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Isolation and Characterization of Detergent-Solubilized Human HLA-DR Transplantation Antigens†

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ABSTRACT: HLA-DR antigens have been isolated from spleens, in vitro grown Daudi cells, and leukocytes obtained from patients suffering from chronic lymphatic leukemia. Highly purified detergent-solubilized HLA-DR antigens were obtained by affinity chromatography on a *Lens culinaris* lectin column, two gel chromatographies, immunosorbent purifications, and diethylaminoethyl-Sephadex chromatography. In the gel chromatography steps the HLA-DR antigens separated into two fractions. The material of the larger size, fraction I, was converted to the smaller form, fraction II, on storage. The two HLA-DR antigen fractions, I and II, were each shown to be highly purified by chemical, physical–chemical, and immunological criteria. Material in both fractions appeared equally reactive with xenoantisera and alloantisera, suggesting that antigenic differences did not account for the size separation. HLA-DR antigens in fractions I and II displayed identical profiles on isoelectric focusing, and both were heterogeneous with regard to charge. Molecular weight determinations by gel chromatography in 6 M guanidine hydrochloride and by sodium dodecyl sulfate–polyacrylamide gel electrophoresis indicated that the HLA-DR antigens in fractions I and II were composed of two types of polypeptide chains with the apparent molecular weights 28 000 and 34 000. The two HLA-DR antigen subunits could be dissociated and separated by isoelectric focusing in 9 M urea. The separated chains were both shown to bind detergents in micellar form as revealed by charge shift electrophoreses and gel chromatography separations. This result strongly suggests that both HLA-DR antigen chains are integrated into the hydrocarbon matrix of the cell membrane. Some physical–chemical properties of the isolated HLA-DR antigens in fractions I and II were determined.

The human major histocompatibility complex (MHC)[1] consists of at least four loci that code for cell surface molecules involved in several immunological reactions (for a review, see Thorsby, 1974). Three loci, HLA-A, -B, and -C, control the expression of the classical transplantation antigens. The HLA-D locus, primarily defined as the main locus responsible for the stimulation in the mixed leukocyte culture reaction, also controls the expression of cell surface antigens (Wernet et al., 1975). The HLA-D locus is believed to be the human counterpart to the better defined murine H-2 Ir region. Thus,

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[1] Abbreviations used: MHC, major histocompatibility complex; CTAB, cetyltrimethylammonium bromide; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; TDGH, tartryl bis(glycylhydrazide); DTT, dithiothreitol.

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genetical studies have shown that the susceptibility to some diseases is closely linked to certain HLA-D haplotypes (Möller, 1975), and it has been claimed that the IgE response toward some antigens is at least in part controlled by the HLA-D locus (McDevitt & Bodmer, 1974). Moreover, some structural information about gene products of the HLA-D locus of man and of the Ir region of the mouse has revealed striking similarities in the gross structure (Cullen et al., 1974; Wernet, 1976; Humphreys et al., 1976; Klareskog et al., 1977; Springer et al., 1977a–c; Snary et al., 1977a,b; Cresswell, 1977; Nilsson et al., 1977).

HLA-DR antigens have recently been purified from in vitro grown lymphoblastoid cell lines. The HLA-DR antigens are, however, also expressed on a variety of other cell types (Wiman et al., 1978; Forsum et al., 1979). It therefore seemed to be of importance to develop a purification procedure for the HLA-DR antigens which could be applied to different types of tissues. This communication describes the isolation of HLA-DR antigens from chronic lymphatic leukemia cells, in vitro grown lymphoblastoid cell lines, and surgically removed spleens. Some chemical and physical–chemical data of the HLA-DR antigens are also given.

Experimental Procedures

Materials

Cells. Lymphocytes were isolated by leukocytophereses from patients suffering from chronic lymphatic leukemia. Raji and Daudi cells were grown in vitro. Surgically removed spleens were obtained from patients suffering from hemolytic anemia, esophageal cancer, or abdominal trauma.

Antisera. The antisera against the HLA-A, -B, -C, and -DR antigens have been described (Rask et al., 1976; Klareskog et al., 1978). An antiserum was raised in a rabbit against a crude membrane fraction isolated from thymocytes, obtained from a surgically removed human thymus. The reactivity of the alloantisera directed against HLA-DR antigens has been described (Klareskog et al., 1977).

Special Materials. Lens culinaris hemagglutinin was isolated as described (Hayman & Crumpton, 1972). The cleavable cross-linker TDGH was synthesized as described (Lutter et al., 1974).

Methods

Concentration of Proteins. Concentration of proteins during the isolation procedure was accomplished by ultrafiltration (Berggård, 1961a).

Immunological Techniques. Immunodiffusion in gel was performed in 1% agarose gels containing 0.01 M Tris-HCl buffer, pH 8.0, 0.1 M NaCl, and 0.01% Tween-80 as described (Berggård, 1961b). Immunoelectrophoresis was carried out according to the Scheidegger micromethod (Scheidegger, 1955). Also, in this case the buffer contained 0.01% Tween-80. HLA-DR antigens were followed during the isolation procedure by a quantitative radioimmunoassay technique (Klareskog et al., 1978). In some cases the occurrence of HLA-DR antigens in highly purified preparations was analyzed by indirect immunoprecipitation (Östberg et al., 1976). β_2-Microglobulin was estimated by means of a solid-phase radioimmunoassay (Evrin et al., 1971).

Affinity Chromatography and Immunosorbent Purification. Lens culinaris hemagglutinin and IgG fractions isolated by gel chromatography from various antisera were coupled to cyanogen bromide activated Sepharose 4B (Axēn et al., 1967; Cuatrecasas, 1970). Desorption of material bound to the lectin column was achieved by 10% α-methyl mannoside in the eluant. Protein bound to the immunosorbent columns was eluted with 0.1 M sodium citrate buffer, pH 2.9, containing 0.5 M NaCl and 0.01% Tween-80.

Detergent Exchange. Deoxycholate was replaced by Tween-80 by dialysis. Trace amounts of [^{14}C]deoxycholate (Amersham, England) were added to the protein sample which was then made 0.1% with regard to Tween-80. After dialysis against three changes of 0.01% Tween-80 in the relevant buffer, the content of radioactivity in the sample was negligible.

Nonionic detergents were changed for deoxycholate by affinity chromatography on Sepharose-coupled *Lens culinaris* hemagglutinin columns. The protein sample containing Tween-80 was applied to the lectin column. Tween-80 was washed off the column with five column volumes of 20 mM deoxycholate and one column volume of a 10 mM deoxycholate solution. The bound material was desorbed by elution with 10% α-methyl mannoside in the 10 mM deoxycholate solution. The monosaccharide was removed by dialysis.

Electrophoresis and Isoelectric Focusing. Polyacrylamide gel electrophoresis in $NaDodSO_4$ was performed in disc gels (Laemmli, 1970). Charge shift electrophoresis in agarose gels was carried out essentially as described (Helenius & Simons, 1977). Isoelectric focusing was performed in 4% polyacrylamide gels in the pH intervals 3.5 or 4.0 to 8.0 (O'Farrell, 1975). In some cases urea was omitted from the separating gel.

Radioactive Labeling. Highly purified HLA-DR antigens were labeled with ^{125}I by the chloramine-T procedure (Hunter & Greenwood, 1962). ^{3}H was introduced by reductive methylation into lysines of the HLA-DR antigens (Rice & Means, 1971).

Cross-Linking of HLA-DR Antigens. Cross-linking with the cleavable cross-linker TDGH was carried out essentially as described (Lutter et al., 1974). HLA-DR antigens (approximately 1 μg/mL) were treated in 0.1 M sodium borate buffer, pH 8.5, containing 0.01% Tween-80 with TDGH of concentrations varying from 0.5 to 5 mM. Cross-linked proteins eluted from polyacrylamide gels were cleaved by incubation in 0.02 M triethanolamine buffer, pH 8.0, containing 30 mM $NaIO_4$.

Analytical Gel Chromatography. The molecular weights of the HLA-DR antigens and their isolated polypeptide chains were determined by gel chromatography on columns of Sepharose 6B, equilibrated with 0.1 M sodium acetate buffer, pH 5.0, containing 6 M guanidine hydrochloride (Fish et al., 1969). The proteins used as references were the β chain of haptoglobin, retinol-binding protein, prealbumin, human β_2-microglobulin, heavy and light chains of human immunoglobulin G, and human serum albumin. Stokes' molecular radii were determined by analytical gel chromatography on Sephadex G-200 columns (100 × 1 or 140 × 2 cm) equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.05 M NaCl and 10 mM deoxycholate. All experiments were performed at 4 °C, and analyses were carried out at least in duplicate (Karlsson et al., 1972).

Determinations of Diffusion Coefficients, Sedimentation Constants, Molecular Weights, and Frictional Ratios. Apparent diffusion coefficients ($D_{20,w}$) were computed from Stokes' radii by use of the Stokes–Einstein equation (Gosling, 1956). The sedimentation constants were determined by sucrose density gradient ultracentrifugation. ^{125}I-Labeled HLA-DR antigens were applied to linear sucrose gradients from 5 to 20% in 0.02 M Tris-HCl buffer, pH 8.0, containing 0.05 M NaCl and 10 mM deoxycholate. After 16–18 h of centrifugation at 280000g, the centrifuge tubes were eluted with a micromodification of the device described (Pertoft &

Laurent, 1969). In some cases the distribution of the HLA-DR antigens in the gel chromatography and ultracentrifugation experiments was assessed by indirect immunoprecipitation. Molecular weights were calculated from sedimentation coefficients, diffusion coefficients, and partial specific volumes by the equation of Svedberg (Svedberg & Pedersen, 1940). On the assumption that HLA-DR antigens contain about 10% carbohydrate by weight, the partial specific volume based on carbohydrate and amino acid content[2] (Edsall, 1943) is about 0.725. Since molecular weights for the HLA-DR antigens were estimated in the presence of deoxycholate, it is the partial specific volume of the protein–detergent complex that is relevant. Deoxycholate has a partial specific volume of 0.78 (Helenius & Simons, 1975), so the true partial specific volume of the protein–detergent complex should be between 0.726 and 0.78. Since lack of material precluded actual measurements of the amount of deoxycholate bound to the HLA-DR antigens, a partial specific volume of 0.74 was assumed for the protein–detergent complex.

Frictional ratios (f/f_0) were calculated from Stokes' radii, sedimentation coefficients, molecular weights, and partial specific volumes (Svedberg & Pedersen, 1940).

Other Methods. Protein concentrations in unpurified fractions were estimated by the modified Folin–Lowry procedure (Lowry et al., 1951) as described by Dulley & Grieve (1975) for detergent-containing samples. Bovine serum albumin served as the reference substance. More highly purified fractions were assayed for protein content by measuring the absorbance at 280 nm. It was arbitrarily assumed that HLA-DR antigens at a concentration of 1 mg/mL give an absorbance of 1.5 at 280 nm. Phosphate analyses were carried out by a micromodification of the method of Chen et al. (1956).

Results

Isolation of the HLA-DR Antigens. The isolation procedure adopted was reproducible and yielded HLA-DR antigens of high purity regardless of whether the starting material was in vitro cultured cells, peripheral blood leukocytes from patients suffering from chronic lymphatic leukemia, or surgically removed human spleens. However, during the isolation of the HLA-DR antigens from spleens it was found that considerably more contaminating material was present prior to the last purification step than was present in the preparations from the other sources. An additional fractionation step was therefore used in the isolation of the HLA-DR antigens from spleens, namely, immunosorbent chromatography on a Sepharose 4B column to which had been coupled antibodies directed against a crude membrane fraction of thymocytes from surgically removed human thymuses. The breakthrough material obtained from this column was then subjected to the final purification steps. By this modification the HLA-DR antigens from spleens were obtained with the same degree of purity as those derived from other sources. The recoveries of highly purified HLA-DR antigens varied between different preparations. Generally, the yield was unacceptably low when the starting material consisted of less than about 30 g of B-lymphocytes. In the typical isolation procedure that is described in detail below, the starting material was leukocytes from a patient suffering from chronic lymphatic leukemia. When not otherwise stated, the data are valid also for the isolation of HLA-DR antigens from the other sources.

Solubilization of Membrane Proteins. Leukocytes, 60 g, were repeatedly frozen and thawed in 50 mL of 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl and 2 mM PMSF. The cells were then subjected to nitrogen cavitation at 800 psi for 20 min and finally homogenized in a Potter-Elvehjelm homogenizer. The nitrogen cavitation step was omitted when spleens were used as starting material. Nuclei, unbroken cells, and debris were removed by centrifugation at 10000*g* for 10 min. The pellet was resuspended in the same buffer and recentrifuged. The combined supernatants were centrifuged at 105000*g* for 60 min. The pellet was washed twice by suspension and centrifugation. The final pellet, representing the crude membrane fraction, was suspended in 0.02 M Tris-HCl buffer, pH 8.0, to a protein concentration of 10 mg/mL. Deoxycholate was added to a final concentration of 20 mM. After 60 min particulate material was sedimented by centrifugation at 105000*g* for 60 min. The whole procedure was carried out at 4 °C.

Table I: Purification of Detergent-Solubilized HLA-DR Antigens

fractionation step	total protein (mg)		amount of HLA-DR antigen[a] (mg)		yield (%)		purity (%)	
	I[b]	II[b]	I[b]	II[b]	I[b]	II[b]	I[b]	II[b]
solubilized material	850[c]		3.4		100		0.6	
affinity chromatography	65[c]		3.2		94		4.9	
first Sephadex G-200	7.3[c]	9.4[c]	0.95	1.56	28	46	13	17
second Sephadex G-200	3.4[c]	6.6[c]	0.67	1.38	20	41	20	21
immunosorbent chromatographies	1.7[d]	2.7[d]	0.62	1.29	18	38	37	48
DEAE-Sephadex chromatography	0.60[d]	1.15[d]	0.45	0.95	13	28	75	83
Sepharose 6B	0.30[d]	0.90[d]	0.30	0.88	8.7	26	100	98

[a] Measured by a radioimmunoassay technique. [b] I and II denote the two fractions of HLA-DR antigens that were subjected to separate purification procedures after the first Sephadex G-200 gel chromatography step. [c] Determined by a modification of the Folin method. [d] Estimated from the optical density at 280 nm.

Affinity Chromatography on a Lens culinaris Hemagglutinin Column. The solubilized membrane macromolecules, comprising 850 mg of total protein and 3.4 mg of HLA-DR antigens, were applied to a column of Sepharose 4B containing covalently bound *Lens culinaris* hemagglutinin. The column was equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 10 mM deoxycholate and 2 mM PMSF. Material not bound to the lectin was washed off the column by exhaustive elution with the same buffer. Bound glycoproteins were then desorbed with α-methyl mannoside and concentrated. Less than 10% of the total protein bound to the column, whereas more than 90% of the HLA-DR antigens was present in this fraction (Table I).

First Gel Chromatography on Sephadex G-200. The material obtained from the lectin affinity chromatography step, comprising 65 mg of total protein and 3.2 mg of HLA-DR antigens, was subjected to gel chromatography on a Sephadex G-200 column (Figure 1). The HLA-DR antigens were eluted at two positions with the K_{av} values 0.15 and 0.25. β_2-Microglobulin occurred in three elution positions, suggesting the presence of HLA-A, -B, and -C antigens as tetramers (K_{av} ~0.19) and dimers (K_{av} ca. 0.31) (Peterson et al., 1977). In addition, free β_2-microglobulin emerged at a K_{av} of 0.75.

The relative proportions of HLA-DR antigens in the two elution positions varied considerably between different preparations. The fractions comprising the HLA-DR antigens

[2] B. Curman and P. A. Peterson, unpublished experiments.

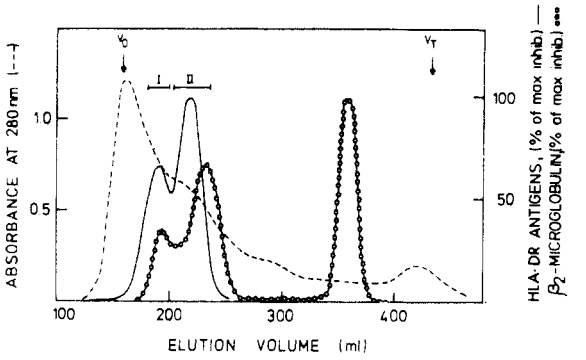

FIGURE 1: Gel chromatography on Sephadex G-200 of the HLA-DR antigen containing fraction obtained after the lectin affinity chromatography step. The column (140 × 2 cm) was equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 10 mM deoxycholate and 2 mM PMSF. Fractions of 2.8 mL were collected at intervals of 20 min. The distribution in the effluent of HLA-DR antigens and of β_2-microglobulin was monitored by radioimmunoassay techniques. The fractions containing the HLA-DR antigens were pooled into two portions as indicated by the bars.

were pooled and concentrated. The earliest eluted HLA-DR antigen fraction will henceforth be termed fraction I and the later eluted material, fraction II.

Second Gel Chromatography on Sephadex G-200. The two HLA-DR antigen containing fractions were separately rechromatographed on columns of Sephadex G-200 (120 × 2 cm) under conditions identical with those described above. An appreciable purification was obtained (Table I). The majority of the HLA-DR antigens in fraction I occurred in an elution position corresponding to a K_{av} of 0.15, but an unexpectedly large proportion emerged from the column with a K_{av} of 0.25. To rule out that the earlier eluted HLA-DR antigens represented poorly solubilized molecules attached to mixed detergent–phospholipid micelles, phosphate analyses were carried out over the entire chromatogram. No phosphate, i.e., less than two phospholipid molecules per HLA-DR antigen molecule, could be detected, suggesting that the HLA-DR antigens in fraction I were not bound to annulus lipids.

HLA-DR antigens in fraction II emerged in the expected elution position. The HLA-DR antigens of fraction I, occurring at a K_{av} of 0.15, and of fraction II, eluted at a K_{av} of 0.25, were separately pooled and concentrated.

Immunosorbent Chromatographies. The HLA-DR antigen containing fractions were passed consecutively over three immunosorbent columns containing covalently bound rabbit antibodies against human IgM, β_2-microglobulin, and heavy HLA-A, -B, and -C chains, respectively. All columns were equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.05 M NaCl and 0.01% Tween-80. At this stage it was necessary to exchange the deoxycholate for Tween-80 to allow charge separations (see below).

Material in the two HLA-DR antigen containing fractions which passed the three columns unretarded was separately pooled and concentrated. The yield of the HLA-DR antigens was excellent in this purification step (Table I).

Chromatography on DEAE-Sephadex. Fractions I and II were chromatographed individually on DEAE-Sephadex under identical conditions. In both cases the HLA-DR antigens displayed heterogeneity, but the elution positions for the antigens were very similar if not identical for the two materials (Figure 2). Fractions were combined to yield HLA-DR antigens contaminated only with minor amounts of unrelated proteins.

Gel Chromatography in Tween-80. The HLA-DR antigen fractions I and II were separately subjected to gel chroma-

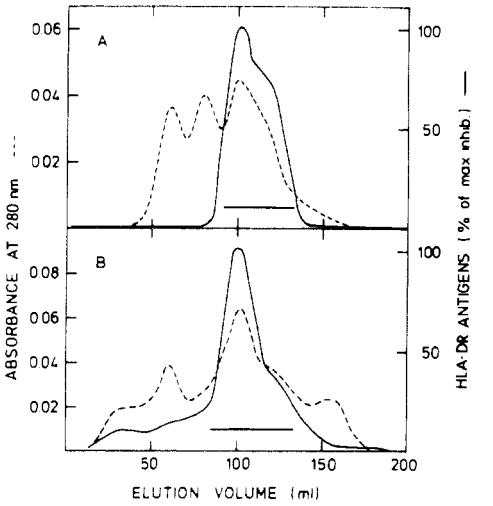

FIGURE 2: Chromatography on DEAE-Sephadex of the HLA-DR antigen containing fractions I (A) and II (B) obtained after the immunosorbent purification steps. The columns (12 × 1.4 cm) were equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.05 M NaCl and 0.01% Tween-80. Prior to application the samples (containing 1.7 and 2.7 mg, respectively, of total protein) were dialyzed exhaustively against the same buffer. Elution was performed at pH 8.0 with 200-mL linear gradients of NaCl from 0.05 to 0.5 M. Fractions of 2.1 mL were collected at 15-min intervals. The distribution of the HLA-DR antigens was monitored by a radioimmunoassay. The fractions containing the HLA-DR antigens were combined as indicated by the bars.

tography on columns of Sepharose 6B equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl and 0.01% (w/v) of Tween-80. The HLA-DR antigens in both fractions were eluted well ahead of the unrelated protein species. The HLA-DR antigens in fraction I again resolved into two peaks (see Figure 1). The elution position of the smaller HLA-DR antigen material coincided with the elution position for the HLA-DR antigens in fraction II. It is noteworthy that on storage for several weeks the HLA-DR antigens of the large size were partly converted into the small size in an irreversible manner.

Purity of the Isolated HLA-DR Antigens. The purity of the HLA-DR antigens from fractions I and II was assessed by $NaDodSO_4$–polyacrylamide gel electrophoresis. Both fractions gave rise to the typical two-chain pattern (Figure 3) when stained with Coomassie Brilliant Blue (Klareskog et al., 1977; Springer et al., 1977a–c; Snary et al., 1977a,b; Cresswell, 1977; Nilsson et al., 1977). Gel electrophoresis of samples of the two HLA-DR antigen fractions labeled either with ^{125}I in tyrosine (and histidine) residues or with ^{3}H in lysine residues also demonstrated the same electrophoretic pattern, thereby emphasizing the high degree of purity of the isolated components.

HLA-DR antigen fractions I and II were examined as to charge homogeneity by isoelectric focusing. ^{125}I-Labeled fraction I and ^{131}I-labeled fraction II gave superimposable profiles. The occurrence of several peaks suggested a high degree of charge heterogeneity. This heterogeneity did not seem to result from the presence of contaminating proteins as material from different parts of the peaks reacted equally well with an antiserum raised against highly purified HLA-DR antigens.

Immunological Properties of the Isolated HLA-DR Antigens. The HLA-DR antigens present in fractions I and II were indistinguishable by Ouchterlony immunodiffusion analyses when tested against a rabbit anti-HLA-DR antigen serum (Figure 4A). HLA-DR antigens in the two fractions displayed identical electrophoretic mobilities and gave rise to

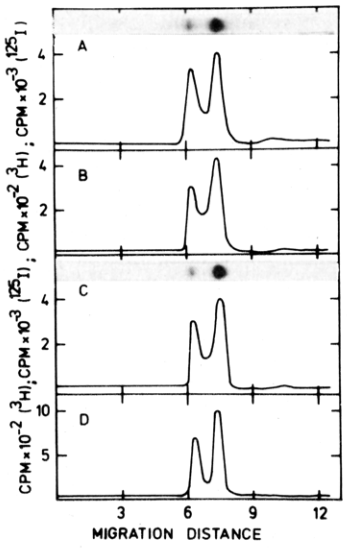

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of HLA-DR antigens in fraction I labeled with ^{125}I in tyrosine and histidine residues (A) and with ^{3}H in lysine residues (B). The inset in (A) depicts a gel of the same material stained with Coomassie Brilliant Blue. ^{125}I- and ^{3}H-labeled HLA-DR antigens in fraction II are shown in (C) and (D). The inset in (C) represents HLA-DR antigens in fraction II after staining with Coomassie Brilliant Blue. The anode is to the right.

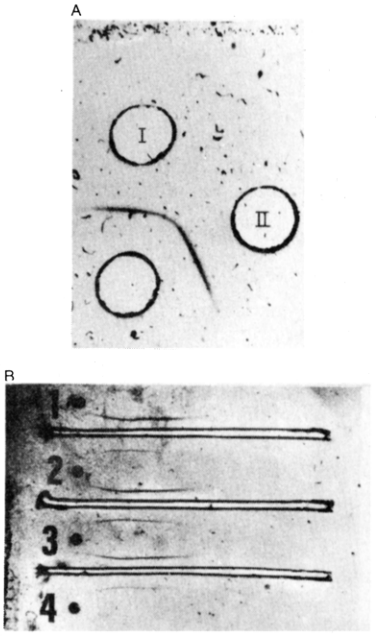

FIGURE 4: (A) Ouchterlony immunodiffusion analysis of highly purified HLA-DR antigens in fractions I and II. The lower cell contained a rabbit antiserum against HLA-DR antigens. (B) Immunoelectrophoretic analysis of solubilized membrane molecules [(1) and (4)] and highly purified HLA-DR antigens in fractions I (2) and II (3). The antiserum basins contained a rabbit antiserum against highly purified HLA-DR antigens.

single precipitin arcs against the same antiserum on immunoelectrophoresis (Figure 4B). The highly purified HLA-DR antigens also exhibited similar electrophoretic mobilities as the HLA-DR antigens in the starting material, thus indicating that the isolated HLA-DR antigens were representative for all HLA-DR antigens present in the crude membrane fraction.

HLA-DR antigens display alloantigenic determinants recognized by sera obtained from some multiparous women. To ascertain that the isolated HLA-DR antigens were reactive with the relevant alloantisera, material from fractions I and II were separately labeled with ^{125}I and reacted with the antisera. Table II shows that antisera directed against the specificities expressed by the cells of the starting material (DRw3 and DRw4) reacted with from 25 to 39% of the labeled material. A combination of the alloantisera did not react with more than about half of the labeled molecules, whereas more than 90% of the label was recovered in the precipitate when the rabbit anti-HLA-DR antigen serum was added. Preprecipitations with the rabbit antiserum eliminated all molecules reactive with the alloantisera, whereas preprecipitations with the alloantisera only removed a fraction of the molecules reactive with the rabbit antiserum. When preprecipitations were performed with one alloantiserum followed by precipitation with the other, no or only small amounts of material were recovered. The reason for this is most probably that the dilution caused by the preprecipitation step renders the reaction kinetics of the second incubation too unfavorable to ensure complex formation. The data in Table II ascertain that the immunological reactivity of the HLA-DR antigens of fractions I and II is similar if not identical.

Table II: Antigenic Reactivity of the Highly Purified HLA-DR Antigens[a]

antiserum[b]		amount precipitated (% of total)	
preprecipitation	precipitation	fraction I[c]	fraction II[c]
	R-α-HLA-DR	92	94
	NRS	2.8	2.0
	DRw3	39.5	28.6
	DRw4	26.1	25.0
	DRw3 + DRw4	54.5	45.1
	DRw2	4.0	2.3
R-α-HLA-DR	DRw3	2.8	1.6
R-α-HLA-DR	DRw4	2.4	1.6
R-α-HLA-DR	DRw2	3.1	1.5
NRS	DRw3	29.1	21.6
NRS	DRw4	17.9	19.3
NRS	DRw2	2.6	1.8
DRw3	R-α-HLA-DR	54.1	63.7
DRw3	NRS	2.2	1.6
DRw4	R-α-HLA-DR	56.2	59.9
DRw4	NRS	2.4	1.5
DRw2	R-α-HLA-DR	89.4	96.2
DRw2	NRS	1.8	1.8
DRw3	DRw4	4.1	3.9
DRw4	DRw3	5.2	4.2
DRw2	DRw3	3.2	3.7

[a] HLA-DR antigens in fractions I and II were separately labeled with ^{125}I, and aliquots (4600 cpm of fraction I and 6900 cpm of fraction II) were incubated with 25-μL portions of the alloantisera and 3-μL portions of the rabbit sera, as indicated above. After 5 h at room temperature immune complexes and free immunoglobulins were precipitated (Rask et al., 1976). The precipitates were washed three times with ice-cold saline and the radioactivity was measured. Preprecipitations were performed by the same technique. In this case the supernatants were incubated for another 5 h with the second antiserum, and the isolation of immune complexes was performed as described above. [b] The following are the designations for the sera: R-α-HLA-DR, rabbit antiserum against HLA-DR antigens; NRS, normal rabbit serum; DRw2, DRw3, and DRw4, alloantisera against antigenic specificities DRw2, DRw3, and DRw4, respectively. The antigenic specificity DRw2 was not detected in tissue typing of the patient. [c] I and II denote the two HLA-DR antigen fractions.

It was of interest to examine the possible immunological cross-reactivity between the isolated HLA-DR antigens and the molecules controlled by the A, B, and C loci of the MHC region. This was performed by radioimmunoassay techniques. The isolated HLA-DR antigens did not interfere in radioimmunoassays specific for the heavy alloantigenic HLA-A, -B, and -C antigen chains or for β_2-microglobulin (Figure 5).

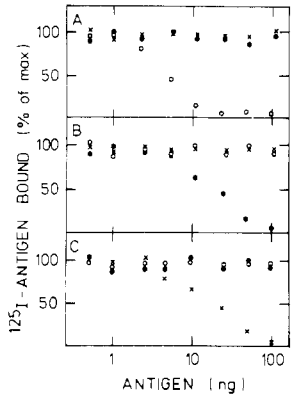

FIGURE 5: Inhibition of radioimmunoassays for β_2-microglobulin (A), the alloantigenic HLA-A, -B, and -C chains (B), and HLA-DR antigens (C) with highly purified β_2-microglobulin (○), alloantigenic HLA-A, -B, and -C chains (●), and HLA-DR antigens (×).

Isolated HLA-A, -B, and -C antigens or free β_2-microglobulin did not cause any inhibition in the HLA-DR antigen radioimmunoassay. Furthermore, the rabbit anti-HLA-DR antigen serum could not directly bind to ^{125}I-labeled HLA-A, -B, and -C antigens, and antisera directed against the two HLA-A, -B, and -C antigen subunits did not recognize ^{125}I-labeled HLA-DR antigens in direct binding experiments.

Subunit Structure of the HLA-DR Antigens. It is evident from Figure 3 that the HLA-DR antigen fractions I and II contain two types of dissimilar polypeptide chains. Data from several laboratories suggest that these chains form a noncovalent complex (Klareskog et al., 1977; Snary et al., 1977a; Springer et al., 1977c). HLA-DR fractions I and II were investigated by cross-linking experiments in order to ascertain that the difference in size between the two materials was not caused by differences in stoichiometry of the constituent chains. Material from fractions I and II, labeled with ^{125}I, was separately treated with the cleavable cross-linker TDGH and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. Fraction II gave rise to a new component with an apparent molecular weight of about 60 000 after cross-linking. Also, when the molar ratio of the cross-linker to the protein was varied, no evidence was obtained for the occurrence of additional components with higher molecular weights. The 60 000-dalton species was recovered from the gels, the cross-linker was cleaved, and the material was resubjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. The two HLA-DR antigen subunits were again visualized.

Fraction I contained HLA-DR antigens of the two size classes. This fraction was therefore examined as to the distribution of the two types of subunits in the two gel chromatography peaks. Accordingly, after cross-linking, the material of fraction I was subjected to gel chromatography. The resulting chromatogram was essentially identical with that obtained with the same material in the absence of the cross-linker. Cross-linked material from each of the two peaks was subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. The electropherograms were identical. In both cases the expected 60 000-dalton component was present, but no molecular species of larger weight. De-cross-linking and $NaDodSO_4$–polyacrylamide gel electrophoresis of the 60 000-dalton component gave rise to the expected pattern with the two dissimilar subunits.

Separation of the HLA-DR Antigen Subunits and Examination of Their Membrane Integration. The ^{125}I-labeled HLA-DR antigens of fractions I and II were subjected to isoelectric focusing in 9 M urea. Figure 6 shows that the material in fraction II resolved into two components which

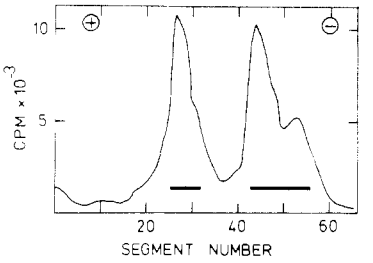

FIGURE 6: Isoelectric focusing in urea of ^{125}I-labeled HLA-DR antigens in fraction II. The two peaks were separately pooled as indicated by the bars.

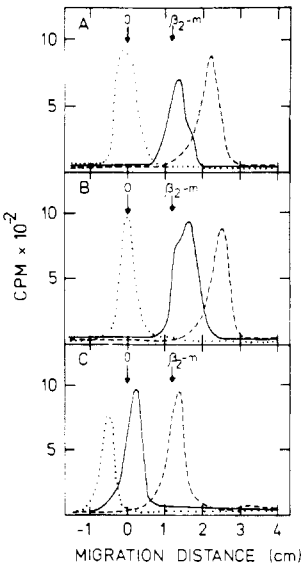

FIGURE 7: Charge shift electrophoresis of ^{125}I-labeled intact HLA-DR antigens (A), the separated 34 000-dalton subunit (B), and the isolated 28 000-dalton polypeptide chain (C). The figure is a composite representation of three electrophoretic experiments performed in Triton X-100 (—), in Triton X-100 and deoxycholate (- - -), and in Triton X-100 and CTAB (···). The arrows denote the origin and the migration position of β_2-microglobulin. Since β_2-microglobulin does not bind detergents its electrophoretic mobility is the same in all three detergent mixtures.

each displayed microheterogeneity. The HLA-DR antigens were pooled, as indicated in the figure, and aliquots from each pool were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. The acidic component corresponded to the larger HLA-DR subunit, whereas the smaller one was more basic. The behavior on isoelectric focusing was indistinguishable for material from fractions I and II. The two subunit pools were extensively dialyzed against 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl and 0.5% Triton X-100 to remove the urea, and concentrated.

It is well-known that detergents in micellar form preferentially, if not exclusively, bind to the hydrophobic portion of membrane proteins but not to their hydrophilic parts nor to water-soluble proteins in the native or the denatured state (Tanford & Reynolds, 1976). Thus, to examine if both HLA-DR antigen subunits are integrated into the hydrophobic matrix of the membrane, they were separately subjected to charge shift electrophoresis. Figure 7 shows that the electrophoretic mobilities of the two HLA-DR antigen subunits were enhanced in the presence of the anionic detergent deoxycholate and retarded in the presence of CTAB, a cationic detergent. These data suggest that both chains bind detergent equally well. There are reports that some hydrophilic proteins may bind one or a few monomers of certain detergents (for a review, see Helenius & Simons, 1975). However, membrane proteins bind detergent micelles. Since nonionic detergent

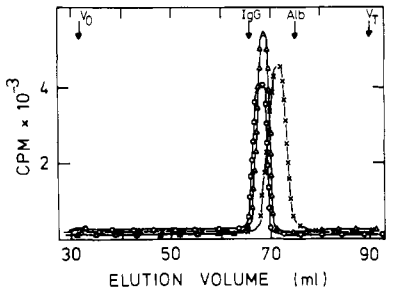

FIGURE 8: Gel chromatography of the separated ^{125}I-labeled HLA-DR antigen subunits on a column (99 × 1 cm) of Sepharose 6B equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.05 M NaCl and 0.01% Tween-80. The 34 000-dalton (○) and 28 000-dalton (△) subunits were analyzed in separate runs. The elution position of the free Tween-80 micelles (×) is included for comparison. The column was calibrated with immunoglobulin G (IgG) and albumin (Alb), and their elution positions are indicated by the arrows.

micelles are large in comparison to the detergent monomer, proteins binding the micellar form should have a relatively large size on gel chromatography compared to the expected size based on the protein molecular weight. Figure 8 shows that the two HLA-DR antigen subunits emerged at an earlier elution position than that recorded for the free Tween-80 micelles, when analyzed by gel chromatography on a column of Sepharose 6B.

The elution positions of the two HLA-DR antigen chains were, thus, well ahead of that of albumin, which only binds the monomer form of the detergent. In contrast, the electrophoretic mobilities of separated, papain-solubilized HLA-DR antigen chains were not influenced by the different detergents. Moreover, on gel chromatography the separated, papain-solubilized chains were eluted considerably later than the protein-free detergent micelles.[3]

Some Physical–Chemical Properties of the Isolated HLA-DR Antigens. Some physical–chemical properties of the fractions I and II HLA-DR antigens are summarized in Table III. The data given are those for HLA-DR antigen–deoxycholate complexes.

The HLA-DR antigens in fractions I and II were examined with regard to the sedimentation behavior of molecules expressing DRw3 and DRw4 antigenic determinants. DRw3 and DRw4 antigens sedimented as single homogeneous components, and no separation of the two types of antigens was observed when fraction II was analyzed. The alloantigens resolved, however, into two peaks when fraction I was subjected to analysis. Also, for this fraction the sedimentation profiles for the two alloantigens were indistinguishable, and the sedimentation constant for the more slowly sedimenting peak was very similar to that recorded for the material in fraction II (see Table III).

Stokes' molecular radii for DRw3 and DRw4 HLA-DR antigens in fraction II were indistinguishable. Both alloantigens gave rise to two gel chromatography peaks when fraction I was analyzed.

The frictional ratios (f/f_0) for the DRw3 and DRw4 HLA-DR antigens in fraction II were similar to that computed for the more slowly sedimenting material of fraction I. The frictional ratio 1.55 of the more rapidly sedimenting component in fraction I and the value 1.40 for the HLA-DR antigens in fraction II are both above the values normally found for globular proteins.

The molecular weights of HLA-DR antigens and their constituent polypeptide chains were estimated by several methods. It can be seen in Table III that the sum of the weights for the subunits fell well below the values estimated for the intact HLA-DR antigens. This is the expected result since the intact HLA-DR antigen molecular weights were estimated in the presence of deoxycholate. The larger component in fraction I had a molecular weight that was almost twice that of the material in fraction II.

[3] L. Trägårdh, L. Klareskog, and P. A. Peterson, unpublished experiments.

Table III: Physical–Chemical Characteristics for Detergent-Solubilized HLA-DR Antigens[a]

	HLA-DR antigens	
	I	II
sedimentation constant (S)	6.0 (4.3)	4.3
Stokes' molecular radius (Å)[b]	55 (40)	40
diffusion constant ($\times 10^7$ cm^2 s^{-1})[b]	3.9 (5.4)	5.4
frictional ratio, f/f_0	1.55 (1.40)	1.40
molecular weight		
sedimentation-Stokes' radius	147 000 (76 000)	76 000
gel chromatography[c]	28 000; 31 000	28 000; 31 000
electrophoresis[d]	28 000; 34 000	28 000; 34 000

[a] Separate analyses were performed for DRw3 and DRw4 antigens in the two fractions. The values were indistinguishable. Values in parentheses represent data for the smaller HLA-DR antigen component present in fraction I. [b] Estimated by analytical gel chromatography. [c] Determined in 6 M guanidine hydrochloride on reduced and alkylated polypeptide chains. [d] Data from NaDodSO$_4$-polyacrylamide gel electrophoresis.

Discussion

The adopted isolation scheme for the HLA-DR antigens is reproducible, and the antigens are obtained in a highly purified form in reasonable yield. It has proven difficult to isolate HLA-DR antigens in highly purified form without resorting to the use of denaturing detergents (Springer et al., 1977a–c) or immunosorbent techniques, which require desorption under nonphysiological conditions (Cresswell, 1977). However, detergent-solubilized HLA-DR antigens in a relatively pure state have been isolated previously under mild conditions from a lymphoblastoid cell line (Snary et al., 1977a,b). The present purification procedure has two distinct advantages. First, the method yields highly purified HLA-DR antigens from sources as diverse as chronic lymphatic leukemia cells, spleens, and in vitro grown lymphoblastoid cell lines. Second, the starting material consists of crude membrane fractions, which obviates the need for a plasma membrane isolation step. This should improve the yield of the HLA-DR antigens considerably since in most plasma membrane isolation procedures only about half of the membranes are recovered (Snary et al., 1977b). The critical step in the described isolation procedure appears to be the use of two types of detergents with different micellar sizes. Thus, on gel chromatography in deoxycholate some contaminating proteins have the same size as the detergent–HLA-DR antigen complexes. In Tween-80 the HLA-DR antigen–detergent complexes are eluted ahead of these contaminating proteins, which are not integral membrane proteins as they do not bind detergent micelles.

The HLA-DR antigens resolved into two size classes on gel chromatography. On storage in detergent solutions the larger material in fraction I displayed a tendency to become of a size identical with that of the HLA-DR antigens in fraction II. Molecular weight estimations demonstrated that the larger HLA-DR antigen species had a molecular weight almost twice that of the HLA-DR antigens in fraction II. A number of explanations for this finding could be afforded. Thus, HLA-DR antigens of the larger size could have been associated with some non-HLA-DR antigen material, thereby increasing

the molecular weight. This does not seem all that likely since several lines of evidence argue against the HLA-DR antigens being associated with unrelated protein or phospholipid material. The high molecular weight is, however, also consistent with the HLA-DR antigens in fraction I being composed of four polypeptide chains, i.e., a dimer of the HLA-DR antigen component in fraction II. Alternatively, HLA-DR antigens may bind detergents like deoxycholate and Tween-80 in two discrete modes. Detailed measurements of the amount of detergent bound to the two size classes of HLA-DR antigens were precluded due to scarcity of material. It should be emphasized that even in the event that HLA-DR antigens containing four polypeptide chains in solution can be unambiguously demonstrated this does not necessarily imply that the antigens occur as tetramers in the intact cell membrane.

Snary et al. (1977a,b) used deoxycholate to solubilize HLA-DR antigens but obtained antigens of a single size only. The reason why these workers did not encounter HLA-DR antigens of the large size remains unexplained. However, subtle differences in the preparation of the starting material and in the solubilization procedure may account for the differences.

^{125}I-Labeled HLA-DR antigens reacted poorly with alloantisera. This may have depended on several factors, like that the titers of the alloantisera used were low or that iodination partly deranges the alloantigenic determinants. It is, however, also possible that the HLA-DR antigen containing fractions contained HLA-DR antigen-like material which did not exhibit the DRw3 and DRw4 alloantigenic determinants. This is reasonable to assume in as much as the human HLA-D locus, in analogy with the murine counterpart (Cullen et al., 1976), may encompass several genes controlling the expression of similar molecules, to some of which alloantisera have not yet been obtained.

With use of heteroantisera no immunological cross-reactivity between the HLA-DR antigens and the HLA-A, -B, and -C antigens could be observed. This, of course, does not exclude the possibility that the two types of molecules may be evolutionarily related. In this context it is noteworthy that antibodies against β_2-microglobulin do not generally react with immunoglobulin G despite a considerable similarity in primary structure (Peterson et al., 1972).

The HLA-DR antigens are composed of two types of noncovalently linked, dissimilar subunits (Klareskog et al., 1977; Springer et al., 1977a,c; Snary et al., 1977a,b; Cresswell, 1977). The cross-linking experiments reported here confirm and extend previous observations, by use of the same cross-linker (Klareskog et al., 1977) or a disulfide-containing reagent (Snary et al., 1977a; Springer et al., 1977c), that the subunits most probably form a complex with 1:1 stoichiometry. The cross-linking experiments did not give evidence that the HLA-DR antigens may occur as tetramers. However, caution has to be exercised in the interpretation of this finding. If reactive lysine residues were not situated at a proper distance from each other, cross-linking would not occur. Likewise, if tetramer formation results mainly from protein–protein interactions in the hydrophobic region embedded in the micelles, cross-linking with the reagent used is unlikely to occur.

Available evidence strongly suggests that only the heavy chain of the HLA-A, -B, and -C antigens is integrated into the hydrocarbon matrix of the membrane (Peterson et al., 1977; Walsh & Crumpton, 1977; Springer et al., 1977d). The present results show that both HLA-DR antigen subunits behave as membrane-integrated proteins. These results are in excellent agreement with those of Walsh & Crumpton (1977). These workers also demonstrated that both chains have their COOH termini on the cytoplasmic side of the membrane.

Since actual measurements of the detergent binding to the HLA-DR antigens could not be performed, the molecular weight determinations do not give precise information as to the glycoprotein weight. From measurements under denaturing conditions a minimum molecular weight of about 60 000 for the HLA-DR antigen dimer can be deduced.

The quite high frictional ratios obtained for the HLA-DR antigens do not necessarily imply a significant asymmetry of the molecules but may be compatible with a rather globular structure and a high degree of hydration, especially as both HLA-DR antigen chains contain carbohydrate and the values obtained are estimates of the protein–detergent complexes. However, HLA-DR antigens should display some type of asymmetry since they are composed of a major portion that is water soluble (Humphreys et al., 1976) and a minor portion that is mainly hydrophobic.

The recorded values for the physical–chemical parameters of the HLA-DR antigens are in good agreement with those reported by Snary et al. (1977a) for HLA-DR antigens obtained from a lymphoblastoid cell line. However, these data like the present ones have been obtained with trace amounts of HLA-DR antigens. High-precision physical–chemical measurements have to await the isolation of HLA-DR antigens in larger quantities. Such studies are now underway.

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