

## ANALYSIS OF THE STRUCTURAL HETEROGENEITY AND POLYMORPHISM OF HUMAN Ia ANTIGENS

Four Distinct Subsets of Molecules Are Coexpressed in the Ia Pool of  
Both DR 1,1 Homozygous and DR 3,W6 Heterozygous B Cell Lines

By ROBERTO S. ACCOLLA

*From the Ludwig Institute for Cancer Research, Lausanne Branch, 1066 Epalinges, Switzerland*

In recent years considerable interest has been focused on the family of polymorphic human major histocompatibility complex (MHC)<sup>1</sup>-encoded products designated class II or Ia antigens because of their central role in the homeostasis of the immune system (reviewed in reference 1). These antigens are expressed at the cell surface of B cells, macrophages/monocytes, and activated T cells as heterodimers comprising a large subunit ( $\alpha$  chain) of 33–36,000 daltons and a small subunit ( $\beta$  chain) of 24–29,000 daltons (2). A third glycoprotein of ~31,000 daltons appears to be associated to the  $\alpha$ - $\beta$  heterodimer intracellularly (3). This glycoprotein has been called invariant because it exhibits no electrophoretic polymorphism (4). The apparent involvement of human Ia antigens in a variety of biological functions as well as in the susceptibility to certain diseases (reviewed in reference 5) has led many investigators to analyze the structural heterogeneity of the human Ia pool as an important step toward possibly correlating a given structure with a specific function. From the studies of many groups it is now well accepted that the human Ia antigens consist of a heterogeneous family of molecules with distinctive structural characteristics (6–18). These include the classical HLA-DR molecules of which at least 10 allospecificities have been identified (19); the DC molecules (9, 10), with more limited polymorphism than DR antigens and in strong linkage disequilibrium with them; the polymorphic SB molecules (11, 20) encoded by genes located centromerically to the DR genes (21).

Recently our group reported on the biochemical characterization of two human Ia subsets, the NG1 and NG2 (7). These subsets are present in all individuals irrespective of their DR phenotype (6), they differ at the level of both  $\alpha$  and  $\beta$  subunits and are coexpressed at the single cell level (22).

The availability of monoclonal antibodies (Mab) recognizing distinct subsets of Ia molecules, such as NG1, NG2, or DC1, prompted us to investigate the structural relationships of the various subsets within the same Ia pool. In this report we describe the biochemical analysis of these subsets in two distinct cell

<sup>1</sup> Abbreviations used in this paper: D1, monoclonal antibody D1-12; D4, monoclonal antibody D4-22; 2DPM, two-dimensional peptide maps; H40, monoclonal antibody H40, 315.7; Mab, monoclonal antibody produced by hybridoma cell lines; MHC, major histocompatibility complex; SDS-PAGE, NaDodSO<sub>4</sub>, polyacrylamide gel electrophoresis; 3/4, monoclonal antibody BT3/4.

lines, LG2 (DR 1,1-DC1) and Raji (DR 3,W6-DC1). Four Mab were used to isolate the various Ia molecules: D1-12, a putative anti-NG1 antibody (6, 7); D4-22, a putative anti-NG2 antibody (6, 7); and two putative anti-DC1 antibodies, BT3/4 (10) and H40.315.7 (23-25).

This study has permitted us to assess the degree of heterogeneity as well as the degree of structural polymorphism of the distinct components of the human Ia family analyzed.

### Materials and Methods

*Monoclonal Antibodies.* The monoclonal antibodies D1-12, D4-22, BT3/4, and H40.315.7 used in this study have been previously described (6, 10, 26). For sake of simplicity we will refer to them in the text as D1 (D1-12), D4 (D4-22), 3/4 (BT3/4), and H40 (H40.315.7).

*Human Cell Lines.* The human cell lines used in this study were the Raji cells (DR3,W6-DC 1) and the LG-2 cells (DR1,1-DC1). The latter was kindly donated by Dr. W. Leibold, Erlangen, FRG. The cells were propagated in RPMI 1640 medium supplemented with 10% fetal calf serum, glutamine and antibiotics as previously described (6).

*Preparation of Purified Ia Molecules and Crossabsorption Procedures.* The Ia molecules were prepared by the immunoabsorption method as described (27); briefly 10 mg of purified IgG fraction of each Mab was covalently bound to 1 g dried weight CNBr-activated Sepharose 4B beads (Pharmacia, Uppsala, Sweden) by standard procedures.  $20 \times 10^6$  cells were lysed by incubation at 4°C for 15 min in 0.5% NP40 in PBS. After elimination of the insolubilized material by centrifugation, the cell lysate was incubated for 3 h at 4°C with 200  $\mu$ l of packed normal mouse IgG-Sepharose beads to remove aspecifically bound material and then for 3 h at 4°C with 100  $\mu$ l of packed Mab-specific Sepharose beads under rotation. After the Sepharose was washed, the specific bound material was eluted with an equal vol of 5% (wt/vol) NaDodSO<sub>4</sub> (SDS). The eluted material was labeled with <sup>125</sup>I by the chloramine T method (28) and was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in nonreducing conditions following Laemmli's technique (29). For crossabsorption studies, after incubation with the packed Mab-specific Sepharose, the cell lysate was reincubated twice with the same immunoabsorbent following the procedure described above. By using this protocol complete depletion of the corresponding Ia molecules was always achieved with all 4 Mab used in this study. The resulting "cleared" cell lysate was then incubated with either one of the three remaining Mab-specific Sepharose immunoabsorbents for an additional 3 h at 4°C. After washes, the specific bound material was eluted, labeled, and separated by SDS-PAGE as described above. The preparative electrophoresis in SDS polyacrylamide gel we used (11% polyacrylamide concentration in nonreducing condition, running gel 10 cm high) allowed us a separation between  $\alpha$  and  $\beta$  chains of ~3 cm. To recover the distinct subunits from the dried gel we used as template the autoradiographic film and only 1/2-cm strips corresponding exactly to the width of the band, were cut and eluted (see below). The accuracy of this procedure rules out the possibility that the preparations of either  $\alpha$  or  $\beta$  subunits of the various molecular subsets analyzed in this study were contaminated with  $\beta$  or  $\alpha$  subunits, respectively.

*Two-dimensional Peptide Maps (2DPM) of Separated  $\alpha$  and  $\beta$  Subunits.* The technique has been previously described in detail (27). Briefly,  $\alpha$  and  $\beta$  subunits of Ia molecules recognized by the distinct Mab in the various experimental conditions (before and after absorption) were separately cut from the dried gel and eluted in 0.1% SDS in PBS. The eluted material was reduced with 20 mM dithiothreitol for 60 min, and alkylated with 60 mM iodoacetamide for 30 min. Peptic digestion was performed in 100  $\mu$ l of formic acid/acetic acid/water 1:4:45 (vol/vol) with pepsin in the presence of bovine serum albumin carrier (1:50 enzyme/protein ratio) for 16 h at 37°C. 2DPM on silica gel plates (Merck cat. 5748) were obtained by spotting side-by-side aliquots of the various digests to be compared. Electrophoresis was performed as first dimension in a Desaga Desaphor

apparatus (Desaga, Heidelberg, FRG) equipped with Desaphor cooling plates (cat 146621) to avoid overheating during migration. After electrophoresis the plates were cut into halves and chromatography was performed as second dimension at right angles in n-butanol/acetic acid/water/pyridin 75:15:40:50 (vol/vol).

### Results

*Crossabsorption Studies Using the Four Distinct Mab D1, D4, 3/4, and H40.* The monoclonal antibodies D1 and D4, which have been previously used to define the NG1 and the NG2 subsets, respectively, were used to purify the corresponding Ia molecules from two different cell lines, LG-2 (DR1,1-DC1) and Raji (DR3,W6-DC1).

Fig. 1 shows that the molecules of the LG2 Ia pool that were eluted from D1 (lane *a*) or D4 (lane *b*) Mab coupled to Sepharose beads both appeared as 34–26,000 daltons heterodimers.

In order to assess the relationships between D1- and D4-reactive molecules, crossabsorption experiments were performed. Samples of LG2 cell lysates were first depleted of either D1- or D4-reactive molecules and then absorbed to and eluted from D4 or D1 immunoabsorbents, respectively. It can be seen that after absorption of D1-reactive molecules, there were still Ia molecules which reacted with D4 Mab (lane *c* in Fig. 1). On the contrary, after absorption of D4-reactive molecules, no residual reactivity with D1 Mab was observed (lane *d*). Similar results were obtained when Raji cell lysates were analyzed (data not shown).

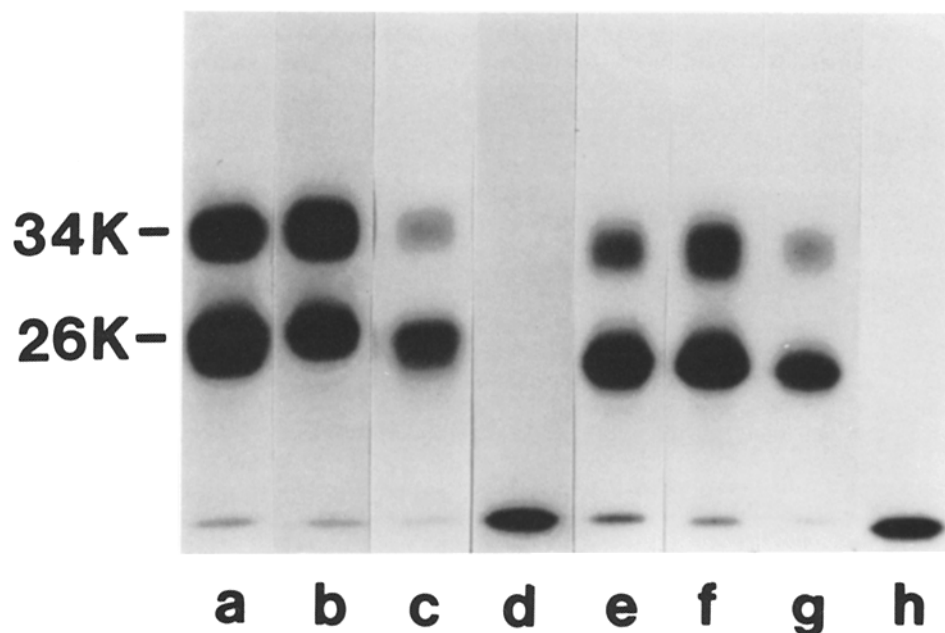


FIGURE 1. Autoradiograph of 11% SDS-polyacrylamide slab gels of unreduced  $^{125}\text{I}$ -labeled Ia antigens from LG2 cell lysate. The electrophoresis was carried out by using the discontinuous Tris buffer system (29). Lanes-Ia molecules eluted from the following Mab-immunoabsorbents: *a*) D1; *b*) D4; *c*) D4, after absorption of the D1 reactive population; *d*) D1, after absorption of the D4-reactive population; *e*) H40; *f*) 3/4; *g*) 3/4, after absorption of the H40-reactive population; *h*) H40, after absorption of the 3/4-reactive population.

As shown in Fig. 1, the molecules in LG2 cell lysates that were recognized by the putative anti-DC1 Mabs, H40 (lane *e*) and 3/4 (lane *f*), displayed a  $\alpha$ - $\beta$  heterodimeric structure with an apparent mol wt of 33,000 ( $\alpha$  chain) and 24,000 ( $\beta$  chain) daltons. Thus, in agreement with previous findings,  $\alpha$  and  $\beta$  subunits of Ia molecules recognized by H40 and 3/4 Mab were of faster electrophoretic mobility with respect to the corresponding subunits of Ia molecules recognized by D1 and D4 Mab (10, 25). The distinction between the H40- and 3/4-reactive molecules vs. the D1- and D4-reactive molecules was confirmed by crossabsorption studies. The depletion of either D1- or D4-reactive molecules had no effect on Ia molecules reacting with H40 and 3/4 Mab; similarly, depletion of either H40- or 3/4-reactive molecules had no effect on the Ia molecules defined by D1 and D4 Mab. These results were obtained with both LG2 and Raji cell lysates (data not shown).

Crossabsorption experiments were then performed to analyze the relationships between H40- and 3/4-reactive molecules. Fig. 1 shows the results obtained with LG2 cell lysates. Absorption with H40 Mab reduced but did not eliminate the Ia molecules reacting with 3/4 Mab (lane *g*). However, after absorption with 3/4 Mab, no residual reactivity of the lysate with H40 Mab was observed (lane *h*). Similar results were obtained with Raji cell lysates (data not shown).

To summarize, the crossabsorption experiments suggested the existence of four distinct subsets of molecules: (a)  $D1^+D4^+$  with mol wt 34–26,000 daltons; (b)  $D1^-D4^+$  with mol wt 34–26,000 daltons; (c)  $H40^+3/4^+$  with mol wt 33–24,000 daltons; (d)  $H40^-3/4^+$  with mol wt 33–24,000 daltons.

*Structural Analysis of the Human Ia Pool.* In the following description of the structural analysis of the various Ia subsets mentioned above, the data concerning the Ia populations recognized by D4 Mab and 3/4 Mab before the absorption of the D1- and H40-reactive molecules, respectively, are included for comparison. Therefore, the  $D1^+D4^+$  subset will be described as the one recognized by D1 Mab; similarly the  $H40^+3/4^+$  subset will be described as the one recognized by H40 Mab. Furthermore, comparisons between homologous subunits of distinct Ia molecules will be discussed on the basis of the actual number of major peptides identifiable in the corresponding fingerprints. The computation of peptides and the evaluation of those peptides shared between the various fingerprints have been carefully made by considering several parameters.

To rule out the possibility that quantitative differences might influence the determination of the total number of peptides as well as the number of the shared ones, several autoradiographic exposures of the same fingerprint have been analyzed. Furthermore we have taken into account the fact that small variations in the peptide migration pattern may occur in the 2DPM technique used in this study. For this reason the relative migration pattern of sets of peptides, unambiguously identifiable as common ones in two fingerprints to be compared, was adopted as a reference to assess the relative migration pattern of the remaining peptides. In this way we could minimize the error of enumerating as "distinct peptides" those which, indeed, were shared between two fingerprints.

*$\beta$  Chains of the  $D1^+D4^+$  and  $D1^-D4^+$  Ia Subset.* The Ia molecules recognized by D1 Mab as well as those recognized by the D4 Mab before and after absorption of the D1-reactive molecules were analyzed at the structural level by using a very

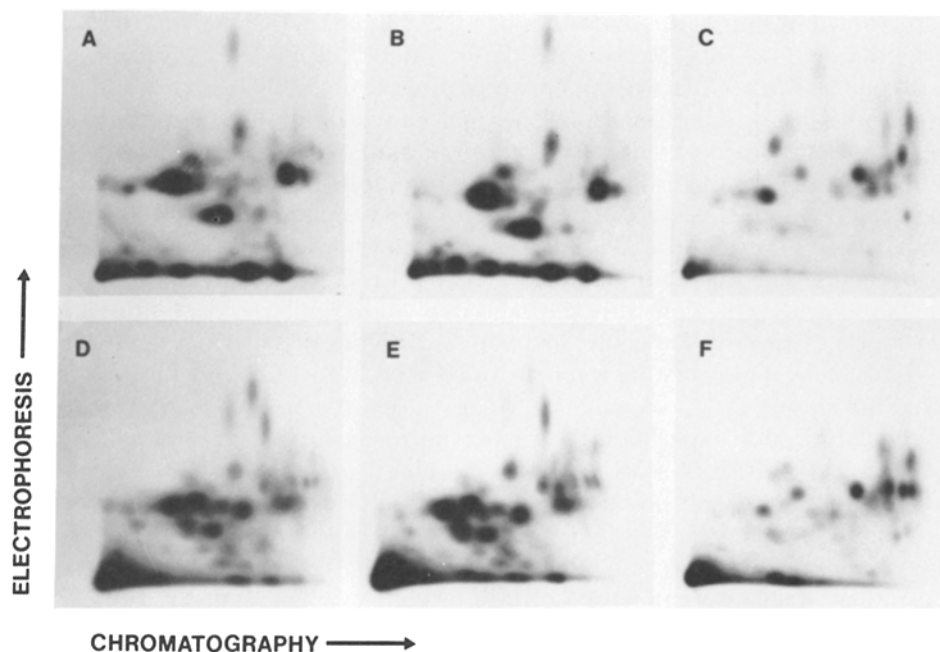


FIGURE 2. Autoradiographs of peptide maps of  $^{125}\text{I}$ -labeled  $\beta$  chains from LG2 (upper panels) and Raji (lower panels) Ia molecules bound by the following Mab: D1 (A and D); D4 (B and E), before absorption of the D1-reactive population; D4 (D and F), after absorption of the D1-reactive population. For detailed description of the fingerprints, see the text and Table I.

sensitive 2DPM technique. Fig. 2 shows the 2DPM of the Ia  $\beta$  chains from LG2 cells (upper panels) and Raji cells (lower panels) which reacted with D1 Mab (A and D), or D4 Mab before (B and E) and after depletion (C and F) of the subset reacting with D1 Mab.

The D1-specific  $\beta$  chains displayed distinct 2DPM when LG2 and Raji Ia pools were compared (panels A and D, respectively). Both qualitative and quantitative differences were observed. Among the 25 recognizable major peptides in the D1  $\beta$  chain of LG2 cells and the 29 peptides in the corresponding subunit of Raji cells, only 12 could be unambiguously defined as shared peptides. Similarly, the 2DPM of the D4  $\beta$  chains before absorption of D1-reactive molecules showed qualitative and quantitative differences between LG2 and Raji cells (compare panels B and E). However the D4 $\beta$  chain 2DPM showed only limited structural differences when compared to the 2DPM of the D1 $\beta$  chains within the same Ia pool (compare panel B with panel A, and panel E with panel D).

It was therefore important to assess the structural characteristics of the  $\beta$  chains recognized by D4 Mab after absorption of D1-reactive molecules. Fig. 2 shows the results obtained. It can be seen that both in LG2 and Raji cells the D1 $^-$ -D4 $^+$   $\beta$  2DPM was strikingly different with respect to that of the D1 $^+$ -D4 $^+$   $\beta$  chains (compare panel C with A, and F with D, respectively). Many of the major peptides present in the D1 $^+$ -D4 $^+$   $\beta$  chains 2DPM were absent or highly reduced in intensity in the D1 $^-$ -D4 $^+$  corresponding fingerprints. Furthermore a series of peptides barely visible, if not undetectable, before absorption of the D1-specific

Ia subset, were clearly represented in the 2DPM of the  $D1^-D4^+$   $\beta$  population. The differences observed are expressed in numbers in Table I: 24 and 27 major peptides were present in the  $D1^-D4^+$   $\beta$  chain fingerprints of LG2 and Raji, respectively. Of those, only 11 and 13 were shared with the  $D1^+D4^+$  corresponding 2DPM.

However, the most striking characteristics of the  $D1^-D4^+$   $\beta$  chains consisted in the relatively high homology between the corresponding 2DPM of LG2 and Raji cells (compare panels *C* and *F* in Fig. 2). In fact, 23 out of 24 and 27 major peptides were shared.

*$\alpha$  Chains of the  $D1^+D4^+$  and  $D1^-D4^+$  Ia Subsets.* Fig. 3 shows the 2DPM of  $\alpha$  chains from LG2 (upper panels) and Raji (lower panels) Ia molecules recognized by D1 Mab (panels *A* and *D*) and D4 Mab before (panels *B* and *E*) and after (*C* and *F*) absorption of D1-reactive molecules. Unlike the corresponding  $\beta$  chains, D1  $\alpha$  chains in both LG2 and Raji cells did not display structural differences (compare panel *A* with panel *D*). In both cases 17 major peptides were found and all of them were shared.

Very similar characteristics were also found when the D4-specific chains, before absorption of the D1-reacting molecules, were analyzed (compare panel *B* with panel *E*). The corresponding 2DPM shared more than 90% of peptides. However, when such D4  $\alpha$  chains were compared with the D1 $\alpha$  chains within the same Ia preparation (compare panels *B* and *A* and panels *E* and *F*, respectively) it was found that the peptides present in the D4 2DPM included all the D1-specific peptides and presented 6–7 additional peptides. Therefore it was of interest to analyze the structural features of the D4  $\alpha$  chains after the Ia pool had been depleted of the D1-reactive subset. Fig. 3 illustrates the results obtained. The  $D1^-D4^+$   $\alpha$  chain population displayed a reduced number of peptides with respect to the  $D1^+D4^+$   $\alpha$  chain counterpart. In particular, only 11 and 9 major peptides were detectable in the  $D1^-D4^+$   $\alpha$  2DPM of LG2 and Raji, respectively, as compared with 17 peptides in the corresponding 2DPM of the  $D1^+D4^+$   $\alpha$

TABLE I  
*Evaluation of the Structural Similarities in Terms of Peptides Between  $D1^+D4^+$  and  $D1^-D4^+$  Ia Subsets*

Ia subunit	Ia subset		
	$D1^+D4^+$	$D1^-D4^+$	Shared <sup>‡</sup>
$\beta$ —LG2	25*	24	11
—Raji	29	27	13
—Shared <sup>§</sup>	12	23	
$\alpha$ —LG2	17	11	4
—Raji	17	9	4
—Shared <sup>§</sup>	17	8	

\* Values are expressed in number of major peptides detected in the corresponding fingerprints (see Fig. 2 for  $\beta$  chains and Fig. 3 for  $\alpha$  chains).

<sup>‡</sup> This column gives the number of major peptides shared between the homologous subunits of the two distinct subsets within the same cell line.

<sup>§</sup> These columns give the number of major peptides shared between two homologous subunits of the same subset in distinct cell lines.

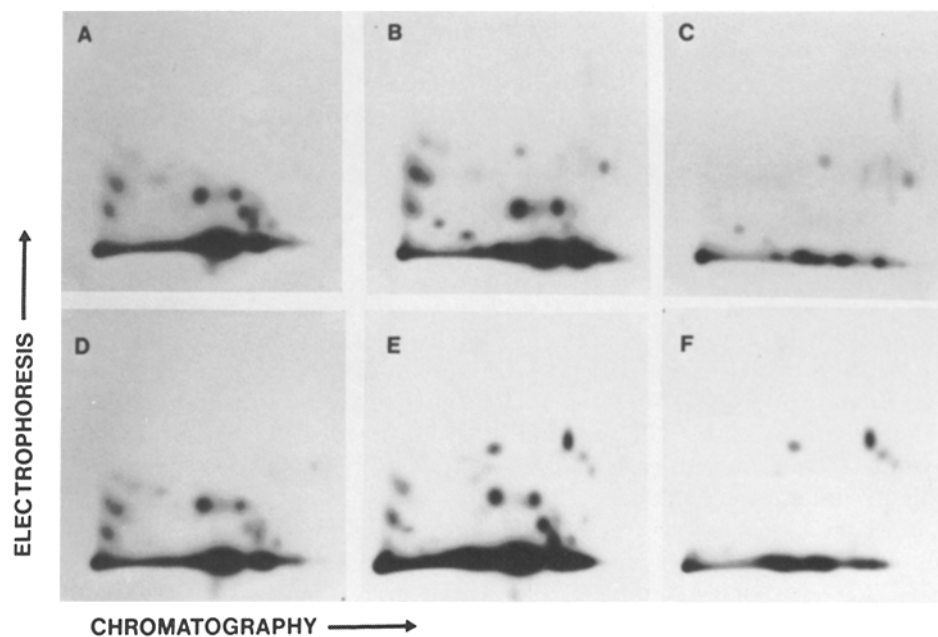


FIGURE 3. Autoradiographs of peptide maps of  $^{125}\text{I}$ -labeled  $\alpha$  chains from LG2 (upper panels) and Raji (lower panels) Ia molecules bound by the following Mab: D1 (A and D); D4 (B and E), before absorption of the D1-reactive population; D4 (C and F), after absorption of the D1-reactive population. For detailed description of the fingerprints see the text and Table I.

subunits (compare panels C and A, and panels F and D, respectively). From such a comparison, it became clear that important qualitative differences were observed between  $\text{D1}^-$ - $\text{D4}^+$  and  $\text{D1}^+$ - $\text{D4}^+$   $\alpha$  chains, since only a minority of peptides (4 out of 11 in LG2, and 4 out of 9 in Raji) were shared. It is noteworthy that the non-shared peptides found in the  $\text{D1}^-$ - $\text{D4}^+$   $\alpha$  chains were all present in the D4-specific fingerprints before absorption of D1-reactive molecules.

When the comparison was made between  $\text{D1}^-$ - $\text{D4}^+$   $\alpha$  chain fingerprints obtained from two distinct Ia pools (LG2 and Raji), a high degree of homology was observed. Among the 9 and the 11 peptides representative of the  $\text{D1}^-$ - $\text{D4}^+$  fingerprints, 8 peptides were shared.

In Table I the numerical relationships between the various fingerprints of the  $\alpha$  chains are summarized.

*$\beta$  Chains of the  $\text{H40}^+$ - $\text{3/4}^+$  and  $\text{H40}^-$ - $\text{3/4}^+$  Ia Subsets.* The  $\beta$  chains of Ia molecules recognized by H40 Mab and 3/4 Mab before and after preclearing of the H40 reacting population were analyzed by 2DPM technique.

Fig. 4 shows the corresponding fingerprints obtained from LG2 (upper panels) and Raji (lower panels) Ia pools, respectively.

When 2DPM of H40  $\beta$  chains in LG2 and Raji cells were compared, considerable similarities were observed (compare panels A and D). A careful analysis of the peptides present in the two corresponding fingerprints revealed that 39 and 36 major peptides were present in LG2- and Raji-specific 2DPM, respectively (Table II); 33 of these peptides were shared between the two  $\beta$  chains. If one takes into account the fact that the two-dimensional peptide mapping technique

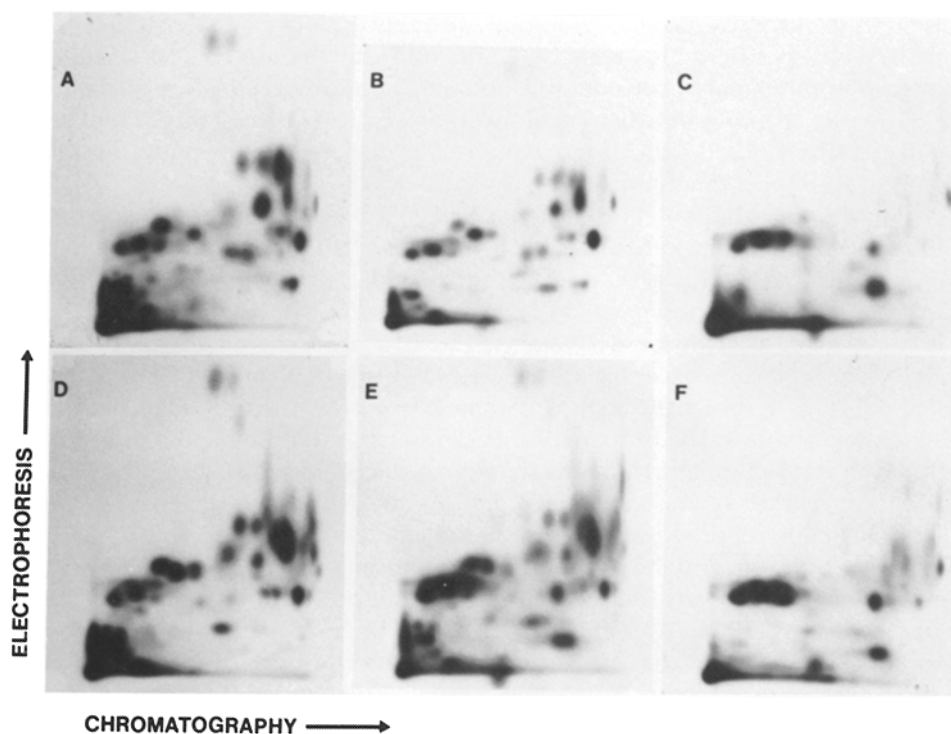


FIGURE 4. Autoradiographs of peptide maps of  $^{125}\text{I}$ -labeled  $\beta$  chains from LG2 (upper panels) and Raji (lower panels) Ia molecules bound by the following Mab: H40 (A and D); 3/4 (B, E), before absorption of the H40-reactive population; 3/4 (C and F), after absorption of the H40-reactive population. For detailed description of the fingerprints see the text and Table II.

TABLE II  
Evaluation of the Structural Similarities in Terms of Peptides Between  
 $H40^+-3/4^+$  and  $H40^-3/4^+$  Ia Subsets

Ia subunit	Ia subset		
	$H40^+-3/4^+$	$H40^-3/4^+$	Shared <sup>‡</sup>
$\beta$ —LG2	39*	19	17
—Raji	36	20	17
—Shared <sup>§</sup>	33	18	
$\alpha$ —LG2	21	20	13
—Raji	22	21	13
—Shared <sup>§</sup>	21	18	

\* Values are expressed in number of major peptides detected in the corresponding fingerprints (see Fig. 4 for  $\beta$  chains and Fig. 5 for  $\alpha$  chains).

<sup>‡</sup> This column gives the number of major peptides shared between the homologous subunits of the two distinct subsets within the same cell line.

<sup>§</sup> These columns give the number of major peptides shared between two homologous subunits of the same subset in distinct cell lines.



utilized in this study tends to overamplify differences, it can be safely concluded that the H40-specific  $\beta$  chains in LG2 and Raji show relatively low structural polymorphism. Similarly, considerable homology was observed in the  $\beta$  chains of the 3/4-reactive molecules before absorption of the H40 reacting population (compare panels *B* and *E*) with more than 90% of detectable peptides between LG2- and Raji-specific subunits being similar.

Within the same Ia pool, H40  $\beta$  chains and 3/4  $\beta$  chains before absorption of H40-reactive molecules were also similar (compare panels *A* and *B* and panels *D* and *E*, respectively). However, a few major peptides clearly present in the 3/4-reactive material were absent in H40 chains.

It was therefore of interest to analyze the structural characteristics of the  $\beta$  chains of Ia molecules recognized by 3/4 Mab after depletion of H40-reactive molecules. Fig. 4 shows the 2DPM obtained from the H40<sup>-</sup>-3/4<sup>+</sup>  $\beta$  chains of LG2 (panel *C*) and Raji cells (panel *F*). It can be seen that the actual number of peptides in the top fingerprints was strongly reduced with respect to the H40<sup>+</sup>-3/4<sup>+</sup>  $\beta$  fingerprints (compare panels *C* and *A*, and *F* and *D*, respectively). Only 19 and 20 major peptides were present in the LG2 and Raji H40<sup>-</sup>-3/4<sup>+</sup>  $\beta$  2DPM with respect to 39 and 36 of their corresponding H40<sup>+</sup>-3/4<sup>+</sup> fingerprints. Furthermore the majority of such peptides (17 out of 19 and 17 out of 20) were shared with the H40<sup>+</sup>-3/4<sup>+</sup>  $\beta$  chains of the same Ia pool.

It must be noted that the few nonshared peptides present in the fingerprints of the 3/4-specific  $\beta$  chains before absorption of H40-reactive molecules, were also present in the H40<sup>-</sup>-3/4<sup>+</sup>  $\beta$  fingerprints. When the comparison was made between the LG2 and Raji H40<sup>-</sup>-3/4<sup>+</sup>  $\beta$  chains, a very high degree of similarity was found. In fact, out of 19 and 20 peptides present in LG2 and Raji specific fingerprints, 18 peptides were shared. In Table II are summarized the relationships between the different subunits described in this section.

As suggested by the crossabsorption studies, very little homology was observed when comparisons were made between H40<sup>+</sup>-3/4<sup>+</sup> or H40<sup>-</sup>-3/4<sup>+</sup>  $\beta$  chains and D1<sup>+</sup>-D4<sup>+</sup> or D1<sup>-</sup>-D4<sup>+</sup>  $\beta$  chains in the two Ia pools analyzed in this study (compare Fig. 4 with Fig. 2).

Therefore at least four subunits could be positively defined at the structural level: (a) D1<sup>+</sup>-D4<sup>+</sup>  $\beta$  chain with relatively high structural variation between Raji and LG2 cells, (b) D1<sup>-</sup>-D4<sup>+</sup>  $\beta$  chain, (c) H40<sup>+</sup>-3/4<sup>+</sup>  $\beta$  chain, (d) H40<sup>-</sup>-3/4<sup>+</sup>  $\beta$  chain, the last three expressing relatively low structural variation when analyzed in DR-different cell lines.

*$\alpha$  Chains of the H40<sup>+</sup>-3/4<sup>+</sup> and H40<sup>-</sup>-3/4<sup>+</sup> Ia Subsets.* Fig. 5 shows the 2DPM of the  $\alpha$  chains of Ia molecules from LG2 cells (upper panels) and Raji cells (lower panels) recognized by the H40 Mab and by the 3/4 Mab before and after depletion of H40-reactive population. When the H40  $\alpha$  chains of LG2 and Raji were compared, a very strong homology was observed. 21 and 22 major peptides were identifiable in the 2DPM of these  $\alpha$  chains in LG2 and Raji, respectively. All the detectable peptides in LG2  $\alpha$  chains were also present in the Raji  $\alpha$  chains (compare panels *A* and *D*).

The same considerations could also be made for the  $\alpha$  subunits of Ia molecules recognized by 3/4 Mab before absorption of H40 reacting population (compare panels *B* and *E*). Furthermore, when H40  $\alpha$  chains were compared to 3/4  $\alpha$

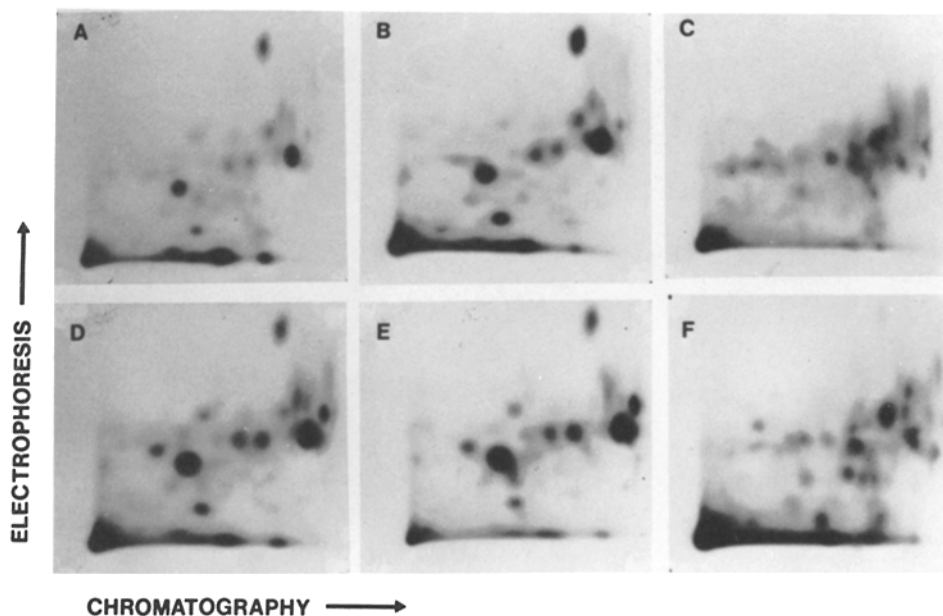


FIGURE 5. Autoradiographs of peptide maps of  $^{125}\text{I}$ -labeled  $\alpha$  chains from LG2 (upper panels) and Raji (lower panels) Ia molecules bound by the following Mab: H40 (A and D); 3/4 (B and E), before absorption of the H40-reactive population; 3/4 (C and F), after absorption of the H40-reactive population. For detailed description of the fingerprints see the text and Table II.

chains before absorption of H40-reactive Ia molecules either in Raji or in LG2 cells, strong homology was also observed, (compare panels A and B or panels D and E, respectively).

Experiments were then performed to assess the structural characteristics of the 3/4-specific  $\alpha$  chains after the Ia population had been depleted of the H40-reactive molecules. As shown in Fig. 5, considerable differences were observed between the  $\text{H40}^-$ -3/4 $^+$  and  $\text{H40}^+$ -3/4 $^+$   $\alpha$  chains within the same Ia pool (compare panels C and A, or panels F and D, respectively). These differences were both quantitative and qualitative; several major peptides present in the  $\text{H40}^+$ -3/4 $^+$  populations were either absent or strongly reduced in intensity; conversely, several other peptides barely visible or absent in the  $\text{H40}^+$ -3/4 $^+$   $\alpha$  fingerprints, were clearly recognizable as major peptides in the  $\text{H40}^-$ -3/4 $^+$   $\alpha$  2DPM. As shown in Table II, 20 and 21 major peptides were present in  $\text{H40}^-$ -3/4 $^+$   $\alpha$  fingerprints of LG2 and Raji cells, respectively. In both cases only 13 peptides were unambiguously shown to be shared with the corresponding  $\text{H40}^+$ -3/4 $^+$   $\alpha$  chains. In contrast, higher homology was observed between the two  $\text{H40}^-$ -3/4 $^+$   $\alpha$  chain fingerprints (compare panels C and F). 18 out of 20 and 21 peptides were shared. It is interesting to note that the similarity between the 2DPM of  $\text{H40}^-$ -3/4 $^+$   $\alpha$  chains in LG2 and Raji involved the majority of those peptides that were scarcely visible or absent in the 2DPM of  $\text{H40}^+$ -3/4 $^+$   $\alpha$  chains. However, a few peptides, clearly well represented in 2DPM of the  $\text{H40}^-$ -3/4 $^+$   $\alpha$  chain of Raji cells, were absent in the corresponding fingerprint of LG2 cells,

probably suggesting a higher heterogeneity of the latter with respect to the former population.

Finally, as was the case for the  $\beta$  chains, very limited homology was observed between the  $H40^+-3/4^+$   $\alpha$  and  $H40^-3/4^+$   $\alpha$  subunits when compared with the  $D1^+-D4^+$  and  $D1^-D4^+$   $\alpha$  subunits (compare Fig. 5 with Fig. 3).

Thus, by structural analysis at least four distinct subunits could be positively defined: (a)  $D1^+-D4^+$   $\alpha$  chain, (b)  $D1^-D4^+$   $\alpha$  chain, (c)  $H40^+-3/4^+$   $\alpha$  chain, (d)  $H40^-3/4^+$   $\alpha$  chain. All of the four distinct  $\alpha$  chains displayed limited structural variations when compared between two distinct cell lines.

### Discussion

In this study we have analyzed at the structural level the human Ia molecules recognized by four distinct Mab: D1, D4, 3/4, and H40. The first three Mab were derived from BALB/c mice immunized with human Ia-positive cell membranes (D1 and D4) (6), or MLC-activated human T cells (3/4) (10), whereas the fourth Mab (H40) was derived from A.TH mice immunized with A.TL mouse cells. This antibody was shown to cross-react with human Ia antigens (23).

In previous studies D1 and D4 Mab have been used to assess the degree of heterogeneity of the human Ia pool. Two subsets of molecules, named NG1 and NG2, respectively, were identified by these two reagents. In the present study, we have extended this analysis by performing crossabsorption experiments that allowed a better definition of the NG1 and NG2 subsets. Human Ia pools derived from two distinct cell lines, LG2 (DR 1,1-DC1) and Raji (DR 3,W6-DC1) were analyzed. In the two cell lines it was possible to show that the molecules reacting with the D1 Mab (which was used to define the NG1 subset), also reacted with D4 Mab. Therefore, the D4 Mab (which was used to define the NG2 subset) appears to react with a family of molecules, all of 34–26,000 daltons, comprising at least two subsets on the basis of expression (or lack thereof) of the D1 Mab defined antigenic determinant. On the basis of these results, we propose to redefine the molecules belonging to the NG1 and NG2 subsets as  $D1^+-D4^+$  and  $D1^-D4^+$  molecules, respectively (Fig. 6).

*NG1 Molecules Display Distinct  $\beta$  but Similar  $\alpha$  Subunits.* The structural analysis of the NG1 subset confirmed that the  $\beta$  chains of these molecules displayed a

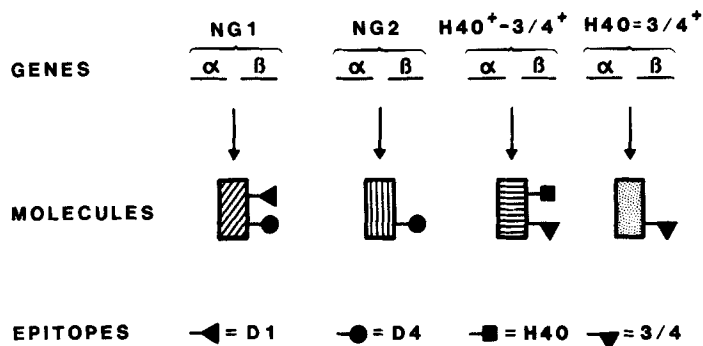


FIGURE 6. A model for genes encoding the  $\alpha$  and  $\beta$  polypeptides of the human Ia molecules described in this study. The gene arrangement is arbitrary.

substantial diversity when analyzed in two cell lines of different DR phenotype. The structural differences in the NG1  $\beta$  chains were also observed when the Ia molecules of other DR homozygous cell lines, distinct from DR1,1 were compared with each other and with the DR 1,1 cell line (reference 30 and unpublished results), thus indicating that the variability of the  $\beta$  chains of the NG1 subset observed in the present study did not result from the fact that comparison was made between a DR homozygous and a DR heterozygous cell line. In contrast, the  $\alpha$  chains of the NG1 subset showed no detectable structural variation. Taken together, these results suggest that NG1 molecules might be similar to the classical serologically defined HLA-DR antigens in which the allelic polymorphism is confined to the  $\beta$  subunits (9, 31).

*Analysis of the NG2 Subset Reveals the Existence of a New Type of Class II Antigens, with Limited Structural Polymorphism, Distinct from NG1 and DC1.* Analysis of the  $\beta$  chains of Ia molecules reacting with D4 Mab after absorption of the NG1 subset (the NG2 molecules) indicated that they were significantly different from those of the NG1 subset in both LG2 and Raji Ia pools. However, limited structural differences were observed when the NG2  $\beta$  chains of LG2 and Raji cells were compared to each other. Furthermore, the NG2  $\alpha$  chains were very different from those of the NG1 subset; however, minimal structural variability was observed between DR-different cell lines. Taken together, these results indicate the existence of another locus distinct from NG1 that codes for Ia molecules with more limited structural polymorphism. One other subset with these characteristics had been previously identified by serological and structural studies, the DC1 molecule (9, 10). In one of these studies it was shown that the 3/4 Mab recognized the same molecules as those defined by allo anti-DC1 antisera (10). It was therefore important to compare the structural characteristics of the NG2 molecules to those of the DC1 molecules as defined by the 3/4 Mab. The results presented here clearly showed that the NG2 subset differed from the DC1 subset by several parameters. First, the electrophoretic mobility of the NG2  $\alpha$ - $\beta$  heterodimer was different from that of the DC1  $\alpha$ - $\beta$  heterodimer (slower: 34–26,000 mol wt vs. faster 33–24,000 mol wt, respectively). Second, crossabsorption studies indicated that absorption of the NG2 subset did not affect the molecules recognized by two distinct Mab directed against DC1-related structures. Similarly, depletion of DC1 molecules was not accompanied by any significant reduction of NG2 molecules. Finally, the structural analysis of the NG2  $\alpha$  and  $\beta$  chains demonstrated important differences with respect to the homologous chains of the DC1 subset. Thus the NG2 molecules represent a third class of Ia antigens, present in all individuals irrespective of their DR or DC phenotype (6). It will be interesting to assess the structural relationships between the NG2 subset and other types of class II molecules that have been recently described, like the SB (17) and the DS (15) subsets. Studies in this direction are in progress in our laboratory.

*The Molecules Defined by the 3/4 and H40 Mab Are a Heterogeneous Population with Very Restricted Structural Polymorphism.* The DC antigens are MHC-encoded products having the characteristics of class II antigens. However, with respect to DR molecules the DC  $\alpha$ - $\beta$  heterodimer shows a faster electrophoretic mobility in SDS-PAGE and more limited allelic polymorphism, which has been deduced

from both serological and structural studies (9, 10).

Crossabsorption studies and structural analysis confirmed that the DC1 molecules, as defined by 3/4 Mab (10), were distinct in both their  $\alpha$  and  $\beta$  subunits from the corresponding subunits of the NG1 subset. Moreover, the DC1 molecules were also structurally distinct from NG2 Ia antigens, as discussed in the previous section.

A second monoclonal antibody, H40, originally produced in the murine A.TH anti A.TL system has been recently shown to cross-react with human Ia antigens that by serological analysis (23), binding competition studies (24), and structural characteristics (25) resemble the DC1 molecules. It was therefore important to establish the structural relationships between the molecules reacting with H40 and 3/4 Mab.

Crossabsorption experiments indicated that the 3/4-reactive molecules included those recognized by H40 Mab. In contrast, depletion of H40-reactive molecules only slightly reduced the molecules reacting with 3/4 Mab, thus suggesting the existence of  $H40^+-3/4^+$  and  $H40^-3/4^+$  molecules. In agreement with previous reports (9, 10) it was found that the  $\beta$  chains of  $H40^+-3/4^+$  Ia molecules did not show important structural variation when analyzed in LG2 and Raji cell lines. Similar considerations could be made for the  $\beta$  chains of the  $H40^-3/4^+$  molecular population.

These results indicate the presence of very limited structural polymorphism in the  $\beta$  chains of the molecules recognized by the 3/4 and H40 Mab. However, within the same Ia pool, the  $H40^+-3/4^+$  and the  $H40^-3/4^+$   $\beta$  chains strongly differed from each other, the  $H40^-3/4^+$  subunits displaying a 2DPM relatively simpler than the  $H40^+-3/4^+$  corresponding subunits.

Taken together, the results obtained indicated the presence of a heterogeneous family of  $\beta$  chains in the Ia molecules recognized by the 3/4 Mab.

Similarly to the small subunits, the  $\alpha$  chains of the  $H40^+-3/4^+$  and  $H40^-3/4^+$  subsets of the LG2 Ia pool did not show appreciable structural variation when compared with their homologous counterpart in the Raji Ia pool. However, when  $H40^+-3/4^+$   $\alpha$  chains were compared with the  $H40^-3/4^+$   $\alpha$  chains, appreciable differences were observed, suggesting (as was the case with the  $\beta$  chains) the existence of at least two distinct  $\alpha$  chain families, with virtually no structural polymorphism, as well. Among the several hypotheses that could explain the results obtained in the present study, one would be that the DC1 subset as recognized by the 3/4 Mab, includes at least two families of molecules, the  $H40^+-3/4^+$  and the  $H40^-3/4^+$ .

The model depicted in Fig. 6 tries to summarize the personal view of the minimal gene number encoding human Ia antigens as it stems from the results we have obtained. The expression of Ia molecules in the two cell lines analyzed is envisaged under the control of at least eight genes:  $\alpha$  and  $\beta$  NG1,  $\alpha$  and  $\beta$  NG2,  $\alpha$  and  $\beta$   $H40^+-3/4^+$ , and  $\alpha$  and  $\beta$   $H40^-3/4^+$ .

It is important to stress that this model constitutes a minimal estimate of the actual repertoire of class II genes. In fact, recent works performed both by amino acid sequencing analysis (14) and genomic cloning (32) have indicated the existence of a more heterogeneous Ia gene pool.

A striking feature that can be deduced from previous studies (7, 18) and

confirmed here, consists in the selective subunit association of the distinct  $\alpha$ - $\beta$  heterodimers. Whether this selective association plays a role in the functional properties of the various Ia molecules, remains to be determined.

Preliminary studies to investigate a possible functional role of some of the Ia subsets described in this report (33-35) suggest that structural heterogeneity of class II antigens may correspond to peculiar functional properties of these molecules.

### Summary

Four monoclonal antibodies reacting with distinct human Ia antigenic determinants have been used to demonstrate the coexpression of four distinct subsets, NG1, NG2, DC1 H40<sup>+</sup>-3/4<sup>+</sup>, and DC1 H40<sup>-</sup>-3/4<sup>+</sup>, in the Ia pool of DR heterozygous or homozygous B cell lines. By two-dimensional peptide mapping the four subsets within the same Ia pool displayed structurally different  $\beta$  as well as  $\alpha$  subunits. The  $\beta$  chain of the NG1 subset was shown to display considerable structural polymorphism when analyzed in two cell lines with distinct DR but similar DC phenotype, LG2 (DR 1,1-DC1) and Raji (DR 3,W6-DC1). In contrast, the  $\beta$  chains of NG2, DC1 H40<sup>+</sup>-3/4<sup>+</sup>, and DC1 H40<sup>-</sup>-3/4<sup>+</sup> subsets of LG2 cells were shown to be very similar to their homologous Raji cell counterparts, thus indicating a relatively low structural polymorphism. Furthermore, the  $\alpha$  chains of either one of the four subsets expressed in LG2 cells displayed very high structural similarities to the homologous counterparts in the Raji Ia pool, thus suggesting a relatively low polymorphism for the large Ia subunits described in this study. A striking feature deduced from this study was the selective subunit association of the distinct  $\alpha$ - $\beta$  heterodimers.

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