RECOMBINATION WITHIN THE HLA-D REGION

Correlation of Molecular Genotyping with Functional Data

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The major histocompatibility complex (MHC)[1] has been shown to play a crucial role in the control of a variety of immune responses. The MHC consists of discrete regions, the products of which are distinguished by structural and functional features. Genes within the murine H-2I region have been shown to control such functions as immune responsiveness and immune suppression as well as to mediate certain interactions that occur between T lymphocytes and B lymphocytes and macrophages (1–2). Similar functions have been mapped to the homologous MHC region in man, HLA-D/DR, and, in addition, this region has been implicated in the susceptibility to certain disease states (3–4).

The human HLA-D/DR region and the murine H-2I region have been shown to contain genes that encode a family of related molecules collectively known as class II products. Class II products consist of two MHC-encoded chains: an alpha chain with a molecular weight of 33–34,000 and a beta chain with a molecular weight of 27–30,000 (5–6). In the murine system it has been possible to design experiments to effectively investigate the role of subregions of the H-2I region using inbred congenic and recombinant strains of mice. It has been more difficult to obtain a functional description of the HLA-D/DR region that is equal in detail to that of the murine I region due to the heterogeneity of the human population and furthermore due to ethical considerations in human experimentation. In addition to the relative difficulties in studies of human class II gene function there is considerable evidence that the HLA-D/DR region is more complex than the H-2I region. The HLA-D/DR region consists of two subregions respectively designated DR and DC/MB (6–7) and there is in addition a third subregion encoding class II molecules that has been designated SB (8). Furthermore, there appear to be more genes within each human subregion than have been found in the H-2I region (9–13). Recent investigations at the molecular level have provided new information concerning human class II genes and have also furnished experimental techniques to further investigate the complexity of the HLA region.

DNA polymorphisms can be detected using specific HLA class II gene probes and restriction endonucleases (14).

The present report describes the results of molecular genotyping studies designed to investigate the arrangement of class II genes in the HLA complex and to relate the presence of specific genes to their respective functions. The family studied in this report had been shown to include an individual who inherited an HLA haplotype recombinant between HLA-B and HLA-D/DR through extensive characterization of HLA antigens using serological and cellular methodologies. Human cDNA probes corresponding to class II alpha and beta chain genes were used in Southern blotting experiments and the segregation of restriction fragments was analyzed in the family. Polymorphic restriction fragments observed for every cDNA probe tested segregated in the family according to previously defined haplotypes in all family members, including the individual known to have inherited the recombinant HLA haplotype. There was, however, a single exception. Molecular genotyping studies using an SBβ gene probe indicated that an additional crossover event had occurred in the family. Subsequent testing of lymphocytes from the individual identified by the molecular genotyping studies revealed that gene products were expressed and could be detected both by cellular and serological reagents.

Materials and Methods

Lymphoblastoid Cell Lines. Lymphoblastoid cell lines (LCL) were prepared by culturing 5×10^6 peripheral blood lymphocytes in the presence of supernatant from the Epstein-Barr virus–producing cell line B95-8 and cyclosporin A (2 μg/ml). RPMI 1640 culture medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco Laboratories, Grand Island, NY) and 10% heat-inactivated fetal calf serum (Dutchland Laboratories Inc., Denver, PA) was used to maintain the cell lines.

HLA Typing. HLA-A, B, and C typing was performed using a modification of the standard microcytotoxicity assay (15). HLA-DR typing was performed using B lymphocytes positively selected with goat antiserum to the F(ab')$_2$ fragments of human IgG in a monolayer. Lymphocytes were incubated for 60 min with antisera and 60 min with complement. Antisera used were capable of detecting all currently defined World Health Organization HLA and provisional workshop (w) specificities at the HLA-A, B, C, and DR loci. In addition, sera capable of detecting MB1, 2, and 3 and MT1, 2, and 3 were used (16). The identity of all LCL was confirmed by typing for HLA-A, B, C, and DR.

Primed Lymphocytes. Lymphocytes were primed in vitro using previously described methods (17–18). Essentially 10×10^6 responder lymphocytes and 10×10^6 irradiated stimulator lymphocytes (2,000 rad ^{37}Ci) were co-cultured in vitro in RPMI 1640 (Gibco Laboratories) with 50 μg/ml gentamycin (Elkins-Sinn, Inc., Cherry Hill, NJ) and 10% human serum in 50-ml tissue culture flasks (Falcon Labware, Oxnard, CA). Responder and stimulator lymphocyte donors were selected to be compatible for all HLA antigens with the exception of one nonidentical SB specificity present on the stimulator lymphocytes. Lymphocytes were initially cultured for 10 d followed by a restimulation with an equal number of original stimulator lymphocytes for an additional 7 d or until <10% blasts cells remained in the culture. SB typing reagents 1–5 (SB1–5) were kindly provided by S. Shaw, National Cancer Institute. SB-6 was developed using similar methods at Georgetown University. These reagents were expanded using SB-specific feeder/stimulator lymphocytes (fivefold excess) and interleukin 2 (quality controlled for specificity) and were provided to the Ninth International Histocompatibility Workshop as standard reagents.

SB typing was performed in matrix experiments with two reagents for each specificity responding to each of 60 stimulator lymphocytes of unknown type and control stimulator

lymphocytes of known SB type. Cells (1×10^4 primed lymphocytes and 5×10^5 stimulator or test lymphocytes) were cultured in round-bottomed, 96-well tissue culture plates (Costar, Cambridge, MA) for 60 h with 1 μCi [^{3}H]thymidine per well (5 Ci/mM) (Amersham Corp., Arlington Heights, IL) added during the last 16 h of culture. Cultures were harvested onto glass fiber filters (19) and analyzed for counts per minute β emission. Specificity was assigned based on the maximized t difference described by Mendell (20) for paired SB reagents (SB1a + SB1b).

Primed cells using lymphocytes from family members were generated by co-culturing 3×10^6 responder and 3×10^6 irradiated stimulator lymphocytes for 10 d. Cells were cryopreserved and later tested against lymphocytes from family members and a panel of well-characterized individuals.

Flow Cytometry.   Indirect immunofluorescence was performed on LCL using monoclonal antibody ILR1 (21) and fluorescein-conjugated sheep anti-mouse IgG (not cross-reactive with human Ig; Cappell Laboratories, West Chester, PA). Flow cytometry was performed on a fluorescence-activated cell sorter (FACS-Analyzer; B-D FACS Systems, Sunnyvale, CA). ILR1 ascites fluid was the kind gift of L. Nadler, Sidney-Farber Cancer Institute, Boston, MA and S. Shaw, National Cancer Institute.

DNA Preparation.   DNA was isolated from LCL by suspending 2×10^8 cells in 15 ml 10 mM Tris-HCl, pH 8.0, 0.4 M NaCl, 10 mM EDTA, 0.2% sodium dodecyl sulfate (NaDodSO$_4$). Proteinase K was added to a final concentration of 100 μg/ml and the mixture incubated for 3 h at 37°C. After extraction with phenol and chloroform, the DNA was precipitated with ethanol. Precipitated DNA was resuspended in 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA. RNAse A (25 μg/ml) was added and the mixture incubated at 37°C for 1 h. The NaCl concentration was increased to 500 mM and, after extraction with phenol and chloroform, the DNA was precipitated with ethanol. The DNA was suspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

cDNA Probes.   cDNA clones corresponding to DRα (22), DRβ (23), DCβ (23), and SBβ (24) have been described previously. The probe for DCα was a cDNA clone kindly provided by C. Auffray (25). Inserts were prepared from Pst1-digested plasmid DNA by electroelution of fragments separated in agarose gels. Inserts (200 ng DNA) were nick translated using the Nick Translation Kit and 100 μCi deoxycytidine 5'-[α-^{32}P]triphosphate (3,000 Ci/mM; Amersham Corp.) and had a specific activity of $\sim 10^8$ cpm/μg DNA.

Digestion of Genomic DNA with Restriction Endonucleases.   High molecular weight DNA (24–48 μg) was digested in the appropriate buffer with 2.5 U of enzyme/μg DNA at 37°C overnight. EcoRI, HindIII, and Pst1 were purchased from New England Biolabs, Beverly, MA. BGlII was purchased from Bethesda Research Laboratories, Bethesda, MD.

Southern Blot Analysis.   Restriction endonuclease–digested DNA (10 μg/lane) was electrophoresed in 0.6% agarose gels for 36–48 h in 40 mM Tris, 10 mM sodium acetate, 1 mM EDTA brought to pH 7.2 with acetic acid. Lambda DNA digested with HindIII was used for size markers. Blots were performed according to the method of Southern (26) using nitrocellulose filters (Millipore Corp., Bedford, MA).

Blots were routinely prehybridized in the presence of 10% dextran sulfate and 40% formamide (27), then hybridized for 18 h at 40°C using a probe at a concentration of $3.5–5.0 \times 10^6$ cpm/ml. After three low stringency washes at room temperature, the blots were washed twice in 15 mM sodium chloride, 1.5 mM sodium citrate, pH 7.0, 0.05% NaDodSO$_4$ at 65°C for 30 min. For the final washes of blots hybridized with the SBβ probe, 75 mM sodium chloride, 7.5 mM sodium citrate, pH 7.0, 0.05% NaDodSO$_4$ was used. Blots were exposed for 24–72 h to Kodak Xomat AR film at −70°C using Dupont Lightening Plus screens.

Results

The HLA genotypes obtained for family 7 using classic HLA typing techniques are indicated in Table I. One of the children, S2, was shown to have inherited a paternal HLA haplotype recombinant between the HLA-B and HLA-D regions. Offspring S2 inherited HLA-ABC of the paternal a haplotype, the HLA-D region

TABLE I

Family 7 HLA Genotypes

Father	Mother	Offspring			
		S1	S2	S3	S4
a		a	a		
b			b	b	b
	c		c		
	d	d		d*	d
a	A2	Cw5	Bw44	DR4	DW4	MB3	MT3	SB2ᵛᵗ
b	Aw24	Cw−	B8	DR3	DW3	MB2	MT2	SB4
c	A2	Cw1	B27	DR3	DW3	MB2	MT2	SB4
d	A11	Cw−	B7	DR2	DW2	MB1	MT1	SB4

Genotypes deduced from HLA typing are shown for each family member. The description of each haplotype is given in the lower portion of table.

* Individual S2 inherited an HLA haplotype recombinant between HLA-B and DR as indicated by (___). Individual S3 will be shown to have inherited a previously undetected recombinant HLA haplotype tentatively designated d* and indicated by (---).

ᵗ SB2ᵛ is the tentative designation for the SB allele of the a haplotype. SB2ᵛ is closely related to but not identical with SB2.

of the paternal b haplotype, and the maternal c haplotype. Because the paternal b haplotype and the maternal c haplotypes both carried DR3, Dw3, MB2, MT2, and SB4, individual S2 was homozygous for these antigens.

All family members were positive for SB4, which was the only clearly typable SB allele in the family using standard primed lymphocyte typing reagents for SB. The father and S1 were both positive for the a haplotype and had low but positive reactions with both SB2 typing reagents. On this basis, the a haplotype is postulated to carry a variant SB2 allele tentatively designated herein as SB2ᵛ.

DNA samples obtained from LCL derived from lymphocytes from members of family 7 were analyzed for the segregation of restriction fragments hybridizing to cDNA probes that correspond to genes that encode class II antigens. Patterns were scrutinized for polymorphic fragments and for segregation of these fragments according to HLA haplotypes in the family. Special attention was paid to the recombinant haplotype. Restriction fragments of DNA from this individual (S2) were, on the basis of typing results, predicted to segregate with the b haplotype; restriction fragments segregating with the a haplotype would indicate the occurrence of recombination within the D region and map the gene telomeric in respect to the crossover.

Southern blots of DNA from members of family 7 were hybridized with ^{32}P-labeled cDNA probes that correspond to DCα, DRα, DRβ, DCβ, and SBβ genes. Characteristics of the cDNA clones from which the hybridizing probes were isolated have been published previously (22–25). The cDNA fragments used as probes in the following studies were Pst1 fragments identified in Fig. 1. Mixtures of A and B fragments were used for DRα, DRβ, and DCβ probes as shown in Fig. 1.

Segregation of DRα and DCα Restriction Fragments. Hybridization patterns of DRα and DCα probes to digested DNA from family members are depicted in
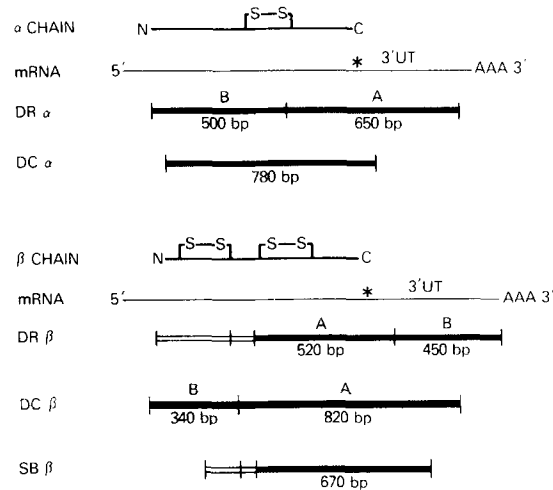
FIGURE 1. Schematic representation of human class II alpha and beta chains, the corresponding mRNA, and the cDNA probes used in these studies. The cDNA clones are marked with vertical lines to indicate PstI restriction sites and darkened areas indicate regions used as probes. Fragment designations (A and B) are indicated above cDNA and the approximate sizes in base pairs (bp) are given below.

Fig. 2. In all cases the assignment of restriction fragments of haplotype was concordant with previously obtained HLA haplotype designations. In Fig. 2A, genomic DNA was digested with BglII, an enzyme previously shown to detect a polymorphism in the 3' untranslated region of the DRα chain gene (28). DNA from both parents have the 4.8 kb fragment, and DNA from the father has an additional 4.4 kb fragment. All the children inherited the 4.8 kb fragment from the mother. The 4.8 kb paternal fragment can be assigned to the a haplotype; S1 (a/d) has only a 4.8 kb fragment. The other three children (S2, S3, S4) inherited the paternal b haplotype and all have in addition the 4.4 kb fragment. Consistent with serological typing, the recombinant S2 inherited the paternal b haplotype restriction fragment. Although the b and c haplotypes are identical as characterized by classical typing techniques, they can be distinguished by restriction enzyme fragments.

Fig. 2B shows hybridization of EcoRI-digested DNA samples with the DCα probe; four fragments that hybridize with the DCα probe were observed (14, 11, 6.8, and 5.4 kb). The 5.4 kb fragment was present in DNA from all family members and therefore was not informative. The father and S1 were positive for the 6.8 kb fragment indicating that it segregated with the a haplotype. This fragment was absent from DNA from the recombinant, S2, characterizing the recombinant haplotype for DCα as non-a. The 11 kb fragment was present in DNA from both the mother and the father. The absence of the 11 kb fragment in S1 (a/d) indicated that it segregated with both the b and c haplotypes. The 14 kb fragment segregated with the d haplotype as it was observed in the mother (c/d), S1 (a/d), S3 and S4 (b/d), but not the father (a/b) nor S2 (a-b/c). In other experiments genomic DNA was digested with BamHI and HindIII and tested with the DCα probe (data not shown). Fragments specific for the a haplotype

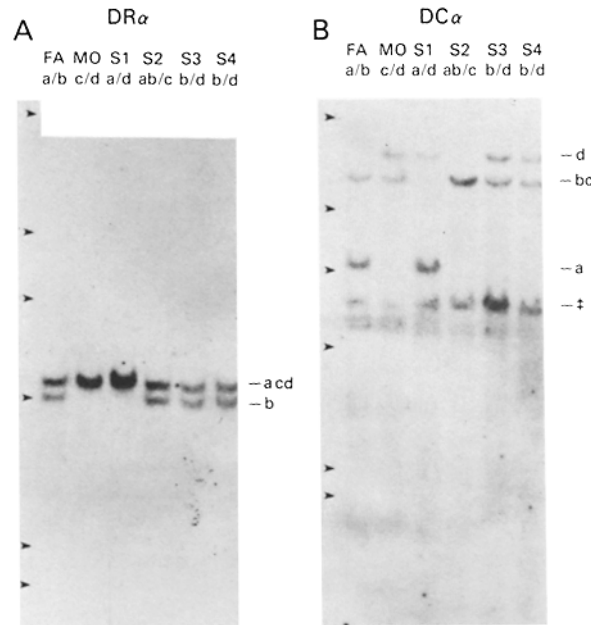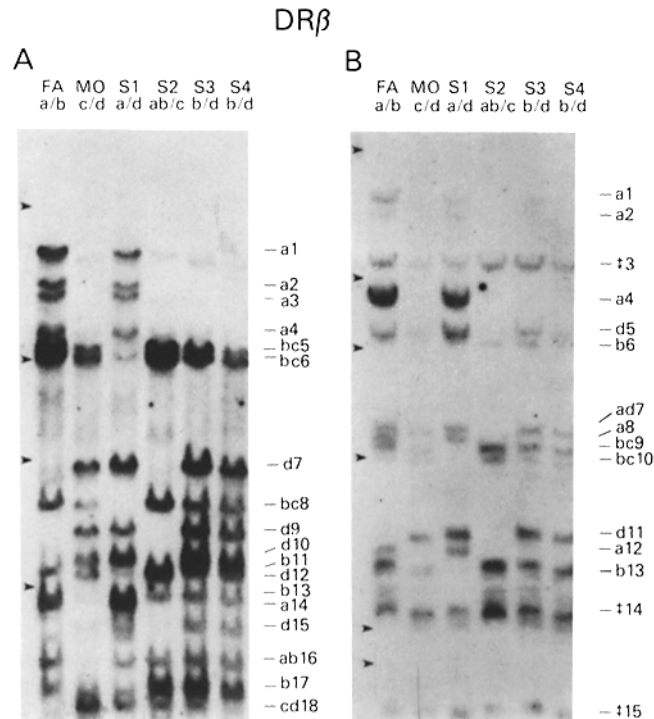FIGURE 2. Southern blot analysis of family 7 using DRα and DCα probes. Lanes of gel are headed by the identification of DNA donors and haplotype designations, as detailed in Table I. Lines to the right of each blot mark restriction fragments and letters indicate the haplotype assignment of the fragment. ‡ designates bands common to all family members. Arrows to the left of each blot mark lambda DNA digested with HindIII having restriction fragments of sizes 23.1 kb, 9.4 kb, 6.7 kb, 4.4 kb, 2.3 kb, and 2.0 kb, respectively. (A) Southern blot of DNA derived from members of family 7 digested with BglII and hybridized with the DRα probe. (B) Southern blot of DNA derived from members of family 7 digested with EcoRI and hybridized with the DCα probe.

were observed with both enzymes. These fragments were not present in DNA from the recombinant S2. In contrast to the results obtained using the DRα probe, these data using the DCα probe indicate that the b and c haplotypes have common restriction fragments.

Segregation DRβ and DCβ Restriction Fragments. Southern blots hybridized with probes corresponding to DRβ and DCβ genes display considerably more complex patterns than those hybridized with class II α chain probes (14). Genomic DNA samples from members of family 7 were digested with EcoRI and HindIII and hybridized with ^{32}P-labeled DRβ (Fig. 3) and DCβ probes (Fig. 4). The autoradiograms in Figs. 3 and 4 were exposed for 24 h; longer exposure times revealed additional fragments. In all cases it was possible to correlate fragments with haplotypes as previously assigned.

In Fig. 3A, 18 different EcoRI fragments that hybridized with the DRβ probe were observed. Five of the fragments (a1, a2, a3, a4, and a14) were specific for the a haplotype segregating in the father and sibling S1. One fragment (ab16) was common to both a and b paternal haplotypes and was inherited by all the children but was not observed in the mother even at longer exposure times. Three fragments (b11, b13, and b17) were specific for the b haplotype and three fragments (bc5, bc6, and bc8) were common to the b and c haplotypes. There

DRβ

FIGURE 3.   Southern blot analyses of family 7 (as in Fig. 2) using the DRβ probe. (A) Genomic DNA samples were digested with EcoRI. (B) Genomic DNA samples were digested with HindIII.

were no fragments specific for the c haplotype. Five fragments (d7, d9, d10, d12, and d15) segregated with the d haplotype in DNA from the mother, S1, S3, and S4. One fragment (cd18) was shared by both c and d maternal haplotypes. All fragments could be assigned to haplotypes, which confirms the assignment of DRβ hybridizing sequences to the HLA-D/DR region.

Fig. 3B shows HindIII DNA digests hybridized with the DRβ probe. 15 fragments were observed and all but 3 (‡3, ‡14, and ‡15), which were present in all family members, could be assigned to haplotypes. Five fragments (a1, a2, a4, a8, and a12) were specific for the a haplotype, two fragments (b6 and b13) for the b haplotype, and two fragments (d5 and d11) for the d haplotype. The cluster of fragments 7–10 were less sharply defined than other fragments, making haplotype designations somewhat difficult. However, each of these fragments could be assigned as follows: fragment ad7 is common to the a and d haplotypes and fragments bc9 and bc10 are common to the b and c haplotypes.

These data further distinguish the b and c haplotypes at the genomic level. Although classic typing results did not distinguish these DR antigens, the b haplotype had DRβ hybridizing fragments that were not present in the c haplotype.

Autoradiograms of Southern blots of family 7 hybridized with a ^{32}P-labeled probe that corresponds to the DCβ chain are shown in Fig. 4. Fig. 4A shows
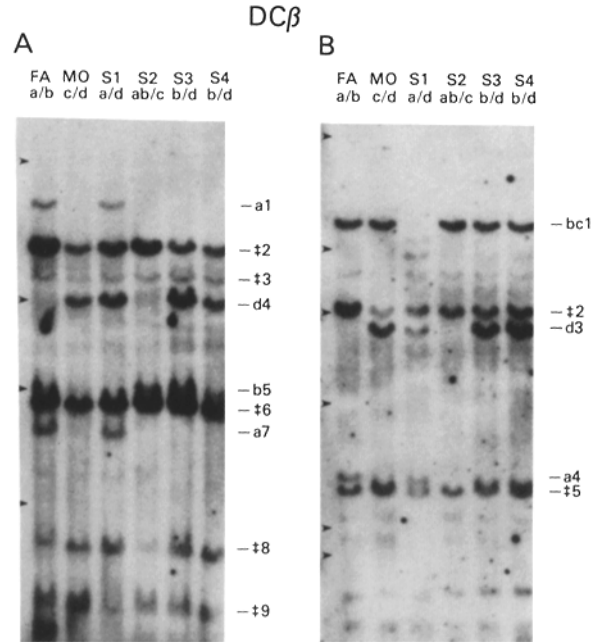
FIGURE 4. Southern blot analyses of family 7 (as in Fig. 2) using the DCβ probe. (A) Genomic DNA samples were digested with EcoRI. (B) Genomic DNA samples were digested with HindIII.

results using genomic DNA digested with EcoRI. Five of the nine fragments observed were present in DNA from all individuals and therefore were not informative in haplotype assignments (‡2, ‡3, ‡6, ‡8, and ‡9). Two fragments (a1 and a7) specific for the a haplotype were not present in DNA from individual S2 that inherited the recombinant haplotype. Fragments d4 and b15 were specific for the d and b haplotypes, respectively.

Fig. 4B depicts further DCβ blots in which the genomic DNA was digested with HindIII. Fragment bc1 was shared by the paternal b and maternal c haplotypes. Fragment d3 was specific for the d haplotype and fragment a4 segregated with the a haplotype. Two fragments (‡2 and ‡5) were present in DNA from all members of the family, as were several weakly hybridizing fragments not given designations in this figure. These weakly hybridizing fragments could be intensified by longer exposures and were consistently found in all family members. These weakly hybridizing fragments may represent cross-hybridization with DRβ chain genes; however, analyses of the segregation of informative fragments accorded with previous haplotype assignments. These data further confirm the assignment of DRβ and DCβ chain genes to the HLA-DR region. As was observed with the DRα and DRβ chain gene probes, a DCβ hybridizing restriction fragment difference between the b and c haplotype was observed.

Segregation of SBβ Restriction Fragments. It is known that, in addition to the SBβ gene, there is a second nonallelic and closely related β gene (13). Under the conditions used in our experiments, the SBβ probe hybridizes to both of these β

genes. Southern blot analyses of family 7 using the SBβ probe produced unexpected results. Fig. 5A depicts genomic DNA from the members of family 7 digested with EcoRI and hybridized with ^{32}P-labeled probe corresponding to the SBβ gene. The 10.5 kb fragment ($\ddagger$) was present in DNA from all family members and therefore could not be assigned to a haplotype. The presence of the 7.6 kb (b) and 7.2 kb (d) fragments was haplotype related in the family, with DNA from the father having only the 7.6 kb fragment and DNA from the mother only the 7.2 kb fragment. Segregation of the fragments in DNA from the children revealed that the fragments were present in only one of each of the parental haplotypes. The 7.2 fragment was present in S2, S3, and S4 but not S1, indicating that this fragment segregated with the paternal b and not with the a haplotype. The maternal 7.2 kb fragment was found in DNA from S1 and S4, suggesting that this fragment segregated with the d haplotype. There was an exception to this assignment in that S3, who was positive for the d haplotype, was negative for the 7.2 kb fragment. This result suggests that S3 inherited an HLA haplotype recombinant between HLA-DR and SBβ.

To evaluate SBβ restriction fragment polymorphisms in family 7, digests using other restriction enzymes were tested. Experiments using HindIII digests are shown in Fig. 5B. The 7.4 and 1.9 kb fragments ($\ddagger$) were present in DNA from all family members but the 5.8 kb (b) and 5.3 kb (acd) fragments were polymorphic. DNA from the father had both 5.8 and 5.3 kb fragments. S1 (a/d) inherited only the 5.3 kb fragment and S2 (a-b/c), S3 (b/d*), and S4 (b/d) inherited both 5.8 and 5.3 kb fragments. These results indicate that the 5.3

SBβ
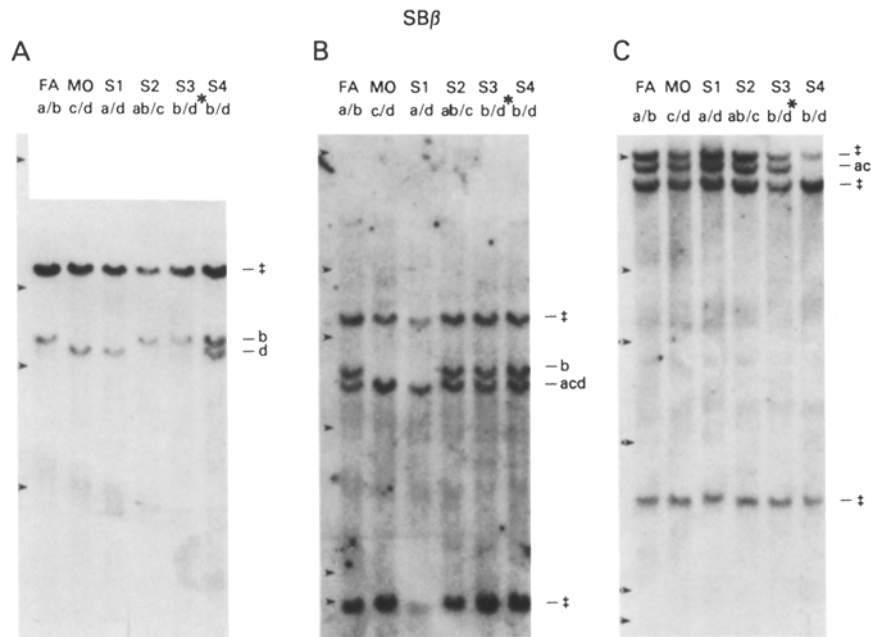
fragment was carried by the a haplotype and the 5.8 fragment by the b haplotype. Only the 5.3 kb fragment was present in DNA from the mother and all of her children inherited this fragment, indicating that this fragment segregates with both the c and d haplotypes. Therefore, although results with HindIII were not informative in analyzing the putative crossover in S3, they did help characterize the SB4 haplotypes in this family.

BglII was also used as a restriction enzyme to detect polymorphism in SBβ hybridizing fragments (Fig. 5C). Fragments of approximately 23, 16.5, and 3.3 kb were present in DNA from all family members. The fragment of ~20 kb (ac) was present in all family members with the exception of S4 (b/d), which indicates that this fragment is carried by both the a and c haplotypes. The presence of this fragment in DNA from S3 further supports the results with EcoRI, indicating that S3 has inherited a recombinant haplotype between DR, DC/MB of the d haplotype and SBβ of the c haplotype.

Cellular Detection of the Recombinant SBβ Haplotype. To determine whether the differences in SBβ restriction fragment sizes correlated with differences in expressed SB products, primed lymphocytes were tested against lymphocytes from family members (Table II). SB2a and SB2b reagents were partially responsive to the father (a/b) and S1 (a/d) as compared with the positive control and other family members, which indicates that the a haplotype carries a SB2-related antigen designated herein as SB2^v. SB4a and SB4b reagents were positive with all family members and the positive control, indicating that all family members

TABLE II

Primed Lymphocyte Analyses of Family

Stimulator-primed lymphocytes*	FA a/b	MO c/d	S1 a/d	S2 a-b/c	S3 b/d*	S4 b/d	Positive control[‡]
			cpm				
SB2a	5,749	169	4,150	635	159	219	25,954
	(1.3)	(1.3)	(1.2)	(1.4)	(1.2)	(1.2)	(1.1)
SB2b	11,521	207	8,141	904	178	307	42,599
	(1.1)	(1.3)	(1.1)	(2.1)	(1.1)	(1.2)	(1.1)
SB4a	38,186	44,802	35,645	73,689	32,382	60,842	59,265
	(1.2)	(1.1)	(1.0)	(1.3)	(1.1)	(1.1)	(1.0)
SB4b	12,897	29,492	14,181	30,158	14,302	27,733	24,746
	(1.1)	(1.1)	(1.1)	(1.2)	(1.1)	(1.1)	(1.1)
S4(S3)$_x$	4,012	13,596	7,478	15,585	12,607	8,368	12,607
	(1.1)	(1.1)	(1.0)	(1.1)	(1.1)	(1.1)	(1.1)
S3(S4)$_x$	1,247	2,288	1,006	1,156	1,516	1,534	1,534
	(1.4)	(1.1)	(1.1)	(1.3)	(1.1)	(1.1)	(1.1)

Primed lymphocyte analysis of family 7 using SB typing reagents. Data are given as the geometrical mean of [³H]thymidine uptake as measured by cpm in three replicate cultures for each primed cell/stimulator pair. The standard error factor is given below in parentheses. Boxes: (———) positive response, (---) partial response.

* SB2a, SB2b, SB4a, and SB4b are standard SB typing reagents. S4(S3)$_x$ and S3(S4)$_x$ are reagents derived from family 7 members; ()$_x$ designates irradiated lymphocytes used as stimulators to generate primed lymphocytes.

[‡] Lymphocytes from an individual positive for SB2 and SB4 were used as the positive control for SB reagents. The positive control for primed lymphocytes reagents S4(S3)$_x$ and S3(S4)$_x$ were lymphocytes autologous to the respective stimulator cells.

are positive for SB4. SB4 can be assigned to the b and d haplotypes because both the father (a/b) and S1 (a/d) were positive. The c haplotype does not segregate independently of the b and d haplotypes but is also thought to carry SB4.

Primed lymphocytes were prepared between S3 and S4 to determine if the differences detected by restriction fragment segregation reflected differences in expressed products that would be cellularly recognized. When lymphocytes from S4 were stimulated by S3 lymphocytes and then restimulated by lymphocytes from family members, significantly enhanced proliferation was observed in cultures restimulated by S3 as well as by individuals positive for the c haplotype (MO and S2). Note that the response of S4 (S3)$_x$ to the negative control, S4, is relatively high. If the control value (8,386 cpm) is subtracted from other experimental values, the positive reactions with lymphocytes from the mother, S2, and S3 become apparent. Although the levels of [^{3}H]thymidine incorporation were low, there were significant differences between responses considered positive and those considered negative (Table II). Low responses are to be expected in situations where the differences are limited.

No significant stimulation was observed in the reciprocal culture where lymphocytes from S3 were stimulated by lymphocytes from S4. These data are consistent with the b and d haplotypes having the same or very similar SB4 alleles and the c haplotype having a slightly different SB4 allele.

Serological Detection of the Recombinant SBβ Haplotype.   To determine whether these SB4 haplotypes could be distinguished serologically, LCL from family members were tested with monoclonal antibody ILR1, previously reported to recognize DR5, SB2, SB3, and certain SB4 alleles (29). Profiles of indirect immunofluorescence using ILR1 and LCL from S3 and S4 are shown in Fig. 6. ILR1 reacts with S3 LCL (Fig. 6A) and not with S4 LCL (Fig. 6B). SB4 is the only antigen carried by S3 and S4 known to be recognized by ILR1. These data indicate that ILR1 reacts with the c haplotype and can distinguish between the various SB4 alleles in family 7.
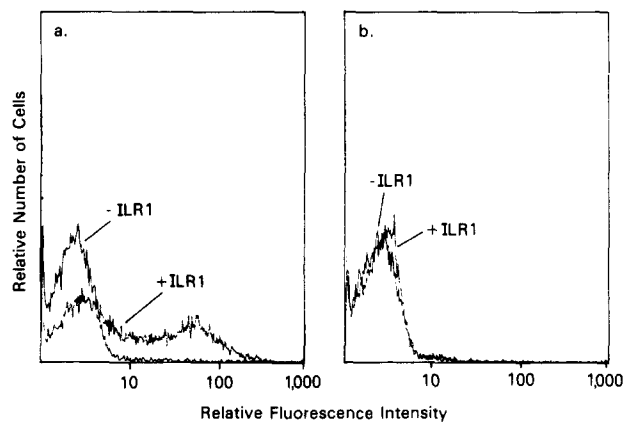

FIGURE 6.   Flow cytometry analysis of LCL from individuals S3 (a) and S4 (b) using monoclonal antibody ILR1 in indirect immunofluorescence. Trace designated: ILR1 indicates samples incubated with fluorescein isothiocyanate-sheep anti-mouse Ig in the absence of ILR1.

Discussion

This report describes the analysis of the HLA haplotypes of family 7 using molecular genetic, serological, and cellular approaches. Family 7 was known to include an individual who inherited an HLA haplotype recombinant between HLA-B and HLA-D/DR, and all data accorded with the location of this recombination. Unexpectedly, the molecular genetic analyses identified a second individual in the family who inherited a recombinant HLA haplotype that had escaped detection by classic typing techniques. The second crossover occurred between the region encoding genes HLA-D/DR, DC/MB, and SBβ and was first detected by the segregation of a polymorphic restriction fragment of the SBβ gene in the family. Extensive testing using primed lymphocyte reagents and a monoclonal antibody supported the observation that there was a second recombinant haplotype in family 7.

Although a great deal of information concerning the genetic organization and function of the HLA-D/DR region has been obtained using serological, cellular, biochemical, and molecular approaches, a number of fundamental questions remain unanswered. The arrangement of the genes in the genome and the functions of individual products are issues that remain unresolved.

Number and Arrangement of Human Class II Genes. Current information indicates that there are at least three subregions encoding human class II antigens, HLA-DR, DC/MB, and SB (6–8). Sequence comparisons of class II cDNA and protein indicate that HLA-DR is homologous to the murine H-2IE subregion and DC/MB is homologous to the H-2IA subregion (30). SB has diverged from DR and DC to the same extent (24). A counterpart to the SBβ gene may be present in the murine genome; a DNA probe corresponding to SBβ has been shown to hybridize with I-Eβ_2 (24).

The relative map order of human class II subregions is thought to be DR-DC/MB and SB; DR-DC/MB is telomeric. SB has been mapped by recombination frequency to ~1.0 centimorgan centromeric to HLA-D/DR (17). DC/MB has been separated from HLA-DR by irradiation mutants selected for the loss of specific HLA determinants (31). However, since no recombinants between HLA-D/DR and DC/MB have been identified, the map distance between them is not known.

Certainly, map distances calculated by recombination frequency may not relate to the actual distance between genes if there are recombinational "hot spots" within the HLA complex.

Class II products are composed of two chains, alpha and beta, both of which are thought to be encoded by genes within the MHC. In this report, the observation that polymorphic restriction fragments hybridizing with class II gene probes segregated according to HLA haplotype provides further support to the localization of class II genes to the HLA complex. However, the precise number and arrangement of human class II alpha and beta chain genes remains to be determined. It is not known whether the genes that encode products of a subregion are clustered together or, alternatively, are interspersed among genes encoding different subregion products.

Whether all of the genes identified by hybridization with currently available probes are expressed or not remains to be determined. This information is

directly relevant to functional analyses. Furthermore, gene expression may vary according to HLA haplotype. For example, the murine H-2I haplotypes vary in their expression of class II products; the b, s, q, and p haplotypes do not express I-E antigens due to a number of genetic defects (32).

The most direct way to determine gene order within the HLA complex and assign specific functions to individual regions and genes is through studies of families that include individuals who have inherited recombinant HLA haplotypes. In families, haplotypes are of a common origin and distal regions and/or genes can be isolated by crossover events in certain individuals. Functional analyses of well-characterized recombinant families make it possible to localize specific functions to isolated HLA regions or genes. Furthermore, lymphocytes from such family members provide the means of generating highly specific cellular reagents.

Molecular Genotyping of Family 7. With a single exception involving the segregation SBβ fragments in S3, molecular genotyping studies of family 7 indicated that class II genes (DRα, DRβ, DCα, and DCβ) segregated according to haplotype assignment as determined by classic serological and cellular typing reagents (Fig. 2, 3, and 4). Individual S2, who inherited an HLA haplotype recombinant between HLA-B and HLA-D/DR, also inherited the full complement of polymorphic restriction fragments hybridizing with class II gene probes that were assigned to the b haplotype as defined by classic typing. Polymorphic SBβ restriction fragments did not segregate according to haplotype as assigned by classic typing but analysis of the family made it possible to identify an individual (S3) who inherited an HLA haplotype recombinant between DR-DC/MB and SBβ. These data indicated that the recombinant individual (S3) inherited at least one SBβ chain of the c haplotype. However, it was not known if the crossover occurred between DR-DC/MB and the SB region or within the SB region itself; it is possible that the crossover isolated a single SBβ gene.

The results from Southern blot analyses made it possible to design cellular reagents capable of detecting the recombinant c haplotype SB4 allele (Table II). The reason for the low level of stimulation of primed lymphocytes [S4(S3)$_x$] when lymphocytes from S4 were stimulated by lymphocytes from individual S3 may be that there are limited differences between the SB4 alleles carried by c and d haplotypes or, alternatively, it may reflect the presence of only a single SBβ chain difference between siblings S3 and S4. This primed lymphocyte culture [S4(S3)$_x$] is being cloned to isolate cells specific for the alloantigenic differences between individuals S3 and S4. Results of studies using a monoclonal antibody confirmed the existence of differences between the SB4 alleles carried by the c and the b and d haplotypes. Further analysis of the differences between S3 and S4 will be possible when SBα probes are available.

The present results illustrate the power of molecular genotyping in studies of the HLA region in families. However, it must be pointed out that such results are possible only with families for which extensive information obtained by classic techniques is available. The Southern blot data alone would not have been as effective in obtaining informative results.

Although haplotype assignment of polymorphic restriction fragments in family

7 was clear, correlation of specific restriction fragments with particular DR, DC/ MB, and SB alleles (defined by classic procedures) was potentially confusing. The paternal b and the maternal c haplotypes were identical for DR3, DW3, MB2, MT2, and SB4 by classic typing, yet there were DRα, DRβ, DCβ, and SBβ hybridizing restriction fragments that segregated differentially for the b and c haplotypes. These data distinguish the b and c haplotypes at the genomic level. Furthermore, there were restriction fragments commmon to haplotypes that did not type identically, which further complicates attempts to correlate restriction fragments with specific HLA alleles.

The most clear example of the discrepancy between allele designation and polymorphic restriction fragment segregation was observed in studies using the SBβ probe. The size of polymorphic restriction fragments for three restriction enzymes that hybridized with the SBβ probe and their respective haplotype assignments are summarized in Table III. The three SB4 haplotypes (b, c, and d) were each represented by unique profiles. The a and c haplotypes character- ized as SB2^v and SB4, respectively, had identical profiles using these three restriction endonucleases. Thus, SB alleles that were characterized as identical by classic typing had different restriction fragment profiles, and SB alleles that were characterized as different had similar restriction fragment profiles. The present results do not support previous reports (33) suggesting that HindIII restriction fragment polymorphisms could help define SB alleles.

The present studies point out the advantage of using complementary tech- niques to analyze the HLA haplotype and the importance of using families for such analyses. Southern blot analysis of families has an advantage in being able to detect recombinational events that are not observed by classical typing. Subsequent functional analyses using molecular genotyping data make it possible to determine correlations with expressed products. These studies demonstrate that it is possible to functionally detect different SB4 alleles in family 7 that were identified by molecular genotyping analyses. Correlation of serological cellular and molecular genetic analyses in families make it possible to further dissect the HLA complex and determine the respective roles of each gene product within this complex.

TABLE III

Polymorphic SBβ Restriction Fragments in Family 7

Haplotype	Restriction enzyme		
	EcoRI	HindIII	BglII
		kb	
a (SB2^v)		5.3	20
b (SB4)	7.6	5.8	
c (SB4)		5.3	20
d (SB4)	7.2	5.3	

Data gives approximate size of polymorphic restriction fragments hybrid- izing with the SBβ probe as determined for each haplotype (based on data depicted in Fig. 5).

Summary

Molecular genotyping of the HLA-D/DR region in a family correlated with serologic and cellular typing data. It was further possible to predict a subtle difference in SB region–related functions from such molecular studies. A family that included an individual who inherited an HLA haplotype with a paternal recombination between HLA-B and the HLA-D/DR region was identified by classic HLA typing techniques. Segregation of HLA-D/DR region genes in this family was studied by Southern blot analysis using cDNA probes for DRα, DRβ, DCα, DCβ, and SBβ. Restriction enzyme fragment polymorphisms observed for every gene tested were in concordance with assigned HLA haplotypes (including the individual known to have inherited a paternal recombinant haplotype) with one exception: two HLA identical siblings were observed to have different SBβ restriction fragment patterns. Further testing revealed that one individual inherited a maternal HLA haplotype recombinant between the HLA-D/DR region and SBβ. Although both maternal SB alleles typed as SB4, allelic differences could be detected cellularly by primed lymphocytes and by the differential expression of a class II cell surface antigen using monoclonal antibody. Therefore, predicted and nonpredicted recombinant haplotypes were detected in a family by molecular genotyping.

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