Purification and Characterization of Urinary Choriogonadotropin from Patients with Hydatidiform Mole*

Yuen-Min Choy, Kit-Man Lau, and Cheuk-Yu Lee

From the Department of Biochemistry, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong

Human choriogonadotropin was isolated from urine of patients with hydatidiform mole by acid and salt precipitation, immunofinity, and DEAE-Sephadex chromatography. Polyacrylamide gel electrophoresis, immunodiffusion, immunoelctrophoresis, and NH2-terminal amino acid analysis showed that the product obtained is essentially homogeneous. This choriogonadotropin was found to resemble the choriogonadotropin from urine of normal pregnant women in amino acid composition but to differ from it in having a lower content of N-acetylglucosamine and mannose.

Hydatidiform mole is a disorder of the human placenta characterized by hyperplasia of the trophoblasts, hydropic degeneration of the villous stroma, and disappearance of villous blood vessels, resulting in early death of the embryo. The disease is relatively prevalent in the Far East (1) and is potentially malignant. In the choriocarcinoma cases reported, 40 to 45% have been found to have mole history (2, 3).

The most striking biochemical change associated with the development of hydatidiform mole is the excess secretion of choriogonadotropin by the syncytiotrophoblasts. Choriogonadotropins from different origins were found to have different biological and physiochemical properties (4). Choriogonadotropin from urine of normal pregnant women (hCG)1 has been purified and characterized by a number of workers (5-14). Canfield et al. (12) had isolated choriogonadotropin from urine of patients with choriocarcinoma and reported its amino acid composition; they also indicated that this choriogonadotropin had the same amino acid composition as choriogonadotropin in normal pregnancy. Lewis et al. (15) demonstrated that no antigenic difference was apparent between the two choriogonadotropins. Existence of differences in the physicochemical properties of these choriogonadotropins was first suggested by Reisfeld and Hertz (16). The difference in chemical properties might be attributed to the variations in the carbohydrate moieties of these urinary choriogonadotropins (12).

However, with the exception of choriogonadotropin in normal pregnancy, little is known about the detailed carbohydrate composition of choriogonadotropins from other sources. Therefore, it is our objective to obtain choriogonadotropin from urine of patients with hydatidiform mole (hCG-hydadiform mole) in order to compare the physicochemical properties, particularly the carbohydrate moieties, of this material with other choriogonadotropins so as to understand more about the etiology of the development of hydatidiform mole and choriocarcinoma.

Attempts have been made by a few workers to purify choriogonadotropin from trophoblastic tissues of hydatidiform mole by the classical chromatographic steps, yet very low yields were obtained (17, 18). At present, we have employed a simpler method of purifying choriogonadotropin from the urine of patients with hydatidiform mole. The most significant step was the immunofinity chromatography. This report describes the purification of this choriogonadotropin and its characterization.

EXPERIMENTAL PROCEDURES

Purification of hCG-Hydadiform Mole

All steps of purification were carried out at 4°C unless otherwise specified. The immunological activity of each fraction was measured using the Pregnosticon Planotest Kit (Organon Inc., Holland). Biological potency of the final product was determined by the mouse uterine weight assay (19, 20). Protein contents were determined according to Lowry et al. (21) using bovine serum albumin as standard.

Preparation of Crude hCG Hydatidiform Mole

Urine was collected in a 24-h period from patients with moles before the removal of the hydatidiform moles. An aliquot of 500 ml of the urine having a total immunological activity of approximately 100,000 IU was centrifuged at 5,000 × g for 10 min. The supernatant was brought up to 30% saturation with ammonium sulfate and the suspension was stirred overnight. The precipitate formed was discarded and ammonium sulfate was added to the supernatant to give 70% saturation. After stirring overnight, the precipitate containing the bulk of the gonadotropic activity was collected by centrifugation and dissolved in about 100 ml of distilled water. This protein solution was dialyzed against distilled water for 2 days. Any precipitate formed during dialysis was removed and the supernatant was lyophilized to give the crude product.

Preparation of Antiserum—Albino rabbits were immunized against hCG. For the first 4 weeks, each rabbit received weekly subcutaneous injections of 1000 IU of hCG in 1 ml of a 1:1 (v/v) mixture of saline and Freund's complete adjuvant. From the 5th week onward, booster injections were given every 3rd week. The animals were bled 7 days after each booster shot. The sera were tested for anti-hCG titer using the hCG-sensitized latex preparation supplied with the Pregnosticon Planotest Kit. The sera became useful when a

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: hCG, choriogonadotropin from urine of normal pregnant women; hCG-hydadiform mole, choriogonadotropin from urine of patients with hydatidiform mole; hCG-choriocarcinoma, choriogonadotropin from urine of patients with choriocarcinoma; IU, international unit of human choriogonadotropin activity; NaCl/Pi, phosphate-buffered saline (0.01 M phosphate buffer, pH 7.3, 0.9% NaCl); dansyl, 5-dimethylaminonaphthalene-1-sulfonil.
10-fold dilution was still sufficient to give positive latex agglutination. **Preparation of Immunoadsorbent**—Before coupling to the Sepharose, the antiserum was first absorbed with urinary proteins obtained from hCG-free urine. This was necessary in order to remove IgM molecules directed against those proteins present in commercial hCG which might cross-react with proteins in the crude hCG-hydatidiform mole preparation. The urinary proteins were obtained by collecting urine samples from nonpregnant women, pooled, and concentrated in an ultrafiltration apparatus. The concentrated urine was then dialyzed exhaustively against several changes of distilled water and lyophilized. Just before use, the proteins were dissolved in a minimum amount of 0.1 M borate/saline buffer (0.1 M borate buffer, 0.9% NaCl), pH 8.4.

An aliquot was mixed with antisera (50 ml) to give a concentration of 0.4 mg of protein/ml of serum. The mixture was left standing at room temperature for 6 h and then in a refrigerator for 1 week with occasional shaking. The precipitate formed was removed by centrifugation and the supernatant was allowed to stand for another week or two. Any precipitate formed during this interval was again removed. The IgG in the supernatant was then concentrated by precipitation with 37% saturation ammonium sulfate according to Gospodarowicz (22). After the precipitate was dissolved in phosphate-buffered saline (NaCl/Pi, 0.01 M phosphate buffer, pH 7.5, 0.9% NaCl) and dialyzed against the same buffer overnight, its volume was adjusted to that of the original antisera (50 ml) with NaCl/Pi. The IgG was then coupled to the cyanogen bromide-activated Sepharose 6B as described by Cuatrecassas (23) at a ratio of 1 g of wet Sepharose/ml of serum.

**Affinity Chromatography of Crude hCG-Hydatidiform Mole**

This was performed according to Gospodarowicz (22) and Saumy et al. (24) with slight modification. Anti-hCG IgG Sepharose was packed into columns of 0.8 x 12 cm and thoroughly washed with NaCl/Pi, followed successively by 2 M MgCl₂ (pH 6), 2 M sodium trichloroacetate, pH 7, and again NaCl/Pi, until no protein could be detected in the runoff. Typically, 2000 IU (immunological activity) of crude hCG-hydatidiform mole dissolved in 5 ml of NaCl/Pi were charged onto each affinity column. Stepwise elution was performed by successive application of 50 ml of NaCl/Pi, 20 ml of 2 M MgCl₂, 20 ml of 1 M sodium trichloroacetate, and 20 ml of 2 M sodium trichloroacetate. The flow rate was maintained at 20 ml/h. The fractions obtained from each elution solution were promptly pooled.

The flow rate was maintained at 20 ml/h. The fractions obtained from each elution solution were promptly pooled. The two fractions eluted by 1 M sodium trichloroacetate and 2 M sodium trichloroacetate were dialyzed respectively with distilled water. Each of these fractions was dialyzed against several changes of 0.1 M ammonium bicarbonate for 2 days. For each fraction, the protein content was determined and the immunological activity was measured. The fractions were lyophilized and stored at 4°C.

The affinity column, after washing and equilibration for 3 days with NaCl/Pi, was ready for another chromatographic run.

**Chromatography on DEAE-Sephadex**—The immunologically active fraction eluted by 2 M sodium trichloroacetate from the affinity column was further purified by DEAE-Sepharose A-50 chromatography. In a typical experiment, about 40,000 IU (immunological activity) of the active fraction were dissolved in 5 ml of 0.04 M Tris/phosphate buffer, pH 8.6. The resulting solution was then applied to a DEAE-Sepharose column (1 X 30 cm) previously equilibrated with 0.04 M Tris/phosphate buffer. The column was initially eluted with 20 ml of the same buffer and then with a linear gradient of NaCl, from 0 to 0.2 M, in 400 ml of Tris/phosphate buffer, pH 8.6. The flow rate was 20 ml/h. Appropriate fractions were pooled, dialyzed against 0.01 M ammonium bicarbonate, and lyophilized to yield the final product.

**Characterization Studies**

**Analytical Polyacrylamide Gel Electrophoresis**—It was carried out according to Davis (25), using Tris/glycine (pH 8.3) as reservoir buffer. The running gel of 7% (w/v) of monomer concentration had a pH of 8.9. The gels were stained by 0.5% Coomassie brilliant blue R250 in a solution of methanol/glacial acetic acid/water (3:1:10).

**NH₂-terminal Analysis**—The protein was dansylated following the procedure of Gray (26) and then hydrolyzed to its constituent amino acids. The dansylated derivatives were analyzed by thin layer chromatography on polyamide sheet (27).

**Immunological Studies**—Immunoelectrophoresis was performed as described by Hamashige and Arquilla (28) with some modifications. The microslides were coated with 0.8% agarose in sodium barbital buffer, pH 8.6, ionic strength 0.025. Ouchterlony double immunofixation in 1% agar was carried out in a Petri dish.

**Molecular Weight Determination**—The sodium dodecyl sulfate electrophoresis was as described by Dunker and Ruechert (30) in 10% acrylamide gels. Cytochrome c, chymotrypsinogen, bovine serum albumin, myoglobin, and ovalbumin were used as calibration markers. Before electrophoresis, all samples and markers were incubated at 37°C for 2 h in 0.1 M phosphate buffer, pH 7, containing 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, and 4% urea.

**Amino Acid Composition**—The procedures of Spackman et al. (31) were adopted. A sample of about 1 mg was hydrolyzed with 1 ml of constant boiling HCl in an evacuated sealed tube at 110°C for 22 h. The hydrolysate was dried over NaOH pellets in a vacuum desiccator. The resulting residue dissolved in sodium/citrate buffer (0.2 M Na⁺, pH 2.2) was analyzed by a Beckman model 120C amino acid analyzer.

**Carbohydrate Composition**—For determination of neutral sugars, the sample was hydrolyzed with 1 N HCl at 100°C for 2 h (32). The released neutral sugars were then analyzed as their alditol acetates (33) by gas-liquid chromatography, using a Hewlett-Packard gas chromatograph (model 402B, equipped with flame ionization detectors), glass column filled with 3% ECNSS-M on Gas-chrom Q (100/120 mesh), and a column temperature of 200°C. Hexosamines were released by hydrolysis of the sample with 2 N HCl at 110°C for 5 h (34). Their derived alditol acetates were again analyzed by gas-liquid chromatography using a column packed with 3% poly(A) 103 on Gas-chrom Q (100/120 mesh) at 230°C.

Sialic acid was assayed by the thiobarbituric method of Warren after the sample was hydrolyzed with 0.05 N sulfuric acid at 80°C for 1 h (34).

**RESULTS**

The results of the purification of hCG-hydatidiform mole are summarized in Table I. When 2000 IU (immunological activity) of crude hCG-hydatidiform mole were charged onto an anti-hCG IgG Sepharose column (0.8 x 1.2 cm) and washed with NaCl/Pi, the bulk of the nonspecific proteins passed through the column unadsorbed. Small amounts of proteins with no immunological activity were desorbed from the column by successive elution with 2 M MgCl₂ and 1 M sodium trichloroacetate. The last elution with 2 M sodium trichloroacetate resulted in a 70% recovery of the input choriogonadotropin activity. Fig. 1 shows a typical elution pattern from an affinity column. When the above immunologically active fraction eluted by 2 M sodium trichloroacetate was subjected to DEAE-Sepharose chromatography, the elution pattern shown in Fig. 2 was obtained. It is seen that the gonadotropic activity is segregated into two chromatographically distinct peaks, designated D-1 and D-2. Peaks D-3 and D-4 contain but little immunological activity. While the specific activity is highest in the D-1 fraction, most of the recoverable hCG-hydatidiform mole activity is segregated into two chromatographically distinct fractions D-1 and D-2. It is seen that the gonadotropic activity is segregated into two chromatographically distinct peaks, designated D-1 and D-2. The specific activity is highest in the D-1 fraction, most of the recoverable hCG-hydatidiform mole activity is segregated into two chromatographically distinct fractions D-1 and D-2.

**TABLE I**

<table>
<thead>
<tr>
<th>Fraction procedure</th>
<th>Fraction</th>
<th>Total activity (IU)</th>
<th>Specific activity (IU/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salt precipitation</td>
<td>Urine (500 ml)</td>
<td>100,000</td>
<td>92</td>
<td>100.0</td>
</tr>
<tr>
<td>Crude hCG-hydatidiform mole</td>
<td>73,000</td>
<td>115</td>
<td>73.0</td>
<td></td>
</tr>
<tr>
<td>Affinity chromatography</td>
<td>DEAE-Sepharose A-50</td>
<td>50,800</td>
<td>1,260</td>
<td>50.8</td>
</tr>
<tr>
<td>DEAE-Sepharose chromatography</td>
<td>D-1</td>
<td>4,000</td>
<td>5,250</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>D-2</td>
<td>23,720</td>
<td>4,090</td>
<td>23.7</td>
</tr>
<tr>
<td></td>
<td>D-3</td>
<td>4,500</td>
<td>540</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>D-4</td>
<td>1,330</td>
<td>130</td>
<td>1.3</td>
</tr>
</tbody>
</table>
Choriogonadotropin from Patients with Hydatidiform Mole

FIG. 1. Elution pattern of crude hCG-hydatidiform mole (extracted from urine of patients with hydatidiform mole) from an anti-hCG IgG Sepharose column (0.8 x 12 cm). Stepwise elutions were performed with NaCl/PI, 2 M MgCl₂, 1 M sodium trichloroacetate, and 2 M sodium trichloroacetate. Flow rate, 20 ml/h.

FIG. 2. DEAE-Sephadex chromatography of hCG-hydatidiform mole. About 40,000 IU (immunological activity) of hCG-hydatidiform mole were charged onto the column (1 x 30 cm) equilibrated with 0.02 M Tris/phosphate buffer, pH 8.6. Elution was done with a linear gradient of NaCl, from 0 to 0.2 M in 0.04 M Tris/phosphate buffer, pH 8.6. ---, fractions pooled; ----, absorbance at 280 nm; -----, molarity of NaCl; , immunological activity.

hydatidiform mole was found in the D-2 fraction (5.5 mg from 500 ml of urine) (Table I). The small amount of material in the D-1 fraction did not permit characterization studies. These were, therefore, confined to the material in the D-2 fraction, the biological activity of which was found to be 24,400 IU/mg.

Fig. 3 shows the disc electrophoretic patterns of the various fractions obtained in the purification scheme. It is seen that most of the proteins present in the crude product were removed by affinity chromatography and existed in the NaCl/PI fraction. However, the fraction eluted by 2 M sodium trichloroacetate was shown to still contain extraneous proteins. It was only after the chromatographic step through DEAESephadex that two relatively pure fractions of hCG-hydatidiform mole (D-1 and D-2) could be obtained.

NH₂-terminal amino acid analysis showed that serine and alanine were the major NH₂-terminal acids in hCG-hydatidiform mole (Fig. 4). Besides, a small amount of valine with a trace of aspartic acid was observed. These findings are the same as that reported for hCG (35).

The purity of the final product was further examined by immunological studies. The hCG-hydatidiform mole developed only one precipitin line in both double immunodiffusion (Fig. 5) and immunoelectrophoresis (Fig. 6). It is also interesting to note in Fig. 5 that the precipitin lines of hCG-hydatidiform mole and hCG fused completely with each other, without any spur, suggesting that the choriogonadotropins from the two sources are immunologically identical.

The molecular weight of the purified hCG-hydatidiform mole, determined by sodium dodecyl sulfate electrophoresis of the dissociated subunits, is 51,000 with 20,000 for the α subunit and 31,000 for the β subunit.

Table II compares the amino acid composition of hCG-hydatidiform mole, hCG (5), and hCG-choriocarcinoma (12), while Table III compares the carbohydrate composition of...
hCG with a smaller amount of mannose and hexosamines. The carbohydrate content of hCG-hydatidiform mole differs from that of different preparations of gonadotropins, while the carbohydrate composition between the amino acid compositions of choriogonadotropins from three different sources are thought to be chemically different, many of the differences noted might be accounted for by variations in the carbohydrate moieties. However, when compared with normal pregnancy choriogonadotropin (Table III), the carbohydrate constituents of our hCG-hydatidiform mole are similar but with smaller amounts in mannose and hexosamines. These findings are consistent with the observation of Ashitaka et al. (17) that choriogonadotropin originated solely responsible for the immunological properties of choriogonadotropin.

The main difficulty encountered in the purification process has been the selection of a good desorbing eluant for affinity chromatography. This is probably due to the stable binding of hCG-hydatidiform mole to its specific antibody. Even 8 M urea was insufficient to elute the hCG-hydatidiform mole molecules from the affinity column. 4 M MgCl₂, reported to dissociate most of the antibody-antigen complex (36), only eluted 10% of the applied activity. When 6 M guanidinium chloride was used, both the yield and extent of purification were found to be low. The use of trichloroacetic acid in neutral solution as desorbing eluant (24) appeared to be suitable for the present purpose. The results in Table I show that the affinity step raised the specific activity of the crude hCG-hydatidiform mole about 13-fold. Further purification was achieved by DEAE-Sephadex chromatography. The final product showed one band in disc electrophoresis (Fig. 3). The diffusion of this band reflects the microheterogeneity of hCG-hydatidiform mole arising from differences in sialic acid content of the molecules (12).

The immunodiffusion pattern (Fig. 5) shows that hCG-hydatidiform mole and hCG are immunologically identical. Bahl and Marrz (37) have suggested that the protein moiety is solely responsible for the immunological properties of choriogonadotropin. Our findings that amino acid compositions of hCG-hydatidiform mole and hCG are remarkably similar would seem to support this contention. As can be seen from Table II, the amino acid compositions of choriogonadotropins from three different sources are basically alike. Although choriogonadotropins from different sources were thought to be chemically different, many of the differences noted might be accounted for by variations in the carbohydrate moieties. Comparison of carbohydrate composition between choriogonadotropins from urine of patients with hydatidiform mole and choriocarcinoma is not feasible due to the absence of detailed carbohydrate analysis of the latter. However, when compared with normal pregnancy choriogonadotropin (Table III), the carbohydrate constituents of our hCG-hydatidiform mole are similar but with smaller amounts in mannose and hexosamines. These findings are consistent with the observation of Ashitaka et al. (17) that choriogonadotropin originated

### Table III

<p>| Carbohydrate composition of hCG-hydatidiform mole and hCG |
|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>hCG-hydatidiform mole</th>
<th>hCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucose</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Galactose</td>
<td>6.7</td>
<td>5.3</td>
</tr>
<tr>
<td>Mannose</td>
<td>2.1</td>
<td>5.3</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>0.5</td>
<td>2.2</td>
</tr>
<tr>
<td>N-Acetylglucoamin</td>
<td>5.4</td>
<td>8.9</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>6.1</td>
<td>9.0</td>
</tr>
<tr>
<td>Total carbohydrate</td>
<td>21.2</td>
<td>31.3</td>
</tr>
</tbody>
</table>

* Data taken from Bahl (5).

## DISCUSSION

In the present study, human choriogonadotropin with high immunological and biological activity was isolated from the urine of patients with hydatidiform mole by immunoadfinity chromatography in conjunction with conventional purification methods. The final product assayed immunologically at 4,090 IU/mg, representing a 45-fold purification of the original starting material, and the biological activity was found to be 24,400 IU/mg. Disc electrophoresis, NH₄OH-terminal amino acid determination, immunoelectrofhoresis, and immunodiffusion demonstrated that the preparation of hCG-hydatidiform mole was essentially homogeneous on macroscopic scale.

The main difficulty encountered in the purification process has been the selection of a good desorbing eluant for affinity chromatography. This is probably due to the stable binding of hCG-hydatidiform mole to its specific antibody. Even 8 M urea was insufficient to elute the hCG-hydatidiform mole molecules from the affinity column. 4 M MgCl₂, reported to dissociate most of the antibody-antigen complex (36), only eluted 10% of the applied activity. When 6 M guanidinium chloride was used, both the yield and extent of purification were found to be low. The use of trichloroacetic acid in neutral solution as desorbing eluant (24) appeared to be suitable for the present purpose. The results in Table I show that the affinity step raised the specific activity of the crude hCG-hydatidiform mole about 13-fold. Further purification was achieved by DEAE-Sephadex chromatography. The final product showed one band in disc electrophoresis (Fig. 3). The diffusion of this band reflects the microheterogeneity of hCG-hydatidiform mole arising from differences in sialic acid content of the molecules (12).

The immunodiffusion pattern (Fig. 5) shows that hCG-hydatidiform mole and hCG are immunologically identical. Bahl and Marrz (37) have suggested that the protein moiety is solely responsible for the immunological properties of choriogonadotropin. Our findings that amino acid compositions of hCG-hydatidiform mole and hCG are remarkably similar would seem to support this contention. As can be seen from Table II, the amino acid compositions of choriogonadotropins from three different sources are basically alike. Although choriogonadotropins from different sources were thought to be chemically different, many of the differences noted might be accounted for by variations in the carbohydrate moieties. Comparison of carbohydrate composition between choriogonadotropins from urine of patients with hydatidiform mole and choriocarcinoma is not feasible due to the absence of detailed carbohydrate analysis of the latter. However, when compared with normal pregnancy choriogonadotropin (Table III), the carbohydrate constituents of our hCG-hydatidiform mole are similar but with smaller amounts in mannose and hexosamines. These findings are consistent with the observation of Ashitaka et al. (17) that choriogonadotropin originated

from hydatidiform mole may have more galactose than mannose as well as low amino sugar content. As it has been suggested that the chemical differences in choriogonadotropins between trophoblastic diseases and normal pregnancy are mainly due to their variations in carbohydrate (12, 17), our present findings appear to further support this postulation.

REFERENCES
Purification and characterization of urinary choriogonadotropin from patients with hydatidiform mole.
Y M Choy, K M Lau and C Y Lee


Access the most updated version of this article at http://www.jbc.org/content/254/4/1159

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/254/4/1159.full.html#ref-list-1