

## CHORIOCARCINOMA SCANNING USING RADIOLABELED ANTIBODY TO CHORIONIC GONADOTROPHIN

José Quinones, Gerald Mizejewski and William H. Beierwaltes

*University Hospital, University of Michigan Medical Center, Ann Arbor, Michigan*

Considerable work has been done in producing antibodies to substances in neoplasms, giving the radiolabeled antibody intravenously and following the distribution of the antibody in vivo by various isotope detection methods (1). Korngold has reviewed the limited success in this area and clearly pinpoints much of the difficulty to the problem of finding a tumor antigen that is relatively unique to the tumor (2). It occurred to us that human chorionic gonadotropic hormone (HCG) is an antigenic hormone that is relatively unique in a choriocarcinoma in a male. In this sense, it is a "cancer specific" antigen.

We report here our studies in Syrian hamsters, with human choriocarcinomas transplanted to their cheek pouches; these hamsters were given  $^{125}\text{I}$ -labeled immunoglobulin G (IgG) from rabbits immunized with human chorionic gonadotropic hormone (HCG).

### METHODS

**Antigen immunization.** Mature male New Zealand rabbits were immunized with commercial grade HCG (Antuitrin-S, Parke Davis, 500 IU/ml) mixed with equal parts of complete Freund's adjuvant. The immunization procedure consisted of biweekly subcutaneous injections (0.5 ml antigen-adjuvant mixture) throughout a 2-month period (3). Fourteen days after the last injection, the animals were bled, the serum separated, and the globulin fraction precipitated with  $(\text{NH}_4)_2\text{SO}_4$  (4). The globulin fraction (8 parts) was absorbed with adult human plasma (1 part) and urine (1 part) to remove nonspecific entities. Lastly, a highly purified antuitrin-S HCG\* (1,700 IU/mg) was obtained as antigen for hemagglutination procedures, passive cutaneous anaphylaxis, and studies of immunological specificity.

**Radioiodination and IgG fractionation.** The globulin fraction of the anti-HCG serum and the control

serum of a nonimmunized rabbit were simultaneously labeled with  $^{125}\text{I}$  by a modified method of Hunter and Greenwood (5). The radiolabeled globulin fractions were then subjected to sephadex column chromatography (G-200) to isolate the IgG fraction and remove free radioiodine† (6). The resultant radioglobulin preparations contained 450  $\mu\text{g}$  protein/ml and had a specific activity of 400  $\mu\text{Ci}/\mu\text{g}$ , suspended in 0.1 M Tris buffer and 0.1 M NaCl (pH = 8.0). The IgG eluates displaying the highest radioactivity counts were pooled and stored at 4°C. The pooled radioglobulin samples were not concentrated before use. The free iodine of the stored radioglobulin was determined periodically by paper radiochromatography in a methanol-acetic acid-water (75:10:15) solvent system.

**Characterization of the radioglobulin.** Immunological specificity and potency of the antiserum both pre- and postlabeled with radioisotope was tested by immunodiffusion, hemagglutination, immunoelectrophoresis, and passive cutaneous anaphylaxis.

**Immunodiffusion.** Agar diffusion studies employed the micro-Ouchterlony method (7) which uses a system of double diffusion in two dimensions. Ready poured and cut diffusion plates were commercially obtained‡ for this procedure. Immunodiffusion was also performed in tubes (1.6–1.8 mm i.d., 40 mm long) according to the method of Preer (8). This method, more sensitive than the Ouchterlony plates, employed double diffusion in one dimension in a layer of 0.6% agar sandwiched between antigen and antibody solutions. The reactions required an incubation of 96 hr at room temperature before precipitin bands could be visualized.

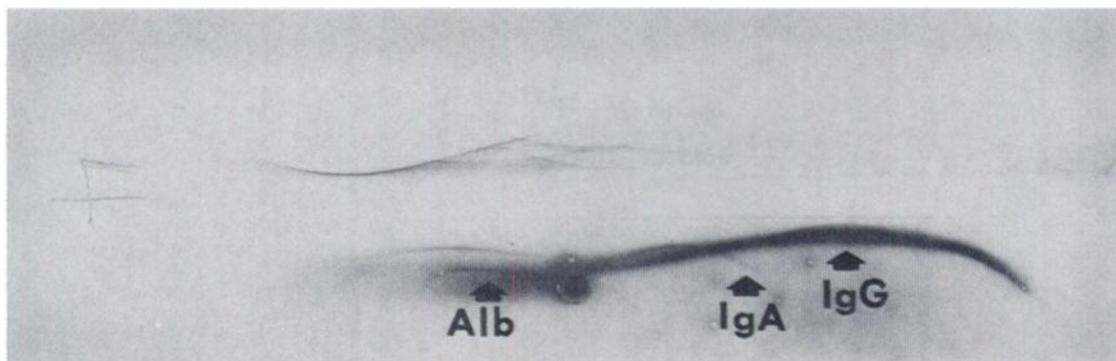
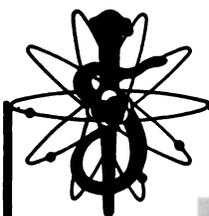
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For reprints contact: W. H. Beierwaltes, University Hospital, Univ. of Michigan Medical Center, Ann Arbor, Mich. 48104.

† Radiolabeling and isolation procedures were performed by B. J. Green, Research Chemist, under the supervision of Jonathan Miller, Abbott Radiopharmaceuticals, North Chicago, Ill.

‡ Immunoplates (pattern C), Hyland Laboratories Inc., Los Angeles, Calif.

\* This HCG was kindly supplied by Merritt R. Callantine, Section of Endocrinology, Parke Davis, Ann Arbor, Michigan.



**FIG. 1.** Immunoelectrophoretic analysis of sephadex G-200 eluates from rabbit immunized to HCG showing presence of predominant IgG moiety with traces of IgA, albumin, and possibly haptoglobin. Practically all radiolabel by autoradiography is in region of IgG fraction.

**Passive hemagglutination (HA).** Sheep red blood cells, preserved in Alsever's solution, were tanned with pyruvaldehyde according to the method of Butt (9). The pyruvaldehyde-tanned red cells were sensitized with 200  $\mu$ g of HCG (1,700 IU/mg) for 1 hr at 50°C. The antiserum was subsequently titrated in micro-Takasky plates.\* After obtaining a hemagglutination titer, a hemagglutination inhibition (9) titration was used with standard amounts of commercial HCG (500 IU/ml) to determine the HCG concentrations in hamster urine, serum, and tumor saline extracts.

**Immunoelectrophoresis.** Micro-immunoelectrophoresis was performed according to Scheidegger (10) using 1.5% agar suspended in tris-baribital-sodium barbital buffer-distilled water (25-75) solution, pH = 8.8. The current applied was 1.5 mA/slide for a 2-hr duration. Following an overnight incubation, the slides were washed, dried, and stained with amidoschwartz. Radio-immunoelectrophoresis followed the method employed by Yagi et al (11).

**Passive cutaneous anaphylaxis (PCA).** The demonstration of immunospecificity using immediate hypersensitivity was performed in the manner described by Ovary (12). Radioglobulin (0.1 ml) in varying dilutions was injected intradermally into the depilated skin of a Guinea pig at several different sites. After a time interval of 6-24 hr, 200  $\mu$ g of purified HCG (1,700 IU/mg) dissolved in 0.1 ml of 10-mg/ml Evans blue dye in saline was injected intravascularly. A positive test of antigen-antibody specificity is designated by "bluing" of the tissues immediately adjacent to blood vessels as a result of altered vascular permeability. Since the crystallizable fraction of the fragmented immunoglobulin (Fc) is

known to be responsible for the PCA reaction (13), this test would indicate whether the Fc tissue binding sites of the radioglobulin were intact.

**Gonadotrophic biological activity.** The gonadotrophic function of commercial grade (Antuitrin-S, 500 IU/ml) HCG was demonstrated by ovarian hyperemia in immature rats by the method of Abert and Berkson (14). HCG was then incubated at 37°C with appropriate amounts of whole anti-HCG serum and  $^{125}$ I anti-HCG IgG, respectively, for 30 min before i.p. injection of the mixture. The same procedure was carried out using nonimmunized rabbit IgG,  $^{125}$ I-IgG, and commercial HCG injected controls. Five rats were used for testing each mixture, totaling 25 test animals. Five saline injected animals served as negative controls. Six hours later, the rats were sacrificed, and the ovaries inspected for hyperemia.

**Choriocarcinoma-hamster biologic-model.** Golden Syrian female hamsters were obtained initially from Roy Hertz, Endocrinology Branch of the National Cancer Institute. The choriocarcinoma transplanted into the cheek pouches of these animals was originally from a female human choriocarcinoma. This line has been maintained in male and female hamsters through 50 serial passages. For transplantation, the tumor tissue was cut into small pieces (approx. 8 mm<sup>3</sup>) in 5 ml of Hank's BSS. A 14-gage needle with obturator was used to inoculate a piece of tumor tissue into the apex of each hamster cheek pouch.

Hamster cheek pouches were inspected 1 week after implantation and definite "takes" were separated for study. Sixty to 80  $\mu$ g of anti-HCG IgG labeled with 25-35  $\mu$ Ci of  $^{125}$ I in a volume of 0.1-0.2 ml were injected i.p. into the tumor-bearing animals. As controls, an equal amount of radiolabeled IgG from nonimmunized rabbits was injected into similar hamsters. At 1, 2, 3, and 4 days after injection, the hamsters were anesthetized with sodium pentobarbital i.p., and total-body scans were made with a 5-in. NaI(Tl) crystal scanner using a 3-in. focus, low-energy, fine-focus collimator. The ani-

\* Microtiter System, Medical Research Div., Cocke Engineering Co., Alexandria, Va.

mals were then injected with Uthol solution\*, and a blood sample was immediately removed from the heart cavity. Nonperfused tissue samples of tumor, ovaries, uterus, pituitary, liver, kidney, lung, muscle, and spleen were removed, rinsed in saline, and weighed. The cut tissue segments were placed in counting tubes and radioactivity assays were made in an automatic well counter. The tissues were counted against a 100- $\mu$ l blood sample from the same hamster. Results were expressed as tissue/blood ratios and tumor/tissue ratios.

To correct for individual differences in tumor growth, another series of experiments was conducted in which the same hamster was injected with both anti-HCG  $^{125}$ I-IgG and nonspecific rabbit  $^{131}$ I-IgG. These animals were studied at the same time intervals as the previous group, and the tissues were analyzed in a dual-channel automatic well counter correcting in each instance for the  $^{131}$ I radioactivity appearing in the  $^{125}$ I channel (15). Results were expressed as in the above group.

**In vitro studies.** To test the ability of the  $^{125}$ I-IgG to unite directly with the human choriocarcinoma without the interference of HCG in the circulating blood, in vitro studies were performed with cells from the tumor and other Syrian hamster tissues. Preparation of the tissues was performed by the method of Martin and Miller (16). Portions of choriocarcinoma from cheek pouch and samples of ovaries, liver, and kidneys were teased and pressed through a 250-micron wire mesh into Tissue Culture Medium 199. The clumps of tumor syncytia and of liver, ovary, and kidney cells were then washed with Medium 199 and resuspended at a concentration of 100 mg/ml. Radiolabeled anti-HCG (5–20  $\mu$ g) and IgG from nonimmunized rabbits were added to duplicate samples of each tissue, incubated at 37°C for 1 hr and washed three times with Hank's BSS; the concentration of radioactivity in the cells and in the washings were then determined. The radioactivity in the cellular fractions was expressed as a percent of tracer dose absorbed.

#### RESULTS

**Antibody characterization.** Immunodiffusion studies in agar using the micro-Ouchterlony and Preer methods revealed the presence of three precipitation zones when nondiluted anti-HCG serum was tested against commercial HCG. Following radioiodination, only two precipitation zones could be detected. This observation suggested that some immunoglobulin was either lost or further diluted due to preparative procedures. The radioglobulin anti-HCG did not

\* Sodium pentobarbital 320 mg/ml in alcohol-propylene glycol base with water, W. A. Butler Co., Columbus, Ohio.

react with pregnant mare serum, human and porcine growth hormone, bovine corpus luteum, and placenta powder, and porcine LH and FSH as determined by radioimmunoassay. Labeled and nonlabeled non-immunized IgG did not react with either commercial or purified grade HCG.

Passive hemagglutination (HA) of HCG-sensitized red blood cells tanned with pyruvaldehyde provided a highly sensitive index of antibody po-

TABLE 1. ISOLATION AND PREPARATION OF RADIOGLOBULIN

Steps in preparing $^{125}$ I-labeled IgG from whole serum	Fraction resulting	Protein concentration of fraction (mg/ml)	Antibody titer of fraction (TRBCH)*	TRBCH titer per mg protein
Centrifuge fresh whole blood	Serum	67.6	1:2560	38.0
NH <sub>4</sub> SO <sub>4</sub> precipitation	Serum globulins	6.7	1:640	96.0
$^{125}$ I-labeling and sephadex G-200 chromatography	$^{125}$ I-labeled IgG	0.47	1:240	511.0

\* Tanned red blood cell hemagglutination.

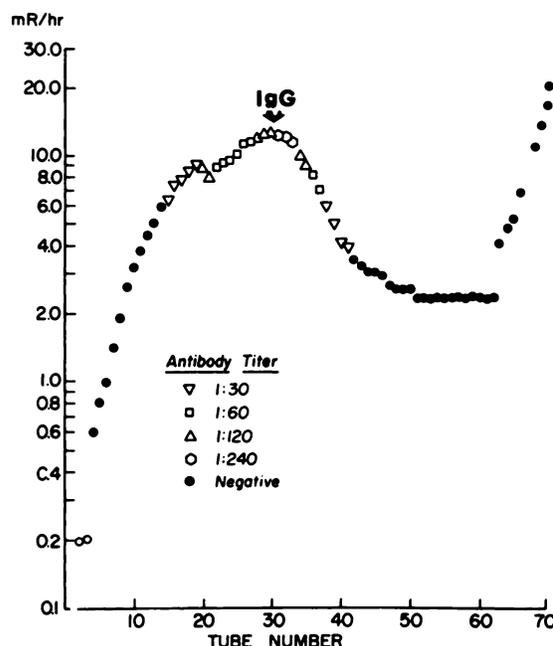
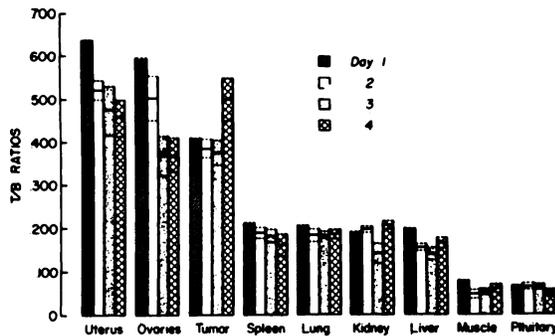
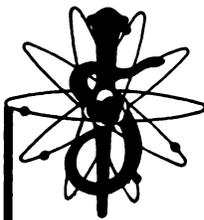
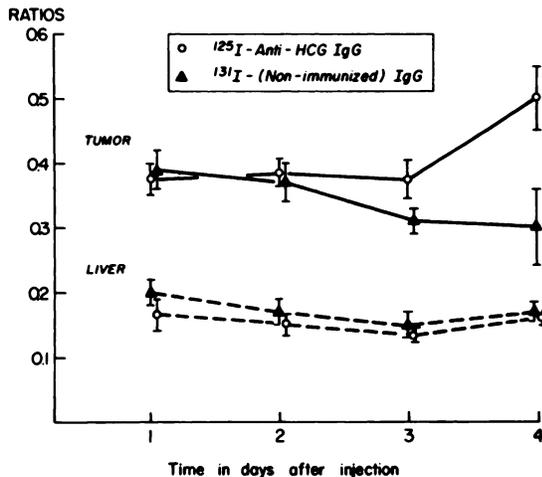


FIG. 2. Plot of radioactivity content of each fraction of radiolabeled globulin obtained with sephadex (G-200) gel filtration. Radioactivity concentration is seen in log ordinate in mR/hr and tube fraction number on abscissa. Maximum antibody titer is associated with maximum radioactivity concentration in IgG fraction.



**FIG. 3.** Bar graph summarizing tissue-to-blood ratios (cpm/mg) in all hamsters given  $^{125}\text{I}$ -labeled anti-HCG IgG and sacrificed at 1, 2, 3, and 4 days after injection. Concentration of radioactivity in tumor remains constant to Day 3 and then increases on Day 4. It may be noted that uterine and ovarian radioconcentrations decrease with passage of time while tumor increases. Each bar represents mean of eight animals.



**FIG. 4.** Plot of mean tissue-to-blood radioactivity ratios in tumor and in liver, showing that at 3 and 4 days significant difference develops between immune and non-immune IgG in tumor, but not in liver. Each point represents mean of eight animals. Both isotopes were injected into same hamster.

tency. The molecule was apparently intact in regard to the reactant sites of the antibody binding portion of the immunoglobulin (Fab) as demonstrated by persistent HA activity. Radiolabeled anti-HCG IgG, titrated using the HCG tanned red cells, demonstrated a titer of 1:2560 before radiolabeling, and 1:240 postlabeling. Table 1 shows that as the IgG is isolated, the antibody titer per milligram of protein rises. Antibody titer was recorded as the reciprocal of the maximal two-fold dilution displaying hemagglutination.

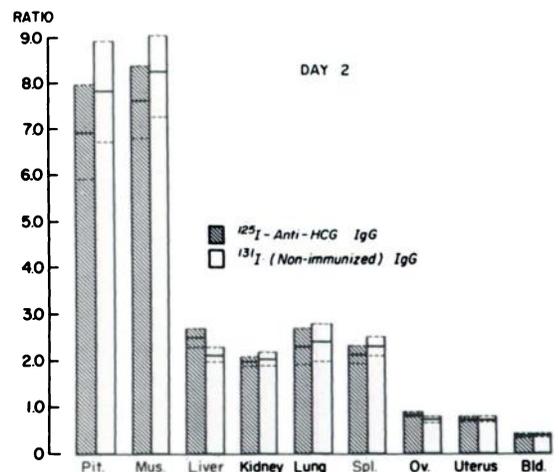
Immunoelectrophoretic analysis of both radioglobulin preparations revealed the presence of a predominant IgG moiety with traces of IgA, albumin, and possibly haptoglobin. Practically all the radiolabel was attributed to the IgG fraction as demon-

strated by autoradiography (Fig. 1) and a plot of radioactivity as a function of the eluate fractions of radiolabeled globulin using sephadex G-200 gel filtration (Fig. 2). Using the radiolabeled IgG from the nonimmunized rabbit, no antibody-antigen reaction was obtained to commercial HCG following electrophoresis. IgG from nonimmunized rabbits was otherwise identical to that of the anti-HCG both by immunoelectrophoretic and by autoradiographic analysis.

Guinea pig skin, sensitized with  $^{125}\text{I}$ -anti-HCG and  $^{125}\text{I}$ -RGG, demonstrated a classical PCA response to purified HCG only at the site of homologous antibody. Titration of the radioglobulin in the PCA response roughly paralleled that of the HA antibody titer. These results indicated no detectable alteration of the Fc portion of the immunoglobulin molecule due to radiolabeling.

**Inhibition of biological activity.** The gonadotrophic function of commercial grade HCG was suppressed in immature rats by whole anti-HCG serum and its corresponding radioglobulin. However, nonimmunized rabbit IgG and its radiolabeled counterpart failed to inhibit hyperemia. These data demonstrated that anti-HCG serum and  $^{125}\text{I}$ -anti-HCG IgG are directed against HCG and both are capable of blocking gonadotrophic function.

Blockage of immunological activity of tumor saline-extracts was also performed using hemagglutination inhibition. With this method, 30–50 I.U. HCG/gm of tumor saline homogenate was detected in hamster-borne tumors. Correspondingly, these hamsters possessed average urine and serum HCG



**FIG. 5.** Bar graph showing that tumor-to-tissue radioactivity concentration ratios (cpm/mg) at all intervals are 2–9 times greater than that of all tissues except ovary, uterus, and blood at 2 days postinjection. Radioactivity concentrations of  $^{125}\text{I}$ -labeled IgG from nonimmunized rabbits does not differ significantly from that of  $^{125}\text{I}$ -IgG from immunized rabbits used in same hamster.

titers of 20–40 I.U./ml. These values, determined by hemagglutination inhibition, are similar to those initially reported by Hertz (17).

**Tissue distribution following  $^{125}\text{I}$ -labeled anti-HCG IgG administration.** Figure 3 is a bar graph summarizing the tissue-to-blood ratios (cpm per mg/cpm per  $\mu\text{l}$ ) in all hamsters given  $^{125}\text{I}$ -labeled anti-HCG IgG and sacrificed at 1, 2, 3, and 4 days postinjection. Each bar graph represents the mean  $\pm$  1 sem value of eight animals studies. The concentration of radioactivity was less in all tissues at all intervals than in blood. The tumor radioactivity concentration increases with passage of time, while uterus and ovary concentrations correspondingly decrease. The tumor radioconcentrations maintain constancy to Day 3 and then increase on Day 4. On the fourth day, however, the tumor value is significantly higher than ovary but not of uterus. For all time intervals, there was an obvious difference in the concentration of radioactivity found in tumor, uterus, and ovaries compared with all other tissues shown in the bar graph.

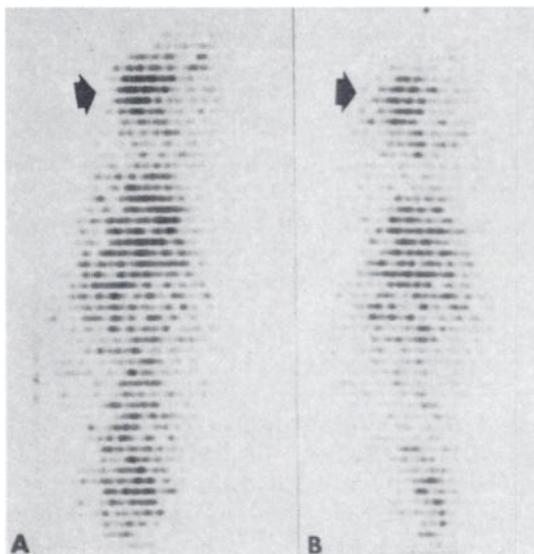
Figure 4 is a plot of the mean tissue to blood radioactivity ratios in tumor and in liver throughout a 4-day interval. A significant difference ( $p < 0.05$ , t-test) was found at Days 3 and 4 between anti-HCG IgG and control IgG in tumor but not liver. This difference may be due to specific tissue binding of the anti-HCG immunoglobulin by the tumor. In contrast to the above, Fig. 5 shows that the tumor-to-tissue radioactivity ratios for both preparations were similar on Day 2 using the double-isotope method. It is probable that the radioactivity concentration was secondary to the vascular supply to each organ at this time interval. Although there was a significant difference between the experimental and control tumor radioactivity concentrations on Days 3 and 4, this difference was not apparent from scanning. With both preparations, tumor-to-tissue activity was sufficient to allow visualization by scintillation scanning. Figure 6 presents the total-body scans at Day 2 on a representative hamster given  $^{125}\text{I}$ -anti-HCG IgG (A) and another hamster given  $^{125}\text{I}$ -IgG from a non-immunized rabbit (B). The tumor is visualized as an increased concentration of radioactivity at the top left (right cheek pouch) in each hamster.

**In vitro absorption.** Figure 7 is a bar graph of six representative experiments displaying the percent of radioactivity (mean  $\pm$  sem) remaining on teased-screened tumor syncytia without interference from circulating HCG present in the serum of the host (hamster). It may be noted that the tumor cell syncytia retained a significantly higher amount ( $p < 0.05$ ) of anti-HCG IgG as compared with control IgG and with all other tissues tested. The

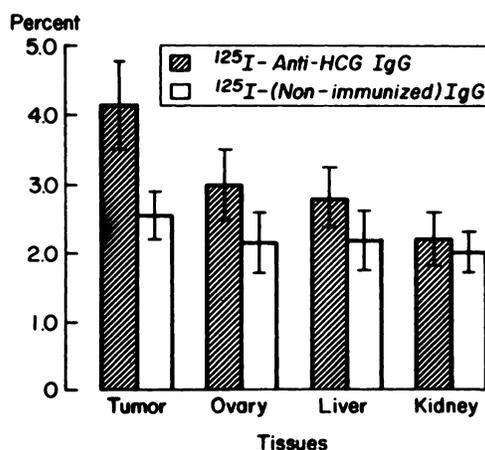
in vitro results indicate an interaction between the antibodies and the tumor cells and supports the findings on Days 3 and 4 in the in vivo experiments mentioned above.

#### DISCUSSION

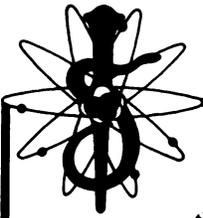
Although a primary weakness of the radiolabeled antibody approach is the lack of evidence that the radiolabeled antibody enters the cancer cell in vivo,



**FIG. 6.** Total-body scans, 48 hr after injection, on: A, representative hamster given  $^{125}\text{I}$ -labeled anti-HCG IgG, and B, hamster given  $^{125}\text{I}$ -labeled IgG from nonimmunized rabbit. Human chorioncarcinoma in hamster cheek pouch is clearly visualized in each (arrow).



**FIG. 7.** Bar graph of absorption of  $^{125}\text{I}$ -anti-HCG by teased-screened cells in vitro, showing percent radioactivity remaining on 100 mg tissue/ml after 1 hr of incubation. Concentration of  $^{125}\text{I}$  IgG from immunized rabbits is significantly greater than from non-immunized rabbits. This significant difference is not seen with ovary, liver, or kidney.



the choriocarcinoma may be unique. Brambelle (18) has shown that only IgG among the immunoglobulins traverses the human placenta to reach the fetal circulation. This is due, not to simple filtration, but to an active transport process across the trophoblastic cells of the placenta; the selectivity is mediated by certain transmission sites present on the Fc portion of the IgG heavy chain. The selective passage of IgG may be due to pinocytotic activity along a system of transcellular canals lined with microvilli (19). Such a system of canals is also found in the syncytiotrophoblast of human choriocarcinoma (20). This placenta mode of transmission, as seen in primates, is unique.

Two innovations were used in this study to encourage radioiodine-labeled antibody to combine with cancer for diagnostic purposes. A "cancer specific" antigen was used, using the hormone produced by a cancer of an organ not ordinarily found in the male (placenta-choriocarcinoma-HCG). Although HCG is not a surface antigen, the presence of this hormone has been demonstrated within giant cells comprising the syncytiotrophoblast (21). Antigens within cells are capable of reacting with specific antibodies, provided such antibodies can reach them by some transport mechanism such as pinocytosis (19). Also, anti-HCG IgG is cytophilic toward choriocarcinoma syncytia as demonstrated by the *in vitro* absorption studies in the present report. Therefore, antigen-antibody interaction is conceivable at the cell surface or in the intercellular compartment of the tumor. Secondly, we used the radioiodine-labeled immune globulin IgG directed against HCG. Specificity of the antibody was determined by: (A) inhibition of HCG-induced ovarian hyperemia in the immature rat, and (B) hemagglutination inhibition by HCG saline extracts from tumor homogenates.

The most encouraging result of this attempt was the significantly increased concentration of radioiodine from immune IgG in choriocarcinoma syncytia as compared to kidney, ovary, and liver in the absorption studies on tissue cells *in vitro*. This method of testing eliminates the possibility of immune IgG being lost by complexing with HCG of tumor origin present in the hamster serum. It was also of interest that at 3 and 4 days postradioinjection, there was a significantly greater retention of  $^{125}\text{I}$ -anti-HCG IgG than  $^{131}\text{I}$  from nonimmunized rabbit IgG in tumor in the *in vivo* studies using tissue:blood ratios. This was not observed in the *in vivo*.

The *in vivo* studies showing significantly higher concentrations of radioiodine from both specific and nonspecific IgG in tumor, uterus, and ovaries during

the first few days after *i.p.* injection must be related to a greater vascularity of these tissues than of the other tissues studied. This statement is substantiated by preliminary studies using radioiodine-labeled albumin (IHSA) which showed this same tissue distribution pattern.

The possibility that only about 10–15% of the IgG from even the immunized rabbits is specific (22) raises the question whether the specific combination of this antibody with HCG in choriocarcinoma is masked by the presence of nonspecific IgG. It is possible that at 3 and 4 days sufficient nonspecific IgG has left the tissues so that the antigen-antibody union of specific IgG becomes apparent. This possibility is also suggested by the data obtained in the *in vitro* study.

Studies beyond 4 days could not be reliably performed. This solid tumor becomes palpable in the hamster at about 7 days after implantation and unfortunately shows necrosis at 11 days. In our experience the cutaneous metastases from choriocarcinoma in the human do not become necrotic for several weeks. It would be of interest to study the concentration of radioactivity from anti-HCG versus nonimmune IgG in the human by doing serial biopsies of the metastases to determine if radioactivity persists longer in tumor with anti-HCG IgG than nonimmune IgG. If radioactivity from specific IgG persisted after radioactivity from nonspecific IgG had decreased in concentration in serum, the resultant improved target to nontarget ratio of radioactivity from specific IgG in tumor might allow the detection of small lesions with the scanner.

We are also interested in increasing the percent of specific IgG in the IgG fraction from the serum of immunized rabbits. This attempt is in progress in our laboratory by the use of specific immunoadsorbents (23). Studies will then be conducted to determine if radioactivity persists longer in tumor with anti-HCG than nonimmune IgG.

#### ACKNOWLEDGMENT

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