Comparative Chemical Analyses of the Alloantigenic Fragments of HL-A Antigens*

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OLE HENRIKSEN AND ETTORE APPELLA
From the National Institutes of Health, Laboratory of Cell Biology, National Cancer Institute, Bethesda, Maryland 20014

DAVID F. SMITH‡
From the American Red Cross Blood Research Laboratory, Bethesda, Maryland 20014

NOBUYUKI TANIGAKI AND DAVID PRESSMAN
From the Department of Immunology Research, Roswell Park Memorial Institute, Buffalo, New York 14203

Papain-solubilized HL-A antigens have been shown to contain two polypeptide fragments: β₂-microglobulin with a molecular weight of approximately 12,000 and a larger fragment with a molecular weight of about 34,000. The large fragments isolated from two HL-A preparations carrying different specificities appeared homogeneous both by immunoelectrophoresis and sodium dodecyl sulfate-acrylamide electrophoresis. Both HL-A antigen preparations contained the same NH₂-terminal (glycine) and the same COOH-terminal residue (serine). The carbohydrate content of the large fragment was 12.9%, making the carbohydrate-free molecular weight approximately 30,000. Small but significant differences have been found in the amino acid compositions and tryptic peptide maps of the two large fragments containing different specificities.

HL-A antigens are membrane glycoproteins which belong to the major histocompatibility locus of man (1). The proteins can be solubilized by treatment of the membranes with papain or with detergents. They are composed of two noncovalently bound subunits with molecular weights of about 44,000 and 12,000 for detergent-solubilized antigens, and of about 34,000 and 12,000 for papain-solubilized antigens. In both cases, the polymorphic alloantigenic specificities are associated with the larger chain; the smaller chain represents β₂-microglobulin (2, 3). It has been shown that β₂-microglobulin has marked homology with the amino acid sequence of the C₃₅ domain of immunoglobulin IgG and it has been suggested that histocompatibility antigens and immunoglobulins have a common evolutionary origin (4, 5). However, whether the alloantigenic polypeptide chain has characteristics similar to the immunoglobulins is not known. More recently, Peterson et al. (6) have proposed that the histocompatibility antigens are tetrameric structures with two basic units containing one each of the large and small polypeptide chains linked by a disulfide bridge.

In this paper chemical data are reported for the larger subunit of purified HL-A preparations containing different alloantigenic specificities from the cell line RPMI 1788.

EXPERIMENTAL PROCEDURES

Materials—Ultrapure guanidine HCl and urea were purchased from Schwarz/Mann, Sephadex G-75 and G-100 from Pharmacia, Ultrapure HCl from Baker, dithiothreitol and iodoacetamide from Calbiochem, L-rhamnose and galactose from Pfanstiehl Laboratories. TPCK-trypsin¹ and carboxypeptidase A were from Worthington. Special grade acrylamide and N,N'-methylenebisacrylamide were obtained from Bio-Rad, Fluram from Roche Diagnostics, 2,4-dinitro-[3,5-³H]fluorobenzene (24.1 Ci/mmol) from New England Nuclear Corp. The proteins used as standards for molecular weight determinations were Bence Jones protein dimer (M, = 45,000), light chain of human IgG (M, = 23,000), cytochrome c (M, = 12,400), and insulin (M, = 5,700).

Purification of HL-A Antigens—The procedures for the solubilization and isolation of the HL-A antigens have been fully described (7). Briefly, the antigens were solubilized by papain digestion of a crude cell membrane material from a human lymphoid cell line, RPMI 1788,¹ and purified by hypotonic dialysis followed by ion exchange chromatography, ultrafiltration, gel filtration, and column electrophoresis.

¹The abbreviations used are: TPCK-trypsin, L-1-tosylamido-2-phenylethyl chloromethyl ketone treated trypsin; dansyl, B-dimethylamino-1-naphthalene sulfonyl; N,N', dinitrophenyl.

‡Postdoctoral Fellow of the National Cancer Institute (1 F02 CA 66287).
Two different antigen preparations, HL-A2 and HL-A7, were isolated. The indicated HL-A specificity of the preparation is the evident specificity followed during isolation. Other HL-A specificities carried by the cells are probably present in these preparations, but were not followed. Also, the HL-A2 preparation was slightly contaminated with HL-A7, and vice versa, but the problem of cross-contamination has not been fully examined. Yet, we estimate, based on results from our previous work, that the cross-contamination was between 10 and 20% in the two preparations. The HL-A phenotype of RPMI 1788 was kindly determined by Dr. P. I. Terasaki, University of California, Los Angeles, and found to be 2,7 and W4. Preparation of the large and small polypeptides by acid dissociation of papain-solubilized HL-A antigens has been described in detail elsewhere (9). 

Purity of HL-A Antigens—The HL-A preparations were checked for purity by immunoelectrophoresis (9) and disc gel polyacrylamide electrophoresis (10). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was done essentially by the method of Shapiro et al. (11). Antiserum used for immunoelectrophoretic studies were those raised in rabbits against a purified preparation of papain-solubilized HL-A antigens described in detail elsewhere (8). Determinations.

Amino Acid Analysis—Lyophilized samples were hydrolyzed in 1.0 ml of 2 N HCl at 105° under nitrogen for 1 to 4 hours. Maximal release of neutral sugars during acid hydrolysis was attained after 3 hours, and these values were used in the calculations of neutral sugar in Table I. After hydrolysis, 1-ribose was added as an internal standard, the samples were dried in vacuo over NaOH and the individual monosaccharides were separated and quantitated by ion exchange chromatography of their borate complexes on a JEOL model JLC-5AH amino acid analyzer modified for neutral sugar analysis. Total hexose was determined by the phenol-sulfuric acid method of Dubois et al. (15) with galactose as a standard. Sialic acid was determined by the method of Aminoff (16) following hydrolysis in 0.1 N H2SO4 for 1 hour at 80°. The glucosamine and galactosamine content was determined by hydrolysis with 2 N methanesulfonic acid in the presence of 0.1% 3-(2-aminoethyl)indole in vacuo at 100° for 6 and 12 hours and the hydrolysates were analyzed on the basic column (20 cm) of a JEOL amino acid analyzer at 57°.

Amino Acid Analysis—Lyophilized samples were hydrolyzed in vacuo in 6 N HCl at 105° for 24, 48, and 72 hours in duplicate, dried by rotary evaporation at 35°, and then analyzed on a Durrum D 500 analyzer. The observed amounts of threonine, serine, and tyrosine were corrected for destruction during hydrolysis by extrapolation of the experimental values to zero time of hydrolysis. Data for each of the other components were averaged over the six runs, except for valine and isoleucine where only data from 72-hour or 48- and 72-hour hydrolysates, respectively, were averaged. Half-cystine content was determined by acid hydrolysis after complete reduction in 5 M guanidine HCl/0.2 M Tris-HCl, pH 8.9, with 0.01 M dithiobisulphite for 1 hour at 37° and then alkylolation with 0.02 M iodoacetamide at 37° for 1 hour.

Amino acid composition in terms of residues (see Table II) is expressed as products of the mole fraction and a corresponding total number of residues. The latter value was estimated to be 267.2. This number was calculated in the following way: a molecular weight of 94,000 for HL-A large component has been derived from sodium dodecyl sulfate gel electrophoresis. If one subtracts a molecular weight of 4,000 for the carbohydrate portion of the large component, one obtains 30,000 for the polypeptide portion of this chain. Division of the latter by 112.3, the observed average residue weight, gives 267.2 residues for the chain length.

Peptide Maps—Three nanomoles of each of the large antigen fragments were reduced under nitrogen with 5 μmol of dithiobisulphite at 37° for 90 min in 0.5 ml of 0.2 M Tris-HCl, pH 8.2/5 M guanidine HCl. The proteins were then alkylolated with 11 μmol of [14C] iodoacetamide (1.5 x 106 cpm/μmol at 80% counting efficiency) at 37° for 40 min and, after completion of the reaction, dialyzed against 50 ml of 0.2 M ammonium bicarbonate (two changes of buffer). To the dialyzed solutions were added TPCK-trypsin (enzyme/substrate ratio, 1/100, w/w) and the samples were digested for 3 hours at 37° and finally lyophilized. The lyophilized samples were dissolved in 5 μl of electrophoresis buffer, pH 1.9 (formic acid/acetic acid/water, 1/3.5/40), and applied to cellulose sheets (20 x 20 cm). Electrophoresis was carried out at 470 V for 1 hour at 14°, which was followed, after drying of the plates, by chromatography in 1-histidine/acetic acid/pyridine/water (15/3/10/12). After drying, the plates were wetted with 3% pyridine in acetone and sprayed with 0.02% fluorescamine in acetone (17).

NH2-Terminal Residues—One nanomole of the large fragments was allowed to react with 280 nmol of [3H]fluorodinitrobenzene (1.5 x 106 cpm/nmol) according to Sanger (18), but in the presence of 2% sodium dodecyl sulfate, and after hydrolysis in 6 N HCl for 6 hours, the dinitrophenyl amino acids were chromatographed on polyester-supported polyamide sheets according to Wang and Weinstein (19). Proteins were also reacted with dansyl chloride according to Gray (20) in the presence of 2% sodium dodecyl sulfate. The labeled proteins were precipitated by addition of acetone, dried after washing several times, and hydrolysed with 6 N HCl for 12 hours. The dansyl-amino acids were chromatographed on polyamide sheets according to Woods and Yang (21).

COOH-Terminal Residues—Protein, 1.1 nmol, in 0.2 M 2-N-ethylmorpholine, pH 8.3, was digested with carboxypeptidase A (enzyme/substrate ratio, 1/50, w/w) for different times at 37°. A blank sample (enzyme alone) was also analyzed. After digestion, the samples were acidified with 1 drop of glacial acetic acid, dried, and analyzed in an amino acid analyzer equipped for high sensitivity (10 pmol).4

1. We thank Dr. P. E. Hare, Geophysical Laboratory, Carnegie Institution of Washington, Washington, D. C., for these analyses.

2. Calculated as N-acetylgalactosamine.

3. Calculated as N-acetylgalactosamine.

4. The indicated specificity is the evident specificity followed during isolation of the respective antigens.

### Table I

<table>
<thead>
<tr>
<th>Components</th>
<th>Residue weight</th>
<th>Mol/mol of HL-A7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sialic acid</td>
<td>1.95*</td>
<td>3.08</td>
</tr>
<tr>
<td>Mannose</td>
<td>1.29</td>
<td>3.64</td>
</tr>
<tr>
<td>Fucose</td>
<td>Trace</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>3.08</td>
<td>8.72</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>3.24*</td>
<td>7.11</td>
</tr>
<tr>
<td>Total carbohydrate</td>
<td>9.55</td>
<td></td>
</tr>
</tbody>
</table>

*a Based on a molecular weight of 46,000.

*b Calculated as N-acetylneuraminic acid.

*c Calculated as N-acetylgalactosamine.

### Table II

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Number of residues</th>
<th>Residue difference (HL-A2) - (HL-A7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>9.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>10.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Arginine</td>
<td>22.3</td>
<td>-0.6</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>23.5</td>
<td>-0.8</td>
</tr>
<tr>
<td>Threonine</td>
<td>20.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Serine</td>
<td>17.3</td>
<td>0.0</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>40.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Proline</td>
<td>12.7</td>
<td>-1.2</td>
</tr>
<tr>
<td>Glycine</td>
<td>23.0</td>
<td>-0.2</td>
</tr>
<tr>
<td>Alanine</td>
<td>22.6</td>
<td>0.7</td>
</tr>
<tr>
<td>Valine</td>
<td>14.5</td>
<td>1.8</td>
</tr>
<tr>
<td>Methionine</td>
<td>4.5</td>
<td>1.4</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>6.7</td>
<td>2.8</td>
</tr>
<tr>
<td>Leucine</td>
<td>16.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>13.9</td>
<td>-0.4</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>9.0</td>
<td>0.6</td>
</tr>
</tbody>
</table>

4. The indicated specificity is the evident specificity followed during isolation of the respective antigens.
Chemical Analysis of HL-A Antigens

Results

Disc gel electrophoresis of the purified HL-A antigens (Fig. 1) shows a clear difference in mobility of the proteins with specificities 2 and 7, respectively. Both the bands were diffuse, presumably due to microheterogeneity within a single HL-A species or to the presence of very closely related HL-A molecules which may or may not have complete immunological identity. All of the microheterogeneous proteins apparently have the same molecular weight, since only two bands were resolved after sodium dodecyl sulfate gel electrophoresis of the HL-A2 and HL-A7 preparations (Fig. 1), and only one band was seen after electrophoresis of the isolated large and small fragments, respectively. The faintly stained extra bands are due to artifactual dimer formation. Immunoelectrophoresis (Fig. 2) confirmed that the antigen preparations were immunochemically pure, or consisted of very closely related proteins, since only one precipitation arch is seen after diffusion against both rabbit anti HL-A antiserum and rabbit anti-human cell membrane antiserum. The lower panels of the figure show reaction with rabbit anti-β2-microglobulin antiserum. Except for the small amount of free β2-microglobulin this diffusion also demonstrated the purity of the HL-A preparations.

The carbohydrate composition of HL-A7 is given in Table I. The total value for the neutral sugars was 0.27 μmol/mg of HL-A7, while the total hexose content as determined by the phenol-sulfuric acid method was 0.29 μmol/mg. This good agreement indicates that the hydrolytic conditions utilized for neutral sugars were optimal. Earlier, Sanderson et al. (22) studied the carbohydrate content of different HL-A preparations. The main qualitative difference between the results of their investigation and ours is the total absence of glucose and galactosamine and the presence of only trace amounts of fucose in our preparations. It can be calculated from our data that the large component contains 12.9% carbohydrate (Table I) since β2-microglobulin purified from spent culture medium was shown to be free of carbohydrates.

Amino acid analyses were carried out in duplicates of approximately 1.3 nmol of each of the isolated large alloantigenic fragments on a Durrum D500 amino acid analyzer after hydrolysis for 24, 48, and 72 hours (Table II). The number of residues was calculated based on a molecular weight of 30,000 as described under “Experimental Procedures.” Comparison of the amino acid composition of the two proteins shows that although they are very similar several significant differences are apparent. These compositional differences appear to involve 1 or 2 residues of the particular amino acid affected. The figures given in Table II indicate that the two HL-A preparations differ by 15 residues (sum of differences irrespective of sign) involving 13 amino acids. A further demonstration of the similarities between the two preparations is seen after tryptic digestion of fully reduced and alkylated proteins, and separation of the peptides by electrophoresis and chromatography (Fig. 3). The peptide maps are very similar, but small differences can be located at the right hand side, the lower left hand side, and in the middle of the maps. Since the carboxymethylation of the proteins was carried out with 14C-labeled iodoacetamide, identification of half-cystine-containing peptides by autoradiography was made possible (Fig. 4). The map of the large fragment from the HL-A2 preparation showed 5 half-cystine peptides while the map of the large fragment from the HL-A7 preparation had 6 half-cystine peptides.

An independent determination of the total half-cystine content of the polypeptide chains was carried out by amino acid analysis, after complete reduction and carboxymethylation. Extrapolation to zero time gave values of 4.0 and 4.4 S-carboxymethyl cysteine residues/mol of M, = 30,000 for preparations containing HL-A2 and HL-A7 specificities, respectively.

It is important for a comparative study of the HL-A gene products to determine the COOH- as well as the NH₂-terminal amino acid residues. Both a complete and a time-dependent digestion of the large antigen fragments by carboxypeptidase A demonstrated (Fig. 5) that serine is the COOH-terminal amino acid in both proteins. This residue was liberated in molar quantities after complete digestion. The next residue was leucine, followed by threonine. It should be noted that the stoichiometric release of serine during carboxypeptidase A digestion provides further evidence of the purity of the two protein preparations. The NH₂-terminal residue was determined by two independent methods: dansylation and dinitrophenylation. With both methods glycine was the only reacting NH₂-terminal residue that could be detected. Table III gives an account of the radioactivity that could be eluted from the polyamide plate after chromatography of the [3H]NpPh derivatives of the HL-A2 protein. It is apparent that the only radioactive α-NpPh-amino acid present was NpPhGly. These results thus confirm the observation by Parham et al. (23) that glycine is the NH₂-terminal amino acid residue of three different HL-A preparations (HL-A2; HL-A7, 12; and HL-A3, W25; 12, 27).
Chemical Analysis of HL-A Antigens

DISCUSSION

In comparative studies of histocompatibility antigens it is important to consider chemical as well as immunological purity of the preparations. The homogeneous behavior during gel chromatography, gel electrophoresis, and immunoelectrophoresis of the antigens under study in this paper as well as the presence of only 1 NH$_2$-terminal and 1 COOH-terminal amino acid residue is good evidence of chemical purity. However, this does not rule out that each of the antigen preparations actually is a mixture of very similar antigen molecules in which a few amino acid substitutions could account for differences in immunological specificity.

The carbohydrate content of the antigens is of considerable interest since, in combination with dry weight determinations, it can give support to an estimation of the peptide molecular weight determined by amino acid analysis. The amount of total carbohydrate was 9.55% in a sample of HL-A7 preparation on which a dry weight determination had been carried out. The amount of carbohydrate which can be calculated for the large component is 12.9%, since no carbohydrate has been found on $\beta_2$-microglobulin. The molecular weight of 34,000 for the large component of HL-A2 or HL-A7 can be corrected for the estimated carbohydrate content in order to obtain the molecular weight of the polypeptide chain. Using this approach a molecular weight of approximately 30,000 was calculated for the large component of papain-solubilized HL-A antigens. These results are at variance with the estimation of the peptide molecular weight of 22,000 by Cresswell et al. (24) who did not perform any carbohydrate analysis but deduced their estimation from a radioactive labeling experiment.

Several authors have suggested that histocompatibility antigens might be structurally related to immunoglobulins. Recently, Peterson et al. (6) suggested that the large components of both papain-solubilized H-2 and HL-A antigens contain two domains with an intrachain disulfide bridge in each. The finding in the present study of 4 to 5 cysteine residues in each of the large components of HL-A2 and HL-A7 preparations supports the notion of two intrachain disulfide bridges, but since immunoglobulin domains studied so far invariably have molecular weights of approximately 10,000 without major stretches of loosely folded peptide chain in between, the demonstration in this investigation of a peptide molecular weight of approximately 30,000 does not necessarily support a two-domain model which depicts a close similarity between histocompatibility antigens and immunoglobulins.

It has been demonstrated in this report that the large components of HL-A2 and HL-A7 preparations are very similar. The NH$_2$-terminal as well as the COOH-terminal amino acid residues are identical and only small differences were noted in the overall amino acid composition and in the tryptic peptide maps. In this connection it is of interest to draw attention to a study by Brown et al. (25) who reported that the products of the H-2K and H-2D genes of the H-2 histocompatibility complex of the mouse were extremely different. Since the two histocompatibility loci of man are considered analogous to the H-2D and H-2K loci of the mouse, it would have been
Fig. 4. Autoradiography of half-cystine-containing peptides. Tryptic peptide maps were prepared as described, after full reduction and alkylation with [35S]iodoacetamide. To each of the plates was applied approximately 4,000 cpm. The autoradiographs were developed for 10 days.

Table III

Recovery of radioactivity from polyamide sheet after chromatography of dinitrophenyl derivatives of large fragment of HL-A2

The procedure was carried out as described in "Experimental Procedures." To the polyamide sheet, 3,700 cpm was applied, together with a standard NPh-amino acid mixture. After chromatography the indicated spots were cut out and incubated in 0.2 ml of NCS (New England Nuclear Corp.) for 10 min before addition of 10 ml of Liquifluor scintillation fluid (New England Nuclear).

<table>
<thead>
<tr>
<th>NPh derivatives</th>
<th>Radioactivity (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPh-Glycine</td>
<td>1076</td>
</tr>
<tr>
<td>NPh-Asp,Glu,Ser,Trp</td>
<td>34</td>
</tr>
<tr>
<td>NPh-Leu,His,Phen,Val,Met,Pro</td>
<td>263</td>
</tr>
<tr>
<td>NPh-Lys,Arg,NPh-Tyr</td>
<td>250</td>
</tr>
<tr>
<td>Dinitrophenol</td>
<td>120</td>
</tr>
</tbody>
</table>

expected, based on the results by Brown et al. (25), that HL-A antigens containing "LA" or "Four" specificities were structurally very different and this does not seem to be the case. However, detailed chemical analyses of various HL-A preparations of a definite single specificity is needed to obtain further insight in this problem.

Fig. 5. Time-dependent hydrolysis of the large component of HL-A2 by carboxypeptidase A. At time intervals aliquots were withdrawn, adjusted to pH 2.2 and applied to the amino acid analyzer. ●—●, serine; ○—○, leucine; ■—■, threonine.
Acknowledgment—The excellent technical assistance of Mrs. Karen Higgins is gratefully acknowledged.

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Comparative chemical analyses of the alloantigenic fragments of HL-A antigens.
O Henriksen, E Appella, D F Smith, N Tanigaki and D Pressman


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