STRUCTURAL ANALYSIS OF A HUMAN I-A HOMOLOGUE USING A MONOCLONAL ANTIBODY THAT RECOGNIZES AN MB3-LIKE SPECIFICITY*

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Cells of the immune system interact with each other or with specific antigen in the context of molecules controlled by the human *HLA-D/DR* region (1–3). For example, *HLA-D/DR* region-encoded molecules regulate immune responsiveness by acting as restriction elements in the proliferative responses of T lymphocytes to antigens (3) or by serving as target antigens for alloreactive T cell killing (4). 10 alleles of the *HLA-DR* locus have been identified and the presence of certain of these alleles has been associated with susceptibility to a number of human diseases. Included in this group of diseases are many conditions that have aberrant immunological processes as a major component, such as multiple sclerosis, systemic lupus erythematosus, and rheumatoid arthritis (5). A comprehensive understanding of the structure of the molecules encoded by the *HLA-D/DR* region could provide valuable insights into both the basic mechanisms underlying normal immune regulation, and the role of these antigens in disease association.

The *HLA-D* region-encoded glycoproteins are composed of two noncovalently associated chains of 34,000 mol wt (alpha chain) and 29,000 mol wt (beta chain) and, in this respect, are similar to the murine Ia (immune response-associated) antigens. The identification and characterization of two distinct Ia molecules encoded by the *I-A* and *I-E* subregions in mice (6) and the association of specific immune responses with molecules encoded by each of these loci has prompted the search for similar alloantigens in man. The predominant family of human Ia molecules, known as HLA-DR, has been shown to be homologous to the murine *I-E* subregion molecules on the basis of amino acid sequence analysis (7). Evidence now suggests that rather than existing as a single species, HLA-DR represents a family of molecules that is distinguished by several structurally distinct beta chains (8, 9). A second homologue of the murine I-E antigens, known as SB, has recently been described in humans (10). This set of antigens, controlled by a subregion separate from the *DR* subregion (11), has been demonstrated by amino-terminal amino acid sequence analysis to be I-E-

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like but, nevertheless, distinct from HLA-DR (12). In addition to these I-E-related molecules, a human equivalent of the murine I-A molecule has recently been described and designated HLA-DS for "second *D* region locus" (13).

The already complex HLA-DR alloantigen system has been complicated further by the description of at least four additional HLA-linked series of B cell alloantigens, MB, MT, Te, and DC (14–17). These specificities were invoked to explain reactivity patterns of some alloantisera that did not correlate with known *HLA-DR* alleles but instead included two or more of the *HLA-DR* alleles. It is not clear if these "supertypic specificities" represent shared (public) determinants on the associated HLA-DR molecules (18) or if they represent specificities present on molecules distinct from DR (17). Recent evidence indicates that at least one of the supertypic specificities, DC1, may be present on the HLA-DS family of molecules. By amino acid sequence analysis, the alpha chain of the molecule carrying this specificity is homologous to the alpha chain of the HLA-DS molecule (19).

The present study describes the use of monoclonal antibody IVD12 for the isolation and partial structural characterization of a molecule present primarily on human B cells having DR4 or DR5 phenotypes. This molecule was purified from a DR4/4 cell line by adsorption on IVD12 and was shown by amino-terminal amino acid sequence analysis to be distinct from the HLA-DR molecules isolated from the same cell line. Furthermore, the IVD12-reactive molecule was found to be homologous to the murine I-A and human HLA-DS molecules. The pattern of binding obtained by indirect fluorescent staining and direct cellular binding with IVD12 is strikingly similar to that pattern obtained using alloantisera against the MB3 specificity (20). These findings suggest that the IVD12-reactive molecules not only represent a human I-A equivalent but also bear the MB3 specificity.

Materials and Methods

Monoclonal Antibodies. Monoclonal antibody IVD12 was the result of a fusion between spleen cells from mice immunized with the human B cell line PRIESS (21) (DR4/4) and the mouse hybridoma line SP2/0-Ag-14 (22). 8–10-wk-old BALB/c mice were immunized intraperitoneally with 10^7 intact cells once a week for 3 wk. 33 d after the last injection, the mice were given 10^7 cells intravenously, and the fusion was performed 3 d later as described by Kennett et al. (23). The antibody IVD12 is a $\gamma 1\kappa$, as determined by Ouchterlony analysis. The monoclonal antibodies L203, L227, IIIE3, and I-LR1 have been described (9, 12, 24).

Immunoglobulins used for affinity columns were isolated from ascites using ammonium sulfate at 50% saturation. After dialysis, preparations were coupled to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) (25). Spent culture media containing the antibodies were concentrated 5–10 times and used directly for immunoprecipitations.

Normal B Cell Panel. Peripheral blood B cells from 75 unrelated members of the tissue-typing laboratory reference panel were screened by indirect immunofluorescence (26) for the presence of IVD12-reactive cell surface antigens. Reactivity with IVD12 or the control monoclonal antibodies was determined by counting positive cells using epifluorescence optics. The HLA-DR phenotype of the cells used in the B cell panel had been previously determined by microcytotoxicity using typing reagents from the Eighth International Histocompatibility Workshop, as well as local alloantisera. Likewise, B cells from three informative families were examined by fluorescence for their ability to bind IVD12.

B Lymphoblastoid Cell Lines. HLA-DR homozygous cell lines were used in the cellular binding assays. The ability of IVD12 to bind the cell lines was examined using the method of Williams (27). Briefly, individual wells of polyvinyl microtiter plates were coated with glutaraldehyde-fixed human B cell lines at a concentration of 5×10^5 cells per well. 50 μl of culture supernatant containing IVD12 or a 1:100 dilution of ascites containing IVD12 was added to each well.

After a 1-h incubation, the microtiter plates were washed and 1–1.5 × 10^4 cpm of ^{125}I-radiolabeled affinity-purified rabbit anti-mouse immunoglobulin (Cappel Laboratories, Cochranville, PA) was added to each well. After a 3-h incubation, the plates were washed and the radioactivity in each well was measured. Alternatively, cellular binding was measured by indirect immunofluorescence. The cell line PRIESS was used for isolation and purification of IVD12-reactive molecules for structural analysis.

Isolation of Radiolabeled Antigens. PRIESS cells were radiolabeled in culture with [^{3}H]amino acids, lysed with 0.5% Nonidet P-40 (NP-40)[1], and passed over a lentil-lectin-coupled Sepharose column to isolate a glycoprotein pool as previously described (9). Glycoprotein pools isolated from 1–3 × 10^8 PRIESS cells were first depleted of DR antigens by two passages over a 10-ml anti-DR affinity column containing three anti-DR monoclonal antibodies, L203, L227, and IIIE3 (9). In one experiment, the DR-depleted glycoprotein pool was chromatographed on a 6-ml anti-SB affinity column containing the monoclonal antibody I-LR1 (12). The DR-SB-depleted glycoprotein pool was then chromatographed on a 10-ml IVD12 immunoadsorbent, and the adherent antigens were eluted with 1.5 M ammonium thiocyanate in Tris-buffered saline (TBS) (0.01 M Tris, 0.15 M NaCl, pH 7.5). HLA-DR antigens were isolated by passage over a IIIE3 antibody affinity column. Pools of eluted antigens were dialyzed against TBS and the antigens were recovered by precipitation with ten volumes of cold acetone.

For some experiments, radiolabeled antigens were isolated by immunoprecipitation. In these experiments, radiolabeled immunoglobulin was removed by preclearing the glycoprotein pool with protein A-bearing *Staphylococcus aureus* cells (Pansorbin, Calbiochem, San Diego, CA) (28), which had been extensively prewashed with TBS containing 0.25% NP40 and 0.1% SDS. The immunoglobulin-depleted pool was then incubated with monoclonal antibody IVD12 for 15 min at 37°C and overnight at 4°C. Rabbit anti-mouse immunoglobulin was then added and the same incubation protocol was followed. The complexes were removed by centrifugation, then washed with TBS containing 0.25% NP-40 followed by TBS without NP-40.

SDS-Polyacrylamide Gel Electrophoresis. IVD12- and IIIE3-reactive radiolabeled antigens purified by either affinity chromatography or immunoprecipitation were electrophoresed under reducing conditions on 12.5% SDS-polyacrylamide tube gels (29). The gels were fractionated into 0.05% SDS in water, and 50-μl aliquots of each sample were counted to determine the positions of the alpha and beta polypeptides. Appropriate fractions were pooled and dialyzed against distilled water before amino acid sequence analysis.

Amino Acid Sequence Determination. Isolated alpha and beta chains labeled with a single [^{3}H]amino acid were sequenced on a Beckman 890C sequencer (Beckman Instruments, Fullerton, CA) as previously described (9). Butyl chloride fractions were evaporated using a stream of N_2, scintillation fluid was added to each fraction, and the radioactivity in each fraction was determined using a liquid scintillation counter.

Results

Monoclonal Antibody IVD12 is Directed against a Specificity Encoded by a Gene Closely Linked to HLA-DR4 and HLA-DR5 Loci. The allospecificity of antibody IVD12 was determined by testing against a panel of B cells from 75 normal unrelated donors (Table I). Antibody IVD12 reacted with the cells of all 20 DR4-positive individuals and with 14 of 16 DR5-positive donors, giving a correlation of 0.81 with these two antigens. IVD12 was also found to react with B cells from three other individuals other than these positive DR4 and DR5 donors. Two of these individuals were typed as DRw6 and the third was typed as DRw9. Additional information regarding the specificity of antibody IVD12 was obtained when IVD12 was used in cellular binding assays with 19 human lymphoblastoid cell lines homozygous for HLA-DR (Table II). IVD12 reacted with all four of the cell lines that were DR4-positive and two of three lines that were DR5-positive. All other cell lines were negative. This reactivity pattern

[1] *Abbreviations used in this paper:* NP-40, Nonidet P-40; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline.

TABLE I

Correlation Between IVD12 Reactivity and HLA-DR Phenotype on B Cells from a Panel of 75 Unrelated Donors

HLA-DR phenotype	IVD12 reactivity/presence of DR phenotype					
	+/+	+/−	−/+	−/−	χ^2	r
DR1	3	32	7	33	0.63	−0.09
DR2	9	26	16	24	1.13	−0.12
DR3	6	29	8	21	0.00	0.00
DR4	20	15	0	40	28.32	0.61
DR5	14	21	2	38	11.62	0.39
DRw6	5	30	9	31	0.38	−0.07
DR7	1	34	16	24	12.65	−0.41
DRw8	2	18	3	22	0.07	−0.03
DRw9	1	19	3	22	0.08	−0.04
DRw10	0	20	2	23	0.32	−0.08
DR4 + DR5	31	4	2	38	49.57	0.81

* χ^2 with Yates' correction; r values were obtained using the formula $r = \sqrt{\chi^2/n}$ and positive or negative correlation by the formula ad = bc.

TABLE II

Reactivity of IVD12 with Human B Lymphoblastoid Cell Lines Homozygous for HLA-DR

DR genotype	Cell lines	IVD12 reactivity
1/1	3104, 3099, HOM2	−
2/2	3161, 3107, KCAR	−
3/3	Mat3, Hal	−
4/4	3103, 3164, WALK, PRIESS	+
5/5	Sweig, 3105	+
	3106	−
w6/w6	Daudi,* Eld2	−
7/7	3163, New7	−

* The Daudi cell line may be DRw6/−.

obtained with IVD12 indicates that this monoclonal antibody is directed against an antigen encoded by a gene closely linked to the genes encoding DR4 and DR5.

Monoclonal Antibody IVD12 Reactivity Segregates with HLA Haplotypes Positive for DR4 or DR5. Segregation of the IV12-reactive determinant with DR4 and DR5 was observed in three families, as shown in Table III. The mother of one of the families was positive for DR5 but negative for IVD12 reactivity. All other DR4 or DR5 individuals were positive with IVD12. These data substantiate the segregation of IVD12 reactivity with DR4 and/or DR5.

Monoclonal Antibody IVD12 Recognizes an Ia-like Molecule Distinct from HLA-DR. After passage over an anti-DR and an anti-SB affinity column, the reactive antigens from a [^{3}H]leucine-labeled PRIESS (DR4/4) glycoprotein pool were isolated using a column containing IVD12. These IVD12-purified antigens were shown to be a bimolecular complex that migrated under reducing conditions in a molecular weight range similar to that of Ia antigens (i.e., ~34,000 for the alpha chain and 29,000 for the beta chain) on a 12.5% polyacrylamide gel (Fig. 1). Since these IVD12-reactive

TABLE III

Segregation of IVD12 Reactivity with HLA Haplotypes in Three Informative Families

Family	Father	Mother	Children			
			1	2	3	4
Gom	a Aw24, Bw44, Cw5, DR2	c Aw24, Bw35, Cw4, DR5	Se	An	Lu	No
	b Aw31, Bw38, Cw-, DR4	d A3, Bw57, Cw-, DR7	b/c	a/d	b/c	a/d
Erw	a A2, B8, Cw-, DR3	c A1, Bw57, Cw-, DRw6	Ro	Je		
	b A1, Bw57, Cw-, DR7	d A2, Bw62, Cw3, DR4	a/d	a/c		
Nia	*a A1, B8, Cw-, DR3	c A29, B7, Cw-, DRw10	Me	Mar	Si	Mah
	b Aw23, Bw49, Cw-, DR4	d Aw32, Bw51, Cw-, DR5	b/d	b/d	b/c	a/c

Boxes indicate reactivity with IVD12 by immunofluorescence.
* Not tested for IVD12 reactivity.

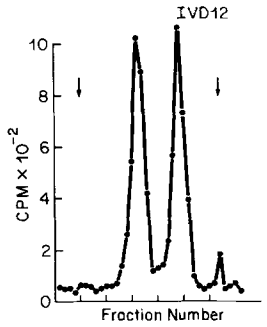

FIG. 1. SDS-polyacrylamide gel electrophoresis under reducing conditions of IVD12-reactive antigens isolated from the cell line PRIESS. Migration was from left to right. Arrows indicate the positions of immunoglobulin heavy and light chain markers.

molecules were not substantially removed from a glycoprotein pool with a series of antibodies known to be directed to HLA-DR or HLA-SB specificities, the data suggest that the Ia molecules reactive with IVD12 are separate and distinct from HLA-DR or HLA-SB molecules.

Monoclonal Antibody IVD12-reactive Alpha and Beta Chains Are Homologous to Murine I-A and Human HLA-DS by Amino Acid Sequence Analysis. PRIESS cells were radiolabeled with [^{3}H]isoleucine, [^{3}H]leucine, [^{3}H]phenylalanine, [^{3}H]tyrosine, and [^{3}H]valine, independently. Alpha and beta chains were isolated from the IVD12-reactive preparations and their amino-terminal amino acid sequences were determined. These sequences were compared with sequences from murine I-E (30), DR (12), I-A (30), and DS (13) molecules (Table IV). The DR sequence was obtained using IIIE3-reactive molecules isolated from the cell line PRIESS. Obvious differences were demonstrated when the amino acid sequences of IVD12-reactive alpha chains were compared with murine I-E alpha chains and human DR alpha chains. Using the 5 radiolabeled amino acids, 18 differences were found between DR and IVD12-reactive alpha chains within the first 29 amino acids. When the sequence obtained for DS alpha chains was compared with the IVD12-reactive alpha chain sequence, however, no differences were observed. Only six differences were demonstrated between IVD12-reactive and I-A alpha chains. These data indicate that alpha chains isolated from IVD12-reactive molecules are quite different from murine I-E and human HLA-DR alpha chains but are homologous to human HLA-DS and murine I-A alpha chains.

TABLE IV

Amino Acid Sequence Comparison of I-E-, DR-,‡ I-A-,* DS-,§ and IVD12-Reactive Antigens*

Part A: Alpha Chains

Position:	1	3	4	6	7	8	11	12	13	14	15	16	19	22	24	25	26	29
I-E	I	-‖	-	-	I	I	-	F	Y	L	L	-	-	F	F	-	F	-
DR	I	-	-	V	I	I	-	F	Y	L	-	-	-	F	F	-	F	-
I-A	-	I	-	-	V	V	Y	-	-	-	V	Y	-	I	·¶	Y	·	·
DS	-	I	V	-	-	V	Y	-	V	-	L	Y	Y	-	-	·	·	·
IVD12-reactive	-	I	V	-	-	V	Y	-	V	-	L	Y	Y	-	-	Y	-	F

Part B: Beta Chains

Position:	7	8	9	10	11	14	16	17	18	24	26	27	30	31	32
I-E	F	L	-	Y	V	L	-	F	Y	V	F	L	·	F	·
DR	F	L	-	-	·	-	-	F	F	·	·	·	Y	·	Y
I-A	F	V	Y	-	F	F	Y	F	-	I	Y	·	Y	·	·
DS	F	V	-	-	F	-	Y	F	-	·	·	·	·	·	·
IVD12-reactive	F	V	Y	-	F	-	Y	F	-	V	-	V	Y	-	Y

* I-E alpha and beta and I-A alpha and beta composite (30).
‡ IIIE3-reactive molecules from PRIESS.
§ DS from reference 13.
‖ Absence of particular amino acid(s) found in corresponding chains of other molecules.
¶ Not determined.

Comparison of the sequences of DR and IVD12-reactive beta chains revealed several differences. Using the 5 radiolabeled amino acids, eight differences could be demonstrated in the first 32 amino acids. Although only limited information is available on the DS beta chain sequence, a comparison of the IVD12-reactive beta chain sequence with this partial sequence demonstrated only minor differences between the two chains. As with the alpha chains, beta chains isolated from IVD12-reactive molecules are considerably different from murine I-E and human HLA-DR beta chains, but exhibit striking homology with murine I-A and human DS beta chains.

Discussion

This study describes the isolation and partial structural characterization of an Ia-like molecule present on human B cells or B cell lines that generally display the DR4 and/or DR5 phenotypes. Using the monoclonal antibody IVD12, this molecule has been specifically purified from a DR4 homozygous cell line and has been demonstrated by amino-terminal amino acid sequence analysis of both chains to be distinct from HLA-DR molecules but homologous to the murine I-A and human HLA-DS molecules.

The strong association of the IVD12 specificity with both DR4 and DR5, as shown in Tables I–III, suggests that the IVD12-reactive molecules also bear the MB3 specificity described by Duquesnoy and Marrari (20). Complete inclusion of DR4 (i.e., all 20 DR4 were IVD12 positive) and high frequency of reactivity with DR5-bearing cells (i.e., 14 of 16 DR5 were IVD12 positive), as well as the reactivity with some cells having DRw6 or DRw9 specificities, correlates well with findings obtained using MB3 allosera defined in the Eighth International Histocompatibility Workshop (20). Alternatively, the IVD12-reactive molecule could be controlled by genes closely

linked to MB3. Comparison of IVD12-reactive products with products reactive with MB3 alloantisera are in progress.

Results from studies in which IVD12 reactivity was followed in three families indicate that IVD12 reacts with a determinant encoded within the *HLA* chromosomal region. In each case the haplotypes positive for IVD12 were also positive for DR4 or DR5. In family Nia, the mother was typed as HLA-DR5 but was not positive for IVD12. Unfortunately, separate segregation of this haplotype could not be observed because the two children that received the DR5 haplotype also had a DR4-positive, IVD12-positive haplotype from the father.

Biochemical analysis of the IVD12-reactive antigens revealed that both the alpha and beta chains from these molecules are distinct from HLA-DR but homologous to the murine I-A molecules rather than the I-E molecules. Likewise, the alpha and beta chains are homologous to the DS molecule (13). With the 5 radiolabeled amino acids used in this study, no differences were observed in the first 20 amino acids when the IVD12-reactive alpha chain from a DR4 line was compared with the DS alpha chain from a DR7 cell line (13). Variation between the IVD12-reactive and DS beta chains is seen at position 9, where a tyrosine is found in the IVD12-reactive beta chain that is absent in the DS beta chain. While the data are limited, this kind of structural variation among beta chains and not alpha chains is typical for class II gene products of both humans and mice (31, 32). Although additional sequence data are needed to determine the extent of variation among both alpha and beta chains of IVD12-reactive and DS molecules, these data are consistent with the hypothesis that these two Ia molecules are, in fact, encoded by the same alpha and beta chain loci, and that the variation observed between the beta chains is due to allelic polymorphism of the locus. Furthermore, amino acid sequence homology of the HLA-DS alpha chain with the DC1 alpha chain (19) suggests that all of these molecules, DS, DC1, and now MB3, are allelic products of a single gene.

This description of a molecule that is homologous to murine I-A is consistent with recent evidence suggesting the presence of multiple loci within the *HLA-D* region (13, 17, 19, 33–37). Previous studies from this laboratory using the cell line PRIESS, coupled with the present study, have now structurally characterized three distinct *HLA-D* region encoded molecules from the same cell line, DR, SB, and MB/DS. All three molecules have structurally distinct alpha and beta chains. DR and SB are I-E-like and MB/DS is I-A-like.

Although a number of studies suggest that the supertypic specificities, such as MB, may be present on the same molecule as the HLA-DR specificities (18, 38), this study supports the view that at least some supertypic specificities are found on molecules encoded by loci separate and distinct from those loci which encode HLA-DR molecules. More evidence obtained with monoclonal antibodies and homozygous cell lines is needed to determine if other supertypic specificities (MT, Te, etc.) represent molecules encoded by even additional loci or if they are determinants present on molecules already described.

If further findings support the assignment of IVD12 as an anti-MB3 monoclonal antibody, this antibody should be invaluable for the precise characterization of these *HLA-D* region products and for studying their tissue distribution, functional significance, and role in disease association. Preliminary experiments (G. Nunez, R. Giles, E. Ball, C. Hurley, J. D. Capra, and P. Stastny, manuscript in preparation) suggest

that IVD12-reactive molecules are expressed on all DR-positive cells with one notable exception. Although a large percentage of peripheral blood monocytes appear to be reactive with antibodies against DR antigens, only a small subpopulation of these cells is reactive with IVD12. Experiments to determine whether the expression of MB/DS molecules on monocytes and macrophages can be modulated, and whether these determinants stimulate allogeneic T cells and serve as restriction elements for antigen-dependent T cell proliferation are currently in progress. In addition, recent findings demonstrating strong association of IVD12 reactivity in patients with chronic lymphocytic leukemia (39, 40) provide a potentially rewarding approach for the analysis of the structural basis of disease association at the molecular level.

Summary

Monoclonal antibody IVD12 was used to isolate and characterize a human Ia molecule present on B cells that generally display DR4 or DR5 phenotypes. The specificity of binding of IVD12 to human peripheral blood B cells from 75 normal individuals and 19 homozygous human lymphoblastoid B cell lines was identical to the supertypic specificity MB3 previously defined. Furthermore, IVD12-reactivity was shown to segregate with HLA in three informative families. In each family, individuals positive for IVD12 binding were also positive for DR4 or DR5. Using IVD12, a molecule has been isolated from the homozygous cell line PRIESS (DR4/4) and has been shown by amino acid sequence analysis to be homologous to the murine I-A and human HLA-DS molecules. These findings suggest that the MB3 specificity is found on a molecule encoded by loci distinct from those loci which encode HLA-DR molecules.

This molecule represents the third family of *HLA-D* region molecules isolated from the cell line PRIESS. Both HLA-DR and HLA-SB molecules from this cell line were previously shown by amino acid sequence analysis to be I-E-like but distinct from one another. Collectively, these data provide evidence that the *HLA-D* region contains at least six loci encoding distinct alpha and beta chains for the HLA-SB, HLA-DR, and HLA-DS molecules.

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