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Immunology

# Distinct forms of both α and β subunits are present in the human Ia molecular pool

(human Ia subsets/monoclonal antibodies/two-dimensional peptide maps)

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**ABSTRACT** **Two distinct subsets of human Ia molecules, called NG1 and NG2, present in all individuals irrespective of their HLA-DR phenotype, which were previously defined by their reactivity with two monoclonal hydridoma antibodies, D1–12 and D4–22, were analyzed by two-dimensional peptide mapping techniques. Results show that, in the Ia molecular pool from a single individual, small β subunits of the NG1 and NG2 subsets display significant differences from each other. In addition, β subunits of the same subset from two different allotype Ia molecular pools are also different from each other, thus indicating that NG1 and NG2 subsets carry polymorphic specificities. Moreover, large α chains of NG1 and NG2 subsets are different from each other; however, no significant differences are observed in α chains of the same subset when different allotype Ia pools are analyzed. The possible genetic implications of these findings are discussed.**

Human Ia molecules are glycoproteins that consist of two noncovalently linked polypeptide chains: a large subunit (α) of 34,000 daltons, and a small subunit (β) of 28,000 daltons (1, 2). It has been reported that these chains are coded for by two distinct loci (3). At least one of these loci is located within the major histocompatibility complex (MHC) and is responsible for the different polymorphic specificities defined by alloantisera. This locus is referred to as *HLA-DR*. In contrast to earlier reports (4), recent biochemical studies have shown that polymorphism is a characteristic of the β subunit (5–7). Thus, it has been concluded that the *HLA-DR* locus codes for the small Ia chain. Until now no clear evidence was available on the location of the gene(s) coding for the large chain, although, in analogy with the mouse Ia system, the possibility exists that these genes are also included in the MHC (8, 9).

By using two monoclonal hydridoma antibodies, D1–12 and D4–22, we have demonstrated the existence of two distinct subsets or isotypes of Ia molecules (10, 11). The two isotypes, named NG1 and NG2, are present in all Ia preparations tested so far irrespective of their DR phenotype. Because each isotype reacts with its corresponding monoclonal antibody through its β subunit (11), it is possible that serologically defined allospecificities are associated with both isotypes. Therefore, the purpose of this study was to determine the extent of structural differences between Ia subsets and to demonstrate the presence of allelic polymorphism in both isotypes. The results indicate that β chains of both the NG1 and NG2 subsets carry the same allospecificity and that two invariant α chains, $\alpha_1$ and $\alpha_2$ (different from each other), are always associated to the corresponding subset.

## MATERIALS AND METHODS

**Hybridoma Monoclonal Antibodies.** D1–12 and D4–22 monoclonal hybridoma antibodies were obtained by fusing spleen cells of BALB/c mice immunized with membrane-enriched fractions of the human lymphoblastoid cell line Daudi and the mouse myeloma cell lines P3-X63-Ag8 (D1–12) and P3-NSI/1 Ag4 (D4–22). The analysis of specificity and the demonstration that the two monoclonal antibodies recognize two different subsets of human Ia molecules have been described (10, 11).

**Preparation of Purified Ia Molecules.** The Ia molecules were prepared by the immunoabsorption method as described (12). About $2 \times 10^7$ Raji or IBW9 cells were lyzed with 0.5% Nonidet P-40 and centrifuged to remove nuclei and cell debris; the lysate was rotated with either D1–12 or D4–22 antibody bound to Sepharose 4B (50 μl of packed immunoadsorbent) for 3 hr at 4°C. To avoid nonspecific adsorption, especially of actin, which is known to bind nonspecifically to all immunoprecipitates, incubation with specific immunoadsorbents was preceded by incubation of the cell lysate with purified mouse IgG fraction coupled to Sepharose.

After washing, the antigens were eluted by boiling with an equal volume of 10% (wt/vol) $NaDodSO_4$.

The eluted proteins were labeled with $^{125}I$ by the chloramine T method (13) and were separated on 10% $NaDodSO_4$/polyacrylamide gel electrophoresis in nonreducing conditions with the discontinuous Tris buffer system (14).

**Two-Dimensional Peptide Maps of Separated α and β Chains from Ia Molecules.** The bands corresponding to α and β chains after electrophoresis were separately cut from the dried gel and eluted with phosphate-buffered saline/0.1% $NaDodSO_4$.

After complete reduction with 20 mM dithiothreitol followed by alkylation with 60 mM iodoacetamide in the presence of bovine serum albumin carrier (50 μg/ml), α and β chains were digested with pepsin (1:50 enzyme/protein ratio) 18 hr at 37°C in 100 μl of formic acid/acetic acid/water, 1:4:45 (vol/vol). Two-dimensional peptide maps on silica gel plates, based on Feinstein's modification (15) of the method of Mole (16), were obtained by spotting side-by-side aliquots of the different digests to be compared. After electrophoresis in one dimension at pH 3.5, the plates were cut into halves, and chromatography was performed at right angles in *n*-butanol/acetic acid/water/pyridine, 75:15:40:50 (vol/vol), as described (12).

## RESULTS AND DISCUSSION

IgG fractions from ascites of monoclonal hydriboma antibodies D1–12 and D4–22 were covalently bound to CNBr-activated Sepharose 4B (Pharmacia). Nonidet P-40 cell lysates of Raji cells (HLA-DR 3,6) and IBW9 cells (HLA-DR 7,7; gift of M. Trucco, Basel Institute for Immunology, Basel, Switzerland) were incubated with either antibody D1–12 or D4–22 immunoadsorbents. The specifically bound material was eluted with 10% $NaDodSO_4$, labeled immediately with $^{125}I$ by the chloramine

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Abbreviation: MHC, major histocompatibility complex.

T method (13), and then analyzed on $NaDodSO_4$/polyacrylamide gel electrophoresis as described (14). The electrophoretic pattern of the material purified from the Raji cell lysate by either immunoadsorbent (Fig. 1) showed two bands of 28,000 and 34,000 daltons, respectively, corresponding to the $\beta$ and $\alpha$ subunits. The same pattern was observed when IBW9 cell lysate was used (data not shown).

The bands were cut from the dried gels, eluted, and digested with pepsin. Peptide maps were obtained by spotting an aliquot ($\approx$100,000 cpm) from each of the digests to be compared side-by-side on a silica gel plate. Following electrophoresis in one dimension, the plates were cut into halves and chromatography was performed at right angles.

**Analysis of NG1 and NG2 $\beta$ Chains.** Fig. 2 shows that substantial differences existed between the $\beta$ chain of Raji cell Ia molecules purified on D1–12 (anti-NG1 antibody) immunoadsorbent (Fig. 2*A*) and that purified on D4–22 (anti-NG2 antibody) immunoadsorbent (Fig. 2*B*), the arrows pointing to peptides unique to either $\beta$ chain. A similar degree of diversity was observed when the peptide maps of NG1 and NG2 $\beta$ chains from the homozygous cell line IBW9 were compared (Fig. 2 *C* and *D*, respectively), arrows again pointing to peptides unique to either NG1 or NG2 $\beta$ chain.

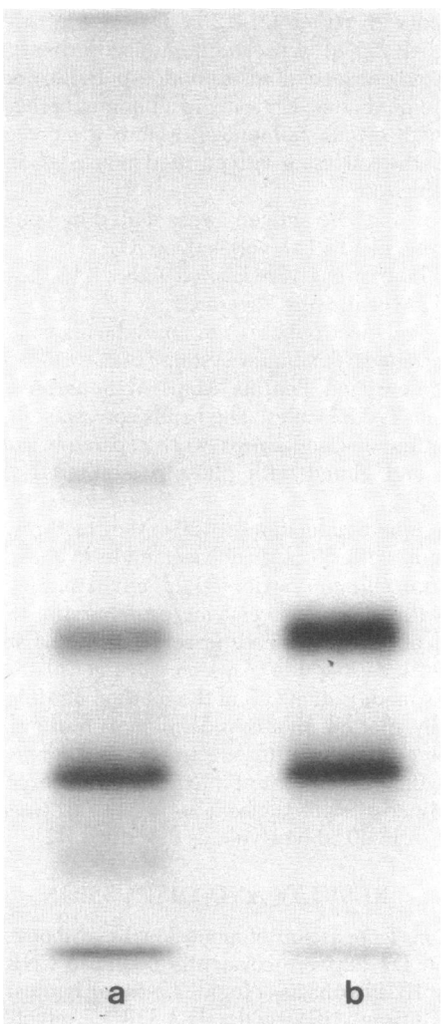


FIG. 1. Autoradiograph of a 10% $NaDodSO_4$/polyacrylamide slab gel of unreduced $\alpha$ and $\beta$ chains of $^{125}$I-labeled Ia antigens from Raji cell lysate. The electrophoresis was carried out in slab gels using the discontinuous Tris buffer system (14). Lanes: a, Ia molecules eluted from D1–12 (anti-NG1 antibody) immunoabsorbent; b, Ia molecules eluted from D4–22 (anti-NG2 antibody) immunoabsorbent.

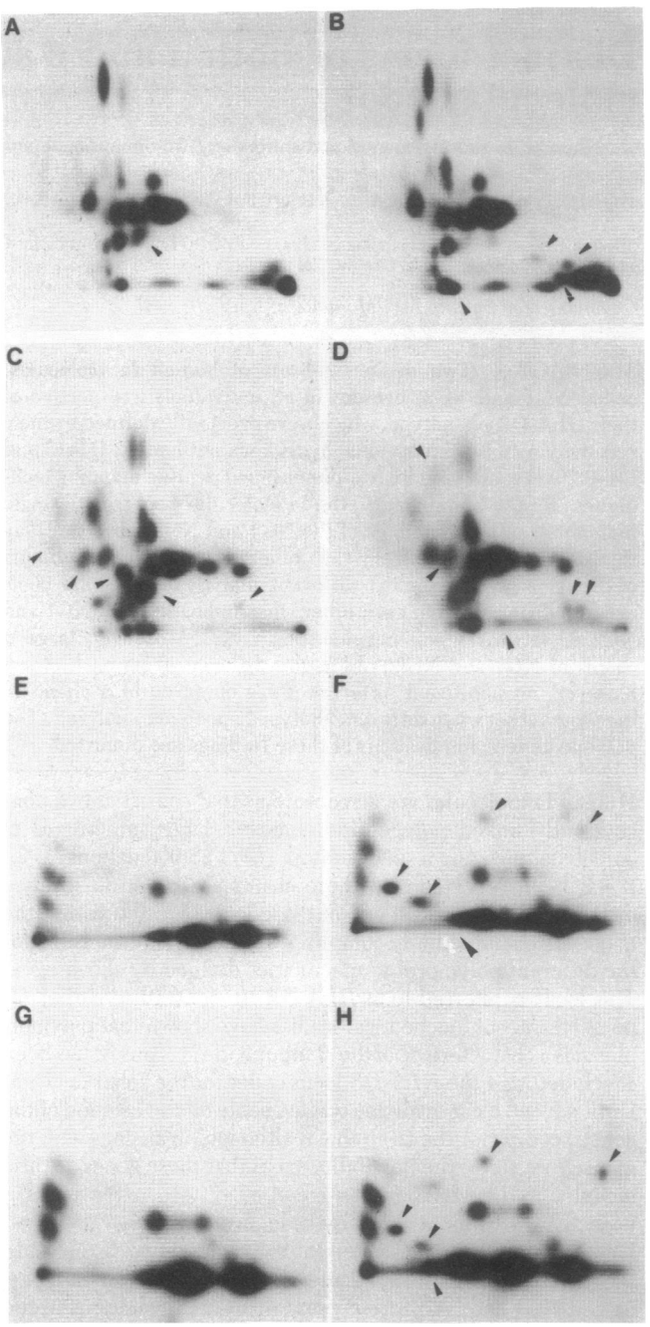


FIG. 2. Autoradiographs of peptide maps of $^{125}$I-labeled $\alpha$ and $\beta$ chains of NG1 and NG2 Ia subsets purified from Raji (HLA-DR 3,6) and IBW9 (HLA-DR 7,7) cells: NG1 $\beta$ chains from Raji (*A*) and IBW9 (*C*) cells; NG2 $\beta$ chains from Raji (*B*) and IBW9 (*D*) cells; NG1 $\alpha$ chains from Raji (*E*) and IBW9 (*G*) cells; NG2 $\alpha$ chains from Raji (*F*) and IBW9 (*H*) cells. Arrows point to unique peptides of either NG1 or NG2 subsets within the same Ia preparation.

These results confirm that monoclonal antibodies D1–12 and D4–22 react with two distinct Ia molecular species which differ in their $\beta$ chain (11). In addition, the comparison between $\beta$ chains of the same subset (compare Fig 2 *A* and *C* or *B* and *D*) reveals also important structural differences. In most cases, such differences are due to peptides shared between the NG1 and NG2 subsets within the same Ia preparation. These results are compatible with the allotypic variations expressed by the two Ia preparations, namely from Raji and IBW9 cell lysates,

and, therefore, indicate that NG1 and NG2 Ia subsets carry polymorphic specificities.

Because NG1 and NG2 $\beta$ chains can carry polymorphic specificities, one could imagine the nonpolymorphic part of each subset to be shared among various Ia molecules with different DR specificities, so that isotype differences between NG1 and NG2 should be represented by the same set of spots irrespective of the DR allospecificity analyzed. Results show that this assumption is only partially true because few NG1 isotype-specific peptides or NG2 isotype-specific peptides are in fact shared in the two Ia preparations analyzed. One possible explanation for this apparent discrepancy could be the different "ratio" between allotype portion and isotype portion present in Ia molecules from Raji and IBW9 cells.

The analysis of a larger panel of Ia molecules, especially from DR homozygous cell lines will better clarify this problem. Experiments to determine whether D1–12 and D4–22 monoclonal antibodies bind also to other minor subsets of Ia molecules with differences in $\beta$ chain structures, such as DC1 (5) and BR 4 × 7 (17) molecules, may add new elements to the observed structural variations.

**Analysis of NG1 and NG2 $\alpha$ Chains.** When the peptide maps of $\alpha$ chains of either NG1 and NG2 isotypes were analyzed, a series of important features were observed. Like $\beta$ chains, distinct $\alpha$ chains were associated to NG1 and NG2 subsets in each of the Ia preparations analyzed (Fig. 2 *E* and *F* from Raji cells, *G* and *H* from IBW9 cells). However, in contrast to $\beta$ chains, $\alpha$ chains associated to the NG1 subset (Fig. 2 *E* and *G*) had a strikingly similar peptide pattern. In fact, the two-dimensional chromatographs of the $\alpha$ subunits of Ia molecules from both Raji and IBW9 cells reacting with D1–12 antibody were virtually superimposable. The same was true for the $\alpha$ chains of the NG2 subset.

These results indicate that only an "isotype-like" and not an "allotype-like" type of diversity exists for the large Ia subunits. The absence of an allotype-like type of polymorphism in the $\alpha$ chains strongly supports the idea that the observed serological allospecificities are indeed confined to the $\beta$ subunit (5–7). In addition, because the two monoclonal antibodies recognize distinct antigenic determinants on two different $\beta$ chains (11), the above findings suggest a specific association between a given $\alpha$ and a given $\beta$ chain within the same Ia subset.

Taken together, these observations may help to understand the arrangement of the genes responsible for Ia polymorphism at the *HLA-DR* locus. Among the models which can be envisaged to account for these results, we favor those depicted in Fig. 3. A conservative view of gene organization is proposed.

The allotype locus (*DR*) is envisaged as a gene separated from the *NG* isotype cluster (Fig. 3, model a) because both $\beta$-NG1 and $\beta$-NG2 carry allospecific determinants. Alternatively (Fig. 3, model b), each different $\beta$-*NG* gene can code for the entire $\beta$ chain, including the allotype portion; in this case, a common allotype-related DNA sequence must be present in each single $\beta$-*NG* gene.

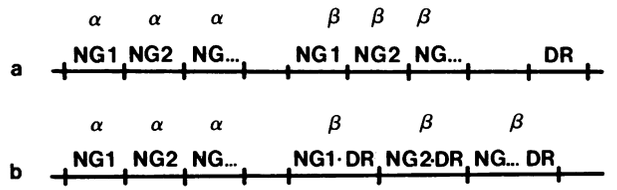


FIG. 3. A model for genes encoding the $\alpha$ and $\beta$ polypeptides of human HLA-DR antigens. Models: a, The *DR* locus is assumed to code only for the allospecific determinants of the $\beta$ chain, and it is separated from both the $\beta$ and the $\alpha$ isotype clusters; b, the $\beta$ isotype cluster includes as an integral part of its single components a repeated DNA sequence specific for coding the allopolymorphic part of the $\beta$ chain.

Within the isotype cluster, we include the genes responsible for the different $\alpha$ chains observed. In analogy with the mouse Ia system, the genetic system proposed is thought to be located inside the MHC, in the human chromosome 6. However, there is no existing evidence opposing the possibility that $\alpha$ chains are encoded by an autonomous set of genes located outside the MHC in a separate region of the chromosome 6 or even in a different chromosome.

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