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HLA-DR4 subtype frequencies in rheumatoid arthritis indicate that *DRB1* is the major susceptibility locus within the HLA class II region

(polymerase chain reaction/oligonucleotide/epitope/major histocompatibility complex)

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ABSTRACT **Susceptibility to rheumatoid arthritis (RA) may be due to the presence of shared functional epitopes common to the HLA-DR β chains of several RA-associated haplotypes. We have obtained direct evidence for this hypothesis by using the polymerase chain reaction and sequencing the *DRB1* and *DQB1* genes from RA patients. A highly conserved epitope present on DR β chains of DR4 and DR1 haplotypes was found in 83% of 149 patients with classical or definite RA but was found in only 46% of 100 control individuals ($P < 0.0001$). Two Dw subtypes of DR4 (Dw4 and Dw14) were associated with disease susceptibility but two other subtypes (Dw10 and Dw13) were not. Sequence differences between these subtypes implicate those residues around the putative antigen binding site of the DR β molecule in the pathogenesis of RA. These data provide a basis for understanding host susceptibility to RA at a molecular level.**

The polymorphism in class II gene products from the major histocompatibility complex (MHC) is known to be localized in the N-terminal domain of these molecules (1), particularly in discrete regions termed allelic hypervariable regions (AHVRs) (2, 3). These influence peptide binding and T-cell recognition by their position on the α-helices and β-strands that form the sides and floor of the putative antigen binding site (4). AHVRs are often shared between several different class II alleles, suggesting that the polymorphism in these molecules has been in part generated by recombination events that have shuffled these AHVRs between haplotypes (3, 5).

Identification of the exact locus within the MHC responsible for particular disease susceptibilities has become more feasible now that sequences are available for most of the class II alleles (3, 6). Attribution of susceptibility to a particular locus relies heavily on comparison of sequences between haplotypes that confer susceptibility or protection (7, 8). Haplotypes associated with rheumatoid arthritis (RA) share a common third AHVR located between residues 67 and 74 of the β chain of the HLA-DR protein (DRβ); this finding has given rise to the shared-epitope hypothesis for susceptibility to this disease (9–11). This hypothesis holds that the third AHVR of certain DR4 subtypes (Dw4 and Dw14) and the DR1 allele is, in part, responsible for the susceptibility to RA seen with these haplotypes. There are two corollaries to this hypothesis. (*i*) DR4 subtypes (Dw10 and Dw13) that differ by nonconservative substitutions in the third AHVR should not confer susceptibility to RA. (*ii*) Other rare alleles with the same third AHVR on other haplotypes might also confer susceptibility and be overrepresented within the RA population. We have tested this hypothesis directly by establishing the frequency of such epitopes in the normal and RA populations.

MATERIALS AND METHODS

Patients and Controls. We recruited 149 Caucasian patients with classical or definite RA (12). All had an erosive arthropathy and had received disease-modifying drugs, but extraarticular features were not a prerequisite for inclusion. IgM rheumatoid factor was assayed (rheumatoid arthritis particle agglutination test; Fujirebio Inc., Tokyo) and considered positive in a titer of >1:40 but negative only if absent on three occasions during active disease. One hundred healthy unrelated Caucasian individuals served as controls for the DR genotyping studies. The frequencies of DR4 subtypes in 178 DR4-positive patients were compared with those in 185 healthy DR4-positive blood donors.

Amplification of HLA Class II Alleles. *DRB* and *DQB1* alleles were amplified from 1 μg of genomic DNA by using *Thermus aquaticus* (*Taq*) polymerase (13). A 232-base-pair segment of the first domain of all known *DRB* alleles was amplified using the primers GLPDRβ and GAMPDRβ as previously described (7). Specific amplification of DR4-associated *DRB1* alleles was achieved using the primers GAMPDRβ (as above) and DR4/337C (5′-TCTTGGAGCAGGTTAAACA-3′), which is homologous with the first AHVR of all DR4 *B1* alleles. *DQB1* alleles were amplified in a two-stage procedure. The primers GLPDQβ1 and GAMPDQXβ2 were used as previously described (7). Two microliters of the reaction mix was then subjected to a second round of amplification using the primers GAMPDQXβ2 and GLPDRβ, which amplify a 210-base-pair fragment of *DQB1*.

Sequence-Specific Oligonucleotide (SSO) Probes. Ten microliters of the appropriate reaction mix was transferred to a nitrocellulose filter (Schleicher & Schuell) in a BRL Hybri-Dot manifold. Baked filters were incubated for 30 min at 31°C in 5 ml of 1.8× standard saline citrate (SSC, 0.15 M sodium chloride/0.015 trisodium citrate, pH 7)/0.2% Ficoll/0.2% polyvinylpyrrolidone/0.2% bovine serum albumin/0.5% SDS containing yeast tRNA at 400 μg/ml. Filter hybridization was performed overnight at 31°C with the appropriate SSO end-labeled with [γ-^{32}P]ATP to give 3×10^6 cpm per filter in 2.5 ml of 1.8× SSC. The filters were washed in 6× SSC at room temperature for 10 min and at the calculated duplex melting temperature (38) for 30 min to remove mismatched probe. Filters were then exposed to Fuji RX film for 4 hr.

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Abbreviations: RA, rheumatoid arthritis; MHC, major histocompatibility complex; AHVR, allelic hypervariable region; SSO, sequence-specific oligonucleotide.

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SSO probes specific to sequences in the *DRB1* alleles encoding the classic HLA-DR specificities (DR1–DRw14) allowed a DR phenotype to be inferred for each individual. Likewise, the subtypes of HLA-DR4 were established by using SSO probes recognizing the individual sequences within the third AHVR that distinguish the Dw4, Dw10, Dw13, Dw14, and Dw15 subtypes (Fig. 1). Confusion arising from cross-hybridization of these probes with sequences on other haplotypes was completely avoided where necessary by specific amplification of the DR4 *B1* alleles; e.g., the third AHVRs of Dw10 and DRw13 are identical. The results of HLA-DR typing obtained by SSO probes were validated in 45 subjects previously typed by the standard National Institutes of Health microlymphocytotoxicity assay. The accuracy of Dw subtyping of DR4 alleles with SSO probes was tested on homozygous typing cell lines. *DQB1* alleles associated with the DR4 haplotype were determined using SSO probes as described (7).

Dideoxy Sequencing of Amplified Products. The DNA sequence of the second and third AHVRs (nucleotides 78–258) was determined from the *DRB* alleles amplified from five RA subjects (four non-DR1/DR4 and one DR4/7). After purification by agarose gel electrophoresis, 20% of the amplification product was ligated into a blunt-ended *Eco*K-selection M13 cloning vector (14). *Escherichia coli* JM101 cells were transformed and plaques were screened for *DRB* expression by nylon filter lifts probed with a full-length radiolabeled *DRB1* probe. All four *DRB* alleles were sequenced in duplicate from these five individuals.

RA Susceptibility. The relative risks for developing RA associated with the various HLA types were calculated by the method of Mantel and Haenszel (15). Adjusted risk estimates for DR1 were calculated with stratification on DR4. The significance of differences between the groups was calculated using a one-tailed Fisher's exact test.

RESULTS

Accuracy of HLA-DR and Dw Typing Using SSO Probes. The DR phenotypes inferred from the results of SSO probing agreed exactly with those obtained by serological testing in the 45 patients tested by both methods. The ability of these probes to distinguish a single nucleotide mismatch was confirmed (Fig. 2). Likewise, this strategy was effective in distinguishing the subtypes of DR4 accurately and in defining the nucleotide sequences of the corresponding third AHVR. The expected third AHVR sequences were observed on all the relevant haplotypes, i.e., Dw4, Dw10, Dw13, Dw14, DR1, and DRw13. Twenty-five RA patients were negative for both DR1 and DR4 but none of them exhibited a Dw4 or Dw14 third AHVR in the context of a novel allele. This result was confirmed from all four *DRB* alleles in 5 patients by DNA sequencing of nucleic acid residues 78–258. These were

	81		97
DR1	5'-	GGAAAGATGCATCTATA-	3'
	89		105
DR4	5'-	ACTTCTATCACCAAGAG-	3'
	204		222
Dw4	5'-	GGAGCAGAAGCGGGCCGCG-	3'
	205		222
Dw10 (DRw13)	5'-	GAAGACGAGCGGGCCGCG-	3'
	205		222
Dw14 (DR1)	5'-	GAGCAGAGGCGGGCCGCG-	3'
	231		212
Dw13	5'-	GGTGTCCACCTCGGCCCGCC-	3'

FIG. 1. SSO probes used in DR and Dw typing. Nucleotides are numbered from the first base pair of the sequence encoding the mature protein.

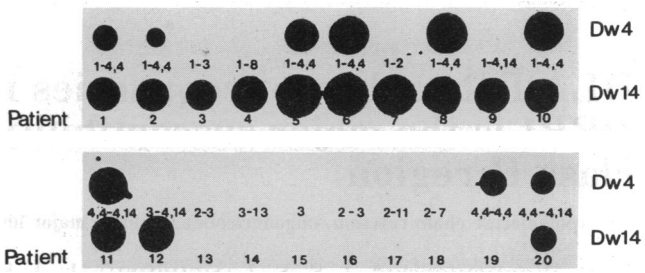

FIG. 2. Dot blots from 20 RA patients probed sequentially with the Dw4 and Dw14 SSOs. The DR types and Dw subtypes of DR4 are illustrated. In addition to the samples from patients 11, 12, and 20, who are Dw14-positive, all the samples from the DR1-positive patients (nos. 1–10) hybridized with the Dw14 SSO, which shares identical sequence with DR1. Patient 9 typed positively for DR1 and DR4, but the Dw14 subtyping could be inferred from negative reactions with the Dw4, Dw10, Dw13, and Dw15 SSO probes.

normal for the corresponding haplotypes, DR2/7, DR3/w13, DR2/w11, DR2/3, and DR4/7 (data not shown).

DR, Dw, and DQ Typing and the Relative Risks for RA. The expected association between seropositive RA and DR4 was seen ($P < 0.0001$) and no DR haplotype was absolutely protective against the disease (data not shown). The relative risks for RA were similar for DR4, Dw4, and Dw14 and were also maintained in the small seronegative group (Table 1). Both Dw4 and Dw14 were significantly increased in the RA group (Table 2). In contrast, none of the RA group was Dw10, compared to 4% of controls ($P < 0.01$), and only two of the RA group were Dw13, compared to 8% of controls ($P = 0.01$). The proportion of Dw4 homozygotes, Dw14 homozygotes, and Dw4/Dw14 heterozygotes was not increased in the RA population. There was only a slightly increased risk of RA associated with DR1, which did not reach significance. Overall, those patients exhibiting the conserved third AHVR, either on a DR1 or on a DR4 haplotype, accounted for 83% of the RA group. The relative risk for RA associated with this epitope was 5.35, but it was clear that this effect was much stronger for the Dw4 and Dw14 alleles than for DR1 (Table 1).

It was possible to assign *DQB1* alleles to DR4 haplotypes in 24 DR4-positive controls (29 haplotypes) and 27 DR4-positive RA patients (30 haplotypes). *DQB1* alleles associated with the DRw11 haplotype were not included in this analysis. Thirteen of 30 RA *DQB1* haplotypes (43%) were DQw7, compared with 16 of 29 control *DQB1* haplotypes (55%). This difference was insignificant.

DISCUSSION

Analysis of the *DRB1* sequences in 149 RA patients in this study provides direct evidence that the sequences within the third AHVR of DRβ are likely to be responsible for the increased susceptibility to RA seen with DR4 and DR1 haplotypes. This region of DRβ, between amino acid residues 67 and 74, differs by only a single conservative change (position 71, arginine to lysine) between DR4 Dw14 or DR1 and DR4 Dw4 (16). This sequence was present in 83% of our rheumatoid population compared to 46% of controls ($P < 0.0001$). The sequence was seen only in the context of the DR4 Dw4, DR4 Dw14, and DR1 alleles, all previously recognized to be associated with RA. Five patients not possessing these alleles were shown by sequencing of the first domains of their *DRB* alleles not to possess this sequence motif, suggesting that if other rare alleles existed with this sequence they were not being selected for in this population. These sequencing studies included patients with the specificities DR2, DR3, DR7, DRw11, DRw52, and DRw53. The 17% of patients lacking the shared third AHVR epitope

Table 1. DR and Dw associations with seropositive and seronegative RA

	n	Total DR4	Dw4	Dw14	Dw13	Dw10	DR1
Controls	100	17	12	5	0	1	33
Seropositive RA	139	95	70	38	1	0	36
Relative risk		10.5 (5.4–21.1)	11.0 (5.2–24.5)	14.3 (5.1–49.2)	—	—	1.2 (0.6–2.3)
Probability		<0.0001	<0.0001	<0.0001	—	—	NS
Seronegative RA	10	7	7	2	0	0	3
Relative risk		11.4 (2.3–72.8)	16.1 (3.0–105)	11.1 (0.7–116)	—	—	0.9 (0.1–4.1)
Probability		<0.001	<0.0005	0.005	—	—	NS

Risk estimates for the DR4 subtypes were calculated relative to all DR4-negative patients and controls. The 95% confidence intervals for the risk estimates are given in parentheses. NS, not significant.

presumably represent examples of the genetic heterogeneity of this disease. Similar heterogeneity has been documented in other polygenic autoimmune diseases such as insulin-dependent diabetes mellitus (7, 8). Ten patients with classical seronegative RA exhibited the same frequency of this conserved epitope as the seropositive group. This result is in keeping with most but not all studies of seronegative RA (17) and suggests that the MHC-encoded genetic susceptibility is relevant to both seronegative and seropositive disease.

Our findings of increases in both Dw4 and Dw14 haplotypes in RA were consistent with many previous studies in Caucasians (18–21). Although DR1 showed the strongest non-DR4 association with the disease, this did not reach statistical significance. This finding was consistent with many other studies, which have been reviewed by Woodrow *et al.* (22). They calculated that the relative risk for RA and DR1 was only 1.46, but because of the large numbers of patients and controls considered, this result was highly significant ($P = 2.5 \times 10^{-10}$).

Shared epitopes have previously been described among RA patients by using a variety of techniques. Duquesnoy *et al.* (23) used alloantisera to define a crossreacting epitope on DR1 and DR4 individuals that was highly enriched in a small rheumatoid population; the distribution of this epitope suggested that it might represent the third AHVR of DRβ. Similarly, Goronzy *et al.* (24) used T-cell clones to identify multiple HLA class II epitopes that were shared among RA patients. Among these was an epitope shared between DR4 and DR1. It has also been suggested that the monoclonal antibody 109d6, which recognizes DRw53, DRw10, and sometimes DR1, may react with an epitope associated with RA susceptibility (25, 26). However, the failure of this antibody to recognize DR1 consistently and the lack of a DRw53 association with RA independent of DR4 (i.e., DR7, DR9) argue strongly against this hypothesis. Oligonucleotide probing of Southern blots is the most direct approach previously used to address this question. One study using this strategy was able to determine that the frequencies of two DR4 subtypes, Dw4 and Dw14, were increased in the patient population (21). However, the difficulty in obtaining data on a large number of patients prevented analysis of the less common DR4 subtypes, restricting the overall conclusions that could be drawn.

By using amplified DNA, we obtained sequence data on a large number of RA patients and thereby indirectly confirmed the role of a shared DRβ epitope in influencing susceptibility to the disease. In addition to detecting such an epitope in 83% of RA individuals, we could evaluate the role of different DR4 subtypes in susceptibility. This is a critical component of the hypothesis of shared epitopes, as the four major DR4 subtypes found in Caucasians—Dw4, Dw14, Dw13, and Dw10—differ by only a few residues across the third AHVR. The Dw10 subtype has two nonconservative substitutions in this region (position 70, glutamine to aspartic acid, and position 71, arginine to glutamic acid). Likewise, Dw13 has a nonconservative substitution of glutamic acid for alanine at position 74 (Fig. 3). These changes would be expected to change this epitope considerably, and it has been suggested that were the third AHVR important in DR4-associated susceptibility, Dw10 and Dw13 would not be increased in rheumatoid populations (10). This possibility has never previously been tested, although some ethnic data indirectly support the hypothesis (22, 28).

Our data have confirmed the strong associations of both Dw4 and Dw14 subtypes of DR4 with RA. Crucially, similar increases in Dw10 and Dw13 were not seen. Among 185 control individuals, 7 Dw10 and 15 Dw13 were detected. Dw10 accounted for none of the 178 DR4 phenotypes in RA patients, while Dw13 was present in only 2 individuals, who coincidentally were also positive for Dw4. One would have expected at least 12 individuals to possess these subtypes. This significant reduction in these subtypes provides direct evidence that different DR4 subtypes confer different risks of developing RA. Because the class II sequence differences between these subtypes are restricted to the third AHVR, this is strong support for the role of *DRB1* in susceptibility.

The role of *DQ* alleles in RA susceptibility has been controversial. Initial studies indicated that no variation in the normal distribution of DQw7 and DQw8 was to be found in DR4 RA patients (29). Some recent studies contradicted these earlier data, suggesting an association between DQw7 and RA (30, 31). Our data strongly suggest that the *DQB1* locus is not associated with RA, as the distribution of the DQw7 and w8 *DQB1* alleles were not significantly different from those seen in the control DR4 population.

Table 2. Inferred Dw subtypes from DR4-positive patients and controls

	n	Dw4	Dw14	Dw10	Dw13
Controls	185	119 (64%)	53 (29%)	7 (4%)	15 (8%)
RA	178	133 (74%)	66 (37%)	0	2 (1%)
Probability		0.02	0.05	<0.01	0.01

		66				70				74	
Susceptible	DR1	Asp	Leu	Leu	Glu	Gln	Arg	Arg	Ala	Ala	Val
Susceptible	DR4 Dw14	-	-	-	-	-	-	-	-	-	-
Susceptible	DR4 Dw15	-	-	-	-	-	-	-	-	-	-
Susceptible	DR4 Dw4	-	-	-	-	-	Lys	-	-	-	-
Susceptible	DRw10	-	-	-	-	Arg	-	-	-	-	-
Not Suscep	DR4 Dw53	-	-	-	-	Arg	-	-	-	Glu	-
Not Suscep	DR4 Dw10	-	Ile	-	-	Asp	Glu	-	-	-	-
Not Suscep	DR4 Dw13	-	-	-	-	-	-	-	-	Glu	-
Not Suscep	DR7	-	Ile	-	-	Asp	-	-	Gly	Gln	-
Nucleotide Sequences	DR1	GAC	CTC	CTG	GAG	CAG	AGG	CGG	GCC	GCG	GTG
	DR4 Dw4	---	---	---	---	---	-A-	---	---	---	---

FIG. 3. *DRB1* third AHVR sequences in RA-associated and -unassociated haplotypes. The susceptible *DRB1* alleles show marked homology with only conservative amino acid substitutions at position 70 or 71. In addition to the well-described associations with DR1, Dw4, and Dw14, other studies have suggested associations with DRw10 (24) and Dw15 (27). In contrast, in the examples of alleles not associated with RA demonstrated here, there are multiple amino acid substitutions, most of which, such as the substitution of aspartic acid for glutamine at position 70 (DR4 Dw10 and DR7) and glutamic acid for alanine at position 74 (DRw53 and DR4 Dw13), are likely to have great functional significance.

Confirmation of a major role for a shared DRβ epitope in RA susceptibility can be derived from studies of ethnic groups. In populations where the strong susceptibility alleles DR4 Dw4 and DR4 Dw14 are uncommon, the weaker DR1 susceptibility allele becomes more prominent (27, 32). This is true in the Israeli population, where Dw10 is the most prominent DR4 allele, and in Yugoslavians, where DR4 is a relatively rare specificity (33). A prediction from this would be that the risk of rheumatoid arthritis might also be less prevalent in these populations where the major susceptibility allele is rare. Indeed, the lifetime risk of RA in both Israel and Yugoslavia is $\approx$25% that seen in English Caucasians (34). This is consistent with the relatively weak susceptibility seen with the DR1 allele (22).

The availability of a three-dimensional crystal structure for the class I molecule HLA-A2 allows residues 67–74 of DRβ to be localized in a class II structural model based on the HLA-A2 data (4, 35, 36). This model suggests that these residues lie along the α-helix equivalent to that encoded by the α2 domain of HLA-A2. This α-helix bounds the foreign antigen binding site, and the polymorphic residues in the helix may play an important role in determining which peptides bind in this site. DR molecules have a conserved α chain; hence the variation that functionally distinguishes DR molecules is confined to one α-helix and four β-strands in the floor of the binding site. The nonconservative changes in the α-helix that distinguish DR4 Dw4 from DR4 Dw10 and DR4 Dw13 should have a substantial effect in determining which peptides are bound in the groove or in dictating patterns of T-cell recognition.

The hierarchy of RA susceptibility with different DR alleles is reflected in the three-dimensional structure inferred from the structure of HLA-A2 (4, 35). DR4 Dw4 and DR4 Dw14 have very similar structure, with only a single conservative amino acid substitution at position 71. DR1 is the most variant of the susceptibility alleles, even though it is identical to DR4 Dw14 along both putative α-helices and the β-strands corresponding to the HLA-A2 α1 domain on the floor of the groove (4). However, it differs from DR4 in the three β-strands corresponding to the HLA-A2 α2 domain, although even here it is more similar to DR4 than most of the other DRβ alleles. It differs from DR4 Dw14 by a change in charge at three residues in the floor of the putative binding site (Fig. 4). Most other DRβ molecules show much greater variation

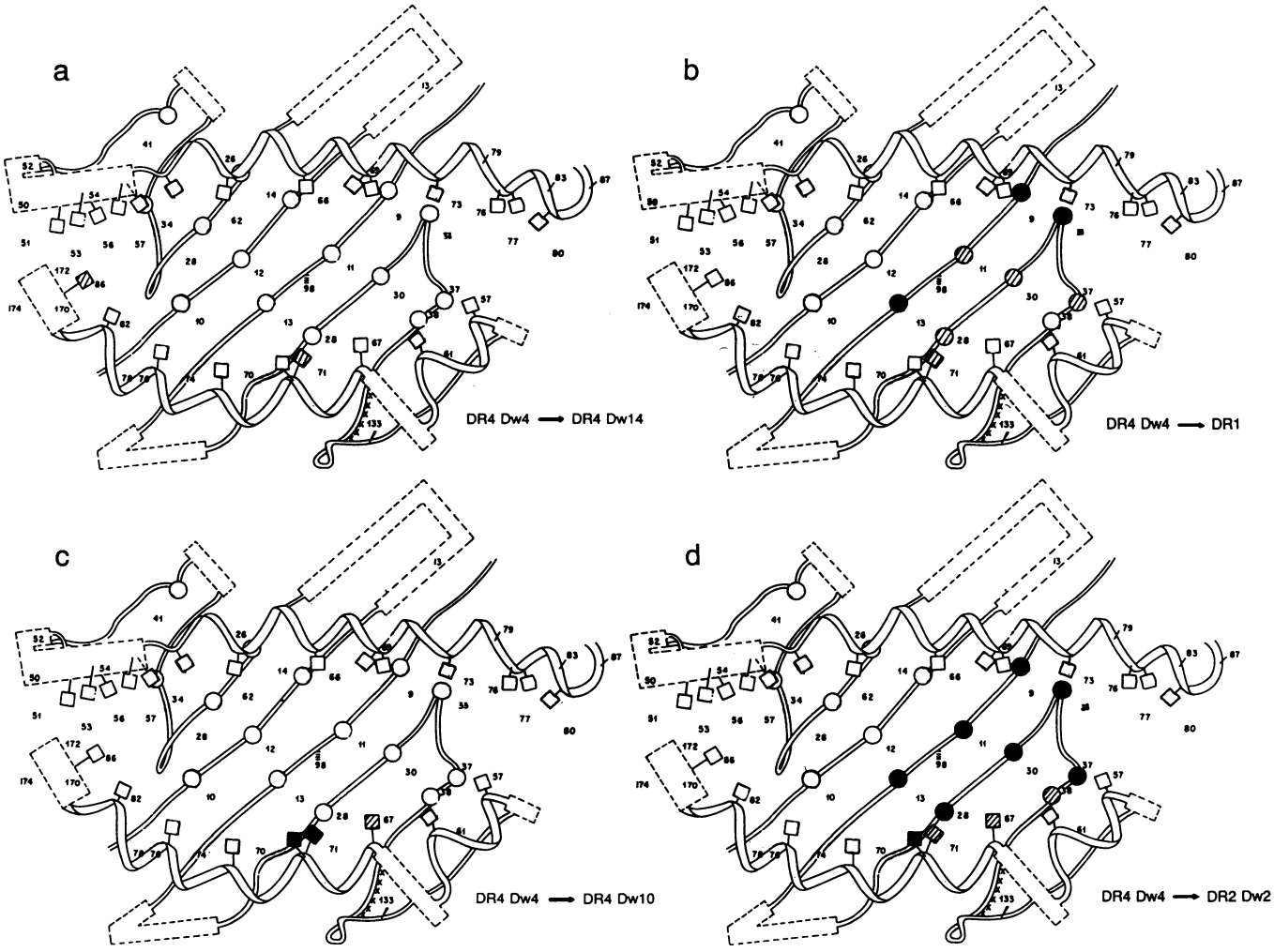

FIG. 4. Comparisons of the sequence of HLA-DR4/Dw4 with two other molecules associated with RA, DR4 Dw14 (*a*) and DR1 (*b*) and two molecules not associated with RA, DR4 Dw10 (*c*) and DR2 Dw2 (*d*). Nonconservative (charge) changes are illustrated by solid symbols and conservative (non-charge) changes are shown as hatched symbols. Circles represent residues in the floor of the binding site and squares represent residues in the α-helices. Only conservative changes distinguish Dw4 and Dw14 (position 71, lysine to arginine; 86, glycine to valine) and DR1 (71, lysine to arginine) in the α-helices. Nonconservative changes are present in the α-helix of Dw10 (70, glutamine to aspartic acid; 71, lysine to glutamic acid). The floor of the binding site is shared by DR4 subtypes, while DR1 shows relatively few changes compared to other nonsusceptibility haplotypes such as DR2. The changes are restricted to the four β-strands and α-helix derived from DRβ. The other residues, derived from the DRα chain, are nonpolymorphic. (Modified from ref. 4 with permission from Macmillan Magazines Ltd.)

from DR4 and are not associated with any susceptibility to RA. For example, DR2 has seven substitutions that alter the charge in the floor of the binding site, as well as further variation in the α-helix. The hierarchy of susceptibility therefore reflects the degree of variation of the whole binding site for foreign antigen and is consistent with the view that functionally, DR1 is a distant member of the family that includes DR4 Dw4 and DR4 Dw14.

The refinement of a linkage to a precise localization of disease-susceptibility sequences has become a major challenge in human genetics. Susceptibility to RA is complex and multifactorial. As in other autoimmune diseases, many genes are likely to be involved (37). Formal confirmation that *DRB1* is the susceptibility locus within the MHC will require both further genetic mapping and functional support. Because of their functional role in regulating the immune response, the class II loci have been the major candidate susceptibility loci. Structurally, the sharing of the DRβ third AHVR between the susceptibility haplotypes DR4 and DR1 provides evidence that *DRB1* might be the relevant locus responsible for disease susceptibility. The observation that some DR4 subtypes are not associated with susceptibility provides further strong evidence that *DRB1* functions as the major susceptibility locus within the MHC. Because the sequences that correlate with susceptibility lie adjacent to the putative antigen binding site, it is likely that the mechanism by which DRβ mediates susceptibility is by specificity of peptide binding or T-cell recognition. This leads directly to the possibility that the functional role played by this molecule can be manipulated.

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