STRUCTURAL ANALYSIS OF HUMAN Ia ANTIGENS REVEALS THE EXISTENCE OF A FOURTH MOLECULAR SUBSET DISTINCT FROM DP, DQ, AND DR MOLECULES

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In recent years the study of MHC-encoded class II or Ia molecules has received considerable attention because of their central role in the homeostasis of the immune system. The crucial role of these molecules stems from the fact that T cells of the helper/inducer phenotype can recognize a foreign antigen only when it is presented on a cell surface in association with self class II molecules. This phenomenon is referred to as MHC restriction of T cell specificity (reviewed in reference 1). An extreme allelic polymorphism is one of the most striking features of class II molecules. Such polymorphism has functional consequences in that T cells can recognize certain antigens only or more efficiently when these antigens are in association with certain allelic products of Ia. Class II molecules have a restricted cell distribution. They are mainly expressed on B cells, macrophages, and activated T cells as well as in a variety of tumor cells (reviewed in reference 2). They are composed of heterodimers with a large subunit (α chain) of 33,000–36,000 daltons and a small subunit (β chain) of 24,000–29,000 daltons (3). It is now well documented that within a single individual, Ia antigens consist of a heterogeneous family of molecules with distinctive structural characteristics and various degrees of polymorphism (4–9). In man, molecular studies at the DNA level have established the existence of at least three class II subregions designated DP, DQ, and DR, respectively (reviewed in references 10 and 11). The actual number of α and β genes residing in each subregion is not yet fully established.

Presently, available data indicate the possibility to express per haploid genome one α and one β gene in the DP subregion, two α and two β genes in the DQ subregion, and one α and two β genes in the DR subregion. Further biochemical studies have indicated the existence of expressed mRNA of a fourth type of β chain, denominated DOβ (12), as well as a fourth type of α chain denominated DZ (13). These studies suggest that additional α/β heterodimers distinct from classical DP, DQ, and DR molecules could be expressed on the cell surface and thus may increase the repertoire of restriction elements the individual can use during the immune response. The isolation of mAbs specific for human Ia molecules has greatly facilitated the analysis of the heterogeneity of the human Ia pool. However, in many cases, these mAbs recognize epitopes shared between

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various Ia subsets. Furthermore, it has been shown that mAbs specific for a given subset of Ia antigens in certain haplotypes may react with additional or even distinct Ia subsets in other haplotypes, thus suggesting sharing and/or acquisition of specific antigenic epitopes between members of the same family (reviewed in reference 14). The above findings must be taken in serious consideration if one wants to correlate a given biological function with a specific structural entity.

Earlier, our group reported (4) on the biochemical characterization of two distinct human Ia subsets, which were designated NG1 and NG2. These subsets were found in all individuals irrespective of the class II haplotype. Recently (15), refined biochemical analysis by two-dimensional peptide mapping demonstrated that NG1 molecules are the equivalent of DR antigens. NG2 molecules, instead, constituted a heterogeneous family of Ia antigens comprising DR and additional α/β heterodimers structurally distinct from DR and DQ1 molecules (15). Additional studies showed that a large panel of anti–class II mAbs reacted with the shared epitope. However, no antibody was found that reacted with epitopes specific for the $DR^-/NG2^+$ molecules (16–17). As mentioned previously, the DP subset constitutes a third Ia molecular family distinct from DR and DQ and could therefore be a candidate for the $DR^-/NG2^+$ subset. In the present study, we took advantage of the availability of an mAb highly specific for DP antigens, the B7/21 mAb (18), to investigate the relationships between $DR^-/NG2^+$ and DP subsets. It will be shown that DP antigens defined by the B7/21 mAb constitute the vast majority of the $DR^-/NG2^+$ molecular pool. However, a fourth population of Ia antigens, constituting a small proportion of the total Ia pool, as assessed by our technique, is still present in the NG2 pool after depletion of DR, DQ, and DP subsets. This fourth class II subsets shows structural characteristics distinct from the previously defined Ia antigens and may represent the product of a fourth class II genetic subregion.

Materials and Methods

Monoclonal Antibodies. The following mAbs were used in this study: D1-12 mAb specific for DR molecules (4, 15); 2-72 mAb specific for DR and NG2 molecules (16); BT3/4 mAb recognizing DQ1 molecules (6); and B7/21 mAb specific for DP antigens (18).

Human Cell Lines. The human cell lines used in this study were the LG-2 cells (DR 1,1 - DQ 1,1 - DP not determined) and Raji cells (DR 3,W6 -DQ 1,2 - DP not determined). LG-2 cells were kindly donated by Dr. W. Liebold, Dept. of Pathology, Medical School, Erlangen, Federal Republic of Germany.

Cells were propagated in RPMI 1640 medium supplemented with 10% FCS, glutamine, and antibiotics.

External Cell Surface Radiolabeling. Cells were surface labeled with ^{125}I by the lactoperoxidase–glucose oxidase (LPGO)[1] catalyzed iodination method essentially as described (19). All reaction steps were rigorously carried out at 4°C to avoid cytoplasmic internalization of the labeling enzymes. Briefly, 2×10^7 cells were extensively washed in cold RPMI medium followed by several washes in PBS (0.01 M sodium phosphate; 0.14 M NaCl, pH 7.4). Cells were then resuspended in 1 ml of PBS plus 5.5 mM glucose. To the cell suspension were added: KI (final concentration, 10^{-6} M), 1 mCi of $NaI^{125}I$ carrier free (Amersham Corp., Amersham, United Kingdom), and 40 μl of lactoperoxidase

[1] *Abbreviations used in this paper:* 2D-PAGE, two-dimensional PAGE; 2D-PM, two-dimensional peptide mapping; In, invariant; LPGO, lactoperoxidase–glucose oxidase catalyzed iodination; NEPHGE, nonequilibrium pH gradient gel electrophoresis.

solution (1 mg/ml; Sigma Chemical Co., St. Louis, MO). Reaction was started by addition of 10 μl of glucose oxidase at 19.55 U/ml (Sigma Chemical Co.). The glucose oxidase step was repeated twice at 10-min intervals. The reaction was stopped by adding 10 ml of cold RPMI supplemented with 5 mM L-cystein hydrochloride (Fluka AG, Buchs, Switzerland). Cells were washed extensively with cold PBS before lysis.

Biosynthetical Radiolabeling. 2×10^7 cells were biosynthetically labeled for 4 h at 37°C with 500 μCi of [^{35}S]methionine (Amersham Corp.) in 5 ml of RPMI medium lacking methionine and containing 5% dialysed FCS. After this period, cells were washed extensively with cold RPMI and lysed in presence of detergent as described below.

Cell Lysis and Purification of Ia Molecules. Cell lysates were prepared by incubating the cells for 20 min at 4°C in 1 ml of Tris-buffered saline containing 1% NP-40 detergent according to the method of Owen et al. (20). Insoluble material was removed by centrifugation at 100,000 *g* for 30 min. Cell extracts were then incubated for 2 h at 4°C with protein A–Sepharose beads (Pharmacia Fine Chemicals, Uppsala, Sweden) under rotation to remove nonspecifically bound material. This step was repeated three times. Ia molecules were then purified by using mAbs specifically bound to CNBr-activated Sepharose 4B beads (15). All incubation steps were performed for 2 h at 4°C under rotation using 100 μl of packed beads. Washes were done with washing buffer (125 mM Tris-HCl, 0.5 M NaCl, 0.5% NP-40, 10 mM EDTA, pH 8.2) at 4°C, and we performed elution from immunoabsorbents by adding 80 μl of SDS-sample buffer to the packed immunoabsorbent (21) and boiling this for 5 min for one-dimensional electrophoresis, or by adding IEF sample buffer (22) for two-dimensional electrophoresis (2D-PAGE).

The following purification scheme was adopted. Cell lysates were first incubated with D1-12-immunoabsorbent to purify DR molecules. The purification step was repeated until we completely eliminated D1-12-reacting material. The DR-depleted cell extract was then incubated with BT3/4-Sepharose immunoabsorbent to first purify and then deplete the DQ1 molecules from the lysate, as described above. At this stage, the cell extract was divided into two aliquots that were separately incubated with 2-72-Sepharose beads and B7/21-Sepharose beads, respectively. The corresponding Ia-reacting molecules were purified as described above until complete depletion was achieved. The 2-72-depleted and B7/21-depleted cell extracts were then reacted with B7/21- and 2-72-Sepharose immunoabsorbents, respectively. Retained material was eluted as above.

Analysis of Ia Molecules. Ia molecules purified as above were separated by SDS-PAGE in nonreducing conditions as described (15). α and β subunits of the various Ia heterodimers labeled by the LPGO method were separately cut from the dried gels, eluted, reduced, alkylated, and then pepsin-digested. The digest was then analyzed by two-dimensional peptide mapping (2D-PM) as described (15).

The Ia molecules intrinsically labeled with [^{35}S]methionine were analyzed by 2D-PAGE, following the method of O'Farrell et al. (22), to separate polypeptides according to charge in the first dimension (nonequilibrium pH gradient electrophoresis [NEPHGE]) and according to size in the second dimension (SDS-PAGE).

Results

Crossabsorption Studies. In previous studies (15) we have shown that the epitope recognized by anti-NG2 antibodies was shared with DR molecules (as recognized by the D1-12 mAb) but not with DQ1 molecules (as recognized by the BT3/4 mAb). Thus, extracts from detergent-solubilized cells labeled with either ^{125}I by lactoperoxidase-catalyzed iodination or intrinsically labeled with [^{35}S]methionine were first depleted of DR and DQ1 molecules. The resulting cell lysates were analyzed for reactivity with B7/21 (anti-DP) and 2-72 (anti-NG2) mAbs before and after depletion of the 2-72- and B7/21-reactive material, respectively. Fig. 1 shows the results obtained with LG-2 cell extracts, and Fig. 2 the results obtained with Raji cell extracts after external ^{125}I–cell surface labeling.

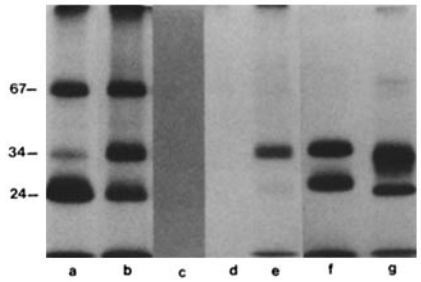

FIGURE 1. Autoradiographs of 11% SDS-polyacrylamide slab gels of unreduced ^{125}I-lactoperoxidase-labeled Ia antigens from LG-2 cells. The electrophoresis was carried out by using the discontinuous Tris buffer system (21). Lanes show Ia molecules eluted from the following mAb immunoabsorbents: (*a*) B7/21; (*b*) 2-72; (*c*) B7/21 after depletion of the B7/21-reactive population; (*d*) B7/21, after depletion of the 2-72-reactive population; (*e*) 2-72, after depletion of the B7/21-reactive population; (*f*) D1-12; and (*g*) BT3/4. Note that to maximize visual comparison, autoradiographs in lanes *a*, *b*, *f*, and *g* were obtained by running a portion, approximately equivalent in counts, of the total immunoprecipitated material. Conversely, autoradiographs in lanes *c*–*e* represent the totality of immunoprecipitated counts obtained from corresponding samples. $M_r \times 10^{-3}$ of the major detectable bands are indicated.

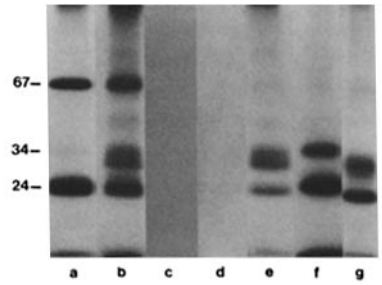

FIGURE 2. Autoradiographs of 11% SDS-polyacrylamide slab gels of unreduced ^{125}I-lactoperoxidase-labeled Ia antigens from Raji cells. The electrophoresis was carried out as described in the legend of Fig. 1. Lanes show Ia molecules eluted from the following mAb immunoabsorbents: (*a*) B7/21; (*b*) 2-72; (*c*) B7/21, after depletion of the B7/21-reactive population; (*d*) B7/21, after depletion of the 2-72-reactive population; (*e*) 2-72, after depletion of the B7/21-reactive population; (*f*) D1-12; and (*g*) BT3/4. Note that to maximize visual comparison, autoradiographs in lanes *a*, *b*, *f*, and *g* were obtained by running a portion, approximately equivalent in counts, of the total immunoprecipitated material. Conversely, autoradiographs in lanes *c*–*e* represent the totality of immunoprecipitated counts obtained from corresponding samples. $M_r \times 10^{-3}$ of the major detectable bands are indicated.

In LG-2 cells, Ia molecules recognized by B7/21 mAb (the DP antigens) consisted of a heavily labeled band of 26,000 daltons, corresponding to the β chain, and of a poorly labeled band of 34,000 daltons corresponding to the α chain (Fig. 1, lane *a*). The labeling pattern was reproducible in several experiments and had been reported previously in the literature (18). The most likely explanation is that tyrosine residues present in the α chain of DP molecules are not easily accessible on the cell surface and therefore cannot be efficiently labeled by the technique used here.

Another reproducible finding in DP-specific immunoprecipitates consisted in the presence in gels run in nonreducing conditions of an additional heavily labeled band of 67,000 daltons, which after reduction migrated like the classic

DP molecules (intense β chain, poorly represented α chain). This band may constitute an artifact of the detergent extraction.

Fig. 1 *b* shows the profile of Ia molecules recognized by the 2-72 mAb. Several peculiar features must be noted. In contrast to DP antigens, the α and β subunits were labeled equally well by ^{125}I. The M_r of these subunits was of 34,000 for α and 24,000 for β chain. Similar to DP-specific immunoprecipitates, a distinct heavily labeled band of 67,000 M_r was consistently observed.

Fig. 1 *c* shows that complete depletion of B7/21-reactive molecules was achieved after absorption of the lysate on B7/21 immunoabsorbent. After depletion of 2-72-reactive molecules, no residual activity of the lysate for the B7/21 mAb was observed (Fig. 1 *d*). In contrast, after depletion of B7/21-reacting molecules, the reactivity of 2-72 mAb, although drastically reduced, was not totally eliminated, as Ia molecules could still be immunoprecipitated. In terms of total precipitable counts these Ia molecules represented 2.5% of the total Ia pool of LG2 cells. By SDS-PAGE these molecules appeared as a relatively well-labeled α chain and a very faint, almost undetectable, β chain (Fig. 1 *e*). In Fig. 1 are also shown, for comparison, the DR molecules recognized by the D1-12 mAb (lane *f*) and DQ1 molecules recognized by the BT3/4 mAb (lane *g*). As previously reported (15) and confirmed here, DQ1 heterodimers migrated with an M_r of 33,000 (α chain) and 24,000 (β chain) clearly distinguishable from the DR heterodimers that migrated with an M_r of 34,000 (α) and 26,000 (β). It must be stressed that to maximize visual comparison, the autoradiographs in lanes *a*, *b*, *f*, and *g* have been obtained by running a portion, approximately equivalent in counts, of the total immunoprecipitated material. Conversely, autoradiographs in lanes *c–e* represent the totality of immunoprecipitated counts obtained from the corresponding samples.

Similar crossabsorption experiments were then performed on extracts of the class II heterozygous (DR 3,W6 - DC1,2 - DP undefined) Raji cells. After depletion of DR and DQ1 molecular subsets, DP antigens as recognized by B7/21 mAb (Fig. 2 *a*) displayed an SDS-PAGE profile very similar to the homologous one in LG-2 cells. We saw a heavily labeled band at 24,000 M_r and a very faint band at 34,000 M_r corresponding to β and α subunits, respectively. Furthermore, the band of 67,000 M_r observed in nonreducing conditions in LG-2-derived DP molecules was also observed in the homologous counterpart of Raji cells.

The 2-72 mAb recognized an α/β heterodimer (Fig. 2 *b*) closely resembling the one observed in LG-2 cells. Fig. 2 *c* shows that complete depletion of B7/21-reactive molecules was achieved also on Raji cells after absorption of the lysate on B7/21 immunoabsorbent. After depletion of 2-72-reactive molecules, no reactivity of the cell extract with the anti-DP B7/21 mAb was observed (Fig. 2 *d*). After depletion of DP molecules, instead, the 2-72 mAb still reacted with a small proportion of molecules, ~2% of them resolved by SDS-PAGE in a heterodimer (Fig. 2 *e*) in which the α subunit migrated as a broader band and the β subunit was comparatively more labeled than its homologous counterpart in LG-2 cells.

For comparison, we also show the SDS-PAGE profiles of DR (lane *f*) and DQ1 (lane *g*) molecules. Again it must be stressed that to maximize visual comparison, we obtained autoradiographs as described above for LG-2-derived Ia molecules.

To summarize, the crossabsorption experiments showed that after DR and

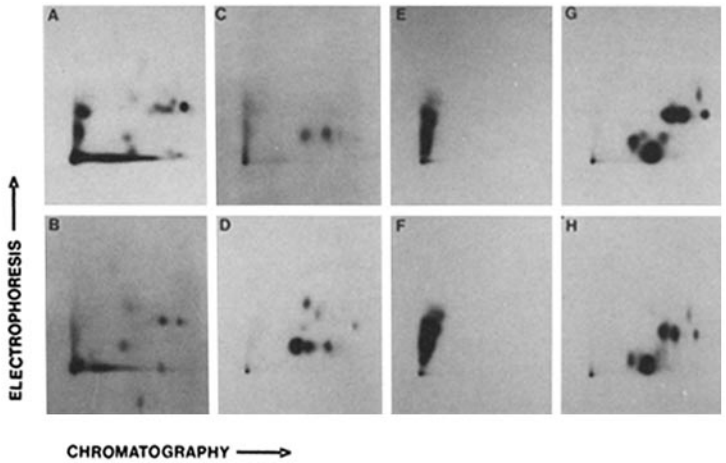

FIGURE 3. Autoradiographs of peptide maps of ^{125}I-lactoperoxidase-labeled α chains from LG-2 (panels *A*, *C*, *E*, and *G*) and Raji (panels *B*, *D*, *F*, and *H*). Ia molecules were bound by the following mAbs: (*A* and *B*) B7/21; (*C* and *D*) 2-72, after depletion of B7/21-reactive population; (*E* and *F*) D1-12; (*G* and *H*) BT3/4. For a detailed description of the fingerprints, see the text and Table I.

DQ1 depletion, the vast majority of NG2 molecules in both LG-2 and Raji cells were indeed DP antigens and thus demonstrate the existence of shared epitopes between DR and DP antigens. Such epitopes must be highly immunogenic in mice to justify the relevant number of available mAbs with similar specificities (16, 17). More important, the above experiments suggested the existence of a fourth family of Ia molecules distinct from DR, DQ, and DP antigens and present in low amounts on the cell surface of both class II homozygous and heterozygous cell lines. For sake of simplicity we will refer herein to these additional molecules as the fourth subset.

Structural Analysis of the Human Ia Pool. In the following description of the structural analysis of the various Ia subsets, comparison between homologous subunits of the same subset in the two distinct cell lines will be discussed on the basis of the actual number of major peptides identifiable in the corresponding fingerprint. Computation of peptides and evaluation of those peptides shared between fingerprints were carefully made after several autoradiographic exposures of the same fingerprint and following the rules previously described (15). Furthermore, since in certain cases α and/or β subunits migrated in SDS-PAGE as broad bands, the entire region corresponding to a given subunit was cut from the dried gel and pepsin digested to minimize errors of interpretation.

The DP α Chain. Fig. 3 shows the DP α chain fingerprints obtained from LG-2 (panel *A*) and Raji (panel *B*) cells. In LG-2 cells, we observed a series of heavily labeled peptides migrating in electrophoresis only. These peptides were virtually superimposable to the peptides found in the DR α chains (compare *A* with *E*). Moreover a series of other peptides, less intensely labeled, migrated in both electrophoresis and chromatography without any appreciable correlation with the migration of α chain–specific peptides of different Ia subsets. Comparison between LG-2 and Raji DP α fingerprints (panels *A* and *B*, respectively) revealed

TABLE I

Evaluation of the Structural Similarities in Terms of Peptides between Pairs of Homologous Ia Subunits from LG-2 and Raji Cells

Ia subset	Ia subunit	Cell line		Shared*
		LG-2	Raji	
DR	α	6‡	6	6
	β	14	12	10
DQ1	α	8	9	8
	β	16	15	15
DP	α	20	8	7
	β	14	16	10
Fourth subset	α	4	8	4
	β	—§	14	—§

For further explanation see the text.

* This column gives the number of major peptides shared between two homologous subunits of the same subset in the two distinct cell lines.

‡ Number of major identifiable peptides in the corresponding fingerprint.

§ Not available.

several important differences. 20 major peptides were present in the former, whereas only 8 were appreciable in the latter fingerprint. 7 peptides were shared (see Table I). Interestingly, the DR-like peptides seen in the LG-2 DP α fingerprint were absent in the homologous fingerprint of Raji cells. From these results we conclude that DP α chains of Ia molecules recognized by the B7/21 mAb are structurally distinct in the two cell lines analyzed.

The DR α Chains. Fig. 3 shows the DR α fingerprints obtained in LG-2 (panel *E*) and Raji (panel *F*) cells. As previously reported (15) and confirmed here, the DR α chains in the two cell lines analyzed, showed virtually superimposable 2D-PM. Six major peptides were observed in either of the two fingerprints and all of them were shared (see Table I). These results further substantiate the notion of absence of allelic polymorphism for the DR α subunits. Interestingly, all peptides showed almost exclusive migration in electrophoresis and not in chromatography. This particular migration pattern was reminiscent of the one obtained with the same subunits and in the same cell lines when DR molecules were labeled after denaturation by SDS and boiling (15). However, labeling after denaturation resulted in the generation of additional peptides, besides the ones described above. These additional peptides migrated in both electrophoresis and chromatography dimensions. Thus it is likely that in the native conformation only certain tyrosine residues of DR α subunits are available to labeling by the LPGO method.

The DQ1 α Chains. Fig. 3 also shows the DQ1 α fingerprints obtained in LG-2 (panel *G*) and Raji (panel *H*) cells. Comparison between the two fingerprints revealed striking similarities. Eight and nine major peptides were identifiable in DQ1 α chains of LG-2 and Raji, respectively; with the exception of the additional peptide in Raji, all remaining peptides were indeed shared.

Thus, the α chains of the DQ1 allele present in LG-2 cells and the DQ1 allele present in Raji cells that, according to Tonnelle et al. (12), we can designate

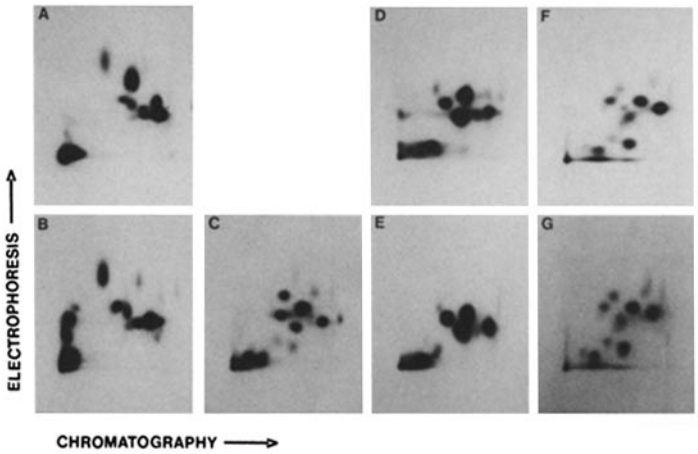

FIGURE 4. Autoradiographs of peptide maps of ^{125}I-lactoperoxidase-labeled β chains from LG-2 (panels *A*, *D*, and *F*) and Raji (panels *B*, *C*, *E*, and *G*). Ia molecules were bound by the following mAbs: (*A* and *B*) B7/21; (*C*) 2-72, after depletion of B7/21-reactive population; (*D* and *E*) D1-12; (*F* and *G*) BT3/4. For a detailed description of the fingerprints, see the text and Table I.

DQ1.1 and DQ1.W6, respectively, showed an extremely low level of structural polymorphism when analyzed by 2D-PM.

The Fourth Subset α Chain. We then performed the structural analysis by 2D-PM on the α chains of Ia molecules recognized by the 2-72 mAb after depletion of DR, DQ1, and DP subsets. In Fig. 3 are depicted the corresponding fingerprints obtained in LG-2 (panel *C*) and Raji (panel *D*) cells. The fourth subset α chain fingerprints in both cell lines showed a family of spots that apparently did not overlap with α chain spots obtained in DP, DR, and DQ1 homologous fingerprints. Comparison between LG-2 and Raji cells showed several important differences. Four and eight major spots were identifiable in the fourth subset α chain fingerprints of LG-2 and Raji cells, respectively (see Table I). All four peptides present in LG-2 were also present in the Raji α chain fingerprint. From these results we conclude that the α chains of the fourth Ia subset are not only distinct from those of DR, DQ1, and DP molecules, but also distinct from each other when analyzed in the two Ia pools considered in this study, thus suggesting the existence of structural polymorphism.

The DP β Chains. Fig. 4 shows the fingerprints of DP β subunits obtained in LG-2 (panel *A*) and Raji (panel *B*) cells. We observed both qualitative and quantitative differences. Among the 14 and 16 recognizable major peptides (see Table I) in LG-2 and Raji DP β fingerprints, respectively, only 10 could be unambiguously defined as shared peptides. These results indicate that a structural variation exists between the DP β chains of the two Ia pools analyzed. Comparison of the DP β fingerprints with either DR (panels *D* and *E*), DQ1 (panels *F* and *G*) or fourth subset (panel *C*) homologous fingerprints indicated very limited homology.

The DR β Chains. Fig. 4 shows the DR β fingerprints obtained from LG-2 (panel *D*) and Raji (panel *E*) cells.

Comparison of these two peptide maps reveals a remarkable degree of similar-

ity. Among the 14 and 12 recognizable major peptides (see Table I) in LG-2 and Raji corresponding fingerprints, respectively, 10 peptides were shared. If these results are compared with those obtained for the DP β subunits, one must conclude that the allelic variations between DR β subunits of LG-2 cells (DR1,1) and Raji cells (DR 3,W6) are not resolved as well as those of the DP β subunits. This rather unexpected finding probably correlates with the peculiar pattern of tyrosine iodination of DR β subunits in their native configuration, allowing detection of very limited structural polymorphism after pepsin digestion.

The DQ1 β Chains. In Fig. 4 are also presented the DQ1 fingerprints obtained from LG-2 (panel *F*) and Raji (panel *G*) cells. Comparison of the two DQ1 β fingerprints demonstrated a striking degree of homology. Among the 16 and 15 recognizable major peptides (see Table I) in the LG-2 and Raji 2D-PM, respectively, 15 peptides were shared. This finding confirms and extends previous results of our group (15) indicating a very low level of structural variability between the DQ1.1 and the DQ1.W6 β alleles.

The Fourth Subset β Chains. As found by crossabsorption studies, the Ia molecules recognized by the 2-72 mAb after absorption of DR, DQ1, and DP molecules in LG-2 cells did not show appreciable radioactivity associated to the small Ia subunits. This fact prevented analysis of the fourth subset–specific β chain by 2D-PM. On Raji cell, however, sufficient counts could be obtained to perform fingerprint analysis. The results are reported in Fig. 4*C*. We observed important structural differences with either DP or DQ1 corresponding β chain fingerprints. (compare *C* with *B* and *G*). More similarities were instead found with the Raji DR β fingerprints: 6 out of 14 major peptides and particularly those highly represented in terms of radioactivity were indeed shared (compare *C* with *E*).

To summarize, the results indicated that the small subunit of the fourth Ia subset displays structural characteristics distinct from those of DR, DP, and DQ1 homologous subunits. Structural differences were significantly higher when compared with DP and DQ1 β chain than with DR β chain.

Biosynthetically Labeled Ia Subsets. We then investigated whether the fourth subset could be evidenced by immunoprecipitation of cell lysates from [^{35}S]-methionine biosynthetically labeled cells. As found in extracts of surface-labeled cells, after complete depletion of DR, DQ1, and 2-72-reactive molecules no reactivity of the cell lysate with the anti-DP B7/21 antibody was observed. After complete depletion of DR, DQ1, and DP molecules, instead, the 2-72 mAb still reacted with a small proportion of molecules, representing in terms of counts, 2–4% of the total Ia precipitable counts obtained by grouping together the various Ia subsets analyzed in this study.

These molecules were analyzed by 2D-PAGE and the results obtained with Raji cells are illustrated in Fig. 5. It can be seen that the fourth subset resolved by this technique resulted in a series of acidic spots corresponding to the α chain and a series of basic spots corresponding to the β chains of a typical Ia heterodimer (Fig. 5*A*). Both the migration and the intensity of the fourth subset β chain spots were distinct from those of the DP (panel *B*) and DR (panel *D*) β chain spots. More similarity was instead found between fourth subset and DQ1 β chain spots (compare *A* and *C*), although the latter were clearly more heterogeneous.

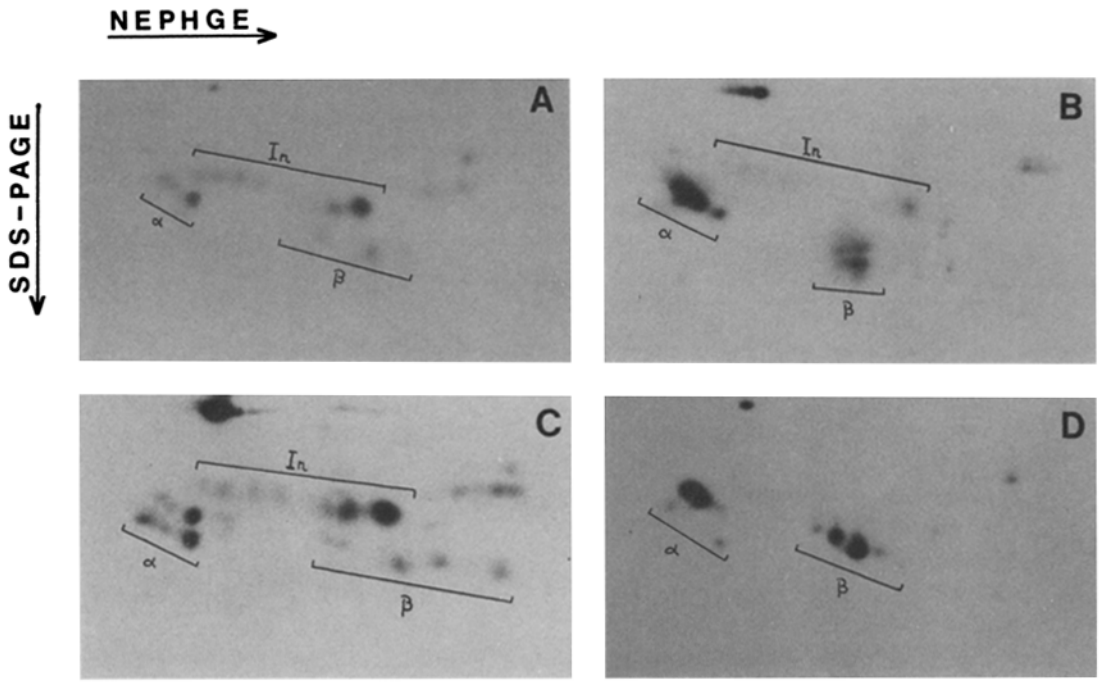

FIGURE 5. Two-dimensional gel analysis in reducing conditions of immunoprecipitates from NP-40 extracts of [^{35}S]methionine–labeled Raji cells. First dimension was NEPHGE, with acidic end to the left and basic end to the right; second dimension was SDS-PAGE, from the top to the bottom. The various autoradiographs show the Ia molecules recognized by the following mAbs: (*A*) 2-72 (anti–fourth subset) after depletion of DR, DQ1 and DP molecules; (*B*) B7/21 (anti-DP), after depletion of DR and DQ1 molecules; (*C*) BT 3/4 (anti-DQ1), after depletion of DR molecules; (*D*) D1-12 (anti-DR). Spots corresponding to the α and β subunits of Ia antigens, as well as to the In chain glycoprotein, are indicated by *brackets*. See text for further details.

The fourth subset α chain resolved as a series of three distinct spots, some of which were superimposable to the DR α and to the DQ1 α chain spots.

A third molecular subunit, the invariant (In) chain, is found associated intracellularly to the α/β Ia heterodimer (20, 23–26). This subunit and its biosynthetic intermediates were also found to be associated with the fourth subset (Fig. 5*A*) and were clearly distinct from both the α and the β chain spots.

Similar patterns of In chains spot distribution were found in DP- (panel *B*) and DQ1- (panel *C*) specific immunoprecipitates. Interestingly, the DR molecules recognized by the D1-12 mAb did not show association with the In chain glycoprotein (panel *D*), suggesting that this antibody recognizes mature forms of DR antigens. Similar results were obtained in LG-2 cells (data not shown).

Thus, even with the more intrinsic limitations of the 2D-PAGE analysis used here in assessing fine structural differences between related Ia proteins (presence of several biosynthetic intermediates of the same polypeptide, poor resolution of the acidic region where the α subunits migrate), the results presented in this section confirm and extend those obtained by the high-resolving 2D-PM technique and indicate that the fourth subset: (*a*) is actually made by the cell; (*b*)

displays a pattern distinguishable from DP and, to a lesser extent, from DR and DQ1 molecules; and (*c*) is associated to the In chain during biosynthesis.

Discussion

In this study we have analyzed at the structural level the human Ia molecular pool expressed on the cell surface of two distinct lymphoblastoid cell lines, LG-2 and Raji, that differ in their class II phenotype.

Purification of Ia molecules after vectorial labeling of externally disposed plasma membrane proteins was selected as a method to specifically analyze mature Ia products at the structural level by 2D-PM and thus minimize problems due to comparison of molecular entities at various steps of biosynthesis. We used four distinct mAbs (D1-12, BT3/4, B7/21, and 2-72) to purify the corresponding Ia molecules. Previous studies (4, 6, 18) have shown that D1-12, BT3/4, and B7/21 mAbs recognize determinants expressed on DR, DQ1, and DP molecules, respectively. On the other hand, the 2-72 mAb is included in a family of anti-Ia mAbs recognizing a determinant shared between DR and other molecular subsets that we had previously defined as NG2. The NG2 α/β heterodimer presented structural features distinct from both DR and DQ1 heterodimers (15). It was therefore of interest to investigate whether NG2 molecules displayed structural similarities with DP antigens.

In the present study we have shown that in cell lysates depleted of DR and DQ1 molecules, preclearing of 2-72-reactive molecules completely abrogated the reactivity of the anti-DP B7/21 mAb. This result clearly indicated that NG2 molecules did include the DP subset. However, preclearing of the same cell lysates with B7/21 mAb drastically reduced but did not completely abrogate the reactivity of the 2-72 mAb, suggesting that additional molecules, other than DP antigens, were included in the NG2 subset. We provisionally designated these additional class II molecules as fourth Ia subset.

From the results of the crossabsorption experiments, several important considerations could be made. The relative contribution to the total Ia pool of the fourth subset, as well as DR, DQ, and DP subsets, could not be precisely extrapolated from the number of ^{125}I counts obtained by specific immunoprecipitation. Substantial disproportions were indeed observed, for example between α- and β-associated counts for the DP subset, as well as for the fourth Ia subset, the α chain of DP antigen labeling very poorly on both LG-2 and Raji cell lysates, and the β chain of fourth subset almost undetectable on LG-2 and, instead, relatively well labeled on Raji cell lysates.

However, even with this limitation, it was clear that the fourth subset represented only a minor population compared with the other three Ia subsets. Calculations based on the relative proportion in terms of ^{125}I-associated counts of the fourth subset with respect to the total Ia pool analyzed in this study, suggest that the fourth Ia subset represents ~2–2.5% of the Ia molecular family. We then performed more refined biochemical analyses to establish the degree of structural variation and polymorphism of the fourth subsets as compared with DR, DQ1, and DP molecules. The results obtained by 2D-PM of separated α and β subunits indicated that the fourth subset was indeed distinct from DR, DQ1, and DP molecules. We observed striking differences in both LG-2 and

Raji cells, particularly between the α subunit of the fourth subset and the homologous counterparts of the other Ia subsets.

Furthermore, fourth subset α chain fingerprints of LG-2 and Raji cells were clearly distinct from each other, strongly suggesting the existence of a structural polymorphism for this subunit that sets it apart from the DR α chain known to be nonpolymorphic.

As shown by SDS-PAGE analysis, β subunits of the fourth subset were almost undetectable in LG-2 cells, and this fact prevented analysis by 2D-PM. Such analysis was instead possible for the homologous subunit in Raji cells. In this case, the fingerprint revealed remarkable differences, as compared with DQ1 and DP homologous fingerprints. Many peptides were instead shared with the DR β subunit, suggesting a certain degree of homology between the small subunits of the two Ia subsets.

Further studies by 2D-PAGE of intact molecules, although not as discriminatory at the fine structural level as the studies by 2D-PM, clearly indicated that the fourth Ia subset is indeed made by the cells and is found associated to the In chain glycoprotein family during biosynthesis. The results obtained by 2D-PAGE analysis in conjunction with previous studies (24) and with the results obtained by 2D-PM of the fourth subset α chains described above, make it unlikely that the large subunit of the fourth subset consists of a putative, very acidic form of In chain associated with the fourth subset Ia heterodimer.

Indeed, we and others (24, 25) have demonstrated the absence of the In chain on the cell surface either associated or not associated to the α/β Ia heterodimer. Furthermore, if particular forms of In chain are expressed on the cell surface, then they should display structural differences between LG-2 and Raji cells to justify the results obtained in the present study (Fig. 3, cf. *C* and *D*), and this possibility argues against all the available data indicating lack of polymorphism of the In chain (reviewed in reference 26).

Taken together, the results indicate the existence of a fourth Ia heterodimer expressed on the cell surface of B cells with structural characteristics distinct from those of DR, DQ1, and DP molecules. The fact that this subset represents only a minority of the total Ia pool (at least as judged by its susceptibility to iodination by the LPGO method and by biosynthetic labeling) and is recognized by mAbs crossreacting with other Ia subsets is probably the reason for it escaping detection in previous studies.

By molecular cloning, various mRNAs and corresponding genes distinct from DR, DQ, and DP have been isolated and described, such as DX α (27) and DX β (28), DZ α (13) and DO β (12). The DX genes are very similar to DQ in their structure, and they have the potential to give rise to an α/β heterodimer, although, to date, no corresponding protein product has been identified. Recent results from our group (15) on DQ molecules recognized by a pair of mAbs (BT3/4 and H40.315.7) indicated the existence of two separated α/β heterodimers that might be candidates for the DQ and DX molecules associated with the DQ1 superspecificity. Interestingly, the BT3/4 mAb recognized both subsets, whereas the H40.315.7 mAb seemed to recognize the classical DQ1 molecules only. The lack of structural correlation at the level of peptide maps between

these two subsets and the fourth subset described here suggests that the latter is not included in the DQ/DX family.

DZ α and DO β genes may constitute additional candidates for a new Ia heterodimer. However, recent results (12) indicate a distinctive pattern of regulation of expression of the two genes that makes it unlikely that they are partners in making up a specific Ia molecule.

It has been shown (12) that the DO β mRNA is expressed in Ia^- variants originally isolated in our laboratory (29). However, preliminary studies suggest that these Ia^- variants do not express the fourth class II subset described in this study (our unpublished results), and thus it is likely that the fourth Ia subset constitutes the product of yet undefined genes.

The analysis of DR, DQ1, and DP subsets by 2D-PM of cell surface–labeled molecules revealed additional important features. Comparative structural analysis of DP α chains isolated from two distinct Ia pools enabled us to show, for the first time, the existence of structural variability of these subunits at the protein level. Although restriction enzyme analysis has detected no polymorphism associated to the functional DP α gene, more recent sequence studies (11) at the cDNA level have indeed revealed limited but significant differences in the primary sequence of DP α chains isolated from distinct cell lines.

Taken together, these results demonstrate the existence of an allelic polymorphism of DP α subunits.

Comparison of DP β subunits of LG-2 and Raji cells indicated the existence of structural differences between them. Although the DP haplotypes of LG-2 and Raji cells have not been defined, the results presented in this report strongly suggest the presence of distinct DP β alleles in the two cell lines. It was of interest to observe that the structural variations between the two β chains were relatively important compared with the other subunits analyzed in this study. It is known from sequence studies at nucleic acid level that allelic polymorphism for DP β genes is limited if compared with the ones observed, for example, in DR β and in DQ α and β genes (reviewed in references 10 and 11). It would therefore appear that the method of analysis reported here is particularly appropriate to detect the discrete polymorphism of DP β gene products.

The DQ1 antigens recognized by the BT3/4 mAb did not show significant differences when compared in LG-2 and Raji cell lines. We saw a lack of appreciable structural polymorphism in both α and β subunits. These results suggest that the allelic forms of α and β chains of DQ antigens present in LG-2 cells (designated as DQ1.1 α and β alleles) are very similar to the corresponding DQ1.W6 allelic products in Raji cells. Alternatively, polymorphic variations between DQ1.1 and DQ1.W6 alleles are confined to areas of the molecular subunits either not accessible to vectorial labeling or lacking tyrosine residues. The possibility that the structural homology observed by 2D-PM reflects lack of polymorphism is strengthened by the recent observation that cDNA clones specific for DQ1.1 and DQ1.W6 β alleles are indeed identical except for a single silent base change (12, 30).

Results obtained by 2D-PM analysis of DP and DQ1 subsets closely correlated with results obtained by gene and protein sequence studies. Similarly, strong correlation between lack of polymorphism at gene or amino acid level and lack

of structural differences by 2D-PM was also observed for the α subunits of DR molecules. A partial exception to this rule was found for the DR β chains known to be among the most polymorphic subunits of human Ia molecules. In fact, fingerprint analysis revealed very limited structural variations between DR β chains of LG-2 and Raji, although sequence studies at nucleic acid level have shown that DR β cDNAs from Raji (31) differ substantially from two virtually identical DR1 β cDNAs isolated in different laboratories (12, 32).

In the above studies, most of the differences have been found in the high polymorphic β-1 domain. It is of interest that tyrosine substitutions were also found in the nonconserved amino acid residues of the β-1 domain of DR 3,W6 and DR1 haplotypes.

The reasons of the partial discrepancy between our results and nucleic acid sequence data are probably related to the tridimensional structure assumed by the DR β chains when they are exposed on the cell surface. It is possible that under native configuration, specific tyrosine residues in, or close to, polymorphic sites of the molecule are not accessible to vectorial labeling because of a particular folding of the β-1 domain of the DR subunit under the influence of or concomitant to, for example, the disulfide bridge generated between cysteine residues 15 and 79. Partial support to such hypothesis comes from recent results reported by our group (15), which showed a larger degree of structural differences between LG-2 and Raji DR β chains when 2D-PM was performed on pepsin digests of Ia molecules labeled by ^{125}I after complete denaturation by SDS and boiling.

In conclusion, we have shown in this report that the combination of vectorial labeling of externally disposed Ia molecules, their isolation by means of specific mAbs and subsequent 2D-PM of separated α and β subunits constitutes a valuable method to analyze the structural polymorphism and heterogeneity of the human Ia pool expressed on the cell surface. In this respect, a new Ia heterodimeric structure with polymorphic characteristics has been defined and provisionally designated fourth Ia subset.

It is likely that the availability of such an heterogeneous array of class II antigens at single cell level increases the capacity of a given individual to present foreign antigens to T cells in the most appropriate environment, thus favouring the correct homeostasis of the immune system.

Summary

Structural analysis by two-dimensional peptide maps (2D-PM) of the human Ia molecular pool expressed on the cell surface of two distinct lymphoblastoid cell lines, LG-2 and Raji, revealed the existence of a novel MHC class II molecular heterodimer that differs at the level of both α and β subunits from the previously described DP, DQ, and DR antigens. These differences were also seen at the level of two-dimensional electrophoresis (2D-PAGE) of biosynthetically labeled intact molecules, although to a lesser extent, due to the intrinsic limitations of this technique in resolving fine structural differences. We have designated this new class II antigen as the fourth Ia subset. The fourth Ia subset seems to represent a small proportion of the human Ia pool. Comparative analysis by 2D-PM of the two cell lines showed the presence of structural variations in the α

chains of the fourth Ia subset, suggesting the existence of polymorphism for these subunits. Cell surface iodination did not show appreciable labeling of the fourth subset β chain in LG-2 cells, and this prevented analysis of the structural polymorphism of this subunit. Furthermore, for the first time, we have shown that DP α chains display distinct peptide maps in LG-2 and Raji cells, thus suggesting the presence of structural polymorphism for these Ia subunits also.

The DQ1 α and β allelic products present in LG-2 cells (DQ homozygous) did not show appreciable structural variation when compared with the homologous allelic products present in Raji cells (DQ heterozygous). Finally, we have confirmed the absence of polymorphism for the DR α subunits. By 2D-PM, relatively low structural variation was instead found for the highly polymorphic DR β subunits expressed in the two cell lines, suggesting that cell surface iodination preferentially labels constant domains of DR β chains.

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