DIRECT DEMONSTRATION OF AN HLA-DR ALLOTYPIC DETERMINANT ON THE LOW MOLECULAR WEIGHT (BETA) SUBUNIT USING A MOUSE MONOCLONAL ANTIBODY SPECIFIC FOR DR3*

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The Ia antigens in mice and the HLA-DR antigens in man play a critical role in the interaction of T lymphocytes and macrophages in antigen recognition (1, 2). In contrast to the murine I region, resolution of the complexity of the human HLA-D/ DR region has been difficult because of limited information from genetic recombination. Furthermore, the exact nature of the determinants involved in cellular interactions is uncertain because they can be defined only with polyclonal antisera. Clarification of the role of DR antigens in various functions controlled by this region would be facilitated by monoclonal antibodies directed against polymorphic determinants. Whereas many monoclonal antibodies precipitating DR-like antigens have been described, the relationship between these molecules and DR antigens defined by alloantisera remains unclear, mainly because these xenogeneic antibodies detect monomorphic (3–6), supertypic (4, 6), or other determinants on DR-like molecules (7). To date, no monoclonal antibody directed against an epitope with the genetic and biochemical characteristics of a single DR alloantigen has been reported (8, 9). In this report, we characterized a mouse monoclonal antibody (16.23) detecting a DR-type molecule that is correlated with the HLA-DR3 alloantigen.

Materials and Methods

Reagents. The origin and maintenance of the cell lines have been described (10). The Rewi cell line was produced by Epstein-Barr virus transformation of peripheral blood lymphocytes (PBL)[1] from a homozygous HLA-Dw1, DR1 donor. The cytotoxic monoclonal antibody 16.23 (IgG3) was obtained from a BALB/c mouse immunized with a single injection of human melanoma cell line Mel JuSo. When tested in a binding assay with a panel of 27 cell lines, it bound only to the immunizing tumor and to an autologous B cell line (10). The JuSo B cell line and PBL were typed as HLA A1, 2; B7, 8; Cw-; DR2, 3. The specificities and numbers of individual HLA-D homozygous typing cells (HTC) used were: HLA-Dw1 (5), Dw2 (7), Dw3 (5), Dw4 (2), Dw5 (1), Dw6 (3), Dw7 (1), Dw8 (1), Dw9 (2), and undefined (1). The HLA-A,

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HTC, homozygous typing cells; M_r, relative molecular mass; PBL, peripheral blood lymphocytes, PBS, phosphate-buffered saline; PMSF, phenyl methyl sulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SpA, protein A.

B, C, D, and DR antigens of the JuSo cells, families, unrelated panel, and HTC were defined with local reagents or those of the VII and VIII International Histocompatibility Workshops. Dr. G. B. Ferrara (Hopital St. Louis, Paris, France), Dr. V. Lepage (Ruhr University, Essen, Federal Republic of Germany), and Dr. H. Grosse-Wilde (Immunohematology Research Center, Massa, Italy) kindly provided many of the HLA alloantisera. A rabbit antiserum against HLA-DR β chains was the kind gift of J. F. Kaufman, Basel Institute of Immunology, Switzerland.

Typing of PBL for Reactivity with Antibody 16.23. PBL were tested with 16.23 by microcytotoxicity, indirect immunofluorescence, or autoradiography. B- and T-enriched lymphocyte fractions were obtained using polymethylacrylate bead columns (11) (Degalon Perlen, Degussa, Frankfurt, Federal Republic of Germany) and characterized by E-rosetting and direct immunofluorescence (fluorescein isothiocyanate [FITC]-goat anti-human IgG, Medac, Frankfurt, FRG). Immunofluorescence with 16.23 was performed on 72-h phytohemagglutinin (PHA) blasts (1% PHA-m, Difco Laboratories, Detroit, MI) using culture supernatant and FITC-goat anti-mouse Ig (Medac). 16.23 was purified from culture fluid by affinity chromatography on protein A (SpA) Sepharose CL (Pharmacia, Upsalla, Sweden) eluted with 0.1 M glycine, pH 3, and iodinated with chloramine T (12) for use in autoradiography. An IgG3 monoclonal antibody (15.95) reacting with melanoma and carcinoma cell lines (10) and anti-HLA-DR monomorphic monoclonal antibodies (3) were used as controls.

Isolation of Antigen Subunits and Direct Binding of Antibody 16.23. Cell surface iodination, lysate preparation, immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and autoradiography were performed as described (10). Iodinated HLA-DR-like antigens were eluted from 16.23-SpA Sepharose (10) with 8 M urea (RT, 1 h), and the subunits were separated by SDS-PAGE (10%) under nonreducing conditions. The unfixed gel was dried and exposed for 4–6 h. Three bands of radioactivity were obtained, two of which (M_r 35,000 and 25,000) were shown by subsequent SDS-PAGE to correspond to the α (M_r 37,000) and β (M_r 31,000) subunits. The 35 and 25 kd bands were eluted from the gel in 0.01 M Tris-HCl, pH 8, containing 0.1% Nonidet P-40, 1 mM benzamidine HCl, 1 mM phenyl methyl sulfonyl fluoride (PMSF), and 1% bovine serum albumin (BSA) (7 h at 4°C). 70–89% of the radioactivity was recovered. To assess antibody binding, subunits in 50% fetal calf serum (FCS) were incubated with myeloma supernatant MPC 11-SpA Sepharose (1 h, 4°C, 10 μl packed gel) followed by 16.23-SpA Sepharose (2 h, 4°C, 10 μl packed gel). The bound radioactivity was measured and analyzed on a 10% SDS-PAGE under reducing conditions. The material in each lane was estimated by scanning the autoradiographs at 405 nm (ISCO gel scanner, model 1310, type 6 optical unit; ISCO, Instrumentation Specialties Co., Lincoln, NE).

Western Blotting and Detection of Antigen in Cell Lysates. Cell lysates (5×10^4 to 5×10^6 cells) containing 8 M urea or 1% SDS were heated for 30 min at 45°C or for 2 min at 100°C and electrophoresed under nonreducing conditions in 10% SDS-PAGE with or without 6 M urea. The separated proteins were electrophoretically transferred (13) to a nitrocellulose filter (Schleicher and Schüll, BA 0.45μ, Dassel, Federal Republic of Germany) for 1.5 h at 30 V using two carbon plates as electrodes and foam rubber pads soaked in transfer buffer (0.025 M Tris, 0.192 M glycine, pH 8.3, plus 20% methanol). The filter was incubated overnight in 0.1% gelatin in phosphate-buffered saline (pH 7.2) containing 0.1% NaN_3 and 0.05% Tween-20 (Fluka AG, Buchs, Switzerland) (PBS-Tween). The blocked filter was incubated for 1 h in 16.23 culture supernatant (plus 0.05% Tween-20), washed in PBS-Tween, incubated 1 h with [I^{125}]SpA in 10% FCS (10^6 cpm/ml; iodinated with chloramine T) (12), washed (PBS-Tween), and autoradiographs prepared.

Results

Antibody 16.23 Is Directed against an HLA-linked Antigen Closely Associated with HLA-D/DR3. Fresh PBL from the melanoma patient JuSo was analyzed using immunofluorescence and autoradiography for the presence of cells binding 16.23. The reactivity observed was restricted to the B cell-enriched fraction (15–28% of the Ig^+ cells), and these cells were shown by double marker studies to be non-E-rosetting, surface Ig-positive cells.

In an analysis of PBL from seven unrelated individuals selected from an HLA-typed panel, only two reacted with 16.23, suggesting that the antigen detected is polymorphic in the population. Subsequent analysis of four families established that the antigen defined by 16.23 segregates in tight linkage with HLA (Table I).

Because the tissue distribution suggested that antibody 16.23 might detect a DR antigen, 32 unrelated HLA-DR typed individuals (specificities 1–8) and 28 HTC (Dw specificities 1–9) were analyzed. A strong association between reactivity with 16.23 and the HLA-Dw/DR3 phenotype was found (Table II). Antibody 16.23 was positive with cells of the eight DR3 heterozygotes and the five homozygous Dw3 individuals examined. It also reacted with two of three Dw6 homozygous cells and with cells of three of five DRw6 heterozygotes. Reactivity with other specificities was not observed.

Biochemical Characterization of the 16.23 Antigen.   The iodinated surface polypeptides precipitated by antibody 16.23 from Mel JuSo, the autologous B cell (JuSo B), and the allogeneic B cell Raji (HLA-DR3, w6) are compared in Fig. 1. Two components of M_r 37,000 (α chain) and 31,000 (β chain) were obtained from all three cell lines. (Under nonreducing conditions, these subunits migrated with apparent 35,000 and 25,000 mol wt, respectively; Fig. 3). The 44,000 mol wt component observed in the JuSo B precipitate (lane c) might be actin, which frequently contaminates B lympho-

TABLE I

Segregation of 16.23 Epitope with HLA

Family	Father		Mother		Children						
					1	2	3	4	5	6	7
Lü	a	A3,B18,Cw5,Dw3/DR3*	c	A23,B27,Cw3,Dw1/DR1	Ma	Co	Ch	Wo	Ro	Rl	
	b	A2,B13,Cw6,—/—	d	A2, B5, —,Dw5/DR5	a/d	a/d	b/d	a/c	b/c	b/c	
Le	a	A2,B27,Cw1,Dw2/DR2	c	Aw24,Bw35,Cw4,Dw3/DR3	Ka	Vi	Ma	Kt	Jo	Ti	Ja
	b	A2,B13,—,—/—	d	A3, B7,—,Dw2/DR2	b/c	a/d	a/d	b/d	a/d	b/c	a/d
Wö	a	A2,Bw35,Cw4,—/DR5	c	A1,B8,—,—/DR1,Glo2	Sa	Lo					
	b	—, B7,w6,—,—/DR2	d	A1,B8,—,—/DR3,Glo1	a/d	a/c					
Ke	a	A2,B12,—,—/DR4			Co	Ei	Ca	Da	Dv		
	b	A11,Bw35,—,—/DR4	d	A2,Bw18,—, Dw3/DR3	a/c	a/c	b/c	b/c	b/c		

* Boxes indicate reactivity with 16.23.
Linkage was determined using the method of lod scores (θ_{max} = 0.0, lod score = 3.607; odds, 4,000:1; $z_{Lü}$ = 1.506 z_{Le} = 1.80, and $z_{Wö}$ = 0.301) (14).

TABLE II

Correlation between 16.23 Epitope and HLA-Dw/DR3

Cells	++*	+—	—+	——	N‡	χ^2	P value
Heterozygotes	8	3	0	21	32	16.67	P < 0.005
Homozygotes	5	2	0	21	28	13.72	P < 0.005

* 16.23 positive, Dw/DR3 positive. All individuals in category +— were typed as HLA-Dw/DRw6.
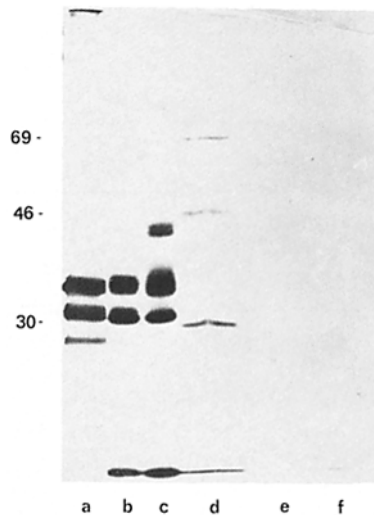‡ Number of individuals tested.

Fig. 1. Immunoprecipitates with antibody 16.23. Cells were labeled by surface iodination and immunoprecipitated with MPC 11 or 16.23, and the antigens were analyzed by 10% SDS-PAGE as described (10). Antibody 16.23 immunoprecipitates from Mel JuSo (a), Raji (b), and JuSo B cells (c). MPC 11 immunoprecipitates from Mel JuSo (e) and JuSo B cells (f). Lane d gives molecular weight markers: bovine serum albumin (69,000), ovalbumin (46,000), and carbonic anhydrase (30,000). The numbers on the ordinate indicate the migration of the marker proteins with molecular weight in kilodaltons.

cyte immunoprecipitates (5); it was not observed in nonreducing conditions nor in preclearing precipitates (lanes e and f). In addition to these DR-like α and β subunits, 16.23 consistently precipitated a smaller 28,000-mol wt component from Mel JuSo cells (lane a).

Subunit Localization of the HLA-DR3-associated Determinant. To localize the epitope defined by this antibody, iodinated antigen was isolated from JuSo B cells, and the subunits were separated and tested directly for reactivity with 16.23. Antibody 16.23 and control Ig MPC 11 bound equivalent amounts of the α subunit (Fig. 2). In contrast, 16.23 bound >50% of the β chain radioactivity, whereas MPC11 binding remained at background levels. This bound material was eluted and analyzed in SDS-PAGE (Fig. 2). Labeled polypeptides were observed only in the 16.23-β chain eluate, and this material migrated in the position of the smaller molecular weight β subunit.

In a second approach, the electrophoretically separated unlabeled cell lysate proteins from JuSo (DR2, 3) and Rewi (DR1) were transferred to nitrocellulose filters. These filters were then incubated with 16.23, MPC 11, or rabbit antiserum against DR β chains, followed by $[I^{125}]$SpA. Location of the α-β complex and the separated α and β subunits was determined by running immunoprecipitated ^{125}I-labeled antigen on the same gel. In lysates containing 1% SDS and heated to 45°C, the majority of the 16.23-binding activity was found in a position corresponding to the undissociated α-β complex, although substantial binding was also observed to a band in the position of the β chain (Fig. 3, lane c). When these lysates were heated to 100°C (separating the α and β chains completely, lane b), binding of 16.23 was still obtained but restricted to the lower molecular weight band (lane d). The rabbit antiserum bound to a band in a similar position in both JuSo and Rewi cells (lanes e and g), although
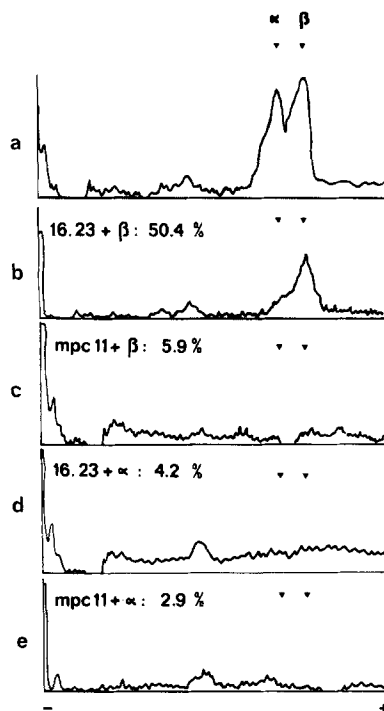
Fig. 2.   Direct binding of 16.23 to isolated subunits of DR-like antigen. Iodinated DR-like subunits precipitated by 16.23 from JuSo B cells were isolated and incubated with 16.23 or MPC 11, as described in Materials and Methods. The eluted material was separated on SDS-PAGE (10%, reduced samples), and the autoradiographs were analyzed by densitometry. The optical density (405 nm, OD_{max} = 1) is presented on the ordinate, and the abscissa indicates the migration distance from the top (−) of the gel. The percent of radioactivity bound by the antibody is indicated for each sample: a, α, and β subunits precipitated by 16.23 from JuSo B cells; b, material eluted from 16.23-β subunit precipitate (input 1158 cpm); c, material eluted from MPC 11-β subunit precipitate (input 2048 cpm); d, material eluted from 16.23-α subunit precipitate (input 1120 cpm); e, material eluted from MPC-11-α subunit preciptate (input, 1,800, cpm).

no binding with 16.23 was observed in Rewi lysates (lane f). In 8 M urea containing JuSo lysates heated to 45°C and separated in a urea containing gel, all of the binding activity was found in a single band migrating in the position of the β subunit (lane h).

Discussion

The unique allotypic specificity of antibody 16.23 should help to resolve several problems that have not yet been clarified in studies using alloantisera or the existing monoclonal antibodies. Although two-dimensional electrophoresis (15, 16) and tryptic digest peptide mapping (17, 18) have demonstrated far more heterogeneity in the light (β) subunit than in the heavy (α) subunit, the actual location of the HLA-DR allotypic determinants has remained unclear. Analysis of mouse-human somatic cell hybrids indicated that the allotypic determinants were associated with the large molecular weight (α) subunit (19). In two independent studies, incubation of alloantisera with isolated subunits resulted in binding exclusively to the α chains (20) or to the β chains (21). More recently, immunoprecipitation with alloantisera and absorbed
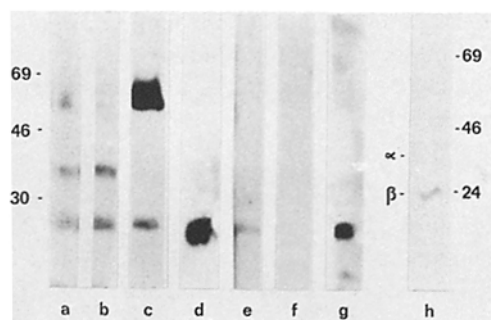
Fig. 3.   Detection of 16.23-binding material in unlabeled cell lysates. Proteins were separated on
SDS-PAGE (10%, nonreducing conditions), transferred to nitrocellulose filters, and incubated with
antibody and [^{125}I]SpA as described in Materials and Methods. Samples in lanes a and b are 16.23-
precipitated material from surface iodinated JuSo B cells heated to 45°C for 30 min or to 100°C for
2 min, respectively. Lanes c–h are nitrocellulose filters. Lanes c, d, and e contain JuSo B lysate with
1% SDS: c, lysate heated to 45°C for 30 min, filter incubated with 16.23; d, lysate heated to 100°C
for 2 min, filter incubated with 16.23; e, as in d, but filter incubated with rabbit antiserum to DR
β chains (1:500). Lanes f and g contain Rewi cell (Dw/DR1) lysate with 1% SDS: f, lysate heated
to 45°C for 30 min, filter incubated with 16.23; g, lysate heated to 100°C for 2 min, filter incubated
with rabbit antiserum to DR β chains. Lane h contains JuSo B lysate with 8 M urea, heated to
45°C for 30 min, filter incubated with 16.23. The location of the separated α and β subunits in the
urea gel are noted, and BSA (69,000), ovalbumin (46,000), and trypsinogen (24,000) were used as
molecular weight markers; the molecular weights are indicated in kilodaltons on the ordinate.
Molecular weight markers on the SDS gel are as in Fig. 1.

xenoantisera provided indirect evidence that the DR3 determinant(s) were present on
the heavy (α) chain (22). Whereas interpretation of these results is complicated by the
use of polyclonal antisera, discordant observations could be explained by the existence
of several alloantigen-associated determinants, some of which are present on the α
and others on the β subunits but that are inherited together because of linkage
disequilibrium and are detected to varying degrees by different alloantisera. Using
two independent approaches, antibody 16.23 was shown to bind to the smaller 31,000
mol wt β subunit, clearly establishing the control of this subunit by HLA and
demonstrating that it carries an epitope of the DR3 phenotype. The relationship of
the 16.23-bearing subunit to the β subunits bearing monomorphic and supertypic
determinants defined by other monoclonal antibodies (3, 6) remains to be investigated.

Monoclonal antibodies directed against individual allotypes will be useful in
dissecting the serological cross-reactivities that have been observed between different
HLA specificities. In an analysis of more than 120 individuals from the German
population, antibody 16.23 reacted with all HLA-D/DR3 and with a fraction of
HLA-Dw/DRw6 individuals (additional typing data from V. Lenhard, Institute of
Immunology, University of Heidelberg, Heidelberg, Germany). The lack of reactivity
with DRw5, 7, and 8 individuals shows that this antibody does not correlate with the
broad MB and MT specificities, MB2 or MT2 (23), and suggests that the defined
epitope is shared between DR3 and at least a portion of DRw6 cells. Operationally
monospecific alloantisera for DRw6 are not available (24), and assignment of this
specificity is presently based on reactivity with three groups of multispecific antisera,
one of which also includes activity against HLA-DR3 (25). An analysis of the
molecules precipitated by antibody 16.23 from cells of these two phenotypes should
clarify the relationship between HLA-DR3 and DRw6.

Finally, antibody 16.23 is an important tool for examining the expression of a clearly defined HLA-DR allospecificity on different cell types, particularly on macrophages, T and B lymphocytes, and tumor cells, as well as for studying its functional involvement in cellular interactions.

Summary

A murine monoclonal antibody directed against a human B cell surface antigen with the characteristics of HLA-DR is described. The antigen detected is tightly linked to HLA and is correlated with the alloantigen HLA-Dw/DR3. Reactivity with a fraction of Dw/DRw6 cells is also observed. The determinant recognized by this antibody has been shown to be present on the smaller molecular weight β subunit of the HLA-DR antigen.

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