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The Bw4 Public Epitope of HLA-B Molecules Confers Reactivity with Natural Killer Cell Clones that Express NKB1, A Putative HLA Receptor

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Summary

Although inhibition of natural killer (NK) cell–mediated lysis by the class I HLA molecules of target cells is an established phenomenon, knowledge of the features of class I molecules which induce this effect remains rudimentary. Using class I alleles HLA-B*1502 and B*1513 which differ only at residues 77–83 which define the Bw4 and Bw6 serological epitopes, we tested the hypothesis that the presence of the Bw4 epitope on class I molecules determines recognition by NKB1$^+$ NK cells. HLA-B*1513 possesses the Bw4 epitope, whereas B*1502 has the Bw6 epitope. Lysis by NKB1$^+$ NK cell clones of transfected target cells expressing B*1513 as the only HLA-A, -B, or -C molecule was inhibited, whereas killing of transfectants expressing B*1502 was not. Addition of an an anti-NKB1 monoclonal antibody reconstituted lysis of the targets expressing B*1513, but did not affect killing of targets bearing B*1502. The inhibitory effect of B*1513 could be similarly prevented by the addition of an anti-class I monoclonal antibody. These results show that the presence of the Bw4 epitope influences recognition of HLA-B molecules by NK cells that express NKB1, and suggest that the NKB1 molecule may act as a receptor for Bw4$^+$ HLA-B alleles. Sequences outside of the Bw4 region must also affect recognition by NKB1$^+$ NK cells, because lysis of transfectants expressing HLA-A*2403 or A*2501, which possess the Bw4 epitope but are in other ways substantially different from HLA-B molecules, was not increased by addition of the anti-NKB1 antibody. Asparagine 86, the single site of N-linked glycosylation on class I molecules, is in close proximity to the Bw4/Bw6 region. The glycosylation site of the Bw4-positive molecule B*5801 was mutated, and the mutant molecules tested for inhibition of NKB1$^+$ NK cells. Inhibition that could be reversed by addition of the anti-NKB1 monoclonal antibody was observed, showing the presence of the carbohydrate moiety is not essential for class I recognition by NKB1$^+$ NK cell clones.

NK cells are a subset of lymphocytes which display spontaneous cytotoxicity for a variety of target cells (1). Although cytolysis by NK cells is MHC unrestricted, recent evidence indicates that interactions with class I HLA antigens can regulate NK cell function. In contrast to MHC-restricted T cells, which require HLA antigens for target cell lysis, class I expression appears to inhibit lysis by NK cells. Initial observations correlated low class I MHC expression with the sensitivity of tumor cell lines to natural killing (2). Transfection of class I genes into susceptible target cells provided protection from lysis by NK cells, demonstrating that inhibition was caused by class I (3, 4). Inhibition is dependent upon the class I allele, since in one study several transfected HLA-A and -B alleles provided protection, but HLA-A*0201 did not (5). Characterization of the patterns of lysis of a panel of class I transfected target cells by NK cell clones, demonstrated that killing is usually inhibited by multiple class I alleles, and indicated the existence of considerable heterogeneity in the HLA specificity of NK cell clones (6). From these observations it appears NK cells recognize polymorphic HLA antigens, and this interaction inhibits target cell lysis.

Attention has been focused on the role of HLA-C antigens in governing NK cell lysis, by observations that expression of self or related HLA-C molecules exerts a dominant inhibitory effect on target cell lysis by NK cells (7). NK cells appear to distinguish two groups of HLA-C alleles based on a diallelism at residues 77 and 80 of the heavy chain sequence. Two potential receptors of 58 kD molecular mass, which may be involved in recognition of the HLA-C groups, have been identified by Moretta and colleagues (8). NK cell clones which

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express these p58 molecules fail to lyse targets bearing certain HLA-C alleles, and killing is restored in the presence of anti-p58 monoclonal antibodies (8).

Recently, another putative NK cell receptor for HLA has been identified (9). This molecule, called NKB1, is distinct from the p58 molecules, in that it has a molecular mass of 70 kD and appears to confer reactivity with certain HLA-B alleles. NK cell clones bearing NKB1 were always inhibited by target cell expression of HLA-B*5801, B*5101, and B*2705, but not by other HLA-B alleles, or by HLA-A and -C alleles (9). Sequence analysis of the inhibitory alleles suggests that reactivity might correlate with the Bw4 public epitope.

Every HLA-B allele possesses one of two mutually exclusive serological epitopes known as Bw4 and Bw6 (10). Bw6 is present on approximately 2/3 of HLA-B alleles, while Bw4 is on the remaining 1/3. These epitopes appear to be evolutionarily ancient features of class I molecules, since related determinants are found on class I molecules from non-human primate species (11). Bw4 and Bw6 were originally thought to define two HLA-B antigens, but were later recognized to represent shared determinants of a much larger family of polymorphic alleles (12). Such shared determinants are known as public epitopes (13). Bw6 is only found on HLA-B alleles; Bw4 is found on HLA-B and some HLA-A alleles.

Sequence comparisons of alleles possessing the Bw4 or Bw6 epitope indicated that the residues involved in forming these determinants map to positions 77–83 of the class I molecule (14, 15). Analysis of HLA-A and B molecules which react with Bw4 alloantisera, reveals some heterogeneity in the amino acids comprising this determinant, while alleles that possess the Bw6 epitope are invariant in this region, with the exception of one allele, B*7301 (16, 17). Serology has identified many pairs of HLA-B alleles which appear to differ only at the Bw4/Bw6 region, and determination of the primary structures of these antigens has proved this prediction correct. Thus this region has apparently been the subject of frequent gene conversion events.

These observations have led to much speculation as to the possible function of the Bw4 and Bw6 epitopes (10). Some of the polymorphic residues that define these epitopes are thought to interact with bound peptides, so they may influence peptide specificity (18). Differences in this region can also affect T cell recognition (19). However, to date no clear function has been assigned to the Bw4 and Bw6 epitopes. The potential association of Bw4 with NK cell reactivity may therefore help to clarify the function of these determinants.

Residues 77–83 which determine the Bw4/Bw6 epitopes are in close proximity to asparagine 86, the highly conserved site of N-linked glycosylation on class I molecules (20). Indeed, for human class I molecules, this is the only known site of glycosylation. The carbohydrate moiety is not required for cell surface expression of class I molecules (21, 22) and its function remains unclear. The finding that Ly-49, a murine NK cell receptor which binds H-2D^d (23, 24), contains an extracellular domain with homology to mammalian C-type lectins, raises the possibility that carbohydrate binding may be involved in NK cell interactions with class I molecules.

The experiments described here were designed to investigate class I recognition by NK cells bearing the NKB1 surface antigen. To test the hypothesis that the putative NK cell receptor NKB1 is influenced by the Bw4/Bw6 diallelism, we examined the inhibitory effect of two HLA-B molecules which differ only at these epitopes. The importance of the carbohydrate moiety in class I recognition by NKB1$^+$ NK cells was also investigated using class I molecules mutated at the site of glycosylation.

Materials and Methods

Preparation of NK Cell Clones. Mononuclear cells from the peripheral blood of two healthy adults were isolated by density centrifugation with Ficoll-Hypaque (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). PBMC were stained with FITC-conjugated anti-CD56 mAb (Leu 19), and PE-conjugated anti-NKB1 (DX9), and Cychrome-conjugated anti-CD3 (Leu 4). NK cells (CD56$^+$-CD3$^-$) which expressed NKB1, were cloned at one cell per well using a single cell deposition system of the FACStar^{PLUS} flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Clones were established using culture conditions described previously (25). All clones generated by this procedure had the cell surface phenotype CD3$^-$, CD56$^+$, NKB1$^+$, and mediated NK cytolytic function.

Target Cell Panel. Class I HLA cDNA was prepared from EBV-transformed cell lines, subcloned into the phage M13 vector and sequenced as described (26). cDNAs encoding HLA-A*0201, A*0211, A*2403, A*2501, A*3601, B*0702, B*1501, B*1502, B*1508, B*1513, B*3505, B*4801, B*5501, B*5801, Cw*0102, Cw*0304, Cw*0401, Cw*0801, and Cw*1503 were subcloned into the expression vector pBJ1-neo (27, kindly provided by Dr. Mark Davis, Stanford University, Palo Alto, CA). The class I HLA-A, B, and C negative B lymphoblastoid cell line, 721.221 (28), was transfected by electroporation at 250V and 960 μF in a 0.4-cm gapped cuvette with pBJ1-neo containing the class I HLA cDNAs, and grown under G418 drug selection (1 mg/ml). Cells were stained with the monomorphic anti-HLA-A, B, and C mAb W6/32 conjugated to fluorescein (W6/32-F1), and those cells expressing high surface levels of class I antigen were sorted using a FACStar^{PLUS} flow cytometer (Becton Dickinson). A construct containing the HLA-B*2705 gene subcloned into the pHEBo expression vector (29) was transfected into the 721.221 cell line by electroporation and cells were grown under 300 μg/ml hygromycin drug selection. The B*5101/721.221 transfectant was generously provided by Dr. Robert DeMars (University of Wisconsin, Madison, WI).

Flow Cytometry. Methods of immunofluorescent staining and flow cytometric analysis were as described (30). Analysis was performed on a FACScan flow cytometer (Becton Dickinson). For flow cytometric analysis the W6/32 mAb was conjugated to fluorescein (W6/32-F1) by reaction with fluorescein-X-NHS (Molecular Probes, Inc., Eugene, OR). 721.221 cells transfected with the class I HLA alleles listed above were stained with W6/32-F1 and monitored by flow cytometry on a regular basis to assess the level and uniformity of class I HLA antigen surface expression.

Preparation of mAb and F(ab')$_2$. The DX9 mAb precipitates a single 70-kD band from NK cells (9). The DX15 mAb precipitates a non-disulfide-linked dimer consisting of polypeptides identical in mobility to the class I HLA heavy chain and β_2-microglobulin, and specifically stains 721.221 HLA transfectants but not the untransfected 721.221 cell line. The DX9 and DX15 IgG$_1$ hybrid-

omas were generated by immunization of BALB/c mice with with the human NK clone VL186-1 ($CD3^-$, $CD16^+$, $CD56^+$) and fusion of their splenocytes with the Sp2/0 myeloma cell line (9). Ascites was obtained, and IgG was purified by caprylic acid precipitation, followed by saturated ammonium sulfate precipitation. $F(ab')_2$ fragments were prepared by digesting DX15 IgG with immobilized pepsin (10 mg/ml in 10 ml of 0.2M sodium citrate, 0.15 M NaCl buffer, pH 3.5, with 2.5 ml immobilized pepsin for 2 h at 37°C) (Pierce Chemical Co., Rockford, IL). Fc-containing species were then removed by protein A affinity chromatography. The $F(ab')_2$ fragment preparation was determined to be pure by SDS-PAGE analysis.

Cell-mediated Cytotoxicity. Cell killing was measured using a standard 4-h ^{51}Cr-radioisotope release assay (31). Percent lysis was calculated by the formula:

$$\text{(Experimental cpm} - \text{spontaneous cpm)/(Total cpm} - \text{spontaneous cpm)} \times 100 = \%\ \text{lysis}$$

All assays were carried out using an effector to target ratio of 6:1. Antibodies, DX9 mAb or DX15 $F(ab')_2$, were added where specified to a final concentration of 6.7 μg/ml. Assays were performed in duplicate or triplicate, and were repeated from two to five times for each clone. Only assays in which the specific lysis of the untransfected 721.221 cell line was ≥30% were included in the analysis.

Site-directed Mutagenesis of HLA-B5801. Two mutants were made: in one Asn 86 was replaced by Gln, and in the other Ser 88 was replaced by Ala. Purified B*5801 cDNA was subcloned into the phage M13 vector and used as a template for site-directed mutagenesis. Two first step PCR reactions were performed with primers encoding the desired mutation and primers that bind either to the 3′ end or the 5′ end of the B*5801 cDNA, outside of the coding region. The PCR products from this reaction were then combined, and a second PCR reaction was performed, using only the outside primers. The complementary primer pairs used were as follows: for the B58.Q86 mutant, 5′-CTCCGCTACTACCAACAGAGCGAGGCCGGG-3′ and 5′-CCCGGCCTCGCTCTGTTGGTAGTAGCGGAG-3′. For the B58.A88 mutant, 5′-CTCCGCTACTACAACCAGGCCGAGGCCGGG-3′ and 5′-CCCGGCCTCGGCCTGGTTGTAGTAGCGGAG-3′. For binding to the 5′ end of the B*5801 cDNA the following primer encoding a SalI restriction site was used: 5′-GGGCGTCGACGGACTCAGAATCTCCCCAGACGCGAG-3′. For binding to the 3′ end of the B*5801 cDNA the following primer encoding a HindIII restriction site was used: 5′-CCGCAAGCTTCTGGGGAGGAAACACAGGTCAGCATGGGAAC-3′. The final PCR products were digested with SalI and HindIII restriction endonucleases and subcloned into the pBJ1-neo expression vector. DH5α *Escherichia coli* bacteria were then transformed with the vector containing the insert cDNA. Vector DNA was prepared from individual bacterial colonies, and the class I cDNA insert was sequenced completely to ascertain the presence of the desired mutations and the absence of nucleotide misincorporations introduced during the PCR reactions. 721.221 cells were then electroporated with the vector containing the mutated class I insert, as described above.

Immunoprecipitation, SDS-PAGE, and IEF Analysis. 721.221 class I HLA transfectants were biosynthetically labeled with ^{35}S-Cys and ^{35}S-Met (Amersham Corp., Arlington Heights, IL), the cells were then lysed and class I HLA molecules immunoprecipitated with the anti-class I monoclonal antibody W6/32 as described (32). Immunoprecipitates were divided into four aliquots, and subjected to digestion with neuraminidase type VI (Sigma Chemical Co., St. Louis, MO) for 3 h, or *N*-glycanase (Genzyme Corp., Cambridge, MA) for 16 h, or mock treated with 0.05 M EDTA or PBS, respectively. Samples treated with and without neuraminadase were subjected to one-dimensional isoelectric focusing gel electrophoresis, and autoradiography. Samples treated with and without *N*-glycanase were subjected to 10 and 12% Tris-glycine polyacrylamide gel electrophoresis, and autoradiography.

Results

The molecules chosen for study were B*1502 and B*1513, which encode the serological specificities HLA-B75 and HLA-B77, respectively. These antigens are characteristically found in southeast Asian populations (33). The B*1502 and B*1513 heavy chain sequences differ only within residues 77–83 of the α_1 helix, the region which determines the Bw4 and Bw6 epitopes (33). Cells of the mutant cell line 721.221, which does not express HLA-A, -B, or -C, were transfected with class I HLA cDNA, resulting in cell cultures expressing just one HLA-A, -B, or -C antigen. These cells were used as targets for a panel of NK cell clones that express the NKB1 molecule. A reduction of lysis of the transfectant compared to the untransfected 721.221 cell line indicated an inhibitory effect of the class I allele. To assess the involvement of the NKB1 molecule, assays were performed in the presence and absence of the anti-NKB1 monoclonal antibody, DX9.

*$NKB1^+$ NK Cells Are Inhibited by B*1513 But Not by B*1502.* NK cell clones bearing the NKB1 surface antigen were generated from two peripheral blood donors, and the HLA specificity of ten clones from each donor examined. Untransfected 721.221 cells were killed effectively by all of the NK cell clones, and this killing was unaffected by the presence of the anti-NKB1 antibody (Fig. 1 *a*). By comparison, all of the clones showed reduced lysis of transfectants expressing B*5801, a known inhibitory allele (9). In the presence of the anti-NKB1 antibody, however, lysis of the B*5801 transfectants was increased to a level comparable to that of the untransfected 721.221 cell line (Fig. 1 *b*). The pattern of lysis seen for 721.221 cells expressing the B*1502 molecule was the same as that seen for untransfected cells: each clone effectively lysed the B*1502 transfectant and no change was seen on inclusion of the antibody (Fig. 1 *c*). In contrast, the pattern of lysis obtained with the B*1513 transfectant was similar to that of the B*5801 transfectant: lysis was reduced compared to the untransfected cells, but was elevated to equivalent levels in the presence of the anti-NKB1 monoclonal antibody (Fig. 1 *d*). These different patterns of lysis were not due to differences in the class I expression levels of the B*1502 and B*1513 transfectants, since the two have comparable expression as monitored by flow cytometry (data not shown).

This experiment shows that B*1513 is able to inhibit lysis by $NKB1^+$ NK cell clones, and that this inhibition can be prevented by addition of the anti-NKB1 monoclonal antibody. In contrast, killing is unaffected by the closely related B*1502 molecule. Since these alleles differ only at the Bw4/Bw6 epitope, this result indicates that the presence of

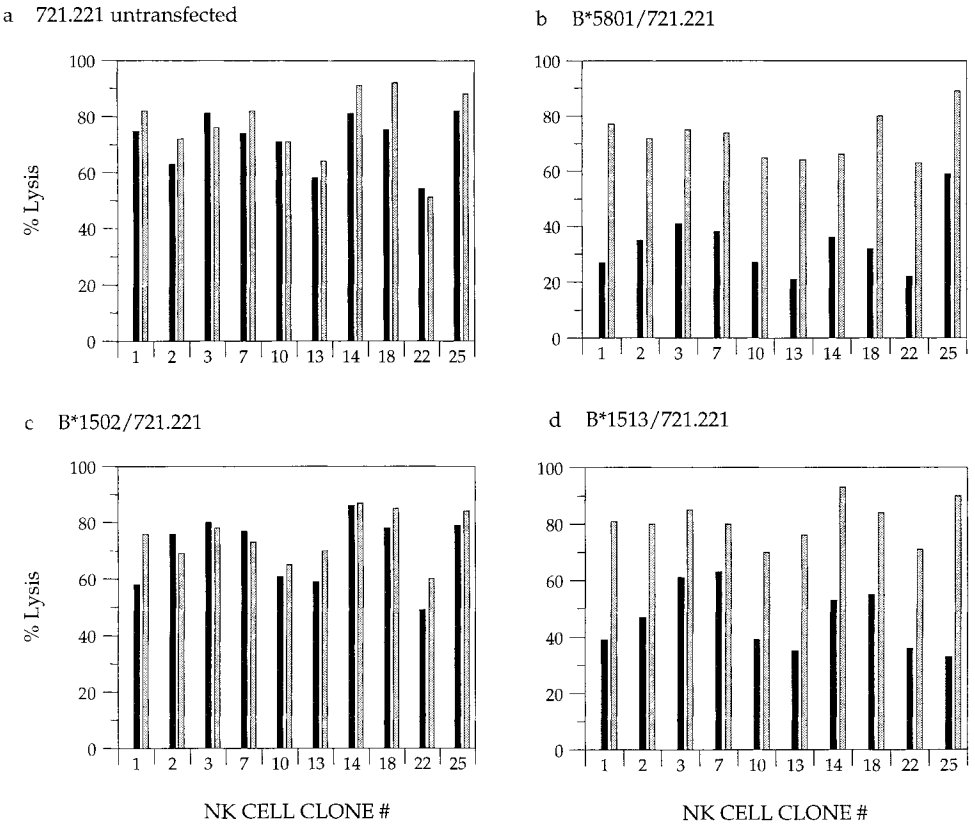

Figure 1. Recognition of HLA-B*1513 but not B*1502 class I alleles by NKB1+ NK cell clones. Percent lysis of class I HLA transfected 721.221 target cells by NKB1+ NK cell clones: (*a*) Untransfected; (*b*) B*5801 tranfectant; (*c*) B*1513 transfectant; (*d*) B*1502 tranfectant. Dark bars show killing in the absence of the anti-NKB1 mAb, DX9. Light bars show killing in the presence of DX9. All of the NK cell clones were established from a single blood donor.

the Bw4 epitope on HLA-B molecules confers reactivity with NKB1+ NK cells.

This thesis is supported by examination of NKB1+ NK cell reactivity with a panel of transfected 721.221 target cells each expressing a different HLA-A, -B, or -C molecule (Table 1). All of the NKB1+ NK clones showed diminished lysis in the presence of each of the Bw4+ HLA-B alleles tested, and lysis was increased by the addition of the anti-NKB1 monoclonal antibody. In contrast, while certain Bw6 reactive HLA-B alleles provided protection from lysis from some of the NKB1+ NK cell clones, none of these alleles inhibited all of the clones, and no clone was inhibited by all of the Bw6+ HLA-B alleles. Significantly, addition of the anti-NKB1 monoclonal antibody had no effect on lysis of these transfectants. Similarly, although HLA-A and HLA-C alleles inhibited some of the clones, this inhibition did not affect every clone, and was not altered by the presence of the antibody (Table 1).

The observation that inhibition of NKB1+ clones requires an appropriate class I molecule and is prevented by the addition of an antibody directed against the NKB1 molecule is suggestive of a receptor/ligand-like interaction between the NKB1 molecule and the class I molecule on the target cell. Further evidence in support of this model is the demonstration that addition of anti-class I monoclonal antibodies augments lysis of transfectants to an extent identical to that obtained with the anti-NKB1 antibody (Fig. 2, *a* and *b*). One anti-class I monoclonal antibody to give this effect was DX15, which emerged from the same fusion and selection as the anti-NKB1 monoclonal antibody DX9. The augmentation of NK cell mediated lysis seen in the presence of DX15 was not due to ADCC, since $F(ab')_2$ fragments were used for all experiments. Several other well characterized anti-class I monoclonal antibodies including MEI, MA2.1, and PA2.6 (34), also increase lysis of the HLA transfected target cells to which they bind (data not shown).

The Effect of the Bw4 Epitope Is Modulated by Sequences Elsewhere in the Class I Molecule. If NKB1 is an HLA receptor, then the simplest interpretation of our finding that HLA-B*1513 inhibits lysis whereas B*1502 does not, is that the amino acid substitutions at positions 77–83 of the B*1502 and B*1513 heavy chains influence the affinity of the interaction with NKB1. This could be due to direct recognition of residues within this region, or to a conformational effect that substitutions within this region have on another site of the class I molecule. To investigate the influence of sequences outside the Bw4/Bw6 region on recognition by NKB1+ NK cell clones, we examined lysis of tranfectants expressing HLA-A*2403 and A*2501. These alleles are recognized by Bw4 alloantisera, and analysis of their heavy chain sequences reveals that A*2403 is identical to B*1513 from positions 75–83, while A*2501 is identical from residues 78–83 (Fig. 3). However, both A*2403 and A*2501 differ substantially from HLA-B alleles in other parts of the heavy chain sequence (Fig. 3).

Expression of A*2403 or A*2501 inhibited lysis by 9 of

Table 1. *Comparison of HLA-A, -B, and -C Alleles Which React or Do Not React with NK Cells Bearing the NKB1 Molecule*

Transfected allele	Serological specificity	NKB1$^+$NK cell reactivity
B*5801	Bw4	+
B*5101	Bw4	+
B*2705	Bw4	+
B*1513	Bw4	+
B*1502	Bw6	−
B*1501	Bw6	−
B*3505	Bw6	−
B*4801	Bw6	−
B*0702	Bw6	−
B*5401	Bw6	−
B*5501	Bw6	−
Cw*0102	Neither	−
Cw*0304	Neither	−
Cw*0801	Neither	−
Cw*0401	Neither	−
Cw*1503	Neither	−
A*0201	Neither	−
A*0211	Neither	−
A*3601	Neither	−

Alleles that react with NKB1 + NK cells (shown by +) inhibit all NK cell clones that express NKB1, and addition of the anti-NKB1 monoclonal antibody augments lysis of these transfectants. Alleles that do not react with NKB1 + NK (shown by −) cells do not inhibit all of the NKB1 + clones, and addition of the anti-NKB1 monoclonal antibody fails to increase killing. (Data include transfectants tested in a previous study, reference 9).

10 NKB1$^+$ clones from each donor (18 out of 20 clones total), but killing was not increased by addition of the anti-NKB1 antibody (Fig. 4, *a–c*). Thus two HLA-A alleles that possess the Bw4 epitope, interact differently with NKB1$^+$ NK cells than do HLA-B alleles having this epitope. This could be due to qualitative differences in binding or to quantitative differences in affinity of these alleles for the NKB1 molecule, such that the presence of the anti-NKB1 antibody does not prevent interaction with A*2403 and A*2501. Alternatively, the NKB1$^+$ NK cells which are inhibited by these alleles might possess another receptor which interacts with the two HLA-A alleles, but is not blocked by the anti-NKB1 monoclonal antibody.

The Presence of the Carbohydrate Moiety on the Class I Molecule Is Not Essential for Inhibition of NKB1$^+$ NK Cells. To investigate whether the absence of the carbohydrate moiety affects class I mediated inhibition of NKB1$^+$ NK cells, we performed site-directed mutagenesis on the glycosylation site of the B*5801 heavy chain (see Fig. 5). This class I molecule was chosen because NKB1$^+$ NK cells were originally isolated from a B58$^+$ donor, and clones bearing NKB1 were known to be specifically inhibited by B*5801 expression (9). Two mutants were made, one in which asparagine 86 was replaced by glutamine (B58.Q86) and one in which serine 88 was replaced by alanine (B58.A88). The two mutants were transfected into 721.221 cells, and expression of the mutant molecules characterized. To assess the glycosylation state of the mutant heavy chains, immunoprecipitates were analyzed with and without neuraminidase and *N*-glycanase treatment. From the reduced molecular mass compared to the wild-type B*5801, and the lack of neuraminidase sensitivity, it was apparent that the B58.Q86 mutant was devoid of carbohydrate. A small proportion (~10%) of the B58.A88 mutant heavy chains were glycosylated.

Both mutants demonstrated an inhibitory effect on lysis by NKB1$^+$ NK cells (Fig. 6, *a* and *b*). Addition of the anti-NKB1 monoclonal antibody reconstituted the level of killing to that of the untransfected 721.221 cells. Although both mutants clearly reduced lysis compared to the untransfected 721.221, the inhibitory effect was not as strong as that observed with the wild-type B*5801 transfect. To investigate this difference further, the class I cell surface expression levels of the mutants were assessed by flow cytometric analysis using the monomorphic anti-class I antibody, W6/32. Both mutants had reduced cell surface expression compared with the

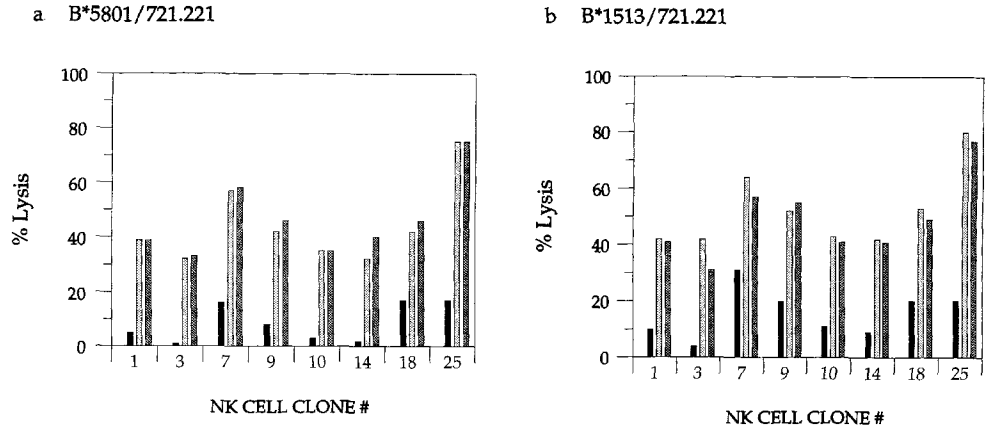

Figure 2. Cell killing is augmented to the same degree in the presence of anti-NKB1 antibodies and anti-class I antibodies. Addition of both mAbs increases lysis of the transfectants to the level of killing of the untransfected 721.221 cell line. Percent lysis of 721.221 transfectants by NKB1$^+$ NK cell clones: without mAb (*dark bars*), with the addition of the anti-NKB1 mAb, DX9 (*light grey bars*), with the addition of the anti-HLA mAb, DX15 (*dark grey bars*). (*a*) B*5801 transfectant; (*b*) B*1513 transfectant. All of the NK cell clones were established from a single blood donor.

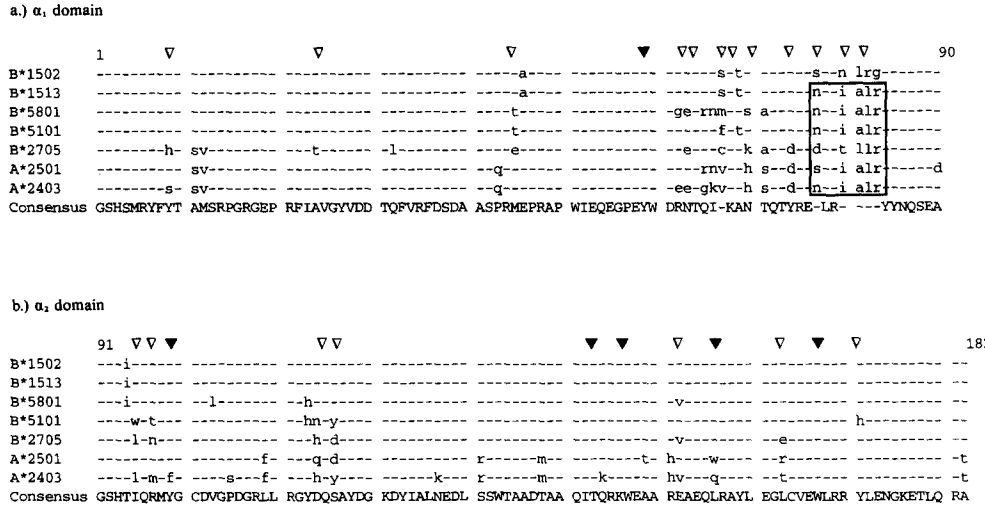

Figure 3. Alignment of the amino acid sequences of the α_1 and α_2 domains of HLA-A, and B heavy chains. Dashes indicate homology with the consensus sequence. The boxed residues confer reactivity with Bw4 alloantisera. Polymorphic residues of HLA-B alleles which are thought to interact with bound peptides are indicated by ▽, those which are shared among the alleles which inhibit NKB1$^+$ NK cells are marked by ▼.

wild-type B*5801 transfectant. The fluorescence intensity of the B58.A88 transfectant was ~5-fold lower than that of the wild type, whereas the B58.Q86 mutant was ~10-fold lower (Fig. 7, *a–c*). From assessment of the results from multiple experiments, the degree of protection from lysis by an NKB1$^+$ clone could be correlated with the level of class I expression at the target cell surface (R^2 = 0.88, n = 21), suggesting that the reduced inhibition by the mutant class I molecules was due to their decreased expression.

To rule out the possibility of a protective effect mediated by elevated expression of class I molecules lacking β_2-microglobulin, the amount of free class I heavy chain on the cell surface of the B58 mutant transfectants was assessed by indirect flow cytometry using Q1/28. This monoclonal antibody binds predominantly to class I heavy chains that are not associated with β_2-microglobulin (35). The intensity of staining with the Q1/28 antibody was proportional to the total amount of class I on the cell surface as measured by W6/32 staining, and neither mutant appeared to have elevated levels of free heavy chain compared to the wild-type B*5801 transfectant (data not shown).

These experiments demonstrate that the inhibitory effect of B*5801 on NKB1$^+$ NK cells does not require the presence of the carbohydrate moiety. The inhibitory effect of the B58.A88 mutant could be due to recognition of the subpopulation of glycosylated forms of this molecule, however inhibition was also observed with the B58.Q86 mutant which completely lacks glycosylated forms, indicating that the carbohydrate moiety is not essential for recognition. Moreover, it is striking that the mutant B58 molecules provided protection from lysis by NKB1$^+$ NK clones, despite being present at significantly reduced cell surface expression levels.

Discussion

The data presented here demonstrate that the Bw4 epitope is critical for recognition by NK cells which express the NKB1 antigen. All of the HLA-B alleles tested which bear the Bw4 epitope inhibit NKB1$^+$ NK cells, while HLA-A and -C alleles and HLA-B alleles with the Bw6 epitope do not. Furthermore, inhibition can be prevented by the binding of an antibody either to the NKB1 molecule, or to the class I molecule. These observations suggest NKB1 is an inhibitory receptor with specificity for the Bw4 epitope of HLA-B alleles, however proof of this thesis awaits direct demonstration of NKB1 binding to the class I molecule.

If the NKB1 molecule is a class I receptor, the residues at positions 77–83 of the class I heavy chain must influence recognition, since HLA-B*1502 and B*1513 differ only in this region. Recognition of inhibitory HLA-C alleles by NK cells which express the p58 molecules also appears to be based on differences at positions 77 and 80 of the class I molecule (8). Therefore, NKB1 and the p58 molecules may recognize a similar region of the class I molecule. Moreover, Storkus et al. found that the presence of His at position 74 of the class I heavy chain affected recognition of HLA-A*0201 by NK cells (5). Thus the carboxy-terminal half of the α_1 helix is consistently implicated in NK cell recognition of class I molecules.

Residues 77–83 comprising the Bw4 epitope may represent the NKB1-binding site, or substitutions in this region may affect the conformation of an NKB1-binding site elsewhere on the class I molecule. Residues within the 77–83 segment can contribute to interactions with the bound peptide, and it is also possible that differences in the peptides bound by HLA-B*1502 and B*1513 contribute either directly or indirectly to the conformation of the NKB1-binding site. Viral infection of HLA transfectants renders them susceptible to lysis by NK cells (36), and hence the ability to detect differences in bound peptides might be a mechanism by which NK cells recognize virally infected cells.

Class I molecules bind peptides in an allele-specific manner, with polymorphic residues within the peptide-binding groove conferring selectivity. Residues 77, 80, and 81 within the Bw4/Bw6 region are predicted to interact with bound peptides. The COOH-terminal amino acid of the peptide binds

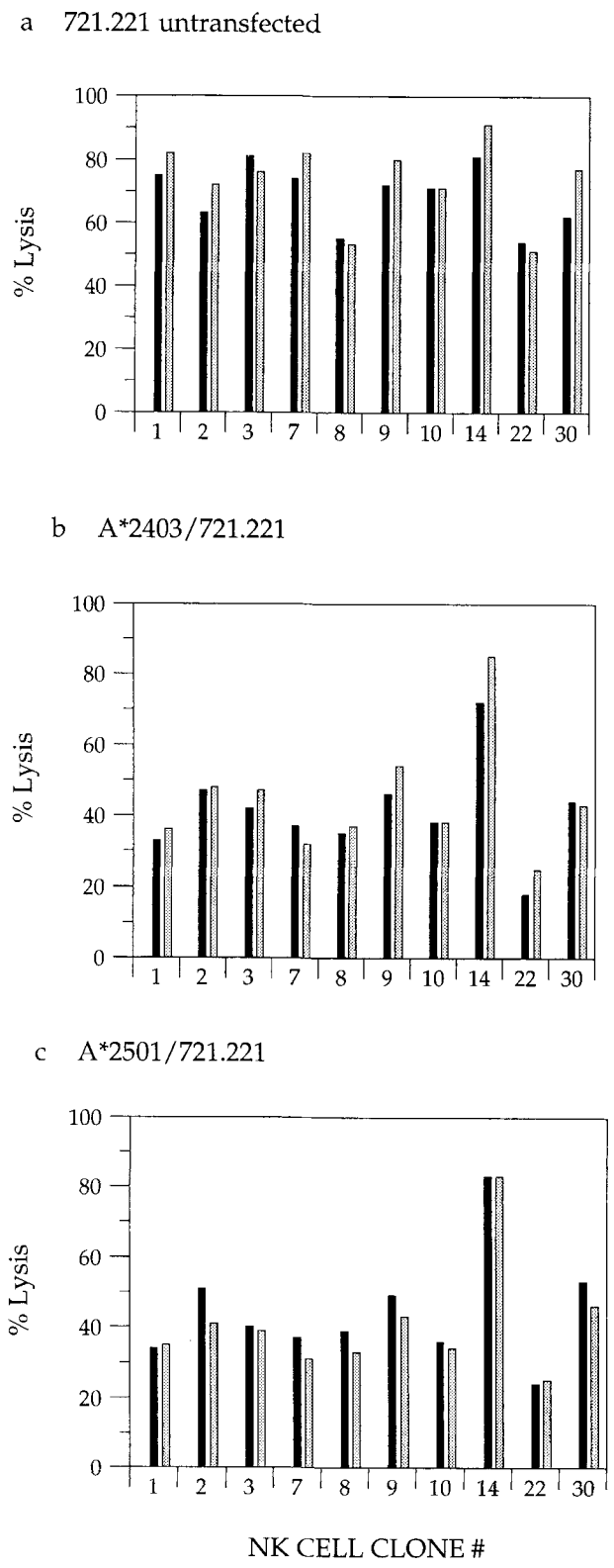

Figure 4. The inhibitory effect of HLA-A alleles which possess the Bw4 epitope is not affected by the addition of the anti-NKB1 monoclonal antibody. Percent lysis of class I HLA transfected 721.221 target cells by NKB1$^+$ NK cell clones: (*a*) untransfected 721.221; (*b*) A*2403 transfectant; and (*c*) A*2501 transfectant. Dark bars show killing in the absence of the anti-NKB1 mAb, DX9. Light bars show killing in the presence of DX9. All of the NK cell clones were established from a single blood donor.

near these positions, so the substitutions which define the Bw4 and Bw6 epitopes may affect the identity of the last residue of the peptide. Peptide binding motifs have been analyzed for HLA-B*3501 and B*5301 which differ, like B*1502 and B*1513, only in the 77–83 region. B*5301 possesses the Bw4 epitope; B*3501 has the Bw6 epitope. Hill et al. found by sequence analysis of the pool of peptides eluted from B*3501 that tyrosine was the only amino acid selected at the COOH terminus, whereas B*5301 had no amino acid preferences at this position (37). However, a subsequent analysis by Falk et al. identified hydrophobic residues as well as tyrosine at the COOH-terminal residue of B*3501-binding peptides (38). Thus the influence of polymorphism within the Bw4/Bw6 region of the class I heavy chain on the bound peptide repertoire is not yet understood.

Apart from the shared Bw4 epitope there is little overall similarity in the peptide-binding site of the HLA-B molecules recognized by NKB1$^+$ NK cells. B*5801, B*5101, B*2705, and B*1513 have the same residue at only 6 of the 25 polymorphic peptide binding positions of the α_1 and α_2 domains of HLA-B molecules (Fig. 3). In pairwise comparisons B*5801 and B*5101 each differ from B*1513 at several peptide binding positions, and differ from each other at 11. Furthermore analysis of peptides bound to B*2705 showed that this allele has a preference for peptides with arginine at the second position, while B*5101 binds peptides with proline at this position (37, 39, 40). Therefore it seems unlikely that these alleles bind a common set of peptides. Chadwick and Miller (41) and Storkus et al. (42) have suggested that self peptides complexed with class I molecules are required for inhibition of NK cell-mediated lysis. The diverse peptide binding characteristics of the four alleles recognized by NKB1$^+$ NK cells suggest that class I binding of particular peptides derived from endogenous proteins is probably not necessary for recognition by this receptor. Likewise, recognition of HLA-C may not depend on the presence of specific bound peptides, since the sequences of the peptide pool eluted from HLA-C molecules reveal no correlation with NK cell reactivity (43). However, changes to the overall repertoire of bound peptides, such as could occur upon viral infection, might affect NKB1 recognition of class I molecules.

It is also possible instead that bound peptides do not influence HLA recognition by NK cells, and that the receptors recognize the class I molecule directly. Five related sequence motifs are found at positions 77–83 among HLA alleles with the Bw4 epitope, while alleles possessing the Bw6 epitope are invariant in this region, with the exception of B*7301 (Table 2). Two of the more divergent motifs are included among the alleles which inhibit NKB1$^+$ NK cells (Table 2 and Fig. 3), suggesting that conserved features of the Bw4 epitope may account for the observed NK cell reactivity. Class I alleles with the Bw4 epitope all possess leucine and arginine at positions 82 and 83, respectively, while those alleles which do not possess the Bw4 epitope express arginine and glycine respectively at these positions. Positions 82 and 83 are located on the "outer" face of the α_1 helix, and are postulated to be solvent accessible (44). Therefore these positions are candidates for direct recognition, and the presence

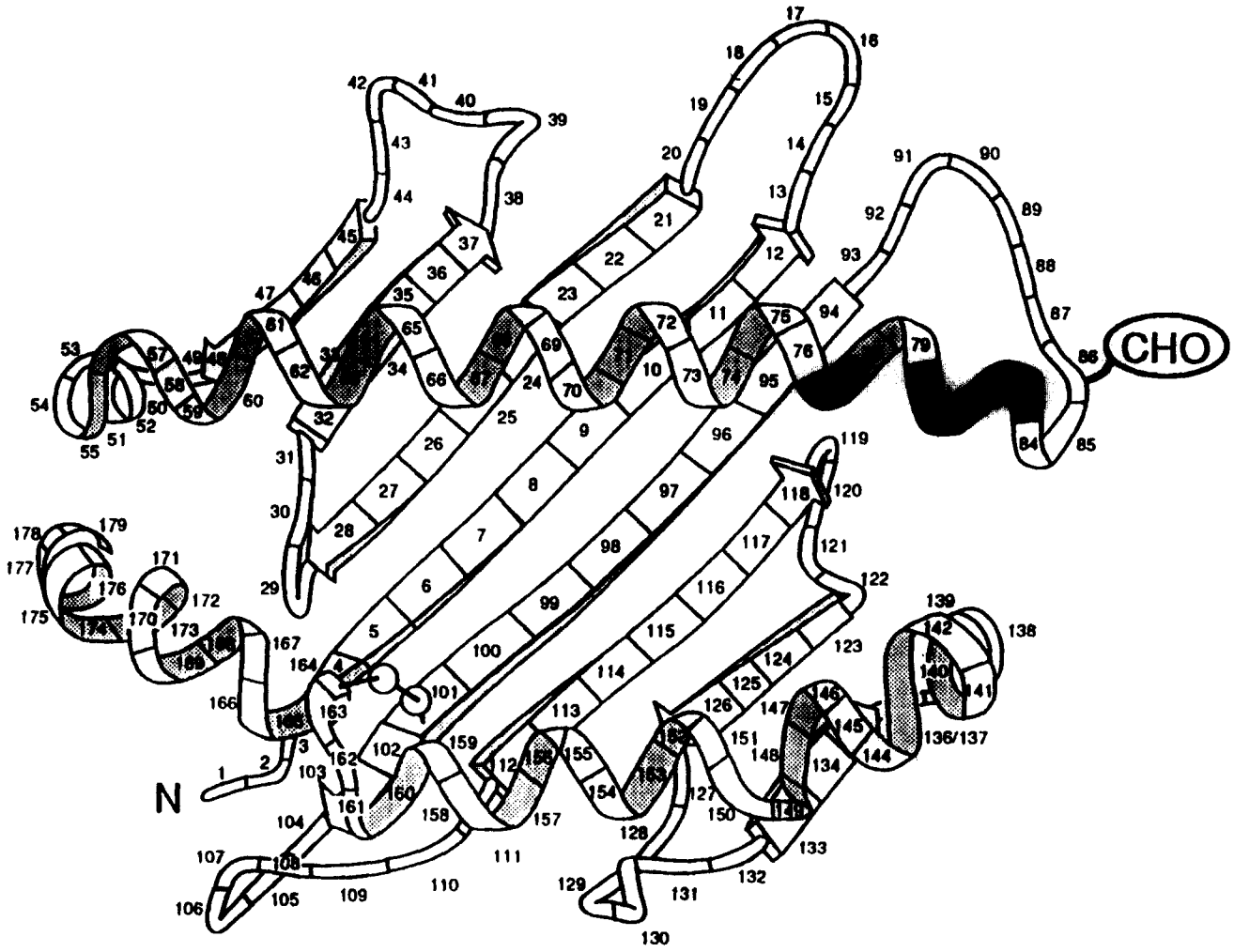

Figure 5. Ribbon diagram showing the α_1 and α_2 domains of the class I molecule. Numbers correspond to amino acid positions of the mature heavy chain. The polymorphic residues which determine the Bw4 serological epitope are darkened. The single site of *N*-linked glycosylation at position 86 is indicated by "CHO".

of leucine and/or arginine at positions 82 and 83 of the class I heavy chain may be key in determining NKB1 reactivity.

Employing a genetic approach to study a recombinant family, Cella et al. (45) have also observed a pattern of HLA recognition by NK cell clones which appears to focus on the Bw4 epitope. Using mitogen stimulated lymphocytes from a panel of HLA-typed donors as target cells, resistance to lysis was correlated with the presence of the Bw4 epitope on either HLA-A or -B molecules. The authors concluded that Bw4 motifs which have an isoleucine at position 80 are strongly inhibitory, whereas those that have threonine at this position provide only moderate inhibition. Our results show that although Bw4$^+$ HLA-A molecules are inhibitory, this inhibition is not prevented by addition of the anti-NKB1 monoclonal antibody. Therefore the inhibitory effect of Bw4$^+$ HLA-A alleles could well be due to the presence of another HLA receptor on the NK cell clones, in addition to NKB1. In the analysis by Cella et al. this second receptor might show specificity for the Bw4 motif of HLA-A molecules. Most of the target cells tested by Cella et al. which are strongly refractory to lysis express a Bw4$^+$ HLA-A allele. If these samples were to be excluded from the analysis in order to assess just HLA-B alleles, then there is no longer a strong correlation of resistance to lysis with the presence of isoleucine at position 80, since HLA-B alleles having either isoleucine or threonine gave strong inhibition in their investigation. Similarly, we find moderate inhibition by an allele which has isoleucine at position 80 (B*1513) as well as by B*2705 which has threonine at this position (Table 2). On the other hand, HLA-B*5101 and B*5801 both of which have isoleucine at position 80 usually mediate strong inhibition in our experiments. Thus, the degree of inhibition mediated by the Bw4 epitope of HLA-B molecules may be influenced by additional features of the class I molecule outside of the Bw4/Bw6 epitope. It is clear that there a number of different NK cell receptors for class I molecules and that individual NK cell clones can express more than one receptor. Given this potential complexity at the level of the NK cell,

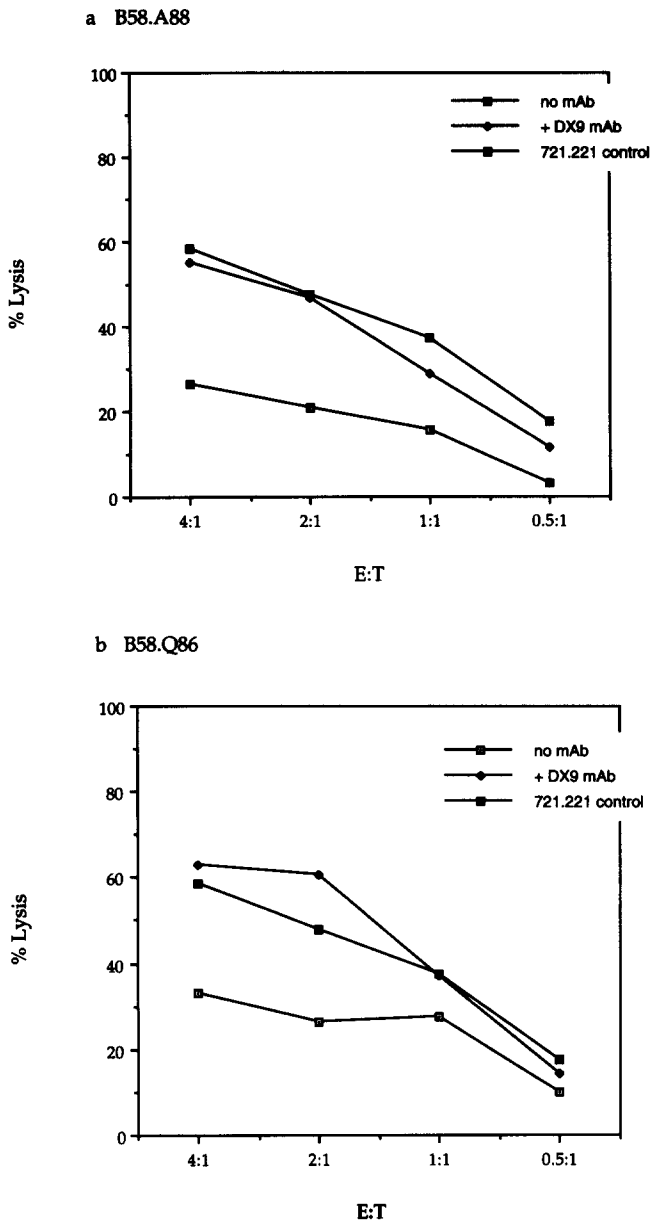

Figure 6. Lysis by an NKB1$^+$ NK cell clone is inhibited by expression of mutant HLA-B58 molecules which are not glycosylated. Effector to target titration curves of cell killing assays performed with an NKB1$^+$ NK cell clone, in the presence and absence of the anti-NKB1 monoclonal antibody DX9. (*a*) Percent lysis of the B*58.A88 mutant. (*b*) Percent lysis of the B*58.Q86 mutant. The level of killing of the untransfected 721.221 cell line is shown for comparison.

there are additional difficulties in analyzing the class I specificities of any one receptor using target cells expressing up to six potential class I HLA ligands, as is the case for mitogen-stimulated lymphocytes. For this reason we elected to analyze the specificity of the NKB1 receptor for class I molecules using transfectants each expressing a single class I allele.

We find that the presence of the carbohydrate moiety is not essential for class I recognition by NK cells expressing the NKB1 molecule. This result is in agreement with the finding by Storkus et al. that polyclonal NK cells were inhibited by the expression of a mutant HLA-B*0702 molecule lacking the glycosylation site at position 86 of the heavy chain (46). Previous studies have also observed reduced cell surface expression of class I molecules lacking glycosylation (21, 22). The observation that class I glycosylation is not needed for NKB1$^+$ NK cell recognition suggests that the carbohydrate moiety is not bound by the receptor. Since the gene for NKB1 has yet to be cloned, it is not known whether NKB1 is homologous to the murine NK cell receptor for H-2D Ly-49, which encodes a lectin-like domain. However, structural distinctions between Ly-49 and NKB1 are already apparent; Ly-49 is a disulfide-linked homodimer with subunits of 44 kD, whereas NKB1 is a single chain of 70 kD (9, 47). It will also be of interest to determine whether the lectin-like domain of Ly-49 binds a carbohydrate moiety of the class I molecule.

Recognition of class I HLA by NK cells which bear the NKB1 antigen appears to be fundamentally different from class I recognition by T cells. Antigen-specific T cells recognize individual peptide + MHC complexes, whereas NKB1$^+$ NK cells appear to recognize a public determinant on class I molecules. Kärre et al. (2) have proposed that the function of NK cell recognition of MHC antigens is to eliminate cells which lack a "self" marker. However, this hypothesis does not account for the observation that NK cells discriminate class I alleles, since elimination of pathogens or cells lacking normal class I expression could be accomplished by monomorphic recognition of HLA molecules. One function for NK cell recognition of polymorphic class I epitopes could be to facilitate detection of differences in peptide presentation that result from infection by intracellular parasites. However, the ability to discriminate polymorphic self antigens could also be beneficial without involving peptide presentation. NK cells are part of the early immune response to an invading pathogen. If NK cells kill target cells because they lack self HLA antigens, pathogenic microorganisms could evade detection by mimicking conserved HLA epitopes. However, if subsets of NK cells recognize polymorphic epitopes of different HLA molecules effective molecular mimicry would be more difficult, because a pathogen would need to mimic epitopes of 3–6 HLA-A, -B, and -C molecules to escape detection by all NK cells. Additionally, recognition of polymorphic determinants allows detection of autologous cells which have lost expression of only one or two class I alleles, as occurs in certain tumors and virus infections.

During the evolution of HLA-B polymorphism, gene conversion events appear to have focused on the region of class I genes which encodes the Bw4 and Bw6 epitopes, resulting in the formation of at least 10 pairs of recombinant HLA-B alleles differing only in this region. The ability to interact with a certain subpopulation of NK cells may contribute to the selection pressures which have driven these gene conversion events. The Bw4 epitope is present on ~35% of the HLA-B antigens identified thus far, and HLA-B molecules that possess the Bw4 epitope are present at a gene frequency

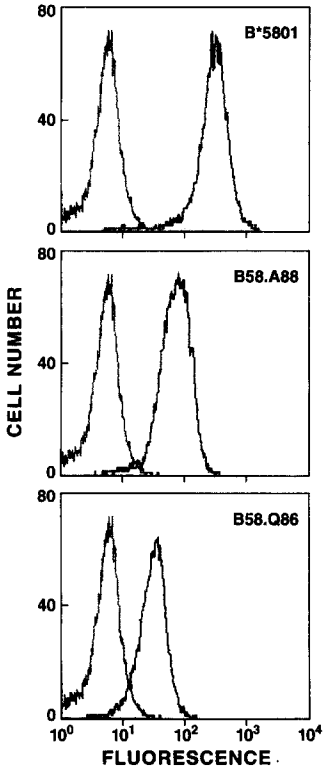

Figure 7. Two HLA-B58 glycosylation site mutants transfected into the 721.221 cell line have reduced cell surface expression, compared to the wild-type B*5801 transfectant. FACS histograms of 721.221 transfectant staining with the anti-class I antibody W6/32. The light histogram trace on the left indicates background staining of the untransfected 721.221 cell line. Transfectant staining is shown by the dark histogram trace. Fluorescence and cell number are presented in arbitrary units. The median fluorescence intensities were as follows: 721.221 = 5.6; B*5801 transfectant = 299.6; B58.A88 transfectant = 75.0; B58.Q86 transfectant = 30.8.

of ~40% in many populations, including North American whites and blacks, and non-Chinese Thais (48). It is possible that selection by NKB1+ NK cells has contributed to maintenance of these gene frequencies.

Understanding features of class I molecules that are important in NK cell recognition, and the distinctions between class I recognition by NK cells and T cells may be helpful in medical practice. Both NK cells and T cells are cytotoxic for hematopoietic cells in vivo, and are potential mediators of bone marrow graft rejection. In rodent models the study of bone marrow rejection suggests that interaction between NK cells and class I molecules of the target cell may be important in determining the success of the transplant (49, 50). In these models, grafts lacking certain self alleles may be rejected by the recipient, although no foreign alleles are present on the donated marrow cells (51). The rejection is mediated by host NK cells, that are more resistant to radiation than T cells or B cells, and is thought to result from a lack of inhibition provided by the "missing" self determinants (51). Further investigation is necessary to determine the role of NK cells in human bone marrow graft rejection, GVHD, and in eliminating leukemic cells. HLA-matching procedures and immunosuppressive drugs have made rejection uncommon, however GVHD and recurrence of leukemia remain a problem. T cell depletion reduces the likelihood of GVHD, but increases the incidence of graft rejection and leukemic relapse (52). If NK cells prove to be involved in these processes, clarification of the class I epitopes recognized by NK cells may facilitate assessment of the extent of matching required for successful bone marrow transplantation between unrelated donors and recipients.

Table 2. *Class I HLA Sequence Motifs Determining the Bw4 and Bw6 Serological Epitopes*

Serological epitope	Class I locus	Class I position					Tested HLA allele
		77	80	81	82	83	
Bw4	HLA-A,B	N	I	A	L	R	B*5801, B*5101, B*1513, A*2403
	HLA-B	N	T	A	L	R	
	HLA-A	S	I	A	L	R	A*2501
	HLA-B	S	T	L	L	R	
	HLA-B	D	T	L	L	R	B*2705
Bw6	HLA-B	S	N	L	R	G	B*1502
	HLA-B	G	N	L	R	G	

Only polymorphic positions within these epitopes are shown.

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