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Immunology

Susceptibility or resistance to lysis by alloreactive natural killer cells is governed by a gene in the human major histocompatibility complex between *BF* and *HLA-B*

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ABSTRACT **The specificity recognized on normal allogeneic cells by a given alloreactive (1-anti-A) natural killer clone is controlled by a gene locus termed *EC1*. Because the *EC1* locus was previously shown to be located on chromosome 6, families characterized by a recombinant major histocompatibility complex haplotype were analyzed to map this locus more precisely. The breakpoint of recombination was studied by standard HLA typing, complement typing, and restriction fragment length polymorphism analysis of a series of genes located between the complement cluster genes and *HLA-B* within the major histocompatibility complex region. Three of 10 families analyzed were informative. From the data obtained, the *EC1* locus maps between *BF* and *HLA-B* and presumably is one of the normal genes recently described in this region.**

Although natural killer (NK) cells are generally known as cells expressing a non-major histocompatibility complex (MHC)-restricted cytotoxicity against tumor target cells (1), recent data have demonstrated that these cells may also mediate specific recognition (2). Thus, both mixed lymphocyte culture-derived $CD3^-$ $CD16^+$ polyclonal populations and clones were found to specifically lyse normal allogeneic cells [phytohematoglutinin (PHA)-induced blasts] derived from the stimulating donor (2). This specific cytolytic function is clearly distinguishable from the conventional non-MHC-restricted lysis of tumor cells (3). The character—susceptibility to lysis—by a given alloreactive NK clone (1-anti-A) was previously shown to be inherited in an autosomic recessive fashion (thus differing from conventional MHC products) and to be carried by chromosome 6 (3). The locus regulating susceptibility or resistance to lysis by 1-anti-A NK clones has been termed *EC1*. Thus, allogeneic target cells susceptible to lysis are presumed homozygous for the recessive allele (*EC1* $-/-$), whereas the character—resistance to lysis—is conferred by a dominant allele, either in an heterozygous (*EC1* $+/-$) or in an homozygous (*EC1* $+/+$) configuration. In this paper, mapping of the *EC1* locus in the MHC region between *HLA-B* and *BF* in the complement gene cluster is described. This region has recently been shown to carry 15 normal genes of presently unknown function (4).

MATERIALS AND METHODS

Isolation of $CD3^-$ $CD16^+$ Lymphocytes, Mixed Lymphocyte Cultures, Cell Cloning, and Evaluation of Cytolytic Activity. Peripheral blood lymphocytes derived from normal donors were isolated on Ficoll/Hypaque gradients, and cells were then incubated with a mixture of anti-CD3 (OKT3, Ortho Pharmaceuticals, Raritan, NJ), anti-CD4 (HP26), and anti-CD8 (B9.4) monoclonal antibodies followed by treatment with rabbit complement for 1 hr at 37°C (2, 3). Viable cells were isolated by Ficoll/Hypaque gradients and cultured in microwells (5×10^4 cells per well) in the presence of allogeneic irradiated mononuclear cells; after 4 days, the cultures were supplemented with recombinant interleukin 2 at 100 units/ml. The mixed lymphocyte culture-derived cells were cloned under limiting-dilution conditions in the presence of allogeneic irradiated feeder cells and recombinant interleukin 2 at 100 units/ml (2, 3, 5). Ten to twenty days later the cytolytic activity was tested in a 4-hr ^{51}Cr-release assay, in which effector cells were tested against PHA blasts derived from different allogeneic (or autologous) donors. PHA blasts were obtained by culturing peripheral blood lymphocytes for 4 days with 0.5% PHA (vol/vol) and recombinant interleukin 2 at 100 units/ml. In some experiments, fresh uncultured tumor cells were the target cells; target cells were used at 5×10^3 cells per well for a final effector/target cell ratio of 5:1. Percent specific lysis was determined as described (6). 1-anti-A alloreactive NK clones were selected as has been described (3).

HLA Typing. Serological typing for HLA-A, -B, and -C antigens was done by the standard National Institutes of Health technique (7). Serological typing for HLA-DR and HLA-DQ was performed on B-lymphocyte-enriched suspensions from peripheral blood by using the long-incubation lymphocytotoxicity technique with reagents obtained both locally and abroad (8).

Complement Typing. For analysis of complement polymorphisms, serum was separated from fresh blood and preserved at −80°C. C4 phenotypes were determined in neuraminidase- and carboxypeptidase B-treated serum samples (9) by using the technique of immunofixation electrophoresis in 0.45% (wt/vol) agarose gels (Seakem ME, FMC). Buffer for the analysis was a discontinuous Tris/glycine/barbital system. Immunofixation of the C4 bands was achieved with sheep anti-human C4, nephelometric grade (Serotec). C4-dependent lysis was performed with sensitized sheep erythrocytes in 1% (wt/vol) agarose overlay (Seakem ME) containing C4-deficient guinea pig serum (provided by G. Hauptmann, Strasbourg, France). The presence of the null gene at the C4A or C4B loci was determined from the different intensity of the bands for the two loci after staining with Coomassie blue.

BF phenotypes were determined by high-voltage electrophoresis of serum in 0.6% (wt/vol) agarose gels (ICN and

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Abbreviations: MHC, major histocompatibility complex; NK, natural killer; PHA, phytohemagglutinin.

Litex HSA, Denmark) followed by immunofixation with goat anti-human factor B (Atlantic Antibodies, Scarborough, ME) (10).

Restriction Fragment Length Polymorphism Analysis. Genomic DNA was prepared by standard techniques. DNA samples were digested with *Taq* I (New England Biolabs), according to the manufacturer's specifications. The DNA fragments were electrophoresed on 0.8% agarose gel and blotted into nylon membrane (Hybond-N, Amersham). The BAT3 probe consisted of a *Bam*HI–*Xba* I fragment from the BAT3 cDNA clone (11). The BAT3 probe was radiolabeled with [α-^{32}P]dCTP (NEN) by the random-primer procedure. Filters were hybridized 16 hr at 65°C in 6× SSC (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7), washed 10 min at room temperature in 5× SSC for three times, 10 min each, at 65°C in 0.1× SSC buffer, and exposed to XAR films (Kodak) for 16–48 hr.

RESULTS

To map the *EC1* locus on chromosome 6, 10 families exhibiting recombinant MHC haplotypes were analyzed. PHA-induced blast cells from each family member were tested for susceptibility to lysis by a set of NK clones displaying 1-anti-A specificity. Three of these families were informative, and the relevant HLA marker combinations of their members are shown in Fig. 1. Analysis of the HLA markers in the R family, including the complement genes, indicated that donor R3 carried a recombinant haplotype. The recombination involved the maternal gametes, and the breakpoint could be localized between the *BF* gene in the complement cluster and *HLA-B*. Blast cells from donor R1 were susceptible to lysis (Table 1), thus suggesting that this individual was homozygous for the recessive allele of the locus *EC1* (−/−). Cells from the R2 donor were resistant to lysis. In this donor, locus *EC1* was in a heterozygote configuration (+/−), as indicated by the fact that both resistant and susceptible individuals were present in the progeny. The family segregation analysis indicates that the recessive *EC1*$^-$ allele segregates with the c haplotype, whereas the dominant *EC1*$^+$ allele segregates with the d haplotype. Because donor R3 (carrying the recombinant c/d haplotype) was susceptible to lysis, she must be homozygous for locus *EC1* (−/−). Thus, it appears that the *EC1*$^-$ allele is inherited in association with markers that are telomeric to the *BF* gene.

Table 1. Cytolytic activity of CD3$^-$ CD16$^+$ 1-anti-A clones against PHA blasts from individuals of three families, including individuals inheriting HLA recombinant haplotypes

^{51}Cr-labeled target cells*			Cytolytic activity† of 1-anti-A CD3$^-$ CD16$^+$ clones‡		
Family	Donor	HLA haplotype	CA 8.25	CA 2.100	CA 6.50
R	1	a b	75‡	53	73
R	2	c d	2	0	0
R	3	a c/d	76	52	71
R	4	a c	82	60	77
R	5	b d	0	0	2
R	6	a c	74	55	65
M	1	a b	0	1	0
M	2	c d	69	67	49
M	3	a c	34	38	25
M	4	a c	38	51	43
M	5	a/b c	3	3	1
G	1	a b	75	31	46
G	2	c d	0	0	1
G	3	a c/d	61	59	38
G	4	a c	0	0	0

Markers for HLA in each family are shown in Fig. 1.
*Target cells were represented by PHA-blasts derived from donors R1–R6, M1–M5, and G1–G4.
†Results are expressed as % of specific ^{51}Cr release at an effector/target ratio of 5:1.
‡1-anti-A clones were derived from individual 1 upon stimulation in mixed lymphocyte culture against donor A.

Further analysis of the two other informative families allowed mapping of the *EC1* locus between class I and complement cluster genes. In family M, donor M5 carried a recombinant haplotype of paternal derivation. In this donor, the breakpoint was located between the *A* and *C* loci of class I genes (Fig. 1). Analysis of susceptibility of lysis by 1-anti-A NK clones revealed that donor M1 was heterozygous for the *EC1* locus (+/−), whereas his mate (donor M2) was susceptible to lysis and, thus, homozygous (−/−). Donor M5 was resistant to lysis and, thus, the locus *EC1* must be heterozygous (+/−). In this case, the *EC1*$^+$ allele was carried by the recombinant haplotype. Analysis of segregation of the pater-

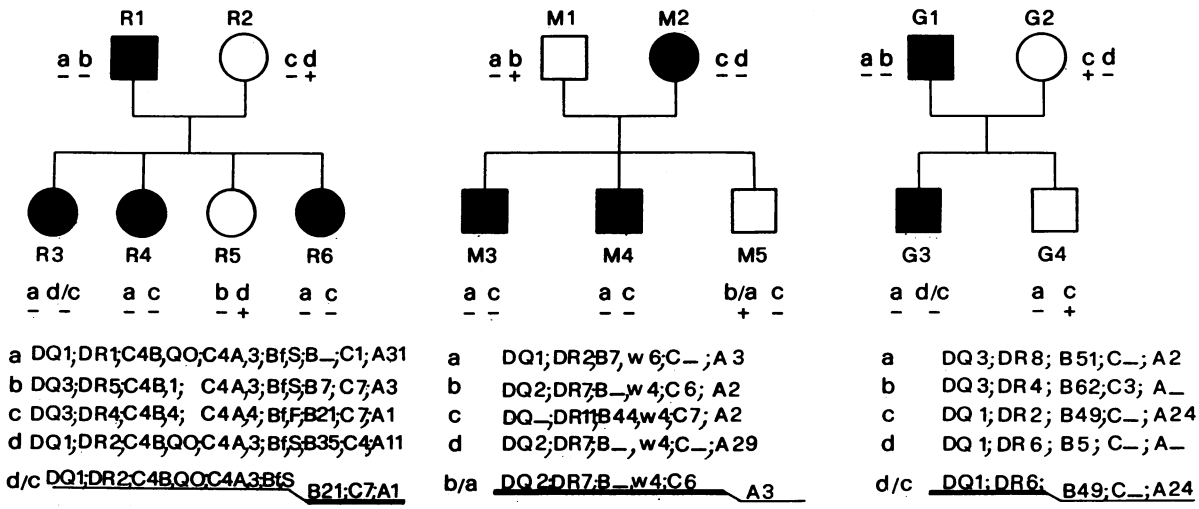

FIG. 1. Mapping of *EC1* locus in the human MHC region. The segregation of the characters susceptibility or resistance to lysis by 1-anti-A clones is analyzed in three representative families (R, M, and G) that include individuals inheriting HLA recombinant haplotypes. Black symbols refer to individuals whose PHA blasts were susceptible to lysis, whereas resistance to lysis is represented by white symbols; circles represent female donors, and squares represent male donors. Small letters (a, b, c, and d) indicate MHC haplotypes; +, allele of *EC1* locus that confers resistance to lysis; −, allele of *EC1* locus that confers susceptibility to lysis. Segregation of *EC1* in relationship to HLA markers in these families indicates that the *EC1* locus maps between class I and class III genes. Bf, BF.

nal haplotype clearly indicated that the *EC1*$^+$ allele was carried by the b haplotype. Therefore, it is possible to conclude that the *EC1* locus maps centromeric to *HLA-A*.

In the third family (G,) the donor G3 carried a recombinant haplotype derived from the mother (G2). In this case, the breakpoint was localized between the *HLA-B* and *HLA-DR* loci by HLA typing. Donor G2 was heterozygous for *EC1* (+/−), whereas donor G1 was homozygous (−/−). Because PHA blast cells of donor G3 were susceptible to lysis by 1-anti-A NK clones, we conclude that the *EC1*$^-$ allele is carried by the recombinant haplotype. Analysis of the segregation in the family members indicated that the recessive *EC1*$^-$ allele, of maternal derivation, is associated with the d haplotype. Thus, the *EC1* locus appears centromeric to the *HLA-B* locus.

To better define the breakpoint in families G and R, the restriction fragment length polymorphism using a set of probes derived from the MHC between *HLA-B* locus and the *C2* gene was studied. Analysis with a BAT3 cDNA probe was informative in family G. The cDNA/restriction enzyme combination BAT3/*Taq* I identified polymorphic bands at 2.5 and 2.7 kilobases (kb), which showed allelic behavior and codominant segregation (Fig. 2). In the G family, the 2.5-kb band cosegregated with the d haplotype (Fig. 2). Because the recombinant G3 donor showed a d haplotype for the BAT3 gene and a c haplotype for the class I HLA genes, the breakpoint of recombination was between the BAT3 and *HLA-B* loci. Thus, the breakpoint is clearly centromeric to *HLA-B*.

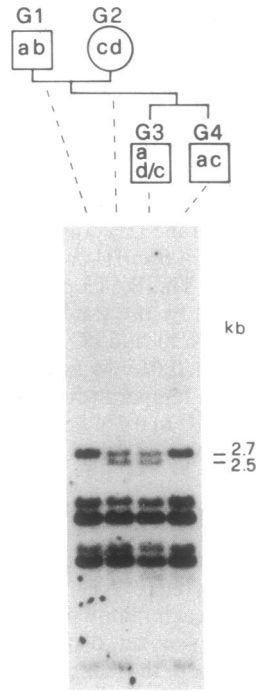

FIG. 2. Restriction fragment length polymorphism analysis of donors from family G. PHA blasts from the various donors were used. DNA samples were extracted, as described, and digested with *Taq* I restriction enzyme. The filter was hybridized with the BAT3 probe.

DISCUSSION

In the present paper, locus *EC1* governing the expression of susceptibility to lysis by 1-anti-A NK clones is shown to be contained in the MHC region between the class I and complement clusters (Fig. 3). This region includes at least 15 genes of unknown structure and function as well as two tumor necrosis factor and two heat shock protein genes (4, 11–14). One of these genes might be involved in the expression of the alloantigen recognized by NK cells. Further studies of DNA polymorphisms in the recombinant families may make it possible to localize *EC1* in a narrower genetic region.

It should be stressed that the character—susceptibility to lysis—is inherited in a recessive fashion. A possible explanation is that the *EC1* locus is not coding for the alloantigen itself but rather represents a regulatory gene that controls expression of the alloantigen. In this hypothesis, the activity of the dominant allele (*EC1*$^+$) determining resistance to lysis in a given individual would inhibit expression of the putative alloantigen. The recessive allele *EC1*$^-$ would be permissive for expression of the alloantigen. Thus, only individuals homozygous for this allele would express the alloantigen and would be susceptible to lysis. Alternatively, a protein-modifying gene that would mask the determinant recognized by the alloreactive NK clone would meet the requirements of this system. Carbohydrate modification, as exemplified by the ABO blood group system, would be an example, without implying that the system is, in fact, ABO. In such a system, an incomplete glycan (group O in the blood group system) would be the target antigen. Its masking by terminal glycosylation (e.g., group A or B) would provide for a dominant nonsusceptibility character and a recessive susceptibility

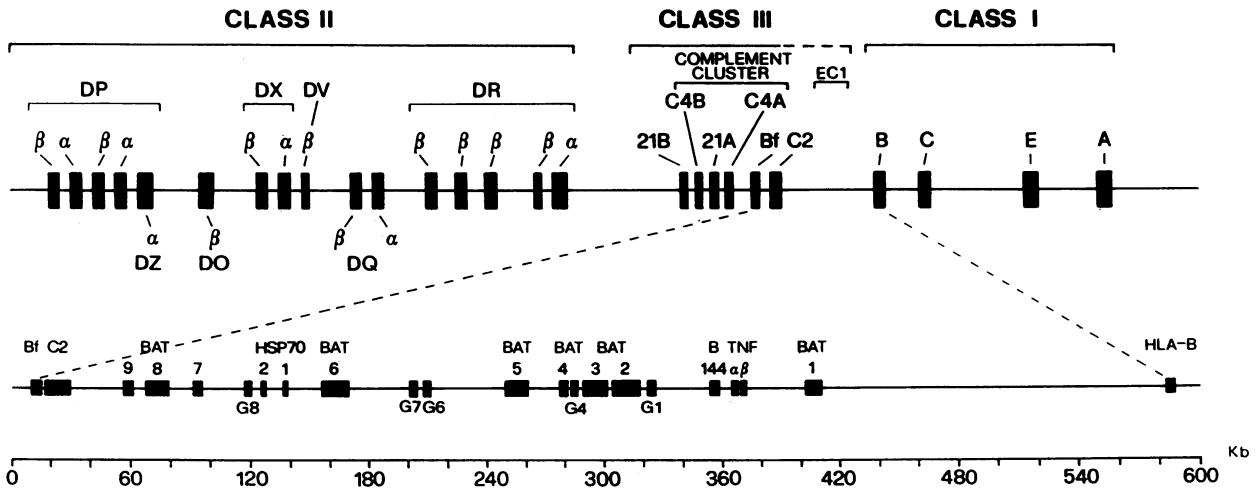

FIG. 3. Map of HLA region. Localization of the known genes in the region delimited by class I and the complement gene cluster are shown in greater detail. According to data of Fig. 1, the *EC1* locus maps in this region between the *BF* (Bf) and *HLA-B* locus. HSP70, 70-kDa heat shock protein; TNF, tumor necrosis factor.

trait. Several distinct susceptibility antigens could be accounted for by different degrees of incompleteness of the glycan, as the Lewis trait in the blood group system. Other types of protein modification could also meet the criteria for this system, but a recessive susceptibility trait is not easily accounted for by an amino acid polymorphism in the protein itself.

The ability of human NK cells to recognize and lyse normal allogeneic cells may have a murine counterpart in the so-called "hybrid resistance" (*Hh-1*) phenomenon. This phenomenon refers to the ability of murine NK cells to reject H-2-compatible *Hh-1*-incompatible bone-marrow grafts. The Hh-1 alloantigens were shown to be recessively inherited and encoded in the murine MHC on chromosome 17 (15, 16). It is evident that the data obtained in the murine system are consistent with the human data presented here. In this study, locus *EC1* was mapped between the complement and class I gene clusters. Interestingly, also in mice, the *Hh-1* genes were mapped between *H-2S* (complement) and *H-2D* (class I) (17).

The present data further support the concept that, as in mice, another family of NK-defined alloantigen exists in humans. Preliminary experiments also suggest that the *EC1* locus is polymorphic, as indicated by the existence of several NK-defined specificities (ref. 3 and E.C., unpublished work). Although the biological relevance of NK-mediated alloantigen recognition is presently unknown, this phenomenon could have important implications in human bone-marrow transplantation.

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1. Trinchieri, G. (1990) *Adv. Immunol.* **47,** 187–376.
2. Ciccone, E., Viale, O., Pende, D., Malnati, M., Biassoni, R., Melioli, G., Moretta, A., Long, E. O. & Moretta, L. (1988) *J. Exp. Med.* **168,** 2403–2408.
3. Ciccone, E., Pende, D., Viale, O., Tambussi, G., Ferrini, S., Biassoni, R., Longo, A., Guardiola, J., Moretta, A. & Moretta, L. (1990) *J. Exp. Med.* **172,** 47–52.
4. Spies, T., Bresnahan, M. & Strominger, J. L. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 8955–8958.
5. Melioli, G., Ciccone, E., Mingari, M. C., Pende, D., Viale, O., Tambussi, G., Merli, A., Moretta, A. & Moretta, L. (1989) *Int. J. Cancer* **4,** 56–57.
6. Moretta, A., Pantaleo, G., Moretta, L., Mingari, M. C. & Cerottini, J. C. (1983) *J. Exp. Med.* **158,** 571–583.
7. Terasaki, P. I. & McClealland, J. D. (1964) *Nature (London)* **204,** 998–1007.
8. Pellegrino, M. A., Ferrone, S., Dierich, M. P. & Reisfeld, R. A. (1975) *Clin. Immunol. Immunopathol.* **3,** 324–331.
9. Sim, E. & Cross, S. J. (1986) *Biochem. J.* **239,** 763–767.
10. Alper, C. A., Boenisch, T. & Watson, L. (1962) *J. Exp. Med.* **135,** 68–80.
11. Spies, T., Blank, G., Bresnahan, M., Sands, J. & Strominger, J. L. (1989) *Science* **243,** 214–217.
12. Spies, T., Morton, C. C., Nedospasov, S. A., Fiers, W., Pious, D. & Strominger, J. L. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 8699–8702.
13. Sargent, C. A., Dunham, I., Trowsdale, J. & Campbell, R. D. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1968–1972.
14. Sargent, C. A., Dunham, I. & Campbell, R. D. (1989) *EMBO J.* **8,** 2305–2312.
15. Cudkowicz, G. & Bennet, M. (1971) *J. Exp. Med.* **134,** 1513–1528.
16. Bennett, M. (1987) *Adv. Immunol.* **41,** 333–445.
17. Rembecki, R. M., Kumar, V., David, C. S. & Bennet, M. (1988) *J. Immunol.* **141,** 2252–2260.