HLA CLASS II REGULATION AND STRUCTURE

Analysis with HLA-DR3 and HLA-DP Point Mutants

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HLA class II antigens are developmentally regulated, polymorphic, cell surface glycoproteins that function as restriction elements in cell-to-cell interactions in the immune response (1). They are heterodimeric, composed of α and β chains of about 34 and 29 kilodaltons, respectively (2). The HLA-DR β chain is polymorphic, whereas the DR α chain has little or no polymorphism; both DQ α and β chains exhibit polymorphism (2). The regions of class II molecules involved in the restriction phenomenon are unknown. There are at least three well-defined class II antigens, HLA-DR, -DQ (formerly MB/DC/DS), and -DP (formerly SB), each with different α and β chains. Southern blots made with DR, DQ, and DP α and β cDNA probes suggest that the HLA region contains at least twice as many class II genes as are necessary to code for the three well-defined dimers (3), but it is not known how many or which of these genes are expressed. Class II molecules are present on cells involved in the immune response, including B cells, macrophages, and activated T cells. They are also expressed transiently on erythroid and granulocytic precursor cells (4) and on nonhematopoietic cells (5, 6), suggesting that class II molecules may have a more general role in cell-cell interactions in development and, also, that nonlymphoid cells may have functional roles in the immune response. In addition to developmentally regulated expression, class II gene expression is modulated in some cells by agents such as γ interferon (7) and prostaglandins (8).

One approach to studying questions of major histocompatibility complex (MHC)[1] structure and regulation is to isolate and characterize immunoselected mutants (9). We previously isolated a class II regulatory mutant, 6.1.6 (10), which appears to have a defect in a positive transactive regulator of transcription of class II genes (11, 12). In the present studies, we have used mutagenesis with the alkylating agent ethyl methanesulfonate (EMS) and the anti-HLA-DR3 monoclonal antibody (mAb) 16.23 (13) to isolate a large number of HLA-DR3 mutants. To simplify the subsequent analysis, the selection was carried out on a previously isolated deletion mutant missing all DR and DQ α and β genes on one

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[1] *Abbreviations used in this paper:* EMS, ethyl methanesulfonate; FCS, fetal calf serum; mAb, monoclonal antibody; MHC, major histocompatibility complex; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RFLP, restriction fragment length polymorphism; SDS, sodium dodecyl sulfate.

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haplotype. Several of the mutants isolated were found to have defects in the epitope reactive with 16.23. Others have defects in several different steps in the pathway leading to expression of the DR α or β genes, including DR gene transcription and mRNA processing. These mutants allowed us to study various aspects of the regulation of the class II gene expression and the structure of class II gene products.

Materials and Methods

Cell Lines. Deletion mutants 8.1.6 and 9.28.6 were derived from T5-1, a clonal derivative of the B cell line PGLC33H, which is DP4 positive and has the HLA haplotypes DQ1, DR1, B27, CW1, A2 and DQ2, DR3, B8, A1 (10). 8.1.6 is an immunoselected deletion mutant of T5-1 that has lost the *cis* specificities DQ1 and DR1 (10). By Southern blotting it is missing all DQ and DR genes on the DQ1, DR1, B27, CW1, A2 haplotype (3). 9.28.6 is an immunoselected deletion mutant of T5-1; it is missing all HLA specificities on the DQ2, DR3, B8, A1 haplotype and, by Southern blotting, all class I, II, and III genes on that haplotype (3, 14). The DR null mutant 4.36.84 was derived from 9.28.6 by mutagenesis with diepoxy octane and selection with the anti-HLA-DR monomorphic antibody, VI.15 (see Results).

Antibodies Against HLA Class II. mAb 16.23 has been shown in population and family studies to be HLA-DR3 specific, but it reacts with some HLA-Dw/DRw6 individuals as well (13). mAbs VI.15 (14), HB10A (15), L227 (16), L243 (16), LKT (17), and DA6.147 (18) are class II monomorphic antibodies that are thought to react with HLA-DR and possibly with other class II antigens; further characterization of the class II molecules reactive with these antibodies is presented in Results. mAb B7/21.2 reacts with a cell surface class II molecule structurally distinct from HLA-DR and -DQ (19); transfection experiments have established that it reacts with HLA-DP (20). mAb Genox 3.53 is an allospecific antibody recognizing DQ1 (21). mAbs VI.15 and L243 react only with dimeric HLA-DR, whereas HB10A, L227, and LKT react with dimer as well as with DR β monomer. DA6.147 reacts with HLA-DR and with monomeric DR α. All mAb were concentrated 10–20-fold from hybridoma supernatants by $(NH4)_2SO4$ precipitation.

Mutagenesis and Mutant Isolation. Cells were mutagenized with 100 μg/ml EMS for 24 h, washed, and grown in normal medium for 7 d, then used for selection. Selection was by brief exposure to an mAb, followed by complement, followed by cloning as previously described (22).

Quantitative Cell Surface Antibody Binding. This was done as described (14). ^{125}I-labeled protein A or ^{125}I-labeled 187.1, which is a rat mAb with specificity for mouse κ chains (23), were used as second-step reagents. All antibodies were used in saturating amounts.

Class II Gene Clones. The cDNA probes used encode the entire DR α (24) and β (25) chains.

Northern and Southern Blot Analysis. These methods have been described (12). Each Northern blot was repeated two to three times and the blots shown are representative. Densitometry was performed using an Ultrascan densitometer (LKB Instruments, Inc., Gaithersburg, MD). The band intensities shown are ratios of the peak height of the mutant compared with that of 8.1.6, normalized to the ratio of peak heights of class I mRNA.

Western Blot Analysis. Cell lysates were prepared by solubilizing cells at a concentration of 5×10^7 cells/ml in 1.5% Nonidet P-40 (NP-40) in 10 mM Tris-HCl, pH 7.8, with 0.2 mM phenylmethylsulfonylfluoride. Lysates were diluted with an equal volume of 2× sodium dodecyl sulfate (SDS) sample buffer (2.5% SDS final) and heated for 2 min at 100°C. In those experiments in which detection of undissociated dimers was desired, the sample, containing only 0.2–0.4% SDS, was not boiled. Samples were electrophoresed in 12% SDS-polyacrylamide gel electrophoresis (PAGE), and then electrophoretically transferred to a nitrocellulose filter for 2 h at 50 V; 0.1% SDS was included in the transfer buffer. The filter was incubated for 1 h at 4°C with phosphate-buffered saline (PBS) containing 25% fetal calf serum (FCS).

The filters were reacted for 2 h with saturating amounts of first antibody (HB10A or DA6.147, which, respectively, recognize DR β and α chains), for 1 h with saturating amounts of second antibody (rabbit anti-mouse IgG), and for 1 h with subsaturating amounts of ^{125}I–protein A at 10^6 cpm/ml. Anti-actin antibody was added with the HB10A or DA6.147 antibody to provide an internal control for the amount of cell extract in each lane. Preliminary experiments established that under these conditions, the band intensities were proportional to the amount of antigen in the extract. After each incubation, filters were washed three times for 5 min each with 100 ml PBS containing 5% FCS and 0.05% NP-40. Autoradiographs were prepared using Kodak X-Omat AR film at −70°C. Densitometry was performed as for the Northern blots except that the ratios of band intensities of mutant to 8.1.6 were normalized to the ratio of the actin band intensities.

Results

Mutant Selection and Screening. A deletion mutant of T5-1, 8.1.6, which had lost all the DR and DQ α and β genes on the DQ1, DR1, B27, CW1, A2 haplotype, was chosen as a target cell for selection to simplify the interpretation of HLA-DR protein and mRNA levels in any mutants isolated. After mutagenesis of 8.1.6 with EMS, immunoselection was carried out with the HLA-DR3 mAb 16.23, at two levels, one approximately saturating (stringent selection), and the other, less than saturating (relaxed selection) (Table I). Relaxed selection maximizes the likelihood that mutants which retain some capacity to bind the selecting antibody will survive selection, but it also increases the likelihood that nonmutant cells will survive (26). Colonies arising after selection were screened for cell surface binding of antibody 16.23 by radioimmune binding assay. Only colonies with binding ratios <0.5 were studied further (23 of the 62 tested clones).

The mutant frequencies for clones with binding ratios <0.5, corrected for cloning efficiency, were 1.6×10^{-4} and 3×10^{-4} for the stringent and relaxed selections, respectively. These mutant frequencies are similar to those of class I mutants induced by comparable exposure to EMS (26). Given the mutant frequencies, the probability that any mutant contains more than one mutation affecting an HLA-DR gene is equal to the observed mutant frequency multiplied by the number of HLA-DR genes, or ~0.001.

Surface Binding Patterns of Antibodies 16.23, VI.15, and HB10A Divide Mutants into Two Classes. To define the phenotype of these mutants further, the cell

TABLE I
Selection of 16.23 Mutants

Tube No.	16.23 Antibody	No. of surviving colonies	No. of colonies with binding ratios* of:		
			<0.1	0.1–0.5	>0.5
	μl				
1	500	21	3	5	8
2	250	64	6	9	31

EMS-mutagenized 8.1.6 cells, 5×10^5 per tube, were immunoselected with 16.23 hybridoma supernatant in the volumes shown and with complement, and were then cloned as described.

* Binding ratio was calculated as (cpm bound by surviving clone − cpm bound by negative control/(cpm bound by 8.1.6 − cpm bound by negative control). For details on the binding assay, see Table II.

surface binding of various mAb, including 16.23 and several monomorphic anti-HLA-DR antibodies, was examined. 15 mutants were studied in detail. A striking feature of these data is that the mutants fall into two distinct, nonoverlapping groups with respect to the pattern of cell surface binding of 16.23 and VI.15 (Table II). One group consists of seven mutants (9.2.3, 9.5.3, 9.10.3, 9.25.3, 9.26.3, 9.28.3, and 9.29.3), which had markedly reduced or absent 16.23 binding but normal or near normal binding of the HLA-DR monomorphic antibody VI.15. We interpret this pattern as indicative of expression on the cell surface of a normal quantity of HLA-DR3 antigen, as measured by VI.15 binding, but with an alteration in the epitope recognized by the 16.23 antibody (epitope defective). The other broad class consists of eight mutants with parallel reductions in binding of 16.23 and VI.15. The parallel reduction in cell surface binding of 16.23 and VI.15, as well as studies to be described, indicate that these latter mutants have a reduced amount of HLA-DR3 antigen expressed at the cell surface (expression defective). Cell surface binding of 16.23, VI.15, and L243 in expression-defective mutants varied from at or near background (mutant 9.22.3) to ~40%, close to the upper range of binding considered mutant; the binding of HB10A, another class II monomorphic antibody, paralleled but was in most cases somewhat higher than that of VI.15.

In contrast to the 16.23 and VI.15 binding, the binding of two other class II

TABLE II

Cell Surface Antibody Binding by 16.23 Mutants

Mutant clone	Antibody binding ratio (×100)						
	16.23	VI.15	L243	HB10A	LKT	L227	B7/21.2
(8.1.6)	(100)	(100)	(100)	(100)	(100)	(100)	(100)
(6.1.6)	15 ± 3	8 ± 3	11 ± 1	4 ± 1	12 ± 4	9 ± 2	10
9.2.3	3	73	ND	ND	ND	ND	ND
9.5.3	0	102	ND	65	88	100	ND
9.10.3	0	73	ND	82	97	101	ND
9.25.3	0	102	ND	ND	ND	ND	ND
9.26.3	12	106	ND	ND	ND	ND	ND
9.28.3	0	82	ND	ND	ND	ND	ND
9.29.3	7	97	ND	86	ND	ND	ND
9.4.3	9	13	11	34	55	43	117
9.7.3	28	36	ND	33	ND	ND	ND
9.12.3	15	25	20	26	75	58	122
9.13.3	25	22	ND	29	ND	ND	ND
9.15.3	17	20	17	34	55	41	105
9.20.3	41	31	32	ND	ND	ND	137
9.22.3	1	3	0	6	45	50	87
9.27.3	7	12	ND	24	ND	ND	ND

Binding ratio: (cpm bound by mutant − cpm bound by negative control)/(cpm bound by 8.1.6 − cpm bound by negative control). The negative control is 6.1.6, a regulatory mutant of T5-1 that has a reduction of ~99.5% in expression of class II molecules (10, 12). Binding ratio of 6.1.6 (cpm bound by 6.1.6)/(cpm bound by 8.1.6). The binding ratios represent the median values from three or more independent assays. The standard errors given for 6.1.6 are representative. The binding to 6.1.6 reflects nonspecific binding to the assay tubes.

monomorphic antibodies, LKT and L227, was reduced at most to 40% of the parental value in the expression-defective mutants, even in 9.22.3, which had >94% reduction in 16.23, VI.15, L243, and HB10A binding. These findings suggest that L227 and LKT might recognize at least two class II surface molecules, only one of which is affected in these mutants.

mAbs LKT and L227 Bind to HLA-DP as Well as HLA-DR. The identity of the other molecule besides HLA-DR recognized by LKT and L227 was studied using another set of mutants derived from T5-1. Mutant 9.28.6 is an immunoselected, hemizygous deletion mutant of T5-1 that has lost the complete HLA complex on the DR3 side (3, 14). Selection on 9.28.6 with the anti-DR monomorphic antibody VI.15 yielded the DR null mutant 4.36.84 (Table III); selection on 4.36.84 was then carried out with the anti-DP antibody B7/21.2. All three mutants from the B7/21.2 selection that are B7/21.2 null (11.6.84, 11.11.84, and 11.13.84) also lost LKT and L227 binding, indicating that loss of LKT and L227 binding is coincident with loss of HLA-DP. The fact that Genox 3.53 binding was retained in these mutants argues against a deletion extending from HLA-DP to -DQ. Thus, LKT and L227 must detect a determinant shared by DR and DP β chains.

Expression-defective Mutants Have Alterations or Reductions in Either DR α or β mRNA, But Not in Both. To investigate the nature of the lesions in mutants with reduced levels of HLA-DR3 at the cell surface, Northern blotting with HLA-DR α and β cDNA probes was used to determine levels of DR α and β chain mRNA. Representative blots are shown in Figs. 1 and 2. All the expression-defective mutants had a clear reduction or alteration in either DR α or β mRNA, but not in both. Mutants 9.4.3, 9.15.3, 9.16.3, 9.20.3 (and 9.7.3 and 9.13.3, not shown) had reductions in DR3 β mRNA ranging of 70–95%, whereas their DR α mRNA levels are essentially normal. Mutant 9.22.3, in contrast, had no detectable DR α mRNA but had normal levels of DR β mRNA. Mutant 9.12.3 had a reduced amount of DR α mRNA of normal size (1.2 kilobase [kb]), but, in addition, had a second band of 2.7 kb, not seen in 8.1.6 or other mutants, which hybridized to the DR α probe. Thus, in all the mutants that were initially

TABLE III
Cell Surface Antibody Binding by HLA-DR and -DR, -DP Null Mutants

Mutant clone	Antibody binding ratio (×100)					
	B7/21.2	VI.15	B10A	LKT	L227	Genox
(9.28.6)	(100)	(100)	(100)	(100)	(100)	(100)
(6.1.6)	14	2	4	9	11	14
4.36.84	128	0	3	40	18	79
11.6.84	0	0	0	0	1	133
11.11.84	0	0	0	0	0	62
11.13.84	0	0	0	0	1	108

For details on the binding assay, see legend to Table II. For the origins of the DR null mutant 4.36.84, and the DR and DP null mutants 11.6.84, 11.11.84, and 11.13.84, see Results.

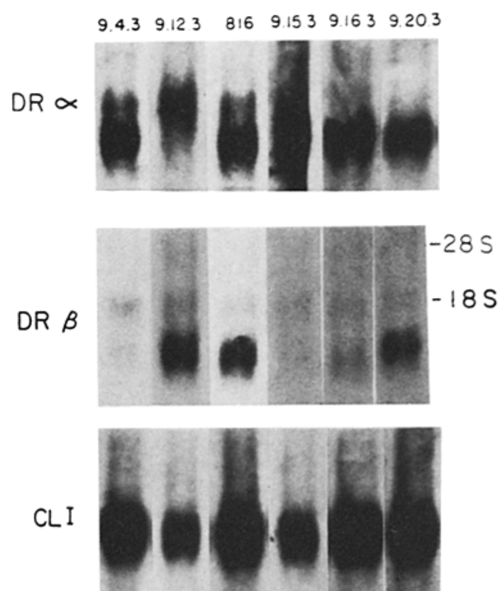

FIGURE 1. Northern blot analysis of 8.1.6 and mutant RNAs with DR α and β and HLA class I cDNA gene probes. The 18 and 28 S ribosomal RNAs can be seen faintly on the DR β blots. The ratios of band intensities of mutants to 8.1.6 for the DR α and β mRNA, respectively, are: 9.4.3, 0.66 and 0.06; 9.15.3, 0.81 and <0.06; 9.16.3, 1.42 and 0.07; 9.20.3, 1.00 and 0.21; 9.22.3, <0.06 and 0.72. The alterations in 9.12.3 DR α mRNA can be seen more clearly in Fig. 2.

grouped together on the basis of reduced levels of HLA-DR3 protein at the cell surface, the reduction can be accounted for by a reduced level or altered size of DR α or β mRNA.

Mutant 9.22.3 Has a Deletion of the DR α Gene. We investigated the possibility that one or more of the expression-defective mutants resulted from gross deletions of DR structural genes, which could be detected by Southern blotting using DR α and β cDNA probes. The DR α probe hybridizes to a single gene (23) that maps to the HLA class II region (27). A DR α Bgl II restriction fragment length polymorphism (RFLP) has been described; T5-1 is heterozygous for this RFLP, with bands of 4.2 and 3.8 kb (Fig. 3*A*). 8.1.6 is deleted for DQ1 and DR1 and had only the 3.8 kb DR α band. Mutant 9.22.3, derived from 8.1.6, lacked both the 4.2 and the 3.8 kb bands and therefore has undergone deletion of the remaining DR α gene. Southern blots made with the DR β probe were more complex, because each haplotype carries two or more DR β genes, and haplotypes may differ with respect to the number of DR β genes carried. However, we have previously shown (3) that in 8.1.6 all DR β genes on the DR1 haplotype of T5-1 are deleted; thus, all bands hybridizing to the DR β probe in 8.1.6 come from the DR3 haplotype. Fig. 3*B* shows that all bands in 8.1.6 hybridizing with the DR β probe were also present in 9.22.3. Thus, the deletion in 9.22.3 does not extend to the closely linked DR β gene complex, nor to the complement C4 gene (data not shown). No bands hybridizing to the DR β probe were lost in the other DR β expression-defective mutants; therefore, of these mutants, only 9.22.3 has a deletion large enough to be detected by Southern

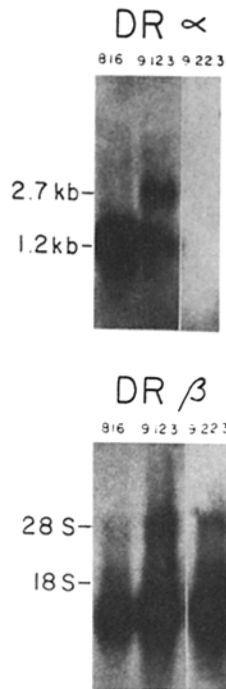

FIGURE 2. Northern blot analyses of 8.1.6, 9.12.3, and 9.22.3 RNAs with DR α and β gene probes. The band intensity ratios for 9.12.3 are: DR α 2.7 kb mRNA, 0.31; 1.2 kb mRNA, 0.28; DR β mRNA, 1.13.

blotting. The fact that there was essentially no binding of 16.23, VI.15, L243, or HB10A to 9.22.3 (Table II), although the deletion did not extend to the DR β genes, suggests that DR β subunits do not form noncognate dimers with DQ or DP α chains.

Methylation of a Single DR β Gene Is Altered in Some DR β Expression-defective Mutants. Class II genes from cells active in class II gene transcription have different patterns of cytosine methylation than those from class II nontranscribing cells (28). It was therefore of interest to determine whether there were changes in the patterns of methylation of DR β genes in the HLA-DR β expression-defective mutants described here. Southern blots were made from Bgl II–Hpa II double digests of DNA from 8.1.6 and from several DR β expression-defective mutants, and were hybridized with a DR β cDNA probe (Fig. 4). Of the six strongly hybridizing bands seen in the Bgl II–Hpa II double digests, five were identical in 8.1.6 and in all three mutants. The sixth band (5.0 kb) was present in 8.1.6 and in mutant 9.20.3, but was missing in mutants 9.4.3 and 9.15.3, and was replaced in 9.4.3 and 9.15.3 by a band of 4.6 kb. Thus, a cytosine in or around one of the DR β genes is methylated in 8.1.6, which expresses DR β, but is demethylated in mutants 9.4.3 and 9.15.3, which have severe reductions in DR β mRNA level.

Expression-defective Mutants Have Reduced Levels of Both DR α and β Protein Subunits. The reductions in DR α or β mRNA in the expression-defective mutants and the cell surface binding data predict that the amounts of the corresponding subunit proteins should also be reduced. To investigate this,

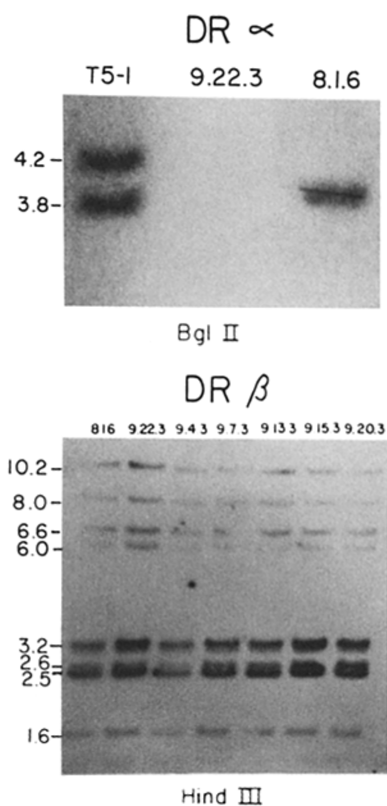

FIGURE 3. Southern blot analysis of 8.1.6 and expression-defective mutants with DR α and β gene probes. The restriction fragment sizes were calculated from a Hind III digest of lambda DNA (not shown).

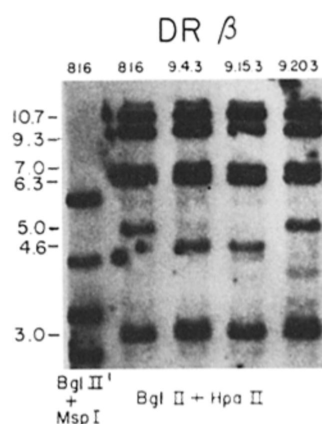

FIGURE 4. Methylation patterns of DR β genes in 8.1.6 and in mutants with reduced DR β mRNA levels. The restriction enzymes Hpa II and Msp I recognize the same sequence (5′ CCGG 3′) but differ in that Hpa II will not cut if the internal cytosine is methylated whereas Msp I will cut regardless of the methylation state of the internal cytosine. In the first lane, 8.1.6 DNA had been digested with Bgl II plus Msp I; in the other lanes the DNAs had been digested with Bgl II and Hpa II.

detergent extracts of the cells were boiled and reduced to dissociate the HLA-DR dimer, then subjected to Western blotting using mAbs DA6.147 and HB10A to detect the DR α and β subunits, respectively. Fig. 5 shows representative Western blots of detergent extracts from equal numbers of mutant and 8.1.6 cells. The most striking finding from these studies is that the quantities of both DR α and β chains were decreased in all the mutants that had reduced expression of HLA-DR3 on the cell surface. Even though in each expression-defective mutant the defect can be related to a reduced level or alteration in size of either DR α or β mRNA, but not of both, the levels of both DR α and β subunits were reduced in all. The fact that all mutants with reductions of either DR α or β mRNA had reductions in *both* DR α and β protein subunits suggests that the protein product from the unaffected DR α or β gene must undergo rapid degradation unless it can form dimers. The apparently greater reduction in DR α than β subunits in all the expression-defective mutants may reflect affinity differences between DA6.147 and HB10A for their respective target antigens. The instability of HLA-DR protein monomers is in contrast to the stability of class I heavy chain monomers, which accumulate intracellularly both in mutants with altered heavy chains that are unable to dimerize (26), and in the Daudi cell line, which lacks β_2 microglobulin (29).

Epitope-defective Mutants Have Altered Subunit Associations. To search for further evidence of structural changes in 16.23 epitope mutants, we altered the conditions of preparation of the cell sample for Western blots so as to visualize the HLA-DR dimer. It is known that HLA-DR antigen migrates as a dimer in SDS-polyacryamide gel electrophoresis (SDS-PAGE) if samples are not boiled (30). Cell lysates were therefore prepared without prior boiling or reduction, in a sample buffer containing 0.2% SDS, and were electrophoresed, blotted, and reacted with HB10A according to standard procedures. As shown in Fig. 6, about two-thirds of the HB10A binding in wild-type 8.1.6 cells was to the HLA-DR dimer, and the remainder was to the DR β monomer. In contrast, under the same conditions no dimer could be seen from any of the 16.23 epitope mutants, and the amount of DR β monomer was increased in proportion to the reduction in dimer. These results indicate that the lesions in the 16.23 epitope mutants

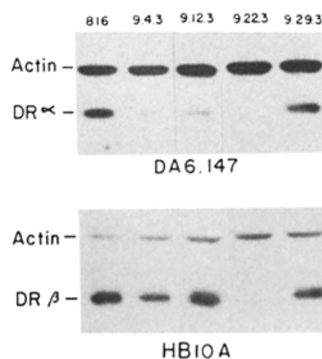

FIGURE 5. Western blot analyses of DR α and β protein subunits in boiled NP-40 extracts of expression-defective mutants. The ratios of DR α and β band intensities of mutants to 8.1.6, normalized to actin, respectively are: 9.4.3, 0.05 and 0.41; 9.12.3, 0.11 and 0.60; 9.22.3, <0.04 and <0.04; 9.29.3, 0.61 and 0.60.

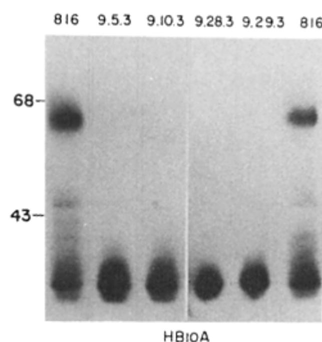

FIGURE 6. Western blot analysis of HLA-DR3 protein dimer and DR β monomer in unboiled NP-40 extracts of epitope-defective mutants.

have reduced the strength of the DR subunit association, rendering the DR3 dimer more susceptible to dissociation by dilute SDS. However, since cell surface binding of the conformation-sensitive antibody VI.15 is reduced at most by 30% in the epitope mutants, the effect of the mutational lesion on HLA-DR3 subunit association, transport, or conformation in the intact cell under physiologic conditions cannot be large in any of the mutants. The fact that the majority of the HB10A binding in the Western blot of 8.1.6 is to dimer whereas, in the epitope mutants, no dimer is seen under the same conditions, suggests that most of the dimer reactive with HB10A contains the DR β chain bearing the 16.23 epitope; otherwise, dimer that formed with the second, unaltered β chain, if present in appreciable amounts, should have been apparent in the Western blots from the epitope mutants.

Discussion

In this report we describe two broad classes of HLA class II mutants, those which express relatively normal amounts of HLA-DR3 at the cell surface, but with an altered structure, and those which express reduced amounts of HLA-DR3. The lesions in the latter, taken together, affect a variety of steps in the pathway leading to class II gene expression. Characterization of the lesions in these mutants therefore offers an approach to elucidating steps involved in class II regulation. We have previously (10–12) described a mutant, 6.1.6, which expresses virtually no class II gene products because of a defect in a transactive regulator of transcription of all class II genes. The expression-defective mutants described here all differ from 6.1.6 in that they only affect expression of HLA-DR α or β. Some of the expression-defective mutants have lesions analogous to those found in mouse strains that are defective in expression of I-E (31), the murine homolog of HLA-DR, whereas others have defects in class II gene expression that have not been previously described.

Among the latter are mutants 9.4.3 and 9.15.3, which have severe reductions in HLA-DR3 protein and DR β mRNA levels accompanied by demethylation of a CpG in one of the DR β genes. We have previously noted (28) a strong correlation between class II gene nonexpression and demethylation of CpG sites in or around class II genes in isogenic pairs of cells, one of which expresses class

II genes and the other of which does not. The demethylation of only one of the DR β genes in 9.4.3 and 9.15.3 is in contrast to the demethylation seen in all class II genes in 6.1.6; it is consistent with the more limited lesions in 9.4.3 and 9.15.3, which appear to affect transcription of a single DR β gene. The selective nature of the transcriptional defect in 9.4.3 and 9.15.3, limited to a single DR β gene, makes it likely that the mutational lesions are in an element *cis* to the affected DR β gene, rather than in a transactive factor, as in 6.1.6. Such a *cis* lesion might be in a promoter or enhancer. Evidence has recently been obtained for an enhancer in the 5′ end or flanking region of the I-E β gene, the murine homolog of DR β (32, 33). By sequencing genomic clones of the affected genes and the 5′ flanking regions of transcription-defective mutants such as 9.4.3, it should be possible to define the *cis* sequences involved in regulation of transcription of HLA class II genes.

Whereas the lesions in mutants 9.4.3 and 9.15.3 are at the level of transcription, those in 9.12.3, 9.20.3, and 9.27.3 appear to affect different posttranscriptional steps. In the HLA-DR3 mutant 9.12.3, the reduced level of normal DR α mRNA and the appearance of a new, larger mRNA species hybridizing to the DR α probe are most easily explained by a nucleotide substitution that alters the processing of the DR α transcript by creating an alternative splice site. These alterations in DR α mRNA in 9.12.3 closely resemble those reported by Mathis et al. (31) in I-E α mRNA from the B10.M strain of mouse. Mutant 9.20.3, which has a reduced level of DR β mRNA without a change in methylation of DR β genes, appears to have a lesion affecting DR β mRNA processing or stability. Mutant 9.27.3 has normal levels of DR α and β mRNA, but in preliminary experiments appears to have reduced levels of DR α and β subunits. These could result from a nonsense or cap site mutation or one affecting a posttranslational step, such as the association of DR α and β subunits to form dimers.

The analysis of mutants such as those described here offers additional insights into issues concerning the class II region. Among these issues are which of the several DR β genes are expressed, and how specific class II genes are related to the specific class II gene products recognized by mAb. Mutant 9.4.3 provides information on both the relative quantities of the different DR β genes expressed per haplotype as well as the gene coding for the gene product recognized by antibodies 16.23, L243, and VI.15. Mutant 9.4.3 has a methylation change in only one DR β gene, and has lost ~94% of the mRNA hybridizing to the DR β probe, indicating that most of the mRNA hybridizing to the DR β cDNA gene probe is transcribed from one DR β gene. The reduction in binding of 16.23, VI.15, and L243 in 9.4.3 makes it likely that these antibodies bind only to the product of the DR β gene affected in 9.4.3. On the other hand, antibody HB10A binding is only reduced by two-thirds in 9.4.3; therefore, HB10A reacts with the same DR β chain as do 16.23, VI.15, and L243, but it also must react with a second β chain not affected in 9.4.3. The deletion of the DR α gene in 9.22.3 virtually eliminates HB10A binding; therefore, the second β chain reactive with HB10A must be a DR β chain. Assuming that the residual binding of ~10% for 16.23, VI.15, and L243 on 9.4.3 reflects the remaining expression of the major DR β chain, the second DR β chain appears to be only 20% as abundant as the major DR β chain. Unlike binding by the other monomorphic antibodies, LKT

and L227 binding are only reduced by 50–60% by the DR α deletion in 9.22.3, suggesting that these antibodies react with non-DR as well as DR β chains. The complete loss of LKT and L227 binding in a DR null mutant that was subsequently selected for and had lost DP expression indicates that the non-DR β chain to which LKT and L227 bind is HLA-DP. This conclusion concerning L227 agrees with that based on studies of deletion mutants (34), but differs from that based on immunoprecipitation experiments (35).

Mutants with single amino acid substitutions in the epitope recognized by the antibody used for selection have proven useful in primary sequence mapping of an alloepitope of an HLA class I molecule (36, 37) as well as in functional studies (38, 39). Comparable class II mutants can be used for similar purposes. The fact that 16.23 binding is lost in mutants such as 9.5.3 whereas VI.15, L243, and HB10A binding remains essentially normal, together with the evidence presented above that 16.23, VI.15, and L243 bind to the same molecule, indicates that mutants such as 9.5.3 are in fact epitope defective. A striking feature of the epitope-defective mutants is that they all have reductions in the strength of their HLA-DR3 subunit associations. One explanation for this similarity among the epitope mutants is that they all derive from the same mutational event, but this seems unlikely based on the finding of a minimum of five distinct phenotypes in the eight expression-defective mutants, and on previous data from a comparable EMS mutagenesis and HLA-A2 selection, which yielded a minimum of nine different phenotypes (including at least four different epitope mutants) of 15 mutants studied. If the epitope-defective mutants arose from different events, then the fact that all are affected in their HLA-DR3 subunit associations suggests two interesting alternatives. Either changes in the several amino acids composing the 16.23 epitope have a high likelihood of affecting dimer stability or, conversely, the 16.23 epitope is perturbed by amino acid substitutions broadly distributed throughout one or both subunits.

DNA sequencing of the epitope mutants offers a way of identifying which of the several DR β genes present on an HLA haplotype is the expressed gene bearing the HLA-DR3 epitope recognized by 16.23, and of locating the amino acids comprising the epitope. These studies should contribute to an understanding of the associations of HLA-DR3 with autoimmune diseases. Recently (40), murine alloantibody-selected class II mutants have been found to be altered in their ability to present soluble antigen. Human Epstein-Barr virus–transformed B cell lines are able to present soluble antigen in a class II–restricted manner (41). These mutants thus should provide a means of assessing the effects of specific amino acid substitutions in class II molecules on antigen presentation in human cells.

Summary

Point mutations that affect HLA-DR structure or expression have not previously been described. In the present study, we isolated such mutants by immunoselection of an ethyl methanesulfonate–mutagenized HLA-DR3 cell line with an anti-HLA-DR3 monoclonal antibody, 16.23. To facilitate analysis, we used a parent cell line with a preexisting deletion of one haplotype encompassing DR and DQ α and β. The selection yielded two sets of mutants, one with defects in

DR3 structure, the other with defects in different steps leading to DR expression. Of the expression-defective mutants, one had undergone a second deletion removing the remaining DR α gene but no other class II genes. It had a normal abundance of DR β mRNA but had lost binding of DR monomorphic antibodies, indicating that DR β chains do not form noncognate dimers. A second mutant had an abnormally large DR α mRNA, probably resulting from a splice site mutation. Several mutants had marked reductions in DR β mRNA levels; in two of these, the lesion appeared to be transcriptional because the reduction in DR β mRNA was paralleled by an altered methylation pattern of one of the DR β genes. Other expression-defective mutants had different posttranscriptional defects. Some of the mutations were similar to those that have been found in mouse strains defective in I-E expression, whereas others have no known natural counterpart. The matrix of reactivities of anti-HLA class II monomorphic antibodies with these and similar mutants allowed us to define the gene products recognized by these antibodies.

A set of seven mutants were "epitope defective," that is, they expressed normal or near normal levels of HLA-DR3 but no longer bound 16.23. Unexpectedly, each of the epitope mutants had decreased DR dimer stability. These mutants should be useful in localizing the DR3 alloepitope and in elucidating its contribution as a restriction element in the presentation of soluble antigen to immune T cells.

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