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Genetics

HLA-DR2, -DR5, and -DRw6 associated Dw subtypes correlate with HLA-DRβ and -DQβ restriction fragment length polymorphisms

(histocompatibility antigens)

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ABSTRACT **DNAs extracted from peripheral blood leukocytes of 24 individuals, selected for their HLA-DR types, -DR2, -DR5, and -DRw6, were analyzed with four restriction enzymes, *Bam*HI, *Eco*RV, *Hin*dIII, and *Taq* I, using the Southern technique. This panel includes 16 individuals with homozygous typing cells and 8 heterozygous individuals who carry rare Dw subtypes or unusual DR–DQ associations. Eighty-five polymorphic fragments were detected and assigned to the *DR* or *DQ* gene families according to their hybridization signals. Thirty-eight fragments (*DR* or *DQ*) were found to correlate with single *DR* or *Dw* specificities or rare associations such as *DRw14–DQw3*. Forty-two fragments correlated with the association of immunologically defined specificities. In total, these 85 fragments constituted 44 different patterns, each comprising 1–9 fragments. For each homozygous typing cell a combination of patterns was observed. Fourteen different combinations of 10–20 patterns were found among the 16 individuals with homozygous typing cells, showing that *Dw18*, *Dw19*, *Dw9*, and *Dw5* are heterogeneous at the genomic level whereas only the Dw2 individuals tested here are identical.**

The *HLA-D* gene region has three subregions, *HLA-DR*, *-DQ*, and *-DP*. Their products are membrane glycoproteins that are heterodimers (α, β). The α chain (33–35 kDa), exhibiting limited polymorphism for DQ and DP, is quasi-invariant for DR; the β chain (26–29 kDa) is extensively polymorphic (1). Each subregion contains several genes as follows: for *DR*, *1α* and *3β* (*β1*, *β2*, *β3*); for *DQ*, *2α* and *2β*; for *DP*, *2α* (*α1*, *α2*) and *2β* (*β1*, *β2*). In addition *DZα* and *DOβ* genes have been characterized but not yet located (2, 3). The numerous antigens encoded by these loci were initially characterized by alloantisera against DR and DQ, homozygous typing cells (HTC), and by primed lymphocytes for D and DP determinants.

The specificities recognized by T-cell proliferation in response to allogeneic stimulation by B cells were initially thought to be controlled by the *HLA-D* locus (4). When serological methods allowed the recognition of B-cell antigens coded by the *HLA* region, these antigens were correlated with their cellular counterpart. The similarity was sufficient to warrant the existence of *DR* locus (D related), and the same nomenclature was applied to the specificities defined by two different procedures (5). Subsequently, this position was no longer tenable as new D specificities had no DR equivalent or were included in broader DR reagents. With this approach, it was demonstrated that HTCs typed as DR4 could be resolved in five different clusters by the level of their proliferative response when tested against each other (6). Availability of proper alloantisera can lead to the recognition of some Dw types by serology. For example, subtypes of DR2, such as FJO or AZH defined by HTCs, can be serologically recognized (7). An alloantiserum has permitted the distinction of a variant of DRw6 (now DRw14) corresponding to Dw9 (8). However, there still remain Dw subtypes like Dw18 and Dw19 (subtypes of Dw6), which evade serological recognition. Alleles at the *DQ* locus (formerly *MB*) are found in strong linkage disequilibrium with DR antigens. DQw1 is usually associated with DR1, DR2, DRw6 (DRw13 and DRw14), and DRw10; DQw2 with DR3 and DR7; DQw3 with DR4, DR5, and DRw9 (20). The rare associations such as DRw13–DQw3 and DRw14–DQw3 raise different cellular response(s) when compared to DRw13–DQw1 or DRw14–DQw1. This dissociation has led to the assumption that Dw specificities could correspond to the summation of epitopes present on DR, DQ, and other molecules (9).

The obtention and use of class II cDNA probes represent an approach to unravel the complexity of this region through restriction fragment length polymorphism (RFLP) studies (10, 11). The degree of polymorphism depends on the locus explored. Almost no polymorphism was observed for *DRα*; limited polymorphism was noted for *DXα*, *DPα*, and *DPβ*; in contrast *DRβ*, *DQα*, and *DQβ* yielded extensive polymorphism (3, 12). Generally, the majority of RFLPs correlate with supertypic specificities whereas fewer fragments are found with single DR specificities (12, 13). Nevertheless, RFLPs specific for *DR*-associated subtypes have been reported (3, 11, 13, 14). The aim of the present study was to investigate *DRβ* and *DQβ* RFLP in various subtypes of *HLA-DR2*, *-DR5*, and *-DRw6*. These subtypes selected from a Caucasoid panel were either HTCs or heterozygous cells.

MATERIALS AND METHODS

HLA-DR, -DQ, and -Dw Typing. The 24 selected cells have been typed for HLA-A, -B, -C, -DR, -DQ, and -D by methods already described (8) with local and 9th Histocompatibility Workshop reagents. Sixteen cells were homozygous for HLA-Dw, eight were heterozygous (see Fig. 2). Cells 1–7 represent DR2 and subtypes: two DR2–Dw2 are local HTCs (cells 1 and 2), Dw12 (cell 3) is a gift from C. Brautbar; FJO (cell 4), a local HTC, and AZH (cell 5, given to us by C. Brautbar) are DR2 short (15, 16); cell 7 is DR1–Dw1/DR2 long-DwX, since this cell isn't Dw2, Dw12, FJO, or DB9 (15). Cells 8–14 represent DR5 and variants; three cells (cells 8, 9, and 10) are local HTCs DQw3–DRw11–Dw5; cell 11, a consanguineous HTC, is also DQw3–DRw11 but is not Dw5.

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Abbreviations: RFLP, restriction fragment length polymorphism; kb, kilobase pair(s); HTC, homozygous typing cells.

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Cell 12 has the rare association DQw1–DRw11 as documented by family typing, the other haplotype untransmitted is DR blank, DQ blank, or DQw1. Cell 13 DQw3–DRw11 is JVM, a new HTC variant subdividing DR5 (17). Cell 14 is heterozygous DQw1–DRw14–Dw9/DQw3–DRw12–DB9. The subtypes of DRw6 are represented by cells 15–24 as follows: of the seven local HTCs, three are Dw18 [cells 15 (9w601), 16, and 17]; two are Dw19 (cells 18 and 19); two are Dw9 [cells 22 (9w903) and 23 (9w902)]. Cell 24 presents the rare combination DRw14–DQw3; the other haplotype is DR3–DQw2. Cells 20 and 21 are unrelated heterozygous presenting the unusual association DRw13–DQw3. However, they are different for Dw as follows: cell 20 is typed by HTC 1802 (1802+) (17); in contrast, cell 21 (1802−) is not typed by this HTC; and the other haplotype of both cells is DQw1–DR2 short-FJO.

Detection of RFLP. Genomic DNA was extracted from peripheral blood leukocytes according to methods described (12, 14). DNA samples were digested by the restriction enzymes, *Hin*dIII, *Eco*RV, *Taq* I, and *Bam*HI, under conditions recommended by the manufacturers. The restriction fragments were separated in 0.6 and 0.9% agarose gel by electrophoresis at 30 V for 40 hr, transferred onto hybridization membranes (Hybond from Amersham), and then hybridized successively with a ^{32}P-labeled *HLA-DR* gene probe (18) and an *HLA-DQβ* gene probe (19).

Membranes were prehybridized for 18 hr at 42°C in the same medium used for hybridization. Hybridization was performed for 40 hr at 42°C in 50% (vol/vol) formamide, 5× NaCl/Cit, 0.1% Denhardt's solution, 5% (wt/vol) dextran sulfate, 1% $NaDodSO_4$, sonicated denatured salmon sperm DNA at 200 μg/ml, and radiolabeled probe at 10 ng/ml (1× NaCl/Cit = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0). Membranes were washed at room temperature with 2× NaCl/Cit for 10 min at 65°C in a water bath with 2× NaCl/Cit, 0.5% $NaDodSO_4$ for 10 min. An additional wash was used after the *DQβ* gene probe hybridization, at 65°C with 0.2× NaCl/Cit, 0.5% $NaDodSO_4$ for 5 min. Labeled fragments were detected by exposing the membranes to x-ray film (XAR5, Kodak) for 2–5 days at −70°C.

RESULTS AND DISCUSSION

The DNA extracted from peripheral blood leukocytes from 24 individuals selected for their HLA-DR types, DR2, DR5, or DRw6, have been analyzed, using the Southern technique, with four restriction enzymes (*Bam*HI, *Eco*RV, *Hin*dIII, *Taq* I) and two cDNA probes, DRβ and DQβ. This panel includes 16 HTCs, of which only 1 HTC (cell 11) is consanguineous. The DNA samples were electrophoresed on 0.6% agarose gels (Fig. 1, *Left*), which gave better resolution of large fragments, and on 0.9% agarose gels (Fig. 1, *Right*) , which usually allowed the detection of small molecular weight fragments. Each membrane was hybridized successively with probe DRβ then probe DQβ to detect cross-hybridizing fragments (12). Ninety-four fragments were recorded; 9 were found in all 24 individuals while the 85 remaining fragments were found only in some individuals. Sixty-one fragments were detected only with DRβ probes, 15 were found only with DQβ probes, and 9 were detected with both probes. Five of the latter gave stronger signals with probe DQβ. Since hybridization with other β chain probes (for *DP* and *DO*) has not been performed, we cannot eliminate the possibility that some faint bands detected here belong to the *DP* and *DO* subregions. Nevertheless, we refer to *DR* fragments as those detected only with the DRβ probe, or which give a stronger signal with the DRβ probe, and *DQβ* fragments as those detected only with the DQβ probe, or which give a stronger signal with the DQβ probe. This is probably particularly justified for *DQβ* fragments, since the corresponding blots were washed under stringent conditions.

Distribution of the 85 "polymorphic" fragments was analyzed on this small panel (Fig. 2), and the results were compared with already published data on a larger sample, which included 82 haplotypes encoding DR1–DR9 specificities (12). Each fragment can be assigned to one of the following categories. (*i*) Fragments associated with a single DR or Dw specificity present in the panel tested, or in the larger sample of 82 haplotypes, were termed "specific" fragments. (*ii*) Fragments found to be associated with more than one DR or Dw specificity were referred to as "broad"

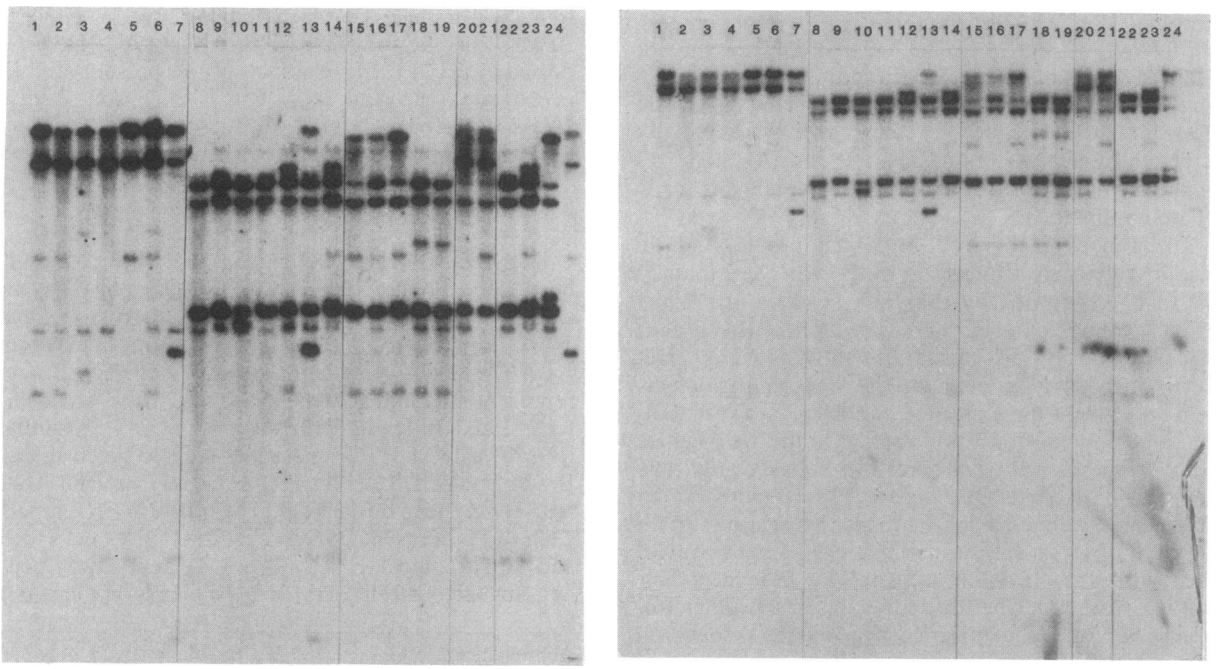

FIG. 1. Autoradiogram of blots from two experiments where 24 DNA samples were digested by *Eco*RV and run on 0.6% agarose gel (*Left*) and 0.9% agarose gel (*Right*). Numbers 1–24 refer to the identification number of the cells appearing in Fig. 2. Autoradiograms were developed after a 20-hr exposure.

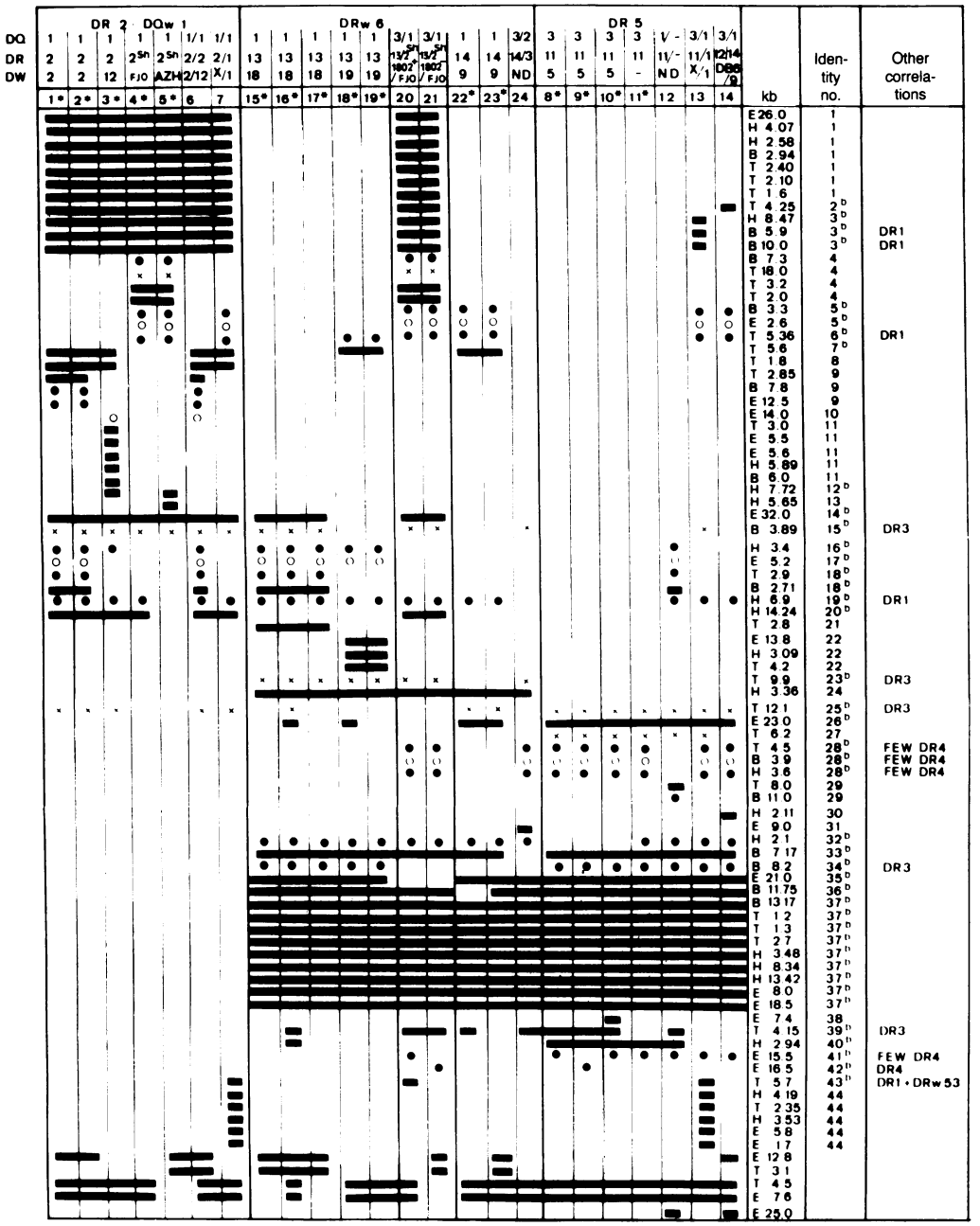

FIG. 2. Distribution of 85 polymorphic restriction fragments on 24 DNA samples. The DQ, DR, and Dw type and identification number (1–24) of each DNA sample are indicated (*Top*). Each fragment (row) is denoted by a capital letter designating the enzyme, followed by its size in kb. The identity number corresponds to the number attributed to each group followed by "b" when it refers to broad fragments. The last column on the right (Other correlations) shows other DR specificities previously reported in a larger panel comprising 82 haplotypes. The five fragments at the bottom do not clearly belong to any specificity. E, *Eco*RV; H, *Hin*dIII; B, *Bam*HI; T, *Taq* I. ■, DRβ; ●, DQβ; ×, strongest hybridization with DRβ; ○, strongest hybridization with DQβ; *, homozygous typing cells.

fragments. Obviously such a correlation should be considered with caution especially when the fragments are found in many individuals—this repeated occurrence deserves further study in another independent sample. (iii) Five fragments did not correspond to either of these two categories.

Thirty-Eight "Specific" Fragments Were Detected Constituting Sixteen Patterns. Each pattern correlates with DR or Dw specificities or rare DR–DQ associations (Table 1). An identification number was attributed to each pattern, which was represented by 1–7 fragments (Fig. 2 and Table 1). Patterns comprising more than one fragment have been termed clusters in previous reports (12). Seven patterns found to be associated with a DR2-related specificity are as follows: all DR2 (pattern 1), DR2 short (pattern 4), DR2 long (pattern 8), Dw2 (pattern 9), hetero- and homozygote Dw12 (pattern 10), HTC Dw12 (pattern 11), and HTC AZH (pattern 13).

Four groups found in DR5-related specificities are as follows: DRw11 (pattern 27), DRw12 (pattern 30), one of the three Dw5 HTCs tested (cell 10) has a specific *DRβ* fragment (pattern 38), cell 12 (DRw11–DQw1) has two specific bands, *1 DRβ, 1 DQβ* (pattern 29), never found in the larger sample of 82 haplotypes. However, they could belong to DRw11–DQw1 or to the other haplotype, which was unfortunately not characterized in this individual.

Four patterns were found in DRw6-related specificities: all DRw6 (pattern 24), Dw18 (pattern 21), Dw19 (pattern 22), DRw14–DQw3 (pattern 31)—this fragment is not found in the larger sample of 82 haplotypes. One group of five *DRβ* fragments (pattern 44) correlates with DR1 and Dw1.

The 42 "Broad" Fragments Detected Constitute 28 Patterns (Single Fragments or Clusters) Correlating with Associations of Immunologically Defined Specificities (Table 2). An identification number followed by "b" (for broad) was attributed to each pattern represented by 1–7 fragments. Two patterns (20b and 12b) correlate with association of several specificities found in DR2 haplotypes, the first associating Dw2, Dw12, FJO, and DwX and excluding AZH, the other Dw12 and AZH only. Eight patterns correlate with association of specificities found in both DR2 and DRw6 haplotypes. For example, pattern 14b is found in all DR2 and Dw18 individuals, pattern 7b is found in DR2 long, Dw9, and Dw19 individuals. Some of these patterns are also found to be

Table 1. Fragments associated with single DR or Dw specificities

Identification number	DR, Dw, or DQ types	Bands, no.
1	DR2 total	7 *DR*
8	DR2 long	1 *DR*
4	DR2 (short) (FJO + AZH)	3 *DR*, 1 *DQ*
13	AZH	1 *DR*
9	Dw2	1 *DR*, 2 *DQ*
10	Dw12	1 *DQ*
11	Dw12 (HTC only)	5 *DR*
	Total DR2 and subtypes	18 *DR*, 4 *DQ*
24	DRw6 total	1 *DR*
31	DRw14–DQw3	1 *DR*
21	Dw18	1 *DR*
22	Dw19	3 *DR*
	Total DRw6 and subtypes	6 *DR*
27	DRw11 total	1 *DR*
30	DRw12	1 *DR*
29	DRw11–DQw1	1 *DR*, 1 *DQ*
38	DRw11–Dw5 (HTC cell 10)	1 *DR*
	Total DR5 and subtypes	4 *DR*, 1 *DQ*
44	DR1	5 *DR*
	Total	33 *DR*, 5 *DQ*

associated with DR1 in the larger panel of 82 haplotypes, i.e., patterns 19b, 6b, and 5b. The latter pattern, previously called DQR1 (12), has been encountered in most DR1 haplotypes, in a few DR2, and some DRw6 haplotypes. In the present study, only DR2 short and Dw9 (DRw14) are found to be DQR1. Interestingly, pattern 6b comprises the same haplotype with the addition of Dw19. Pattern 19b correlating with DQw1 in the larger panel is found to be absent only in AZH. Pattern 17b (previously called DQR2,6), shown to be allelic to DQR1 (pattern 5b) (12), is found to be present in Dw2 and DRw13 (Dw18, Dw19) and in cell 12, which carries a DRw11, DQw1 haplotype. Patterns 16b and 18b represent slight variations of DQR2,6—pattern 18b is not found in Dw19, and pattern 16b is found in Dw12.

One pattern, 23b, found in this study, specially associated with DRw13, was also associated in the larger panel with HLA-B8 positive DR3 haplotypes. Pattern 25b behaves like an allele of pattern 23b and is found in DR2 long, Dw9, DR5, and HLA-B8 negative DR3 haplotypes. Interestingly, it is present in only one (cell 16) of the three Dw18 HTCs, underlying a heterogeneity of this haplotype, at least at the genomic level.

Table 2. Fragments associated with two or more HLA-DR, Dw, or DQ specificities

Identification number	DR, Dw, or DQ types	Bands, no.
3b	DR2 total, DR1	3 *DR*
20b	DR2 long, FJO	1 *DR*
12b	Dw12, AZH	1 *DR*
14b	DR2 total, Dw18	1 *DR*
15b	DR2 total, DR1, Dw18, DR3	1 *DR*
18b	Dw2, Dw18, DR11–DQw1	1 *DR*, 1 *DQ*
17b	Dw2, Dw18, Dw19, DR11–DQw1	1 *DQ*
16b	Dw2, Dw12, Dw18, Dw19, DR11–DQw11	1 *DQ*
19b	DQw1 (except AZH)	1 *DQ*
5b	DR2 short, DR1, Dw9	2 *DQ*
6b	DR2 short, DR1, Dw9, Dw19	1 *DQ*
7b	DR2 long, Dw9, Dw19	1 *DR*
23b	DRw13, DR3	1 *DR*
25b	DR2 long, DR5, Dw9, HTC16 (Dw18)	1 *DR*
2b	DR2 total, DRw12	1 *DR*
26b	DR5, Dw9, HTC16 (Dw18), HTC18 (Dw19)	1 *DR*
40b	DRw11 (except cell 13), HTC16 (Dw18)	1 *DR*
34b	DR5, DRw13–DQw1	1 *DQ*
33b	DR5, DRw13, Dw9	1 *DR*
32b	DR5 (not Dw5), DRw6	1 *DR*
35b	DR5, DRw6 (except DR13–DQw3)	1 *DR*
37b	DR5, DRw6	9 *DR*
36b	DR5, DRw6 (except HTC22, Dw9)	1 *DR*
41b	DR5 [except HTC9 (Dw5)], cell 20 (1802^+)	1 *DQ*
42b	HTC9 (Dw5), cell 21 (1802^-)	1 *DQ*
28b	DQw3	3 *DQ*
43b	DR1, cell 20 (1802^+)	1 *DR*

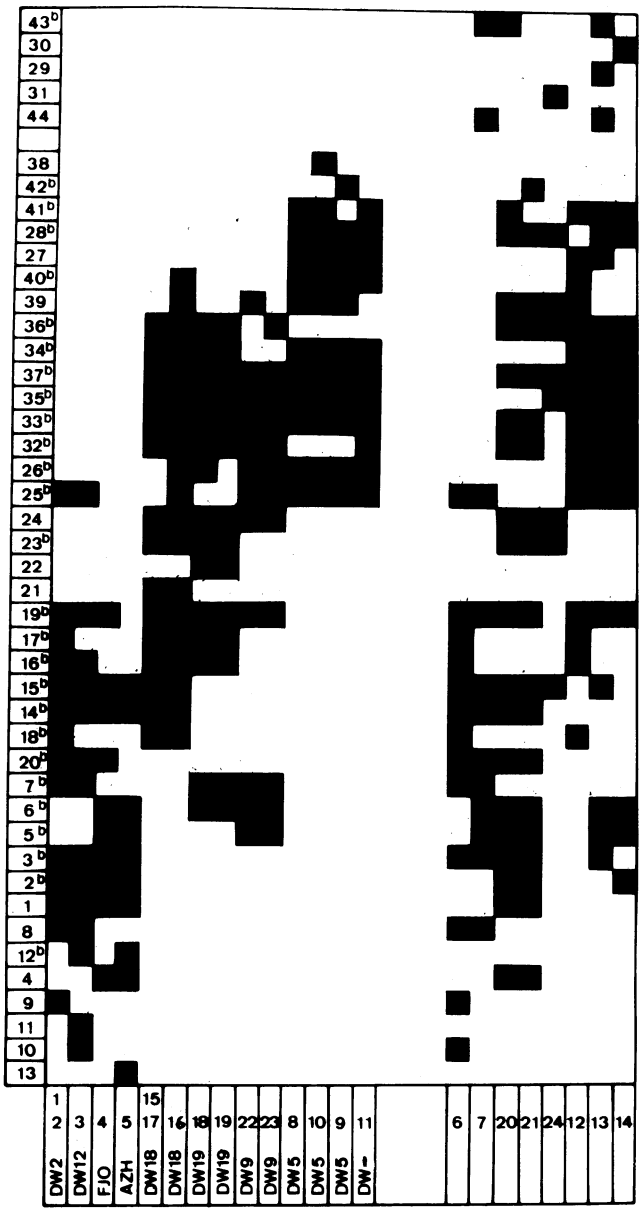

FIG. 3. Distribution of 44 groups in the 24 individuals tested. Each row represents one group, indicated on the left by its identification number as in Fig. 2 and Tables 1 and 2. Each column individualizes the combination of groups; 14 combinations are found among 16 HTCs (*Left*). Number identifying each cell (*Bottom*). Patterns found in heterozygous individuals (*Right*). Note the high divergence between DR2- and DR5-associated subtypes in contrast to the DRw6 subtypes, which share many groups with DR2 and DR5 subtypes.

Eleven patterns are found in both DRw6 and DR5 haplotypes (Table 2). For example, pattern 34b is found in DR5 and DRw13–DQw1. Pattern 40b is found to be associated with all DRw11 except one (cell 13, DRw11–DQw3 associated with the JVM Dw subtype). It must be noted that this pattern is found only in the Dw18 HTC also marked by pattern 25b. This HTC is also individualized by pattern 26b, which is found in all DR5, Dw9, and only one of the two Dw19 HTCs suggesting, here also, a heterogeneity of a Dw19 haplotype, at least at the genomic level.

A heterogeneity of the Dw5 haplotype is also found with patterns 41b and 42b. In the larger panel of 82 haplotypes, patterns 41b and 42b subdivide DQw3 haplotypes. Pattern 41b is found in most DR5 and rarely in DR4, and conversely pattern 42b is found in most DR4 and rarely in DR5 individuals. This subdivision is different from the formerly described DQR4 and DQR5 clusters (12). In this study, patterns 41b and 42b allow three kinds of DQw3 haplotypes to be distinguished: pattern 41b positive, present in all DR5 (except cell 9) and in cell 20, which is DRw13–DQw3–HTC 1802 positive; pattern 42b positive, found in only one Dw5 HTC (cell 9) and in cell 12, which is DRw13–DQw3, but HTC 1802 negative; and patterns 42b and 41b negative, as in cell 24 (DRw14–DQw3). It is noteworthy that pattern 28b correlates with all DQw3 haplotypes irrespective of their linkage disequilibrium with DR types. Five "*DRβ*" fragments cannot be correlated with DR and Dw specificities; they might belong to the *DP* or *DO* gene subregion (see bottom of Fig. 2). Interestingly, four of them constitute two clusters that tend to be mutually exclusive like alleles [12.8-kilobases (kb) *Eco*RV and 3.1-kb *Taq* I on the one hand, and 7.6-kb *Eco*RV and 4.5-kb *Taq* I on the other].

What Is the Genetic Significance of the Correlation Between Polymorphic Restriction Sites and Immunologically Defined Specificities? Each "polymorphic" fragment (specific or broad) corresponds to the variation of the position of restriction sites. These sites occur probably more often in noncoding parts of genes, since the coding parts represent approximately one-tenth of the class II genes. Eighty out of 85 polymorphic fragments detected here are found to correlate with DR, DQ, or Dw specificities known to be independent of *DP* gene subregion polymorphism. Indeed, only weak linkage disequilibria have been found between *DP* alleles and *DR* or *DQ* alleles, themselves in strong linkage disequilibrium. This suggests that these 80 fragments are associated with the *DR* or *DQ* subregion and not with the *DP* subregion.

The correlation between each of these 80 fragments and DR, DQ, or Dw specificities suggests also, with regard to the point discussed above, that the polymorphism of the noncoding parts is in strong linkage disequilibrium with the polymorphism of the coding parts in principle responsible for the immunologically defined specificities. These 80 fragments constitute 44 patterns, each having a different distribution in the panel tested (Fig. 3). Each of these 44 patterns might correspond, in most cases through linkage disequilibrium, to a polymorphic stretch of sequences located in the coding part. In a maximal interpretation, 44 different coding polymorphisms could correspond to these 44 patterns.

For example, Dw2 HTCs are associated with 14 different patterns. These 14 patterns might correspond to 14 different polymorphic stretches of sequences located in *DRβ* and/or *DQβ* exons. The 14 stretches could correspond to a maximum of 14 epitopes in DRβ and/or DQβ molecules, but this number could be less for at least three reasons: (*i*) It is possible that in some haplotypes the noncoding parts diverge independently of the coding part. (*ii*) Some polymorphic stretches of coding sequences might not affect the product (silent mutation) or might be found in genes not expressed. (*iii*) Finally, as nothing is known about the three-dimensional structure of class II molecules, the correspondence between polymorphic coding sequences and epitopes remains obscure—i.e., we do not know if one epitope corresponds to a single stretch of polymorphic sequences or to the spatial association of amino acids encoded by different parts of a single gene or by several genes (α and β).

Among the 16 HTCs tested, 14 different combinations have been found; the only identical HTCs remaining in this study are the two Dw2 HTCs. Hence, *Dw18, Dw19, Dw9,* and *Dw5* are heterogeneous at the genomic level. From the above considerations, it is quite conceivable that this heterogeneity can be found at the product level.

Finally, an interesting point is underlined by the study of rare DR–DQ associations such as DRw14–DQw3 for which we have probably found a specific marker (pattern 31). We have reported specific markers for the rare DR7–DQw3 haplotypes (12). These rare associations raise different cellular responses from the DR14–DQw1 or DR7–DQw2 haplotypes. We suggest that these cellular responses are not merely due to the summation of epitopes found in the associated alleles (*DRw14* and *DQw3* or *DR7* and *DQw3*) but also are due to the presence of epitopes found only in these rare haplotypes.

This report essentially raises the relationship between the polymorphism of coding and noncoding regions. Further knowledge needs accumulation of gene sequences and their comparison with the structure of gene products (including three-dimensional studies).

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