Brief Definitive Report

Amino Acid Substitutions Can Influence the Natural Killer (NK)–mediated Recognition of HLA-C Molecules. Role of Serine-77 and Lysine-80 in the Target Cell Protection from Lysis Mediated by "Group 2" or "Group 1" NK Clones

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Summary

Natural killer (NK) cells have been shown to express a clonally distributed ability to recognize HLA class I alleles. The previously defined NK clones belonging to "group 1" recognize HLA-Cw*0401 (Cw4) and other HLA-C alleles sharing Asn at position 77 and Lys at position 80. Conversely, the "group 2" NK clones recognize HLA-Cw*0302 (Cw3) and other HLA-C alleles characterized by Ser at position 77 and Asn at position 80. We assessed directly the involvement of these two residues in the capacity of NK cell clones to discriminate between the two groups of HLA-C alleles. To this end, Cw3 and Cw4 alleles were subjected to site-directed mutagenesis. Substitution of the amino acids typical of the Cw3 allele (Ser-77 and Asn-80) with those present in Cw4 (Asn-77 and Lys-80) resulted in a Cw3 mutant that was no longer recognized by group 2 NK cell clones, but that was recognized by group 1 clones. Analysis of Cw3 or Cw4 molecules containing single amino acid substitutions indicates roles for Lys-80 in recognition mediated by group 1 clones and for Ser-77 in recognition mediated by group 2 clones. These results demonstrate that NK-mediated specific recognition of HLA-C allotypes is affected by single natural amino acid substitutions at positions 77 and 80 of the heavy chain.

NK cells recognize HLA class I alleles via clonally distributed surface receptors (1–6). The NK receptor/HLA class I molecule interaction results, in most instances, in the generation of a negative signal that inhibits the cytolytic activity of NK cells, thus preventing target cell lysis. As shown by the analysis of NK cell clones, a subset of NK cells specifically recognizes HLA-C molecules. In particular, a group of NK clones ("group 1") recognizes Cw4 and other HLA-C molecules (Cw2, Cw5, and Cw6) having Asn at position 77 and Lys at position 80 of the heavy chain sequence. A second group of NK clones ("group 2") recognizes Cw3 and related alleles (Cw1, Cw7, and Cw8) sharing Ser at position 77 and Asn at position 80 (5–7). Of note is that residues 77 and 80 are localized in the peptide-binding groove and more precisely in the F pocket (8). Given the correlation between NK-mediated recognition of HLA-C allotypes and the dimorphism defined by amino acid substitution at positions 77 and 80, we have assessed whether directed mutagenesis at these two sites affects NK-mediated recognition of HLA-C. We find that single amino acid substitutions are sufficient to modify recognition of HLA-C alleles by group 1 or group 2 NK clones.

Materials and Methods

Cloning of $CD3^-16^+56^+$ Lymphocytes. PBL were isolated on Ficoll-Hypaque gradients and then incubated for 30 min at 4°C with a mixture of anti-CD3 (OKT3; Ortho Pharmaceuticals, Raritan, NJ), anti-CD4 (HP2.6), and anti-CD8 (B9.4) mAbs followed by treatment with rabbit complement for 1 h at 37°C, as described previously (9). Viable cells were isolated and cloned under limiting dilution conditions in the presence of irradiated feeder cells, 0.5% (vol/vol) PHA (Gibco Ltd., Paisley, Scotland), and exogenous IL-2 (rIL-2; Cetus Corp., Emeryville, CA), as previously described for both T and NK cell cloning (9–11).

Site-directed Mutagenesis. Site-directed mutagenesis was performed using two-step PCR as described by Ho et al. (12). This

Table 1. *Primers Used for Site-directed Mutagenesis of HLA-Cw3 or HLA-Cw4 Alleles*

Primer	Sequence	HLA-C cDNA nucleotide number
Universal UP*	CGCCAGGGTTTTCCCAGTCACGAC	(M13mp19)‡
Universal DOWN	CTCCGCAGGGTAGAAGC	692-708
A	CGCAGTTTCCGCAGGTTCA	299-317
B	GTGAACCTGCGGAAACTG	298-316
C	CGCAGTTTCCGCAGGCTCA	299-317
D	GTGAGCCTGCGGAAACTG	298-316
E	CGCAGGTTCCGCAGGTTCA	299-317
F	GTGAACCTGCGGAACCTG	298-316

* Each set of primers was used in a 30 cycles PCR (30 s at 94°C, 30 s at 55°C, 30 s at 72°C) followed by a final elongation of 7 min at 72°C.
‡ The universal primer UP has been designed on the sequence of M13mp19 vector and therefore does not correspond to any cDNA nucleotide number.

method requires two specific mutagenic primers (forward and backward) and two universal primers flanking the region to be mutated. Briefly, in separate PCR tubes, two fragments of the target gene were amplified. Each PCR used one universal primer at one end of the gene sequence and one mutagenic internal primer that contained the mismatched base at the site to be mutated. As template, M13mp19-HLA-C*0302 (Cw3) and M13mp19-HLA-C*0401 (Cw4) were used. Amplification fragments obtained in the first step were gel purified and used in the second PCR with universal primers. The oligonucleotide sequences of the primers are shown in Table 1. Primers A and B were designed to generate the Cw3 double mutant (Cw3-DM), in which residues 77 and 80 were changed to Asn and Lys by inducing nucleotide mutations at positions 302 and 312, respectively (indicating cDNA nucleotide number). To generate single mutated Cw3 or Cw4 molecules (Cw3-SM80 or Cw4-SM77) carrying either Ser-77 and Lys-80 or Asn-77 and Asn-80 (Cw3-SM77 or Cw4-SM80), the set of primers C and D or the primer combination E and F were used, respectively. Each set of primers was used for 30 cycles of PCR (30 s at 94°C, 30 s at 55°C, 30 s at 72°C) with a final elongation of 7 min at 72°C using a thermocycler (Perkin-Elmer Corp., Norwalk, CT). Products obtained from the second PCR were digested with the restriction enzymes SalI and PstI, and inserted into M13mp19-HLA-Cw*0302 or M13mp19-HLA-Cw*0401, which had been cleaved with the same enzymes. Sequences of the 600-bp fragments inserted in M13mp19 were determined to verify the fidelity of the mutations.

HLA-C Constructs and Transfections. 1.1-kb SalI-HindIII fragments (blunted at HindIII site) containing HLA-C–coding regions were isolated from M13mp19 constructs and inserted into the pRSV.5 neo (13) expression vector previously digested with SalI-BamHI (blunted at BamHI site). The same approach was used to obtain pRSV.5-HLA-B*4601 from M13mp18-HLA-B*4601. Either the HLA class I constructs or the pRSV.5 neo vector alone (used as negative control) were transfected by electroporation (250 V, 500 μF) into the HLA-A, -B, and -C class I null cell line LCL 721.221 (14) (indicated as 221, for simplicity) using a gene pulser (Bio-Rad Laboratories, Hercules, CA). After electroporation, cells were seeded in 75-cm^2 flasks and selected, after 96-h culture, in medium containing 0.6 mg/ml G418. Surface expression of HLA-C and HLA-B molecules was analyzed by indirect immunofluorescence using the HLA-C–specific mAb F4/326 (15) or the HB115 mAb (anti-HLA-B) (16). Cells expressing class I molecules were isolated by immunomagnetic purification using anti-HLA-C or -B mAbs and magnetic microspheres coated with anti-mouse Ig (Dynal, Oslo, Norway). Enriched cell populations were cloned by limiting dilution and selected clones were used as target cells in cytolytic assays.

Cytolytic Assays. The cytolytic activity of cloned NK cells was assessed in a 4-h ^{51}Cr release assay in which effector cells were tested against K562 target cells, the murine P815 cell line (either transfected with the HLA-Cw3 gene or untransfected) (3), or the Cw4$^+$ C1R human cell line (17). NK cell clones of defined HLA-C allele specificity, indicated as group 1 (Cw4 specific) or group 2 (Cw3 specific), were tested against the HLA class I–negative cell line 221; either untransfected, transfected with the pRSV.5 neo expression vector alone or transfected with one of the following native or mutated HLA class I alleles: HLA-Cw3, HLA-Cw4, HLA-B46, HLA-Cw3-DM, HLA-Cw3-SM77, HLA-Cw3-SM80, HLA-Cw4-SM77, and HLA-Cw4-SM80.

Results and Discussion

221 Cells Transfected with HLA-Cw3 or HLA-Cw4 Are Protected from Lysis by Different Groups of NK Clones. The human 221 cell line, which lacks surface expression of HLA-A, -B, and -C molecules (14), was transfected with cDNA coding for different HLA class I heavy chains. 221 cells transfected with HLA-C alleles were analyzed for surface expression of HLA-C molecules using F4/326, an mAb specific for an epitope shared by all HLA-C allotypes (15). As shown in Fig. 1, 221 cells transfected with either Cw3 (Fig. 1 *C*) or Cw4 (Fig. 1 *D*) cDNA expressed high levels of HLA-C. On the contrary, no HLA-C expression was detected in untransfected 221 cells (Fig. 1 *A*) or 221 cells transfected with the RSV.5 neo vector (Fig. 1 *B*).

Previous studies have indicated that target cells expressing the Cw3 allele are protected from lysis by NK clones belonging to group 2, whereas expression of Cw4 confers protection from group 1 NK clones (3, 5–7). In the present study, we examined whether the HLA class I–negative, NK-sensitive, 221 cell line similarly acquired resistance to lysis upon expression of appropriate HLA-C alleles. As shown in Table

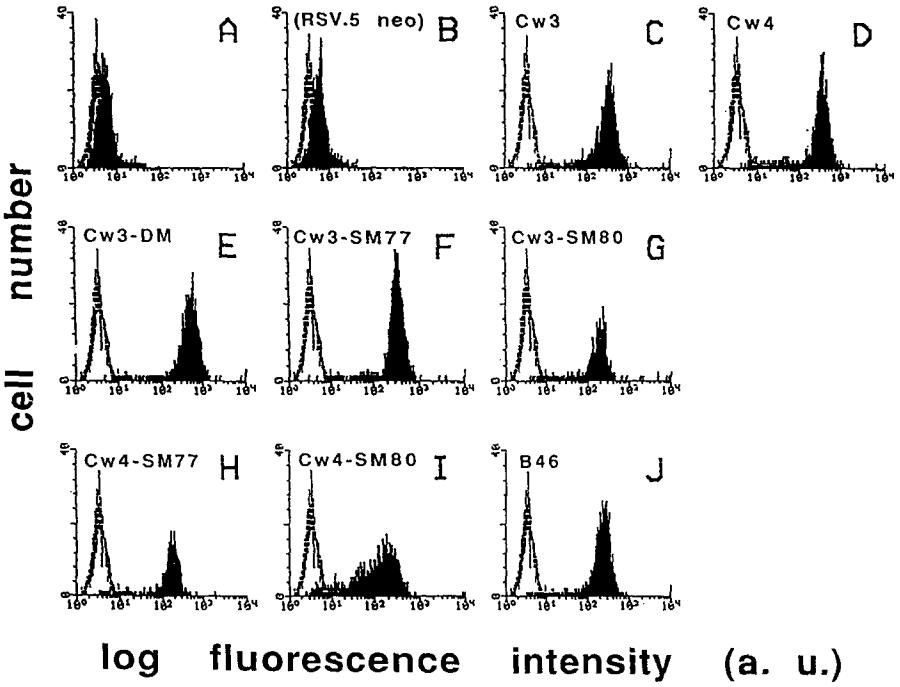

Figure 1. Surface expression of native or mutant HLA-Cw3 or HLA-Cw4 alleles transfected in the HLA class I–negative 221 cell line. The HLA class I–negative 221 cell line was transfected with HLA-Cw3 (*C*), HLA-Cw4 (*D*), and with mutants obtained by site-directed mutagenesis at positions 77 and/or 80 (*E–I*), as indicated in the text. A natural chimeric molecule (HLA-B46) containing in a backbone of HLA-B62 molecules, the relevant 77 and 80 amino acid positions of Cw3 has also been transfected (*J*). (A) Untransfected 221 cells; (*B*) 221 cells transfected with the expression vector RSV.5 neo alone. Cells were stained with the F4/326 mAb specific for HLA-C molecules or HB115, specific for HLA-B molecules, followed by a fluorescein-conjugated goat anti–mouse second reagent. *a.u.*, arbitrary units.

2, while 221 cells transfected with the pRSV.5 neo vector alone were lysed by representative clones belonging to either group 1 or group 2 NK clones, Cw3-transfected 221 cells (221/Cw3) were resistant to lysis by group 2 clones and susceptible to group 1 NK clones. Conversely, Cw4-transfected 221 cells were lysed by group 2 but not group 1 NK clones.

Protective Effect of HLA-Cw3 Molecules Mutated at Positions 77 and/or 80. To investigate the role of residues 77 and 80 in the differential protective effect exerted by HLA-C allotypes we generated, using site-directed mutagenesis, a mutant Cw3 in which positions 77 and 80 were substituted with the amino acids present in the Cw4 heavy chain. The resulting mutant, termed Cw3-DM, had Asn (instead of Ser) at position 77 and Lys (instead of Asn) at position 80. 221 cells transfected with Cw3-DM (221/CW3-DM) were analyzed for the surface expression of the mutated HLA-C allele. As shown

Table 2. *Protective Effect of Native or Mutagenized HLA-Cw3 and HLA-Cw4 Molecules from Group 1 or Group 2 NK Clone–mediated Lysis*

		221*	221 Cw3	221 Cw4	221 Cw3 DM	221 Cw3 SM-77	221 Cw3 SM-80	221 Cw4 SM-77	221 Cw4 SM-80	221 B46
Aa positions	77/80	– / –	Ser/Asn	Asn/Lys	Asn/Lys	Asn/Asn	Ser/Lys	Ser/Lys	Asn/Asn	Ser/Asn
NK clones										
Group 1‡	2F3	95§	100	5	14	100	2	0	100	83
	1F23	100	46	5	3	51	4	5	72	94
	PIC12	64	62	5	2	52	4	2	71	31
Group 2	AM72	95	2	46	70	31	7	9	70	8
	G15	100	8	65	89	74	21	2	95	14
	B5	85	1	38	58	44	6	8	61	7

* Target cells were represented by the HLA class I–negative human 221 cell line transfected with either the RSV.5 neo–expressing vector alone simply indicated as 221 or with the indicated HLA-C or HLA-B molecules.

‡ Alloreactive clones belonging to the two groups of specificities were obtained as described in a previous report (9).

§ Results are expressed as percent of ^{51}Cr release at an E/T ratio of 10:1.

in Fig. 1 *E*, the HLA-C expression of 221/Cw3-DM was comparable to that of 221/Cw3 or 221/Cw4 cells. We analyzed the ability of Cw3-DM molecules to protect 221 cells from lysis by group 1 or group 2 clones. As shown in Table 2, 221/Cw3-DM cells were resistant to lysis by group 1 clones, but were lysed by group 2 clones. Evident is that 221/Cw3-DM cells display the same pattern of susceptibility and resistance to lysis as 221/Cw4 cells.

To investigate further whether one of the two positions play a predominant role in the protective effect, we made Cw3 mutants in which either residue 77 or 80 was mutated to the residue found at the homologous position in Cw4: mutant Cw3-SM77 has Asn at position 77 (substituted from Ser) and mutant Cw3-SM80 has Lys at position 80 (substituted from Asn).

After transfection, both Cw3-SM77 and Cw3-SM80 were expressed at the surface of 221 cells at levels comparable to those seen for the Cw3 and Cw4 transfectants (Fig. 1, *F* and *G*). As shown in Table 2, the 221/Cw3-SM77 cells were lysed by both group 1 and group 2 NK clones. In contrast, the 221/Cw3-SM80 cells were resistant to both group 1 and group 2 NK clones. It thus appears that the presence of a Ser-77 is crucial for protection from lysis by group 2 NK clones. These results also show that the introduction of Lys at position 80 confers to Cw3 molecules the capacity to protect target cells from lysis by group 1 NK clones. Moreover, Cw3-SM80 maintained the ability to protect 221 target cells from group 2 NK clones.

Protective Effect of HLA-Cw4 Molecules Mutated at Position 77 or 80. Since single amino acid substitutions are sufficient to modify the protective effect of Cw3 molecules, we examined whether mutant Cw4 molecules, possessing the amino acids typical of the Cw3 allele at positions 77 or 80 (Ser and Asn, respectively), acquired a Cw3-like protective effect. Two Cw4 mutants, having a substitution at either position 77 or 80, were made and transfected in 221 cells. As shown in Fig. 1, *H* and *I*), 221 cells expressing the made Cw4-SM77 mutant or the Cw4-SM80 mutant expressed levels of HLA-C comparable to the Cw3 or Cw4 transfectants. In cytolytic assays, the 221/Cw4-SM80 cells were lysed by both group 1 and group 2 NK clones (Table 2), whereas the 221/Cw4-SM77 cells resisted to lysis by both groups of NK clones (Table 2). These results confirm that Lys-80 plays a crucial role in the protective effect from group 1 clones, and they also indicate that Ser-77 is important for conferring protection from group 2 clones.

221 Cells Transfected with HLA-B46. We further analyzed the role of the 77 and 80 amino acid positions in an unusual HLA-B allele (HLA-B*4601). This allele has a chimeric primary structure that is identical to that of HLA-B62, except in residues 66-77 of the α1 helix, where it is identical to Cw3 (18). Thus, at positions 77 and 80, B46 has the same residues as Cw3. HLA-B46 molecules were brightly expressed on 221 transfectants (Fig. 1 *J*). In addition, 221/B46 cells were found to be resistant to group 2 but not to group 1 NK cell clones, thus mimicking the protective effect of Cw3 molecules (Table 2).

Concluding Remarks. The data presented here demonstrate that single amino acid substitutions at position 77 or 80 in Cw3 and Cw4 molecules are sufficient to alter the protection of target cells from lysis by the group 1 and 2 NK cell clones. Lys 80 is essential for protection from group 1 clones and Ser 77 is essential for protection from group 2 clones. In addition, when Cw3 mutant molecules having the Asn 77 and Lys 80 residues present in Cw4 alleles were made, they were found to have lost the protective effect typical of Cw3 and instead acquired that typical of Cw4. Our results are consistent with the finding by Storkus et al. that amino acid substitution at position 74, in the α1 helix of HLA-A2, modified the capacity of this molecule to protect target cells from lysis by polyclonal NK cells obtained from peripheral blood (19).

Noteworthy is that residues 77 and 80 are in the peptide-binding groove and, more precisely, contribute to the formation of the F pocket. According to Zemmour and Parham, both amino acids have potential contact with a bound peptide (20). Thus, one may visualize a role for bound peptide in the protective effect. In this context, NK cells may recognize directly the bound peptide or, alternatively, they could sense changes in the conformation of class I MHC molecules caused by peptide binding.

Of relevance is the protective capability of an unusual HLA-B allele, HLA-B*4601. In this molecule, the peptide binding groove appears to be largely composed of amino acids belonging to the HLA-B allele. Transfectants of 221 cells with B46 allele were found to be resistant to group 2 but not to group 1 NK clones, thus behaving like 221/Cw3-transfected cells. Since one may speculate that, in HLA-B46, peptide binding is influenced primarily by HLA-B–encoded structures, we suggest that the type of bound peptide is not crucial for the NK-mediated recognition.

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