parallel with the cytolytic results, NK–target cell conjugation decreased as a function of increasing HLA, but not H-2, class I antigen expression (Table 1). When unlabeled transfectants were used to compete with chromium-labeled C1R target cells in 4-hr NK cytotoxicity assays, the high HLA class I expressors were found to compete with C1R much less effectively than low HLA class I expressors (Fig. 2). H-2 class I transfectants were indistinguishable from C1R in their ability to compete. These results suggest that the protective effect occurs at the level of target cell binding but do not rule out additional effects at the lytic level of NK-mediated cytotoxicity.

Protection Does Not Extend to LAK-Mediated Cytotoxicity. As many investigators use NK purification procedures that include poly(I·C) or IL-2 activation, we examined the sensitivity of these transfectants to cytotoxicity mediated by NK

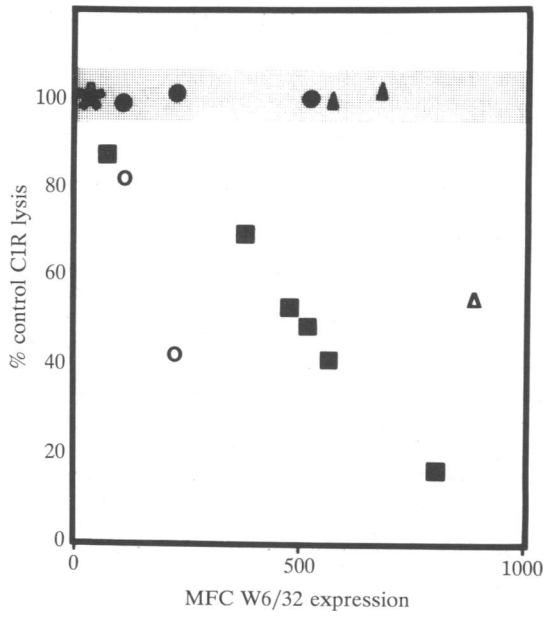

FIG. 1. The NK sensitivity of C1R class I transfectants as a function of class I antigen expression. The NK sensitivities of C1R and the transfectants were determined by 4-hr chromium release assays. Cytolytic results are reported as percent control C1R-specific release. This value represents the mean of percent control release determinations made at effector/target cell ratios of 20:1, 10:1, and 5:1. Control C1R-specific release at these ratios was 39% ± 2%, 20% ± 1%, and 12% ± 2%, respectively. Standard deviations for these reported mean values ranged from 0% to 5%. The range of control C1R lysis is indicated by the shaded region. W6/32 expression is reported as the MFC number determined from immunofluorescence assays. *, C1R; ●, C1R.D^p; ▲, C1R.K^b; ■, C1R.Bw58; ○, C1R.A3; △, C1R.B7.

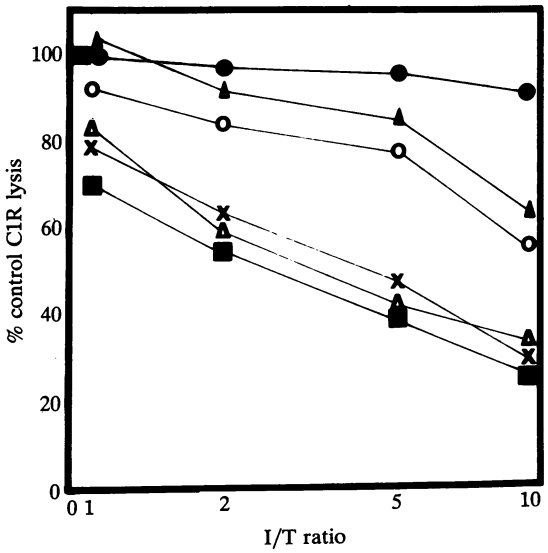

FIG. 2. Transfected HLA class I gene products reduce target cell sensitivity at the conjugation level. Cold-target inhibition assays were performed at the indicated inhibitor/target (I:T) ratios. Results are reported for an effector/target cell ratio of 10:1. Control C1R lysis (i.e., inhibitor/target ratio = 0) was 23% ± 2%. Standard deviation values ranged from 0% to 5%. ■, C1R; ●, CEM.NK^R; △, C1R.D^P; ▲, C1R.Bw58.2; ○, C1R.Bw58.12; X, C1R.Bw58.18.

cells that had been activated with rIL-2 (Table 2). In contrast to the results obtained for endogenous NK cells, expression of transfected HLA class I genes by C1R resulted in no protection from activated NK effector cells, suggesting that lymphokine-activated NK are capable of circumventing the protective effects of HLA class I antigens.

DISCUSSION

In a previous report we showed that the progressive elimination of functional class I genes from a human B-cell line rendered it increasingly sensitive to NK-mediated conjugation and cytolysis (3). The mutant B-cell line most deficient in class I expression, C1R, which is HLA-A,-B null, served as the recipient of exogenous HLA or H-2 class I genes. Cell-surface expression of the HLA class I antigens resulted in enhanced sensitivity to alloantigen-reactive CTL cytolysis but reduced sensitivity to NK. Resistance was displayed at the level of effector–target cell conjugation as the HLA class I transfectants were inefficiently bound by highly purified NK effectors in single cell-binding assays and competed poorly in cold-target inhibition assays against C1R. Resistance varied quantitatively as a direct function of the amount of HLA class I antigens expressed at the target cell surface.

Table 2. Expression of transfected class I antigen does not affect target cell sensitivity to rIL-2-stimulated NK effector cells

Target	% specific chromium release at E/T of 10:1	5:1	2:1
	Experiment 1		
C1R	64 ± 4	54 ± 5	25 ± 3
C1R.Bw58.2	63 ± 2	54 ± 3	22 ± 1
C1R.Bw58.5	67 ± 3	52 ± 4	23 ± 1
C1R.Bw58.8	56 ± 4	48 ± 4	19 ± 2
C1R.Bw58.12	56 ± 4	53 ± 1	19 ± 2
C1R.Bw58.18	55 ± 5	49 ± 2	21 ± 2
	Experiment 2		
C1R	44 ± 2	NT	11 ± 1
C1R.Bw58.2	46 ± 3	NT	9 ± 3
C1R.A3.3	46 ± 2	NT	15 ± 2
C1R.B7.1	41 ± 3	NT	17 ± 4
C1R.D^P.16	46 ± 2	NT	15 ± 2

Highly purified NK cells were stimulated with rIL-2 (5 units/ml) for 24 hr. The resulting effectors were then used against the indicated targets in 4-hr chromium release assays. NT, not tested; E/T, effector/target cell ratio.

A somewhat surprising result was obtained when murine H-2 class I genes were expressed in C1R. In contrast to the HLA class I genes, the H-2 class I genes failed to affect the NK-sensitive phenotype of the C1R cells. This was so despite their giving rise to class I antigen levels comparable to their HLA counterparts. The explanation for H-2 class I ineffectiveness in this system is not readily apparent. It may involve either conformational restraints placed on the molecule because of its association with human β_2-microglobulin (17), or it may reflect species specificity in the expression of the required epitopes in the HLA molecule. This clear distinction between the abilities of human and murine class I antigens to protect human targets from human NK-mediated cytotoxicity argues that the effect may be subject to natural selection and is therefore biologically significant.

The protective effect that HLA class I antigens invoke in the C1R transfectants does not extend either to lymphokine (rIL-2)-activated NK cells (Table 2), which are believed to represent the majority of LAK cells (23), or to interferon-activated NK cells (unpublished data). These findings are particularly relevant in light of several recent publications that have used either poly(I·C)- or IL-2-activated NK cells (6, 7, 24–26). While some of these investigations have demonstrated an effect of target cell class I antigen expression on sensitivity to activated NK-mediated cytolysis (6, 7), others have not (24–26). These contrasting findings may relate to subtle differences between endogenous nonactivated NK cells and lymphokine-activated effectors in recognizing target cells.

The mechanism by which class I molecules affect target cell sensitivity to NK is unknown, although various models have been proposed (3–6). One model is that elevated class I antigen expression on the target cell results in a negative signal to the NK effector cell (4, 5). This appears unlikely since monoclonal anti-class I reagents fail to modulate the NK sensitivity of class I-expressing target cells (3, 25), but it cannot be formally ruled out. Another hypothesis is that class I molecules may associate with NK target structures, thereby sterically hindering them from or lowering their affinity for receptors on the NK effector cell (3–5). LAK cells may compensate for this class I protective effect by increasing their "NK receptor" density, modifying their NK receptors to produce higher-affinity "LAK receptors," or expressing LAK receptors that are unaffected, in some systems, by target-cell class I antigen expression. In support of the model that class I molecules physically associate with NK target structures are reports of the cell-surface interaction of class I antigens with a number of membrane proteins—i.e., the receptors for insulin, glucagon, epidermal growth factor, endoperoxide–thromboxane, and somatostatin (27–29). In addition, the postulated lower affinity of the NK receptor for class I-associated target structures has precedent in the insulin receptor system in which class-I-associated receptor binds insulin with a 10 times lower affinity than the nonassociated receptor (30).

If the insulin receptor class I system provides a model system for the NK target structure class I system, a further prediction may be made. Different alleles of class I antigens should associate with target molecules with different affinities, as has been shown for the insulin receptor (31). Additional transfectants in the C1R.A3 and C1R.B7 series must be produced before any legitimate ranking of allelic effective-

ness in generating target cell protection against NK can be made.

The authors wish to thank C. Brissette-Storkus for providing the anti-Bw58 CTL culture. This work was supported by grants from the National Institutes of Health (CA42890 and AI15775). W.J.S. was supported by National Institutes of Health Grant CA09058. J.A. was supported by a grant from the National Science Foundation–Industrial University Cooperative Research Center for Lymphocyte Technology LTC422323.

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