Biochemistry. In the article "Determination of pathways of glycolysis synthesis and the dilution of the three-carbon pool with [U-13C]glucose" by Joseph Katz, P. A. Wals, and W. N. Paul Lee, which appeared in number 6, March 1991, of Proc. Natl. Acad. Sci. USA (88, 2103–2107), the following correction should be noted. On p. 2107, Eq. 4 should read as follows.

\[ E = \frac{3 \sum_{i=1}^{3} m_i}{\sum_{i=1}^{3} m_i n_i} \]  

Immunology. In the article "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" by Ermanno Ciccone, Marco Colonna, Oriane Viale, Daniela Pende, Carolina Di Donato, Daniel Reinharz, Antonio Amoroso, Michel Jeannet, John Guardiola, Alessandro Moretta, Thomas Spies, Jack Strominger, and Lorenzo Moretta, which appeared in number 24, December 1990, of Proc. Natl. Acad. Sci. USA (87, 9794–9797), the authors request that the following correction be noted: Repeat and more extensive HLA typing of family G employed in our study has revealed a typing error that substantially alters interpretation of the data. In particular, the d haplotype of the mother, G2, has now been established as A23(9), B49(21), Cw5 or Cw7, Drw6, DRw52, DQw1. The putative recombinant child, G3, has inherited the maternal d haplotype intact and is thus not a recombinant within HLA. The typing errors were due to cross-reactions of the B5 antiserum employed with B49 and to the relative difficulty in distinguishing the A24 and A23 splits of A9, which were corrected by using a different panel of alloantisera. The G family was critical in appearing to establish the telomeric break point on the short arm of chromosome 6 within which the EC1 gene had to lie. Linkage of this gene to the human major histocompatibility complex and its centromeric limit at BF remain firmly established. It is important to emphasize that, in addition to all of the distinctive genes between BF and HLA-B, none of the genes in the class I region of the human major histocompatibility complex centromeric of HLA-A can now be excluded as the locus for EC1.

Biochemistry. In the article "Escherichia coli kgtP encodes an α-ketoglutarate transporter" by Wongi Seol and Aaron J. Shatkin, which appeared in number 9, May 1991, of Proc. Natl. Acad. Sci. USA 88, 3802–3806, the authors request the following correction be noted. On p. 3803, in Fig. 2A, the restriction site in witA labeled Sac I should read Sca I.

Biochemistry. In the article "Three-dimensional structure of human basic fibroblast growth factor, a structural homolog of interleukin 1β" by Jiandong Zhang, Lawrence S. Cousens, Phillip J. Barr, and Stephen R. Sprang, which appeared in number 8, April 1991, of Proc. Natl. Acad. Sci. USA (88, 3446–3450), too much was cropped from the sides of Fig. 2, so that part of the color image was lost. The complete figure and its legend are shown below.

FIG. 2. Ribbon diagram illustrating the tertiary and secondary structure of bFGF. β-Sheet secondary structure is depicted with flattened ribbons. The polypeptide segment (residues 105–115) implicated in receptor recognition is shown in red (see text). The diagram was produced using the computer program RIBBONS by M. Carson (University of Alabama, Birmingham).
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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Contributed by Jack L. Strominger, September 5, 1990

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 ECI. Because the ECI 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 breakopoint 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 ECI 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 [phytohemagglutinin (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 autosomal 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 ECI. Thus, allogeneic target cells susceptible to lysis are presumed homozygous for the recessive allele (ECI \(-/-\)), whereas the character—resistance to lysis—is conferred by a dominant allele, either in an heterozygous (ECI \(+/-\)) or in an homozygous (ECI \(+/+\)) configuration. In this paper, mapping of the ECI 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 × 10⁴ 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 ⁵¹Cr-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 × 10⁴ 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). 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 (Serakem 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

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 Tag 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 BamHI-Xba I fragment from the BAT cDNA clone (11). The BAT3 probe was radiolabeled with [α-32P]dCTP (NEN) by the random-primer procedure. Filters were hybridized 16 hr at 65°C in 6x SSC (1x SSC is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7), washed 10 min at room temperature in 5x SSC for three times, 10 min each, at 65°C in 0.1x SSC buffer, and exposed to XAR films (Kodak) for 16–48 hr.

**RESULTS**

To map the ECI 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 ECI (−/−). Cells from the R2 donor were resistant to lysis. In this donor, locus ECI 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 ECI− allele segregates with the c haplotype, whereas the dominant ECI+ 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 ECI (−/−). Thus, it appears that the ECI− allele is inherited in association with markers that are telomeric to the BF gene.

Further analysis of the two other informative families allowed mapping of the ECI 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 to lysis by 1-anti-A NK clones revealed that donor M1 was heterozygous for the ECI locus (+/−), whereas his mate (donor M2) was susceptible to lysis and, thus, homozygous (+/−). Donor M5 was resistant to lysis and, thus, the locus ECI must be heterozygous (+/−). In this case, the ECI+ allele was carried by the recombinant haplotype. Analysis of segregation of the parental haplotypes in the three families indicated that the ECI locus maps between class I and class III genes.

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**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**

<table>
<thead>
<tr>
<th>Family</th>
<th>Donor</th>
<th>HLA haplotype</th>
<th>51Cr-labeled target cells*</th>
<th>Cytolytic activity† of 1-anti-A CD3+ CD16+ clones†</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>1</td>
<td>a c</td>
<td>75†</td>
<td>CA 8.25</td>
</tr>
<tr>
<td>R</td>
<td>2</td>
<td>c d</td>
<td>25</td>
<td>CA 2.100</td>
</tr>
<tr>
<td>R</td>
<td>3</td>
<td>a c/d</td>
<td>76</td>
<td>CA 6.50</td>
</tr>
<tr>
<td>R</td>
<td>4</td>
<td>a c</td>
<td>82</td>
<td>G 1</td>
</tr>
<tr>
<td>R</td>
<td>5</td>
<td>b d</td>
<td>0</td>
<td>G 2</td>
</tr>
<tr>
<td>R</td>
<td>6</td>
<td>a c</td>
<td>74</td>
<td>G 3</td>
</tr>
<tr>
<td>M</td>
<td>1</td>
<td>a b</td>
<td>0</td>
<td>G 4</td>
</tr>
<tr>
<td>M</td>
<td>2</td>
<td>c d</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>3</td>
<td>a c</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>4</td>
<td>a c</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>5</td>
<td>a/b c</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>1</td>
<td>a b</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>2</td>
<td>c d</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>3</td>
<td>a c/d</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>4</td>
<td>a c</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*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 51Cr 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.

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**Fig. 1.** Mapping of ECI 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 HMC haplotypes; +, allele of ECI locus that confers resistance to lysis; −, allele of ECI locus that confers susceptibility to lysis. Segregation of ECI in relationship to HLA markers in these families indicates that the ECI 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 (G3) 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 1 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.

**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. 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 1 restriction enzyme. The filter was hybridized with the BAT3 probe.

**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 ECI 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 ECI 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.

This work was supported, in part, by grants awarded by the Consiglio Nazionale delle Ricerche (CNR), Piano Finalizzato Oncologia, Associazione Italiana per la Ricerca sul Cancro (AIRC), Istituto Superiore di Sanità (ISS), and by the National Institutes of Health (CA-47554). O.V. is recipient of an AIRC fellowship.