IMMUNOHISTOCHEMICAL LOCALIZATION OF HUMAN CHORIONIC GONADOTROPIN*

By A. R. MIDGLEY, Jr.,‡ M.D., and G. B. PIERCE, Jr.,§ M.D.

(From the Department of Pathology, The University of Michigan, Ann Arbor, Michigan)

Plates 32 and 33

(Received for publication, October 17, 1961)

The relative importance of cytotrophoblast and syncytiotrophoblast in the formation of human chorionic gonadotropin (HCG) has not been determined. Cytotrophoblast usually has been assumed to be the cell of origin of this hormone for two reasons: (*a*) The degree of cytotrophoblastic development during pregnancy closely parallels the urinary and serum levels of HCG (1). (*b*) Placental tissue cultured *in vitro* has been shown to continue synthesizing HCG when, as it was assumed, only a single cell type resembling cytotrophoblast was present (2, 3). Attempts to confirm this theory histochemically, however, have confused rather than clarified the problem, stainable mucoprotein having been reported in cytotrophoblast (4), in syncytiotrophoblast (5), and in both cell types (6).

Precise histochemical localization of HCG requires the use of a reagent with which this hormone will specifically react. By employing the fluorescent antibody technique, we have localized HCG in trophoblastic tissues with an antiserum which had previously been shown to react specifically with biologically active HCG (7).

Method

As reported previously, antiserum to HCG was prepared in adult rabbits by repeated subcutaneous injection of commercial HCG (Follutein) emulsified in incomplete Freund's adjuvants. The antiserum was absorbed with normal human serum and an extract of male urine until a single precipitin band of identity was obtained on immunodiffusion with preparations containing HCG (7). The resulting rabbit anti-HCG serum and normal rabbit serum (used as a control) were each fractionated with one-half saturated ammonium sulfate. The concentrated globulins were conjugated with fluorescein isothiocyanate according to the procedure of Marshall (8) and stored at −20°C following the addition of merthiolate 1:10,000. The conjugates were absorbed once with activated charcoal (25 mg/ml) and once with human liver powder prior to use.

* This work, supported by grants (C-5320 and C-6115) from United States Public Health Service, was initiated at The Department of Pathology, The University of Pittsburgh School of Medicine, Pittsburgh.

‡ Sarah Mellon Scaife Fellow in Pathology.

§ Markle Scholar in Medical Sciences.

Since some of the rare tissues required in these experiments were difficult to obtain fresh for frozen sectioning, we ascertained whether HCG could be localized in formalin-fixed paraffin-embedded tissues. Accordingly in preliminary experiments it was determined whether formalin fixation altered the antigenic or biologic properties of HCG, and whether the localization of this hormone in tissues was altered by paraffin embedding. For this purpose a solution containing 5000 IU of HCG/ml of 0.01 M phosphate buffered saline (PBS, pH 7.0) was dialyzed for 18 hours against 10 per cent formalin in PBS at 25°C and then against PBS at 4°C to remove the formalin. This method of treatment was chosen as it approximated the conditions existing in tissues during fixation. The resulting solution was designated "formalin-fixed HCG" and was compared with "control-HCG," a solution of HCG dialyzed solely against PBS. Anti-HCG was absorbed with each of these solutions and then studied by double diffusion in agar gel using the same methodology as reported earlier (7). Biologic activity of the two preparations was determined by measuring the effects upon prostatic weights of weanling male rats.

Serial sections were cut from formalin-fixed paraffin-embedded tissue at 6 microns, deparaffinized, washed in PBS at 4°C, stained with the fluorescent conjugates, mounted in glycerin-PBS (9:1), and studied by ultraviolet microscopy. Some sections were stained with hematoxylin and eosin. Appropriate blocking and absorption controls were employed.

To confirm results obtained with formalin-fixed tissues and to determine whether localization of specific fluorescence was altered by paraffin embedding, fresh tissues were frozen immediately by immersion in liquid nitrogen, stored at −20°C, and sectioned in a cryostat at 6 microns. The thawed and air-dried sections were fixed in ether and absolute ethanol (1:1) at 25°C for 15 minutes followed by 95 per cent ethanol at 37°C for 15 minutes. The sections were then washed and stained as above.

RESULTS

Formalin fixation had no detectable effect on the capacity of HCG to react with its specific antibody when studied by immunodiffusion techniques. Rabbit anti-HCG gave a single band of identity on double diffusion in agar with formalin-fixed and control HCG. Anti-HCG absorbed with either fixed or control HCG failed to produce a precipitin band with either absorbent. Formalin treatment under the specified conditions decreased the biologic activity of HCG, but not more than 50 per cent. Since this HCG was fixed with the same concentration of formalin for approximately the same length of time and at the same temperature as that in paraffin-embedded tissues, it was concluded that HCG could be localized immunohistochemically in formalin-fixed tissues.

Formalin-fixed and paraffin-embedded sections of trophoblastic tissues stained with fluorescent anti-HCG contained apple-green fluorescent material localized to syncytiotrophoblast whereas adjacent sections stained with fluorescent normal rabbit globulin or with fluorescent anti-HCG absorbed with non-formalin-fixed or formalin-fixed HCG showed no fluorescence (Figs. 1 to 6). In addition, this specific fluorescence was greatly reduced in slides incubated first with non-fluorescent anti-HCG serum when compared with slides incubated first in non-fluorescent normal rabbit serum. No specific fluorescence was found in kidneys, myometrium, decidua, ovaries, testes, brain, or skin. Because

these findings indicate immunohistologic specificity and previous work had shown that anti-HCG reacts specifically with biologically active HCG, it was concluded that HCG is localized in syncytiotrophoblast.

Positive fluorescence was distinctly regional. The majority of syncytiotrophoblastic cells contained no detectable specific fluorescence, the staining frequently being limited to a few cells or villi. For example, in one section of a hydatidiform mole, the syncytiotrophoblastic layer of two villi stained brilliantly while the remainder was negative.

The results from examination of a spectrum of normal and malignant trophoblastic tissues are given in Table I. No specific fluorescence has been detected

TABLE I

Immunohistochemical Localization of HCG in a Variety of Trophoblastic Tissues

Tissue	Total No. examined	No. of specimens showing localization of HCG in:	
		Syncytiotrophoblast	Cytotrophoblast
Immature placenta*	23	9	0
Mature placenta	9	0	0
Erythroblastotic	4	0	0
Toxemic	1	0	0
Normal	4	0	0
Hydatidiform mole	6	4	0
Chorioadenoma destruens	2	1	0
Choriocarcinoma in human hosts	3	3	Equivocal
Choriocarcinoma in heterologous hosts (9)	4	4	(see text)

* Clinical data concerning the number of these placentas actively secreting HCG at the time of abortion was not available.

in cytotrophoblast. Whenever positive fluorescence has been found, it has been localized to definitive syncytiotrophoblast. The most consistent and brightest fluorescence occurred in the choriocarcinomas, many strongly positive cells were seen in all of these tumors. The observation that no stainable HCG was detected in nine term placentas correlated well with the low levels of circulating HCG normally found during the last trimester of pregnancy.

In some heterotransplanted choriocarcinomas, positive fluorescence was found in a few small cells located at the edge of the tumor masses in edematous, inflamed connective tissue of the hamster. Because these cells were too small to appear in adjacent sections, it was impossible to positively identify them.

The syncytium of many immature and mature placentas emitted a moderately intense yellow autofluorescence which made recognition of small amounts of specific fluorescence difficult. Dempsey and Wislocki (10) ascribed this auto-

fluorescence to placental estrogenic steroids, as the fluorescent emission was characteristic of estrogenic substances associated with thecal ovarian tissue. Yellow autofluorescence was absent in all choriocarcinomas studied; an observation in keeping with the previous demonstration that heterotransplanted choriocarcinomas do not secrete estrogens in appreciable amounts (11).

Studies on non-formalin–fixed, fresh frozen placentas, and heterotransplanted human choriocarcinomas confirmed the above results.

DISCUSSION

These immunohistochemical studies have shown that fluorescein-labeled rabbit anti-HCG serum reacts with an antigen present in human syncytiotrophoblast in formalin-fixed or fresh frozen tissues. Because the antiserum has been shown to be specific for biologically active HCG, and because all staining controls indicated immunologic and histologic specificity, the conclusion appears warranted that the specific fluorescence observed in syncytiotrophoblast represents the localization of HCG in syncytiotrophoblast.

These results indicate that HCG is either produced by or stored in syncytiotrophoblast. Although we have been unable to determine which of these alternatives is correct, argument by analogy favors production rather than storage. For instance, whenever the fluorescent antibody technique has been applied to an endocrine organ in which a single cell type has been known to synthesize a particular hormone, that hormone has been localized in the cell of origin and not to other cells (12–14). It would seem reasonable, therefore, to assume that syncytiotrophoblast synthesizes HCG.

Electron microscopic examination of the ultrastructure of syncytiotrophoblast has revealed a well developed endoplasmic reticulum—evidence that mechanism exists in these cells for active synthesis of protein for export (15, 16). Although the nature of this secretion has not been determined, the suggestion that it might be fetal serum protein was not supported by the fluorescent antibody study of Bardawil *et al.* (17). The possibility remains, therefore, that at least part of this endoplasmic reticulum may synthesize HCG.

In view of these data, the evidence favoring synthesis of HCG by cytotrophoblast requires re-evaluation. The *in vitro* experiments, in which placental cells resembling cytotrophoblast were reported as synthesizing HCG (2, 3), do not prove that cytotrophoblastic cells synthesize HCG. Identification of cell type *in vitro* is notoriously difficult, and it would not be unreasonable to expect that syncytiotrophoblastic differentiation sufficient to permit synthesis of HCG might remain undetectable by light microscopy.

We have shown that a heterotransplantable embryonal carcinoma, containing no microscopically apparent choriocarcinomatous tissue, secretes HCG (18). This finding has been interpreted as support for the hypothesis that embryonal carcinoma may be a stage in the morphogenesis of choriocarcinoma. It remains

to be determined whether the cells synthesizing HCG are undergoing differentiation toward syncytiotrophoblast.

Our studies were facilitated by the observation that formalin fixation did not alter the ability of HCG to react with its specific antibody. Not only was it possible to use rare specimens accumulated in the routine tissue collections, but as a result of formalin fixation it was possible to make more precise recognition of cell type than was possible with fresh frozen tissue.

SUMMARY

Through the use of immunohistochemical techniques, human chorionic gonadotropin has been localized to syncytiotrophoblastic cells of immature placenta, hydatidiform mole, chorioadenoma destruens, and choriocarcinoma. No gonadotropin has been detected in cytotrophoblast. Evidence is discussed which suggests that syncytiotrophoblast is the cell of origin of human chorionic gonadotropin.

The observation that formalin fixation did not alter the ability of human chorionic gonadotropin to react with its specific antibody permitted the study of formalin-fixed paraffin-embedded tissues stored in the tissue collection. In addition, the excellence of histologic preparations following formalin fixation facilitated cytologic identification.

The authors gratefully acknowledge the help given by Dr. Charles G. Cochrane and Dr. Jacinto J. Vasquez, the technical assistance afforded by Mrs. Nancy Gibson and Miss Caroline Gajewski, and the comments and criticism of Dr. J. Sri Ram during the preparation of the manuscript.

BIBLIOGRAPHY

1. Wislocki, G. B., and Bennett, H. S., The histology and cytology of the human and monkey placenta, with special reference to the trophoblast, *Am. J. Anat.*, 1943, **73,** 335.
2. Jones, G. E. S., Gey, G. O., and Gey, M. K., Hormone production by placental cells maintained in continuous cultures, *Bull. Johns Hopkins Hosp.*, 1943, **72,** 26.
3. Stewart, H. L., Jr., Sano, M. E., and Montgomery, T. L., Hormone secretion by human placenta grown in tissue culture, *J. Clin. Endocrinol.*, 1948, **8,** 175.
4. Pearse, A. G. E., Cytochemistry of the gonadotropic hormones, *Nature, London*, 1948, **162,** 651.
5. Pali, K., and Lajos, L., Histochemische Untersuchungen der innersekretorischen Tätigkeit de Hypophyse und der Plazenta, *Gynaecologia*, 1952, **133,** 37.
6. Zilliacus, H., Polysaccharide structures in normal chorionic villi, hydatiform mole and chorioepithelioma, with special reference to the chorionic gonadotrophic hormone, *Gynaecologia*, 1953, **135,** 161.
7. Midgley, A. R., Jr., Pierce, G. B., Jr., and Weigle, W. O., Immunobiological identification of human chorionic gonadotropin, *Proc. Soc. Exp. Biol. and Med.*, 1961, **108,** 85.

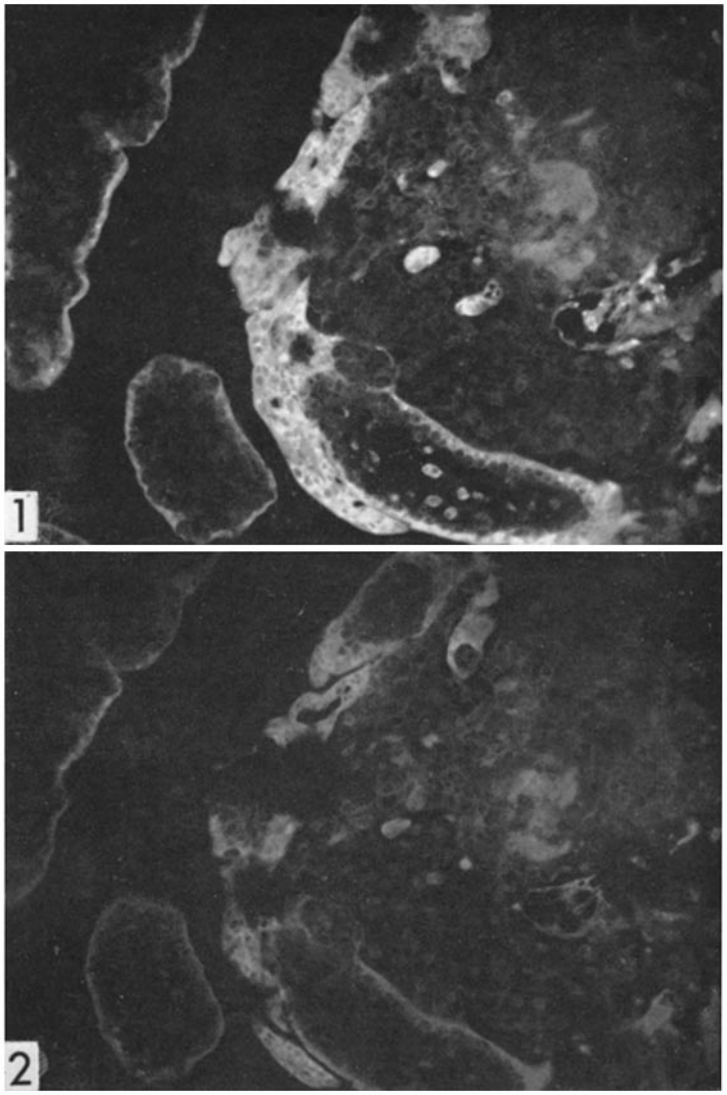
8. Marshall, J. D., Eveland, W. C., and Smith, C. W., Superiority of fluorescein isothiocyanate (Riggs) for fluorescent-antibody technic with a modification of its application, *Proc. Soc. Exp. Biol. and Med.*, 1958, **98,** 898.
9. Pierce, G. B., Jr., Midgley, A. R., Jr., and Verney, E. L., Therapy of heterotransplanted choriocarcinoma, submitted to *Cancer Research.*
10. Dempsey, E. W., and Wislocki, G. B., Observations on some histochemical reactions in the human pancreas, with special reference to the significance of lipoids, glycogen, and iron, *Endocrinology*, 1944, **35,** 409.
11. Verney, E. L., Pierce, G. B., and Dixon, F. J., Jr., The biology of testicular cancer. III. Heterotransplanted choriocarcinomas, *Cancer Research*, 1959, **19,** 633.
12. Leznoff, A., Fishman, J., Goodfriend, L., McGarry, E., Beck, J., and Rose, B., Localization of fluorescent antibodies to human growth hormone in human anterior pituitary glands, *Proc. Soc. Exp. Biol. and Med.*, 1960, **104,** 232.
13. Marshall, J. M., Jr., Localization of adrenocorticotropic hormone by histochemical and immunochemical methods, *J. Exp. Med.*, 1951, **94,** 21.
14. Lacy, P. E., Electron microscopic and fluorescent antibody studies on islets of Langerhans, *Exp. Cell Research*, 1959, *Suppl. 7*, 296.
15. Siekevitz, P., The cytological basis of protein synthesis, *Exp. Cell Research*, 1959, *Suppl. 7*, 90.
16. Wislocki, G. B., and Dempsey, E. W., Electron microscopy of the human placenta, *Anat. Rec.*, 1955, **123,** 133.
17. Bardawil, W. A., Toy, B. L., and Hertig, A. T., Localization of homologous plasma proteins in the human placenta by fluorescent antibody, *Am. J. Obst.*, 1958, **75,** 708.
18. Pierce, G. B., Dixon, F. J., Jr., and Verney, E., Endocrine function of a heterotransplantable human embryonal carcinoma, *Arch. Path.*, 1959, **67,** 204.

EXPLANATION OF PLATES

PLATE 32

FIG. 1. Dark-field photomicrograph of immature placental villi stained with fluorescein-labeled anti-HCG. Positive fluorescence localized to syncytiotrophoblast. Subjacent cytotrophoblastic cells are negative. × 320.

FIG. 2. Adjacent serial section of tissue in Fig. 1 stained with fluorescein-labeled anti-HCG absorbed with HCG. Although no positive fluorescence is seen, the syncytium contains some yellow autofluorescence (see text). × 320.



(Midgley and Pierce: Human chorionic gonadotropin)

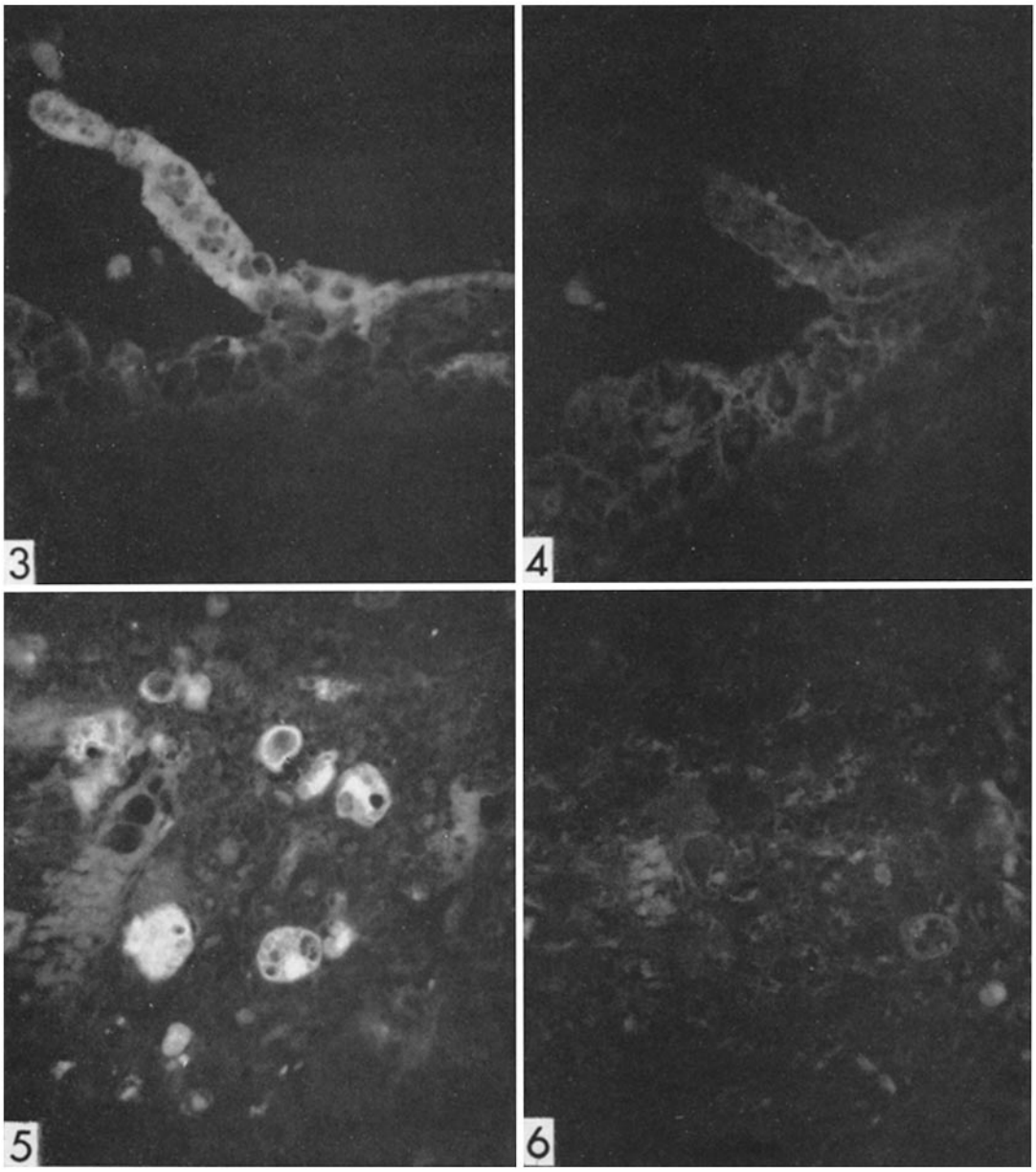
PLATE 33

FIG. 3. Edge of villus of chorioadenoma destruens stained with fluorescein-labeled anti-HCG. Fluorescent material localized solely to syncytiotrophoblastic cells. × 310.

FIG. 4. Adjacent serial section of tissue in Fig. 3 stained with fluorescein-labeled anti-HCG absorbed with HCG. No specific fluorescence is seen. × 310.

FIG. 5. Heterotransplanted human choriocarcinoma stained as in Fig. 3. Positively fluorescent syncytiotrophoblastic giant cells are randomly distributed; some of the giant cells are negative. × 310.

FIG. 6. Adjacent serial section of tissue in Fig. 5 stained as in Fig. 4. No specific fluorescence is seen. × 310.



(Midgley and Pierce: Human chorionic gonadotropin)