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Subunit and Disulfide Structure of Monomeric and Dimeric Forms of Detergent-soluble HLA Antigens*

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The structure of monomeric and disulfide-bonded dimeric forms of HLA antigens has been studied. Detergent-soluble HLA antigen heavy chains contain one or two easily reduced sulfhydryl groups not found in papain-solubilized HLA antigens, as demonstrated by amino acid analysis (Springer, T. A., and Strominger, J. L. (1976) *Proc. Natl. Acad. Sci. U. S. A.* 73, 2481-2485, and Terhorst, C., Parham, P., Mann, D. L., and Strominger, J. L. (1976) *Proc. Natl. Acad. Sci. U. S. A.* 73, 910-914) and by labeling with iodo[^{3}H]acetate. Dimer formation occurred during purification, since it was prevented by pretreatment of membranes containing HLA antigen with iodoacetamide. Cross-linking studies showed that the non-disulfide-bonded form of HLA antigens contains one subunit each of the M_r = 44,000 heavy chain and the M_r = 12,000 light chain (β_2-microglobulin).

Human histocompatibility (HLA) antigens, when solubilized with detergents, contain two noncovalently associated subunits, a glycoprotein (p44) of M_r = 44,000 bearing HLA antigenic specificity, the gene for which is on chromosome 6 (1), and a protein of M_r = 12,000 (p12), the gene for which is on chromosome 15 (2), and which has been shown to be identical with β_2-microglobulin, a protein originally isolated from nephrotic urine (3-7). Detergent-soluble HLA antigens chromatograph at a high molecular weight (400,000) due to bound detergent (8, 9, and see below). Papain solubilization converts p44 to a glycoprotein of M_r = 34,000 (p34) without affecting p12 (8-14). This form of the antigen does not bind detergents, and p34 when compared to p44 has lost a COOH-terminal segment, containing both a hydrophobic and a hydrophilic region, and easily reducible sulfhydryl groups (8, 9). Detergent-soluble HLA antigens and H-2 antigens in mice have been reported to exist in dimeric forms containing S–S-linked heavy chains (15-20), and in monomeric forms (9, 20, 21). Varying amounts of monomeric, dimeric, and higher oligomeric forms have been found in different preparations. This paper reports studies on these different molecular species, using gel filtration, SDS[1]-gel electrophoresis, cross-linking reagents, cysteine determination, and iodoacetamide treatment before purification.

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; Brij 99:97, Brij 99:Brij 97 (2:1); $HSCH_2CH_2OH$, 2-mercaptoethanol; $MalNEt_2$, *N*-ethylmaleimide.

MATERIALS AND METHODS

HLA Antigens

HLA antigens, solubilized with Brij 99:97, were prepared by two techniques. HLA antigens were purified by biochemical techniques as described (9, 22), with or without the presence of 1 mM dithiothreitol during purification. HLA antigens were purified by anti-β_2-microglobulin IgG affinity chromatography in the presence or absence of iodoacetamide as follows. Frozen JY cells (a lymphoblastoid line) (30 g) were thawed and divided in half. To one-half iodoacetamide was added to a final concentration of 10 mM, and membrane preparation and detergent solubilization (9, 22) were conducted with buffers containing 1 mM iodoacetamide. The other half was treated identically, except iodoacetamide was omitted. The detergent-soluble supernatants were purified in 80% yield by adsorption to an anti-β_2-microglobulin IgG affinity column, and elution after standing in the presence of a 10-fold excess of β_2-microglobulin for 48 h at 4° (23, 24).[2] The anti-β_2-microglobulin IgG had been prepared from rabbit serum by adsorption to β_2-microglobulin-coupled Bio-Gel A-50m and elution at pH 2.8. Details of the preparation of the immunoaffinity column and its use are published separately.[2] Pools 1, 3, and 4 (see below) were also further purified by anti-β_2-microglobulin IgG affinity chromotography for some experiments.

SDS-Gel Electrophoresis

SDS-gel electrophoresis (25) was performed after mixing proteins with SDS sample buffer containing either 5% $HSCH_2CH_2OH$ (reduced) or an alkylating agent such as 0.1% $MalNEt_2$ or 1 mM iodoacetamide (unreduced) and heating in a boiling water bath for 5 min. Two-dimensional SDS-gel electrophoresis was done using a modification of the method of Wang and Richards (26).

Cross-linking of HLA Antigens

Detergent-soluble HLA antigen was purified to homogeneity in the presence of 1 mM dithiothreitol (22), alkylated with an excess of iodoacetate in 0.2 M Tris/HCl, pH 8.4 for 2 h at room temperature, and dialyzed against two changes of 50 mM sodium borate, pH 9.0. To 200 μl of HLA (0.2 mg/ml) were added dithiobis(succinimidyl propionate) (Lomant's reagent) (Pierce) freshly dissolved in 4 μl of dimethyl formamide (27), to give a final concentration of 0.1 mg/ml. After 1 h of reaction at room temperature, the samples were dialyzed against two changes of 20 mM NH_4HCO_3, pH 7.4, 1 mM sodium iodoacetate for 3 days, and precipitated overnight by the addition of 0.5 volume of acetone and 0.05 volume 100% w/v trichloroacetic acid. Precipitates were centrifuged at 3,000 × *g*, washed twice with acetone, and dissolved by heating in a boiling water bath 5 min in 50 μl of sample buffer containing 0.1 mg/ml of $MalNEt_2$.

Iodination

Aliquots of Pools 3 and 4 were iodinated using ^{125}I and the lactoperoxidase method (28), and free ^{125}I was removed on a Bio-Gel A-5m column.

[2] R. J. Robb, D. L. Mann, and J. L. Strominger (1976) *J. Biol. Chem.* 251, 5427-5428.

Radioactive Labeling of Half-Cystines

In addition to Pools 3 and 4, the following HLA antigens were used: detergent- or papain-solubilized HLA-A3, W25;B12,27 from the cell line IM-1, and papain-solubilized HLA-A2 and HLA-B7,12 from the cell line RPMI 4265. Control experiments were performed on rabbit IgG and β_2-microglobulin. Two types of experiments were done in order to determine whether the larger polypeptide chain of HLA antigens has any free sulfhydryl groups or easily reduced half-cystines.

Estimation of Free —SH Groups—HLA antigens (5 μM) were treated in the dark with 0.5 mM iodo[^{3}H]acetic acid (35 μCi/μmol) in 0.2 M Tris/HCl pH 8.1, 2 mM EDTA, 6 M guanidine hydrochloride for 1 h at room temperature under an atmosphere of nitrogen. The unreacted iodoacetic acid was removed by dialysis against 0.2 M Tris/HCl, pH 8.1, 2 mM EDTA for 24 h, followed by distilled water for another 40 h. The proteins were then lyophilized and dissolved in 1 mM dithiothreitol, 0.2 M Tris/HCl, pH 8.1, 2 mM EDTA, 6 M guanidine hydrochloride, and incubated for 1 h at 37° under nitrogen. Subsequently they were reacted in the dark with 3 mM iodo[^{14}C]acetic acid for 1 h at room temperature. After addition of 1 drop of $HSCH_2CH_2OH$, the reaction mixture was dialyzed for 24 h against 0.2 M Tris/HCl, pH 8.1, followed by distilled water, and lyophilized.

The ratio of moles of ^{3}H/^{14}C iodoacetic acid incorporated in the heavy chain was calculated after SDS-gel electrophoresis (25). Gels were cut into 2-mm slices and the slices were shaken overnight in 8 ml of 5% Protosol in toluene scintillation fluid before counting. The efficiency of counting in a Beckman liquid scintillation counter and the cross-over between the ^{3}H and ^{14}C channels was measured with ^{14}C- and ^{3}H-labeled toluene under the same conditions. Assuming two intrachain S—S bridges for the papain fragment of the HLA heavy chain, the number of free —SH groups as mole per mole of protein was estimated from the ^{3}H/^{14}C ratio.

Estimation of Easily Reduced S—S Bridges and Free —SH Groups—HLA antigens (5 μM) were incubated with 1 mM dithiothreitol, 0.2 M Tris/HCl, pH 8.1, 2 mM EDTA for 1 h at 37°. Subsequently, they were reacted with 3 mM iodo[^{3}H]acetic acid as described above. After dialysis and lyophilization, the proteins were completely reduced and alkylated in 6 M guanidine HCl with iodo[^{14}C]acetic acid. The ratio of ^{3}H/^{14}C was determined as described under "Estimation of Free —SH Groups," and this ratio was used to determine the number of easily reducible S—S bridges and free —SH groups, assuming two intrachain S—S bridges for the papain-solubilized heavy chain.

RESULTS

Detergent-soluble HLA antigens were examined after preparation in the absence of disulfide-reducing and -blocking agents from the cell line RPMI 4265. Membranes were prepared, solubilized with detergent, and the detergent-soluble supernatant was applied to a *Lens culinaris* lectin affinity column (9, 22). HLA antigens appeared in the α-D-methylmannoside eluate (Eluate I) and also in the flow-through, since the column was overloaded. The flow-through was applied to the washed column, and HLA activity was obtained in a second eluate (Eluate II). The two lectin eluates were filtered on Bio-Gel A-5m (Fig. 1), revealing that HLA antigens were heterogenous in size, and that Eluate I contained a much higher proportion of high molecular weight HLA antigens than Eluate II. Aggregation or disulfide bond formation during lectin chromatography did not occur, since Eluate II, containing a lower proportion of aggregated material than Eluate I, had been subjected to twice as much lectin chromatography. It was therefore concluded that the high molecular weight material predominated in Eluate I because it had a higher affinity for the lectin column, presumably because its aggregated or disulfide-bonded nature allowed multiple site binding to the lectin column. The materials from the Bio-Gel A-5m columns were combined as indicated into Pools 1 to 4 for further study. Pool 1 contained material in the excluded volume, Pool 4 contained a sharp peak of HLA antigen at the lowest molecular weight observed, M_r = 400,000, and Pools 2 and 3 contained material at intermediate molecular weights.

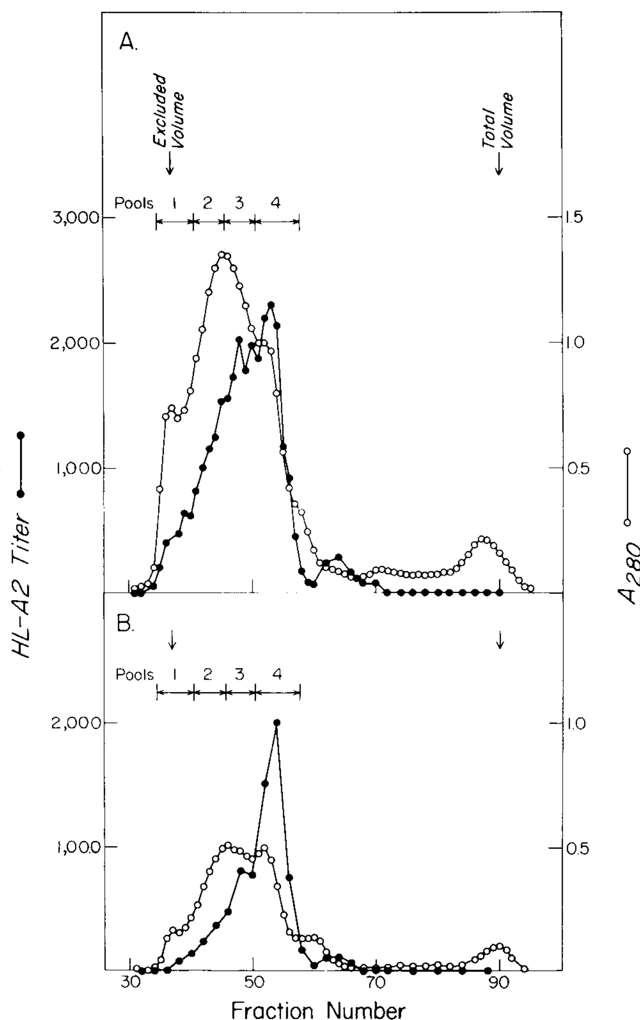

FIG. 1. Bio-Gel A-5m filtration of lectin column eluates. HLA antigens were purified through the lectin chromatography step as described (9, 22), except dithiothreitol was omitted, and the two eluates from the lectin affinity column (see text) were separately chromatographed on Bio-Gel A-5m in 0.02 M Tris/HCl, pH 8.0, 0.02% NaN_3, 0.1 mM EDTA, 2% Brij 99:97, as previously described (22), except dithiothreitol was omitted. The columns were pooled as indicated, and corresponding pools from the two columns were combined.

To determine whether HLA antigens existed in disulfide-bonded multimeric forms, several experiments were done. Pools 3 and 4 were radioiodinated, subjected to double antibody precipitation with rabbit anti-β_2-microglobulin, and goat anti-rabbit IgG, and electrophoresed on SDS-gels in the presence or absence of $HSCH_2CH_2OH$ (Fig. 2). With the lower molecular weight material (Pool 4), peaks of HLA heavy chain (p44) at M_r = 44,000 and β_2-microglobulin (p12) at M_r = 12,000 were obtained whether or not the material had been reduced. With the intermediate molecular weight material (Pool 3) in the absence of $HSCH_2CH_2OH$, a higher multimer of the heavy chain in addition to smaller peaks of dimer (M_r = 82,000) and monomer (M_r = 44,000), and β_2-microglobulin were obtained. In the presence of $HSCH_2CH_2OH$, the multimer and dimer peaks disappeared, and a much larger peak of monomer and β_2-microglobulin was present. Identical results were also obtained using rabbit anti-HLA-A2 serum (29) or a HLA-A2 alloantiserum (Davis) as the first antibody. The multimeric material never resolved into discrete peaks, even when lower

percentage acrylamide gels were used, and, thus, contained HLA antigens with heterogeneity in the number or type of interchain disulfides. This heterogeneity could not be accounted for by disulfide interchange in the presence of SDS, since pretreatment with alkylating reagents did not alter the pattern observed.

Similar results were obtained with stained tube gels of Pools 3 and 4 (Fig. 3). When stained bands at M_r = 82,000 were cut out of unreduced gels, treated with $HSCH_2CH_2OH$, and rerun, the monomer band of M_r =44,000 was obtained (Fig. 3*F*). When electrophoresis of unreduced Pool 3 (Fig 3*A*) and reduced Pool 3 (Fig. 3*C*) were compared, a very large increase in the amount of p44 at M_r = 44,000 was seen after reduction. This large increase of material at M_r = 44,000 cannot be accounted for by the faint dimer band at M_r = 82,000, and probably is largely derived from diffuse stain seen through the upper portion of the unreduced gel, in agreement with the ^{125}I-HLA immunoprecipitation results.

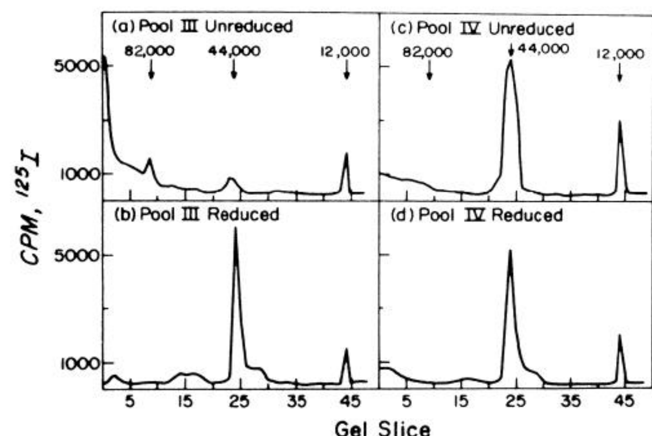

FIG. 2. SDS-gel electrophoresis with and without $HSCH_2CH_2OH$ of immunoprecipitated ^{125}I-HLA from Pools 3 and 4. ^{125}I-HLA antigen was precipitated by incubation with rabbit anti-β_2-microglobulin serum followed by incubation with goat anti-rabbit IgG serum (Cappell Laboratories). Precipitates were dissolved in SDS sample buffer with or without $HSCH_2CH_2OH$ and electrophoresed on Laemmli 10% acrylamide SDS gels (25). Gels were cut into 2-mm slices and counted in 5% Protosol (New England Nuclear).

Disulfide-linked forms of HLA antigens were also detected by two-dimensional SDS-gel electrophoresis (Fig. 4), in the first dimension of which nonreduced samples are electrophoresed in tube gels, and in the second dimension of which the tube gel is placed over an SDS-acrylamide slab gel with an intervening layer of agarose containing mercaptoethanol, in which samples are reduced during electrophoresis before they enter the acrylamide slab gel. Proteins which are not interchain S—S-linked appear on a diagonal line, while S—S-linked dimers and higher multimers lie below this diagonal (26). Pools 1 and 2 contained multimeric (Spot D) and monomeric HLA (Spot A). The monomeric HLA in these pools must have been derived from aggregated, non-disulfide-bonded forms of HLA. Pool 3 contained heavy chain multimer (Spot D), dimer (Spot B), and monomer (Spot A), and Pool 4 contained a dark spot of monomer (Spot A) and a faint spot of dimer (Spot B), the peak of dimer being centered in Pool 3, but overlapping with Pool 4. In these preparations, only one other protein, of M_r = 105,000 (p105) formed dimers (Fig. 4*C*, Spot F) and multimers. P105 could also have formed an S—S bridge with p44, since in Fig. 4*C*, faint spots of p44 (Spot C) and p105 (Spot E), both below the diagonal, are vertically aligned with each other at M_r = 140,000 in the first dimension. The formation of a disulfide bridge between p105 and p44 was confirmed by cutting out a band at M_r = 140,000 from a nonreduced tube gel and rerunning it after $HSCH_2CH_2OH$ treatment, which yielded bands of M_r = 105,000 and 44,000 (data not shown). Since the above pools were contaminated with proteins other than HLA, Pools 1, 3, and 4 were purified on an anti-β_2-microglobulin affinity column, to confirm that the observed p44 spots seen in two-dimensional gels were truly derived from HLA. Strong spots of p44 dimer were again observed (data not shown), although the amount of multimeric p44 was greatly lessened, since multimeric material was excluded from the

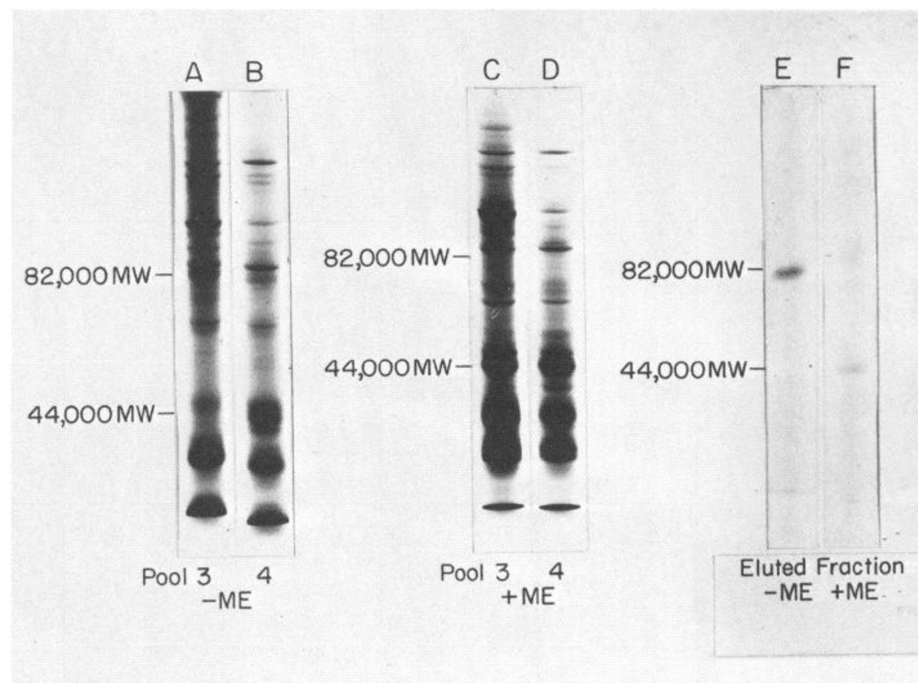

FIG. 3. SDS-gel electrophoresis of Pools 3 and 4 with or without 2-mercaptoethanol (*ME*) pretreatment. *A* to *D*, aliquots of Pools 3 and 4 were dissolved in sample buffer containing either 0.1% $MalNEt_2$ (*A*, *B*) or 5% $HSCH_2CH_2OH$ (*C*, *D*), electrophoresed on Laemmli 8% acrylamide SDS gels, and stained. *E*, *F*, the stained M_r = 82,000 band was cut from gels identical with *A*, boiled 5 min in sample buffer containing either 0.1% $MalNEt_2$ (*E*), or 5% $HSCH_2CH_2OH$ (*F*). The gel slices plus buffer were placed on top of and electrophoresed in another 8% gel.

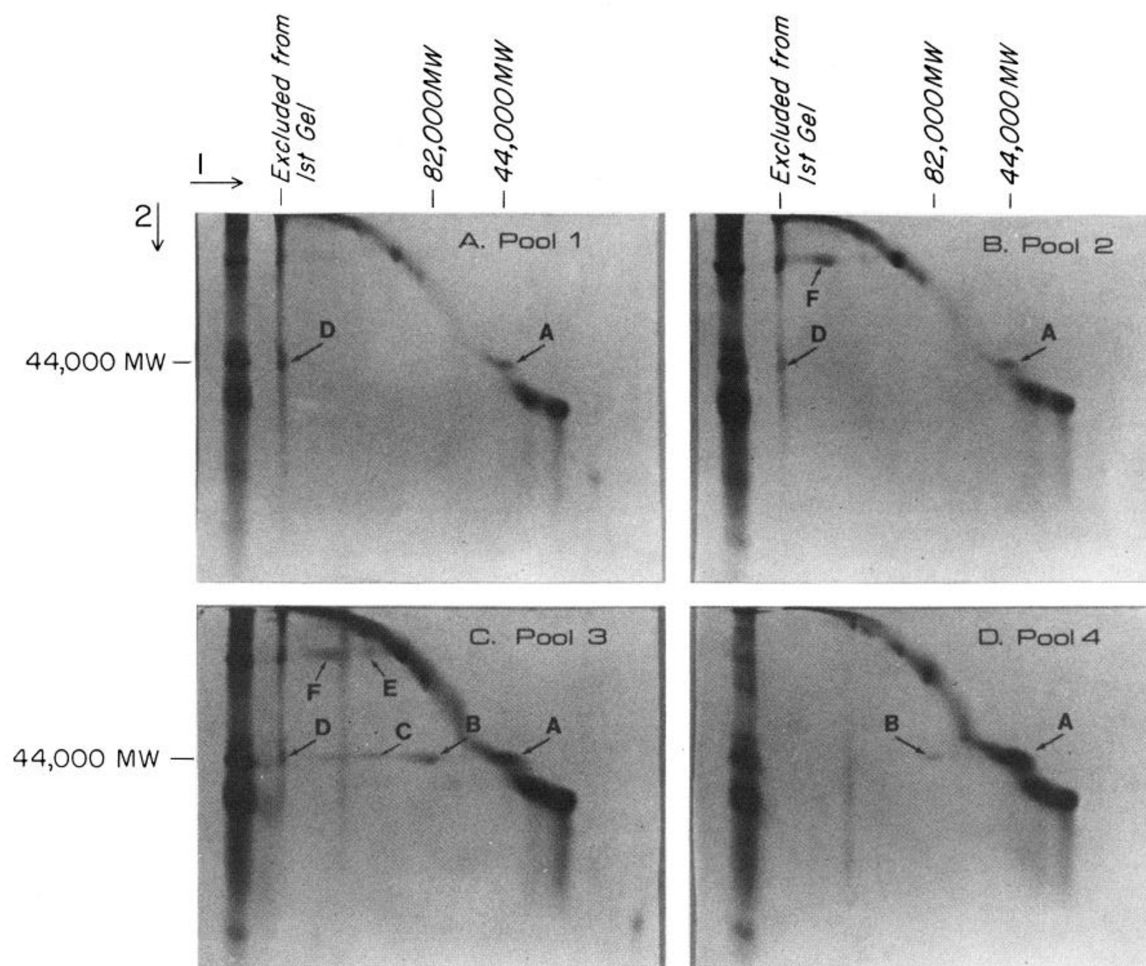

FIG. 4. Two-dimensional SDS-gel electrophoresis of Pools 1 to 4, without $HSCH_2CH_2OH$ in first dimension, $+HSCH_2CH_2OH$ in second dimension. Samples of pools (300 μl) were incubated 20 min with 0.1% $MalNEt_2$, precipitated with 1.2 ml of acetone, dissolved in SDS sample buffer containing 0.1% $MalNEt_2$ by boiling 5 min, and electrophoresed in 6% polyacrylamide first dimension gels and after reduction by $HSCH_2CH_2OH$ in an intervening agarose layer, in 10% polyacrylamide second dimension gels (26). Labeled peptides (and disulfide-linked complexes from which they are derived) are *A*, p44 (p44 monomer); *B*, p44 (p44 dimer); *C*, p44 (p44-p105 mixed dimer); *D*, p44 (p44 multimer); *E*, p105 (p44-p105 mixed dimer); *F*, p105 (p105 dimer). An identical sample in each case was applied to a slot to the left of the tube gel before electrophoresis in the second dimension.

agarose beads of the affinity column. Immunoprecipitation of ^{125}I-labeled HLA using anti-β_2-microglobulin serum (Fig. 2) indicated that the multimeric form was still associated with β_2-microglobulin.

The two noncovalently associated polypeptides of the HLA-linked B lymphocyte antigen (30) at M_r = 29,000 (p29) and 34,000 (p34) are seen in Fig. 4 as dark spots lying diagonally below p44. No spots corresponding to a disulfide-linked form of p29 and p34 were found, in contrast to other results reported for this antigen in humans (31) and the analogous Ia antigen in mice (32).

Quantitative conversion upon disulfide reduction of multimers and dimers of p44 to monomeric p44 was observed in the presence of SDS. However, upon reduction of Pool 3 with 20 mM dithioerythritol using nondenaturing conditions and Bio-Gel A-5m rechromatography in the presence of 1 mM dithiothreitol, only a small proportion of HLA molecules chromatographed at a lower molecular weight (Fig. 5*A*). Upon rechromatography, unreduced Pools 3 and 4 eluted at the same positions as in the first chromatography. The small proportion of Pool 3 HLA activity which eluted at a lower M_r was at an M_r identical with that of Pool 4 (M_r = 400,000). To determine whether disulfide reduction had occurred, Pool 3, with or without reduction with dithiothreitol as above, was alkylated with iodoacetamide before addition of SDS and electrophoresis in SDS gels (Fig. 5*B*). The amount of protein at M_r = 44,000 was greatly increased after reduction and alkylation. It was therefore concluded that upon reduction of interchain disulfide bonds in Pool 3 HLA, most of the HLA remained aggregated due to noncovalent interactions, even though disulfides had been reduced. Noncovalent aggregation also occurs in the absence of S—S bridges in Pools 1, 2, and 3 as is shown by the presence of p44 monomer in these pools (Fig. 4).

Radioactive Labeling of Cysteines—The presence of two intrachain disulfide bonds which are resistant to mild reduction has been demonstrated in papain-solubilized HLA antigen heavy chains (p34) (10). No easily reduced half-cystines were found. In immunoglobulins, interchain disulfides can be distinguished from intrachain disulfides by their ability to be reduced using mild conditions (33). Therefore, HLA antigens from Pools 3 and 4 were tested for the presence of interchain disulfides by initial mild reduction and labeling with iodo[^{3}H]acetate followed by complete reduction in 6 M guanidine HCl and alkylation with iodo[^{14}C]acetate (Table I). With

initial mild reduction (30-fold excess of dithiothreitol), a ratio of 0.5 mol of ^{3}H to 1 mol of ^{14}C was found (Table I), indicating two easily reduced half-cystines are contained in the detergent-soluble molecule, in addition to the four half-cystines in intrachain linkage found in the papain product (10). Five total cysteines were found in the detergent product by amino acid analysis (8), although six are indicated by the labeling experiments. This discrepancy is probably due to the experimental difficulties involved in cysteine measurements using either technique. It was also found, for both Pool 3 and Pool 4, that no free sulfhydryl groups were found when mild reduction with dithiothreitol was omitted. Therefore, the sulfhydryl groups of the monomeric form of HLA antigen must have been either reversibly blocked, as by cysteine, or involved in an easily reducible intrachain disulfide bond.

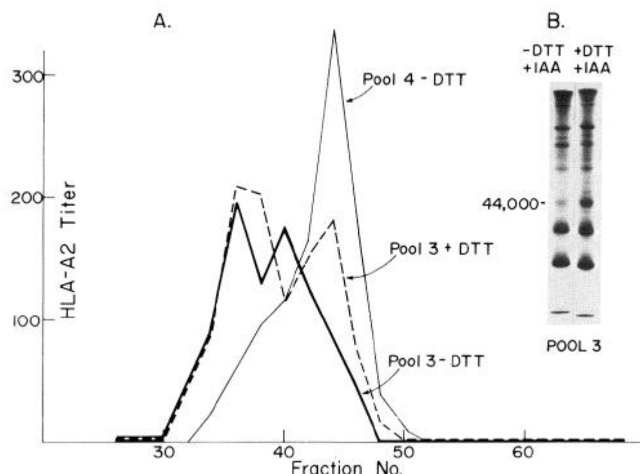

FIG. 5. *A*, Bio-Gel A-5m filtration of Pool 4 in the absence of dithiothreitol (*DTT*) and of Pool 3 both in the absence and presence of dithiothreitol. Samples (1.5 ml) of Pools 3 and 4 were chromatographed on a column (1.4 × 41 cm) Bio-Gel A-5m equilibrated with 0.01 M Tris/HCl, pH 8.0, 0.02% NaN_3, 0.1 mM EDTA, and 1-ml fractions were collected. For reduction with dithiothreitol, the sample was made 20 mM in dithiothreitol and allowed to stand at room temperature 1/2 h, before chromatography in the above buffer containing dithiothreitol. *B*, SDS-gel electrophoresis of Pool 3. Aliquots were removed and alkylated with an excess of iodoacetamide (*IAA*) for 1 h at room temperature before dissolving in SDS sample buffer without $HSCH_2CH_2OH$.

TABLE I

Estimation of free —SH groups and easily reduced S—S bridges in heavy chains of detergent- and papain-solubilized HLA antigens

The number of free —SH groups in HLA_{det} is estimated from the ratio of incorporation of iodo[^{3}H]acetic acid and the incorporation of iodo[^{14}C]acetic acid after complete reduction, assuming four half-cystines in two strong S—S bridges in the papain fragment. The number of free —SH groups plus easily reduced S—S bridges were likewise determined (see "Materials and Methods"). The heavy chains of HLA antigens derived by papain cleavage, β_2-microglobulin, and rabbit IgG served as controls.

Preparation	No. of free —SH groups		No. of easily reduced S—S bridges + free —SH groups	
HLA-A2; B7, $B12_{det}$[a]				
Pool 3	0.08	0.08[b]	2.16	2.20[b]
Pool 4	0.08	0.08[b]	1.96	1.36[b]
HLA-A3, W25;B12, 27_{det}	0.02		1.92	
$HLA\text{-}A2_{pap}$[a]	0.12		0.12	
HLA-B7, 12_{pap}	0.28		0.16	
HLA-A3, W25;B12, 27_{pap}	0.36		0.40	
β_2-Microglobulin	0.08		0.12	
Rabbit IgG				
Heavy chain	0.08		2.14	
Light chain	0.12		0.93	

[a] The subscripts det and pap refer to solubilization with detergent and papain, respectively.

[b] After passage through an anti-β_2-immunoadsorbent column.

Preparation of HLA Antigens after Iodoacetamide Pretreatment of Cells — Since several molecular weight species of HLA antigens were found after preparation in the absence of reducing agents, it was important to establish which of these species most nearly resembles HLA found on lymphocytes. Therefore, cells were treated with iodoacetamide to prevent both disulfide bond formation by oxidation and disulfide interchange catalyzed by free sulfhydryl groups (34). HLA was prepared from such cells and from a parallel batch of untreated cells by preparation of membranes, detergent solubilization, and chromatography on anti-β_2-microglobulin affinity columns, and analyzed by SDS-gel electrophoresis without $HSCH_2CH_2OH$ (Fig. 6). Much more dimer and multimer heavy chain was found in HLA prepared from untreated cells than from iodoacetamide-treated cells. Only a very small proportion of HLA molecules from the iodoacetamide-treated preparation were dimers, and these could certainly have arisen during freezing and thawing of the cells which preceded iodoacetamide treatment.

Cross-linking Experiments — Detergent-soluble HLA antigens have also been purified in the presence of 1 mM dithio-

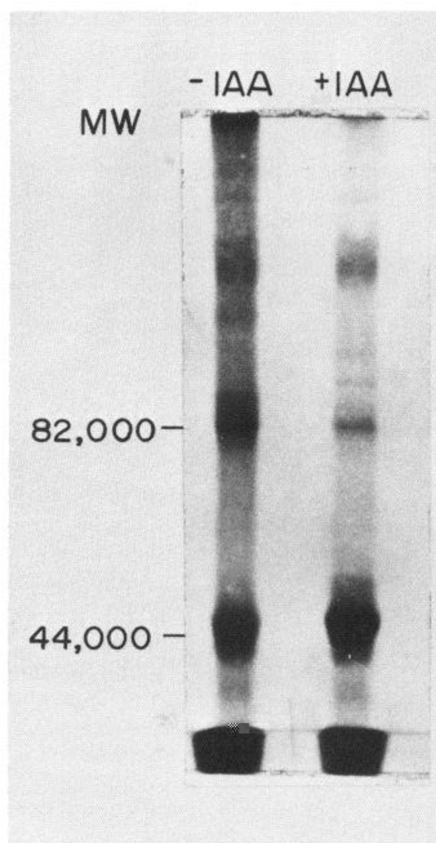

FIG. 6. SDS-gel electrophoresis of HLA prepared in the presence and absence of iodoacetamide (*IAA*). Aliquots of the preparation were electrophoresed on Laemmli 7.5% polyacrylamide SDS gels in the absence of $HSCH_2CH_2OH$.

threitol, and when compared to purification in the absence of dithiothreitol, a much greater percentage (70 to 85%) of the total HLA activity was found in a sharp peak at the same molecular weight as Pool 4 during Bio-Gel A-5m chromatography (22). When these preparations were carboxymethylated, the molecular weight in gel filtration was unchanged. The gel filtration patterns suggested that this major peak of HLA activity at M_r = 400,000 contained monomeric material, since interchain disulfide-bonded material chromatographed at higher molecular weight. The apparent high M_r = 400,000 observed in gel filtration was thought to be due to bound detergent (22, 35).

To analyze the subunit composition of the peak of apparent M_r of 400,000, cross-linking studies were performed. HLA antigens were purified to homogeneity in the presence of dithiothreitol (22) and were carboxymethylated after mild reduction, since the cross-linking reagent had to be used in the absence of dithiothreitol, and a small portion of the molecules became disulfide-linked if dithiothreitol was removed by dialysis. HLA antigens were cross-linked with the cleavable reagent, dithiobis(succinimidyl propionate) (Lomant's reagent) (Fig. 7) and electrophoresed in a two-dimensional SDS-gel system, the reagent being cleaved with 0.1 M dithiothreitol before electrophoresis in the second dimension to dissociate cross-linked products into their component polypeptides. Most of the HLA antigen was cross-linked into a complex of M_r = 54,000, which was shown to contain p44 (Spot B) and p12 (Spot C) in the second dimension, and therefore was a $(p44)_1(p12)_1$ complex. Spots of uncleaved $(p44)_1(p12)_1$ (Spot A), uncross-linked p44 (Spot D), and p12 (Spot E) were also observed. However, no spots of p44 dimer, p12 dimer, or higher oligomers were observed, although when pools from higher molecular weight gel filtration fractions were similarly cross-linked, oligomeric cross-linked products of p44 were observed (data not shown). These data showed that the M_r = 400,000 form of detergent-soluble HLA antigens has a $(p44)_1(p12)_1$ structure,

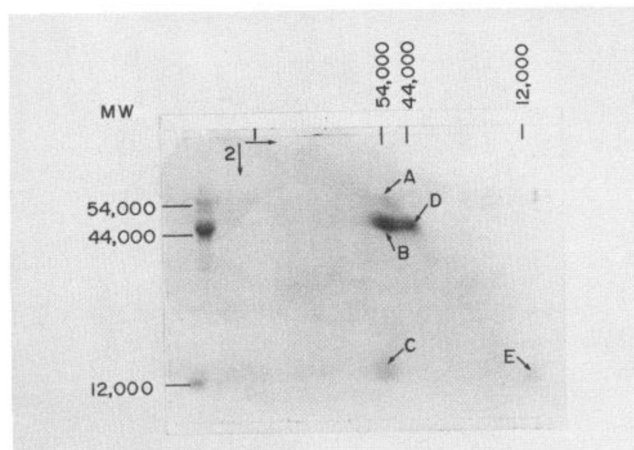

FIG. 7. Two-dimensional electrophoresis in SDS gels of cross-linked, purified HLA antigen. The sample (20 μl) was electrophoresed in a 5.5% acrylamide tube gel (2.5 × 110 mm), which was layered on top of a 10% acrylamide slab gel (0.8 × 90 × 145 mm), with an intervening 20-mm layer of 0.9% agarose containing 0.1 M dithiothreitol, and electrophoresed at 30 V for 2 h and 100 V for 3 h (26). A portion of cross-linked sample (5 μl) was placed in a slot to the left of the top of the tube gel. Molecular weights were determined from a similar, stained tube gel and a tube gel containing calibration proteins. Spots (and products from which they were derived) are: *A*, p56, *i.e.* uncleaved cross-linked p44, 12; *B*, p44 (cross-linked p44, 12); *C*, p12 (cross-linked p44, 12); *D*, p44; *E*, p12.

and that the higher molecular weight observed in gel filtration must be due to a bound detergent micelle.

DISCUSSION

Dimeric forms of histocompatibility antigens containing S—S-linked heavy chains have been observed for both H-2 and HLA antigens after detergent solubilization. However, other studies have failed to reveal the occurrence of dimers (9, 21). The present report shows that detergent-soluble HLA antigens contain 1 to 2 cysteine residues which can be alkylated after mild reduction, in addition to the four half-cystines found in papain-solubilized HLA antigens, which can only be alkylated after reduction using denaturing conditions. The easily reduced sulfhydryl groups in detergent-soluble HLA antigens must be involved in dimer formation, since papain-solubilized HLA antigens do not dimerize. Detergent-soluble HLA antigens contain a COOH-terminal hydrophilic region and a penultimate hydrophobic region which are removed during papain digestion. At least one of the easily reduced cysteines is located in the hydrophilic region, which has been suggested to lie inside the cell (1). If the easily reduced cysteines are intracellular, as suggested, then they would normally most likely exist as free sulfhydryl groups in the strong intracellular reducing environment (36, 37).

The possibility was investigated that dimer formation occurred during purification. Pretreatment with iodoacetamide prevented dimer formation. The vast majority of HLA molecules in the membrane are therefore unlinked by disulfide bonds. If an equilibrium exists between linked and unlinked molecules, it must greatly favor the latter species. Dimerization could result either from sulfhydryl oxidation or disulfide bond interchange, which is catalyzed by free sulfhydryl groups (34). Both are prevented by iodoacetamide treatment. When HLA antigens were prepared in the absence of either reducing or alkylating agents, neither monomeric nor dimeric forms contained free sulfydryl groups. These observations are consistent with either mode of dimer formation, since oxidation could result in mixed disulfide formation, *e.g.* with free cysteine, as well as in dimer formation. Experiments on H-2 antigens in which disulfide formation is induced by oxidation with *o*-phenanthroline/$CuSO_4$ show the cysteines would normally be free rather than intrachain-bonded (20).

In two-dimensional electrophoresis experiments it was observed that the higher molecular weight form of HLA antigens contained aggregated, non-disulfide-linked molecules as well as disulfide-linked dimeric and multimeric molecules. This was confirmed by the finding that dithiothreitol treatment converted only a small portion of the higher molecular weight HLA molecules to the lower molecular weight form. Similar observations have been made on bovine serum albumin dimer preparations (38). Aggregation has been observed to increase when HLA was subjected to repeated purification steps, such as DEAE-cellulose chromatography and gel filtration. It therefore may be the result of partial denaturation, perhaps not involving the antigenic determinant, rather than a representation of the original state of HLA molecules in the membrane, *i.e.* aggregation rather than dissociation, is the process observed during purification.

Two observations show that the HLA antigen peak at M_r = 400,000 contains monomeric HLA antigen, the rest of the molecular weight being accounted for by bound detergent. First, dimeric disulfide-bonded forms of HLA antigens were only found at higher molecular weight. Second, cross-linking experiments failed to reveal any noncovalent association be-

tween heavy chains in the absence of disulfide linkage, while demonstrating a noncovalent association between heavy chains and β_2-microglobulin. When care is taken to prevent sulfhydryl oxidation during purification, HLA antigen are isolated in a largely monomeric form, either after solubilization with nonionic Brij 99:97 detergents as in the present report or after solubilization with deoxycholate (21). However, these experiments do not rule out the possibility that HLA antigens may exist as noncovalently associated dimers in the cell membrane.

The homology of the light chain of HLA antigens, β_2-microglobulin, with immunoglobulin constant domains, coupled with the finding of a disulfide-linked, dimeric, four chain form of HLA antigens, has created new interest in an old hypothesis that HLA heavy chains and immunoglobulins may be derived from a common ancestral gene (15, 18, 39, 40). The finding that disulfide-linked dimers of HLA antigens do not naturally occur in the cell membrane neither supports nor rules out this hypothesis. It can be argued that HLA may more resemble a primitive, cell-bound immunoglobulin, and the evolution of interchain disulfide bonds might not have occurred until immunoglobulins became secreted. Correspondingly, β_2-microglobulin is clearly homologous with immunoglobulin constant domains but it does not possess an interchain S—S bridge such as connects heavy and light chains in immunoglobulins.

REFERENCES

1. Van Someren, H., Westerveld, A., Hagemeijer, A., Mees, J. R., Meera Khan, P., and Zaalberg, O. B. (1974) *Proc. Natl. Acad. Sci. U. S. A.* 71, 962–965
2. Goodfellow, P. N., Jones, E. A., van Heyningen, V., Solomon, E., Bobrow, M., Miggiano, V., and Bodmer, W. F. (1975) *Nature* 254, 267–269
3. Berggård, I., and Bearn, A. G. (1968) *J. Biol. Chem.* 243, 4095–4103
4. Grey, H. M., Kubo, R. T., Colon, S. M., Poulik, M. D., Cresswell, P., Springer, T., Turner, M., and Strominger, J. L. (1973) *J. Exp. Med.* 138, 1608–1612
5. Cresswell, P., Springer, T., Strominger, J. L., Turner, M. J., Grey, H. M., and Kubo, R. T. (1974) *Proc. Natl. Acad. Sci. U. S. A.* 71, 2123–2127
6. Nakamuro, K., Tanigaki, N., and Pressman, D. (1973) *Proc. Natl. Acad. Sci. U. S. A.* 70, 2863–2865
7. Peterson, P. A., Rask, L., and Lindblom, J. B. (1974) *Proc. Natl. Acad. Sci. U. S. A.* 71, 35–39
8. Springer, T. A., and Strominger, J. L. (1976) *Proc. Natl. Acad. Sci. U. S. A.* 73, 2481–2485
9. Springer, T. A., Strominger, J. L., and Mann, D. L. (1974) *Proc. Natl. Acad. Sci. U. S. A.* 71, 1539–1543
10. Terhorst, C., Parham, P., Mann, D. L., and Strominger, J. L. (1976) *Proc. Natl. Acad. Sci. U. S. A.* 73, 910–914
11. Cresswell, P., Turner, M. J., and Strominger, J. L. (1973) *Proc. Natl. Acad. Sci. U. S. A.* 70, 1603–1607
12. Turner, M. J., Cresswell, P., Parham, P., Strominger, J. L., Mann, D. L., and Sanderson, A. R. (1975) *J. Biol. Chem.* 250, 4512–4519
13. Parham, P., Terhorst, C., Herrmann, H., Humphreys, R. E., Waterfield, M. D., and Strominger, J. L. (1975) *Proc. Natl. Acad. Sci. U. S. A.* 72, 1594–1598
14. Tanigaki, N., and Pressman, D. (1974) *Transplant. Rev.* 21, 15–34
15. Strominger, J. L., Cresswell, P., Grey, H., Humphreys, R. E., Mann, D., McCune, J., Parham, P., Robb, R., Sanderson, A. R., Springer, T. A., Terhorst, C., and Turner, M. J. (1974) *Transplant. Rev.* 21, 126–143
16. Strominger, J. L., Humphreys, R. E., McCune, J. M., Parham, P., Robb, R., Springer, T., and Terhorst, C. (1976) *Fed. Proc.* 35, 1177–1182
17. Cresswell, P., and Dawson, J. R. (1975) *J. Immunol.* 114, 523–525
18. Peterson, P. A., Rask, L., Sege, K., Klareskog, L., Anundi, H., and Östberg, L. (1975) *Proc. Natl. Acad. Sci. U. S. A.* 72, 1612–1616
19. Schwartz, B. D., Kato, K., Cullen, S. E., and Nathenson, S. G. (1973) *Biochemistry* 12, 2157–2164
20. Henning, R., Milner, R. J., Reske, K., Cunningham, B. A., and Edelman, G. M. (1976) *Proc. Natl. Acad. Sci. U. S. A.* 73, 118–122
21. Snary, D., Goodfellow, P., Bodmer, W. F., and Crumpton, M. J. (1975) *Nature* 258, 240–242
22. Springer, T. A., Mann, D. L., De Franco, A. L., and Strominger, J. L. (1977) *J. Biol. Chem.* 252, 4682–4693
23. Strominger, J. L., Chess, L., Herrmann, H. C., Humphreys, R. E., Malenka, D., Mann, D., McCune, J. M., Parham, P., Robb, R., Springer, T. A., and Terhorst, C. (1975) in *Histocompatibility Testing 1975* (Kissmeyer-Nielsen, F., ed) pp. 719–730, Munksgaard, Copenhagen
24. Strominger, J. L., Chess, L., Humphreys, R. E., Mann, D., Parham, P., Robb, R., Schlossman, S., Springer, T., and Terhorst, C. (1975) *Proceedings of International Conference at Brook Lodge, Michigan, November 1975*, pp. 621–643, Academic Press, N.Y.
25. King, J., and Laemmli, U. (1971) *J. Mol. Biol.* 62, 465–477
26. Wang, K., and Richards, F. M. (1974) *J. Biol. Chem.* 249, 8005–8018
27. Lomant, A. J., and Fairbanks, G. (1976) *J. Mol. Biol.* 104, 243–261
28. Marchalonis, J. J., Cone, R. E., and Santer, V. (1971) *Biochem. J.* 124, 921–927
29. Robb, R. J., Humphreys, R. E., Strominger, J. L., Fuller, T. C., and Mann, D. L. (1975) *Transplantation* 19, 445–447
30. Springer, R. A., Kaufman, J. F., Terhorst, C., and Strominger, J. L. (1977) *Nature,* in press
31. Wernet, P., and Kunkel, H. G. (1975) in *Histocompatibility Testing, 1975* (Kissmeyer-Nielsen, F., ed) pp. 731–734, Munksgaard, Copenhagen
32. Cullen, S. E., David, C. S., Shreffler, D. C., and Nathenson, S. G. (1974) *Proc. Natl. Acad. Sci. U. S. A.* 71, 648–652
33. Gall, W. E., Cunningham, B. A., Waxdal, M. J., Konigsberg, W. H., and Edelman, G. M. (1968) *Biochemistry* 7, 1973–1982
34. Ryle, A. P., and Sanger, F. (1955) *Biochem. J.* 60, 535–540
35. Helenius, A., and Simons, K. (1975) *Biochim. Biophys. Acta* 415, 29–79
36. Benedict, S. R., and Gottschall, G. (1933) *J. Biol. Chem.* 99, 729–740
37. Bartlett, P. D., and Stevenson, A. (1954) *Fed. Proc.* 13, 594 Abstr.
38. Janatova, J., Fuller, J. K., and Hunter, M. J. (1968) *J. Biol. Chem.* 243, 3612–3622
39. Burnet, F. M. (1970) *Nature* 226, 123–126
40. Gally, J. A., and Edelman, G. M. (1972) *Annu. Rev. Genet.* 6, 1–46