Cancer Immunol Immunother (1999) 48:401–410

ORIGINAL ARTICLE

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HER-2/*neu* peptide specificity in the recognition of HLA-A2 by natural killer cells

Received: 16 March 1999 / Accepted: 3 June 1999

Abstract Although natural killer (NK) cells have been described as non-MHC-restricted, new evidence suggests that NK activity can be either up- or down-regulated after interaction with the peptide–MHC-class-I complex expressed on target cells. However, the epitope(s) recognized by NK cells have remained ill-defined. We investigated NK cell recognition of synthetic peptides representing a portion of a self-protein encoded by the HER-2/*neu* (HER-2) proto-oncogene and presented by HLA-A2. HER-2 nonapeptides C85, E89, and E75 were found partially to protect T2 targets from lysis by freshly isolated and interleukin-2(IL-2)-activated NK cells (either HLA-$A2^+$ or $A2^-$). This inhibition was not solely due to changes in the level of HLA-A2 expression or conformation of serological HLA-A2 epitopes. Using single-amino-acid variants at position 1 (P1) of two HER-2 peptides, we observed that protection of targets was dependent on the sequence and the side-chain. These results suggest similarities in the mechanism of target recognition by NK and T cells. This information may be important for understanding the mechanisms of tumor escape from immunosurveillance and could help explain the aggressiveness of HER-2-overexpressing tumor cells.

Key words Natural killer cells · HER-2/*neu* · Peptides · MHC · Tumor immunity

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Introduction

Natural killer (NK) cells are thought to play an important role in the elimination of virus-infected cells and cancer cells [5, 26, 39]. Although target-cell killing by NK cells has traditionally been described as non-MHC-restricted, interaction of NK-cell-inhibitory receptors with MHC class I molecules often leads to a down-regulation of NK cytolytic function in proportion to the level of MHC class I expression on the targets [20, 27, 40, 42]. Recent reports also indicated that single amino acid mutations within the peptide-binding groove of the MHC molecule can affect target cell sensitivity to lysis, suggesting that NK cells recognize different conformations induced by peptides bound in the MHC class I pockets [19, 37]. This hypothesis has been supported by observations that external loading of target cells with either self or foreign peptides can enhance or inhibit sensitivity to NK-mediated lysis in a peptide-specific manner independent of the level of MHC class I up-regulation [7, 25, 28, 38]. However, the basis for peptide specificity in the induction of lysis or protection is unknown. Further analysis of the mechanism of NK recognition of peptides may provide an important insight into the function of NK cell specificity for tumor cells.

The HER-2/*neu* (HER-2) proto-oncogene product is overexpressed in a variety of human cancers including breast, ovarian, colon, lung, and stomach, and its overexpression by breast and ovarian cancers has been shown to correlate with earlier relapse and a worse prognosis [36]. Since it has been reported that HER-2 overexpression also correlates with decreased NK cell activity [41], we wanted to determine if HER-2 peptides are directly involved in NK cell inhibition. Therefore, we used HER-2 peptides recognized by cytotoxic T lymphocytes (CTL) as targets, where the question of the sequence specificity in NK recognition can be addressed. In this report we investigated the ability of freshly isolated and in vitro interleukin-2(IL-2)-activated NK cells to recognize self-HER-2 peptides that bind to the HLA-

A2 molecule with variable affinities and induce conformational changes in the α1 and α2 domains. We found that these peptides decreased NK-mediated lysis of T2 cells, and the ability to inhibit lysis depended more upon peptide sequence than the ability to up-regulate or induce conformational changes of HLA-A2 on the target cells. Interestingly, the peptide that induced the most HLA-A2 up-regulation and conformational changes inhibited lysis least. Furthermore, targets pulsed with HER-2 peptide variants containing amino acid substitutions at position 1 (P1) showed either side-chain-dependent protection or increased sensitivity to NK-mediated lysis. Again, increased levels of lysis inhibition among the peptides did not correlate with increased levels of expression of HLA-A2, as detected by the W6/32 mAb specific for a monomorphic MHC I epitope (α3 domain), and lysis inhibition did not correlate with conformational changes of HLA-A2 detected by the MA2.1 mAb (α1 domain). Peptides that induced the most change in expression and conformation of HLA-A2 were often less effective at inhibiting lysis. However, the enhanced sensitivity to NK lysis seen with one peptide was paralleled by changes in the conformational epitope recognized by the BB7.2 mAb (α2 domain). These results indicate an important effect of changes in peptide sequence at position 1, and provide further evidence that the mechanism of NK target recognition has some similarity to that of T cells in that it is determined by interactions of peptide side-chains with NK receptors. These findings may also be helpful in explaining why cancer patients with tumors overexpressing HER-2 have a worse prognosis.

Materials and methods

Target cells

The T2 line has been described previously [15] and was a generous gift from Dr. Peter Cresswell (Yale Univeristy School of Medicine, New Haven, Conn.). The B cell line C1R:A2, an HLA-A2-gene-transfected derivative of C1R, was a gift from Dr. William Biddision (National Institute of Neurological Disorders, Bethesda, Md.). C1R:A2 cells were transfected with the plasmid pCMV.HER-2 encoding a full-length HER-2 cDNA (the kind gift of Dr. Mien-Chie Hung, Department of Tumor Biology, M.D. Anderson Cancer Center). C1R:A2:HER-2 transfectants were selected by resistance to hygromycin B by co-transfection of SV2.Hygro plasmids (ATCC, Rockville, Md.).

Effector cells

Peripheral blood buffy coats of normal donors were purchased from a local blood center, and mononuclear cells (PBMC) prepared by Ficoll-Hypaque gradient separation [24]. NK cells were enriched to high purity by negative selection using a MACS NK Isolation Kit (Miltenyi Biotec, Auburn, Calilf.). In brief, PBMC were incubated for 15 min at 4 °C with a cocktail of monoclonal antibodies (mAb) recognizing CD3, CD4, CD19, and CD33, washed, and then incubated for an additional 15 min with colloidal superparamagnetic microbead-labelled antibody reacting to the primary antibodies (Beckton Dickinson, Mountain View, Calif.). The cells were then passed twice through an iron-wool column placed within a strong magnetic field, and the nonadherent cells collected. The effluent population was routinely 91.7%–98.2% $CD56^+$, $CD3^-$ NK cells, 0.1%–1.4% $CD56^+$, $CD3^+$ T cells, and 0.2%–1.3% $CD56^-$, $CD3^+$ T cells as determined by two-color flow cytometry [35].

For IL-2 activation, NK cells were cultured for 5–7 days in RPMI-1640 medium supplemented with 10 mM HEPES buffer, 10% human AB serum, antibiotics, 2 mM glutamine, 2 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 μM 2-mercaptoethanol (complete RPMI medium), and 500 U/ml highly purified human recombinant rIL-2 (18×10^6 IU/mg; Cetus Corp., Emeryville, Calif.). The NK cell line, NKL (kindly provided by Dr. M.J. Robertson, Dana Farber Cancer Institute, Boston, Mass.) was obtained from peripheral blood of a patient with a $CD3^-$, $CD16^+$, $CD56^+$ large granular lymphoproliferative disorder [18]. These cells were maintained in culture in complete RPMI medium supplemented with 30 U/ml IL-2.

In some experiments, the CTL line 41 (CTL-41) was used as a source of effectors. This line was developed by repeated in vitro stimulation of $HLA\text{-}A2^+$ peripheral blood mononuclear cells from a healthy donor with peptide C84: HER-2 (971–979 V) and a longer peptide C43: HER-2 (968–981) [12]. For these studies, CTL-41 were maintained in culture with monthly restimulation with 10 μg/ml C84 peptide and autologous or allogeneic $HLA\text{-}A2^+$ PBMC. The CTL used as effectors were selected on mAb-coated plates (AIS Micro CELLector, Applied Immune Sciences, Menlo Park, Calif.), and were $CD3^+$, $CD4^-$, $CD8^+$. Clones were isolated from the CTL-41 line, as previously described [14].

Synthetic peptides

Synthetic peptides corresponding to sequences in HER-2: E75 (369–377), E89 (851–859), C85 (971–979), and recognized by ovarian tumor-specific CTL, have been reported previously [12–14]. The amino acid sequences of these peptides are shown in Table 1. Variants of the C85 peptide substituted at P1 are designated as G1, F1, T1, and K1 [12]. The E75 peptide substituted at P is designated as peptide F41. The synthetic peptides used in this study were prepared by the Synthetic Antigen Laboratory of M.D. Anderson Cancer Center, purified to 92%–95% by HPLC, and dissolved in phosphate-buffered saline (PBS) at a stock concentration of 1 mg/ml.

Table 1 Sequences of synthetic HER-2 peptides

Code	Position	1	2	3	4	5	6	7	8	9
E75	369–377	K	I	F	G	S	L	A	F	L
F41		G	–	–	–	–	–	–	–	–
E89	851–859	V	L	V	K	S	P	N	H	V
C85	971–979	E	L	V	S	E	F	S	R	M
G1		G	–	–	–	–	–	–	–	–
F1		F	–	–	–	–	–	–	–	–
T1		T	–	–	–	–	–	–	–	–
K1		K	–	–	–	–	–	–	–	–
C84		E	–	–	–	–	–	–	–	U

Cytotoxicity assay

The ^{51}Cr release assay has been described in detail previously [24]. For peptide-pulsing experiments, ^{51}Cr-labelled T2 cells were dispensed into 96-well microtiter plates and preincubated for 2 h in serum-free RPMI medium, to which was added either 10 µl peptide (100 µg/ml final concentration), or an equivalent volume of PBS as a control. Effector cells, suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum, were then added in various E:T ratios (ranging from 40:1 to 1:1), and the culture supernatants were tested for chromium release after 4–5 h of culture. Each experimental condition was tested in triplicate. Results are expressed as the percentage specific lysis according to the formula $(E - S)/(M - S) \times 100$, where E is the radioactivity (cpm) of experimental wells containing both effectors and targets, S is the spontaneous release of ^{51}Cr from targets incubated in medium (with and without peptide), and M represents the radioactivity for targets incubated with 0.2% Triton X-100 (maximum release). In some experiments the cytotoxicity was expressed as lytic units (LU), where 1 LU is the number of effector cells required for lysis of 30% of the target cells [35]; when this calculation is used, the results are expressed as $LU_{30}/10^6$ effector cells.

In studies designed to analyze the sensitivity of HER-2-gene-transfected cell lines to lysis, NK or CTL effector cells were incubated for 4–5 h with ^{51}Cr-labelled C1R:A2:HER-2-transfected target cells at effector-to-target ratios (E:T) ranging from 12:1 to 50:1

Flow-cytometric analysis

Expression of HLA-A2 on T2 target cells was evaluated by flow cytometry, using BB7.2, MA2.1 and W6/32 mAb. W6/32 mAb (Dako, Dakopatts, Denmark) recognizes a monomorphic epitope common to HLA-A, -B, and -C. The anti-HLA-A2 mAb, BB7.2 (mouse IgG2b) and MA2.1 (mouse IgG1) were obtained from the American Type Culture Collection (ATCC). Other antibodies used in this study included anti-CD11a, anti-CD18, anti-CD58, and anti-CD56 (Beckton-Dickinson, Mountain View, Calif.); and Ab2, reacting with the extracellular domain of HER-2 protein (Oncogene Science, Uniondale, New York). Briefly, 5×10^5 cells were incubated for 30 min at 4 °C with primary antibody (or an isotype control antibody nonreactive with human cells), washed, and then incubated for an additional 30 min with fluorescein-isothiocyanate-conjugated goat anti-(mouse Ig). Flow-cytometric analyses were performed on 5000 gated events/sample, using a FACScan flow cytometer (Becton-Dickinson, Mountain View, Calif.) and Consort 30 software.

To analyze the effect of peptide pulsing on HLA-A2 expression, T2 cells were incubated for 2 h at 37 °C with 10–100 µg/ml peptide (or PBS alone as a control), prior to labelling with the primary mAb. All cells tested were positive for HLA-A2 expression; data are reported as the mean channel fluorescence, indicative of the channel number corresponding to the average peak of fluorescence [6, 31, 35].

Statistical analysis

The data were analyzed statistically using Prism 2.01 software (GraphPad Prism for Scientists, Sorrento, Calif.). Multiple groups were compared by the Newman Keuls one-way analysis of variance. When only two groups were compared, Student's t-test was used. Differences were considered significant when P was less than 0.05.

Results

HER-2 peptides inhibit NK-mediated lysis of T2 cells

In the first series of experiments, we investigated the effects of HER-2 self-peptides on the sensitivity of T2 target cells to lysis by NK cells. The T2 cells have a defect in TAP (transporter-associated with antigen presentation) proteins and display "empty" HLA-A2 molecules [33] that can be loaded exogenously with peptides having the proper anchors for binding to HLA-A2, i.e. L/M/I/V (P2) and V/L/M/I (P9). For our studies we used three different synthetic nonapeptides of HER-2 that display these HLA-A2 anchors: E75, E89 and C85 (the amino acid sequences of these peptides are shown in Table 1). These peptides were previously found to reconstitute recognition of $CD8^+$, $CD4^-$ CTL lines derived from ovarian tumor-associated lymphocytes [12], suggesting that HER-2 is naturally processed into identical or similar peptides presented by HLA-A2 on tumor cells.

The T2 targets were pulsed with peptides at a concentration of 100 µg/ml prior to addition of effector cells. In agreement with others [32], we observed that untreated T2 targets were sensitive to lysis by freshly isolated and IL-2-activated peripheral blood NK cells from all healthy donors tested (Fig. 1). However, T2 cells pulsed with HER-2 peptides were significantly protected from killing by unstimulated HLA-$A2^+$ and HLA-$A2^-$ NK cells (Fig. 1A, B). These peptides also protected T2 targets from lysis by IL-2-activated HLA-$A2^+$ and HLA-$A2^-$ NK cells (Fig. 1C, D). The results from four representative donors of eight tested are shown in Fig. 1. The reduction in lysis of peptide-pulsed

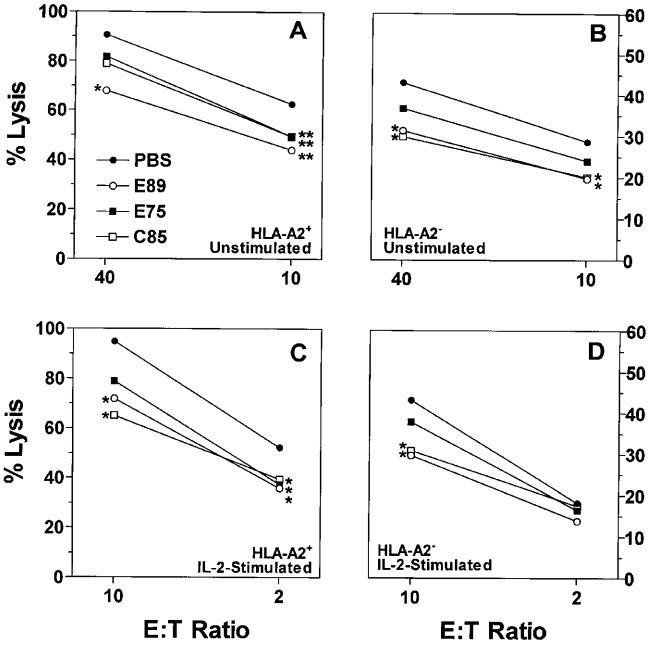

Fig. 1A–D Recognition of HER-2 peptide-pulsed T2 cells by natural killer (NK) cells. Magnetically sorted NK cells from four healthy donors (one per panel) were tested for lytic activity against HER-2-peptide-pulsed T2 cells in a ^{51}Cr-release assay. The NK cells were (**A**) unstimulated HLA-$A2^+$, (**B**) unstimulated HLA-$A2^-$, (**C**) interleukin-2(*IL-2*)-stimulated HLA-$A2^+$, and (**D**) IL-2-stimulated HLA-$A2^-$. Significant inhibition of lysis: $^*P < 0.05$ compared to phosphate-buffered saline (*PBS*) control; $^{**}P < 0.01$ compared to PBS control

target cells was consistently observed at multiple E:T ratios and ranged from 15% to 30%.

Because sensitivity of target cells to NK-mediated lysis has been shown to be inversely related to the levels of MHC class I expression [40], we next determined whether the resistance of HER-2-pulsed T2 targets to lysis by NK cells was associated with an increase in MHC class I molecules caused by peptide-induced stabilization [6]. As shown in Fig. 2, T2 cells incubated with HER-2 peptides displayed an increase in the relative density of surface HLA class I molecules as detected by the HLA-A, -B, -C-specific W6/32 mAb; this was observed as an increase in the fluorescence intensity of mAb-labelled peptide-pulsed T2 cells compared to controls. However, there were marked differences among the peptides in the relative density of class I molecules induced, with an approximately twofold and threefold increase caused by E89 and E75 respectively. The C85 peptide only slightly increased MHC class I expression. Despite these large differences in MHC class I expression, the level of protection afforded by E89 and C85 peptides was comparable. Although E75 increased MHC expression the most, it was consistently least effective at inhibiting target lysis (Fig. 1). These results indicate that the increased resistance of T2 to lysis by NK cells induced by C85 was not simply caused by up-regulation of MHC class I molecules.

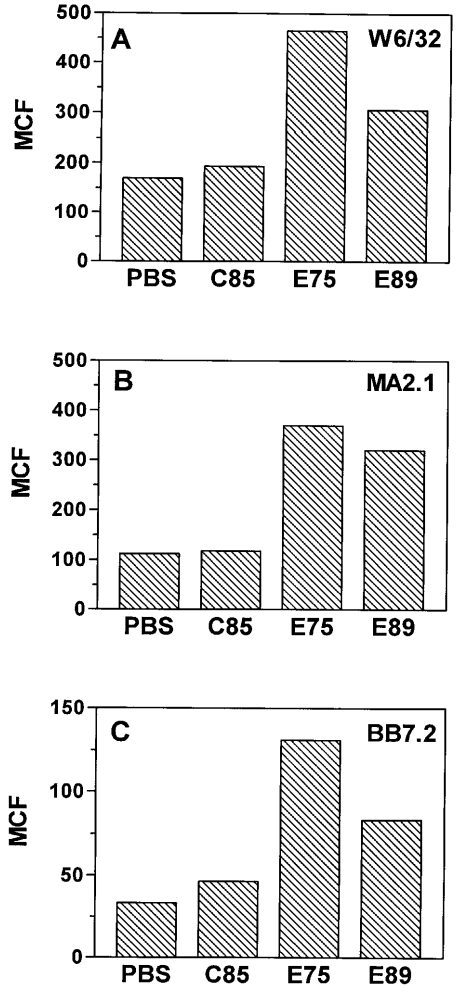

Fig. 2A–C Up-regulation of HLA-A2 expression by HER-2 peptides. T2 cells pulsed with or without (PBS control) HER-2 peptides, were analyzed by flow cytometry for expression of epitopes recognized by the W6/32, MA2.1, and BB7.2 mAb. *Bars* the mean channel fluorescence (*MCF*) value, i.e., the channel corresponding to the mean fluorescence intensity of positively stained cells

To determine if peptide-induced protection was related to changes in the conformation of HLA-A2 molecules, we also analyzed peptide-pulsed and control T2 cells for expression of conformational epitopes recognized by BB7.2 and MA2.1 mAb. The epitope recognized by the BB7.2 mAb is located on the N-terminal loop of the $\alpha 2$ domain (including W108) of HLA-A2, in an area not expected to contact the peptide directly [34]. MA2.1 mAb reacts with the $\alpha 1$ domain of HLA-A2 at residues 64–68, which border the A and B pockets of the peptide-binding groove; mutations of HLA-A2 in this area have been reported to affect T cell recognition significantly [16, 34]. The results in Fig. 2 indicate that the decrease in the sensitivity of T2 to lysis by NK cells, after pulsing with a particular peptide, was not proportional to the increase in the expression of either of these conformational epitopes. On the contrary, while MA2.1 and BB7.2 epitopes were expressed at approximately threefold higher levels on E75-pulsed T2 cells compared to untreated or C85-pulsed targets, the protection induced by E75 was, in most cases, less than that of the other peptides (Fig. 1).

Recognition of peptide variants by NK cells

Crystallography studies have shown that the N-terminal (P1) residue of peptides binds within the A pocket of the MHC molecule, and that the nature of the side-chain of this residue affects peptide binding to HLA-A2 [3]. To address the question of whether a single amino acid substitution at P1 would alter the ability of a HER-2 peptide to protect targets from NK-mediated lysis, we created a series of C85 variants by replacing the glutanic acid at P1 with lysine (variant K1), glycine (variant G1), threonine (variant T1) or phenylalanine (variant F1) (Table 1). These peptide variants do not have changes in the dominant anchors for HLA-A2 at P2 (L/M/I/V) and P9 (V/L/M/I), so they should still bind to HLA-A2. Using a peptide concentration that was protective for C85, we tested the ability of the peptide variants to protect T2 cells from NK-mediated lysis (Fig. 3A). Significant differences between the peptides were observed regarding their ability to affect T2 lysis. Specifically, we found that the K1 variant was as effective as the natural C85 peptide in protecting T2 cells from lysis, while the F1 and G1 variants did not significantly inhibit T2 lysis compared to control T2 cells treated with PBS. In contrast, T2 targets pulsed with the T1 variant not

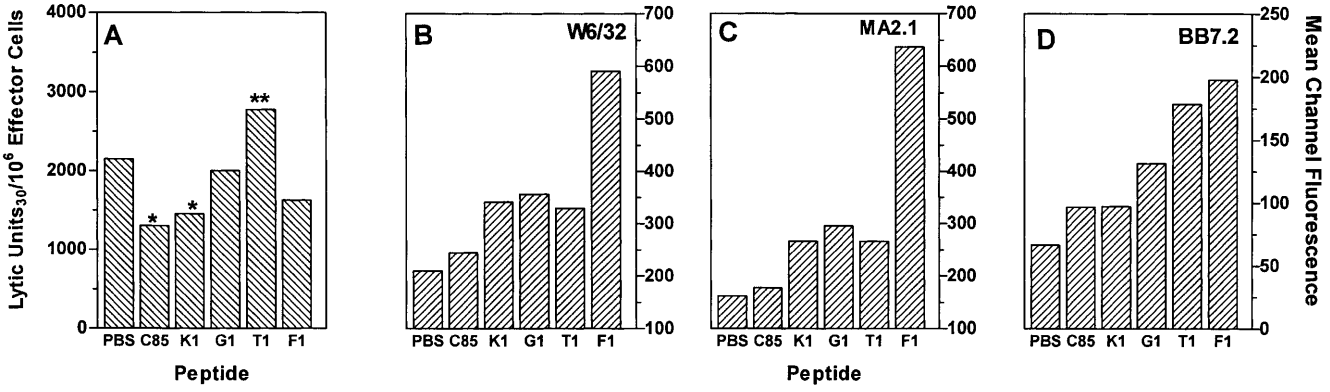

Fig. 3A–D Recognition of HER-2 peptide variants by NK cells. **A** Immunomagnetically isolated HLA-A2$^+$ NK cells were tested for cytolytic activity against T2 targets pulsed with 100 μg/ml C85 and C85 variants, and the results from four separate donors were averaged. Data are expressed as lytic units as described in Materials and methods. * There was significant protection of T2 after pulsing with C85 (E1) or the K1 variant ($P < 0.05$ compared to PBS-treated control T2 targets). ** The T1 variant caused significant enhancement of T2 lysis compared to both C85-pulsed and control targets ($P < 0.05$). **B–D** Three different mAb (W6/32, MA2.1, and BB7.2) were used to detect MHC class I expression by T2 cells pulsed with the same peptides used in **A**

only were not protected, but instead were even more susceptible to lysis by NK cells than were PBS-treated controls. On the basis of a comparison of cytotoxicity (LU), they were also twofold more susceptible to NK lysis than C85-pulsed T2.

All of these C85 variants up-regulated and stabilized MHC class I expression as detected by the W6/32 mAb, albeit to different degrees (Fig. 3B). The conformational epitopes recognized by MA2.1 and BB7.2 mAb were also up-regulated when compared to PBS-treated T2 targets (Fig. 3C, D). The levels of expression of W6/32 and MA2.1 HLA-A2 epitopes on T2 cells pulsed with K1, G1, and T1 variants were similar, and they were higher than the levels induced by C85. The F1 variant induced a twofold higher increase in these epitopes relative to the other variants. The T1 and F1 variants induced the highest levels of BB7.2 epitope expression among the variants tested.

When the levels of expression of MHC class I and the BB7.2 and MA2.1 conformational epitopes were compared to protection from lysis, the ability of a particular peptide to down-regulate target sensitivity to NK lysis was not directly proportional to the increase in the level of HLA-A2 expression. For example, neither the G1 nor F1 variant was significantly protective, even though F1 induced a substantially higher expression of W6/32, MA2.1, and BB7.1 epitopes. Furthermore, compared to C85, the T1 peptide enhanced the susceptibility of T2 cells to NK-mediated lysis, even though this variant induced higher levels of HLA-A2 expression and conformational changes. The K1 variant was as protective as C85, but induced higher levels of MHC class I than did the natural peptide, as detected by the W6/32 and MA2.1 mAb. Again, these data support the observation that increased MHC class I does not always correlate with enhanced resistance to lysis by NK cells.

The results in Fig. 3 show a significant role for the P1 residue side-chain in NK inhibition. Protective peptides in this study (C85 and K1) have charged side-chains, while the nonprotective peptides (F1 and G1) have nonpolar side-chains. T1, which enhanced NK sensitivity, has a hydroxyl group. To confirm that the NK inhibition is dependent on the side-chain of the amino acid at P1, we investigated NK recognition of T2 cells pulsed with the weakly NK protective peptide E75 and its P1 variant, F41. In the latter variant, the lysine at P1 in the natural peptide is substituted with glycine, which lacks a side-chain (Table 1). This K $\rightarrow$ G change at P1 of E75 is identical to the change between the K1 and G1 variants of C85 described above. Thus F41 was expected to be less protective than E75. We observed that both E75 and F41 showed a similar concentration-dependent ability to up-regulate HLA-A2 expression on T2 cells: for example, at 100 μg/ml, the mean channel fluorescence for the expression of the BB7.2 epitope was approximately threefold higher for both E75-pulsed and F41-pulsed targets than for the controls (data not shown). Both peptides also protected T2 targets from lysis by the NK cell line (NKL) when used at concentrations of 10–100 μg/ml (Fig. 4A). However, at 200 μg/ml, E75 (charged P1 side chain) was significantly more protective ($P < 0.001$) against NK lysis than was F41 (nonpolar P1 side-chain), supporting the conclusion from the previous experiment.

To rule out the possibility that the HER-2 peptides were stabilizing non-HLA-A2 MHC or nonclassical MHC molecules that could mediate a decrease in NK sensitivity [1, 2], E75-pulsed T2 targets were treated with either the HLA-A2-specific MA2.1 mAb or control antibody before lysis by the NK cell line was assessed. As seen in Fig. 4B, HER-2-peptide-induced inhibition of lysis was most likely mediated directly through interaction with HLA-A2, because MA2.1 antibody completely blocked the inhibition of T2 lysis at 100 μg/ml E75 ($P < 0.05$) and significantly blocked the higher inhibition at 200 μg/ml E75 ($P < 0.01$).

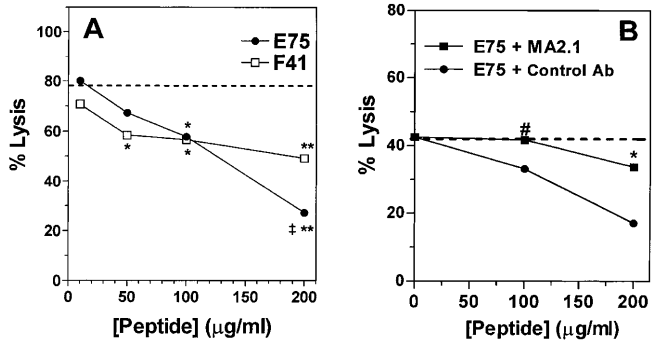

Fig. 4A, B Protection of T2 targets by HER-2 peptides is concentration-dependent and HLA-A2-dependent. T2 cells pulsed with E75 or its variant, F41, were tested for susceptibility to lysis by the HLA-A2$^+$ NK cell line, NKL, at an E:T ratio of 40:1. - - - - The percentage lysis of PBS-treated control T2 targets (78% for **A** and 42% for **B**). **A** Significant protection from lysis: * $P < 0.01$ compared to PBS-treated control; ** $P < 0.001$ compared to PBS control; ‡ E75 inhibited lysis significantly better than F41 at 200 μg/ml ($P < 0.001$). **B** E75-pulsed T2 targets were incubated with HLA-A2-specific blocking antibody (MA2.1) or control antibody. # MA2.1 completely blocked the protection from lysis at 100 μg/ml E75 ($P < 0.05$). * Significant blocking of the protection from lysis also occurred at 200 μg/ml E75 ($P < 0.01$)

Recognition of HER-2-transfected C1R:A2 cells by NK cells

Under physiological conditions, NK cells interact with HLA-A2 molecules presenting peptides processed endogenously. Therefore, it was of interest to determine if endogenously processed HER-2 peptides could also protect target cells from lysis by NK cells. We approached this question using C1R:A2 cells transfected with the HER-2 gene (C1R:A2:HER-2$^+$ cells). C1R:A2:HER-2$^+$ cells were cloned by stringent limiting dilution, and three clones (HER-2:G, HER-2:B, and HER-2:L), expressing different levels of surface HER-2, were tested for sensitivity to lysis by CTL and NK cells. CTL clones (CD3$^+$, CD8$^+$, CD4$^-$) were developed by in vitro stimulation of HLA-A2$^+$ mononuclear cells from a healthy donor with C84, a P9-substituted (M → V) C85 peptide [12]. As shown in Fig. 5, two clones (2C2 and 2C3) recognized the C1R:A2:HER-2:L clone but not C1R:A2 cells lacking HER-2 gene expression, suggesting that an epitope similar to C85 was presented by C1R:A2:HER-2$^+$ cells. However, the HER-2$^+$ clones were more resistant to lysis by IL-2-stimulated NK cells than were the nontransfected

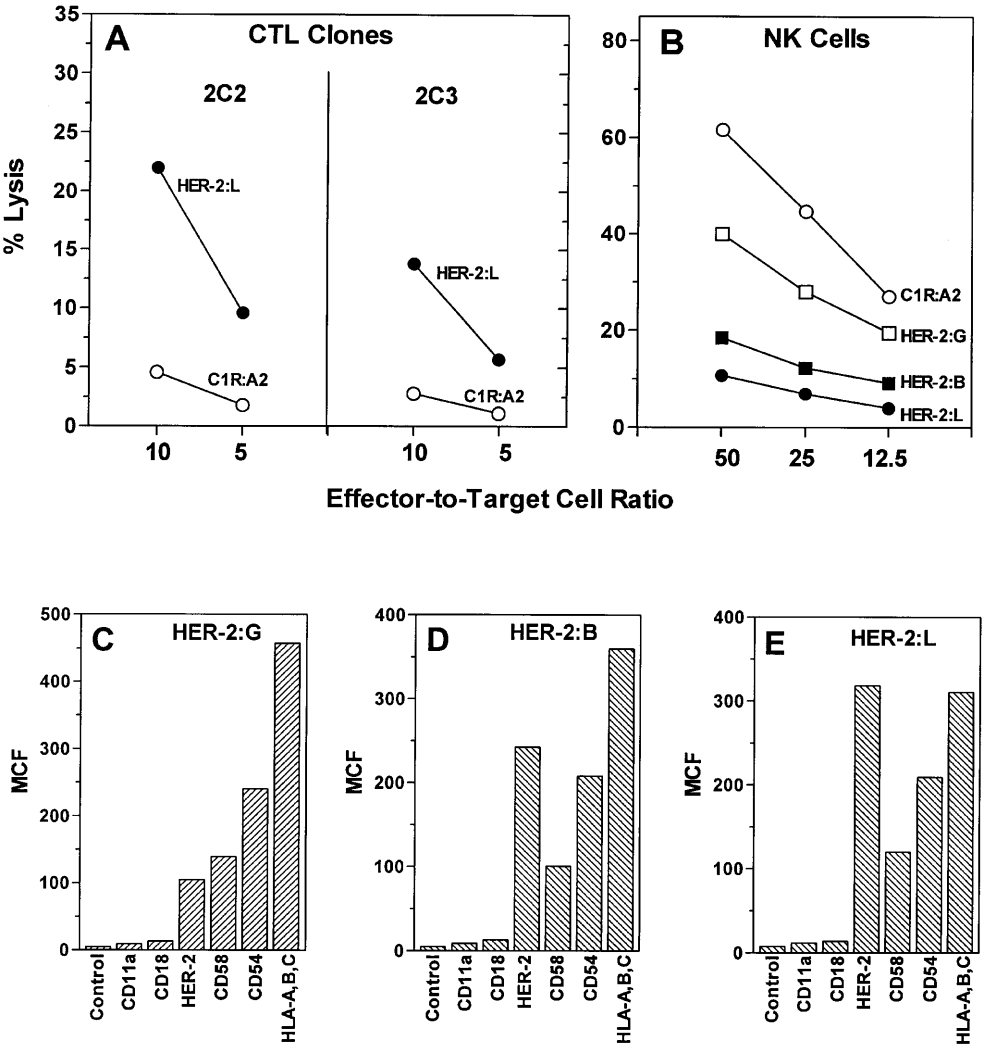

Fig. 5A–E Lysis of the HER-2 gene-transfected target cells by cytotoxic T lymphocytes (CTL) and NK cells. **A, B** Nontransfected and HER-2-transfected CIR:A2 cells (HER-2:G, HER-2:B, and HER-2:L) were tested for susceptibility to lysis by two C85-specific HLA-A2$^+$ CTL clones (2C2 and 2C3) and magnetically sorted, IL-2-activated HLA-A2$^+$ NK cells. The HER-2 transfectants were clones chosen for their high (HER-2:L), medium (HER-2:B), or low (HER-2:G) expression of HER-2. **C, D, E** Surface MHC class I, HER-2, LFA-1a (CD11a), LFA-1b (CD18), LFA-3 (CD58), and ICAM-I (CD54) expression by C1R:A2 and HER-2 transfectants was analyzed by flow cytometry

targets. Furthermore, the sensitivity of the clones to lysis by NK cells varied inversely with the density of HER-2 expression; i.e. the sensitivity of the transfectants to lysis ranked G > B > L, while HER-2 expression ranked L > B > G (Fig. 5).

The HER-2:G targets were most sensitive to NK lysis even though they expressed a higher density of HLA-A2 than did HER-2:B and HER-2:L. Additional phenotypic analyses of these clones revealed that they all expressed only very low levels of CD18 (LFA-1β) and CD11a (LFA-1α), while CD54 (ICAM-1) and CD58 (LFA-3) were expressed at similar levels among the cloned transfectants (Fig. 5C–E). Therefore, there was no correlation between adhesion molecule expression and the sensitivity of HER-2 transfectants to lysis by NK cells. Our data suggest that quantitative and qualititative changes in the composition of the naturally processed HER-2 peptides presented by MHC, rather than alterations in the expression of MHC class I or adhesion molecules, are responsible for the protective effects of HER-2.

Discussion

In this report we present novel evidence that HLA-A2-binding HER-2 peptides, known to form CTL epitopes, can protect targets from lysis by NK cells. This protection was found to be dependent upon (a) peptide concentration, requiring pulsing with peptides at 50–100 μg/ml; (b) peptide sequence, since single amino-acid substitutions could significantly alter the status of target susceptibility; and (c) side-chain charge, with charged side-chains at position 1 generally inducing more protection from NK lysis than uncharged side-chains. In support of previous studies by others [2, 25, 26, 28, 43], this indicates that NK cells recognizing peptide-MHC complexes display a high degree of target specificity. These findings also suggest that CTL epitopes on tumor cells may block NK lysis, a mechanism that may have implications for tumor survival in the absence of CTL. An increase in the relative ability of a peptide to inhibit lysis was, in most cases, not associated with increased expression of HLA-A2 on T2 target cells, or with conformational changes of HLA-A2 detected by BB7.2 and MA2.1, suggesting that these serological epitopes are not solely responsible for inhibition of NK function.

HLA-A2 conformational changes were often seen on targets that were most sensitive to lysis in this study. For example, increased staining with the BB7.2 mAb was associated with enhanced lysis in the case of the T1 peptide and decreased protection from lysis for E75. One possible explanation for the enhanced sensitivity to lysis of targets bearing HLA-A2 conformational changes could be that, although HLA-A2 expression inhibits lysis, it can only do so if the conformation is not altered by the peptide. However, the full explanation is probably more complex, because E89 induced a fair amount of HLA-A2 conformational changes (both MA2.1 and BB7.2) yet inhibited lysis as effectively as C85, a peptide that did not induce such changes in HLA-A2. One alternative explanation for the enhanced sensitivity to lysis caused by T1 is the hydroxylated side-chain (tyrosine) at P1, which may have decreased the recognition of HLA-A2–peptide by an inhibitory NK receptor. Further experiments are necessary to elucidate this mechanism.

In agreement with previous studies, the peptide concentrations required to induce a significant NK-protective effect were higher than the concentrations required to sensitize T2 cells to CTL effectors from breast and ovarian cancer patients [12, 14]. This may indicate that these effects are only relevant in vitro. However, recent studies on peptide binding to HLA-A2 molecules indicate that, during 4–6 h of incubation, the number of class I MHC complexes formed with similar amounts of exogenously added peptides is in the range of 10^3–10^4, which is consistent with the level of expression of a number of endogenous peptides [17]. Therefore our results should be relevant to certain pathological conditions, such as viral infections and cancer, where large amounts of viral or tumor peptides are processed and presented by MHC class I. The observation that NK cells were less effective in lysis of C1R:A2 cells expressing high levels of HER-2, than of those expressing lower levels, is suggestive of this possibility. Thus, protection from NK-mediated lysis may be dependent not only on the presence of self-peptides or MHC, but also on the high-density expression of specific peptide-MHC complexes. These findings are compatible with the use of an NK-inhibitory receptor with low affinity for the recognition of peptide-MHC complexes. Furthermore, the same peptides were capable of inhibiting lysis of HLA-$A2^+$ T2 cells by NK effectors from both HLA-$A2^+$ and HLA-$A2^-$ donors, indicating that the receptor(s) responsible for this inhibition are expressed independently of HLA-A2 expression in the donors.

These studies were performed using highly enriched (up to 98% purity) NK cells, to exclude a role for T cells in any of the observed effects. We also observed that HER-2 peptides protected targets from lysis by an established NK cell line. In no experiment, though, was complete protection of T2 cells by HER-2 peptides observed. This is not surprising, because the NK cells used in our studies were not clones. It has been shown that different NK clones can respond differently to the same peptide-pulsed targets [8, 9, 23, 25], most likely because of expression of different combinations of inhibitory and activation receptors. Bulk NK populations were used in most of our experiments to mimic more closely the effector/tumor conditions existing in vivo. In fact, it is important to realize that the 15%–30% of tumor cells that might be protected from NK cells by HER-2 peptides would represent a substantial number of malignant cells likely to escape NK cell attack.

Our results show that a side-chain charge at P1 of two different HER-2 peptides is important for protection from lysis. It is interest that the requirement for a specific side-chain in the protection of a target against NK-mediated lysis suggests that certain NK receptors, or

structures on NK cells involved in target lysis, directly contact MHC-bound peptide. Importantly, these effects were observed for the first time when peptides known to induce CTL-mediated lysis in the HLA-A2 system were used. Recent studies have shown sequence-specific NK-potentiating effects for P8 of nonapeptides, although the effects were not associated solely with charged residues at P8 [26]. Furthermore, Peruzzi and collaborators identified a role for P7 and P8 of HLA-B*2705-associated peptides in modulation of NK recognition [28]. Charged side-chains in residues at P7 and P8 in their system enhanced NK-mediated lysis. These studies indicate that residues in certain positions of the class-I-MHC-bound peptides can up- or down-modulate NK lysis. Nevertheless, the effects may be dependent upon HLA type or other unknown factors, which may help explain why one donor of four tested (Fig. 3) in our study showed a somewhat different-from-average pattern of NK inhibition by the C85 variants (inhibition by F1 and G1 but not K1; data not shown). It is most likely that HER-2 peptides were inhibiting lysis directly through the interaction of HLA-A2–peptide complexes with NK receptors, since A2-specific mAb significantly blocked the inhibition. Although the inhibition was not completely blocked when high levels of peptide were used, likely explanations are that monomorphic HLA-A2 was up-regulated more than the MA2.1 conformational epitope or that the antibody was not saturating the HLA-A2 at high peptide concentrations. This could also possibly be due to peptide stabilization of non-classical MHC, such as the deletion variants described by Abu-hadid et al. [1].

Positive stimulation (activation) of NK cells may occur through several different activation or costimulatory receptors on NK cells, such as NKR-P1 proteins, CD16 and CD28, but it appears that the specificity of NK target recognition is often not provided by activation signals, but rather by the presence or absence of inhibitory signals induced by recognition of peptide-MHC complexes [20]. It has been suggested that peptide-induced protection from NK cells may be due to stabilization and/or conformational effects of peptides on MHC class I molecules. However, the role of the peptide in NK recognition is probably not simply to stabilize MHC class I or to promote changes in MHC conformation. NK cells express an array of different receptors that inhibit target cell lysis upon recognition of MHC class I. Examples are the C-type lectin superfamily of receptors (e.g. CD94, NKG2) and the killer-cell-inhibitory receptors of the immunoglobulin superfamily (e.g. p 70, p 58) [4, 20, 29]. Several investigators have now demonstrated that inhibitory receptors on NK cells not only recognize specific types of MHC but also recognize a specific subset of peptides on HLA-B or C [4, 25, 28–30, 43]. Our results in the HLA-A2 system also show that NK cell recognition is sensitive to mutations in peptides that minimally affect monomorphic MHC class I expression. Furthermore, changes in the expression of conformational MHC epitopes did not appear to cause the inhibition of NK-mediated lysis in this model, although such epitopes may have caused increased sensitivity to lysis, as discussed above. It is tempting to hypothesize that NK receptors use a similar mechanism of recognition to the one recently proposed for the T cell receptor [10]; i.e., the proper conformation of the MHC-peptide complex is required for the receptor to "land" on the target, while the changes in side-chain moieties (charge, polarity, van der Waals forces), are responsible for initiation of signaling. This will explain why expression of the MA2.1 conformational epitope does not correlate with recognition, since the epitope recognized by MA2.1 mAb is directly affected by side-chains of residues in pocket A (and possibly B) of HLA-A2, while the BB7.2 mAb detects altered conformation induced by the peptide in a different position (α2 domain, W108), which does not interact directly with peptide side-chains. More extensive studies are needed to address this point, but this study suggests that a number of mutations in peptides (including CTL epitopes) presented by MHC class I may interfere with MHC recognition by NK cells. These findings may have implications for understanding the mechanism by which cells infected with viruses (e.g. influenza or AIDS), and displaying a high rate of mutation, might escape immune defenses. This mechanism may also apply to tumor cells where overexpression of certain gene products (e.g. tyrosinase, gp100, or Muc-1) could lead to the presentation of a high density of self-epitopes with inhibitory effect on NK cells. An additional possibility to be examined is that presentation of mutated peptides (e.g. from p53 or p21 *ras*) may protect tumor cells from NK surveillance.

In support of our conclusions, it has been shown previously that HER-2-overexpressing breast and ovarian cell lines were more resistant to NK-mediated lysis than nonexpressing (or HER-2^{low}) targets [21]. As was the case also in our investigations, resistance in the latter studies could not be attributed solely to an increase in MHC class I or to changes in ICAM-1 expression by the HER-2^{+} targets [11, 22]. Taken together, these results suggest that endogenously processed HER-2 peptides expressed in complexes with MHC class I molecules may contribute to the resistance of HER-2-overexpressing tumor cells to NK-mediated lysis. Therefore, further elucidation of how NK cells recognize peptides may help to explain the aggressiveness of some tumors, as well as provide new insight into the nature of NK cell receptors for antigens.

Acknowledgements The authors would like to thank Timothy W. King for his assistance in the preparation of this manuscript. Peptide synthesis was supported in part by core grant 16672. Supported in part by grants DAMD: 4313 and 7098.

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