Involvement of HLA Class I Alleles in Natural Killer (NK) Cell-specific Functions: Expression of HLA-Cw3 Confers Selective Protection from Lysis by Alloreactive NK Clones Displaying a Defined Specificity (Specificity 2)

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Summary

This study was designed to identify the target molecules of the natural killer (NK) cell–mediated recognition of normal allogeneic target cells. As previously shown, the gene(s) governing the first NK-defined allospecificity (specificity 1) were found to be localized in the major histocompatibility complex region between BF gene and HLA-A. In addition, the analysis of a previously described family revealed that a donor (donor 81) was heterozygous for three distinct NK-defined allospecificities (specificities 1, 2, and 5). HLA variants were derived from the B-Epstein-Barr virus cell line of donor 81 by γ irradiation followed by negative selection using monoclonal antibodies specific for the appropriate HLA allele. Several variants were derived that lacked one or more class I antigen expressions. These variants were analyzed for the susceptibility to lysis by NK clones recognizing different allospecificities. The loss of HLA-A did not modify the phenotype (i.e., "resistance to lysis"). On the other hand, a variant lacking expression of all class I antigens became susceptible to lysis by all alloreactive clones. Variants characterized by the selective loss of class I antigens coded for by the maternal chromosome became susceptible to lysis by anti-2-specific clones. Conversely, variants selectively lacking class I antigens coded for by paternal chromosome became susceptible to lysis by anti-1 and anti-5 clones (but not by anti-2 clones). Since the Cw3 allele was lost in the variant that acquired susceptibility to lysis by anti-2 clones and, in informative families, it was found to cosegregate with the character "resistance to lysis" by anti-2 clones, we analyzed whether Cw3 could represent the element conferring selective resistance to lysis by anti-2 clones. To this end, murine P815 cells transfected with HLA Cw3 (or with other HLA class I genes) were used as target cells in a cytolytic assay in which effector cells were represented by alloreactive NK clones directed against different specificities. Anti-2-specific clones efficiently lysed untransfected or A2-, A3-, and A24-transfected P815 cells, while they failed to lyse Cw3-transfected cells. NK clones recognizing specificities other than specificity 2 lysed untransfected or Cw3-transfected cells. Thus, the loss of Cw3 resulted in the de novo appearance of susceptibility to lysis, and transfection of the HLA-negative P815 cells with Cw3 resulted in resistance to lysis by anti-2 clones. Therefore, we can infer that Cw3 expression on (both human and murine) target cells confers selective protection from lysis mediated by anti-2 NK clones.

NK cells are generally thought to lyse tumor cells or virus-infected cells in a nonspecific fashion (1, 2). Recent studies, however, have challenged this concept since peripheral blood–derived $CD3^-CD16^+$ NK cells were shown to be capable of specific recognition of allogeneic cells (3). In addition, the finding that this ability was clonally distributed, and that NK clones derived from single individuals appeared to recognize different allospecificities, supported the notion

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of the existence of an NK cell repertoire (4, 5). So far, five distinct allospecificities have been defined. A major question arising from these studies is the nature of the target molecules recognized by alloreactive NK clones. Genetic analysis performed on three of the NK-defined specificities (termed 1, 2, and 3, respectively) indicated that the corresponding characters "susceptibility to lysis" were inherited in a recessive manner, while the characters "resistance to lysis" were dominantly inherited (5). A similar mode of inheritance was also suggested for specificities 4 and 5. In addition, the analysis of the segregation of these characters and of the HLA haplotypes indicated that the corresponding genes were localized on chromosome 6 (5). Further analysis of informative families, which included donors with recombinant HLA haplotypes, allowed us to map the gene(s) controlling the susceptibility or resistance to lysis by clones recognizing specificity 1. Thus, the gene(s) was found to be localized in the MHC region telomeric to BF gene (complement cluster) and centromeric to the HLA-A locus (6).

In an attempt to identify the genes encoding for the molecules recognized by alloreactive NK clones, we used, as a source of target cells, an EBV-transformed B cell line derived from a donor heterozygous for three distinct NK-defined specificities (specificities 1, 2, and 5). We further analyzed a series of cell variants derived from this cell line and lacking surface expression of one or more HLA class I molecules. The loss of expression of HLA-Cw3 was found to be associated with the de novo appearance of susceptibility to NK clones recognizing specificity 2. In addition, murine P815 cells transfected with HLA-Cw3 (but not with other HLA class I genes) acquired resistance to lysis by anti-2-specific NK clones.

Materials and Methods

Antibodies. mAbs OKT3 (anti-CD3; Ortho Pharmaceutical, Raritan, NJ), HP2.6 (anti-CD4), B9.4 (anti-CD8), W6-32 (anti-HLA class I [7]), 131 (derived in our laboratory, specific for an epitope shared by HLA-A1, A3, A11, and A24), BB7.2 (anti-HLA-A2 [8]), BT3/4 (anti-HLA-DQw1 [9]; kindly provided by G. Corte, IST, Genova), XIII 358.4 (anti-HLA-DQw2 [10]; kindly provided by C. Mazzilli, Universita "La Sapienza", Roma), CCCL11 (anti-HLA-Cw1, 3, 11 [11]; kindly provided by J. Strominger, Harvard University, Boston, MA and W. Brown), F4/326 (anti-HLA-C [11]; kindly provided by J. Strominger and B. Du Pont) were used in this study.

Cell Cultures. PBMC were isolated by Ficoll-Hypaque density gradient centrifugation from peripheral blood of normal donors. B cell–enriched fractions were prepared from PBMC by removing cells capable of forming rosettes with sheep erythrocytes. B cells were infected with EBV according to published methods (12). PHA blasts were obtained by culturing PBL for 4 d with 0.5% PHA (vol/vol) in the presence of IL-2 (100 U/ml).

Derivation of HLA-defective Cell Variants. Cell variants were obtained after γ irradiation of B-EBV cell line with 200–300 rad (1 rad = 0.01 Gy) using a cesium source. Cells were then cultured in the presence of peritoneal macrophages for 4–6 d before immunoselection with mAb and magnetic beads (Dynal, Oslo, Norway). To this end, 5×10^7 mutagenized B-EBV cells were negatively selected using magnetic beads coated with anti-mouse IgG (13) after incubation with saturating amounts of 131 (anti-HLA-A11) mAb to generate variant 81-B2. BB7.2 (anti-HLA-A2) mAb was used to select variant 81.TA. W6-32 (anti-HLA class I) mAb was used to select variant 81.0A. Finally, the combined use of 131 mAb and BT3/4 (anti-DQw1) mAb was used to generate variant 81.G, while the combined use of BB7.2 mAb and XIII 358.4 (anti-DQw2) mAb was used to generate variant 81TB. The recovered cells were cultured in the presence of peritoneal macrophages and the procedures of negative selection described above were repeated at least three times at different culture intervals.

HLA Gene Transfectants. Murine P815 cells transfected with the thymidine kinase (TK)[1] and HLA-Cw3 genes (clone 444/C9.3), with TK and HLA-A24 genes (clone 452/D1), were kindly provided by Drs. Janet L. Maryanski (Ludwig Institut, Lausanne) and B. Jordan (Luminy, Merscilla) (14). P815 cells transfected with HLA-A2 (clone 3.32.3) or with HLA-A3 (clone 2.23.2) (15) were kindly provided by Dr. Giovanna Chimini (Luminy, Merscilla).

Isolation and Cloning of $CD3^-CD16^+$ Lymphocytes and Evaluation of Cytolytic Activity. PBL derived from normal donors were isolated on Ficoll-Hypaque gradients and cells were then incubated with a mixture of anti-CD3 (OKT3; Ortho Pharmaceutical), anti-CD4 (HP26), and anti-CD8 (B9.4) mAbs, followed by treatment with rabbit complement for 1 h at 37°C as previously described (3–5).

Viable cells were isolated by Ficoll-Hypaque gradients and cloned under limiting dilution conditions in the presence of irradiated feeder cells, 0.1% PHA (Gibco Ltd., Paisley, Scotland), and a source of exogenous IL-2 (rIL-2; Cetus Corp., Emeryville, CA), as previously described for both T and NK cell cloning (16, 17).

The cytolytic activity of cloned cells was tested in a 4-h ^{51}Cr-release assay in which effector cells were tested against B-EBV cells or PHA blasts derived from different allogeneic donors. B-EBV cell variants and murine P815 cells transfected with HLA-A2, A3, A24, or Cw3, or untransfected P815 were used as targets in other series of experiments. All these target cells were used at 5×10^3/well, for a final E/T cell ratio of 10:1. Percent specific lysis was determined as previously described (3–5).

Flow Cytofluorometric Analysis. 10^5 cells were stained with the appropriate mAb followed by fluoresceinated goat anti–mouse Ig. Control aliquots were stained with the fluorescent reagent alone. All samples were then analyzed on a flow cytometer (FACStar®; Becton Dickinson & Co., Mountain View, CA) gated to exclude nonviable cells. The percentage of positive cells was calculated on histograms displaying $\log_{10}$ of fluorescence (in arbitrary units) vs. number of cells. An electronic gate was positioned on the basis of 99% of autofluorescent-negative cells. Fluorescent cells trespassing the gate were considered as positive.

HLA Typing. The B-EBV cell lines were typed for HLA-A, B, C using the standard National Institutes of Health lymphocytotoxicity technique (18). A set of 240 well-standardized alloantisera, locally produced or derived from the international exchange, were allowed to type all the HLA class I specificities defined at the 10th Histocompatibility Workshop (18).

A long incubation lymphocytotoxicity technique was carried out in order to type the following DR and DQ specificities: DR1, DR2, DR3, DR4, DR5, DRw6, DR7, DRw8, DRw9, DRw10, DRw53, DQw1, DQw2, and DQw3.

HLA class II typing was performed using 120 well-standardized anti–class II alloantisera and two different batches of complement.

Southern Blotting. 12 μg of the genomic DNA extracted from the cell variants (19) was digested with the PvuII restriction en-

[1] *Abbreviation used in this paper:* TK, thymidine kinase.

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zyme. DNA was size fractionated by electrophoresis on a 0.7% agarose gel, blotted, and hybridized as described (19). The probe used was a 1.4-kb PstI cDNA fragment of pHLA-2 (20) and was labeled by random priming (21).

HLA-C PCR Amplification. Selective amplification of the HLA-C locus was obtained by the use of two degenerated synthetic oligonucleotides primers designed on the basis of the published HLA-C sequences (22). 50-μl reaction mixtures containing 1 μg of genomic DNA, 25 pmol of AC1 primer (CGACGMCGMGAGTCCRAGAG), and AC2 primer (CCGKCCTCGCTCTGGTTGTA) (22), in which M = A or C, R = A or G, and K = T or G, 200 μM or each dNTP, 1 mM DTT, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris/HCl, pH 8.3, 200 μg RNase A, 200 μg of gelatine, and 1.25 U Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) were overlaid with paraffin oil and subjected to 30 rounds of temperature cycling with a DNA thermal cycler (Perkin Elmer Cetus). A typical cycle was 30 s at 96°C (denature), 30 s at 60°C (anneal), and 30 s at 72°C (elongate). The samples were extracted, ethanol precipitated, and resuspended in 50 μl of Tris/HCl EDTA (TE). Dot blots were prepared spotting on nitrocellulose membranes (Nitroplus MSI; Westboro, MA), and the amplified samples were denatured in 0.4 N NaOH. Two oligonucleotides recognizing mutually exclusive sequences were designed: HYBC1 derived from a Cw3-related sequence (GACCGAGTGAGCCTGCGGA) (22) and HYBC2 derived from a Cw4-related sequence (GACCGAGTGAGCCTGCGGA) (22a) were end-labeled (21) and used to hybridize the dot blots. Hybridization was performed at 62°C overnight in 3× SSPE, 5× Denhardt's solution, 0.5% SDS, and 2 × 10^6 cpm/ml. The filters were washed 10 min at room temperature in 0.1× SSPE and 0.1% SDS, and stringent wash was performed with the same solution used before but for 10 min at 50°C. Filters were exposed to a x-ray film (Hyperfilm-MP; Amersham, UK).

Results

Analysis of family A (described in a previous report [10]) for the pattern of segregation of the five NK specificities identified so far revealed the existence of a donor (donor 81) heterozygous for specificities 1, 2, and 5. Donor 81 was generated by the mating between donor 82 (resistant to lysis by clones anti-1 or anti-5, but susceptible to anti-2 clones), and donor 51 (susceptible to lysis by anti-1 or anti-5 clones, but resistant to lysis by anti-2 clones) (Fig. 1). Given the recessive mode of inheritance of the various NK-defined specificities (i.e., susceptibility to lysis by different groups of alloreactive NK clones), and on the basis of the segregation pattern of the HLA haplotypes, it is evident that the maternal (donor 51) chromosome d carries the dominant allele for specificity 2 and the recessive allele for specificities 1 and 5 (Fig. 1). Conversely, the paternal (donor 82) chromosome y7 carries the recessive allele for specificity 2 and the dominant alleles for specificities 1 and 5 (Fig. 1). It should be stressed that donor 81, being heterozygous for specificities 1, 2, and 5, was resistant to lysis by clones recognizing specificities 1, 2, or 5.

Generation of B-EBV-transformed Cell Lines. To obtain immortalized cells from donors 81, 82, and 51, B cell–enriched mononuclear cells derived from these donors were infected with EBV. B-EBV-infected cell lines were tested for their susceptibility to lysis by NK clones expressing different specificities. As shown in Table 1, the EBV-transformed cell lines maintained the same pattern of susceptibility or resistance to lysis as the PHA blasts derived from the same donor. In

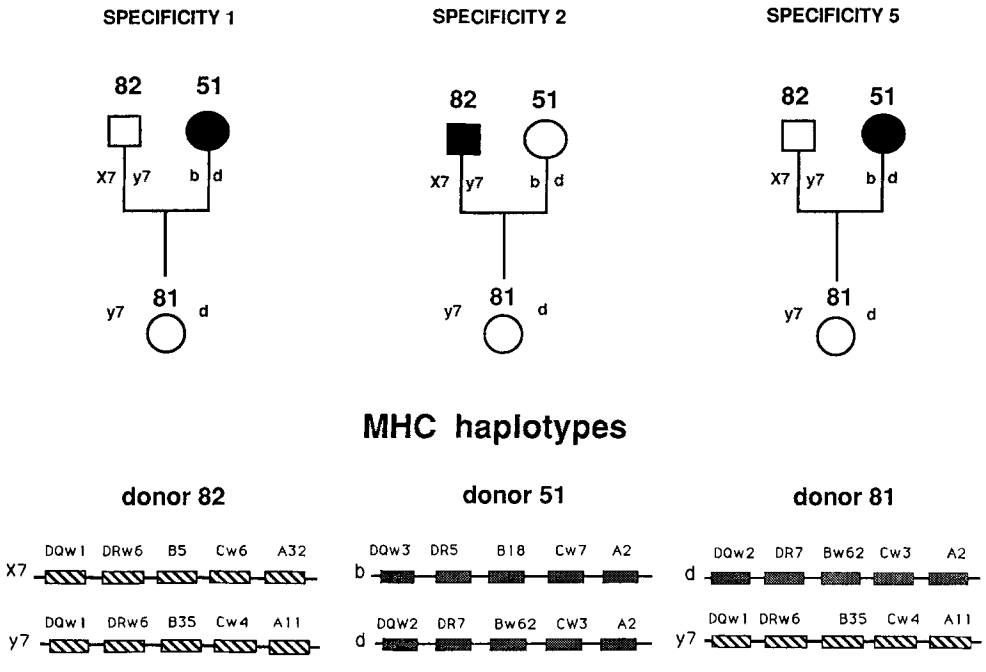

Figure 1. Mode of inheritance of the character "susceptibility to lysis" by NK clones recognizing specificities 1, 2, and 5. Circles indicate female donors and squares indicate male donors. Filled symbols represent susceptible donors and open symbols represent resistant donors. Small letters identify serologically defined MHC haplotypes, including markers for the HLA A, B, C, DR, and DQ loci. The various alleles expressed by donors 82, 51, and 81 are indicated. The effector cells used were represented by the NK clone H12 for specificity 1. Clones ALE-5 and Mary 25 have been used for specificity 2. For specificity 5, clones AGD48 and AO16 have been used (for details, see reference 5). It is important to note that, given the recessive mode of inheritance of the various NK-defined specificities and on the basis of the segregation pattern of the HLA haplotypes, the maternal chromosome d carries the dominant allele for specificity 2 and the recessive allele for specificities 1 and 5. On the other hand, the paternal chromosome y7 carries the recessive allele for specificity 2 and the dominant alleles for specificities 1 and 5.

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Table 1. *Comparison between Susceptibility to Lysis by Alloreactive NK Clones of EBV-transformed Cell Lines and PHA Blasts Derived from the Same Donors*

	Target cells from donor					
	82		51		81	
Clones	PHA blasts*	B-EBV	PHA blasts	B-EBV	PHA blasts	B-EBV
Group 1‡						
EMI 14	2§	0	60	72	0	2
EMI 11	2	2	57	50	0	0
CES26	0	5	66	56	2	2
Group 2						
ANNA33	32	41	2	5	0	5
ANNAP	44	38	0	2	2	2
MARY 25	39	41	0	2	0	8
Group 5						
AB12	0	0	45	55	0	2
A51-17	0	2	70	72	0	2

* In this test target cells were labeled with ^{51}Cr.
‡ Alloreactive clones belonging to the various groups of specificities were obtained as described in a previous report (5).
§ Results are expressed as percent of ^{51}Cr release at an E/T ratio of 10:1.

view of these data, B-EBV cell lines appeared to represent a suitable target for the analysis of the NK-defined allospecificities. The analysis of the HLA haplotypes in donor 81 revealed that the alleles HLA-A2 and DQw2 were inherited from the mother (donor 51), while the alleles HLA-A11 and DQw1 were inherited from the father (donor 82). The occurrence of the above segregation pattern was confirmed in the B-EBV cell lines, by the use of appropriate allele-specific mAbs. Thus, the B-EBV cell line derived from donor 81 was homogeneously stained by mAbs specific for A11, A2, DQw1, and DQw2. On the other hand, the line derived from donor 82 was stained by anti-A11 and anti-DQw1 mAbs but not by mAbs specific for A2 or DQw2. Conversely, B-EBV cells derived from donor 51 were stained by anti-A2 and anti-DQw2 mAbs but not by anti-A11 or anti-DQw1 mAbs.

Generation of Cell Variants Lacking the Expression of MHC Class I Surface Antigens from the 81 B-EBV Cell Line. On the basis of the above data, we generated a series of cell variants induced by γ irradiations of the B-EBV-transformed cell line derived from donor 81. In view of the availability of mAbs specific for HLA antigens coded for by maternal or paternal MHC regions, it was possible to select variants lacking maternal or paternal MHC regions. Thus, γ-irradiated cells were depleted of cells expressing one or another HLA allele by appropriate mAb-coated Dynabeads. The variant termed 81.B2 was negatively selected by depletion of $A11^+$ cells; the 81.G variant was obtained by the combined use of anti-A11 and anti-DQw1 mAbs; the 81.TA variant was selected by the use of anti-A2 mAb, whereas the 81.TB variant was obtained by the combined use of anti-A2 and anti-DQw2 mAbs. Finally, the 81.OA variant was obtained by the use of W6-32 mAb (reactive with a common determinant of MHC class I molecules). The above variants were analyzed by indirect immunofluorescence and FACS® analysis for the expression of the various HLA markers above. We also analyzed the expression of the HLA-Cw3 allele, which is expressed by donor 81 and 51, but not by donor 82 (Fig. 1).

As shown in Fig. 2, the 81.B2 variant lost the HLA-A11 only, whereas the 81.G variant lost both HLA-A11 and DQw1 (both coded for by the paternal chromosome). It should be noted that both 81.B2 and 81.G variants expressed the maternal HLA antigens A2 and DQw2 (Fig. 2). The 81.TA variant lost the HLA-A2 only, while the 81.TB variant lost both HLA-A2 and DQw2 (both of these variants expressed the paternal HLA markers A11 and DQw1). In addition, the analysis of the expression of HLA-Cw3 in the various variants revealed that this HLA marker was lost only in the 81.TB variant. Finally, the 81.OA variant lacked all class I markers (note also the lack of reactivity with the W6-32 mAb), whereas the class II antigens DQw1 and DQw2 were normally expressed.

To gain a more complete representation of the various MHC antigens expressed by the various variants, a conventional HLA typing was further performed. Data are shown in Table 2. It is evident that the 81.B2 variant selectively lost the expression of the A11 allele, while all of the other MHC products analyzed (including Cw4, B35, DRw6, DRw52, and DQw1) were detected. On the other hand, none of the paternal MHC antigens were detected in the 81.G variant. The TA variant selectively lost the A2 antigen (but it expressed Cw3, Bw62, DR7, DRw53, and DQw2), while the 81.TB variant lost all of the MHC antigens of the maternal haplotype. Different from the above variants, which selectively lost one or more of either the maternal or the paternal HLA antigens, the 81.OA variant failed to express all of the class I antigens, whereas both maternal and paternal class II antigens were expressed.

Molecular Characterization of the B-EBV Cell Variants. To better characterize the HLA-defective variants, we performed Southern blot analysis by the use of the PstI fragment (of the pHLA-2 probe) specific for all class I genes (20). The genomic DNA derived from different cell variants was digested with the PvuII restriction enzyme to analyze the HLA class I region (23). RFLP analysis of the parental 81 cell line showed two informative bands of ~4.2 and ~3.7 kb, respectively. The 4.2-kb band was detectable in the mother (donor 51), but not in the father (donor 82) (Fig. 3 *A*). Conversely, the 3.7-kb band was present in the father, but not in the mother. The 81.B2 and 81.TA variants expressed a RFLP pattern identical to that of the parental 81 cell line. On the other hand, the variant 81.TB lost the 4.2-kb band (inherited from the mother), while the variant 81.G lost the 3.7-kb band (inherited from the father) (Fig. 3 *A*). Finally, the 81.OA variant expressed both bands as in the 81 parental cell line. One of the possible explanations regarding the variants 81.TA and 81.B2 may be the occurrence of a point mutation in the class

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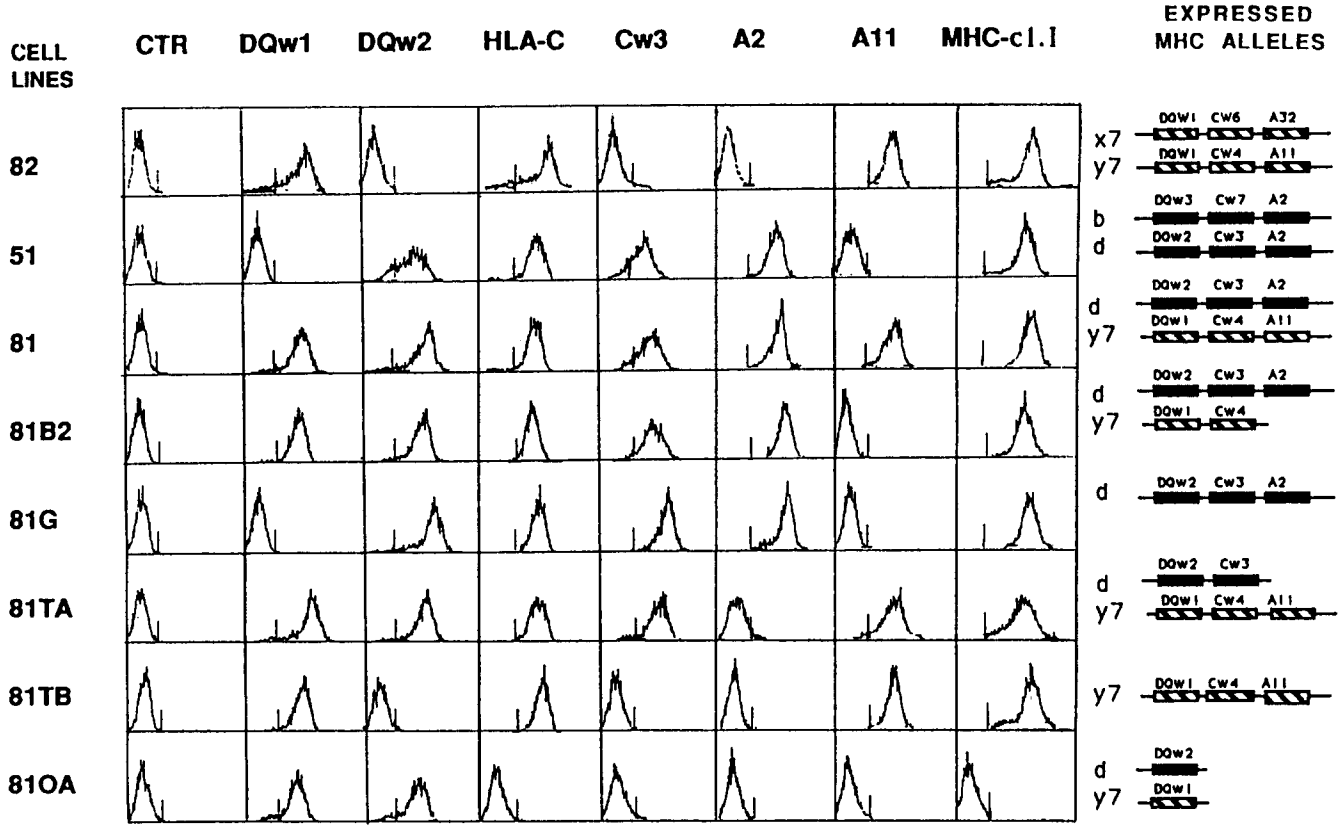

Figure 2. Surface phenotype of B-EBV cell line from donors 82 (father), 51 (mother), and 81 (sibling), and of the HLA-defective cell variants derived from the 81 cell line. The mAbs used in these experiments were represented by BT3/4 (anti-HLA-DQw1), XIII 358.4 (anti-DQw2), F4/326 (anti-HLA-C), CCCL11 (anti-Cw1, 3, 11), BB7.2 (anti-HLA-A2), 131 (specific for an epitope shared by HLA-A1, A3, A11, A24), and W6-32 (anti-HLA class I). The right panel summarized the MHC alleles expressed by the variants. Small letters refer to the MHC haplotype.

I region, undetectable with this type of analysis (24), leading to selective lack of expression of either maternal or paternal HLA-A molecules, respectively. On the other hand, evidence of a deletion of either maternal or paternal MHC region was obtained in the 81.TB and 81.G variants, on the basis of the absence of either the maternal (4.2 kb) or the paternal (3.7 kb) bands. Finally, the expression of both bands in 81.OA variant, which express neither maternal nor paternal class I antigens, suggests a defect in the regulation of class I expression in this variant.

Further evidence that 81.G and 81.TB variants indeed represent deletion mutants of the paternal or maternal MHC

Table 2. *HLA Typing of HLA-defective Variants Derived from the EBV-transformed B Cell Line Obtained from Donor 81*

Cell line	Maternal HLA haplotype						Paternal HLA haplotype					
	A2	Cw3	Bw62	DR7	DRw53	DQw2	A11	Cw4	B35	DRw6	DRw52	DQw1
81	+	+	+	+	+	+	+	+	+	+	+	+
81 B2	+	+	+	+	+	+	−	+	+	+	+	+
81 G	+	+	+	+	+	+	−	−	−	−	−	−
81 TA	−	+	+	+	+	+	+	+	+	+	+	+
81 TB	−	−	−	−	−	−	+	+	+	+	+	+
81 OA	−	−	−	+	+	+	−	−	−	+	+	+

HLA-defective variants were obtained as described in Materials and Methods. HLA typing was performed using a standard complement-dependent microcytotoxicity assay. +, presence of the antigen; −, absence of the antigen.

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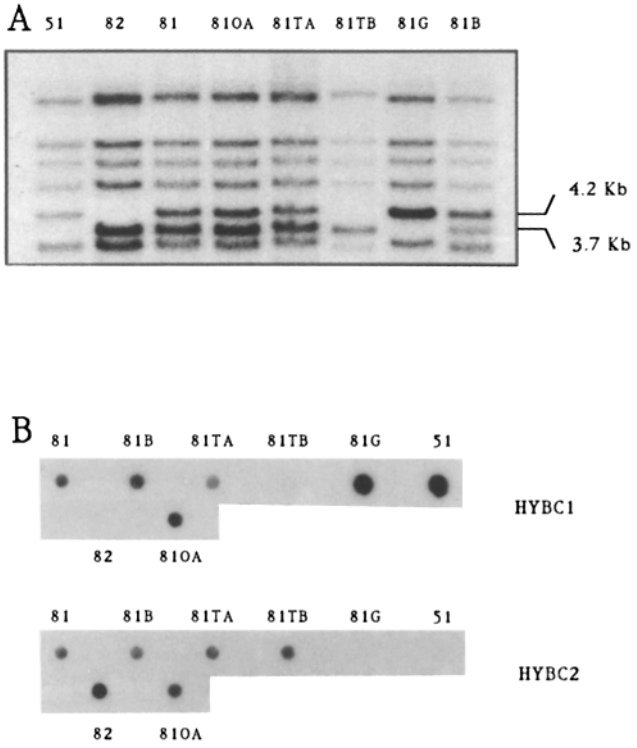

Figure 3. Molecular characterization of cell variants derived from the 81 B-EBV cell line. (*A*) Southern blot analysis of PvuII-digested genomic DNA with a class I probe (HLA-2). From left to right, samples were derived from the BEBV cell line of donors 51 (mother), 82 (father), 81, and the various HLA-defective cell variants derived from the 81 cell line. Note that the 4.2-kb band, present in donor 81, was inherited from the mother and is lost in variant 81 TB. On the other hand, the 3.7-kb band was inherited from the father and is lost in the 81.G cell variant. (*B*) Selective amplification of HLA-C locus by PCR. Two degenerated synthetic oligonucleotide primers designed on the basis of the published HLA-C sequences were utilized for amplification. Amplified DNA from the B-EBV cell line from donors 82 (father), 51 (mother), and 81 and of the HLA-defective cell variants derived from the 81 cell line was spotted on nitrocellulose membranes. Two oligonucleotides recognizing mutually exclusive sequences were synthetized. HYBC1 derived from a Cw3 sequence (GACCGAGTGAGCCTGCGGA) and HYBC2 derived from a Cw4 (22a) sequence (GACCGAGTGAGCCTGCGGA) were end-labeled and used to hybridize the dot blots.

region, respectively, was obtained by PCR analysis of the HLA-C locus. The synthetic oligonucleotides utilized to hybridize the amplified HLA-C locus were found to be informative since they segregated in the mother or in the father, respectively. Therefore, they could discriminate between Cw3 and Cw4 alleles (Fig. 3 *B*). Thus, the lack of hybridization with the HYBC1 oligonucleotide in the 81.TB cell variant (as well as in the paternal 82 cell line) is consistent with a deletion of the Cw3 gene. On the other hand, the lack of hybridization of the HYBC2 oligonucleotide in the 81.G cell variant (and in the maternal 51 cell line) is in agreement with the absence of the gene coding for Cw4 (Fig. 3 *B*).

Analysis of HLA-defective Cell Variants for Susceptibility to Lysis by Alloreactive NK Clones. We next analyzed whether cell variants that selectively lost surface expression of one or more HLA antigens (derived from either paternal or maternal chromosomes) were susceptible to lysis by alloreactive NK clones. Since donor 81 is heterozygous for specificities 1, 2, and 5, we used a series of clones recognizing specificities 1, 2, and 5. Clones recognizing these specificities were previously defined as group 1, group 2, and group 5 (or anti-1, anti-2, or anti-5 clones). As shown in Table 3, both 81.B2 and 81.TA cell variants, which selectively lost HLA-A antigens (either the paternal A11 or the maternal A2 allele), similar to the parental 81 cell line, were both resistant to lysis by anti-1, anti-2, or anti-5 clones. On the other hand, the 81.G variant was susceptible to lysis by anti-1 and anti-5 clones but resistant to anti-2 clones. Conversely, the 81.TB variant was susceptible to lysis by anti-2 clones, but resistant to anti-1 and anti-5 clones. Finally, the 81.OA cell variant was lysed by all groups of alloreactive NK clones tested. Thus, on the basis of these data, it appears that the selective loss of HLA-A (as in variants 81.B2 and 81.TA) does not modify the susceptibility to lysis of these cell variants as compared with the 81 parental cell line. On the other hand, the loss of all paternal HLA antigens resulted in a de novo appearance of susceptibility to lysis by anti-1 and anti-5 clones. Conversely, the loss of all maternal HLA antigens led to the susceptibility to anti-2 clones. Finally, the lack of expression of all class I but not of class II antigens (as in the 81.OA cell variants) resulted in susceptibility to anti-1, anti-2, and anti-5 clones.

Taken together, these data suggest that one or more HLA class I antigens (with the exception of HLA-A) may be involved in the induction of resistance to lysis by alloreactive

Table 3. *Susceptibility or Resistance to Lysis by Alloreactive NK Clones of the HLA-defective Variants Derived from 81 Cell Line*

	Target cells*							
Clones	82	51	81	81.B2	81.G	81.TA	81.TB	81.0A
Group 1‡								
EMI 14	0§	64	2	0	67	4	6	80
EMI 11	0	62	0	0	57	0	0	86
CES26	0	65	0	2	60	2	0	100
Group 2								
ANNA33	38	0	0	2	0	2	38	ND
ANNAP	32	0	2	3	0	0	26	100
MARY 25	78	0	5	2	0	0	49	96
Group 5								
AB12	0	50	0	0	50	0	0	90
A51-17	0	58	0	0	64	0	3	95

* In this test target cells were labeled with ^{51}Cr.
‡ Alloreactive clones belonging to the various groups of specificities were obtained as described in a previous report (5).
§ Results are expressed as percent of ^{51}Cr release at an E/T ratio of 10:1.

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NK clones recognizing specificities 1, 2, and 5. It should be noted that HLA-Cw3 was lost in the 81.TB variant, which acquired the susceptibility to lysis by anti-2 clones. In addition, analysis of the representative family "A" (which includes donors 82, 51, and 81, and which has been extensively described in previous reports [4, 5]) indicated that all family members expressing the HLA-Cw3 allele were resistant to lysis by anti-2 clones, thus suggesting a possible protective role mediated by this HLA allele.

Transfection of P815 Cells with Cw3 Gene Confers Selective Protection from Lysis by Anti-2 NK Clones. We further analyzed whether HLA-Cw3 represented the "protective element" conferring resistance to lysis by anti-2 clones. To directly assess this possibility, we used, as target cells, murine P815 cells transfected with human HLA-Cw3 (14). Control target cells were represented by untransfected P815 cells or by P815 cells transfected with HLA-A2, A3 (15), or A24 (14). As shown in Table 4, anti-2-specific NK clones efficiently lysed untransfected P815 cells. Importantly, transfection with Cw3 conferred a complete resistance to lysis by anti-2 clones. On the other hand, transfection with HLA-A2, A3, or A24 did not modify the susceptibility to lysis of P815 cells by anti-2 clones. Note that clones with other specificities efficiently lysed in Cw3-transfected cells (as well as the other HLA class I transfectants tested).

Table 4. *Susceptibility or Resistance to Lysis by Different Group of NK Clones of P815-transfected Cells*

	Target cells*				
Clones	P815	P815-A2	P815-A3	P815-A24	P815-Cw3
Group 1‡					
ES1	82§	76	72	72	72
ES9	65	63	68	67	68
ES10	42	43	50	53	70
Group 2					
ANNA20	50	43	51	49	2
ANNAP	35	38	30	32	3
ANNA37	32	38	35	35	0
Group 3					
A51.40	65	69	59	71	63
A51.51	68	71	70	73	69
A51.43	70	58	63	75	72
Group 5					
AB.12	70	85	77	81	75
A51.17	62	68	71	70	75
Clones with undefined specificities					
ABM.1	75	72	66	82	77
ABM.12	77	78	80	80	65
A51.11	63	70	82	78	82
A51.25	85	68	75	70	75

* In this test target cells were represented by untransfected P815 murine cell line or by P815 transfected with different HLA class I genes as described in Materials and Methods. The target cells were labeled with ^{51}Cr.
‡ Alloreactive clones belonging to the various groups of specificities were obtained as described (5).
§ Results are expressed as percent of ^{51}Cr release at an E/T ratio of 10:1.

Discussion

A number of previous studies indicated that the expression of class I molecules by target cells may confer resistance to lysis mediated by polyclonal populations of NK cells. In the present study we provide the first direct evidence that a given HLA class I allele selectively protects target cells from lysis mediated by a defined group of NK clones. Indeed, we show that the HLA-Cw3 allele is selectively involved in the protection from lysis mediated by NK clones recognizing specificity 2. The specificity of this phenomenon is further supported by the finding that NK clones recognizing other allospecificities efficiently lysed HLA-Cw3-positive target cells.

In addition, transfection of P815 murine cells with Cw3 but not with other HLA class I alleles conferred protection of these target cells from anti-2 clones. These data indicate that only certain HLA alleles are involved in the protection from NK clones recognizing a defined specificity. This would imply that, similar to T lymphocytes, alloreactive NK cells also may specifically recognize appropriate HLA class I alleles. Our data do not exclude the possibility that other HLA alleles may also function as protective elements from clones with specificity 2, but they provide clear evidence that this property is not shared by all HLA alleles.

Our present findings are consistent with the hypothesis that NK cells recognize either polymorphic epitopes of class I molecules or structures (e.g., peptides) selectively bound to certain HLA alleles. In this context, it is noteworthy that, in several informative families, the character "resistance to lysis" by group 2 clones cosegregated not only with the Cw3 allele, but also with Cw1 and Cw7 alleles. Importantly, these HLA-C alleles were found to share amino acid positions 77 (serine) and 80 (asparagine) in the putative peptide-binding site of HLA-C (25). It is possible to hypothesize that this epitope(s) may be involved in the mechanism of protection from group 2 NK clones. In a similar context, Storkus et al. (26) have identified an epitope characterized by an asparagine at position 74 shared by A3, A11, A24, Aw68, Aw69, B7, and B27 that appears to confer protection from lysis mediated by fresh polyclonal NK cells. According to the hypothesis that NK cells recognize self-polymorphic epitopes, the NK-mediated specific recognition would require the existence of clonally distributed surface receptors delivering a signal resulting in inhibition of NK-mediated cytolysis (27, 28). Thus, the lack of an appropriate HLA allele would result in cytolysis, while the expression of this allele would confer resis-

tance to lysis by NK clones with a given specificity. In accordance with this hypothesis, only allogeneic target cells lacking in both chromosomes the "protective" HLA alleles would be susceptible to lysis by a given NK clone. This explanation is also compatible with the recessive mode of inheritance of the character "susceptibility to lysis" and with the dominant mode of inheritance of the character "resistance to lysis", which have been previously documented for all five NK-defined specificities.

An alternative hypothesis, also compatible with our data, is that appropriate class I alleles may mask a self-epitope that represents the actual target structure recognized by NK cells. The lack of the appropriate HLA allele (as, for example, the Cw3 allele in our defective variants) would result in unmasking of the epitope that would thus be available for binding to receptors on NK cells. According to this hypothesis, the interaction between ligand and receptors would result in NK cell activation leading to triggering of the lytic machinery and target cell lysis.

Whatever the explanation would be, our data further support the notion that different NK cells express surface receptors for different specificities. In this context, we recently described clonally distributed triggering surface molecules termed GL183 and EB6 (29, 30). These molecules belong to a novel 58-kD NK-specific family, which define at least four different NK subsets. More importantly, these subsets correlated with the ability of the cells to recognize different allospecificities, thus suggesting that they may be part of a receptor structure involved in the clonally distributed specific recognition, by human NK cells.

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