THREE Ia SPECIES WITH DIFFERENT STRUCTURES
AND ALLOANTIGENIC DETERMINANTS
IN AN HLA-HOMOZYGOUS CELL LINE*

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The serological analysis of human B cell alloantigens has led to the definition of a
HLA-linked locus, designated HLA-DR (1, 2), which codes for human Ia antigens,
i.e., human homologues of murine Ia antigens. Thus far, 10 allospecificities have been
assigned to this locus.

Evidence has been accumulating over the past few years that human Ia antigens
are controlled by at least two HLA-linked loci. The first indication was the discovery
of a subset of Ia molecules carrying a non-DR specificity DC1 by immunochemical
analysis at the molecular level (3). This determinant is controlled by an allele at a
separate locus in strong linkage disequilibrium with three alleles of the DR locus,
DR1, DR2, and DRw6 (4, 5). Structural characterization by microfingerprinting (6)
and by two-dimensional electrophoresis (7) has shown that DC1 molecules differ from
DR molecules. This identification of a second Ia locus has unveiled a basic homology
of the human Ia system with the murine system in which two subsets of Ia molecules,
I-A and I-E, exist (8).

The elucidation of another Ia specificity, BR4X7 (9) or HON7 (10), has revealed
an additional complexity of the human Ia system. This specificity is controlled also
by an HLA-linked locus different from the DR locus and is transmitted in strong
linkage disequilibrium with the DR4, DR7, and DRw9 alleles (5). BR4X7 could
conceivably be allelic to DC1 and thus be incorporated in a two-loci model. However,
peptide mapping analysis showed that the molecules carrying BR4X7 specificity are
far more similar to DR molecules than to DC1 molecules,1 leading to the assumption
that DC1 and BR4X7 may not be allelic. Consequently, a third HLA-linked Ia locus
would be required to accommodate BR4X7.

Direct evidence for or against a three-loci hypothesis cannot be obtained using the
specificities DC1 and BR4X7 because they are always transmitted in repulsion as a
result of their association with nonoverlapping sets of DR alleles. More complete
immunochemical testing of the three-loci concept requires the consideration of

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1 Sorrentino, R., G. Corte, F. Calabi, N. Tanigaki, and R. Tosi. Human Ia molecules carrying the non-
DR specificities DC1 and BR4X7 differ in both subunits. Manuscript submitted for publication.
additional specificities. Such specificities can be found in the supertypic specificities of two HLA-DR-associated systems designated MB (11) and MT (12). Three MB specificities have been recognized; each shows a strong association with two or more DR specificities: MB1 with DR1, DR2, and DRw6; MB2 with DR3 and DR7; and MB3 with DR4, DR5, DRw6, and DRw9. The MT specificities are, with the exception of MT1, associated with other groups of DR specificities: MT1 with DR1, DR2, and DRw6; MT2 with DR3, DR5, DRw6, and DRw8; and MT3 with DR4, DR7, and DRw9. The DC1 is equivalent to MB1 and MT1, and the BR4X7 is similar and probably identical to MT3.

In this study, the MB2 specificity that frequently forms an HLA haplotype along with DR7 and BR4X7 was analyzed at the molecular level by using an HLA-homozygous cell line expressing these three specificities. We have found that the DR7, BR4X7, and MB2 determinants correspond to three distinct molecular subsets with different structural features as well as with a different population distribution. These data substantiate the three-loci concept for the genetic control of human Ia antigens.

Materials and Methods

Antisera. Most of the alloantisera used were derived from Ferrara and Duquesnoy serum panels. Some were kindly provided by other laboratories: serum HON by Dr. M. Katagiri, Asahikawa Medical College, Asahikawa, Japan; serum T-1514 by Dr. T. Juji, Tokyo University School of Medicine, Tokyo; serum 8-4513 by Dr. K. Tsuji, Tokai University School of Medicine, Isehara, Japan. Serum KIT 13390 is an Eighth Histocompatibility Workshop antiserum, BW455, that was provided by Dr. K. Gelsthorpe, National Blood Transfusion Service, Sheffield, England. Sera Murphy and Gallardo were obtained from the serum bank of the National Institute of Allergy and Infectious Diseases, Research Resources Branch, Bethesda, MD (NIH).

Some of the antisera used were studied during the Eighth Histocompatibility Workshop and their specificity was assessed by cytotoxicity assay. These antisera were tested for their binding properties by radioimmunoassay using a panel of Ia preparations of different phenotypes. In general, a good correspondence was found between the specificities detected by cytotoxicity and by molecular binding (13). The specificity of other alloantisera was defined by radioimmunoassay only. In several cases the definition of specificity was facilitated by typing the immunizer (father, or volunteer blood donor in cases of planned immunization) and the antibody producer (mother, or volunteer blood recipient in cases of planned immunization).

Table I lists the alloantisera used, their known specificities, and other available data.

In addition, two rabbit antisera, anti-human Ia antiserum 7147 and anti-human Ia a-subunit antiserum U115, were used. The former was raised against a preparation of Ia molecules purified from Daudi cells and its specificity has been fully assessed (9, 14). The latter was generated against an a-chain preparation isolated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis from a pooled Ia preparation that was derived from peripheral leukocytes of five patients with chronic lymphocytic leukemia. Aliquots corresponding to ~0.02 A280 units were injected intramuscularly at weekly intervals with complete Freund’s adjuvant. The antiserum obtained after 2 mo reacted only with isolated Ia a-chains and not with Ia b-chains. The antiserum also bound intact Ia molecules irrespective of their DR specificity. This binding was inhibited only by Ia a-chains and not by Ia b-chains. Moreover, the antiserum was found to bind only DR and BR4X7 molecules and not to bind DC1 molecules. In fact, by starting with a Daudi Ia preparation containing ~70% DRw6 molecules and <10% DC1 molecules and depleting the preparation of DRw6 molecules specifically by the use of this antiserum, a preparation was obtained in which these proportions were approximately reversed (15).

Antigen Preparation. The Ia preparation that was analyzed was purified by the procedure described in detail (16) from a HLA-Dw7 homozygous cell line, LG-10, established by Dr. W. Leibold, Institute for Pathology, Hannover, Federal Republic of Germany. This cell line had been typed to be DR7-positive and BR4X7-positive by radioimmunoassay (5, 17).
TABLE I

Alloantisera Used in the Present Study

| Antiserum | Laboratory | Incompatible specificity* | Detected specificity‡ |
|-----------|------------|--------------------------|-----------------------|
| Fe96/19   | Ferrara    | DR1, DR3, DC1, MB2        | DC1, MB2              |
| Fe59/15   | Ferrara    | DR4, BR4X7                | DR4, BR4X7            |
| Fe57/26   | Ferrara    | DR7, BR4X7, MB2           | DR7, BR4X7, MB2       |
| Fe53/23   | Ferrara    | DR7, BR4X7, DC1           | DR7, BR4X7, DC1       |
| Fe118/13  | Ferrara    | DR7, MB2                  | DR7, MB2              |
| Duffy     | Duquesnoy  | DR3, MB2                  | DR3, MB2              |
| Tellier   | Duquesnoy  | DR3, MB2                  | DR3, MB2              |
| Swift     | Duquesnoy  | Not available             | DR4, BR4X7            |
| Eckert    | Duquesnoy  | Not available             | DR7, BR4X7, MB2       |
| Gnadt     | Duquesnoy  | DR7, BR4X7, MB2           | DR7, BR4X7            |
| Keliher   | Duquesnoy  | Not available             | DR7, BR4X7            |
| HON       | Katagiri   | Not available             | BR4X7                 |
| T:1514    | Juji       | Not available             | DR4, BR4X7            |
| 8-4513    | Tsuji      | Not available             | DR4, BR4X7            |
| KIT13390  | Gelsthorpe | Not available             | DR4, BR4X7            |
| Murphy    | NIH§       | Not available             | DR4, BR4X7            |
| Gallardo  | NIH§       | Not available             | DR4, BR4X7            |

* Incompatibilities were established by cytotoxicity typing of immunizer and serum producer. DC1 is equivalent to MB1 or MT1 and BR4X7 to HON7 or MT3.
‡ The specificities were assessed by cytotoxicity assay and/or radioimmunoassay.
§ National Institutes of Health.

Briefly, crude cell membranes were isolated by differential centrifugation from ~13 g (wet weight) of packed LG-10 cells (~6.5 × 10⁹ cells) disrupted by nitrogen decompression and solubilized with 2% Renex 30 (Ruger Chemical Co., Irvington, NJ). The solubilized material was separated by ultracentrifugation and subjected to affinity chromatography on a column of lentil lectin-coupled Sepharose (18). The retarded fraction was concentrated by ultrafiltration and fractionated by gel filtration on a column of Bio-Gel A1.5M (Bio-Rad Laboratories, Richmond, CA; bed vol: 100 ml). The major fractions with high human Ia activity as determined by the binding inhibition assay (19) were pooled. The yield was 3.84 as determined by absorbance at 280 nm.

A small amount (0.020 A₂₈₀ units) of the pool was trace-labeled with ¹²⁵I by the chloramine T method (20). The labeled preparation (~50 mCi/4₈₀₀ unit) was fractionated on a column of Bio-Gel A1.5M (bed vol 50 ml), and the major fractions which gave high binding with rabbit anti-human Ia antiserum as determined by the direct binding assay (21) were pooled. Binding with rabbit anti-human Ia antiserum was 63%. This preparation was used mostly for sequential binding experiments and peptide analyses.

To obtain a preparation depleted of DR7 and BR4X7 molecules, a small amount (0.040 A₂₈₀ units in 40 μl) of the LG-10 Ia preparation was absorbed before radiolabeling, with the Sepharose-bound antiserum U115 (200 μl of a 33% suspension of Sepharose-4B coupled with 10 mg crude immunoglobulins/ml gel) at 4°C for 24 h on a rotating wheel and then radioiodinated. The labeled preparation was subjected to papain digestion at 37°C for 30 min using an enzyme to substrate ratio of 1:40 (10 mg bovine serum albumin as carrier) at pH 7.8 in the presence of 10 mM cysteine-HCl and 1 mM EDTA. The digestion was terminated by addition of iodoacetamide in slight molar excess over cysteine. The digest was further processed by gel filtration on Bio-Gel A1.5M followed by lentil lectin affinity chromatography, a procedure that separates the intact from the papain-split Ia molecules and improves the purity of the final products, as previously described in detail (15, 16, 21). The papain-split, DR- and BR-depleted Ia preparation showed a purity of 85%. It was used as the reference labeled antigen for the radioimmunoassay of MB2 specificity.

Typing of MB2 by the Radioimmune Inhibition Assay. The same procedure as described for
typing of DR specificities and for DC1 and BR4X7 typing was followed (5, 16, 17). Briefly, leukocytes obtained from defibrinated blood were lysed with Renex 30. A portion of lysate corresponding to 7 x 10⁶ cells was added to 2 µl of reference antiserum Fe96/19. After incubation for 16 h at room temperature, ~10,000 cpm of the labeled U115-treated LG-10 preparation, prepared as above, was added. After a further 16-h incubation at room temperature, the immune complexes were precipitated by a goat anti-human Ig antiserum. The precipitate obtained was washed and the radioactivity was determined. The extent of inhibition was calculated as the percentage of the radioactivity precipitated in the absence of inhibitor.

For typing cell lines, the same procedure was adopted, except that 5 µl of a 20% (vol/vol) cell lysate was used and the incubation time for alloantibody with inhibitor was reduced to 4 h at room temperature. Typing of cell lines was also performed using an LG-10 preparation not treated with U115. The percentage of Ia molecules carrying MB2 specificity was low (~10% vs. >40% in the enriched preparation) but still sufficient to clearly distinguish between inhibitors and noninhibitors. An assay was also performed under these conditions using antiserum Duffy (5 µl) instead of Fe96/19.

Direct-binding Assay and Sequential-binding Assay. The direct-binding assay used for measuring the binding activity of alloantisera was carried out as previously described in detail (16). Briefly, equal portions of an ¹²⁵I-labeled Ia preparation having 10,000-20,000 cpm were incubated with 5 µl of test alloantisera in a total reaction vol of 60 µl for 16 h at room temperature and further with an optimum amount of goat anti-human Ig antiserum for 1 h at room temperature. The resultant immune precipitate was washed and the radioactivity was determined. A control with normal human serum was included in each assay. Results were expressed as the percent Ia binding, which indicates the proportion of the maximum binding obtained with rabbit anti-human Ia antiserum 7147.

The sequential-binding assay used to determine the relationship between Ia molecules bound by alloantisera of different Ia specificities was also performed as described previously (9). Briefly, the first binding reaction was carried out by incubating an ¹²⁵I-labeled LG-10 Ia preparation with a selected human Ia alloantiserum for 4 h at room temperature. After further incubation with goat anti-human Ig antiserum for 1 h at room temperature, the resultant precipitate was removed by centrifugation. The supernatant was subsequently assayed by the direct-binding assay for the remaining binding activity with a set of human Ia alloantisera, including the antiserum used in the first binding reaction. The extent of binding in the second binding reaction was expressed as the percentage of the control binding, i.e., the extent of binding given by the labeled Ia preparation treated with normal human serum under the same conditions.

Microfingerprinting. The ¹²⁵I-labeled Ia molecules bound by a given alloantiserum were recovered on protein A-coupled Sepharose (Enzyme Center, Boston, MA) using 300 µl of a 20% suspension for 50 µl of alloantiserum and eluted with 0.5 ml of a 1% SDS solution. The Ia subunits were separated by polyacrylamide gel electrophoresis on a 10% gel (10 x 90 mm) containing 0.1% SDS according to the method described by Laemmli (22). The separated subunits were eluted from the segmented gels by overnight incubation at 4°C with Tris-HCl buffer (Tris 0.037 M, pH 7.8) containing 0.05% SDS.

The peptide analysis of isolated subunits was performed by a two-dimensional mapping, which has a higher resolving power than one-dimensional analysis (6), using a slight modification of the method originally described by Mole (23). The subunit preparations were concentrated to <0.5 ml by Speed Vac, a concentrator (Savant Instruments, Inc., Hicksville, NY), reduced, and alkylated in the presence of 50 µg rabbit IgG and precipitated with an acid mixture at a final concentration of 10% trichloroacetic acid and 4% sulfosalicylic acid. The precipitates were washed with acetone and then with methanol, dissolved with 100 µl of 0.1M ammonium carbonate, and digested with elastase at 37°C overnight using an enzyme to protein ratio of 1:20. The digest was dried by the Speed Vac, dissolved with a small volume of a thin-layer electrophoresis buffer (water/acetic acid/pyridine 189:10:1) and subjected to electrophoresis on a silica gel plate, Silica Gel G60 (E. Merck, Darmstadt, Federal Republic of Germany) followed by thin-layer chromatography as described by Feinstein et al. (24). The map obtained was autoradiographed on a Kodak X-ray film, X-Omat AR, using the regular intensifying screen (Eastman Kodak Co., Rochester, NY).
Results
The Presence of Molecules Reacting with Anti-MB2 Antisera in the LG-10 Ia Preparation. HLA-Dw7 homozygous LG-10 cells, besides being DR7-positive, were also positive for BR4X7, a specificity associated with DR4, DR7, and DRw9, as determined by the radioimmunoassay (5). These two specificities have previously been shown to be carried on different Ia molecules (9), and thus if the human Ia system is controlled by three loci, the LG-10 cells would be expected to express a third Ia subset carrying another distinct alloantigenic specificity. The best candidate as a marker of this third component was the MB2 supertypic specificity, also called Te24 (25), that was known to be associated with DR3 and DR7 at the population level from cytotoxicity studies (121). Therefore, a $^{125}$I-labeled Ia preparation from LG-10 cells was tested with other labeled Ia preparations of various DR phenotypes for reactivity with a series of relevant antisera, including those known or supposed to possess anti-MB2 activity.

The binding data for LG-10 and two other Ia preparations are shown in Table II for the pertinent antisera. The LG-10 Ia preparation gave high binding levels in the reaction with antisera possessing anti-MB2 activity, which were raised against DR7-positive immunizers, e.g., Fe27/26 or Fe118/13, indicating that they contain anti-DR7 antibodies as well. Just as DR molecules are the dominant species of Ia molecules accounting for 70-80% of the total Ia pool, so are DR7 molecules in LG-10 cells; thus these antisera could hardly be used to reveal the minor Ia component carrying MB2 specificity unless the anti-DR7 antibodies are depleted. Only the anti-MB2 antisera raised against a DR7-negative, DR3-positive immunizer were expected to give mean-

| Antiserum | Extent of binding of $^{125}$I-labeled Ia preparation* |
|-----------|--------------------------------------------------|
|           | LG-10 (DR7, DR7) | Chevalier (DR3, DR7) | U698M (DR2, DR4) |
| Fe96/19   | 13.0               | 16.5               | 15.3               |
| Duffy     | 6.6                | 7.8                | 1.4                |
| Tellier   | 7.2                | 7.3                | 1.6                |
| Fe53/23   | 66.0               | 40.2               | 10.0               |
| Fe27/26   | 73.7               | 49.3               | 13.4               |
| Fe118/13  | 52.3               | 31.6               | 1.0                |
| Eckert    | 79.5               | 48.7               | 11.2               |
| Gnadt     | 61.7               | 40.5               | 8.4                |
| Keliher   | 66.5               | 46.0               | 13.9               |
| HON       | 8.7                | 11.0               | 11.3               |
| Fe59/15   | 7.7                | 13.3               | 28.6               |
| Swift     | 9.4                | 11.9               | 49.9               |
| T-1514    | 9.1                | 14.9               | 41.8               |
| 8-4513    | 7.2                | 13.4               | 49.0               |
| KIT13390  | 7.2                | 9.8                | 43.5               |
| Murphy    | 6.3                | 10.4               | 44.2               |
| Gallardo  | 7.1                | 14.2               | 40.5               |

* The extent of binding was determined using 5 μl antiserum by the direct binding assay and is presented as the percent Ia binding (Materials and Methods).
meaningful results. Among the antisera fitting these conditions, Fe96/19, Duffy, and Tellier were found to give a reasonably low, at most 13%, but still significant binding level. It should be pointed out that the producer of serum Duffy was DR7*, MB2*, MT3*, whereas the immunizer was DR3*, MB2*, MT2*. Anti-DR4 antisera also showed low binding, ~8%, similar to that of antiserum HON. This binding has been shown to be due to reaction with the BR4X7 subset (9, 10).

The binding characteristics of LG-10 Ia preparation with three representative alloantisera, Fe53/23 (anti-DR7, BR4X7), HON (anti-BR4X7), and Fe96/19 (anti-MB2), are depicted in Fig. 1. A clear binding plateau which indicates the presence of an Ia subset can be seen at a level of ~9% in reaction of the untreated Ia preparation with serum HON (Fig. 1A). This subset corresponds to the BR4X7 identified previously in the Ia preparations purified from two DR-heterozygous cell lines, one of DR3 and DR7 and another of DR2 and DR4 (9). The deletion of BR4X7 subset by treatment with the serum HON induced little change in the binding pattern with either of Fe53/23 and Fe96/19 (Fig. 1B). The binding level with these two sera was substantially reduced by absorption with serum Fe118/13 of DR7 and MB2 specificities (Fig. 1C). The same procedure produced a marked elevation of the binding level with serum HON: the plateau formation is now seen at a level of ~30% (Fig. 1C). This selective elimination or enhancement of the reactivity with serum HON indicates that the BR4X7 determinant recognized by serum HON is carried by a subset that is immunospecifically separable from those reacting with Fe53/23 or Fe96/19.

A similar increase of the binding level with Fe96/19 was attained by a procedure that was proven effective in increasing the proportion of Ia molecules carrying the DC1 determinant in a Daudi (DRw6, DC1) Ia preparation. This procedure involves two steps: a depletion of DR and BR molecules by the use of rabbit antiserum U115 specific to the α-chains of DR and BR molecules and a limited digestion with papain (Materials and Methods). As shown in Table III, the same procedure was found to considerably increase the proportion of Ia molecules reacting with the Fe96/19.

![Figure 1](image-url)

**Fig. 1.** The binding characteristics of LG-10 Ia preparation with three representative alloantisera. 

[^125I]-labeled LG-10 Ia preparation (220,000 cpm), (A) untreated, or (B) treated with antiserum HON (anti-BR4X7; 0.5 μl), or (C) treated with antiserum Fe118/13 (anti-D7, MB2; 25 μl), was tested for binding activity with varying amounts of antisera Fe53/23 (anti-DR7, BR4X7), HON (anti-BR4X7), and Fe96/19 (anti-MB2) by the direct-binding assay. The binding activity was expressed as the percent Ia binding and plotted against the amount of antiserum used. The antiseras used contain antibodies not reacting with LG-10 Ia preparation as specified in Table I; only the relevant specificities are given in parenthesis (above).
TABLE III

| Antiserum | Relevant specificity§ | Extent of binding of LG-10 Ia preparation‡ |
|-----------|-----------------------|---------------------------------------------|
|           |                       | Untreated | Treated with anti-Ia α subunit |
| Fe96/19   | MB2                   | 12.9      | 41.1                        |
| Fe53/23   | DR7, BR4X7            | 68.2      | 17.7                        |
| KIT 13390 | BR4X7                 | 7.2       | 7.4                         |
| Fe27/26   | DR7, BR4X7, MB2       | 80.8      | 67.0                        |

*MB2 molecules were enriched by a selective depletion of DR and BR molecules by the use of rabbit antiserum U115 specific to the α-chains of DR and BR molecules and a limited papain digestion (Materials and Methods).
‡ The extent of binding was determined using 5 μl antiserum by the direct binding assay and is presented as the percent Ia binding (Materials and Methods).
§ These antisera contain additional antibodies not reacting with LG-10 Ia preparation as specified in Table I.

Antiserum in the LG-10 Ia preparation. Binding with Fe53/23 produced against a DR7-positive, MB2-negative immunizer was reduced, whereas BR4X7 reactivity remained low. This selective increment of the binding level with Fe96/19 indicates that the alloantigenic specificity recognized by anti-MB2 antisera and the DC1 specificity may well be allelic.

Immunogenetic Analysis of the MB2 Specificity by the Radioimmunoassay. It was then determined if the determinant recognized by anti-MB2 antisera in the preceding experiments corresponds to the supertypic specificity MB2 defined by cytotoxicity with respect to population distribution, association with DR allelic specificities, and segregation in families. The results indicated that this specificity defined by the molecular binding conforms in all these aspects to the MB2 specificity.

Individuals can be typed for an Ia allospecificity by testing their peripheral blood leukocytes for inhibitory activity on the binding of a 125I-labeled Ia preparation by a specific alloantiserum (17). For the present MB2 typing by the radioimmunoassay, the U115-treated LG-10 Ia preparation described above was used as the reference antigen in combination with Fe96/19, which was found to possess the highest titer among the available anti-MB2 antisera. As shown in Fig. 2, 121 specimens from a panel of HLA-typed individuals and families were unequivocally distributed in two groups, an MB2-positive group and an MB2-negative group. In accord with the association pattern of MB2 specificity by the cytotoxicity assay, the positive cells included exclusively DR3-positive and/or DR7-positive cells. Within this group, the two phenotypes did not appear to be associated with different degree of inhibition. A substantial proportion of DR3-positive or DR7-positive individuals were found to be in the negative group. These relationships among MB2, DR3, and DR7 at the population level is more clearly illustrated by Fig. 3, which includes only unrelated individuals positive for these three specificities. 37% of DR3-positive individuals (7 out of 19) and also 37% of DR7-positive individuals (10 out of 27), excluding DR3, DR7 heterozygotes from the calculation, were MB2-negative. These proportions are similar to those found by cytotoxicity for the Te24 specificity in its relationship with
DR3 and DR7 (25). Some DR7-positive individuals have been known to be negative for the BR4X7 specificity, which is tightly associated with DR4 and DR7 (5, 9). Three such cases, cell donors 644, 539, and MF83, found in the present panel all turned out to be also MB2-negative, suggesting a linkage disequilibrium condition specifically involving the MB2 and BR4X7 specificities.

30 B cell-type cell lines available from the Roswell Park Memorial Institute, Buffalo, NY were also typed. The results (Fig. 4) are similar to those obtained in the normal population. The MB2 specificity was tightly associated with DR3 and DR7. Also in these cell lines, two DR3-positive cells, RPMI 8392 and 6410, were found to be negative for MB2 and the two DR7-positive, BR4X7-negative cases, RPMI 5287 and 8068, both turned out to be MB2-negative.

The inhibition assay of cell lines was repeated using an 125I-labeled LG-10 Ia preparation, unprocessed for the enrichment of MB2, in combination with either antiserum Fe96/19 or Duffy. The same qualitative results were obtained, suggesting the similarity of the determinants recognized by these two antisera and indicating that the enrichment procedure involving papain digestion did not affect the alloantigenic specificity of Ia molecules (data not shown).

The members of 13 families that were extensively typed during the Eighth Histocompatibility Workshop were also tested. Three families showed segregation of MB2, transmitted in coupling with DR7 in one case and with DR3 in the other two cases (Table IV). Notably, the family 8wFER01 shows segregation of all three non-DR markers: DC1, BR4X7, and MB2. No instance of crossing over between MB2 and any of the other HLA markers was found in any family studied.

Sequential Binding Experiments. The three specificities DR7, BR4X7, and MB2 have different population patterns, but this does not necessarily mean that they are all carried by distinct sets of Ia molecules, although the selective enrichment of BR4X7 molecules (Fig. 1) and of MB2 molecules (Table II) provided evidence suggesting this
Fig. 3. Schematic representation of the MB2-DR3-DR7 relationship. The figure includes only individuals positive for these three specificities. Only panel members and unrelated parents have been included. The total number of individuals typed was 101. Positivities are indicated by dots. Frames are drawn to clarify the relationships between the three specificities. DC1, MB2, and BR4X7 typing was performed by radioimmunoassay. DR typing was done by cytotoxicity assay with Eighth Histocompatibility Workshop antisera and confirmed in most cases by radioimmunoassay.

A direct proof was provided by sequential-binding experiments using the antisera corresponding to the three specificities. The 125I-labeled LG-10 Ia preparation was first allowed to react with the HON antiserum, which possesses a strong anti-BR4X7 reactivity. After removing the immune complexes by reaction with anti-human Ig antiserum, the supernatant was tested with three groups of antisera that possess anti-BR4X7, anti-MB2, or anti-DR7 activity. As shown in Fig. 5, the reactivity with anti-BR4X7 was mostly eliminated, whereas the reactivity of anti-MB2 antisera
**Ia Specificity**

| Cell line | DC1 | DC2 | MB1 | BR4X7 |
|-----------|-----|-----|-----|-------|
| Rej       |     |     |     |       |
| B35       |     |     |     |       |
| RPMI4265  |     |     |     |       |
| RPMI 8235 |     |     |     |       |
| RPMI 7850 |     |     |     |       |
| Chevoller |     |     |     |       |
| B85       |     |     |     |       |
| LG-10     |     |     |     |       |
| RPMI1788  |     |     |     |       |
| NK-9      |     |     |     |       |
| RPMI 5287 |     |     |     |       |
| RPMI 8068 |     |     |     |       |
| RPMI 8592 |     |     |     |       |
| RPMI 6410 |     |     |     |       |
| LG-2      |     |     |     |       |
| RPMI 7760 |     |     |     |       |
| RPMI 8057 |     |     |     |       |
| B89       |     |     |     |       |
| U928M     |     |     |     |       |
| B411-4    |     |     |     |       |
| CCRF-SB   |     |     |     |       |
| AL-T      |     |     |     |       |
| DND-39    |     |     |     |       |
| BT-1      |     |     |     |       |
| MT-1      |     |     |     |       |
| KM-3      |     |     |     |       |
| Reh       |     |     |     |       |
| B46M      |     |     |     |       |
| Daudi     |     |     |     |       |
| NALM-1    |     |     |     |       |

**Fig. 4.** Ia typing of 30 lymphoid cell lines. All typings were performed by radioimmunoassay. Results are presented as described in the legend to Fig. 3.

**Table IV**

*Segregation of MB2 in Three Families Studied in the Eighth Histocompatibility Workshop*

| 8WFER01 haplotypes | 8WFER09 haplotypes | 8WTOS18 haplotypes |
|--------------------|--------------------|--------------------|
| a: A2, B5, C-, DRw6 | a: A1, B8, C-, DR3 | a: A2, B7, C-, DRw6 |
| b: A2, B18, C-, DR5 | b: A26, Bw38, C-, DRw6 | b: A-, Bw50, Cw6, DR3 |
| c: A2, B5, C-, DR5  | c: A25, B3, Cw1, DRw6 | c: Aw32, Bw60, Cw2, DR3 |
| d: Aw33, Bw44, C-, DR7 | d: A26, Bw39, C-, DR2 | d: A2, B18, C-, DR5 |

| DC1 | MB2 | BR4X7 |
|-----|-----|-------|
| +   | +   | +     |
| -   | -   | -     |

| DC1 | MB2 | BR4X7 |
|-----|-----|-------|
| +   | +   | +     |
| -   | -   | -     |

| Sibling |
|---------|
| + +     |
| - -     |

* Sibling.
three Ia subsets were isolated from the 125I-labeled LG-10 preparation with the corresponding antisera and separately analyzed. Fe53/23, HON, and Fe96/19 antisera were used for the isolation of DR7, BR4X7, and MB2 subsets, respectively. For the isolation of DR7 molecules, the LG-10 preparation was pre-absorbed with the HON antiserum to remove BR4X7 molecules, because Fe53/23 possessed anti-BR4X7 reactivity. Immune complexes formed with the three antisera were absorbed on protein A-coupled Sepharose, then the Ia subunits were separated by SDS-polyacrylamide gel electrophoresis and subjected to microfingerprinting analysis after digestion with elastase. The patterns obtained for the three Ia subsets are shown in Fig. 6. The relevant features can be summarized as follows: (a) The three subsets markedly differ from each other in the β-chains. Only a group of four to five spots on the lower right (circled on Fig. 6D) is preserved. This group of peptides is present in all Ia β-chains so far analyzed and may constitute an invariant marker of these molecules. The comparison between DR7 and BR4X7 reveals little or no difference in the α-chains. In both preparations a group of intensely labeled spots is present on the bottom, corresponding to peptides of low chromatographic migration.

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**Fig. 5.** Identification of three Ia subsets by sequential binding assay. 125I-labeled LG-10 Ia preparation (420,000 cpm) was treated with serum HON (anti-BR4X7: 0.5 µl) and tested for binding activity with three groups of antisera; anti-BR4X7, anti-MB2, and anti-DR7. The labeled preparation (280,000 cpm) depleted of BR4X7 by this treatment was subsequently treated either with Fe96/19, (anti-MB2; 50 µl) or with Fe53/23, (anti-DR7, BR4X7; 25 µl) and then assayed for binding activity with two groups of antisera; anti-MB2 and anti-DR7. The binding activity was assayed with 5 µl of each antiserum. Results are expressed as the percentage of the binding given by the 125I-labeled LG-10 preparation treated with normal serum under the same conditions and shown by a histogram. Three of the anti-DR7 antisera used (Fe27/26, Fe118/13 and Eckert) apparently possessed an anti-MB2 activity. It can be seen in a weak but significant reduction (10-20%) of their binding with the LG-10 Ia preparation treated with HON and Fe96/19, and also in a weak but relatively high level (>20%) of their binding with the LG-10 Ia preparation treated with HON and Fe53/23.

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**Microfingerprinting Patterns.** Three Ia subsets were isolated from the 125I-labeled LG-10 preparation with the corresponding antisera and separately analyzed. Fe53/23, HON, and Fe96/19 antisera were used for the isolation of DR7, BR4X7, and MB2 subsets, respectively. For the isolation of DR7 molecules, the LG-10 preparation was pre-absorbed with the HON antiserum to remove BR4X7 molecules, because Fe53/23 possessed anti-BR4X7 reactivity. Immune complexes formed with the three antisera were absorbed on protein A-coupled Sepharose, then the Ia subunits were separated by SDS-polyacrylamide gel electrophoresis and subjected to microfingerprinting analysis after digestion with elastase. The patterns obtained for the three Ia subsets are shown in Fig. 6. The relevant features can be summarized as follows: (a) The three subsets markedly differ from each other in the β-chains. Only a group of four to five spots on the lower right (circled on Fig. 6D) is preserved. This group of peptides is present in all Ia β-chains so far analyzed and may constitute an invariant marker of these molecules. The comparison between DR7 and BR4X7 reveals little or no difference in the α-chains. In both preparations a group of intensely labeled spots is present on the bottom, corresponding to peptides of low chromatographic migration.
These are hydrophilic peptides that may possess a carbohydrate moiety. The MB2 α-chains possess clearly distinctive features. First, it essentially lacks the hydrophilic peptides. Second, the other spots show differences in position and intensity as compared with BR4X7 α- and DR7 α-chains. Third, it has a central spot, indicated by the arrow, which has been found for DC1 α-chains as shown for those isolated from Daudi (DRw6, DC1) cells to facilitate a direct comparison (Fig. 7). This spot has not been seen for other Ia α-chains so far analyzed (15).

Discussion
The present report shows that three antigenic specificities can be identified in Ia molecules from an HLA-homozygous cell line. These three specificities, DR7, BR4X7,
and MB2 are recognized by different alloantisera. They show HLA-linked segregation in families and they are distributed in the population according to different but partially overlapping patterns. Sequential-binding analysis and peptide mapping have shown that these three determinants are carried by distinct sets of Ia molecules; therefore, they must be controlled by different genes.

This conclusion is based on the characterization of the molecular subset carrying the BR4X7 determinant and of that carrying the MB2 determinant and on the demonstration that these two subsets are distinct from the DR7 molecular subset and are also different from each other. The evidence concerning the molecular entity of each of BR4X7 and MB2 subsets will be considered separately.

Most data exploring the relationship of BR4X7 with DR have been reported previously (5, 9). The evidence that BR4X7-carrying and DR7-carrying molecules are different entities is based on the outcome of sequential-binding experiments indicating that these molecules possess distinct alloantigenic determinants recognized by different alloantisera and that the two kinds of molecules can be separated by the use of these antisera. Thus, the molecular subset that carried the BR4X7 determinant existed in the Ia preparation purified from a DR3/DR7, and this subset was not absorbed out by either anti-DR3 or anti-DR7 antisera. Conversely, the pretreatment with anti-BR4X7 did not affect the DR3 and DR7 components. The BR4X7 subset was also demonstrated in the Ia preparation from a DR2/DR4 cell line. Similar sequential binding experiments showed that it is carried by a third molecular subset distinct from the DR2 and DR4 subsets. Therefore, we concluded that BR4X7 is controlled by a separate locus different from the HLA-DR locus. This conclusion was also confirmed here by sequential-binding analysis using an LG-10 Ia (DR7/DR7) preparation (Figs. 1 and 5). In fact, the absorption with HON serum (anti-BR4X7) did not significantly affect the binding of several different anti-DR7 antisera. The same experiment also showed that BR4X7 and MB2 determinants are on different molecules, because the absorption with serum HON left MB2 reactivity unaffected. This set of converging data, in agreement with those reported by others (10), firmly characterizes BR4X7 as an alloantigenic determinant controlled by an allele of a separate locus in linkage disequilibrium with the DR4, DR7, and DRw9 alleles of the DR locus. In the present work, additional information on BR4X7 was provided in respect to the structural features. It should be noted that a proof for the presence of two different Ia subsets primarily depends upon the sequential-binding data. In this
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In respect, the structural data are secondary: they have been used to reveal the structural relationship of Ia subsets and to define the allelic variants.

Molecular binding data on MB2 are reported here for the first time. The data indicate that most individuals who type for DR3 or DR7 are also positive for MB2, conforming to the association pattern of MB2 shown by the cytotoxicity assay. Because DR3 and DR7 were not completely included within MB2, this association must be a result of a strong disequilibrium of linked gene products. After the association of DC1 with DR1, DR2, and DRw6, and the association of BR4X7 with DR4, DR7, and DRw9, this is the third example of multiple linkage disequilibrium in the human Ia system. The degree of linkage disequilibrium is extremely high in the sense that MB2 is found to be invariably associated with either DR3 or DR7. Such an extreme degree of linkage disequilibrium must reflect a close relation of the corresponding loci and, perhaps, a selective advantage of the observed haplotypic combinations in addition.

It is certain that the MB2 specificity is controlled by a third Ia locus differing from the loci that encode DR7 and BR4X7. Also, it is very likely that MB2 and DC1 are allelic products of the same locus. The demonstration that the frequencies of MB1, equivalent to DC1, and MB2 are in Hardy-Weinberg equilibrium supports the notion that DC1 and MB2 are allelic (11). Moreover, the present immunochemical analyses have provided data indicating structural similarities between DC1 and MB2 molecules. First, MB2 molecules differ markedly in both α- and β-subunits from either DR7 or BR4X7 molecules. Likewise, DC1 molecules have been shown to differ in both subunits from Ia molecules carrying different DR specificities (6) and also from the BR4X7 molecules. Second, the microfingerprinting patterns of MB2 α-chains and DC1 α-chains show some common features (Figs. 6C and 7A). They essentially lack labeled glycopeptides and share an invariant spot not possessed by the DR or BR4X7 α-chains so far analyzed. Third, neither DC1 nor MB2 molecules react with rabbit anti-Ia α-subunit antiserum U115, which binds DR and BR4X7 molecules; indeed, this was exploited to obtain Ia preparations enriched in DC1 or MB2 molecules.

The data presented show the presence of a minimum of three HLA-linked loci: DR locus, a locus that encodes DC1 or MB2, and a locus that encodes BR4X7. To assess the real number of HLA genes involved and their correspondence with the Ia subunits, the problem of the localization of the serological markers at the subunit level must be considered. All available evidence indicates that DR determinants are located either predominantly or exclusively on the β-chain. In fact, when dissociated Ia subunits were analyzed for their binding with specific alloantisera, it was invariably found that only the β-subunits could react (3, 9, 15). Supporting evidence has been derived from structural studies showing that the α-chains of Ia molecules carrying different DR specificities are very similar, whereas the β-chains are different (26-28). As for the BR4X7 determinant, anti-BR4X7 antisera were found to react only with isolated Ia β-chains (9). This evidence is supported by the finding that BR4X7 β-chains are different from DR7 β-chains (Figs. 6D and E), whereas their α-chains are very similar (Figs. 6A and B). Similar observations have been made for Ia preparations of different phenotype, i.e., DR3 and DR7 or DR2 and DR4, by the use of a different proteolytic enzyme, pepsin, in the microfingerprinting analysis. Therefore we conclude that the loci controlling DR7 and BR4X7 β-chains must be HLA-linked. As to the gene or
genes controlling the corresponding α-chains, it is possible that, in analogy with the mouse, they are also HLA-linked, but as long as their alloantigenic determinants are not detected this hypothesis cannot be easily tested in the human system.

The situation concerning the DC1 and MB2 determinants must be considered with more caution. Previously reported data showed that the isolated β-chains, but not α-chains, react with anti-DC1 antisera (3). However this lack of reactivity cannot be considered a proof, because the subunits were dissociated by a denaturing procedure that may eliminate some determinants. The microfingerprinting patterns of MB2 and DC1 molecules show clear differences in the β-subunits (Figs. 6F and 7B). The α-subunits show, besides the similarities discussed above, clear differences in several spots that are certainly more conspicuous than those observed between different DR molecules (Figs. 6C and 7A). Therefore the possibility that for DC1 and MB2 molecules the alloantigenic determinants may reside on both subunits should not be disregarded. If this is the case, it will follow that DC α-chains are controlled by an HLA-linked locus because the DC determinants so far defined, wherever they are located, are clearly inherited as HLA markers.

The data presented also confirm a unique feature in the human Ia system, i.e., the phenomenon of selective subunit association. In the mouse, Aβ and Eβ subunits are associated with different α subunits (29). In man, the same can be said at least for DR7 and BR4X7 molecules from one side and DC1 and MB2 molecules on the other side. It is possible that DR7 α- and BR4X7 α-subunits are not identical. In fact, Markert and Cresswell (30) have reported the presence of three α-chains of different isoelectric points in a DR-homozygous cell line. Whatever the mechanism is, it is possible that this selective subunit association, which depends on the linkage between loci for the two subunits, underlies some distinct functional property of the different Ia subsets.

Summary

Three distinct molecular subsets with different structures and alloantigenic determinants were identified in human Ia antigens from cells of an HLA-Dw7 homozygous cell line. The subsets carried DR7 specificity, BR4X7 supertypic specificity and MB2 supertypic specificity, respectively, and were immunospecifically separated by the use of operationally monospecific alloantisera. These specificities showed HLA-linked segregation in families and they were distributed in the population according to different but partially overlapping patterns. On peptide mapping analysis, the three subsets showed marked differences in the β-chains. The α-chains of DR7 and BR4X7 subsets were very similar to each other, whereas the α-chains of MB2 subset were distinctive from those of DR7 and BR4X7. These data indicate the presence of a minimum of three HLA-linked loci; DR locus, a locus that encodes BR4X7, and a locus that encodes MB2, and substantiate the three-loci concept for the genetic control of human Ia antigens.

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