Immunobiological Identification of Human Chorionic Gonadotropin.*† (26853)

A. R. MIDGLEY, JR.,‡ G. B. PIERCE, JR.,‡ AND W. O. WEIGLE§
Dept. of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pa.

Human chorionic gonadotropin (HCG) has been difficult to obtain in an immunochemically pure form. Early workers using crude preparations were unable to determine whether this mucoprotein was capable of eliciting the formation of specific precipitating and neutralizing antibodies(1). That antigenic impurities are present has been clearly shown by Got(2) who prepared rabbit antiserum to HCG assaying 1500 I.U./mg and demonstrated by immunoelectrophoresis the presence of 8 antigen-antibody precipitin bands. Extensive purification resulted in a preparation which assayed 12,000 I.U./mg and gave a single band following immunoelectrophoresis with the same antiserum. McKean(3) developed an antiserum to a purified preparation of HCG assaying 8,000-9,000 I.U./mg and described its use in detecting HCG in urine of pregnant women.

In spite of apparent specificity of antibodies to HCG, and especially of antibodies to anterior pituitary hormones(4), it has been suggested that these "hormone specific" antibodies may be directed against carrier proteins differing in urine and serum(5). This report describes the preparation and characterization of an antiserum against HCG, and demonstrates the ability of this antiserum to identify HCG of both benign and malignant trophoblastic origin and to neutralize the biological activity of HCG in serum or urine.

Materials and methods. Immunization procedure. Adult rabbits were given subcutaneous injections of 10,000 to 20,000 I.U. of commercial human chorionic gonadotropin‖ (HCG-U) emulsified in incomplete Freund's adjuvants. This procedure was repeated 5 times at intervals of 1 to 3 weeks. Thirteen days after the last injection, the animals were bled and the antisera pooled.

Antigens. Pregnant human serum (PHS) and urine (PHU) were collected between the 50th and 90th days of gestation. Normal human serum (NHS) was obtained from Microbiological Associates and normal human urine (NHU) from young adult males. Sera were also obtained from golden hamsters heterotransplanted with choriocarcinomas (Pitt 89 and Pitt 100) and an embryonal carcinoma (Deac 3), all of human testicular origin. Pitt 89 and Pitt 100 have consistently secreted chorionic gonadotropin, whereas no evidence of gonadotropic activity has been detectable in the serum from hamsters bearing Deac 3(6).

The sera were concentrated to enhance gonadotropic activity according to modifications of the procedures of Delfs(7) and Landgrebe(8). Fifty milliliters of each serum were extracted 5 times with ether, precipitated with 6 volumes of 95% ethanol, extracted with 0.1 N acetate buffer (pH 4.8), and precipitated differentially with acetone, the fraction precipitating between 40-70% being saved. This precipitated fraction was dissolved in a small volume of 0.1 N acetate buffer. An extract of urine from young adult males was prepared by the procedure of Katzman(9).

To test possible cross reactions of the antiserum with gonadotropins of pituitary origin, an extract of human anterior pituitary tissue stored in acetone at 4°C was prepared by a modification of the method of Woods(10). The tissue was extracted with acetone, dried and then extracted with 40% ethanol.

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† This is publication No. 291 from Dept. of Pathology, Univ. of Pittsburgh School of Med., Pittsburgh.

‡ Present address: Dept. of Pathology, Univ. of Michigan School of Medicine, Ann Arbor.

§ Present address: Division of Ex. Pathol., Scripps Clinic and Research Foundation, La Jolla, Calif.

‖ The commercial human chorionic gonadotropin (Follutein) employed was supplied in generous quantities by Dr. Aleck Borman, Suibb Inst. for Medical Research, New Brunswick, N. J.

The ethanol extract was dialyzed against 0.1 N acetate buffer (pH 4.8), and the gonadotropin-rich fraction precipitated with 70% acetone and solubilized by dialysis against 0.01 M phosphate buffered 0.15 M NaCl (pH 7.0). The extracts were designated as follows: male urine extract (MUE), anterior pituitary extract (APE), concentrated pregnant human serum (C-PHS), concentrated normal human serum (C-NHS), and concentrated sera from hamsters bearing human tumors, Pitt 89 (C-89), Pitt 100 (C-100), and Deac 3 (C-D3).

All sera and extracts were stored at either 4°C or −20°C after addition of merthiolate (1:10,000).

Double diffusion and immunoelectrophoresis in agar. Double diffusion in agar was accomplished by a modification of the Ouchterlony technic(11), using 0.7% lonagar No. 2¶ in veronal buffered 0.15 M NaCl (pH 8.2) containing 1.0 M glycine and 1:10,000 merthiolate.

Immunoelectrophoresis was performed according to a modification of the method of Grabar and Williams(12), employing 1.25% lonagar No. 2 in veronal buffer at pH 8.2 with an ionic strength of 0.04. The reactants were mixed with an equal volume of 2.5% lonagar No. 2 and placed in wells. Following electrophoresis (1 hour at 18 volts/cm) troughs were cut and filled with undiluted antisera. For development of precipitin bands, the immunoelectrophoretic plates and the double diffusion plates were incubated at 4°C. They were photographed and finally dehydrated and stained with azocarmine G or ponceau S for a permanent record.

To remove antibodies to impurities detected by the immunodiffusion technics, antisera were absorbed by adding 0.05 ml of NHS or MUE to each ml of antiserum. This mixture was incubated at 37°C for 30 minutes, followed by incubation at 4°C overnight. Resulting precipitates were removed by centrifugation. This procedure was repeated until no precipitate could be detected and no precipitin bands could be seen against the NHS or MUE on double diffusion in agar.

¶ Consolidated Laboratories, Inc., Chicago Heights, Ill.

Bioassay and neutralization studies. The biological activity of HCG in various preparations was determined in weanling male rats by measuring changes in mean prostatic weight. The preparations were injected subcutaneously daily for 4 days and the wet weight of the ventral lobes of the prostates determined 24 hours after the last injection. Results were expressed as milligrams of prostate per 100 g of body weight. The commercial HCG used to prepare the antiserum served as the standard. Using this method PHS assayed 64 I.U./ml; C-PHS, 500 I.U./ml; and PHU, 8 I.U./ml. APE contained gonadotropic activity equivalent to 730 I.U. of HCG/ml.

Neutralization studies were performed by mixing 1 ml dilutions of antiserum in normal rabbit serum with 1 ml of either PHU or PHS, the latter preparations diluted in saline to contain approximately 4 I.U. of HCG/ml. The preparations were incubated for one-half hour at 37°C followed by incubation overnight at 4°C. Each rat received 4 I.U. of HCG plus varying amounts of antiserum in a total volume of 2.0 ml over a period of 4 days (0.5 ml × 4 days).

Results. Agar-gel double diffusion studies of pooled rabbit anti HCG demonstrated that the commercial HCG employed for immunization contained several contaminating antigens. From 2 to 5 precipitin bands were formed upon interaction of anti HCG with: MUE, C-NHS, NHS, C-PHS, PHS, C-89, C-100 and HCG-U. No precipitin bands could be detected with APE, with concentrated sera from hamsters heterotransplanted with a non-gonadotropin secreting human embryonal carcinoma (C-D3), or with sera from mice in early or late gestation. A common band of identity was detected against all preparations containing HCG (PHS, C-PHS, C-100, C-89 and HCG-U).

This common band of identity was shown with gel diffusion studies employing antiserum absorbed with either NHS or MUE to be the result of the reaction of HCG with its specific antibody. Antiserum absorbed with NHS produced a single band of identity with all sera or serum concentrates containing HCG, while no bands could be detected against NHS, C-NHS, APE or C-D3 (Fig.

TABLE I. Neutralization of Biological Activity of HCG in PHU and PHS As Determined by the Effect upon Prostate of Rat.*

Gonadotropin source	Antiserum	Control NRS†	Dilutions of antiserum in NRS 1:5	1:25	1:125	1:625	1:3125
PHU‡	anti HCG-U	107 ± 15	—	56 ± 4.8	60 ± 7.8	67 ± 9.4	118 ± 16
PHS§	"	113 ± 20	68 ± 9.7	66 ± 9.5	64 ± 5.7	66 ± 6.7	97 ± 14
PHU	anti HCG-U abs. with NHS	88 ± 11	—	50 ± 4.7	47 ± 6.8	59 ±11	89 ± 14
PHU	anti HSA	104 ± 16	98 ±17	116 ±15	110 ±12	—	—
PHS	"	104 ± 16	85 ±12	99 ±20	109 ±11	—	—
PHU	anti HGG	102 ± 13	107 ±15	96 ±16	112 ±18	—	—
PHS	"	102 ± 13	85 ±16	100 ±15	97 ±14	—	—

* Figures in columns are mean weights of 8 prostates expressed as mg/100 g of body wt ± 1 stand. dev.

† NRS = Normal rabbit serum.

‡ PHU = Pregnant human urine diluted 1:2 with saline to give approximately 4 I.U. HCG/ml.

§ PHS = Pregnant human serum diluted 1:16 with saline to give approximately 4 I.U. HCG /ml.

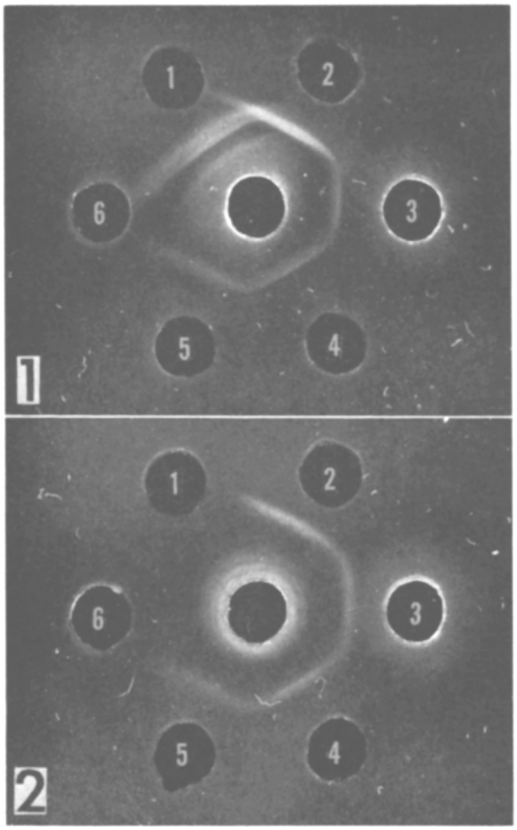

FIG. 1. Diffusion in agar of anti HCG absorbed with NHS (center well). Note common band of identity to all preparations containing HCG and non-related bands to MUE and HCG-U. Well 1 = MUE; Well 2 = HCG-U; Well 3 = PHS; Well 4 = C-100; Well 5 = C-89; Well 6 = NHS.

FIG. 2. Diffusion in agar of anti HCG absorbed with MUE (center well). A single band of identity appeared before all preparations containing HCG. Peripheral wells contain same reactants as in Fig. 1.

1). This common band of identity was also formed with HCG-U. Two or 3 additional bands, however, formed with MUE and HCG-U, indicating that anti HCG-U absorbed with NHS contained antibodies to contaminating antigens of urinary origin. Antiserum absorbed with MUE gave a single band of identity with all preparations containing HCG and no bands with MUE, NHS, C-NHS, C-D3 or APE (Fig. 2). The specific precipitin band appeared with concentrations of HCG as low as 16 I.U./ml. This level of hormone may be expected in serum and urine a week or so after the first missed menstrual period in early pregnancy.

Immunoelectrophoresis of HCG-U with non-absorbed anti HCG-U revealed 8 to 10 antigen-antibody precipitin bands. Similar results were obtained when another commercial preparation of HCG-U was employed (Pregnyl, Organon). After absorption of the antiserum with NHS, 3 bands were still detectable against HCG-U and 2 bands against MUE. This again indicated that HCG-U contained contaminating antigens common only to extracts of urine and not to serum.

When immunoelectrophoresis was performed on C-PHS and C-NHS with non-absorbed anti HCG-U, 2 closely associated bands migrating with alpha globulins were

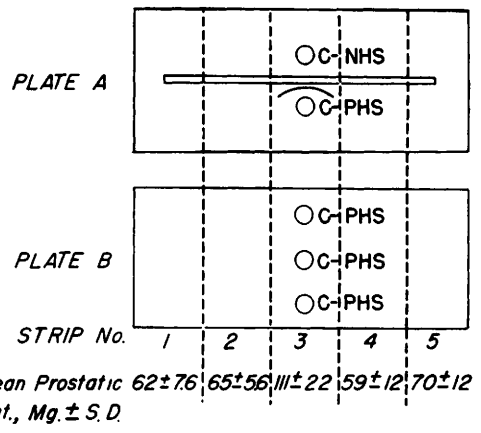

FIG. 3. Simultaneous (A) immunoelectrophoresis of C-PHS and C-NHS with anti HCG absorbed with NHS and (B) agar electrophoresis with subsequent bioassay of C-PHS to determine if the precipitin band to C-PHS migrated with the mobility of biologically active HCG. Saline extracts of each agar strip (Plate B) were bioassayed in 8 weanling rats and chorionic gonadotropic activity expressed as mean wt of ventral prostatic tissue in mg ± one stand. dev. Position of the single precipitin band to C-PHS (immunoelectrophoretic plate) corresponds to the agar strip containing maximal chorionic gonadotropic activity (electrophoresis plate).

formed against each preparation. In addition, a single band migrating with the beta globulins could be discerned against C-PHS. This electrophoretic mobility has been reported previously for HCG in the serum of pregnant women(13). When immunoelectrophoresis was repeated with anti HCG-U absorbed with NHS, only this latter band could be detected (Fig. 3, Plate A).

To determine if this single band migrated in the same manner as biologically active HCG, an agar plate containing C-PHS in 3 wells was subjected to simple electrophoresis in agar. Following the electrophoresis strips of the agar were bioassayed for gonadotropic activity (Fig. 3, Plate B). Simultaneous immunoelectrophoresis using anti HCG absorbed with NHS was performed on a second plate containing C-NHS and C-PHS (Fig. 3, Plate A). Maximal chorionic gonadotropic activity was found in strip 3 of the simple electrophoretic plate which corresponded to the position of the single band in the immunoelectrophoretic plate (Fig. 3). This provided further evidence that the single band was the result of the interaction of biologically active HCG with its specific antibody. A t-test comparison of the difference in mean chorionic gonadotropic activity (prostatic weight) between strips 2 and 3 and between strips 3 and 4 each gave $p<0.001$.

The results of studies to determine the ability of anti HCG-U to neutralize the biological activity of HCG are listed in Table I. The biological activity of 4 I.U. of HCG in either pregnancy serum or urine was fully neutralized by 1 ml of a 1:625 dilution of absorbed or non-absorbed antiserum. To confirm this specificity further pooled rabbit anti-human serum albumin (anti HSA) containing 0.85 mg antibody nitrogen/ml and pooled rabbit anti-human serum gamma globulin (anti HGG) containing 0.93 mg antibody nitrogen/ml were tested for their ability to neutralize the biological activity of HCG. Concentrations as great as 1:5 gave no evidence of neutralizing ability against preparations of urine or serum of pregnancy.

Discussion. Immunization of rabbits with commercial HCG has resulted in production of an antiserum capable of identifying HCG and neutralizing its biological activity in serum of urine. These studies have demonstrated that commercial HCG-U contains contaminating antigens common to normal human urine and a few common to normal human serum. The presence of these nonrelated antigens presents a theoretic objection to employment of non-absorbed antiserum to commercial HCG-U in an immunologic test for pregnancy(14). Our studies clearly indicate, however, that an immunologic test for pregnancy is feasible, employing either an antiserum against a highly purified preparation of HCG-U as done by McKean(3) or, as in this study, a properly absorbed antiserum against commercial HCG-U.

The similarity in immunobiologic behavior between HCG from serum and from urine strongly suggests that the specific antibodies are reacting directly with the biologically active hormone and not with carrier proteins differing in serum and in urine as hypothesized by others(5). These observations also demonstrate an immunobiologic similarity between the gonadotropic secretion of benign

and malignant trophoblast of placental and testicular origin.

Summary. This report describes the immunobiological characterization of an antiserum to commercial human chorionic gonadotropin. This antiserum identified human chorionic gonadotropin of benign or malignant trophoblastic origin in serum or urine and neutralized its biological activity.

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Effect of 2,4-dinitrophenol on Free Fatty Acid Uptake by Skeletal Muscle.* (26854)

JOHN J. SPITZER, WILLIAM T. MCELROY, JR., AND BELA ISSEKUTZ, JR.
Dept. of Physiology, Hahnemann Medical College and Hospital and Division of Research, Lankenau Hospital, Philadelphia, Pa.

It was reported(1) that in the anesthetized dog during direct electrical stimulation the muscles of the hind leg showed a consistent uptake of free fatty acids (FFA) on the stimulated side. A metabolic change very similar to that induced by muscular contraction can be produced by dinitrophenol (DNP) poisoning. Although the detailed mechanism of action of this "uncoupler" on the electron transport system is not yet entirely understood and the question whether it inhibits the formation of energy rich P bonds or enhances the hydrolytic cleavage of the terminal phosphate of ATP is a matter of discussion(2,3,4), it can be safely stated that DNP induces an imbalance between formation and breakdown of the high energy P compounds of the cell. The result of this imbalance is a dramatic rise of the metabolic rate.

Experiments carried out with a method similar to that used in this study had shown that in the anesthetized dog DNP increased blood flow, oxygen uptake and lactic acid production of the muscle 5-9-fold, glucose uptake was increased about 2-fold, and the muscle glycogen decreased about 40% in 60 minutes. A considerable loss of inorganic phosphate and a concomitant rise of plasma inorganic P level were also recorded(5,6).

The muscle creatine phosphate content dropped 59% while ATP content decreased only 13%(5,6). As far as the metabolism of muscle is concerned, DNP poisoning can be considered as exhausting work lasting for about 1-2 hours, the only difference being that the liberated energy is wasted in the form of heat without performing work.

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