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Immunological and Chemical Purity of Papain-Solubilized HL-A Antigens

(radiolabeling/immune complexes/gel filtration/N-terminal amino acids)

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ABSTRACT **Three preparations of purified papain-solubilized HL-A antigens have been radiolabeled by reductive methylation using formaldehyde and potassium boro[^{3}H]hydride, and their reaction with specific HL-A antisera has been investigated. Greater than 99% of the radioactivity in the [^{3}H]HL-A2 preparation could be complexed with several HL-A2 antisera, but not with specificity controls. The other two preparations, which contained mixtures of HL-A antigenic specificities (HL-A7,12 and HL-A3,W25;12,27), showed 63% and 70% complex formation with mixtures of the appropriate HL-A antisera.**

The N-terminal amino acid of both subunits has been determined for the three HL-A antigen preparations. In all cases the only detectable N-terminal amino acids were isoleucine for the small subunit, β_2-microglobulin, and glycine for the larger subunit.

The purity of HL-A antigen preparations has been of concern to those engaged in studying the structure of these molecules, and the criticism that true allqantigens comprise but a small percentage of the molecules in purified preparations has often been made. In the case of papain-solubilized antigens it has been shown that large-scale preparations of HL-A antigens by conventional biochemical methods (1, †) have the same polypeptide structure as HL-A antigens labeled by growth of the cells in radioactive amino acids and prepared by formation of a specific immune complex (2). On sodium dodecyl sulfate/polyacrylamide gel electrophoresis both preparations were shown to contain two polypeptides, β_2-microglobulin of 11,400 daltons and a larger glycopeptide of about 34,000 daltons.

On isoelectric focusing the large subunit shows a charge heterogeneity which is largely due to differences in sialic acid content (3). We now report that both polypeptides show a single N-terminal amino acid, suggesting further the chemical homogeneity of these preparations.

However, such chemical data do not rule out the possibility that the HL-A preparations are contaminated with chemically similar but immunologically distinct molecules. Only a fraction of the total β_2-microglobulin on the surface of lymphocytes co-caps with HL-A antigens (4, 5), suggesting that this polypeptide might form a subunit of cell surface molecules other than HL-A antigens. If these molecules are structurally similar to HL-A antigens, then contamination of HL-A preparations by their papain cleavage products may occur. To show specific reaction with alloantisera one needs to incorporate a radioactive label of high specific activity into the HL-A antigen preparations. The conventional way to achieve this has been by radioiodination. However, we have found that this procedure severely alters the properties of the antigen preparations. A milder method of radiolabeling was, therefore, sought. A procedure using methylation of amino groups with formaldehyde and tritiated potassium borohydride has been adopted (9).

This paper reports that, on radiolabeling three different HL-A preparations with [^{3}H]methyl by this method, the fraction of radioactivity that was complexed by specific HL-A antisera was >99%, 63%, and 70%.

MATERIALS AND METHODS

HL-A Antigens. HL-A antigens were prepared as described (1, †). The three preparations used in this study were HL-A2, a mixture of HL-A7 and HL-A12 from the cell line RPMI 4265 (1), and a mixture of four specificities HL-A3, W25; HL-A12, and HL-A27 from the IM-1 cell line†.

Determination of N-Terminal Amino Acid. The methods used were based on those described by Weiner *et al.* (6). Samples of HL-A antigen (100–200 μg) were dialyzed against water, lyophylized, and taken up in 50 μl of 0.5 M sodium bicarbonate adjusted to pH 9.8 with NaOH and containing 1% sodium dodecyl sulfate. For dansylation, 10 μl of dansyl chloride (5 mg/ml in acetone) was added to 20 μl of the sample and the mixture was incubated at 37° for 30 min. The reaction was stopped by addition of 200 μl of cold 20% trichloroacetic acid. The precipitate was spun down and washed once with 200 μl of cold 1 N HCl. At this stage dansylated samples were either hydrolyzed in 50 μl of 6 N HCl for 20 hr at 105° or electrophoresed on 10% dodecyl sulfate/polyacrylamide gels (7). After electrophoresis fluorescent protein bands were sliced from the gel, mashed, and eluted into 500 μl of distilled water. Elution was carried out at 37° for 20 hr. Eluted samples were lyophylized and then hydrolyzed as described above. Hydrolyzed samples were taken to dryness in a vacuum dessicator, taken up in 50% aqueous pyridine, and analyzed for dansyl amino acids by thin-layer chromatography on polyamide plates (8).

Preparation of Tritiated HL-A Antigens by Reductive Methylation. The method used is a modification of that described by Rice and Means (9). To 100 μg samples of HL-A antigens in 100–200 μl of 0.01 M Tris·HCl buffer, pH 8.0, were added equal volumes of 0.2 M sodium borate buffer, pH 9.0. The samples were cooled to 4° and 10 μl of 0.03% formaldehyde was added. After 30 sec, 5 μl of a freshly prepared potassium boro[^{3}H]hydride solution (approximately 2 mg/ml in borate buffer) was added, followed at 30 sec intervals by three similar additions. After 15 min 10 μl of unlabeled sodium borohydride solution (5 mg/ml in borate buffer) was added. After warming to room temperature samples were neutralized with 1 M sodium acetate buffer, pH 5.6, exhaustively dialyzed against 0.14 M NaCl in 0.01 M Tris·HCl buffer, pH 8.0, and frozen. Preparations of labeled HL-A antigens were analyzed

† R. E. Humphreys, P. Gonzalez-Porque, J. L. Strominger, and D. L. Mann (1974), submitted for publication.

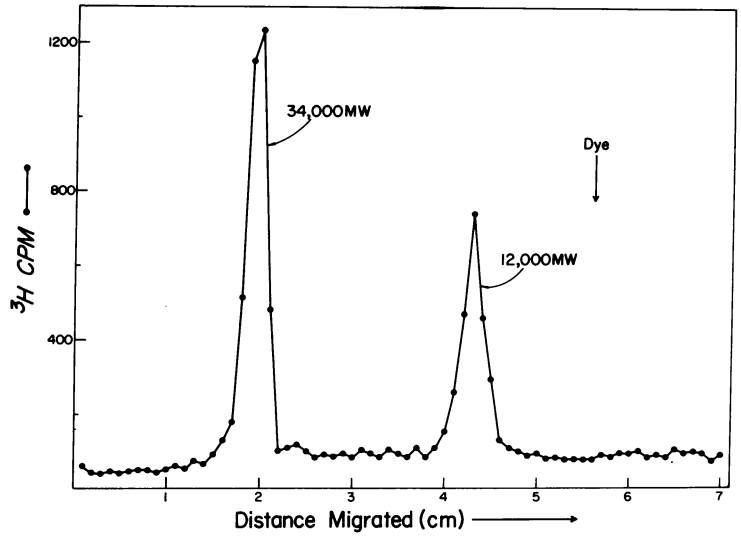

FIG. 1. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of the [^{3}H]HL-A2 antigen preparation. The gel was cut into 1 mm slices, mashed, shaken for 4 hr at 45° in 8 ml of 5% Protosol in toluene scintillation fluid, cooled, and analyzed for radioactivity. MW = molecular weight.

by electrophoresis on 10% dodecyl sulfate/polyacrylamide gels.

Formation and Analysis of Immune Complexes. Immune complexes were formed by incubation of tritiated antigen with an excess of the appropriate alloantiserum for 4 hr at room temperature (see legends to Figs. 3 and 4 for details). The mixture was then applied to a column of Sephadex G-150 equilibrated with 0.14 M NaCl in 0.01 M Tris·HCl buffer, pH 8.0. Fractions were collected and aliquots were measured for radioactivity after dissolving in Aquasol.

As HL-A antisera are nonprecipitating, immune complexes of HL-A antigens are soluble and can be detected as a peak of radioactivity excluded from the gel. Uncomplexed antigen is detected as a peak of radioactivity included in the gel and eluting at a molecular weight of about 55,000. In experiments involving sequential incubations with different antisera the peak of uncomplexed ^{3}H-labeled antigen was pooled, dialyzed against 0.01 M Tris·HCl, pH 8.0, concentrated to a volume of 1 ml or less by surrounding the dialysis bag with either Ficoll or Sephadex, dialyzed, and then incubated with the second antiserum.

Reagents. HL-A antisera were kindly provided by Dr. Bernard Amos, Dr. Arnold Sanderson, Dr. Thomas Fuller, and by the Transplantation Immunology Branch of the National Institute of Allergy and Infectious Diseases.

Potassium boro[^{3}H]hydride (9.3 Ci/mmol) was from Amersham-Searle, dansyl chloride from Pierce, and dansyl amino acids from Sigma.

RESULTS

Immunological Purity of HL-A2 Antigen. Samples (100 μg) of the different HL-A2 antigen preparations were tritium-labeled by reductive methylation and analyzed on sodium dodecyl sulfate/polyacrylamide gels (Fig. 1). All samples had only two peaks of radioactivity corresponding to the 34,000 and 11,400 dalton subunits of HL-A antigens. The ratio of radioactivity in the larger subunit to that in β_2-microglobulin varied with the antigen preparation and with different labeled samples from the same antigen preparation in the range of 1:1 to 2:1. Specific activities of about 5×10^8 cpm/mg were routinely obtained, corresponding to between 3 and 4 moles of [^{3}H]methyl incorporated per mole.

The methylated HL-A2 antigen retained its ability to form specific immune complexes. To determine the amount of antiserum needed to give maximal complex formation, increasing amounts were incubated with aliquots of ^{3}H-labeled antigen and subjected to Sephadex G-150 chromatography. The titration of [^{3}H]HL-A2 by one HL-A antiserum (Meunier) is illustrated in Fig. 2, together with a specificity control (anti-HL-A8, Wagner).

When the [^{3}H]HL-A2 preparation was incubated with an excess of three HL-A2 antisera (Meunier, Phannix, and Davis) more than 99% of the radioactivity was found in the excluded peak containing the immune complex. With a normal control serum from an AB individual, with an anti-HL-A8 serum

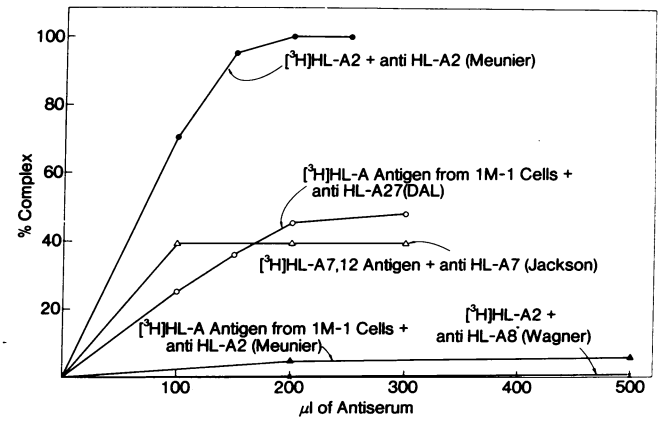

FIG. 2. Titration of [^{3}H]HL-A antigen preparations against HL-A antiserum. Samples of [^{3}H]HL-A antigen containing 10–20,000 cpm were incubated with increasing amounts of HL-A antiserum for 4 hr at room temperature in a volume of 500 μl. The mixture was then applied to a Sephadex G-150 column (105 cm × 1.1 cm) equilibrated with 0.14 M NaCl in 0.01 M Tris·HCl, pH 8.0, at 4°. Fractions of 1 ml were collected and their radioactivity was measured in 8 ml of Aquasol. The degree of complex formation was calculated by dividing the cpm in the excluded peak by the sum of the cpm in the excluded *and included* peaks.

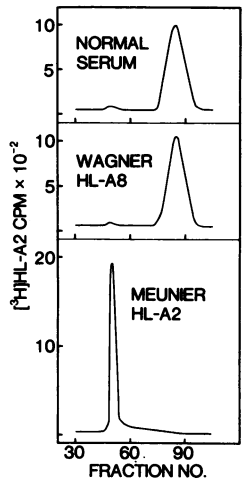

FIG. 3. Elution profile on Sephadex G-150 chromatography of complexes formed between [³H]HL-A2 preparations and either an anti-HL-A2 serum (Meunier), an anti-HL-A8 serum (Wagner), or a normal human serum. Complexes were formed and analyzed as described under Fig. 2.

(Wagner) or with an anti-HL-A7 serum (Jackson), less than 1% complex formation was seen (Fig. 3).

Assessment of Immunological Purity of HL-A7,12 Antigen Preparations from RPMI 4265 Cells and of HL-A3,W25;12,27 Preparations from IM-1 Cells. Similarly, the HL-A7,12 and HL-A3,W25;12,27 antigen mixtures were labeled with [³H]-methyl and in each case the same two polypeptides were found as is illustrated in Fig. 1 for the [³H]HL-A2 antigen. The formation of immune complexes in antibody excess for the [³H]HL-A7,12 preparation is summarized in Table 1. Anti-HL-A7 serum (Jackson) complexes 40% and anti-HL-A12 serum (SLA) 21% of the radioactive preparations. When both sera were used together, 63% of the radioactivity was complexed. In a sequential experiment, an HL-A7 complex was first formed with Jackson antiserum. The peak of uncomplexed antigen was then dialyzed, concentrated, incubated with SLA antiserum, and applied again to the Sephadex G-150 column. The percentage of radioactivity complexed was again 40% for Jackson and 21% for SLA antiserum, showing that these two sera were complexing different populations of radioactive molecules.

TABLE 1. *Complex formation between [³H]HL-A7,12 and [³H]IM-1 preparations and various HL-A antisera*

Antiserum	HL-A specificity of antiserum	Maximum radioactivity complexed, %
1. [³H]HL-A7,12 preparations		
Jackson	7	40
SLA	12	21
Meunier	2	5
Wagner	8	3
Jackson and SLA	7,12	63
2. [³H]HL-A3,W25;12,27 preparations		
DAL	27	48
BC	3	30
EVA	W25	11
MS	10	11
SLA	12	8
N513	12	8
Phannix	2	6
Davis	2	5
Wagner	8	5
DAL/BC/SLA/EVA	3,W25;12,27	70
DAL/BC	27,3	70

Only the major HL-A specificity for each antiserum is listed. Many of the antisera will show reactivity against other HL-A specificities when used in high enough concentrations. For a full description of the reactivity of the HL-A antisera used see the *Catalog of Tissue Typing Antisera 1974–75*, (DHEW Publication no. [NIH] 75–97).

The nature of the material that did not complex with either of these two specific antisera was investigated. It has been reported that the cell line RPMI 4265 types as HL-A2,W32;7,-12 rather than HL-A2,2;7,12 (10). The residual uncomplexed ³H-labeled material could be molecules carrying W32 specificities. With a W32 antiserum (Thompson) 30% of the radioactivity was complexed, although this involved a much larger quantity of serum (400 μl) than was usually employed. However, when a mixture of W32, HL-A7, and HL-A12 antisera was used, only 63% of the radioactivity was complexed, the same result as obtained when the W32 antiserum was omitted. In a sequential experiment, the W32 antiserum complexed with none of the residual radioactivity after [³H]HL-A7 and [³H]HL-A12 antigens had been removed. Therefore, a subset of molecules having HL-A7 or HL-A12 reactivity must also react with the W32 antiserum.‡

Table 1 also summarizes the results obtained with the [³H]-HL-A3,W25;12,27 antigen preparation from IM-1 cells (referred to below as the [³H]IM-1 antigen). Nonspecific sera generally complexed with 6% or less of the radioactivity. HL-A27 antiserum (DAL) and HL-A3 antiserum (BC) complexed 48% and 30% of the radioactivity, respectively. In contrast, although many different W25 antisera (including HL-A10 antisera which contain anti-W25 as a component) and HL-A12 antisera were tried, the degree of complex formation was never more than a few percent above the specificity control. This result agrees qualitatively with the approximate 5-fold difference in cytotoxic inhibitory titer seen between HL-A27 and HL-A3 on the one hand and HL-A12 and W25 on the other hand, for the unlabeled IM-1 antigen preparation. When sera against all four HL-A specificities were used, 70% complex formation was seen, the same as when just the HL-A27 and HL-A3 sera were used together. When HL-A27 and HL-A3 complexes had been removed, a mixture of W25 antiserum (EVA) and HL-A12 antiserum (SLA) formed no significant complex with the remaining radioactivity. HL-A7 cross-reacts with HL-A27. When HL-A7 antiserum (Jackson) was incubated with the [³H]IM-1 antigen, 22% of the radioactivity was complexed.

Sephadex G-150 chromatography of immune complexes from both the [³H]IM-1 antigen and the [³H]HL-A7,12 antigen preparations sometimes revealed a small peak of radioactivity, eluting at a molecular weight corresponding to free β_2-microglobulin; this material was never seen with the [³H]-

‡ Pressman and coworkers (11) have reported the presence of still another HL-A antigen, HL-A1, in papain-solubilized HL-A antigens from RPMI 4265 cells. An HL-A1 antiserum (Raymond) complexed 25% of the radioactivity in the HL-A7,12 preparation, but further work needs to be done to see if this is the result of cross-reaction or whether an independent set of HL-A1 molecules is present.

HL-A2 immune complexes. It is possible that some dissociation of subunits may have occurred and that the unreactive material present in these two preparations was due either to dissociated molecules, or to reassociated molecules having a different conformation without or with greatly reduced HL-A antigenic activity. That separation of subunits can result in considerable loss of HL-A antigenic activity was demonstrated in the following experiment. The [^{3}H]IM-1 antigen preparation was dialyzed against 6 M urea for 12 hr at 4° and applied to a column of Sephadex G-75 equilibrated with 0.14 M NaCl in 0.01 M Tris·HCl, pH 8.0, and containing 0.1% bovine serum albumin. Two peaks of radioactive material were eluted at molecular weights corresponding to the two subunits of HL-A antigen. That separation of subunits had been achieved was confirmed when samples from the two peaks (Fig. 4-IIb and IIIb) were subjected to dodecyl sulfate/-polyacrylamide gel electrophoresis, running the original labeled preparation in a parallel gel (Fig. 4-Ib). Equimolar quantities of separated large subunit (Fig. 4-IIa), β_2-microglobulin (Fig. 4-IIIa), and the intact molecule (Fig. 4-Ia) were separately incubated with a volume of HL-A27 antiserum (DAL) that gave maximal complex formation with the intact molecule. Each sample was analyzed for specific immune complexes on the same Sephadex G-150 column. DAL antiserum complexed 48% of the radioactivity in the original preparation; the same volume of antiserum complexed none of the radioactivity in the β_2-microglobulin preparation and only 11% of the radioactivity of the large subunit.

An identical experiment was attempted using the [^{3}H]-HL-A2 preparation, but separation of subunits was not achieved by this method; after exposure to 6 M urea the radioactive material eluted as a single peak from the Sephadex G-75 column. A similar difference between the HL-A2 and the HL-A3,W25;12,27 antigen preparations was seen when samples were eluted from a column of Bio-Gel P-60 equilibrated with 1 N acetic acid containing 0.1% bovine serum albumin. The [^{3}H]IM-1 antigen preparation eluted as two peaks of radioactivity corresponding to the separate subunits. The [^{3}H]HL-A2 antigen preparation eluted as a single peak corresponding to the intact molecule.

Determination of N-Terminal Amino Acids. The complete amino-acid sequence of the small subunit of HL-A antigen, β_2-microglobulin, is known (12); its N-terminal amino acid is isoleucine. In initial studies dansylation was carried out on mixtures of the two subunits followed by complete acid hydrolysis and chromatographic analysis on polyamide plates (6, 8). For all three of the HL-A antigen preparations studied, two major spots corresponding to dansyl-Ile and dansyl-Gly were seen, indicating that glycine might be the N-terminal amino acid of the larger subunit. However, a large number of minor spots were also seen, some of which could have been due either to contaminating amino acids or to N-terminal heterogeneity of the larger subunit. Moreover, glycine is a well-known contaminant of many laboratory solutions and so further confirmation of a glycyl end group was required. This was achieved by dansylating the mixture of subunits and electrophoresing the neutralized trichloroacetic acid precipitate on dodecyl sulfate/polyacrylamide gels. Fluorescent protein bands corresponding to the large and small subunits were sliced from the gels, eluted, hydrolyzed, and analyzed for dansyl-amino acids. Free amino acids contaminating the dansylation solutions were eliminated in the electrophoresis. Those contaminating solutions used in subsequent steps never

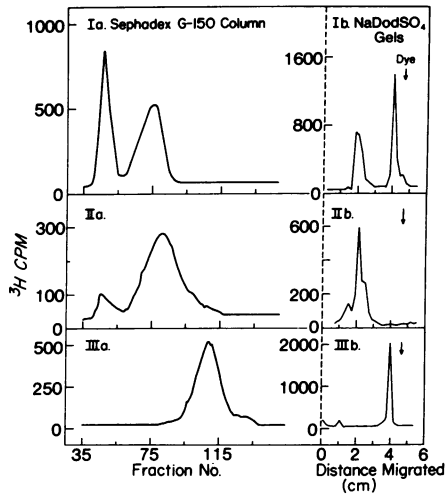

FIG. 4. HL-A antigenic activity of separated subunits. The two subunits of the [^{3}H]IM-1 preparation were separated by dialysis against 6 M urea and gel filtration on a Sephadex G-75 column equilibrated with buffer containing no urea. Sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis of the intact [^{3}H]IM-1 preparation (Ib) and of the separated large (IIb) and small subunits (IIIb) was performed as described in Fig. 1. Equimolar amounts, as determined from the specific activities of the three preparations, were incubated with 300 μl of DAL serum (HL-A27) for 4 hr at 37° in a volume of 500 μl. Elution profiles on Sephadex G-150 of the HL-A27 complexes from the intact [^{3}H]IM-1 preparation (Ia) and the separated large (IIa) and small (IIIa) subunits are shown. The amount of DAL antiserum used was sufficient to give maximal complex formation with the intact [^{3}H]IM-1 preparation.

came into contact with dansyl chloride and would not show up in chromatographic analysis. After this procedure, the polyamide plates from all three HL-A antigen preparations showed single spots of either dansyl-Ile from β_2-microglobulin or dansyl-Gly from the larger subunit.

These results have also been confirmed by initial studies using the Beckman Automatic Sequencer. When 18 nmol of purified large subunit from HL-A2 was subjected to one cycle of Edman degradation, 12 nmol of glycine was recovered after conversion to the phenylthiohydantoin with HCl and back hydrolysis to the free amino acid with HL. Thus, 0.67 mole of glycine per mole was recovered from the HL-A2 antigen preparation in the first round of reaction. Similarly 0.4 mole/mole of glycine was recovered from the HL-A7,12 antigen preparation (8 nmol from 20 nmol of the large subunit of the HL-A7,12 preparation).

DISCUSSION

In this study further evidence for the chemical homogeneity of papain-solubilized HL-A antigen preparations has been provided by showing that each of three preparations has glycine as the sole N-terminal amino acid. Moreover, alloantigenic activity was associated with >99, 63, and 70% of the molecules in the three preparations studied.

For each of the three preparations studied the only detectable N-terminal amino acids were isoleucine for the small subunit. This result for membrane-bound β_2-microglobulin agrees with studies on urinary β_2-microglobulin. It is possible that only some fraction of the large subunit molecules have N-terminal glycine while the rest may be blocked, or that there is considerable N-terminal heterogeneity with glycine

being the major and only detectable residue. This is an important question since a papain cleavage product was being studied. However, there is some evidence against this idea. (*a*) The spots of dansyl-Ile and dansyl-Gly when a mixture of the two chains was analyzed were of roughly equal intensity. (*b*) Quantitative studies by Edman degradation using the Beckman Automatic Sequencer confirmed this result. Further studies on detergent-solubilized HL-A antigen preparations should reveal whether or not papain cleavage takes place from the N-terminal end of the molecule.

When the HL-A2 antigen preparation was labeled by reductive methylation over 99% of the radioactivity complexed with specific HL-A2 antiserum with a specificity ratio in excess of 100. Thus, this preparation appeared to be immunologically pure. The results with the HL-A7,12 and the HL-A antigen preparation from IM-1 cells were less clear-cut, although in both cases in excess of 60% of the radioactivity complexed with an appropriate mixture of HL-A antisera. Specificity ratios were lower than for HL-A2 and there was considerable crossreaction between antisera with different HL-A specificities. Many HL-A antisera are polyspecific. Others that behave monospecifically in a tissue typing system may reveal other minor HL-A antibodies when conditions of antibody excess are used. The number of HL-A antisera used in this study has by a serological criterion been small and any conclusions drawn concerning the relative amounts of different HL-A specificities should be regarded as tentative. The relative proportions of the different HL-A specificities in the HL-A7,12 and HL-A3,W25;12,27 preparations does appear to differ, although one should bear in mind that differential labeling of HL-A specificities could conceivably occur. However, the inhibitory titers of HL-A12 in the HL-A7,12 preparation and of HL-A12 and W25 in the IM-1 antigen preparation do appear to be considerably lower than those of the other specificities.

Both [^{3}H]HL-A7,12 and [^{3}H]IM-1 antigen preparations have a significant fraction of radioactivity (approximately 30%) that cannot be complexed by the HL-A antisera used. This radioactivity could represent: (*a*) HL-A molecules that have lost activity during purification; (*b*) HL-A molecules that have lost activity during labeling; (*c*) non-HL-A molecules having electrophoretic properties similar to those of HL-A; or (*d*) HL-A molecules having antigenic specificities other than those tested.

Dissociation of subunits in urea led to loss of activity for at least one of the HL-A specificities (W27), and the presence of free β_2-microglobulin in the [^{3}H]HL-A7,12 and [^{3}H]IM-1 antigen preparations indicates that dissociation may take place. Whether this dissociation is the result of reductive methylation, incubation with antiserum, or other factors is not known. Reports that HL-A spontaneously dissociates on standing in aqueous solution (13) and that reductive methylation selectively destroys certain H-2 specificities (14) indicate that either might be the case. To separate the subunits of HL-A2 requires stronger conditions than for the mixture of HL-A specificities in the IM-1 preparation. Presumably this reflects a stronger binding between the subunits of this antigenic specificity. As dissociation diminished antigenic activity, it is possible that the apparent differences in immunological purity between HL-A preparations is merely a reflection of the relative strengths of binding between their subunits.

The existence of a third locus of serologically defined HL-A antigens has been established (15, 16), and a limited number of antigenic specificities defined. Whether they resemble the first and second locus HL-A antigens chemically or are different types of molecules is still uncertain. On sodium dodecyl sulfate gel electrophoresis papain-solubilized third locus antigens showed an identical band pattern to that seen with first and second locus antigens in one report (17). On the other hand, detergent-solubilized third locus antigens were not the same as first and second locus antigens in another report (§).

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