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Immunology

Identification of HLA-DP polymorphism with DPα and DPβ probes and monoclonal antibodies: Correlation with primed lymphocyte typing

(major histocompatibility complex/restriction fragment length polymorphism/ELISA)

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ABSTRACT **Thirty-four lymphoblastoid cell lines that had been previously typed for HLA-DP antigens by primed lymphocyte typing (PLT) were tested by Southern blotting and by ELISA. Using two DPβ probes and a DPα probe with a series of enzymes, it is possible to identify restriction fragment length polymorphism (RFLP) patterns characteristic of DPw1, -2, -3, -4, and possibly -5. ELISA typing results, based on two polymorphic DP antibodies DP11.1 and ILR1, were compared with PLT-defined and RFLP-defined types. Thus, using a range of probes and enzymes it is possible to identify DP polymorphism. The value of monoclonal antibodies for such studies is demonstrated, and the molecular data can, in some cases, pinpoint the amino acids responsible for the specificity of the monoclonal antibodies.**

Polymorphism in the HLA-DP subregion† has until recently only been detectable by primed lymphocyte typing (PLT) (1). Two other methods are now available to identify these polymorphic differences, one by Southern blotting with a series of DP DNA probes and restriction enzymes and the other using monoclonal antibodies in an ELISA. In this paper, we describe studies using these two methods to determine both the amount of DP polymorphism they can define and whether the alleles seen correspond to those identified using PLT.

One of the problems with using full-length cDNA clones as probes in the HLA system is that there is cross-hybridization between the sequences at the *DP*, *DQ*, and *DR* loci. We therefore chose to use two short DPβ probes (Fig. 1) to analyze DP restriction fragment length polymorphisms (RFLP) as well as a DPα probe.

Three monoclonal antibodies were used in the study. Two were directed against polymorphic determinants of the DP region and the third was a monomorphic antibody.

MATERIALS AND METHODS

Cell Lines, Media, and Antibodies. The cell lines used were all B lymphoblastoid lines obtained from the following laboratories: FB11, W7, M16, S11, FB6, S. Shaw (Washington, DC); Daudi, G. Klein (Karolinska Institute, Sweden); JVM, H. O. McDevitt (Stanford, CA); SCTA, P. N. Goodfellow and R. O. Payne (Stanford, CA); Akiba, K. Tsuji (Isehara, Japan). Remaining lines: 1296-S, IDF, IBW4, JHH, Mette, TF6, MST, PGF, BBF, WT49, Mann, RAFF-T, FPF, WT46, WVB, ARNT, WT18, LLICRF, JHF, Priess, Maja, Herluf, HOM-2, AHB, Madura-T, and WT52 were all transformed in the authors' laboratory.

The cells were grown in hydrogen carbonate-buffered RPMI 1640 medium (Flow Laboratories) supplemented with 10% fetal calf serum/penicillin (100 units/ml)/streptomycin (100 μg/ml).

The antibodies used in this study are listed in Table 1 (2–4). DP11.1, one of the two polymorphic DP antibodies used, was produced by immunizing a C3H mouse (2) with C3H-derived L cells transfected with cosmid LC11, which contains *DP1A* and *DP1B* genes (5).

The antibody has been shown by immunoblotting to be directed against the DPα chain and reacts by ELISA only with DPw2 and DPw4 cells. The other polymorphic antibody, ILR1, was produced as a result of immunization with ascites tumor cells from a Burkitt lymphoma patient (3). The monomorphic antibody, B7/21, was produced in response to immunization with the pre-B-cell leukemic cell line Nalm-6 (4).

Screening of the Cell Lines by ELISA. The cell lines were screened using an ELISA galactosidase–antigalactosidase complex technique (6) with poly(L-lysine)-embedded gluteraldehyde-fixed cells as antigen.

Extraction of DNA, Southern Blotting, and Hybridization. High molecular weight genomic DNA was extracted from either peripheral blood or from Epstein–Barr virus-transformed B lymphoblastoid cell lines essentially as described (7).

Samples of genomic DNA (10 μg) were digested separately with 50 units of the restriction enzymes *Bam*HI, *Pst* I, *Eco*RI (New England Biolabs), and *Sst* I (Uniscience, London) at 37°C overnight and *Taq* I (New England Biolabs) at 65°C for 3 hr using the buffer conditions specified by the suppliers. The digested samples were electrophoresed in 0.7% agarose at 30 V for 24–40 hr.

DNA was transferred to Hybond-N nylon filters (Amersham) overnight (8) and hybridization was carried out according to the protocol recommended by Amersham.

A [^{32}P]CTP-labeled probe was prepared by the oligolabeling method (9) and was then added to a specific activity of 1–2 $\times$ 10^6 cpm·ml^{-1} of hybridization solution. After hybridization, filters were washed three times with 2$\times$ SSC (1$\times$ SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.1% $NaDodSO_4$ for 5, 15, and a further 15 min, followed by two 20-min washes in 0.2 $\times$ SSC/0.1% $NaDodSO_4$, and finally a 20-sec wash in 2$\times$ SSC. All the washes were carried out at 65°C.

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Abbreviations: PLT, primed lymphocyte typing; RFLP, restriction fragment length polymorphism(s).

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†Nomenclature for *HLA-DP* region genes: The genes in the *DP* region are called *DP1A*, *DP1B*, *DP2A*, and *DP2B* for, respectively, the expressed α and β chains and the pseudogenes for the second, nonexpressed, set of *DP*α- and *DP*β-chain genes.

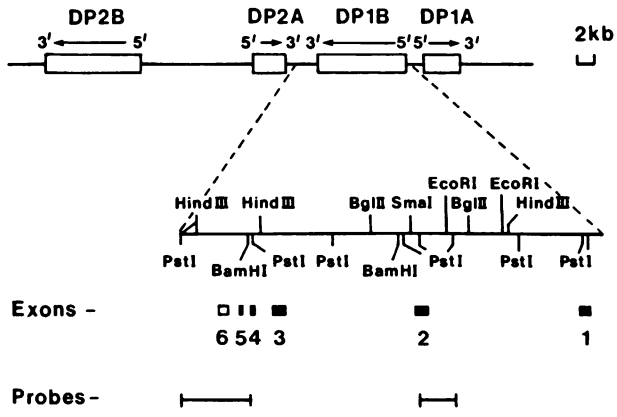

FIG. 1. The *HLA-DP* region showing the positions of the two DPβ probes used. At the 3' end is the *Pst* I 2-kb fragment containing exons 5 and 6 of the *DP1B* gene and the 3' untranslated region. Toward the 5' end is the 0.9-kb fragment of the *DP1B* gene.

The filters were partially dried and then exposed to Kodak XAR-5 film for autoradiography at −70°C for between 3 and 14 days.

Probes. Three probes were used. The first was a *Pst* I 2-kilobase (kb) fragment isolated from cosmid LC11. It encompasses exons 5 and 6 of the *DP1B* gene encoding the cytoplasmic domain and the 3' untranslated region (nucleotides 12869–14777) (10). The other DPβ probe is a 0.9-kb fragment of clone 11–13 from the 5' end of the *DP1B* gene (11). The third probe was a DPα cDNA probe (12).

RESULTS

Thirty-four lymphoblastoid cell lines, which had all been typed for DP by PLT (13), were tested by Southern blotting and in an ELISA using monoclonal antibodies. Twenty-two of the 34 lines, most of which were originally selected as homozygous typing cells for the mixed lymphocyte typing test, have been shown to be homozygous for *HLA-A, -B, -C, DR*, and *DQ*. Fifteen of them were the offspring of consanguineous marriages.

In the Southern blotting tests, several enzymes were used but the most informative results for the DP polymorphism were obtained with the enzymes *Sst* I and *Bam*HI and the 3' β-chain probe, *Pst* I and the 5' β-chain probe, and *Eco*RI and *Taq* I and the α-chain probe. The data are shown in Table 2 and the associations with *DP* alleles are shown in Table 3 (14).

RFLP with *Sst* I. The 34 cell lines were tested with the 3' β-chain probe after digestion with the enzyme *Sst* I. Bands seen were 20 and 12.0 kb and were present in complementary *DP* types. The 20-kb band was seen with 22 cells, including all 11 cells that were either *DPw1* or *-3*, and the two *DPw5* cells, S11 and Mette. The nine other cells showing this band had only one *DP* allele assigned and so it is possible that they carry another unidentified *DP* allele. The 12-kb band was seen in 30 of the 34 lines tested, including all 29 cells that were *DPw2* or *-4*. The one extra reaction was seen with a nonhomozygous cell, typed as *DPw3* (LLICRF).

Fig. 2 shows *Sst* I digests of family B in the first five lanes compared with four cell lines of similar *DP* types. The homozygote *DPw2* mother (second lane) passes the 12.0-kb band associated with *DPw2* to all the offspring (lanes 3–5), while the heterozygote father (first lane) passes the 20.0-kb band associated with *DPw3* to the first child and the 12.0-kb band associated with *DPw4* to the two other children. The pattern of bands is the same as that for the cell lines of matched *DP* type (lanes 6–9). These band patterns correlate with the PLT-assigned *DP* types of the family.

*Eco*RV gave a pattern of association with PLT types similar to *Sst* I with bands of 11.0 kb for *DPw1* and *-3* and 3.2 kb with *DPw2* and *-4* (data not shown).

RFLP with *Bam*HI and *Pst* I. We tested *Bam*HI digests of DNA samples from 34 lines. Two bands of 10.2 and 10.0 kb were seen (Fig. 3) showing a different pattern of association with complementary sets of antigens. Twenty-three lines showed the 10.2-kb band, all but two of which were *DPw1* or *-4*. One of these was Daudi and the other was a *DPw2, -w5* cell S11. As the other *DPw5*-positive cell is also *DPw4*, it is not certain whether this band also identifies *DPw5*. Two cells that were *DPw4* and one that was *DPw1* failed to produce the 10.2-kb band, indicating possible evidence for splits in these antigens.

Twenty-four of the 34 cells showed the 10.0-kb band, including all the *DPw2* and *DPw3* cells except Daudi. Of the 10 other cells showing the band, 8 had a blank *DP* allele, which in this case means an allele other than *DPw1, -2, -3* or *-4*. The other two cells, Mette and TF6, carried *DPw5* and *-6*, respectively. It should be noted that only a minority of the cells have been tested for *DPw5* and *DPw6*, so in some cases the DP blank may include these alleles.

As shown in Table 2 in the 5' β-chain probe on *Pst* I digests, a 4.2-kb band was seen only in *DPw2*-positive cells. One cell, WT46, typed by PLT as *DPw2*, failed to show the band (11).

RFLP with DPα Probe. Thirty-three cell lines digested with *Eco*RI were probed with the DPα probe. Eleven of the samples produced a 2.5-kb band. Of these, three DPw1, two DPw5, two CP63 (15), and two FB11 and FB6 were assigned DP blank, which may correlate with CP63. DPw2, -3, -4, and -6 cell lines did not produce this band. The four other cell lines that did show the band had only one defined *DP* allele and so may also have carried a blank allele. A similar pattern seems to be found on a smaller number of cells digested with *Hin*cII giving a 3.1-kb band and *Bgl* II giving a 3.4-kb band. Preliminary studies on *Taq* I digests show polymorphic bands, which may also be of use in identifying *DP* alleles. In particular, a 7.6-kb band is only present on the two DPw5 cell lines.

ELISA Typing with Monoclonal Antibodies. ELISA typing with monoclonal antibodies DP11.1, ILR1, and B7/21 was carried out to compare their patterns of reaction with the RFLP results (Table 2).

DP11.1 had previously been shown (2) to identify DPw2 and -4 on the panel tested. ILR1 reacts with DPw2 and -3 and with DR5 as will be discussed later. Their correlations with DP antigens on this panel of cells are shown in Table 3. The two RFLP bands that should be compared are therefore *Sst* I (12.0 kb) to be compared with DP11.1, and *Bam*HI (10.0 kb) to be compared with ILR1. These comparisons are shown at the bottom of Table 3. In the 34 cells tested with *Sst* I and DP11.1, there are five discrepancies with the presence of the 12-kb band and in four of the five, the RFLP band is in agreement with the PLT-defined type. In these four cases, the monoclonal antibody failed to react. On the other hand, there are nine discrepancies between the *Bam*HI 10.0-kb band and ILR1. In six of these, where the band is present but ILR1 does not react, the PLT typing result agreed with the antibody reaction—namely, is DPw2, -3, or blank. The seventh exception is IBW4, which is DPw3 but does not react with ILR1. In two cases, FB11 and FB6, *Bam*HI (10.0 kb) and ILR1 are positive and the cells are defined by PLT as carrying a blank *DP* allele, so it is likely that this combination defines a *DP* blank allele not so far identified by PLT. Of the two cells

Table 1. Monoclonal antibodies used

Antibody	Specificity	Ref.
DP11.1	DPw2, DPw4	2
ILR1	DPw2, DPw3, DR5	3
B7/21	DP monomorphic	4

Table 2. RFLP and monoclonal antibody reactions on DP typed cell lines

	DPW1			DPW2									DPW3					DPW4															DPW1, -6
Cell line	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
HLA-DPW1	+	+	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
HLA-DPW2	−	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
HLA-DPW3	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
HLA-DPW4	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
HLA-DPW5	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
HLA-DPW6	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
DP-BLANK	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
ST1DPB20.0*	+	+	+	−	+	+	−	−	+	+	−	+	+	+	+	+	+	−	−	−	−	−	−	+	−	+	+	+	+	−	+	+	+	+
ST1DPB12.0	−	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
BAMDPB10.2	+	+	+	−	−	+	−	−	−	+	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+
BAMDPB10.0	+	−	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	−	−	−	−	+	−	+	+	+
PSTDPB04.2†		−	+	+	+			+	−		+	+				−	−					−			−		−					−		
EC1DPA02.8‡	+	+	+	−	+	+	−		−	+	−		−	−	−	−	−	−	−	+		−	−	+	−	−	−	−				+	−
TAQDPA15.1							−	−		−			−												+		+	+				+	+
TAQDPA07.6							−	−		+			−												−		−	−				+	−
DP11.1	−	+	+	+	−	+	+	+	−	+	+	+	−	−	−	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	−
ILR1	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−	+
B7/21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+, Band present; positive binding with monoclonal antibodies. −, Band absent; negative binding with monoclonal antibodies. Blank space, not tested. ST1, *Sst* I; BAM, *Bam*HI; PST, *Pst* I; EC1, *Eco*RI; TAQ, *Taq* I. Numbers after enzyme abbreviations are the band sizes in kb. Cell lines: 1, FB11; 2, W7; 3, M16; 4, Mann; 5, RAFF-T; 6, Daudi; 7, JVM; 8, FPF; 9, WT46; 10, S11; 11, WVB; 12, ARNT; 13, WT18; 14, LLICRF; 15, JHF; 16, Priess; 17, JHH; 18, Maja; 19, Herluf; 20, HOM-2; 21, AHB; 22, Madura; 23, WT52; 24, FB6; 25, MST; 26, PGF; 27, BBF; 28, WT49; 29, SC-TA; 30, 1296-S; 31, IDF; 32, IBW4; 33, Mette; 34, TF6.
*DPβ 3′ 2-kb probe.
†DPβ 5′ 0.9-kb probe.
‡DPα probe.

that are ILR1 positive and *Bam*HI (10.0 kb) negative, one is Daudi and the other, IDF, is DR5-positive and is therefore reacting to ILR1 for that reason, as will be discussed later.

DISCUSSION

The aim of this study was to see if it was possible to detect DP polymorphism using DP probes, and how the RFLPs would correlate with the PLT-defined antigens and with the reactions of monoclonal antibodies directed at the DP polymorphism. As the results above have shown, in the majority of cases linkage disequilibrium between the α- and β-chain polymorphisms identified by the antibodies and probes used seems to be strong enough to define DP polymorphism, which has a high correlation with the PLT-defined types. A

Table 3. Correlations between RFLP, monoclonal antibodies, and DP antigens

Probe	Enzyme	kb	DPw	++	+−	−+	−−	χ^2	*r*
DPβ 3′	*Sst* I	20.0	3	7	15	0	12	3.1	0.38
			1	4	11	0	12	1.9	0.37
			5	2	9	0	12	0.6	0.32
			One DP*	9	0	0	12	17.1	1.00
	Sst I	12.0	4	20	10	0	4	4.0	0.44
			2	9	1	0	4	6.5	0.85
	*Bam*HI	10.2	4	18	5	2	9	8.7	0.57
			1	3	2	1	8	1.8	0.52
			5	1	1	0	8	0.7	0.67
	*Bam*HI	10.0	3	7	17	0	10	2.1	0.33
			2	8	9	1	9	2.4	0.38
			One DP	8	2	0	9	9.4	0.81
DPβ 5′	*Pst* I	4.2	2	6	0	1	7	7.3	0.87
DPα	*Eco*RI	2.8	5	2	7	0	18	1.7	0.40
			1	3	4	2	18	1.9	0.37
			One DP	4	0	0	18	15.8	1.00
DPα	*Taq* I	7.6	5	2	0	0	7	4.1	1.00
Monoclonal antibodies									
DP11.1			4	18	7	2	7	4.9	0.45
			2	7	0	2	5	5.0	0.75
ILR1			2	10	9	0	15	8.8	0.57
			3	5	4	1	14	4.8	0.55
			One DP	4	0	1	13	9.1	0.86
DPβ	*Sst* I	12.0	DP11.1	25	5	0	4	8.7	0.61
DPβ	*Bam*HI	10.0	ILR1	17	7	2	8	5.5	0.47

These associations are calculated as described (14). The most significant association with an antigen is first listed and then all cells with that antigen are removed before calculating the next association.
*Only one DP antigen has been assigned; implies that in some cases an unidentified *DP* allele may be present.

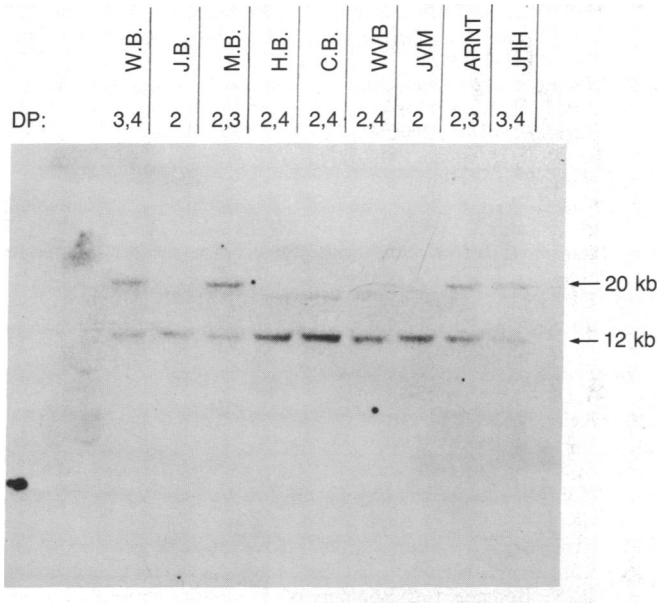

FIG. 2. Southern blot of *Sst* I digests using the *DP* 3′ β-chain probe (2 kb). The first five lanes from a family show the presence of the 20-kb band correlating with *DPw3*. The 12-kb band, present in all the family, correlates with *DPw2* and *DPw4*. The remaining four lanes are digests of random lymphoblastoid cell lines of similar *DP* types. Exposure was for 9 days.

clear exception to this is the result with the cell line Daudi, originating from an African Burkitt lymphoma patient. This indicates the possibility that as well as linkage disequilibrium differences between ethnic groups for *HLA-A, -B, -C, DR*, and *DQ*, which have already been seen, there may be differences within the *DP* subregion. Recent DNA sequencing has shown that Daudi may also carry a *DP1A* gene characteristic of the blank allele (12).

DP Typing and Antigen Assignment. In this study, five restriction enzymes and three DP probes have been used to characterize the DP polymorphism at the DNA level by Southern blotting. This might seem to be an excessive effort

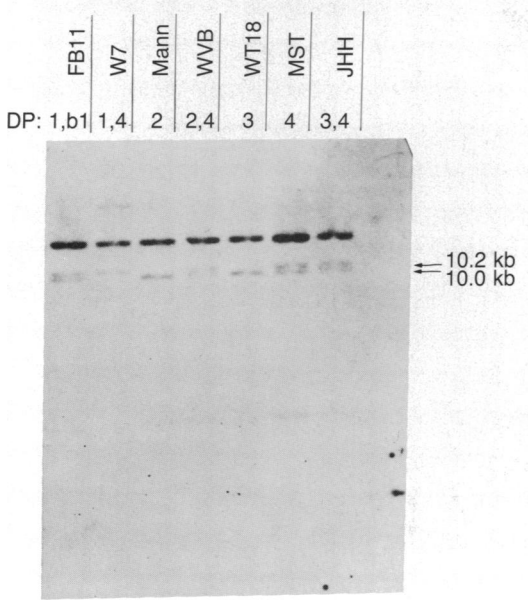

FIG. 3. Southern blot of *Bam*HI digests of PLT-typed lymphoblastoid cell lines using the DP 3′ β-chain probe (2 kb). The 10.2-kb band is seen in cells carrying *DPw1* or *DPw4* and the 10.0-kb band is with *DPw2*- or *DPw3*-positive lines. Exposure was for 11 days.

since it is clear that it is possible in theory to produce unique patterns of bands for *DPw1, -2, -3*, and *-4* and all heterozygote combinations of these alleles with just one probe, the 3′ β-chain probe, and two enzymes, *Sst* I and *Bam*HI. However, there are more than four *DP* alleles. *DPw5, DPw6*, and at least one *DP* blank, which may be the same as or include CP63, have been shown to exist and to complicate the simple pattern identifiable by one probe and two enzymes. As we have seen in the study of families, it is not in practice always possible to identify the alleles of heterozygotes unequivocally with these two enzymes alone.

Using the complete battery of probes and enzymes described above, it seems more probable that a correct *DP* assignment can be achieved, even in the presence of *DP* alleles other than *DPw1, -2, -3*, and *-4*. The patterns of *DPw2*, only seen on *Pst* I digests with the 5′ β-chain probe, and *DPw5*, only seen with *Taq* I digests with the α probe, look to be extremely useful markers for the presence of these antigens and will be confirmed in a larger study.

However, in the long term this method, involving a battery of probes and enzymes, may be considered too time consuming for routine studies and other methods may have to be devised. One method that could be used involves multiple digests, in which the DNA is cut with more than one or possibly all the enzymes at the same time and probed with a combination of probes. A more attractive possibility, since the sequence of many of the alleles is now available (J.Y., unpublished data) and it has been shown that each of the alleles has a unique sequence, is that molecular typing should be carried out on a single digest using a series of nucleotide probes for this region. In each case, a single band should be present or absent and this would produce a more robust method of typing than a complex combination of probes and enzymes.

Molecular Basis of Monoclonal Antibody Reactivity. A further possibility, now that molecular techniques have identified the allelic differences, is that monoclonal antibodies could be made against them and that typing for routine purposes should be carried out with these in an ELISA. That this should be possible is illustrated by the two polymorphic antibodies used in this study.

ILR1 reacts with DPw2, -3, and DR5 cells. Examination of the sequences of these three specificities shows (J.Y., unpublished data) that residues 36, 55, and 56 in DP1B and the corresponding positions 38, 57, and 58 in DR5 are valine, aspartic acid, and glutamic acid. This combination of residues is not found in any other DR or DP type except the DP type CP63. However, since valine and aspartic acid are found in other DP types, it can be suggested that it is the alanine to glutamic acid change at residue 58, giving a charge difference, which produces the specificity recognized by the antibody ILR1 in DPw2, -3, and DR5. As two of the cells, FB11 *DPw1*/blank and FB6 *DPw4*/blank, react to ILR1, it is likely that the blank allele in these cases may be the same as CP63. This suggestion is supported by the fact that the cell line Akiba, which is CP63 (15), reacts with ILR1.

Sequence data can also throw light on the specificity of the antibody DP11.1. As shown by immunoblotting (2), this antibody is directed against the α chain, although by binding and in immunoprecipitation studies it is polymorphic for the DPw2 and -4 specificities. Further studies have shown that after separation of the chains in immunoblotting, the antibody will blot to the α chain of all DP specificities tested and is not limited to DPw2 and DPw4. The polymorphism seen is likely therefore to be due to conformational change imposed on the α chain by the difference between the β chains of DPw2 and -4 and that of the other DP types. Residues 84–87 are glycine, glycine, proline, methionine in DPw2 and DPw4 and aspartic acid, glutamine, alanine, valine in DPw3 and CP63 and are likely candidates (J.Y., unpublished data).

It is clear from the specificity of these antibodies that one can sometimes pinpoint exactly the part of the sequence against which each of them is directed. This is especially the case for the DP sequences, which show relatively limited polymorphism and variation between alleles. The antibody DP11.1 was in fact made using mouse cells transfected with human *DP* genes and there is no reason why transfectants engineered to express restricted parts of *DP* molecules should not be used in the production of such antibodies. These could then be used for typing in an ELISA in the knowledge of which polymorphic region of the molecule is being recognized.

The two methods, a simplified Southern blotting method and the use of genetically engineered monoclonal antibodies in an ELISA, will provide the means to type for DP polymorphism on a far wider scale than has been possible by cellular methods. Now that many of the DP sequences have been obtained, specific oligonucleotide probes may also be powerful tools for detection of allelic variation in the *DP* region. This will provide the opportunity to assess the importance of the *DP* region in the susceptibility to many diseases, particularly those in which a weak but persistent HLA association has been seen.

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