Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 7983–7985, September 1992
Immunology

Alloantigen recognition by two human natural killer cell clones is associated with *HLA-C* or a closely linked gene

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Contributed by Jack L. Strominger, May 22, 1992

ABSTRACT **Human natural killer (NK) cells with the $CD3^-$ $CD16^+$ phenotype recognize allospecificities on normal T-cell blasts. The NK-defined specificity 1 (NK-1) is recessively inherited and has been mapped to the major histocompatibility complex between the complement gene cluster and *HLA-A*. A gene for NK-1, however, has not been identified. Here we demonstrate that NK-1 and the recently defined NK specificity 2 (NK-2) are reciprocally associated with homozygosity for a diallelic polymorphism at amino acid positions 77 and 80 in the putative peptide-binding site of HLA-C ($P < 10^{-5}$). NK-cell recognition of allogeneic cells may, therefore, be controlled by *HLA-C* itself or by a closely linked gene(s), which dominantly prevents (resistance alleles) or recessively permits (susceptibility alleles) recognition of still-unknown target determinants.**

Natural killer (NK) cells lyse tumor and virus-infected cells without restriction by the major histocompatibility complex (MHC) class I or class II molecules (1, 2). The target molecules recognized by NK cells and the receptors for recognition are presently unknown. Independently from tumor-cell lysis, $CD3^-$ $CD16^+$ NK cells also recognize and lyse normal allogeneic T-cell blasts (3, 4). Alloreactive NK-cell lines and clones have been derived from $CD3^-$ peripheral blood lymphocytes and have been shown to define polymorphic specificities (5, 6). Among these, the NK specificity 1 (NK-1) defined by the "1 anti-A" clone is inherited as an autosomal recessive trait in linkage with the MHC (5). Analysis of MHC recombinant families has shown that NK-1 maps telomeric to the complement gene *BF* and centromeric to *HLA-A* (7). This region includes class I genes (*HLA-E, -C, -B*), tumor necrosis factor genes, heat shock protein 70 genes, as well as 15 additional genes [genes for HLA-B-associated transcripts (BAT) and G genes] of unknown function (8–10). To understand which of these genes is involved in NK allorecognition, the analysis of MHC recombinant families was extended by using DNA polymorphisms identified in the cloned interval between *HLA-B* and the complement gene *BF*. The present work describes the precise location of the recombinational breakpoint in one family (family R), which enables the mapping of NK-1 specificity to the class I region of the MHC centromeric of *HLA-A*. Further analysis of class I gene polymorphisms in this interval reveals a complete correlation of NK-1 with homozygosity for a sequence motif in the α1 domain of HLA-C. In addition, a second NK specificity (NK-2) (6) shows highly significant correlation with homozygosity for the alternative motif in the same position of HLA-C. These results provide evidence that susceptibility and/or resistance of T-cell blasts to lysis by these NK alloreactive clones are controlled by *HLA-C* or by a closely linked gene.

MATERIALS AND METHODS

NK Clones. NK clones were derived from $CD3^-$ peripheral blood lymphocytes as described (3, 5–7). The specific cytolitic activity of NK clones against phytohemagglutinin-induced lymphoblasts was tested in a 4-hr ^{51}Cr-release assay. Phytohemagglutinin blasts from random individuals were obtained by culturing peripheral blood lymphocytes for 4 days with 0.5% phytohemagglutinin in the presence of recombinant interleukin 2 (100 units per ml).

Microsatellites and Single-Strand Conformation Polymorphism (SSCP) Analysis. Microsatellites and SSCP loci were amplified from genomic DNAs by PCR. Primer pairs for amplification are available on request. The 10-μl reactions contained 100 ng of DNA, PCR primers (0.5 μM each; 0.05 μM of one primer 5'-end labeled with γ-[^{32}P]ATP), dNTPs (125 μM each), 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, and 0.25 units of *Taq* polymerase. PCR was done for 20–25 cycles, with 1-min intervals at 94°C, 55°–72°C, and 72°C. Microsatellites and SSCP variants were resolved on 6% denaturing and native polyacrylamide gels, respectively. After electrophoresis gels were transferred to Whatman 3MM paper, dried, and exposed overnight to Kodak X-Omat AR film without intensifying screen.

Sequence-Specific Oligonucleotide Analysis. The primers for *HLA-C*-locus-specific amplification were GACG(C/A)CG(C/A)GAGTCC(G/A)AGAGG (5', nucleotides 187–206) and CG(G/T)CCTCGCTCTGGTTGTAG (3', nucleotides 324–343). Degenerate positions are shown in parenthesis. The 5' primer includes a 1 base-pair (bp) mismatch at the 3' end with the DNA sequences of *HLA-A, -B, -E, -F*, and *-G*, to allow for the selective amplification of the second exon of *HLA-C*. Genomic DNA samples were amplified by PCR in a 50-μl standard reaction for 30 cycles, each with 1-min steps at 94°C, 60°C, and 72°C. Approximately 10 ng of the amplified fragments was denatured in 100 μl of 0.4 M NaOH/25 mM EDTA and applied to Hybond-N (Amersham) by using a slot-blot apparatus (Schleicher & Schuell). Membranes were hybridized with ^{32}P-labeled oligonucleotides [ACCGAGTGA(A or G)CCTGCGGAA] (nucleotides 293–311) specific for Ser-77 or Asp-77. Hybridization was done for 2 hr in 5× Denhardt's solution, 5× standard saline phosphate/EDTA (SSPE: 1× SSPE is 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA), 0.5% SDS, and probe at 2×10^6 cpm/ml. Filters were then washed in 0.5% SDS/6× standard saline citrate for 20 min at room temperature and for 5 min at the calculated t_m (melting temperature) for each oligonucleotide.

Statistical Analysis. The significance of associations was determined by Fisher's exact test with three-by-two tables.

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Abbreviations: NK, natural killer; MHC, major histocompatibility complex; SSCP, single-strand conformation polymorphism; NK-1 and NK-2, NK-defined specificity 1 and 2, respectively.

RESULTS AND DISCUSSION

The locus controlling NK-1 is telomeric of a cross-over between the properdin factor B gene (*BF*) in the complement gene cluster and *HLA-B* in the c/d recombinant MHC haplotype of individual R3 in the R family (7). To map this recombinational breakpoint more precisely, polymorphic DNA markers were isolated from previously cloned cosmids containing the entire 550 kilobases (kb) of *BF–HLA-B* interval (8). Three microsatellites showing informative length variability were identified 360, 290, and 140 kb centromeric of *HLA-B*. Polymorphic bands segregating in the R family with individual MHC haplotypes defined by HLA and complement-typing distinguished the c and d haplotypes (Fig. 1). In R3 DNA, all three microsatellite markers detected bands characteristic of the d haplotype, which contributes the centromeric HLA-DR–complement C4–BF segment of the c/d composite haplotype. The telomeric segment from the c haplotype includes the *HLA-B*, *-C*, and *-A* genes by serological typing. This result was confirmed by a DNA sequence polymorphism 30 kb centromeric of *HLA-B*, which was detected by SSCP and yielded a sequence variant characteristic of the c haplotype (Fig. 1). The breakpoint in the c/d recombinant haplotype is, therefore, between 30 and 140 kb centromeric of *HLA-B* (i.e., between the markers III and IV shown in Fig. 1). Susceptibility to NK-1 segregates with the c haplotype, whereas dominant resistance is carried by the d haplotype (7). As the individual R3 is susceptible to NK-cell lysis, the recessive gene for NK-1 in the c/d haplotype is telomeric of the recombinational breakpoint. This gene presumably lies within the class I region centromeric of *HLA-A*, which has been previously established as a telomeric boundary in an informative MHC recombinant family (M family) (7).

Among the genes in this interval, *HLA-B* was unrelated to NK-1: in the previously characterized G family (7), NK-1 is expressed by only one of two haploidentical siblings, who, however, are identical for HLA-B by serological typing. The identity of the *HLA-B* alleles in these two individuals was confirmed by DNA sequencing of their α1 and α2 domains. Also, no differences were found in the corresponding sequences of the class I gene *HLA-E*, which lies between *HLA-C* and *HLA-A*, displays very little polymorphism (11), and is not known to be expressed at the cell surface (12) (data not shown).

These results suggested a possible relationship between NK-1 and *HLA-C*, the only other known functional gene in this region. By comparison of 15 unrelated individuals, NK-1 exclusively correlated with the presence of either HLA-Cw1, -Cw3, -Cw7 or C "blank" in both *HLA-C* alleles and the absence of -Cw2, -Cw4, -Cw5, and -Cw6. Comparison of available amino acid sequences revealed that alleles in the first group share serine and asparagine at positions 77 and 80 in the α1 domain, whereas in the second group asparagine and lysine were found at these positions (13). This diallelic polymorphism is reminiscent of the serological epitopes Bw4 and Bw6, which are located at residues 80–83 in the α1 domain of HLA-B (14, 15). The significance of this association between NK-1 and these polymorphic residues of HLA-C was tested in a panel of 66 random individuals by genomic DNA typing with sequence-specific oligonucleotides (representative samples are shown in Fig. 2). All 18 samples positive for NK-1 were homozygous for Ser-77 of the HLA-Cw1 group of alleles, as opposed to the 48 samples negative for NK-1, all of which were either heterozygous or homozygous for Asp-77 ($P < 10^{-5}$) (Table 1). These results agree completely with the recessive inheritance of NK-1 and indicate its association with an epitope determined by amino acid residue 77 and, presumably, amino acid residue 80 of the HLA-Cw1 group.

Like NK-1, the recently found NK-2 specificity is also recessively inherited, cosegregates with the MHC, and could be allelic to NK-1, as these alloantigens appear to be mutually exclusive (6). Indeed, NK-2 was absent from all cell samples that were positive for NK-1 and correlated with homozygous Asp-77 of the Cw2 group. However, out of 30 samples that

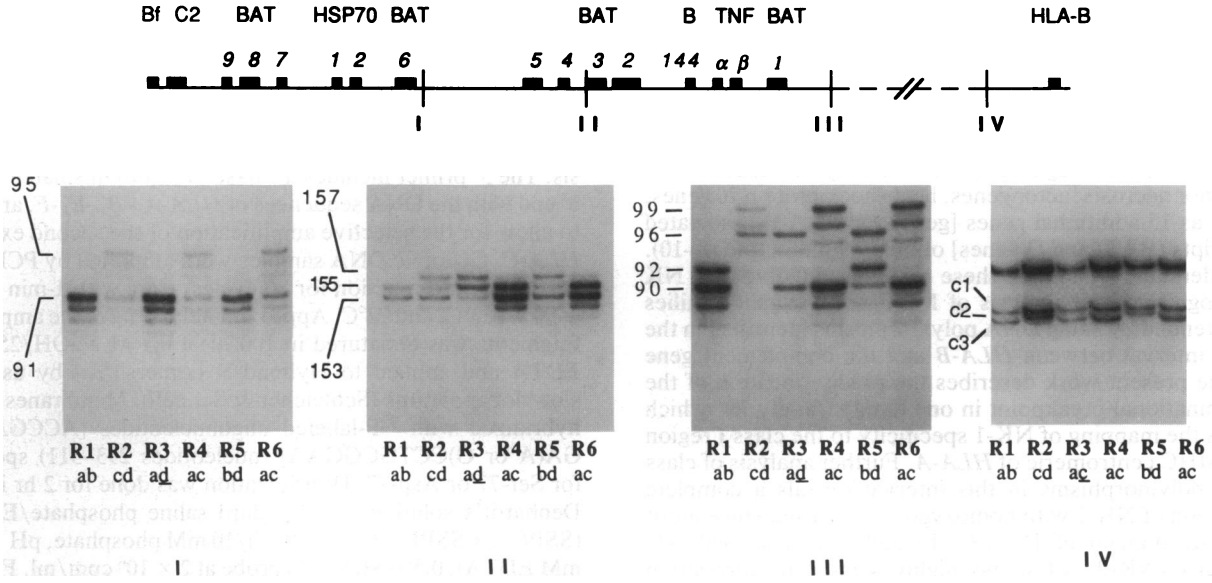

FIG. 1. Breakpoint mapping in the MHC recombinant c/d haplotype informative for location of NK-1. Positions of three microsatellites (*I*, *II*, and *III*) and of a SSCP (*IV*) are shown within the region centromeric of *HLA-B* (*Top*). Closed boxes refer to genes (ref. 8; Bf, BF; TNF, tumor necrosis factor; HSP70, heat shock protein 70; BAT, HLA-B-associated transcript). The region of the crossover in individual R3 is shown as a broken line. Segregation of the polymorphic DNA markers is analyzed in the R family (*Bottom*). MHC haplotypes (small letters below family members) are defined by HLA and complement-typing (7). The c/d recombinant haplotype is underlined. (*I–IV*) Haplotype associations with polymorphic DNA bands: (*I*) a (91-bp), b (91-bp), c (95-bp), and d (91-bp); (*II*) a (155-bp), b (153-bp), c (153-bp), and d (157-bp); (*III*) a (92-bp), b (90-bp), c (99-bp), and d (96-bp); and (*IV*) a (c3 SSCP variant), b (c1), c (c1), and d (c2). The recessive susceptibility allele for NK-1 is carried by haplotypes a, b, and c, whereas the dominant resistance allele is carried by haplotype d (7). As the R3 individual is positive for NK-1, the c/d haplotype carries the susceptibility allele telomeric of the recombinational breakpoint.

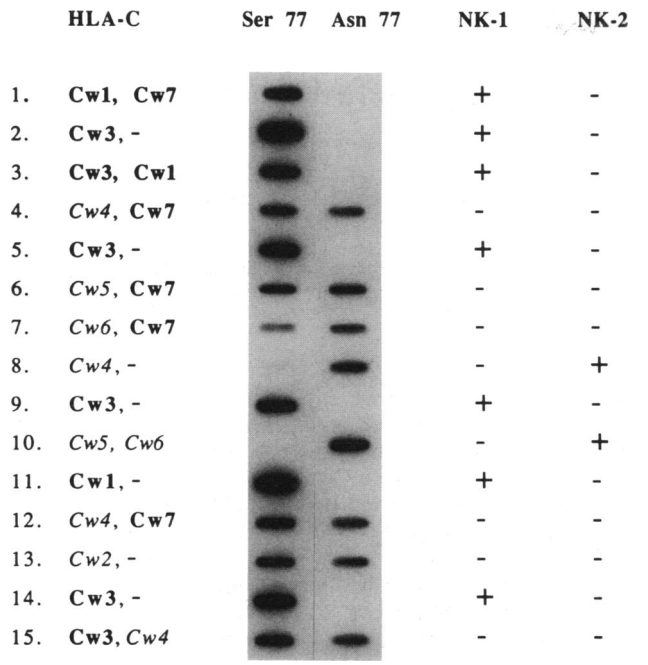

FIG. 2. Genomic oligonucleotide-typing of residue 77 of HLA-C in 15 individuals positive or negative for the NK-1 or NK-2 specificities. Serological typing of HLA-C is indicated where known; alleles belong to the Cw1 (boldface letters) or Cw2 (italic letters) groups.

were heterozygous by DNA typing, 3 were positive for NK-2. Thus, although NK-2 is highly associated with HLA-C ($P < 10^{-5}$) (Table 1), the correlation is, nevertheless, incomplete, possibly indicating the involvement of a closely linked gene.

A role of HLA-C in NK alloreactivity is corroborated by studies suggesting that MHC class I molecules can modulate NK-cell recognition of tumor cells and bone-marrow grafts (16–20). According to previous models, some *HLA-C* alleles could protect target cells from lysis by target interference (17) or by delivering a negative signal to NK cells, thereby mediating effector inhibition (16). Alternatively, a possible function of an *HLA-C*-linked gene could have precedent in the ABO blood-group system, in which the H specificity (blood group O) and the "Bombay" specificity result from recessive null alleles for glycosyltransferases. Similarly, the NK allospecificities could be determined by different carbohydrate moieties, encoded by null (recessive susceptibility) or plus (dominant resistance) alleles of a gene near *HLA-C*. The association of specific HLA-C amino acid residues with the NK-1 and NK-2 specificities identified here is of particular interest because these NK specificities may be equivalent to the MHC-linked hemopoietic histocompatibility antigens defined by bone-marrow grafting in the mouse (21, 22) and thus may be relevant to problems encountered in human allogeneic bone-marrow transplantation.

Table 1. Correlation between HLA-C sequence variants and NK-1 and NK-2 specificities defined by NK-cell clones in a random panel

HLA-C	NK-1$^+$			NK-2$^+$		
amino acid 77	*n*	+	%	*n*	+	%
Ser/Ser	18	18	100	14	0	0
Ser/Asn	38	0	0	30	3	10
Asn/Asn	10	0	0	9	9	100
P value		$< 10^{-5}$			$< 10^{-5}$	

The HLA-C diallelic polymorphism at amino acid 77 was determined by oligonucleotide typing as shown in Fig. 2. In the known HLA-C sequences Ser-77 is always linked to Asn-80, and Asn-77 is linked to Lys-80 (13).

We thank Dr. G. B. Ferrara for his generous support. This work was supported by Consiglio Nazionale delle Ricerche (CNR) Grants 90.00125 PF70, 91.01258 PF70, and 91.0059 PF99 and the American-Italian Foundation for Cancer Research (M.C.); the Cancer Research Institute/Elaine R. Shepard Investigator Award (T.S.); National Institutes of Health Grants AI-30581 (T.S.), CA-47554, and DK-30241 (J.L.S.); Consiglio Nazionale delle Ricerche (CNR), Istituto Superiore di Sanita (ISS) and Associazione Italiana per la Ricerca sul Cancro (AIRC) (E.C., A.M., L.M., D.P., and O.V.).

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