Human Natural Killer (NK) Alloreactivity and Its Association with the Major Histocompatibility Complex: Ancestral Haplotypes Encode Particular NK-defined Haplotypes

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Summary

As ancestral haplotypes of the major histocompatibility complex (MHC) appear to define identical MHC haplotypes in unrelated individuals, unrelated individuals sharing the same ancestral haplotype should also share the same NK-defined allospecificities that have recently been shown to map to the human MHC. To test this prediction, multiple cell lines from unrelated individuals sharing the same ancestral haplotypes were tested for the NK-defined allospecificities. It was found that cells sharing the same ancestral haplotypes do have the same NK-defined specificities. Furthermore, the NK-defined phenotype of cells that possess two different ancestral haplotypes can be predicted from the NK-defined phenotypes of unrelated cells that are homozygous for the ancestral haplotypes concerned. Although the group 1 and 2 NK-defined allospecificities can be explained to some extent by HLA-C alleles, evidence is presented that additional genes may modify the phenotype conferred by HLA-C.

With the expanding use of bone marrow transplantation there is an increasing number of patients for whom an HLA genotypically identical sibling donor is not available. Unrelated donors identified from large panels are being used. Current strategies for donor/recipient matching require detailed matching for alleles at HLA class I and II loci. It is evident that these approaches are inadequate (1). Graft rejection can occur despite apparently good matching whereas successful outcome can occur despite mismatches at these loci (2). Current methods may not allow adequate matching of the class I and II alleles. This has been demonstrated in a case report of T cell rejection involving mismatching at HLA-B (3). In addition, however, other polymorphic non-HLA genes within the MHC may be involved, and matching for HLA alone does not ensure matching for these genes. There is direct evidence in the mouse for the presence of at least one set of such genes.

The hemopoietic histocompatibility (Hh)[1] system in mice has been shown to determine F_1 hybrid resistance to a bone marrow graft from either parent with graft rejection mediated by radio-resistant NK cells (for a review see reference 4). Unlike the classical MHC antigens, the Hh antigens are inherited in a recessive fashion (5). It has been suggested that the major Hh locus (Hh-1) maps within the H-2 complex between H2-S and H2-D and can be dissociated from the class I genes (6, 7). However, the class I MHC antigens may play a role in the function or expression of Hh-1 antigens (for a review see reference 8). One model suggests two genetic loci Hh-1r and Hh-1s control expression of Hh-1 antigens. The Hh-1s genes encode the structural antigen whereas the Hh-1r gene downregulates expression of the Hh-1s genes. Complex Hh-1 haplotypes have been suggested (8). Compatibility between the donor and recipient at Hh-1 is required to prevent NK-mediated graft rejection.

There is now good evidence that the equivalent of the Hh-1 system exists in humans. In a series of papers, Ciccone et al. (9) have demonstrated that NK cells can mediate specific allogeneic target cell lysis. NK clones derived from single donors can recognize different allospecificities (10) and five different allospecificities have been defined (11). Susceptibility to lysis by NK clones recognizing specificities 1, 2, and 3 and probably 4 and 5 has been shown to be inherited in an autosomal recessive manner whereas resistance to lysis is dominantly inherited (11). Segregation studies and mapping

[1] *Abbreviations used in this paper:* AH, ancestral haplotype; Hh, hemopoietic histocompatibility; LCL, lymphoblastoid cell line.

using families with recombinant haplotypes have shown that the genes controlling susceptibility or resistance to lysis are localized within the MHC between complement factor Bf and the HLA-A locus (12). The nature of the target molecules is uncertain. However, in a recent series of studies, Ciccone et al. have provided evidence that HLA-Cw3 can provide specific protection of target cells against lysis mediated by group 2–reactive NK clones (13) and the group 1 and 2 specificities are reciprocally associated with homozygosity for a diallelic polymorphism at amino acid positions 77 and 80 on HLA-C (14).

Given the existence of the human equivalent of Hh-1, it is likely that NK allorecognition is involved in human bone marrow graft rejection. Therefore, simple means of matching for these determinants and to retrospectively analyze cases for such matching are required.

We have previously shown that the polymorphism of the human MHC can be described in terms of a limited number of ancestral haplotypes (AH) and their recombinants (15). Increasingly it is evident that these AH have been maintained en bloc from remote ancestors and that each haplotype defines a continuous specific sequence of DNA (16–18). It follows, therefore, that AH provide markers for alleles at unknown as well as known genes in the MHC. We therefore predicted that each AH would be associated with particular sets of NK-defined determinants (NK haplotypes). Consequently, the identification of AH would provide an effective means of matching for the NK-defined specificities before bone marrow transplantation. Therefore in this study we determined the NK-defined specificities present on target cells carrying various AH and related the findings to the known alleles present on these haplotypes.

Materials and Methods

Target Cells for NK Allorecognition. A panel of 34 EBV-transformed lymphoblastoid cell lines (LCLs) served as targets. These cells were selected from an extensive local panel of LCLs based on being either homozygous or heterozygous for the ancestral haplotypes listed in Table 1. Several of these cells were included in the 10th International Histocompatibility Workshop cell panel held in Princeton, NJ and New York, NY, 12–23 November 1987. Each cell has been characterized using all the MHC markers listed in Table 1 to confirm the presence of the particular AH. HLA-A, -B, -C and DR, DQ serological typing was performed by a complement-mediated microcytotoxicity assay using a panel of antisera extensively characterized against standard cells included in previous International Histocompatibility Workshops. DNA-based HLA class II typing was performed according to the methods detailed in the 11th International Histocompatibility Workshop held in Yokohama, Japan, 6–13 November 1991 using a series of sequence-specific oligonucleotide probes labeled with derivatised horseradish peroxidase suitable for detection by enhanced chemiluminescence (19). Complement components C4 and Bf allotyping was performed by immunofixation using appropriate antisera after electrophoresis as described previously (20). Methods for the typing of the alleles at TNF (21), BAT3 (22), and XYV (19) have been described previously.

Isolation of NK-clones and Evaluation of NK Cytotoxicity. NK alloreactivity against the LCL target cells was evaluated using previously described methods (11). In brief, PBL from normal donors were isolated on Ficoll-Hypaque gradients and NK cells enriched after depletion of T cells using a mixture of mAbs against CD3, CD4, and CD8 (9, 10). The viable cells were then separated on a Ficoll-Hypaque gradient. These viable NK-enriched cells were then cloned under limiting dilution conditions in the presence of irradiated feeder cells, 0.1% PHA and recombinant IL-2. The NK-defined specificities present on the LCL target cells were determined in a 4-h ^{51}Cr-release assay using cloned NK effector cells reacting specifically with group 1 (ES2 or ES10), group 2 (AM25, Mauro P), group 3 (A51–8), and group 5 (OA64) specificities. Target cells were used at 5×10^3/well, for a final E/T ratio of 10:1. Percent specific lysis was determined as described previously (9, 10).

Using this assay cytotoxicity is usually clearly bimodal. Target cells considered negative for a specificity give ≤10% lysis whereas targets considered positive give >20% lysis with the specific NK clone (11).

HLA-C Alleles and Sequencing. All target cells were HLA typed for the presence of the Cw alleles 1–7. The Cw allele associated with each AH has been previously established based on typing of many examples of each AH. The presence of the amino acids at residues 77 and 80 on the α chain of the Cw molecule present on each AH was established by review of published nucleotide sequences (23) and for Cw4 from a sequence submitted to GenBank (sequence number M84386) by Dr. P. Parham. This sequence was derived from a HLA-B35 positive cell and has therefore been provisionally assigned to the 35.1 and 35.4 AH.

The HLA-C allele on the 44.2 AH was sequenced by the following method. HLA-C was specifically amplified by PCR using primers and conditions described previously (14). The resulting PCR product was diluted 1 in 25 in distilled water and reamplified with nested degenerate primer CACAGAAGTACAA(C/G)CGCCAGG (5′, nucleotides 189–209, exon 2) and the same 3′ primers used in the original PCR. The nested PCR was performed in a standard PCR reaction, using 25 μl of diluted product in 50 μl of final reaction mix, with 1-min steps at 94, 60, and 72°C for 30 cycles with 2-s increments every cycle. The resulting PCR product was purified by column centrifugation (Centricom 30 microconcentrator; Amicon W.R. Grace & Co., Beverly, MA) and sequenced using fluorescent labeled dideoxy termination reactions on an automated DNA sequencer (model 373A; Applied Biosystems, Inc., Foster City, CA) with the primers used to produce the nested PCR product.

Results

NK Group 1 and 2 Specificities Are Associated with Particular AH. The cytotoxicity of the NK clones defining the group 1 and 2 specificities against the 34 LCL target cells are shown in Table 2. Several points are evident. The specific lysis is bimodal with most target cells clearly positive (>20% lysis) or negative (≤10%). All cells homozygous for an AH express either a group 1 or 2 specificity and these specificities behave as alleles at a single locus. The results for cells heterozygous for two AH are predictable from the results obtained with the cells that are homozygous for these AH and a recessive model of susceptibility to lysis. For example, both the 8.1 and 7.1 AH carry the group 1 specificity and cells R7/17219 and Q9/20920 which are heterozygous for these two AH are group 1 positive. On the other hand, cell Q6/8187 is heterozygous for AH 8.1 and 57.1 which bear group 1 and 2 specificities, respectively, and expresses neither the group

1 nor 2 specificity. Cell R86/12336, which is homozygous for the 44.2 AH gave indeterminate cytotoxicity of 14% with the group 1 clone. This cell has been considered group 1 positive. This classification is supported by the results of two other cells (R8/5618, Q5/7952) that are heterozygous for 44.2 and another AH which is clearly group 1 positive based on the results for homozygous cells. Both these cells are group 1 positive (37 and 27% cytotoxicity, respectively) indicating that the 44.2 AH must also be group 1 positive under a recessive model of susceptibility to lysis. However the cytotoxicity of both these cells and the homozygous cell is lower than that present in most of the other group 1 positive cells. These data suggest that the Cw allele present on the 44.2 AH is different from the other Group 1 alleles.

The results for the 68 haplotypes present on the 34 cells are summarized in Table 3. All AH tested carry either the group 1 or 2 specificity. All examples of the same AH from unrelated individuals bear the same NK-defined specificity, i.e., these specificities are AH haplotypic. Therefore, AH will identify the specificity present and the NK-defined phenotype of a cell can be predicted by the AH present.

NK-defined Haplotypes Occur and Are Associated with Particular AH. Having shown that the group 1 and 2 determinants are AH haplotypic, we examined whether more complex NK-defined haplotypes are also associated with particular AH. A subset of 12 of the 34 target cells were therefore additionally tested against NK clones defining the group 3 and 5 specificities. These included cells homozygous for the 7.1, 8.1, 18.2, 60.1, 57.1, and 44.1 AH and three heterozygous cells. All 12 cells were positive for the group 3 specificity so the nature of the group 3 inheritance could not be determined. The 7.1, 8.1, and 44.1 homozygous cells were all positive and the 18.2 and 57.1 homozygous cells were negative for the group 5 specificity. However, the results in the three heterozygous cells cannot simply be explained by the same genetic model as the group 1 and 2 specificities.

From the results obtained above, several points are evident: (*a*) target cells homozygous for an AH can encode several specificities; (*b*) sets of NK-defined specificities (i.e., NK-defined haplotypes) occur; (*c*) unrelated individuals homozygous for the same AH express the same NK-defined haplotype, i.e., NK-defined haplotypes are AH haplotypic; and (*d*) the same NK-defined haplotype can be shared between several different AH.

Residues 77–80 on the HLA-C Molecule Do Not Account for All NK-defined Specificities. Having demonstrated that NK specificities associate with particular AH, we determined whether these associations could be accounted for by the two alternate epitopes at amino acid residues 77 and 80 on HLA-C associated with the group 1 and 2 specificities (13, 14). There-

Table 1. *List of MHC Ancestral Haplotypes Included in this Study*

	MHC alleles and RFLP markers																					
Name	A	Cw	B	Ya	Yb	X	V	TNF	B144	BAT3	C2	Bf	C4A	C4B	DR	DRB1	DBR3	DRB4	DRB5	DQA1	DQw	DQB1
7.1	3	7	7	A	S	L	A	L	S	L	C	S	3	1	15	1501			0101	0102	6	0602
8.1	1	7	8	B	S	L	B	S	S	S	C	S	Q0	1	3	0301	0101			0501	2	0201
13.1	30	6	13	D	L	L	C	L		S	C	S	3	1	7	07		0101		0201	2	0201
18.1	25		18	A	S	S	B	L	S	L	Q0	S	4	2	15	1501			0101	0102	6	0602b
18.2	30	5	18	C	S	M	A	L	S	S	C	F1	3	Q0	3	0301	0202			0501	2	0201
18.3			18					L				S	3	1	11	(1102)	(0202)			(0501)	7	(0301)
35.1		4	35	C	S	L	A	S	S	L		S	3	1	11	1104	0202			0501	7	0301
35.4		4	35	G		L	B	S		L		S	3	1	11	1103	0202			0501	7	0301
44.1	2	5	44	A	S	L	C	S	S	L	C	S	3	Q0	4	0401		0101		(0301)	7	(0301)
44.2	29		44	D	L	L	C	L		L	C	F	3	1	7	07		0101		0201	2	0201
46.1	2	1	46	A	S	L	C	S		L	C	S	4	2	9	0901		(0101)		(0301)	9	(0303)
52.1	24		52	A		L	A	L			C	S	3+2	Q0	15	1502			(0102)	(0103)	6	(0601)
54.1		1	54	E	L	M	C	L	S	L	C	S	3	5	4	0405		(0101)		(0301)	4	(0401)
57.1	1	6	57	E	L	S	C	L	S	L	C	S	6	1	7	07		0101		0201	9	0303
58.1	33		58		S	S		S		S		S	3	Q0	3	0301	02			(0501)	2	(0201)
60.1		3	60						S	L	C	S	3	1	4	(0404)		(0101)		(0301)	3	(0302)
60.3	2	3	60	A		L	B	S		S	C	S	Q0	2	13	1302	(0301)			(0102)	6	(0604)
62.1	2	3	62	A		L	B	L	S	L	C	S	3	3	4	0401		0101		0301	8	0302

TNF, B144, and BAT3 determined by RFLP typing. DRB1, DRB3, DRB4, DRB5, DQA1, and DQB1 determined by DNA typing. Ya, probe Y/TaqI + RsaI; Yb, probe Y/Bst Ell; X, probe X/TaqI; V, probeV/TaqI; S, short fragment; M, medium fragment; L, long fragment; ( ), provisional assignment.

Table 2. *Group 1 and 2 Phenotypes of Cell Lines which Are Homozygous or Heterozygous for Ancestral Haplotypes*

Cell ID	AH present	NK reactivity		NK phenotype
		Group 1	Group 2	
Homozygous cells				
Q6/3975	7.1	85*	10	1+, 2−
R6/12367	7.1	67	5	1+, 2−
R5/1518	8.1	65	2	1+, 2−
R5/843	8.1	90	6	1+, 2−
R9/52658	46.1	72	4	1+, 2−
R5/12361	46.1	75	ND	1, (2−)‡
R7/4709	52.1	44	12	1+, 2−
R7/12580	52.1	32	2	1+, 2−
R8/15375	54.1	95	ND	1+, (2−)‡
R6/12317	62.1	48	ND	1+, (2−)‡
R6/12293	18.1	65	2	1+, 2−
R6/12383	60.1	61	0	1+, 2−
R6/12382	60.3	100	5	1+, 2−
R6/12336	44.2	14	ND	1+,(2−)‡
R6/12303	18.2	4	42	1−, 2+
R5/5054	18.2	5	61	1−, 2+
R6/12337	57.1	3	88	1−, 2+
R0/9217	44.1	ND	52	1−, 2+
R6/12333	13.1	11	54	1−, 2+
Q5/8086	35.1	2	ND	1−, (2+)§
R6/12327	35.4	8	ND	1−, (2+)§
Heterozygous cells				
R7/17219	7.1, 8.1	92	10	1+, 2−
Q9/20920	7.1, 8.1	70	0	1+, 2−
R0/23685	62.1, 8.1	63	3	1+, 2−
Q6/2731	62.1, 8.1	83	ND	1+, (2−)‡
R5/10168	62.1, 7.1	70	ND	1+, (2−)‡
R8/5618	60.3, 44.2	37	0	1+, 2−
Q5/7952	62.1, 44.2	27	ND	1+, (2−)‡
R9/52661	46.1, 54.1	63	ND	1+, (2−)‡
R5/13141	60.3, 57.1	0	ND	1−, (2−)
Q6/8187	8.1, 57.1	5	0	1−, 2−
R9/52417	46.1, 13.1	4	2	1−, 2−
Q5/2711	7.1, 44.1	0	ND	1−, (2−)
R0/22825	7.1, 44.1	0	0	1−, 2−

* Results expressed as percent ^{51}Cr-release.
‡ Group 2 negative based on positive reactivity with group 1.
§ Group 2 positive based on negative group 1 reactivity.

fore, the HLA-C allele and the epitope present on each AH was examined. These results are summarized in Table 4. In all cases where data are available, the results fit the hypothesis that the alternative epitopes defined at residues 77–80 are associated with the NK-defined group 1 and 2 specificities. However the NK specificity associated with the 44.2 AH is unusual in expressing group 1 target relatively weakly.

In view of the different phenotype of the 44.2 AH, the

Table 3. *Frequency of Group 1 and 2 Specificities Associated with Each Ancestral Haplotype*

AH	No. of haplotypes tested	Group 1 positive	Group 2 positive
7.1	9*	9	0
8.1	9	9	0
18.1	2	2	0
44.2	4	4	0
46.1	6	6	0
52.1	4	4	0
54.1	3	3	0
60.1	2	2	0
60.3	4	4	0
62.1	6	6	0
13.1	3	0	3
18.2	4	0	4
44.1	4	0	4
57.1	4	0	4
35.1	2	ND	2‡
35.4	2	ND	2‡

* Cells homozygous for a single AH were considered to provide two examples of the same AH.
‡ Based on group 1 reactivity only.

HLA-C allele of this cell was sequenced from nucleotides 190–269 of exon 2, in the expectation that this allele may possess a different epitope from those shown to correspond to groups 1 and 2. Surprisingly, this cell carries the S - - N epitope expected for group 1 positive cells. However, its sequence apparently describes a new HLA-Cw allele. This new allele is identical to Cw1 and HLA-Cw*1401 in this region, except that it has an "A" at nucleotide 267.

It is also evident that the group 3 and 5 specificities are not associated with any particular C allele or either of the two alternative Cw epitopes at residues 77 and 80.

Discussion

In this study we have utilized cells that are homozygous or heterozygous for particular AH as target cells for NK clones defining specificities 1, 2, 3, and 5. We have shown that all cells homozygous for an AH express either a group 1 or 2 determinant and that the phenotypes of heterozygous cells can be predicted from the results in the homozygous cells and a recessive model of inheritance for susceptibility to lysis. Homozygous cells carry several NK-defined specificities, suggesting the presence of NK-defined haplotypes. Without exception, all examples of the same AH carry the same group 1 or 2 specificity, and in the more limited panel of cells tested, carry the same NK-defined haplotype, i.e., the NK-defined specificities are AH haplotypic. Therefore, the identification of AH will allow the identification of the associated NK specificities and provide a simple means of identifying the presence of these specificities in an individual. Based on the particular combination of AH present, the NK-defined phenotype of an individual can be predicted.

Whereas AH provide an excellent means of identifying the presence of a particular NK-defined haplotype, the relevant genes may be encoded anywhere along the haplotype. Elsewhere we have provided evidence that AH consist of several blocks of several hundred kilobases of DNA. Recombination can occur preferentially between these blocks, but has not been observed within them. In fact, there appear to be at least four distinct blocks of polymorphism within the MHC interval, viz. (*a*) the α block which carries HLA-A; (*b*) the β block which carries HLA-C, HLA-B, and CL (24); (*c*) the γ block which carries complement components C4, C2, and Bf and the Cyp21 genes; and (*d*) the δ block which carries the DR and DQ gene clusters. Mapping studies in both humans (12) and in mice (6, 7) and examination of several cells bearing recombinant AH (data not shown) all suggest that the genes encoding the group 1 and 2 NK-defined allospecificities are carried on the β block.

The nature of the target molecules for NK allorecognition has not been determined. Ciccone et al. (13) have recently suggested that HLA class I molecules are directly involved. HLA-Cw3 provides protection against lysis mediated by group 2 reactive clones. This resistance to lysis was inherited in a dominant manner and was specific for the group 2 specificity. Two alternative epitopes defined by polymorphism of amino acid residues 77 and 80 on HLA-C have been shown to be associated with protection against the group 1 and 2 specificities (14). The present data support this hypothesis, but also show that these epitopes are not associated with the group 3 or 5 specificities. HLA-C is included within the β block. In all those cases where data is available, the group 1 and 2–associated AH carry the predicted HLA-Cw epitope. However, cytotoxicity by the group 1 reactive NK clones against the 44.2 AH homozygous cell is only weak, and the two cells heterozygous for this AH gave cytotoxicity considerably less than other susceptible cells. We have shown that this AH carries a new HLA-C allele which may behave similarly to HLA-Cw*1401, as reported by Colonna et al. (25).

Two possible models to account for NK recognition have been suggested (26). These involve either effector inhibition, in which a MHC class I molecule provides an inactivating signal that blocks the NK cell ability to lyse, or target interference, in which an appropriate class I molecule masks a putative self-epitope that is the actual molecular target for NK recognition leading to cell lysis. HLA-C may be one such class I molecule, but there is evidence that other class I molecules may also be involved (27). The phenotype conferred by HLA-Cw*1401 and the HLA-Cw allele carried on the 44.2 AH supports the involvement of additional genes.

Our findings have important practical implications. NK allorecognition is likely to be involved in bone marrow graft rejection in humans given the mouse model and the inadequacy of current matching. We have shown that bone marrow

Table 4. *Ancestral Haplotypes Are Associated with Particular NK-defined Haplotypes and Encode NK Specificities that Cannot Be Explained by the Putative HLA-Cw Associated Epitope*

NK defined haplotype						
1	2	3	5	AH	Associated CW allele	Putative epitope
+	−	+	+	7.1	Cw7	S--N*
				8.1	Cw7	S--N
				60.1	Cw10	ND
−	+	+	−	57.1	Cw6	N--K
				18.2	Cw5	N--K
−	+	+	+	44.1	Cw5	N--K
+	−	ND	ND	60.3	Cw3	S--N
				62.1	Cw3	S--N
				46.1	Cw1	S--N
				18.1	Blank	S--N
				44.2	Blank	S--N‡
−	+	ND	ND	35.1	Cw4§	N--K
				35.4	Cw4§	N--K
				13.1	Cw6	N--K

* Putative epitope defined by amino acid residues 77-80 on HLA Cw α chain.
‡ As determined in this study.
§ Based on sequence data submitted to GenBank by Dr. P. Parham (accession number M84386).

from an HLA-A,B,DR,DQ matched, MLC nonreactive unrelated donor who was mismatched with the recipient for the NK-defined group 1 specificity was rejected (Witt, C. S., F. T. Christiansen, E. Ciccone, R. Herrmann, G. Tay, L. Moretta, and R. L. Dawkins, manuscript submitted for publication). Also, we have preliminary evidence that mismatching within the β block, as shown by mismatching for the CL region, is associated with graft rejection (Tay, G., C. S. Witt, F. T. Christiansen, L. Smith, and R. L. Dawkins, manuscript submitted for publication). Matching for NK-defined allospecificities is therefore likely to be an important factor for successful bone marrow engraftment. Ultimately, the relevant target molecules need to be identified and their genes mapped and characterized. However, it will not be necessary to develop direct typing methods for all these NK-defined specificities. Rather, it will only be necessary to identify a marker specific for the MHC blocks associated with each of the approximately 40 AH present in each major racial group. Matching for these blocks will result in matching for all the NK allospecificities present within these blocks. We have data that the polymorphic CL region provides such a haplospecific marker for the β block. Crossmatching donor and recipient at CL would therefore match for the NK-defined specificities. Work is currently in progress to confirm the validity of this approach.

This is Publication No. 9246 of the Department of Clinical Immunology, Royal Perth Hospital, Sir Charles Gairdner Hospital, and the University of Western Australia, Perth.

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Received for publication 14 December 1992 and in revised form 28 May 1993.

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