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Immunology

Polymorphism of human B-cell alloantigens: Evidence for three loci within the HLA system

(major histocompatibility complex/HLA-DR antigen/Ia antigens)

M. LOUISE MARKERT AND PETER CRESSWELL

Department of Microbiology and Immunology, Duke University Medical Center, Durham, North Carolina 27710

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ABSTRACT **The B-cell alloantigens of an *HLA-DR3*-homozygous, *MB2*-homozygous, MT2-positive lymphoblastoid cell line were studied by two-dimensional gel electrophoresis. Analysis of gel patterns suggested assignment of the HLA-DR3 determinant to the larger (35,000-dalton) subunit (α) of the B-cell alloantigen. MB2 was found to be either a determinant on the small (27,000 dalton) subunit (β) or a crossreactive determinant(s) on the HLA-DR3 α subunit and an additional α subunit. MT2 was found to be a determinant on Ia antigen-like molecules distinct from those carrying the MB and HLA-DR determinants. The results are consistent with the existence in the major histocompatibility complex of at least three loci encoding B-cell alloantigens.**

HLA-DR, a locus in the human major histocompatibility complex (MHC) (1), codes for polymorphic cell surface glycoproteins present on B lymphocytes, macrophages, and certain other populations of cells (2–5). The HLA-DR antigens (DRs) are composed of two noncovalently associated glycoproteins of 35,000 daltons (α) and 27,000 daltons (β) as determined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (3, 6, 7). The two subunits show marked differences in NH_2-terminal amino acid sequence (7) and isoelectric point (7–9).

After serological analysis of data from the Seventh International Histocompatibility Workshop, Duquesnoy *et al.* (10) proposed a second B-cell alloantigen system, *MB*. *MB* is thought to be a gene closely linked to *HLA-DR* although it is possible that it encodes public specificities of DRs. The specificity MB1 is closely associated with DR1, DR2, and DRw6; MB2, with DR3 and DR7; and MB3, with DR4 and DR5 (10).

In the Eighth International Histocompatibility Testing Workshop, three supertypic, presumably crossreactive, specificities were discussed (11, 12). These antigens were designated MT1, MT2, and MT3. MT1 is found in association with DR1, DR2, DRw6, and DRw10; MT2, with DR3, DR5, DRw6, and DRw8; and MT3, with DR4, DR7, and DRw9 (11). These specificities may be determinants present on the molecule carrying the DR determinants or on products distinct from DR.

Murine Ia antigens encoded in the *I* region of *H-2* are analogous to human DR antigens. Recently Jones *et al.* (13) and others (14–16) have documented two-gene control of expression of certain Ia antigens. These investigators have shown that Ia antigens precipitated by antisera directed against certain *I-E/C* haplotypes are composed of an α subunit encoded in the *I-E/C* region and a β subunit encoded in the *I-A* region.

At present in the human, it is not known how many loci code for B-cell alloantigens nor is it known whether genes in the MHC code for one or both of the subunits of B-cell alloantigens.

We have investigated the molecular relationships of DR, MB, and MT antigens by immune precipitation and two-dimensional electrophoretic techniques, using a lymphoblastoid cell line homozygous for *HLA-DR3* and *MB2* and positive for MT2 and a combination of a chimpanzee antiserum and alloantisera specific for these three products.

METHODS

Cells. The Pala B-lymphoblastoid cell line was derived by Epstein–Barr virus transformation of peripheral blood lymphocytes from an individual whose HLA haplotypes were *Aw23, B8, DR3, MB2/Aw24, B8, DR3, MB2*. The individual was also MT2-positive. This typing was verified by 1980 workshop typing of the cell donor, her parents, and related individuals carrying the appropriate *HLA* haplotypes.

Sera. Antiserum JIMOH, produced in collaboration with Richard Metzgar, is a chimpanzee anti-DR xenoantiserum made by sequential intravenous and subcutaneous (with incomplete Freund's adjuvant) injection of 100 μg of affinity-purified B-cell alloantigens in 0.5% sodium deoxycholate/0.15 M NaCl/0.01 M Tris·HCl, pH 8.0 (Tris-buffered saline). The antigens were purified from the Pala cell line by affinity chromatography (6). When screened on a panel of typed lymphocytes, antiserum JIMOH reacted with DR3-, DR5-, DRw6-, and DRw8-positive cells. Antiserum JIMOH/Swei was prepared by absorbing antiserum JIMOH with Swei, a DR5-homozygous cell line (17, 18). In ^{51}Cr-release experiments, JIMOH/Swei retained activity against DR3 but lost activity for DR5 and DRw6. DRw8 was not tested (data not shown). Serological analysis of JIMOH/Swei showed that JIMOH/Swei reacted only with the DR3-positive cells in a panel of 100 individuals. The reactivity of JIMOH is consistent with two antibody populations, one against DR3 and the other against DR5, DRw6, and possibly DRw8. The latter would correspond to MT2.

MAX (7w101) is a human alloantiserum (19) produced by immunization of a father (*A2, B8, C-, DRw6, Dw6, MB1/A3, B7, C-, DR2, Dw2, MB1*) against his son's lymphocytes (*A2, B8, C-, DRw6, Dw6, MB1/Aw31, B14, C-, DR7, Dw7, MB2*). MAX has strong reactivities against DR7 and MB2 and a weak reactivity against Aw31; the activity against Aw31 is removed by absorption of the antiserum with the son's platelets. Our laboratory uses MAX (after absorbtion with Aw31-positive platelets and with DR7-positive MB2-negative DAW cells) to define MB2. This specificity was formerly designated DuB 33. Similar reaction patterns are given by the MB2 serum 7w127 (20). A somewhat longer MB2 pattern has been described by Duquesnoy *et al.* (10). Because the Pala cell line used in all studies

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Abbreviations: MHC, major histocompatibility complex; DR, HLA-DR antigen; α, 35,000-dalton subunit of HLA-DR; β, 27,000-dalton subunit of HLA-DR.

is DR7-negative, the reactivity of interest in MAX which will be emphasized in this paper is anti-MB2.

Serum IA172 is a human multiparous alloantiserum produced by an *A1, A2, B7, B12, DR2, DR4, MB1, MB3* individual against her husband (*A1, Aw32, B27, B18, DR5, DRw6, MB1, MB3*). This serum reacts with all DR3 and most DR5 and DRw6 individuals and therefore is thought to contain anti-MT2 antibodies. Antisera IA172 and MAX were generous gifts of A. H. Johnson.

Preparation of Radiolabeled Solubilized Membranes. The Pala lymphoblastoid cell line was radiolabeled with [^{35}S]methionine by incubation of 2×10^7 cells for 15 hr in 10 ml of methionine-free RPMI-1640 medium (GIBCO) supplemented with gentamycin (50 μg/ml) and 750 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{35}S]methionine (Amersham). Membranes were purified (21) and then solubilized in 0.1% octaethylene glycol dodecyl ether (Nikkol) in Tris-buffered saline prior to centrifugation at 100,000 $\times$ *g* for 90 min. Phenylmethylsulfonyl fluoride was added to 0.5 mM and tosyllysine chloromethyl ketone was added to 0.1 mM at the beginning of membrane purification.

Immunoprecipitation. Immunoprecipitations were performed after preprecipitations with normal rabbit serum as described (22). Formalin-fixed heat-killed *Staphylococcus aureus* strain Cowan I (23) was used to bind the immune complexes and precipitate them from the detergent-solubilized membrane preparation (22). The *S. aureus*–antibody–antigen pellets were washed three times with 2% gamma globulin-free fetal calf serum in 0.05% octaethylene glycol dodecyl ether in Tris-buffered saline and then twice in water. Antigen and antibody were dissociated from the *S. aureus* pellet by incubation for 30 min at 25°C in lysis buffer A of O'Farrell (24).

Two-Dimensional Electrophoresis. The sample was analyzed with the two-dimensional gel electrophoresis system of O'Farrell (24). Gels of pH range 5–7 or 5–8 were used. For the latter, 5/7 Ampholines (Bio-Rad) and 3/10 Ampholines were used in a 1:1 ratio. All other conditions were identical to those described by O'Farrell (24). The sodium dodecyl sulfate/polyacrylamide gels were all 10.5% (wt/vol) in acrylamide for the second dimension. All gels were developed by the fluorographic method of Bonner and Laskey (25).

RESULTS

Various antisera were used to precipitate B-cell alloantigens from the detergent-solubilized Pala membrane preparation. The antisera JIMOH, JIMOH/Swei, MAX, and IA172 all gave simple patterns on two-dimensional gel electrophoresis. In each case there was a series of spots at the acidic end of the gel which corresponded to the α subunit. At the basic end of the gel, there was a series of spots of higher electrophoretic mobility, corresponding to the β subunit.

Analysis of the β Subunits. Gels at pH 5–8 were run to examine the basic β subunits. In the JIMOH/Swei precipitate (Fig. 1*A*), the β subunit appeared to be a doublet. This usually is the case for MAX precipitates (Fig. 2*C*). When the MAX and JIMOH/Swei precipitates were run together on one gel, the β subunits superimposed (Fig. 1*C*). This strongly suggests identity between DR3 and MB2 at the β subunit. Repeated analysis has failed to reveal any differences in the β patterns of MAX and JIMOH/Swei precipitates.

Fig. 1*D* shows the JIMOH (anti-DR3, anti-MT2) pattern for the β subunit. It is complex, with three spots of different isoelectric points. The most prominent basic spot (see vertical arrow, Fig. 1*D*) is identical to the β subunit of Fig. 1 *A* and *B*. The middle β spot appears to be three molecules of the same isoelectric point but different molecular weights. This effect

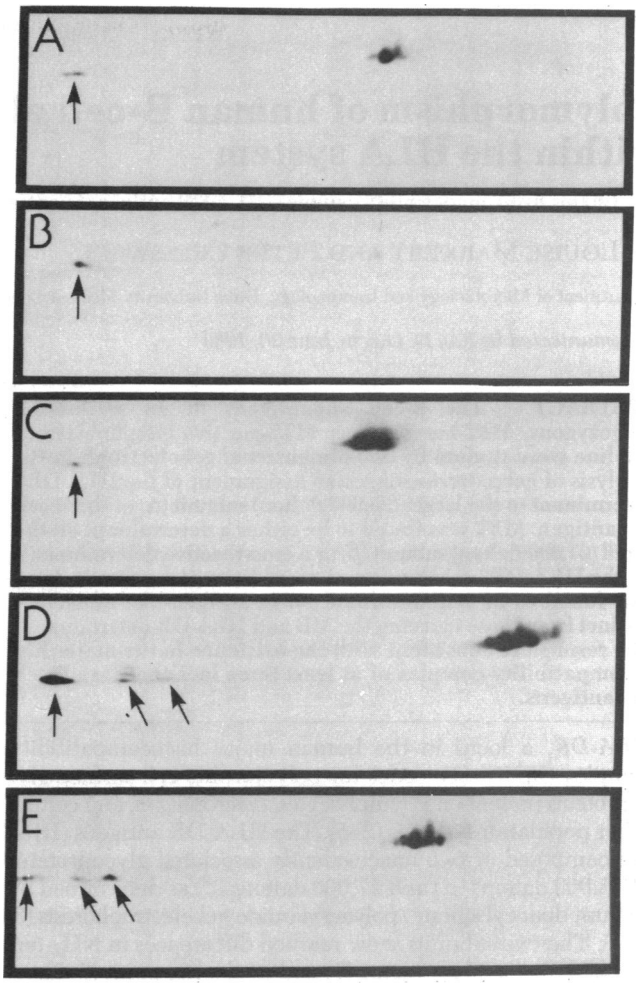

FIG. 1. Two-dimensional gel electrophoresis of Pala-B cell alloantigens. Approximately 4×10^6 cpm of ^{35}S-labeled detergent-solubilized Pala membranes was preprecipitated with normal rabbit serum followed by 20 μl of JIMOH/Swei (anti-DR3) (*A*), 40 μl of MAX (anti-MB2) (*B*), 5 μl of JIMOH (anti-DR3 anti-MT2) (*D*), or 10 μl of IA172 (anti-MT2) (*E*). In *C*, equal amounts of the JIMOH/Swei and MAX precipitations were combined and run together on the same gel. In *A*, *B*, and *C*, samples were applied to two-dimensional gels of pH 5–8; in *D* and *E* the pH was 5–7. For each gel the cathode was at the left and anode was at the right. Migration in the sodium dodecyl sulfate/polyacrylamide gel was from top to bottom. In *B* the α subunit is not visible because it ran off the anodic end of the gel. Vertical arrows show the β subunit which is equivalent in each panel. Diagonal arrows show the MT2-specific β subunits. See text for further details.

was also observed for some α subunit spots (see below). The β subunit pattern obtained with serum IA172 (Fig. 1*E*) was similar to that of JIMOH. The β subunits precipitated by IA172 have been shown to be identical to those precipitated by JIMOH (Fig. 1*D*) by running both precipitates on the same gel and finding that the dots superimpose (data not shown). The β spots present in the JIMOH and IA172 gels and not present in the JIMOH/Swei or MAX precipitates are assumed to be the result of anti-MT2 antibodies present in JIMOH and IA172 and absent in JIMOH/Swei and MAX. We consider these to be MT2-specific β subunits (diagonal arrows, Fig. 1).

Analysis of the α Subunits. Gels with a pH gradient of 5 to 7 were used to examine the α subunit. The JIMOH/Swei (anti-DR3) pattern was the most simple (Fig. 2*A*), with three α subunit spots (diagonal arrows). They are a subset of the spots precipitated by JIMOH (Fig. 2*B*) and IA172 (Fig. 1*E*). The

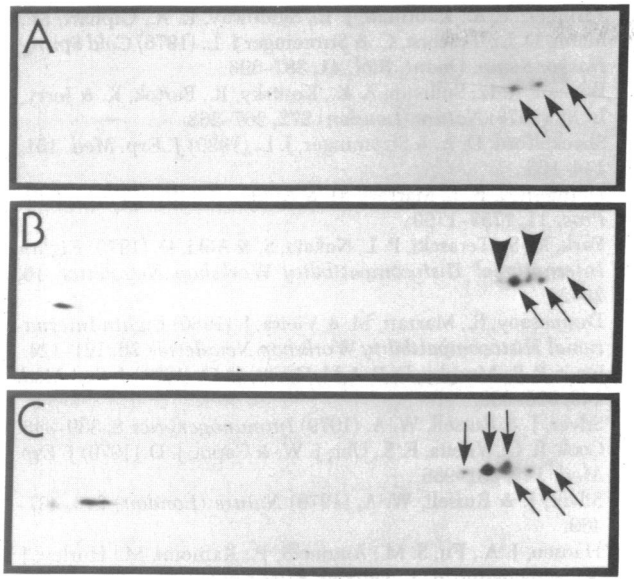

FIG. 2. Two-dimensional electrophoresis of B-cell alloantigens isolated from the Pala cell line. Approximately 2×10^6 cpm of ^{35}S-labeled detergent-solubilized Pala membranes was precipitated with normal rabbit serum followed by 30 μl of JIMOH/Swei (anti-DR3) (*A*), 20 μl of JIMOH (anti-DR3, anti-MT2) (*B*), or 40 μl of MAX (anti-MB2) (*C*). All samples were applied to two-dimensional gels of pH 5–7. Orientation of gels was the same as in Fig. 1. Diagonal arrows, equivalent sets of spots; vertical arrows, MB2-specific α subunits; arrowheads, MT2-specific α subunits.

JIMOH and IA172 gels contained two additional spots (arrowheads, Fig. 2*B*) not found in the JIMOH/Swei (anti-DR3) or MAX (anti-MB2) patterns (Fig. 2*C*). These appeared to be MT2-specific α subunits. The more acidic of these two MT2-specific α spots appeared to be several molecules of the same isoelectric point but different molecular weights.

The MAX (anti-MB2) pattern of α subunits contained two sets of spots (Fig. 2*C*). One set was identical to that in the JIMOH/Swei pattern in Fig. 2*A*. The other set of three spots (vertical arrows, Fig. 2*C*) was not seen in the JIMOH/Swei or JIMOH pattern. These we consider to be MB2-specific α subunits.

If MAX (anti-MB2) and JIMOH/Swei (anti-DR3) do indeed precipitate the same β subunit, then on the cell surface both sets of α subunits precipitated by MAX must be found in combination with the same β subunit, shown by the vertical arrow in Fig. 1. Thus, the anti-DR3 antibodies of JIMOH/Swei presumably must be directed against the α subunit of the HLA-DR3 complex (Fig. 2*A*) because, otherwise, the MB2-specific α subunit should have been precipitated by JIMOH/Swei also.

Furthermore, if the MB2 and DR3 β subunits are indeed identical, then the MB2 determinant(s) either (*i*) are on the β subunit (vertical arrow, Fig. 1*B*) or (*ii*) are crossreactive determinants on the two sets of α subunits shown in Fig. 2*B*. In either case a gene coding for MB determinants exists which is distinct from *HLA-DR*. The first possibility is analogous to the situation with murine Ia antigens in which a gene in the *I-A* region encodes the β subunit and a gene in the *I-E/C* region encodes the α subunit (13).

The additional α and β subunits precipitated by JIMOH and IA172 are distinct from the DR3 and MB2 molecules. They appear to be separate molecules bearing MT2 determinants. We do not know which subunit, α or β, carries the MT2 determinant but, again, the results suggest that a separate gene must code for MT2 determinants.

DISCUSSION

The data presented provide for the presence on one cell of a minimum of five components of human Ia-like antigens, each of which may be a product of a separate gene. This minimum is based upon the assumption that posttranslational variability, such as differential sialic acid content (26), is responsible for the presence of "sets" of spots. These Ia-like antigen components include three α subunits: one MT2-associated, one MB2-associated, and one which is DR3-associated and possibly crossreactive with MB2 antibodies (diagonal arrows, Fig. 2*A*). In addition there are a minimum of two β subunits: one MT2-associated and one shared by the DR3 and MB2 antigens. At least three of these five components—the *HLA-DR* product, the *MB* product, and the *MT* product—are coded for by MHC-linked loci. *HLA-DR* maps near *HLA-D* in the MHC (1). MB2, as defined by MAX/DAW and 7w127, has been shown in family studies to segregate with HLA (20). MT2, as defined by IA172, also segregates with HLA in families (27, 28). Because the Pala cell line used is homozygous for *HLA-DR3* and *MB2* and is positive for MT2, a minimum of three independent loci coding for Ia-antigen-like determinants can be assigned to the human MHC.

If we are correct in our interpretation that the β subunits precipitated by MAX (anti-MB2) and JIMOH/Swei (anti-DR3) are identical, then we can logically identify the α subunit of the DR3 antigen as the *HLA-DR3* product. The MB2 determinants may be restricted to the α chains, with antibodies to MB2 crossreacting with the DR3 α chain. Alternatively, the *MB2* product may be the β chain, which can associate with the DR3 α chain and with a second α chain which we cannot detect serologically (vertical arrows, Fig. 2*C*). Thus, we have two genes linked to HLA which contribute to the structure of a single B-cell alloantigen, the DR3-positive and MB2-positive antigen seen in Fig. 2*A*.

If the DR3 and MB2 β subunits are not identical but merely have the same molecular weight, isoelectric point, and tendency to periodic detection as a doublet, then we cannot assign DR3 determinants to the α subunit. In this case the minimum number of Ia antigen-like components on one cell type has to be raised to six, now including three β chains—the DR3-associated β chain, the MB2-associated β chain, and the MT2-specific β chain. A definitive answer on this point will only come from peptide mapping or sequencing of the β chains.

From our data we cannot determine whether the α or β chain of the MT2 antigen carries the MT2 determinant. MT2 is presumably expressed on one or more of the β spots (diagonal arrows, Fig. 1*D*) or α spots (arrowheads, Fig. 2*B*) unique to the JIMOH gels. Tosi *et al.* (29) found the MT1 (DC-1) (30) and DRw6 determinants to be on separate molecules. In binding studies, MT1 was localized to the β subunit. Our results are similar to theirs in that MT2 determinants are found on molecules separate from those containing the DR3 determinant. Conceivably, some of the α and β spots precipitated by JIMOH but not by JIMOH/Swei (anti-DR3) may be derived from DR-like antigens currently undefined by serology.

Examination of Fig. 1*E* indicates that IA172 does precipitate DR3 in addition to MT2-specific subunits. This may be a result of crossreactivity of DR3 with the anti-DR5 or anti-DRw6 antibodies potentially present in the serum or, alternatively, MT2 and DR3 may themselves be crossreactive.

Several research groups have suggested that the DR allospecificities reside on the small (27,000 daltons) subunit of B-cell alloantigens. These groups have pointed to the binding of alloantisera to isolated β subunits (29) and to the high variability of β subunit tryptic fragments (31). Two-dimensional gel electrophoresis has shown variable patterns for the β sub-

units with more constant patterns for the α subunits (9, 32). From binding studies and analysis of α and β tryptic fragments Klareskog *et al.* (33) came to the contradictory conclusion that the HLA-DR determinant is on the α subunit, a conclusion supported by our data. The DR3 determinant of Pala similarly may reside on the α subunit. The two opposing points of view could be reconciled if some DR specificities were hybrid determinants formed by close association of α and β subunits. Some antisera might have predominantly antibodies to the α-subunit portion and others, to the β-subunit portion, causing difficulties in interpretation when measuring binding to isolated subunits.

Our two-dimensional gel electrophoresis patterns are similar to those of B-cell alloantigens published by others (9, 32). We do not understand the vertical stacks of three spots seen in Figs. 2*B* and 1 *D* and *E*. Given the identical isoelectric point of the spots in each stack, each dot may represent the same polypeptide with varying numbers of high-mannose carbohydrate side chains, but as yet we have no evidence for this. Our gel patterns do not include the basic invariant spot described by Jones *et al.* (34). We have used nonequilibrium pH gradient electrophoresis on DR of several cell lines and never have found such a spot. Shackelford and Strominger (9) found that detergent washing of the precipitated DR resulted in removal of one of their basic invariant spots (M1). Our washings are quite vigorous and may explain this discrepancy.

Our studies were greatly aided by the availability of the MB2-specific human alloantiserum MAX. As can be seen by comparing Fig. 2 *C* and *B*, the MAX serum precipitates the MB2-specific α subunit which is not precipitated by the chimpanzee serum JIMOH. A rabbit anti-DR xenoantiserum used in our laboratory (J_{HSB}) (3) also did not precipitate the MB2-specific α subunit (data not shown). Other investigators using xenoantisera therefore may have found less heterogeneity of the α subunit than actually is present.

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