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Partial Purification of Detergent-Soluble HL-A Antigen and Its Cleavage by Papain

(β_2-microglobulin/Brij 99/histocompatibility)

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ABSTRACT HL-A antigen solubilized with the nonionic detergent, Brij 99, has been purified to about 50% of homogeneity from a cultured human lymphoblast line. It consists of two nonidentical subunits of 44,000 and 12,000 molecular weight (MW). Upon papain proteolysis the 44,000 MW peptide is converted by at least two cleavages to a 34,000 MW peptide, but the 12,000 MW peptide appears to be unchanged. Concomitantly, the apparent molecular weight in gel filtration chromatography under nondenaturing conditions in the presence of Brij 99 is reduced from 460,000 to 45,000. HL-A molecules produced by direct papain proteolysis of membranes and by papain treatment of purified detergent-soluble HL-A are identical.

Histocompatibility antigens (HL-A in man, H-2 in mice) are glycoproteins present on the surface of cells of lymphoid and other tissues (1). They are serologically defined (for example, by antibody and complement-dependent cell lysis) and recently they also have been shown to be the target for cell-mediated killing (2). The major histocompatibility regions of man and mouse are very closely related genetically and with respect to the chemical properties of the antigens they code for. The histocompatibility antigens are specified by two gene loci which are expressed codominantly. These genes are closely linked to immune response genes controlling reactivity to ragweed in man or to some synthetic polypeptides in the mouse and also to mixed lymphocyte reaction genes controlling reactivity in mixed lymphocyte cultures (MLC) (3–5).

Papain (EC 3.4.22.2) (6–8) and 3 M KCl (9) have most commonly been used to solubilize from membranes a form of HL-A which would be amenable to purification and biochemical characterization. Both HL-A and H-2 antigens have been purified extensively after removal from the cell by papain. H-2 antigens from lymphocytes have been solubilized with detergent and purified 2- to 4-fold (10), and HL-A antigens have been solubilized from platelets with Nonidet P-40 (11). Radioactively labeled, detergent-soluble H-2 antigens have been purified on an analytical scale by double antibody immunoprecipitation (12). Purification of detergent-soluble H-2 has been hindered by interference by detergent with the assay (12) and by the formation of polydisperse material after detergent removal (10). This paper concerns the assay and purification of detergent-soluble HL-A, of which a preliminary report has appeared (13), and its relation to the product of papain proteolysis.

Abbreviations: BSA, bovine-serum albumin; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; MW, molecular weight.

MATERIALS AND METHODS

$Na_2{}^{51}CrO_4$ labeled lymphocytes are lysed when incubated with HL-A antisera followed by complement. HL-A antigens are assayed by their ability to inhibit lysis (cytotoxicity) (14, 15). In a typical assay, HL-A antigen in an 0.5% detergent solution was mixed with an equal volume of 30% BSA and incubated for 10 min at room temperature. Then 10 μl of HL-A antigen solution was diluted 2-fold serially through 10 μl of a dilution of antiserum giving 90% of maximal lysis. The antiserum was two times more concentrated in the first than in the remaining 6 × 50-mm culture tubes, prior to antigen addition. After 1 hr at 37°, 5 μl of ^{51}Cr-labeled peripheral human lymphocytes at 5 × 10^6 cells per ml were added and incubated $^1/_2$ hr at 37°. Target cells were from laboratory personnel, selected for the HL-A specificity being measured, and contained 5–50,000 cpm/5 μl. Then 100 μl of rabbit complement diluted 1 to 4 in 0.14 M NaCl, 0.01 M Tris–Bicine [*N,N'*-bis(2-hydroxyethyl)glycine] buffer (pH 8.3), 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, and 0.01 mM ethylene diaminetetraacetic acid (EDTA) was added and the mixture was incubated for 30 min at 37°. After adding 50 μl of 0.14 M NaCl, 0.01 M sodium phosphate (pH 6.8), 20 mM EDTA to inhibit complement, the tubes were centrifuged for 5 min at 1000 × *g*. Aliquots (50-μl) of the supernatant solutions were counted for ^{51}Cr. The inhibitory titer is the inverse dilution of antigen in the assay volume after target cell addition giving 50% inhibition of lysis. Inhibitory units are expressed as ml units, i.e., the inhibitory titer × ml of antigen.

Protein was assayed according to Lowry *et al.* (16) if detergent was absent. Protein, when mixed with detergent, was assayed with ninhydrin after alkaline hydrolysis (17) or estimated from the A_{280} × 0.6 mg/ml per absorbance unit. Organic phosphate was assayed according to Ames and Dubin (18). Laemmli sodium dodecyl sulfate (SDS) stacking gels with 12% acrylamide (19) and nonstacking 7.5% sodium phosphate-buffered SDS gels (20) were prepared essentially as described. For SDS–6 M urea gels, urea was added to running gels but not to the stacking gel or sample buffer. ^{125}I-labeled HL-A was prepared using a modification of the chloramine-T method (21). The lectin from *Lens culinaris*, purchased at a local grocery, was purified (22) and coupled to Biogel A-50m essentially as described (23).

RESULTS

Assay of HL-A in the Presence of Detergents. Since detergents lyse the ^{51}Cr-labeled lymphocytes, the target cells of the cytotoxicity assay for HL-A or H-2 (see *Methods*), a means of

TABLE 1. *Purification of HL-A2 from RPMI 4265 cells*

Step	Volume (ml)	Total HL-A2 (ml units)	Yield of HL-A (%)	Total protein (mg)	Specific activity (ml units/mg of protein)	Purification
Starting cells	114.5	81,300	(100)	3600	22.6	(1.0)
1. Membranes	23	71,900	88	650	111	4.9
2. Detergent-soluble 100,000 × *g* supernatant	34	69,200	85	514	135	6.0
3. Biogel A-5m column pool*	21	60,000	74	68	882	39

* No nonspecific inhibition of Hackett HL-A3 antiserum by the Biogel A-5m column pool was detected (less than 30 units/ml), giving a specificity ratio >100.

avoiding detergent lysis in the assay was sought. BSA inhibited detergent cytolysis, presumably because of its known capacity for fatty acid and detergent binding (24, 25). After addition of 30% BSA to the assay mixture, lymphocytes were lysed only by 100-fold greater concentrations of a variety of detergents, thereby increasing the assay sensitivity 100 times.

Partial Purification of HL-A (Table 1).

Step 1. *Membrane preparation.* Membranes were prepared by freeze-thawing and hypotonic washing (7) of 15 ml of packed RPMI 4265 cells (HL-A2, 7, 12), and finally suspended in 23 ml of pH 7.5 Na *N*-tris(hydroxymethyl)methyl-2-ethanesulfonate (NaTES) buffer. The purification was 5-fold.

Step 2. *Solubilization by detergent.* Extensive studies of solubilization of HL-A by a variety of detergents were carried out. Two criteria of solubilization were examined, ultracentrifugation and, more important, Biogel A-5m gel chromatography.‡ Many detergents, e.g., deoxycholate, solubilized HL-A antigens as well as most other membrane proteins, i.e., both HL-A antigens and these other membrane proteins were not removed by centrifugation and were included in the agarose gel. Brij 98 and Brij 99 appeared to solubilize selectively HL-A by the criterion of agarose chromatography, since much of the other protein remained in the excluded volume of the agarose column, presumably aggregated. Therefore, considerable purification was provided by agarose chromatography. In a typical preparation 13 ml of 10% Brij 99 was added to 23 ml of membranes from step 1 to give a 2:1 w/w ratio of detergent to protein. Insoluble material was removed by centrifugation at 100,000 × *g* for 1 hr.

Step 3. *Agarose chromatography.* The 100,000 × *g* supernatant solution from step 2 was applied directly to a 4.5 × 70-cm column on Biogel A-5m, 200–400 mesh, equilibrated with 0.01 M NaTES (pH 7.5), 0.14 M NaCl, 0.1% Brij 99, 0.02% NaN_3, and run at a flow rate of 50 ml/hr. The elution profile of a similar column with a smaller sample is shown in Fig. 1. Most of the protein appeared in the void volume of the agarose column. A small amount of protein was included in the column and HL-A activity was eluted at the leading edge of this included protein. Phospholipid was eluted at the trailing edge of this protein peak and thus did not coincide with the elution of HL-A. HL-A antigen was eluted at an apparent molecular weight (MW) of about 460,000 based on a calibration of the column; this value is presumably the molecular weight of a complex between the detergent micelle and HL-A antigen. The purification of HL-A antigen was 40-fold. The A-5m pool was concentrated by adsorption to a DEAE-cellulose (DE-52) column in 0.01 M NaTES (pH 7.5), 0.1% Brij 99, and elution with 0.25 M NaCl added to the same buffer.

Treatment of Partially Purified HL-A Antigen with Papain. This material was treated with varying amounts of papain and each digest was run on a Biogel A-5m column. It is noteworthy that treatment with papain under the conditions of these experiments resulted in no loss of activity of HL-A2 or HL-A7. At lower papain concentrations no apparent effect on molecular size was observed (Fig. 2*A* and *B*). At higher concentration, the HL-A activity was partially converted from a form with an apparent molecular weight of 460,000 to a form with a much lower molecular weight of approximately 45,000 (Fig. 2*C*). At still higher concentrations, essentially all of the HL-A was converted to the latter form (Fig. 2*D*). These observations are interpreted as representing the conversion of HL-A from a high-molecular-weight form bound to a detergent micelle to a lower-molecular-weight form no longer bound to the micelle.

Analysis by SDS–urea gel electrophoresis (Fig. 3) of the material in the higher (A-D) and lower (A′-D′) molecular weight HL-A activity peaks of the agarose columns was carried out. The untreated sample from the higher-molecular-weight region (Fig. 3*A*) contained a number of protein bands of MW 30–60,000, and in addition a peptide of molecular weight about 12,000, similar to that previously found in the product prepared after papain treatment of lymphocytes (8). At the lowest concentration of papain (Fig. 3*B*), the only effect observed was the disappearance of a few minor bands. At the intermediate concentration of papain, band(s) at molecular weight about 44,000 (Fig. 3*A*) completely disap-

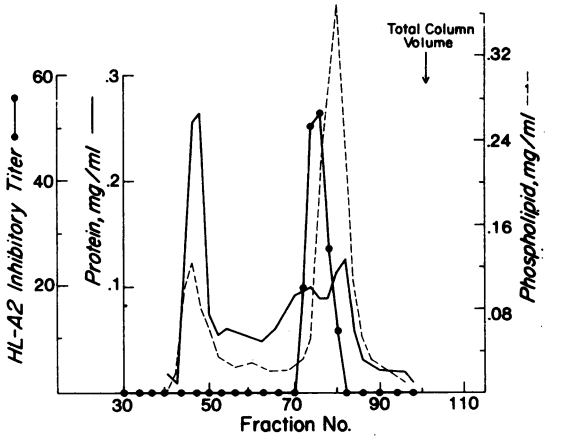

FIG. 1. Filtration of detergent-soluble HL-A antigen (100,000 × *g* supernatant solution) on Biogel A-5m. Phospholipid is calculated as 25 times the weight of phosphorus.

‡ The molecular weight of HL-A antigen in detergent varied from 2,700,000 to 160,000, depending upon the detergent used, and correlated with the detergent micelle size. The smallest value, MW 160,000 in deoxycholate, is then only a maximum MW for HL-A.

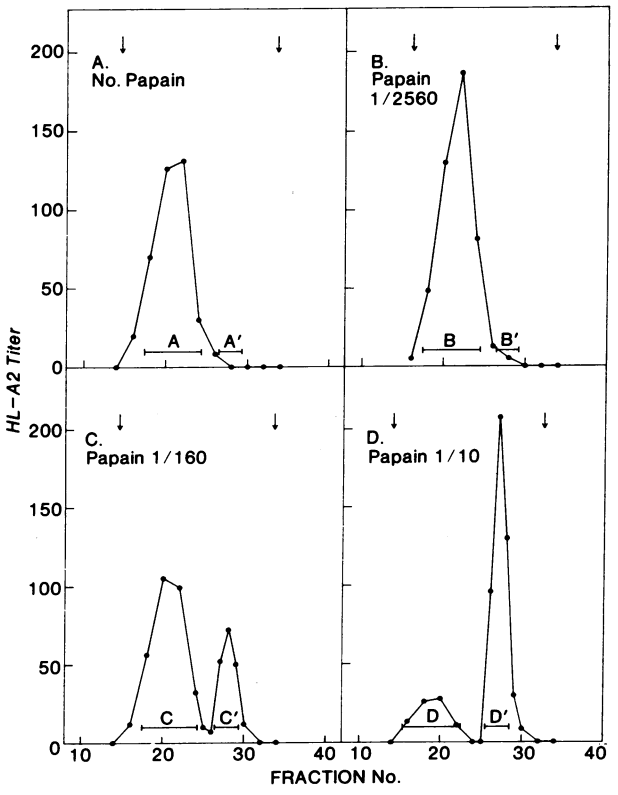

FIG. 2. Filtration of papain digests of HL-A antigen on Biogel A-5m. After papain digestion at the indicated w/w ratios of papain to HL-A, digests were filtered on a Biogel A-5m column (10 ml, 0.6 × 34 cm) equilibrated with 0.01% Brij 99, 5 mM Na phosphate (pH 6.8), 0.05 M NaCl, 0.02% NaN_3, 0.1 mM EDTA. Fractions of 0.3 ml were collected and assayed for HL-A. Total column volume was determined with ^{51}Cr and the excluded volume with ^{125}I-labeled membranes for each sample, and are marked with *arrows*. The pooled fractions are marked with *bars*.

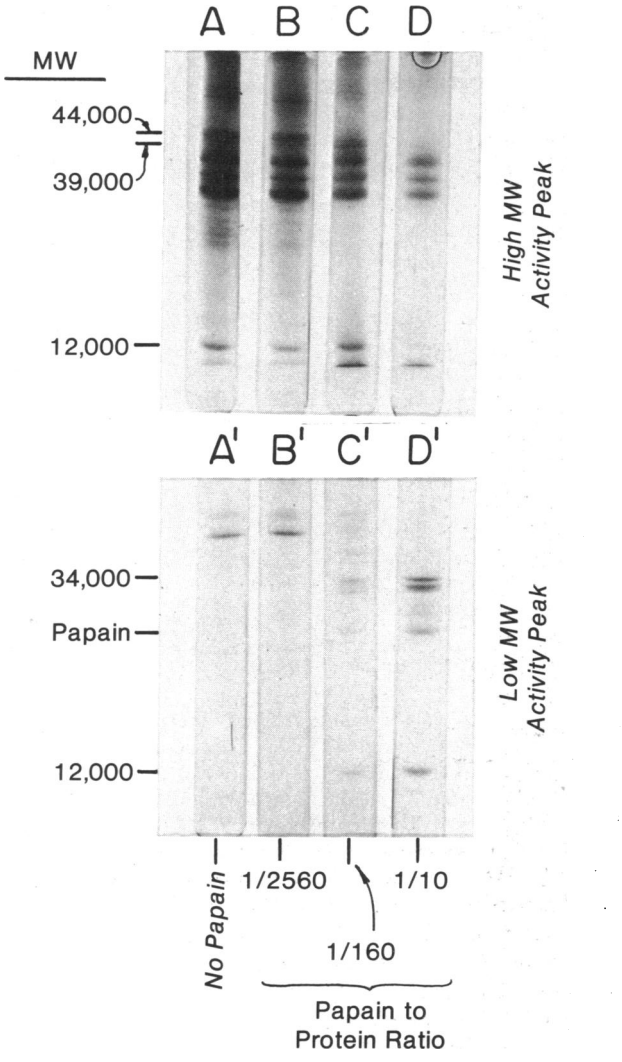

FIG. 3. SDS–urea gels of papain-treated HL-A antigen. The regions corresponding to the two activity peaks from Biogel A-5m chromatography (Fig. 2) were pooled from each column, lyophilized, and dissolved in 200 μl of 1% SDS. One-fifth of each pool (40 μl) was analyzed in the stacking SDS–6 M urea gel system.

peared and were replaced by new band(s) at molecular weight approximately 39,000 (Fig. 3*C*) (see also below). The lower-molecular-weight fraction from the agarose column now contained a small amount of materials of molecular weights around 34,000 and 12,000. At the highest concentration of papain (Fig. 3*D*) all of the materials at molecular weights 44,000 and 39,000 disappeared from the higher-molecular-weight region of the agarose column, and the lower-molecular-weight region of the agarose column now contained prominent bands at around 34,000 and 12,000 MW. In addition, the higher-molecular-weight region of the agarose column appeared to contain additional peptides of molecular weight considerably lower than 12,000 migrating at a buffer front in the Laemmli system, possibly split hydrophobic peptides.

These observations are interpreted to indicate that papain cleavage resulted first in the formation of an intermediate(s) of molecular weight about 39,000, which was still hydrophobic and remained bound to the detergent micelle at a relatively high molecular weight together with the 12,000 molecular weight peptide. At higher papain concentrations, the 39,000 molecular weight intermediate(s) was converted to 34,000 molecular weight peptides which were no longer included in the detergent micelle and together with the 12,000 molecular weight peptide now appeared in the lower-molecular-weight region of the agarose column.

Further evidence that the peptide(s) at molecular weight 44,000 is the HL-A antigen was obtained by indirect ("sandwich") immunoprecipitation. For these experiments, ^{125}I-labeled HL-A was prepared by the chloramine-T method (20). The addition of HL-A antiserum followed by sheep anti-human gamma globulin resulted in the formation of an immune precipitate. Analysis of this precipitate by SDS gel electrophoresis (Fig. 4) revealed that the major component precipitated in the presence of HL-A2, HL-A7, or HL-A12 antisera was at a molecular weight of 44,000 and that little or no precipitate was formed in the presence of an HL-A8 antiserum (a specificity control).§ Specificity of the precipitate was also shown by its competition with appropriate unlabeled antigen.

Purification of HL-A by Use of Lectin Affinity Column. HL-A antigen from step 2 could be further purified by adsorption to a *Lens culinaris* lectin-Biogel A-50m column, elution with a 0–2% methyl-α-D-mannoside gradient, concentration on DE-52, and gel chromatography on Biogel A-5m. The HL-A2 activity was obtained in 2.4% over-all yield

§ Although a 12,000 MW band was not found in the precipitates, other experiments have suggested that dissociation of the small and large subunits of HL-A occurs after iodination.

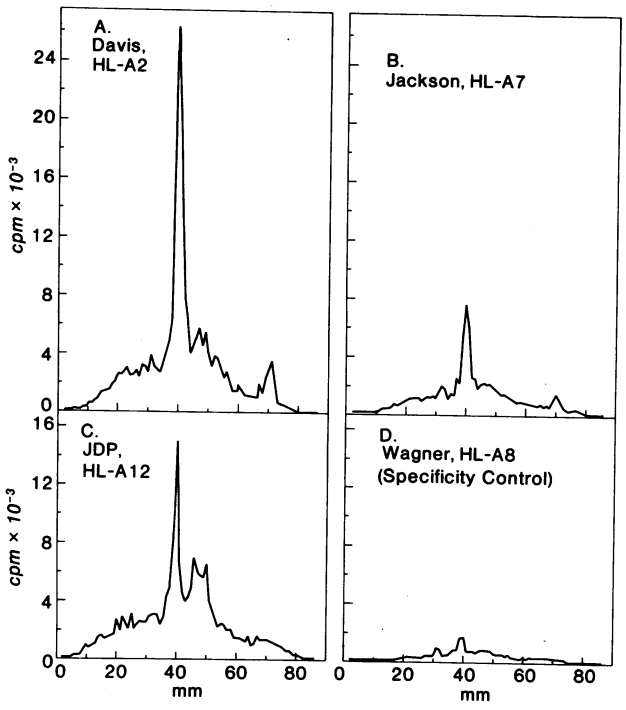

FIG. 4. SDS gels of double antibody precipitates of detergent-soluble [^{125}I]HL-A antigens. Tubes (6 × 50 mm) were presoaked with normal sheep serum. Antigen (20 μl of ^{125}I-labeled HL-A antigen from step 3) was incubated with 5 μl of HL-A antiserum for $^1/_2$ hr at 25°. Then an excess (125 μl) of sheep anti-human gamma globulin was added and incubated $^1/_2$ hr at 25° and then 2 hr at 4°. The precipitate was collected by centrifugation for 5 min at 3,000 × *g* and washed three times with 0.2% Brij 99, 0.14 M NaCl, 0.01 M Tris·HCl (pH 8.2), 0.02% NaN_3, $^1/_{10}$ normal sheep serum. Precipitates were dissolved in 75 μl of 1% SDS, 1% mercaptoethanol by boiling for 2 min, and run on phosphate-buffered SDS gels containing 7.5% acrylamide. Gels were cut into 1-mm slices and the radioactivity measured.

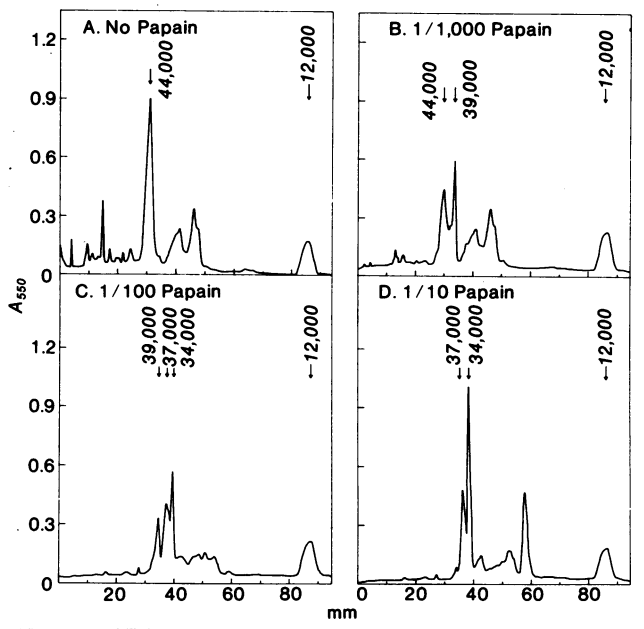

FIG. 5. SDS gels of HL-A antigen purified through lectin affinity chromatography and its papain digestion products. Protein (16 μg in 25 μl) was digested with 0, 1/1000, 1/100, or 1/10 ratios of papain to protein in gels *A-D*, respectively. Samples were run on 12% Laemmli SDS gels and, after staining with Coomassie blue, scanned at 550 nm.

and 220-fold purified over whole cells and had a specific activity of 6700 HL-A2 units/mg of protein. This material is 50% homogeneous by SDS gel electrophoresis (Figs. 5, 6).

SDS gel electrophoresis of this preparation (Figs. 5, 6) revealed the presence of two prominent protein bands, one at MW 44,000 and one at MW 12,000, together with impurities. The gel scan (Fig. 5*A*) showed that these two peaks represented 50% of the total protein. This material was also subjected to papain digestion (Fig. 5 *B–D*). As the amount of papain increased from gels *B* to *D*, the native molecule(s) in gel *A* was again converted to intermediate peptide(s) of molecular weight 39,000 appearing in gels *B* and *C*. This peptide was cleaved further in gels *C* and *D* to three peptides at molecular weights 34–37,000. Moreover, these products were identical to the products obtained by direct treatment of lymphoid cells with papain (Fig. 6) (products kindly given by Dr. Mervyn Turner). Thus, one of the 34,000–37,000 molecular weight peptides obtained from the detergent-solubilized HL-A antigen was identical to the HL-A2 product obtained by papain treatment of lymphocytes. The two products found in the HL-A7, 12 preparation coincided with two of the bands obtained from the papain cleavage of the detergent-solubilized HL-A. The MW 12,000 bands were identical in all cases.

DISCUSSION

Papain-solubilized HL-A has previously been found to contain a 12,000 MW peptide in addition to a 34,000 MW glycopeptide (8). It was, therefore, unclear whether papain releases HL-A from membranes by "nicking" a single 46,000 MW glycopeptide to form the 34,000 MW glycopeptide plus the 12,000 MW peptide or whether two precursor peptides were present, one or both of which was degraded. This report establishes that the detergent-soluble HL-A antigen contains two nonidentical peptides and that the small peptide is apparently unchanged after papain treatment. The cleavage of the larger glycopeptide may be the means of its release from the membrane. The association of the small and large peptides after two different methods of solubilization supports the idea that these peptides are also associated in the membrane. The small peptide has also been found in human serum (26). It has been shown in several laboratories (27–29) to be identical to β_2-microglobulin, a protein first isolated from human urine and homologous in structure to constant region domains of immunoglobulins. H-2 antigens from mice have been reported to contain a glycopeptide chain of MW 43–47,000 after detergent solubilization or 29–44,000 after papain solubilization (30); a small subunit has however not been found associated with H-2 antigens so far. The product of extraction of HL-A from cells by 3 M KCl has also been said to represent the native HL-A molecule (9). However, its molecular weight, 34,000, is considerably lower than that reported here for the detergent product and, as has been suggested (31), it may be an autolysis product.

The cleavage of detergent-soluble HL-A antigen by papain is very similar in rate and extent to the direct cleavage from the intact cell or cell membrane. It, therefore, is unlikely that the nonionic detergent produced much unfolding of the HL-A molecule. Papain cleavage of detergent-soluble HL-A is at least a two-step process which first produces a 39,000 MW intermediate which remains bound to detergent micelles and then produces the 34,000–37,000 MW peptides which are released from detergent micelles. Therefore, at least two small

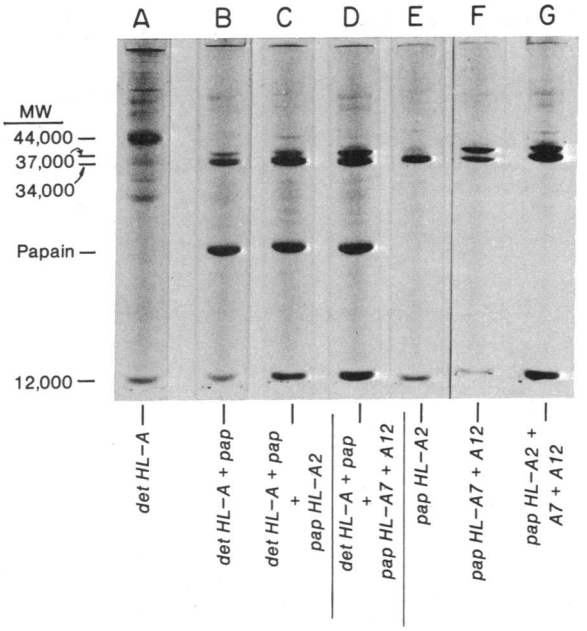

FIG. 6. Identity of detergent-soluble HL-A antigen digested with papain and HL-A antigen solubilized from membranes with papain. Detergent-soluble HL-A antigen (8 μg) was run on SDS–6 M urea Laemmli 12% gels with no papain (pap) digestion (*A*) or after digestion with 1/10 papain (*B,C,D*). HL-A antigen solubilized with papain from membranes and purified (5 μg of either HL-A2 (*C,E,G*) or a mixture of HL-A7 and A12 (*D,F,G*) was run either alone or mixed with the above as indicated on the figure. The relative migration of the three 34,000–37,000 MW peptides is different when 6 M urea is added to the gel system.

peptides totalling about 10,000 MW are cleaved off. These peptides have not yet been identified, although optimal conditions for finding them have not been sought. The papain products of certain specificities in both mouse and man have been found in two different sizes, classes I and II (30, 32, 33). This fact could be related to the finding of papain cleavage at more than one site. The analogous cleavage of immunoglobulins by papain yielding Fab and Fc products has been reported to occur at two sites in the hinge region of most immunoglobulins (34, 35).

Since the detergent-soluble molecule is presumably the same as the molecule in membranes which elicits graft rejection (except that it has a detergent micelle substituted for a membrane matrix) detergent-soluble HL-A should be the antigen of choice in immunological studies such as graft enhancement and the production of high titered HL-A antisera. The procedure is adaptable to large-scale preparations. It should be ideal for preparation of materials for chemical comparisons of different HL-A antigens, because extraneous differences are not introduced by cleavage at several sites, which may differ according to the antigen. Although many antigens are stable to papain, some determinants may be in a papain-susceptible region of the molecule (7). In addition, if HL-A antigen has a normal biological function aside from transplant rejection, the solubilization with detergent is more likely to preserve such a function than solubilization with exogenous or endogenous proteases or denaturing agents. The preparation of a homogenous product would certainly stimulate such studies.

Note Added in Proof. The use of lentil lectin in the partial purification of HL-A antigens has recently been independently reported (36, 37).

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