

THE COMPLEXITY OF HLA-DS MOLECULES

A Homozygous Cell Line Expresses Multiple HLA-DS Alpha Chains

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The human Ia antigens are encoded within the HLA complex on chromosome six and are borne on molecules composed of two noncovalently associated glycoproteins of ~34,000 daltons (α chains) and ~28,000 daltons (β chains). Ia antigens are important in the effective collaboration between immunocompetent cells in the generation of immune responses; they have also been associated with putative immune response genes and the predisposition to the development of certain diseases. Three types of human Ia molecules have been identified and biochemically characterized: DR, DS (or DC), and SB. DR molecules are defined by their N-terminal amino acid sequence homology to murine I-E molecules (1-3). HLA-DR homozygous cells appear to express a single DR α chain that associates with either of two distinct DR β chains to form two heterodimeric DR molecules (2, 3). SB molecules appear to be structurally different from the corresponding DR molecules, although SB α chains, like DR α chains, are homologous to murine I-E α chains (4). DS (or DC) molecules are defined by their N-terminal amino acid sequence homology to murine I-A molecules (1, 5). Although previous studies indicated that HLA-DR homozygous cells express a single DS molecule, we have recently shown that an HLA-DR5 homozygous cell line expresses at least two distinct DS molecules (6). Therefore, existing evidence at the protein level indicates that HLA-DR homozygous cell lines express at least five Ia molecules: two DR molecules, two DS molecules, and one SB molecule.

Serologic testing with anti-Ia allosera has identified two major groups of serologically defined Ia alloantigens: the HLA-DR antigens and the non-DR antigens including the MB (7, 8), MT (9), LB (10), Te (11), DC (12, 13), and BR (14-16) alloantigen systems. The serologically defined non-DR Ia alloantigen systems share the common feature that each specificity is found in association with two or more HLA-DR antigens. The determination of the molecular relationships of these serologically defined Ia alloantigens to the biochemically defined Ia molecules (DR, DC, SB) has been the focus of considerable interest. In general, the biochemically defined DR molecules appear to bear the serolog-

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ically defined HLA-DR allodeterminants. Several of the serologically defined non-DR Ia alloantigens (MB1, DC1, MT1, LB12 [5, 17–19]; MB3 [6, 13, 19, 20]; and MT4 [6]) appear to reside primarily on DS molecules. The MT2 determinant appears to reside on both DR and DS molecules from the same cell (6). The location of the MT3 determinant is variable: on DR4 cells, the MT3 determinant resides on DS molecules, while on DR7 cells, the MT3 determinant is found on DR molecules (18, 19).

The MB1, MT1, DC1, and LB12 allospecificities are expressed in linkage disequilibrium with DR1, DR2, and DRw6. It is thought that the MB1, MT1, DC1, and LB12 specificities are identical (17); therefore, this group of presumably identical specificities will be referred to as MB1. Two monoclonal antibodies, Genox 3.53 (21) and BT3.4 (22), have been described that define specificities with the same distribution (DR1, DR2, and DRw6) as the MB1 allodeterminant. The determinants defined by these monoclonal antibodies will be referred to as MB1-like to denote their possible differences from the MB1 allodeterminant. A previous report (17) indicated that both the MB1 allodeterminant and the MB1-like determinant defined by Genox 3.53 reside on the single DS molecule identified in those studies. In the present study, the molecular specificities of two anti-MB1-like monoclonal antibodies and two anti-MB1 allosera have been examined in light of the recent evidence that HLA-DR homozygous cell lines may express at least two DS molecules (6).

The data presented here indicate that an HLA-DRw6 homozygous cell line expresses at least two distinct DS α chains and two distinct DS β chains that associate to form at least two heterodimeric DS molecules. In addition, these studies document for the first time that anti-Ia reagents which are specific for the MB1 or MB1-like determinants in population studies do not all recognize the same Ia molecules in immunochemical studies.

Materials and Methods

Cells. The B lymphoblastoid cell line ELD, HLA-A2, A2; B40, B40; DRw6, DRw6; Dw6, Dw6; MB1, MT1, MT2, was obtained from Dr. John Hansen (Fred Hutchinson Cancer Research Center, Seattle, Washington) (23).

Anti-Ia Reagents. SG157 is an anti-Ia monoclonal antibody that is specific for DR molecules; the α and β chains of Ia molecules isolated with SG157 have N-terminal amino acid sequences that are homologous to the α and β chains, respectively, of murine I-E molecules (1). RbO3 is a rabbit antiserum specific for human Ia molecules (DS) that have N-terminal amino acid sequence homology to murine I-A molecules (1). RbO3 isolated no DR molecules from multiple cell lines in previous two-dimensional (2-D)¹ gel studies (1, 6, 18, 19). BT3.4 (22) and Genox 3.53 (21) are anti-Ia monoclonal antibodies that bind only to DR1-, DR2-, and DRw6-positive cells. Therefore, BT3.4 and Genox 3.53 appear to define antigenic determinants similar to the MB1/MT1/LB12/DC1 determinants defined by allosera. BT3.4 was provided by Dr. Giorgio Corte (Universita di Genova, Genova, Italy). L243 and L227 are anti-Ia monoclonal antibodies that recognize nonpolymorphic determinants on DR molecules (24); they have been shown to isolate Ia α and β chains having N-terminal amino acid sequence homology to the α and β chains, respectively, of murine I-E molecules (2, 3). The L243, L227, and Genox 3.53 hybridomas were obtained from the American Type Culture Collection, Rockville, MD. Baron and

¹ Abbreviations used in this paper: 2-D, two-dimensional; SaCl, protein A-bearing *Staphylococcus aureus*, Cowan I strain; IEF, isoelectric focusing; NP-40, Nonidet P-40; PBS, phosphate-buffered saline.

Dobbe are anti-MB1 allosera from the Blood Center of Southeastern Wisconsin, Inc. Dobbe was serum 119 in the Eighth International Histocompatibility Workshop (25). The donor of serum Baron types as HLA-A1, Aw31; B8, Bw35; DR3, DR4; MB2, MB3; MT2, MT3; the immunizer types as HLA-Aw26, Aw30; B7, Bw51; DR2, DR4; MB1, MB3; MT1, MT3. The donor of serum Dobbe types as HLA-A2, A3; Bw51, Bw62; DR4, DR5; MB3; MT2, MT3; the immunizing haplotype is HLA-A11; Bw35; DR1; MB1; MT1.

Preparation of Radiolabeled Antigens. Cells of the B lymphoblastoid cell line ELD were resuspended at 3×10^6 /ml in RPMI 1640 lacking leucine and methionine and supplemented with 10% dialyzed fetal bovine serum. [^3H]leucine (130–190 Ci/mM; Amersham Corp., Arlington Heights, IL) and [^{35}S]methionine (>600 Ci/mM; Amersham Corp.) were each added to a concentration of 400 $\mu\text{Ci/ml}$ and the cultures were incubated for 8 h at 37°C in a humidified 7% CO_2 atmosphere. The cells were harvested and washed three times with Hanks' balanced salt solution before they were solubilized for 30 min at 4°C in phosphate-buffered saline (PBS) containing 0.5% of the non-ionic detergent Nonidet P-40 (NP-40) (Particle Data, Inc., Elmhurst, IL), 200 $\mu\text{g/ml}$ phenylmethylsulfonyl fluoride (PMSF), 50 $\mu\text{g/ml}$ L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK), and 50 $\mu\text{g/ml}$ N- α -*p*-tosyl-L-lysine chloromethyl ketone HCl (TLCK) (Sigma Chemical Co., St. Louis, MO). Insoluble material was removed by ultracentrifugation at 100,000 *g* for 60 min. Before immunoprecipitation with the specific anti-Ia reagents, the radiolabeled antigen preparations were precleared with normal human serum, rabbit anti-mouse immunoglobulin, and protein A-bearing *Staphylococcus aureus*, Cowan I strain (SaCI) (Pansorbin; Calbiochem-Behring Corp., San Diego CA) to remove radiolabeled immunoglobulin.

Immunoprecipitation. Aliquots of the antigen preparations were incubated overnight at 4°C with the various anti-Ia reagents. The monoclonal antibodies BT3.4, Genox 3.53, L243, and L227 do not bind to SaCI. Therefore, in immunoprecipitations with these reagents, rabbit anti-mouse immunoglobulin was added to the antigen-antibody mixture before the SaCI immunoabsorbent was added. After the immunoprecipitations, the SaCI pellets were washed three times with PBS containing 0.25% NP-40 before elution according to the method of O'Farrell (26). For the sequential immunoprecipitation experiments, an aliquot of antigen preparation was immunoprecipitated three times with the preclearing anti-Ia reagent. This precleared aliquot was then divided into two equal portions which were immunoprecipitated with the preclearing reagent (negative control) and the test reagent, respectively.

2-D Gel Electrophoresis. 2-D gel electrophoresis was performed according to the method of O'Farrell (26) with some modifications. The first dimension separation by isoelectric focusing (IEF) was performed in 5×125 -mm cylindrical gels containing a mixture of 0.6% (wt/vol, pH 3.5–10), 0.4% (pH 8–9.5), and 1% (pH 5–8) Ampholine (LKB Instruments, Rockville, MD). The IEF gels were electrophoresed at 600 V for 16 h and were then imbedded in agarose on the top of a slab gel with a 3-cm 4% stacking segment and a 13-cm sodium dodecyl sulfate-12.5% polyacrylamide separating segment prepared according to the method of Laemmli (27). The slab gels were fixed in a solution containing 10% (vol/vol) glacial acetic acid and 40% (vol/vol) methanol before fluorography was performed using EN 3 HANCE (New England Nuclear, Boston, MA). The dried gels were exposed to Kodak X-omat AR5 film at -70°C .

Results

A biosynthetically radiolabeled antigen preparation of the DRw6 homozygous cell line, ELD, was immunoprecipitated with several anti-Ia reagents; the precipitated material was analyzed by 2-D gels (Fig. 1). The various Ia α and β chains identified in these studies are indicated in Fig. 1 and are shown schematically in Fig. 7. The β chain pattern of Ia molecules isolated by the DR-specific monoclonal antibody SG157 consists of four major spots and two less intense spots. The β chain pattern of Ia molecules isolated by the anti-Ia monoclonal antibody L227 is identical to the acidic portion of the SG157 β pattern. This is not unexpected

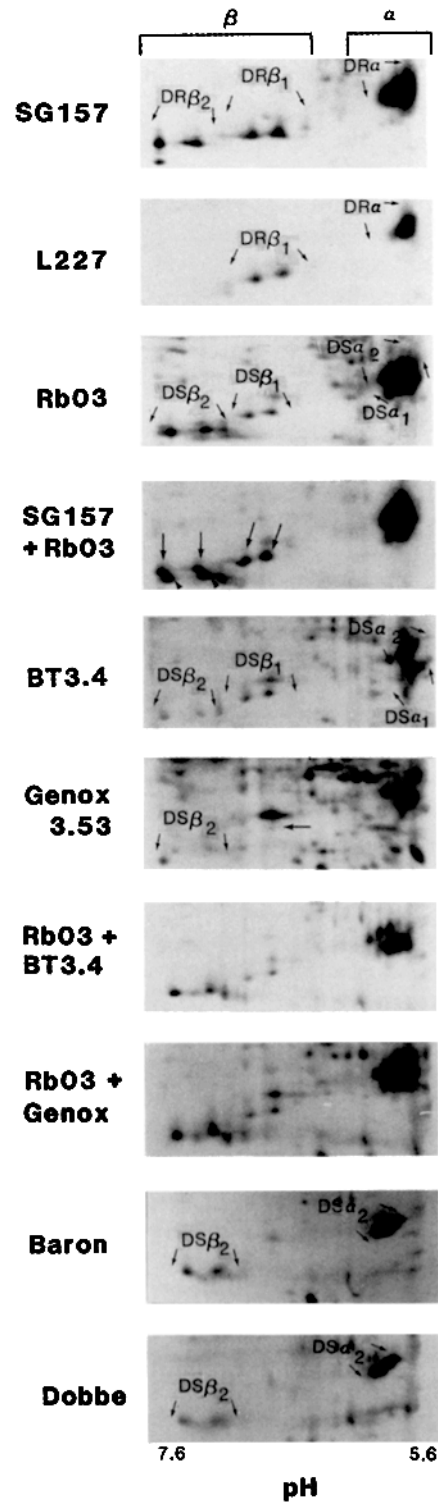


FIGURE 1. Fluorographs of 2-D gels of Ia molecules precipitated from a radiolabeled antigen preparation of the DRw6 homozygous cell line, ELD, by the anti-Ia reagents listed at the left. The α and β chain portions of the gels are designated by brackets at the top. For the SG157 + Rb03, Rb03 + BT3.4, and Rb03 + Genox gels, individual precipitates were mixed and analyzed together by IEF. In the SG157 + Rb03 gel, the two vertical arrows mark DR β_2 , the two diagonal arrows mark the coincident spots of DR β_1 and DS β_1 , and the two lower arrowheads mark DS β_2 . In the Genox 3.53 gel, the horizontal arrow marks the faint spots of DS β_1 .

since SG157 and L227 have both been shown to isolate DR molecules (1-3). However, the L227 pattern lacks the two major basic spots in the SG157 β pattern. These data suggest that the SG157 β pattern may be produced by at least two distinct DR β chains. Additional studies (discussed below) have shown that the SG157 β pattern is produced by at least two distinct DR β chains, which will be referred to as DR β_1 (isolated by L227 and SG157) and DR β_2 (isolated by SG157 but not L227). The DS β chain pattern of RbO3 consists of four major spots with isoelectric points similar to the major spots in the SG157 β pattern. In addition, the RbO3 pattern also includes two less intense spots. Based on additional studies discussed below, the RbO3 β pattern is produced by at least two distinct DS β chains, DS β_1 and DS β_2 .

As expected, the DR and DS α chain patterns in the SG157 and RbO3 gels, respectively, also differ. Because some of the α chain patterns shown in Fig. 1 are more intense than the β chain patterns, shorter exposures of the α chain portions of some gels are shown in Fig. 4 for clarity. The SG157 α pattern consists of three closely spaced spots with similar molecular weights; this pattern is produced by the single DR α chain (DR α) expressed by this cell line. The L227 α pattern is very similar to the SG157 α pattern. Based on additional studies discussed below, the more complex RbO3 α pattern appears to consist of at least two distinct DS α chains as indicated in Fig. 1: a lower molecular weight α chain consisting of four spots (DS α_1) and a slightly higher molecular weight α chain also consisting of four spots (DS α_2).

To more definitively compare the DR and DS Ia molecules, individual SG157 and RbO3 immunoprecipitates were mixed and analyzed together in the same IEF gel. This method eliminates the variables of stretching of the individual IEF gels and differences in separation in the second dimension gels. The SG157 + RbO3 gel demonstrates that the spots of DS β_2 isolated by RbO3 are clearly distinct from the spots of DR β_2 isolated by SG157. These DS β_2 spots (lower arrowheads) are slightly more acidic and are lower in molecular weight than the DR β_2 spots (vertical arrows). By contrast, the two most acidic spots of the RbO3 β pattern appear to co-migrate with the two intense acidic spots in the SG157 pattern (diagonal arrows). A SG157 + BT3.4 mixing gel (data not shown) was identical to the SG157 + RbO3 gel. Because the Ia molecules isolated by SG157 and RbO3 have been shown to have distinct N-terminal amino acid sequences (1), these coincident spots presumably represent glycosylated forms of distinct proteins that have similar physical characteristics when analyzed by this 2-D gel method. The relationship of these coincident spots of Ia molecules isolated by SG157, RbO3, and BT3.4 has been further investigated and will be discussed below. The α chain pattern in the SG157 + RbO3 gel demonstrates that DR α and the DS α chains have very similar isoelectric points and molecular weights when analyzed by this 2-D gel technique.

Two anti-MB1-like monoclonal antibodies, BT3.4 and Genox 3.53, were also used to isolate Ia molecules from the ELD antigen preparation. The BT3.4 pattern consists of five major spots that are similar to the RbO3 pattern. However, compared with the RbO3 gel, the two most acidic, higher molecular weight spots in the BT3.4 pattern are more intense than the basic spots. This differential intensity of the acidic β spots compared with the basic β spots suggests that the

BT3.4 β pattern is produced by two distinct β chains. The Genox 3.53 β pattern also consists of five major spots and is very similar to the RbO3 and BT3.4 β patterns; however, in contrast to the BT3.4 pattern, the two most acidic, higher molecular weight spots in the Genox 3.53 gel are relatively less intense than the two major basic spots. These reciprocal quantitative differences in the intensities of these two pairs of β chain spots in the BT3.4 and Genox 3.53 gels suggest that the two most acidic, higher molecular weight spots isolated by BT3.4, Genox 3.53, and RbO3 belong to one distinct DS β chain ($DS\beta_1$), while the two major basic spots isolated by BT3.4, Genox 3.53, and RbO3 belong to a second distinct DS β chain ($DS\beta_2$). Therefore, as indicated in Fig. 1, the BT3.4 β pattern is formed by $DS\beta_1$ and $DS\beta_2$. The Genox 3.53 β pattern contains $DS\beta_2$ and a lesser amount of $DS\beta_1$ (horizontal arrow).

To more definitively compare the Ia molecules isolated by RbO3, BT3.4, and Genox 3.53, immunoprecipitates with RbO3 and BT3.4 were mixed and immunoprecipitates with RbO3 and Genox 3.53 were mixed and the mixtures analyzed by 2-D gels. The RbO3 + BT3.4 β pattern demonstrates co-migration of the major β chain spots isolated by RbO3 and BT3.4. In addition to the five major DS β chain spots, two other spots are visible in the RbO3 + BT3.4 gel that are only faintly visible in the individual RbO3 and BT3.4 gels. This presumably results from the additive effect of minor components in both individual precipitates. The RbO3 + Genox 3.53 β pattern also demonstrates co-migration of the major β spots isolated by RbO3 and Genox 3.53 and is qualitatively very similar to the RbO3 + BT3.4 gel. As with the RbO3 + BT3.4 gel, two spots that are only faintly visible in the individual gels are better seen in the RbO3 + Genox 3.53 gel. In a BT3.4 + Genox 3.53 mixing experiment (data not shown), the DS β spots are also coincident. Taken together, these mixing experiments document that the DS β spots isolated by RbO3, BT3.4, and Genox 3.53 are coincident and that these reagents isolated Ia molecules containing both major DS β chains.

Two anti-MB1 allosera, Baron and Dobbe, were also used to isolate Ia molecules from the ELD antigen preparation. Both the Baron and Dobbe β chain patterns contain two intense basic spots and two less intense basic spots. The two prominent basic spots in the Baron and Dobbe gels are very similar to the spots of $DS\beta_2$ in the RbO3, BT3.4, and Genox 3.53 gels. The Baron and Dobbe α chain patterns are identical and correspond to the $DS\alpha_2$ chain. Therefore, two anti-MB1 allosera isolated the molecule composed of $DS\alpha_2:DS\beta_2$. The isolation of $DS\beta_2$ in the absence of $DS\beta_1$ provides additional evidence that the RbO3, BT3.4, and Genox 3.53 β patterns are composed of two distinct DS β chains. These data with Baron and Dobbe also establish that the molecular specificities of MB1 allosera differ from the molecular specificities of two MB1-like monoclonal antibodies.

To formally demonstrate that the β chain isolated by Baron and Dobbe is the same as $DS\beta_2$ isolated by RbO3, BT3.4, and Genox 3.53, additional mixing experiments were performed with another ELD antigen preparation. The β chain portions of these gels are shown in Fig. 2. The individual Baron and RbO3 β chain patterns are similar to those in Fig. 1; the Baron β pattern consists of $DS\beta_2$ only, while the RbO3 pattern includes $DS\beta_1$ and $DS\beta_2$. The Baron + RbO3

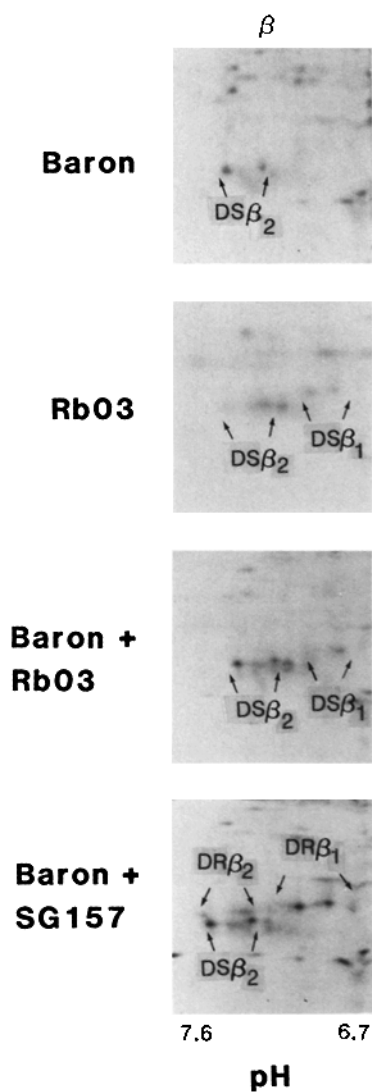


FIGURE 2. Fluorographs of the β chain portions of 2-D gels of Ia molecules isolated by the anti-Ia reagents listed at the left. For the Baron + RbO3 and Baron + SG157 gels, individual precipitates were mixed and analyzed together by IEF.

gel demonstrates that the basic spots in the Baron pattern co-migrate with the spots of $DS\beta_2$ isolated by RbO3. Therefore, Baron and Dobbe clearly isolate Ia molecules containing $DS\beta_2$. The Baron + SG157 gel demonstrates that the spots of $DS\beta_2$ isolated by Baron are clearly distinct from the spots of $DR\beta_2$ isolated by SG157. The relationship between the $DR\beta_2$ and $DS\beta_2$ in the Baron + SG157 gel is identical to the relationship between the same chains in the SG157 + RbO3 gel in Fig. 1.

The β pattern in the Genox 3.53 gel in Fig. 1 suggests that the spots of $DS\beta_1$ are relatively less intense than the spots of $DS\beta_2$. Additional studies demonstrate

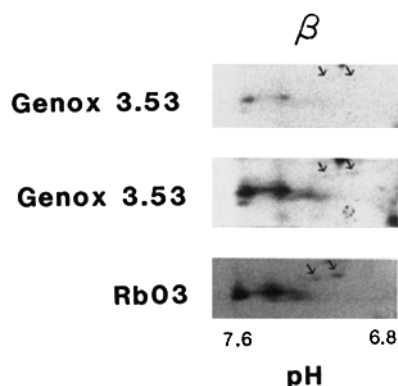


FIGURE 3. Fluorographs of the β chain portions of 2-D gels of Ia molecules precipitated by Genox 3.53 and RbO3 from an ELD antigen preparation. The fluorograph of the Genox 3.53 gel in the second panel was exposed six times longer than the fluorograph in the top panel. The arrows in each panel indicate the position of the spots of DS β_1 .

this point more clearly. The Genox 3.53 β pattern in the top panel of Fig. 3 consists primarily of DS β_2 and a very faint amount of DS β_1 (arrows). The middle panel shows the same 2-D gel, in which the fluorograph has been exposed six times longer than the fluorograph in the top panel; the DS β_1 spots (arrows) are now more easily seen, but they are still much less intense than the DS β_2 spots. The RbO3 gel from the same experiment is shown in the bottom panel to document that DS β_1 (arrows) is adequately radiolabeled in the antigen preparation used in these studies. These data provide additional evidence that DS β_1 and DS β_2 are distinct β chains. Moreover, the finding that DS β_1 may be only faintly visible in some Genox 3.53 gels, while DS β_2 is always clearly visible, may provide an explanation for the previous observation that Genox 3.53 immunoprecipitates from another DRw6 homozygous cell line contained only a single β chain (28) analogous to DS β_2 .

Because the α chain portions of some of the fluorographs shown in Fig. 1 are slightly overexposed and do not permit optimal examination of the α chain patterns, additional exposures of the α chain portion of selected gels from Fig. 1 are shown in Fig. 4. Taken together, these data indicate that the ELD cell line expresses at least two distinct DS α chains. The α chain data are also presented schematically in Fig. 7. The Dobbe α chain pattern consists of four spots that are arranged in a stair-step pattern of increasing molecular weights from the most basic to the most acidic. This pattern is designated DS α_2 and is also found in the Baron gel. There is no evidence of DS α_1 in the Baron or Dobbe gels. The BT3.4 α pattern consists of DS α_2 and another lower molecular weight α chain referred to as DS α_1 . There is some minor electrophoretic distortion of the α chains in the individual BT3.4 gel; a better resolved pattern is shown in the RbO3 + BT3.4 gel. DS α_1 consists of four spots that are also arranged in a stair-step pattern of increasing molecular weights from the most basic to the most acidic. Each spot of DS α_1 has a slightly lower molecular weight than the proximate spot of DS α_2 . Although DS α_1 is not isolated alone by any of the DS reagents, the isolation of DS α_2 without DS α_1 by Baron and Dobbe strongly indicates that DS α_1 is a distinct entity. The Genox 3.53 α pattern consists of three spots and is clearly distinct

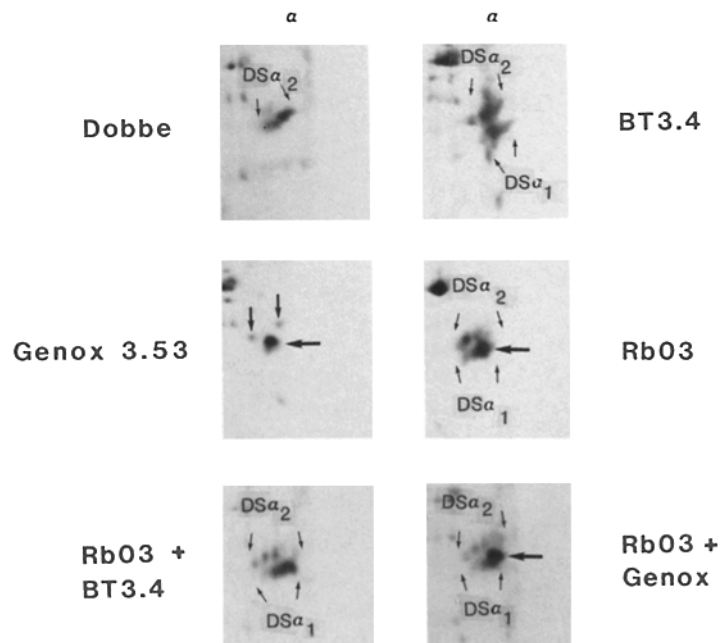


FIGURE 4. Fluorographs of the α chain portions of 2-D gels of Ia molecules isolated by the anti-Ia reagents listed beside each gel. These are shorter exposures of the same gels shown in Fig. 1.

from the DS α patterns in the Dobbe, Baron, BT3.4, and RbO3 gels. The major, lower molecular weight spot (horizontal arrow) in the Genox 3.53 α pattern comigrates with the more acidic spots of the DS α_1 pattern in the RbO3 + Genox gel. The Genox 3.53 α pattern lacks the more basic spots of DS α_1 that are seen in the BT3.4 and RbO3 gels. The two less intense, higher molecular weight spots (vertical arrows) in the Genox 3.53 α pattern are closely related to two of the four spots of DS α_2 ; however, the other spots of DS α_2 are not present in the Genox 3.53 gel. Therefore, the three spots in the Genox 3.53 α pattern may be biosynthetic precursors of DS α_1 and/or DS α_2 or they may represent one or more additional distinct DS α chains. The complex RbO3 α pattern includes DS α_1 and DS α_2 . In addition, the RbO3 α pattern contains a spot (horizontal arrow) analogous to the major spot in the Genox α pattern. The RbO3 + BT3.4 α pattern clearly demonstrates the relationship between DS α_1 and DS α_2 . The RbO3 + Genox 3.53 α pattern shows that the α chain spots isolated by Genox 3.53 co-migrate with the spots isolated by RbO3. The spot indicated by the horizontal arrow in the RbO3 + Genox gel corresponds to the major spot in the individual Genox 3.53 gel. Therefore, at least two distinct DS α chains have been isolated from this DRw6 homozygous cell line.

To further define the DR β chains, a sequential immunoprecipitation experiment was done; the β chain portions of these 2-D gels are shown in Fig. 5. Another ELD antigen preparation was precipitated with the DR-specific reagents L227 and L243. The L243 β chain pattern is identical to the pattern of DR β chains isolated by SG157. The L243 α chain pattern (data not shown) is identical

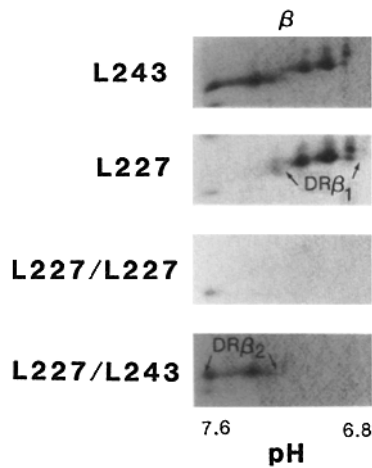


FIGURE 5. Fluorographs of the β chain portions of 2-D gels of Ia molecules precipitated by the anti-DR reagents, L243 and L227, from an ELD antigen preparation. The first two panels show β chains of Ia molecules isolated by direct precipitation with L243 and L227. The two lower panels show a sequential immunoprecipitation experiment in which an aliquot of the antigen preparation was first depleted of L227-reactive material and then divided into two aliquots before immunoprecipitation with L227 (L227/L227) or L243 (L227/L243), respectively.

to the SG157 α chain pattern in Fig. 1. As before, the L227 β pattern consists only of four acidic spots ($DR\beta_1$) that are also present in the SG157 and L243 patterns. An aliquot of the antigen preparation was depleted of L227-reactive material and was then divided into two aliquots that were immunoprecipitated with L227 and L243, respectively. Immunoprecipitation of the antigen preparation with L227 with removal of L227-reactive material (L227/L227) isolated no Ia molecules. This result documents that all of the L227-reactive material had been removed in the initial precipitations. Immunoprecipitation of the L227-depleted antigen preparation with L243 (L227/L243) isolated a β chain pattern that corresponds to the basic portion of the L243 and SG157 β patterns ($DR\beta_2$). These results document that the ELD cell line expresses at least two DR β chains, designated $DR\beta_1$ and $DR\beta_2$.

The SG157 + RbO3 gel (Fig. 1) and a SG157 + BT3.4 gel (data not shown) indicate that the spots of $DS\beta_1$ isolated by RbO3, BT3.4, and Genox 3.53 are coincident with the spots of $DR\beta_1$ isolated by SG157, L243, and L227. As discussed previously, these coincident spots are presumably components of distinct glycoproteins that have the same physical characteristics in the 2-D gel system. To more clearly define the relationships of these coincident spots, a sequential immunoprecipitation experiment was performed with SG157 and BT3.4; the β chain portions of these 2-D gels are shown in Fig. 6. The β chain patterns in the individual SG157 and BT3.4 gels are the same as in Fig. 1. The SG157 β pattern includes $DR\beta_1$ and $DR\beta_2$, while the BT3.4 β pattern includes $DS\beta_1$ and $DS\beta_2$. Immunoprecipitation of the antigen preparation with SG157 after removal of SG157-reactive material (SG157/SG157) isolated no Ia molecules. This result documents that all of the SG157-reactive material has been removed by the initial precipitation. Immunoprecipitation of the SG157-depleted

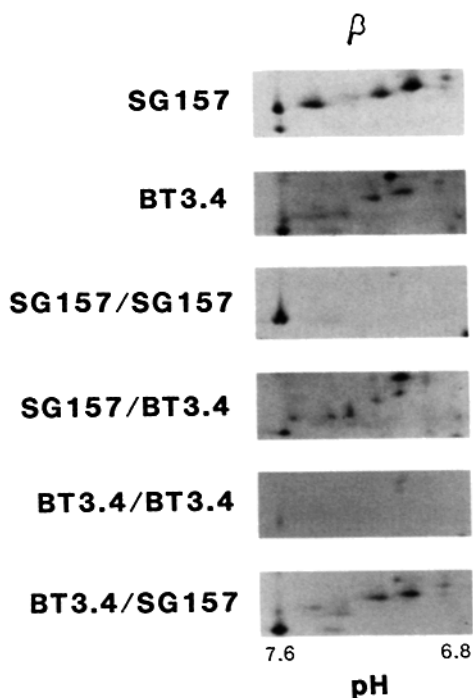


FIGURE 6. Fluorographs of the β chain portions of 2-D gels of Ia molecules precipitated by SG157 and BT3.4 from an ELD antigen preparation. The first two panels show the β chains of Ia molecules isolated by SG157 and BT3.4. The four lower panels show sequential immunoprecipitation experiments in which aliquots of the antigen preparation were first depleted of SG157-reactive material or BT3.4-reactive material and then divided into two aliquots before immunoprecipitation with SG157 (SG157/SG157 and BT3.4/SG157) or BT3.4 (BT3.4/BT3.4 and SG157/BT3.4).

antigen preparation with BT3.4 (SG157/BT3.4) isolated a β chain pattern ($DS\beta_1$ and $DS\beta_2$) that is identical to the direct BT3.4 precipitate. Immunoprecipitation of the antigen preparation with BT3.4 after removal of BT3.4-reactive material (BT3.4/BT3.4) isolated no Ia molecules. Immunoprecipitation of the BT3.4-depleted antigen preparation with SG157 (BT3.4/SG157) isolated a β chain pattern ($DR\beta_1$ and $DR\beta_2$) identical to the direct SG157 precipitate. These results document that the coincident spots of $DR\beta_1$ (isolated by SG157, L227, and L243) and $DS\beta_1$ (isolated by BT3.4, RbO3, and Genox 3.53) are indeed components of distinct Ia β chains.

The relationships of the Ia α and β chains identified in these 2-D gel studies are shown schematically in Fig. 7. The single DR α chain and the two DS α chains are depicted in panels *a-c*. Panel *a* shows the DR α chain that is isolated by SG157, L227, and L243. The $DS\alpha_1$ and $DS\alpha_2$ chains are shown in *b*. BT3.4 and RbO3 isolated $DS\alpha_1$ and $DS\alpha_2$. Panel *c* shows $DS\alpha_2$, which is isolated without other α chains by Baron and Dobbe. Panel *d* shows the α chain pattern isolated by Genox 3.53; the three spots in the Genox 3.53 α pattern may represent additional distinct DS α chains or biosynthetic precursors of $DS\alpha_1$ or $DS\alpha_2$. The relationships of the two DR β chains and two DS β chains are shown in *e*. The

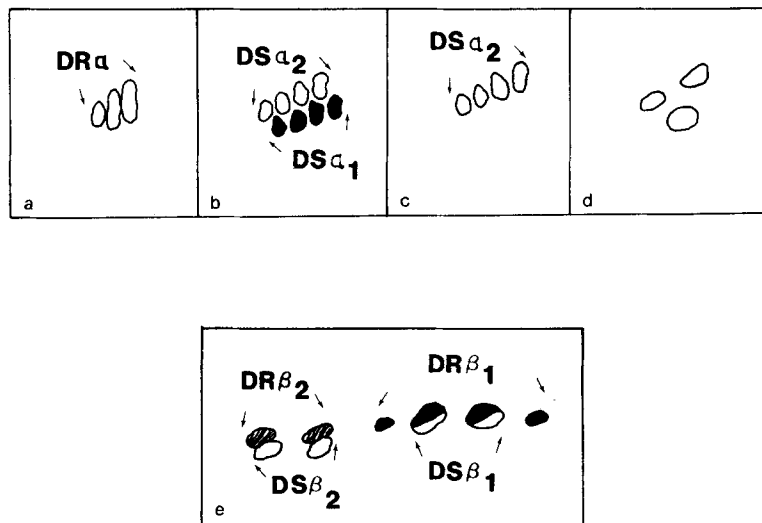


FIGURE 7. Schematic diagrams of the Ia α and β chains isolated from the ELD cell line. The α chain schematics (a-d) depict the patterns seen in these gels; (a) SG157, L227, and L243; (b) BT3.4; (c) Baron and Dobbe; and (d) Genox 3.53. The four β chains are shown in e. DR β_1 is shown as two closed spots and two spots that are half closed and half open to indicate that these latter spots of DR β_1 co-migrate with the two spots of DS β_1 . DR β_2 is shown as hatched spots, DS β_2 as open.

four spots of DR β_1 are shown as two closed spots and two spots that are half-closed and half-open to indicate that these DR β_1 spots co-migrate with the two spots of DS β_1 . The spots of DR β_2 are hatched; those of DS β_2 are open.

Discussion

These studies were initiated to better define (a) the molecular complexity of human DS molecules and (b) the relationship of the DS molecules that bear the MB1 allodeterminant and the MB1-like determinants defined by monoclonal antibodies. The data presented here document for the first time that an HLA-DRw6 homozygous cell line expresses at least two distinct DS α chains. These two DS α chains associate with two DS β chains to form at least two DS molecules. In addition, the cell line was shown to express two DR β chains and one DR α chain that combine to form two DR molecules. The MB1 allosera and MB1-like monoclonal antibodies used in these studies do not all recognize determinants on the same DS molecules. Therefore, these data also document for the first time that there is heterogeneity of the molecular specificities of anti-Ia reagents that define the MB1 and MB1-like antigens in population studies.

The MB1 allosera, Baron and Dobbe, and the MB1-like monoclonal antibodies, BT3.4 and Genox 3.53, were shown to isolate DS molecules, based on comparisons to the DS molecules isolated by the DS-specific heteroserum RbO3. RbO3 has been shown (1) to isolate Ia molecules whose α and β chains have N-terminal amino acid sequence homology to murine I-A α and β chains, respectively. The RbO3 + BT3.4, RbO3 + Genox 3.53, and RbO3 + Baron mixing experiments demonstrate that the α and β chains of Ia molecules isolated by BT3.4, Genox

3.53, and Baron co-migrate with the DS α and β chains, respectively, of Ia molecules isolated by RbO3. These data, plus the previous demonstration that the α chains of Ia molecules isolated by Genox 3.53 have N-terminal amino acid sequence homology to murine I-A α chains (5), establish that BT3.4, Genox 3.53, Baron, and Dobbe are specific for DS molecules.

Comparison of the β chain patterns of Ia molecules isolated by RbO3, BT3.4, Genox 3.53, Baron, and Dobbe indicates that the ELD cell line expresses at least two distinct DS β chains. Baron and Dobbe isolated Ia molecules containing only DS β_2 . BT3.4 isolated Ia molecules containing both DS β chains; however, in the BT3.4 gels, the spots of DS β_1 are more intense than the spots of DS β_2 . Genox 3.53 also isolated Ia molecules containing both DS β chains. However, in contrast to the BT3.4 gel, the spots of DS β_2 in the Genox 3.53 gels are more intense than the spots of DS β_1 . Taken together, these reciprocal patterns of β chain spot intensities and the demonstration that DS β_2 can be isolated without DS β_1 indicate that this DRw6 homozygous cell line expresses at least two distinct DS β chains. In addition to the spots of DS β_1 and DS β_2 , the RbO3, BT3.4, Genox 3.53, Baron, and Dobbe gels contain additional minor spots in their β chain regions; some of these minor components are accentuated in the RbO3 + BT3.4 and RbO3 + Genox 3.53 gels. These less intense spots may be minor components of DS β_1 and/or DS β_2 , or they may represent additional DS β chains that are present in smaller quantities than DS β_1 and DS β_2 or are less well labeled with methionine or leucine than the major DS β chains. The finding that a DRw6 homozygous cell line expresses at least two distinct DS β chains extends our previous observation that a DR5 homozygous cell line expresses two DS β chains (6) and suggests that the expression of at least two DS β chains by homozygous cell lines is a generalized phenomenon.

The studies presented here also indicate that the ELD cell line expresses at least two distinct DR β chains and a single DR α chain that combine to form two DR molecules. Three DR-specific monoclonal antibodies were used to isolate DR molecules in these studies; SG157, L227, and L243 have all been shown (1-3) to isolate Ia molecules that have N-terminal amino acid sequence homology to murine I-E molecules. SG157 and L243 isolated both DR molecules from the ELD cell line, while L227 isolated only the DR β_1 -containing molecule. The DR β_2 -containing Ia molecule was isolated alone when L243 was used to immunoprecipitate an L227-depleted ELD antigen preparation. These results agree with previous studies that demonstrated two distinct DR molecules from DR4 (2), DR5 (6, 29), and DRw6 (3, 28) cells.

Two of the four spots of DR β_1 co-migrated with the two spots of DS β_1 in the SG157 + RbO3 and SG157 + BT3.4 mixing gels. The coincident spots of DR β_1 and DS β_1 were formally shown to belong to distinct Ia chains in sequential immunoprecipitation experiments. After complete removal of both DR molecules by immunoprecipitation with SG157, BT3.4 still isolated Ia molecules containing DS β_1 and DS β_2 . Likewise, after complete removal of Ia molecules containing both DS β chains by immunoprecipitation with BT3.4, SG157 still isolated Ia molecules containing DR β_1 and DR β_2 . Therefore, the two spots of DR β_1 and DS β_1 that co-migrate represent glycosylated forms of distinct proteins which have similar physical characteristics when analyzed by this 2-D gel method.

Comparison of the α chains of Ia molecules isolated from the ELD cell line by the various anti-Ia reagents suggests a degree of molecular complexity that has not been previously appreciated in studies at the protein level. The data shown in Figs. 1 and 4 indicate that the ELD cell line expresses at least three distinct Ia α chains: the DR α chain isolated by SG157, L227, and L243; the DS α_1 chain isolated by both RbO3 and BT3.4; and the DS α_2 chain isolated by RbO3, BT3.4, Baron, and Dobbe. DR α and DS α_2 were each isolated in the absence of some or all of the other α chains; this strongly indicates that each is a distinct Ia α chain. DR α was isolated alone by SG157, L227, and L243. DS α_2 was isolated alone by Baron and Dobbe. The DS α_1 chain was not isolated alone by any of the reagents used in these studies; however, the existence of DS α_1 as a distinct α chain can be reasonably deduced from the DS α patterns in the RbO3, BT3.4, Baron, and Dobbe gels. The isolation of DS α_2 without DS α_1 by Baron and Dobbe suggests that DS α_1 is distinct from DS α_2 . The Genox 3.53 α pattern is distinct from the RbO3, BT3.4, Baron, and Dobbe DS α patterns. The Genox 3.53 α pattern may represent an additional distinct DS α chain; however, it is not possible, based on these data, to exclude the possibility that these spots represent precursors of DS α_1 or DS α_2 . The three Ia α chains identified in these studies have very similar, sometimes partially coincident, isoelectric points and molecular weights. These marked physical similarities, coupled with the technical difficulties of optimally resolving both the basic β chains and the acidic α chains in the same isoelectric focusing gel or nonequilibrium pH gradient electrophoresis gel, have undoubtedly contributed to previous difficulties in resolving the degree of Ia α chain complexity shown here.

The expression of at least two distinct DS α chains and at least two distinct DS β chains by the ELD cell line raises the possibility that one or both of the DS β chains may associate with both of the two DS α chains to form multiple DS molecules. The present data indicate that a minimum of two DS molecules are expressed by this DRw6 homozygous cell line; however, there is the potential for the expression of four different DS molecules (DS α_1 :DS β_1 ; DS α_1 :DS β_2 ; DS α_2 :DS β_1 ; and DS α_2 :DS β_2). Although it seems likely that all of these molecules exist, it is not possible, based on the present data, to identify all of these potential DS molecules because RbO3, BT3.4, and Genox 3.53 all precipitate more than one DS molecule. The possibility that a single DS β chain may associate with more than one DS α chain to form additional distinct DS molecules has important implications for our understanding of the functions of DS molecules because of the amplification of Ia antigenic diversity which would result.

The data presented here document for the first time that there is heterogeneity of the molecular specificities of anti-Ia reagents that define the MB1 or MB1-like antigens in population studies. Analysis of Ia molecules immunoprecipitated by two MB1 allosera and two MB1-like monoclonal antibodies revealed three distinct 2-D gel patterns of Ia molecules. The 2-D gel patterns observed with two MB1 allosera, Baron and Dobbe, include DS α_2 and DS β_2 , but not DS α_1 or DS β_1 . The 2-D gel pattern of BT3.4 includes DS α_1 , DS α_2 , DS β_1 , and DS β_2 . The 2-D gel pattern of Genox 3.53 includes DS β_1 and DS β_2 , and a DS α chain(s) that may be related to DS α_1 or DS α_2 . Importantly, there were reciprocal quantitative differences in the intensities of the spots of DS β_1 and DS β_2 in the BT3.4 and

Genox 3.53 gels, suggesting that these reagents isolated different populations of Ia molecules. Therefore, this DRw6 homozygous cell line appears to express three distinct MB1-related determinants on DS molecules: the MB1 allodeterminant recognized by Baron and Dobbe, the MB1-like determinant recognized by BT3.4, and another MB1-like determinant recognized by Genox 3.53. The present data do not allow assignment of these determinants to α chains, β chains, or combinatorial determinants. These findings underscore the importance of designating in the nomenclature of the serologically defined Ia alloantigens the potential differences between the specificities of alloreagents and monoclonal antibodies that have the same reactivity patterns in population studies. The designation of such monoclonal antibodies as MB1-like or MB3-like (20), for example, would seem to accomplish this goal without unduly complicating the nomenclature. These considerations will undoubtedly assume greater importance as more monoclonal antibodies with specificities similar to recognized Ia allo-specificities become available for clinical tissue typing.

The MB1 allodeterminant is located on only one of the multiple DS molecules expressed by the DRw6 homozygous cell line used in these studies. This finding is in general agreement with our recent observation (6) that the MB3/MT4 allodeterminant is present on only one of the two DS molecules that we identified from a DR5 homozygous cell line. In studies with the DR5 cell line, we found no alloantisera which isolated the non-MB3/MT4-bearing DS molecule (6, 29). Together with the studies presented here, this suggests that the restriction of the MB1 and MB3/MT4 allodeterminants to only one of multiple DS molecules may be an example of the generalized organization of the DS-related, serologically defined alloantigens. Moreover, these data raise the possibility that the DS molecules that bear the MB1 and MB3/MT4 allodeterminants, and perhaps the other DS-related allodeterminants, are more alloimmunogenic than the DS molecules which do not bear these allodeterminants.

The Ia molecules expressed by another DRw6 homozygous cell line, WT46, have been investigated by Shackelford et al. (28) using 2-D gel electrophoresis. These studies demonstrated that the WT46 cell line expresses two DR molecules which could be separated using L243 and L227. Interestingly, the DR β chain patterns from WT46 are very similar to the DR β chain patterns of ELD. They also used several anti-MB1 allosera and the anti-MB1-like monoclonal antibody, Genox 3.53, to isolate DS molecules from WT46. The anti-MB1 allosera and Genox 3.53 all isolated a single Ia molecule composed of a β chain analogous in position to DS β_2 in the present studies and an α chain distinct from the DR α chain. The two spots of this single DS β chain, like DS β_2 in the present study, were very closely related to the major spots of the basic DR β chain. However, in the studies of Shackelford et al., Genox 3.53 did not appear to isolate a second DS β chain corresponding to DS β_1 . It seems most likely that a second DS β chain was not detected in their studies because of the finding shown in Fig. 3: DS β_1 is sometimes not visible or is only faintly visible in Genox 3.53 precipitates, while the spots of DS β_2 are moderately intense. Indeed, our initial Genox 3.53 immunoprecipitate from an ELD antigen preparation revealed only DS β_2 (data not shown). Although it seems unlikely, it is also possible that the WT46 cell line does not express a second DS β chain analogous to DS β_1 .

Because of the relatively recent identification of DS molecules and the paucity of DS-specific reagents, the functional role of DS molecules has not been extensively investigated. The studies reported to date indicate that DS molecules, like the more extensively studied DR molecules, are important in the function of the human immune system. In studies using the DS-specific reagent RbO3, Gonwa et al. (30) demonstrated that there are two populations of adherent peripheral blood monocytes: DS⁺,DR⁺ and DS⁻,DR⁺. Importantly, only the DS⁺ monocytes could present antigen to T cells. Corte et al. (31) found that, in contrast to an anti-DR monoclonal antibody, the anti-MB1-like monoclonal antibody, BT3.4, blocked the generation of cytotoxic T lymphocytes but did not inhibit T cell proliferation in the mixed lymphocyte culture. However, Koide et al. (32) reported that another anti-MB1-like monoclonal inhibited the mixed lymphocyte culture. The evidence presented here for the heterogeneity of molecular specificities of anti-MB1 and anti-MB1-like reagents may provide an explanation for these apparently disparate results regarding the role of DS molecules bearing MB1-like determinants in the mixed lymphocyte culture. The finding that homozygous cells express multiple DS molecules has important implications for future studies of the functional role of DS molecules.

Recent studies at the gene level have greatly accelerated the understanding of the portions of the HLA complex that encode Ia molecules. Genomic clones and/or cDNA clones for multiple Ia α and β chain genes have now been isolated. Present evidence indicates that there are at least three DS α chain genes (33), a single DR α chain gene (34–38), and a single SB α chain gene (39, 40). These β chain genes have been identified: three DR, two DS (39, 40), and one SB (39, 41). The recent identification of at least three DS α chain genes by Auffray et al. (33) is particularly pertinent to the data presented in this paper. They have used a cDNA probe for the DS α chain gene to investigate several deletion mutant cell lines that are DR-null, MB1-positive or DR-null, MB1-negative. When the cell lines were analyzed with the DS α probe under sensitive conditions, at least three different DS α chain genes were identified in the DR-null, MB1-positive mutants. Interestingly, in the context of our data, only one of these DS α chain genes was absent from the MB1-negative mutants. Therefore, the loss of only one of the three DS α chain genes was correlated with, but was not necessarily the sole basis for, the loss of the serologically defined MB1 phenotype. However, these studies did not include analysis of the DS β chain genes in the MB1-negative mutants. Therefore, the correlation of the loss of only one DS α chain gene with the loss of the MB1 phenotype is compatible with three, as yet unresolved, possibilities: (a) the MB1 allodeterminant is located on the α chain that is encoded by the lost DS α chain gene, (b) the MB1 allodeterminant is located on a DS β chain whose gene was lost along with the DS α chain gene in the mutant, or (c) the MB1 allodeterminant is a combinational determinant whose presence is dependent on the expression of both the lost DS α chain gene and an as yet unidentified β chain gene. The data presented in this paper for the expression of at least two distinct DS α chains and the localization of the MB1 allodeterminant to a single DS molecule based on studies at the protein level agree with the studies of Auffray et al. (33) at the gene level. Moreover, the present studies suggest a basis at the level of expressed Ia molecules for the

finding that the loss of only one of three DS α chain genes is associated with the loss of the MB1 phenotype. As discussed previously, the MB1 allodeterminant can be localized to the DS α_2 :DS β_2 molecule from the ELD cell line. Molecules containing DS α_1 or DS β_1 do not bear the MB1 allodeterminant (although these molecules do bear MB1-like determinants defined by the monoclonal antibodies). Therefore, in a hypothetical deletion mutant, the loss of genes that encode DS α_2 and/or DS β_2 would be expected to be associated with the loss of the MB1 phenotype in serologic testing. Moreover, these data predict that a DR-null, MB1-negative mutant which has lost the genes encoding DS α_2 and/or DS β_2 , but still contains the DS α_1 chain gene and the DS β_1 chain gene, would still express Ia molecules recognized by BT3.4 and perhaps Genox 3.53. The data presented here underscore the utility of continued studies of Ia molecules at both the protein and gene levels.

Summary

Ia molecules expressed by an HLA-DRw6 homozygous cell line were immunoprecipitated with anti-Ia allosera and monoclonal antibodies and analyzed by 2-D gel electrophoresis. The DRw6 homozygous cell line was shown to express two DS β chains; this observation extends our previous finding that a DR5 homozygous cell line expresses two DS β chains and suggests that the expression of at least two DS β chains by DR homozygous cell lines is a generalized phenomenon. The data presented here document for the first time that a DR homozygous cell line expresses at least two DS α chains. Therefore, this cell line expresses at least two DS molecules with the potential for the expression of four DS molecules. In agreement with previous reports, the cell line was shown to express two DR β chains and one DR α chain that combine to form two DR molecules. The molecular specificities of two MB1 allosera and two MB1-like monoclonal antibodies were also compared in these studies. Both MB1 allosera isolated a single DS molecule, while the MB1-like monoclonal antibodies isolated at least two DS molecules. Therefore, these studies document for the first time that anti-Ia reagents which are specific for the MB1 or MB1-like determinants in population studies do not recognize the same Ia molecules in immunochemical studies. The data presented here for the expression of at least two DS α chains and the location of the MB1 allodeterminant on only one of multiple DS molecules are in agreement with recent studies at the gene level.

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