Imaging of Tumor in Patients with Indium-111-Labeled Biotin and Streptavidin-Conjugated Antibodies: Preliminary Communication

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Tumor localization in patients has been achieved through the in vivo use of streptavidin and biotin. In these preliminary studies, the monoclonal antibody HMFG1 was conjugated with streptavidin and 1 mg was administered intravenously to each of 10 patients with documented squamous cell carcinoma of the lung. Two to 3 days later, ^{111}In-labeled biotin was also administered intravenously. No evidence of toxicity was observed. Background radioactivity levels were reduced in liver (1% ID at 24 hr) and kidneys (2%) and in all other normal tissues and blood. Images of lung tumor were obtained in as little as 2 hr following administration of labeled biotin. In eight patients, tumor was detected with labeled biotin alone without the previous administration of streptavidin-conjugated antibody but in three of these patients, the images were improved with the prior administration of conjugated antibody. These results suggest that this approach may improve the tumor-to-normal tissue radioactivity ratios in radioimmunotargeting.

J Nucl Med 1990; 31:1791–1796

With the realization that tumor targeting with radiolabeled antibodies is not providing in patients the high tumor and low normal tissue radioactivity levels seen in animals (*1*) has come attempts to improve the biodistribution. For the most part, these attempts have focused on improving ways in which antibodies are radiolabeled (*2*), however, an alternative approach known as pretargeting is also under investigation. Nonradioactive antibody is administered first and allowed time to localize in tumor and to clear from normal tissues before the radioactive label is administered in a chemical form with high affinity for the antibody. Thus, anti-tumor antibodies have been conjugated with biotin and administered to animals several days prior to the administration of indium-111- (^{111}In) or iodine-125- (^{125}I) labeled avidin or streptavidin (*3–5*). Antibodies have also been conjugated with streptavidin and administered to animals prior to the administration of ^{111}In-labeled biotin (*3*). A novel approach employing an antibody nonspecific for tumor but specific for a low molecular weight ^{111}In chelate has been investigated in animals (*6*). The labeled chelate was administered after the antibody had diffused into the interstitial spaces surrounding the tumor and just after a "chase" was used to clear circulating antibody. Avidin has also been used to clear circulating biotinylated antibodies in animals (*7*). Bifunctional antibodies, antibodies specific both for tumor antigens and for the radiolabeled tracer have also been investigated in animals. In one such use, an F(ab')$_2$ antibody was constructed from one Fab fragment directed against a tumor antigen and another directed against a low molecular weight ^{111}In chelate (*8*). A particularly novel adaptation of this idea employs an anti-tumor F(ab')$_2$ to which a Fab fragment specific for a bivalent low molecular weight ^{111}In chelate was conjugated (*9*). It has been suggested that the arrangement of F(ab')$_2$ antibodies on the tumor surface may bring together two Fab fragments such that divalent chelates may have a higher affinity for cellbound rather than unbound circulating antibody (*9*).

Although pretargeting has provided encouraging results in animals, a true test of the concept will require studies in patients. The results of the first such study have recently been reported (*10*). A bifunctional antibody consisting of an anti-CEA Fab' fragment was covalently bound to another Fab' fragment directed against a low molecular weight ^{111}In chelate. Nineteen patients with colon carcinoma received up to 40 mg of this antibody up to 5 days prior to the administration of the labeled chelate. With the exception of kidneys

Received Nov. 28, 1989; revision accepted Apr. 13, 1990.
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(route of excretion of the labeled chelate), tumor-to-normal tissue ratios were improved with respect to the ^{111}In-labeled intact anti-CEA antibody.

This report describes preliminary results in a limited clinical trial of the pretargeting concept in patients in which streptavidin and biotin have been employed because of the high affinity ($K = 10^{15}M^{-1}$) of their association (*11*). Streptavidin, a 60K dalton protein, has been conjugated to the anti-human milk fat globule HMFG1 IgG to serve as the first injectate. Several days later, ^{111}In was administered chelated to biotin via diethylenetriaminepentaacetic acid (DTPA) (*3*). The HMFG1 antibody is a mouse IgG1 antibody which has been shown to bind to a large mucin-like molecule normally produced by the lactating breast but also expressed by the majority (>90%) of ovarian, breast, and other carcinomas (*12*).

Internalization of antibody expression at the tumor surface is obviously a concern in the pretargeting approach and tissue culture studies with a tritium-labeled antibody thought to recognize the same antigenic determinants as HMFG1 showed digestion products of internalization and catabolism, although not appearing before ~6 hr (*13*). Immunohistochemistry and autoradiography performed on patient tumor tissue obtained at biopsy after administration of DTPA-conjugated HMFG1 IgG showed no evidence of internalization (Epenetos AA, unpublished observations).

Ten patients with documented squamous cell carcinoma of the lung have received 1 mg of a streptavidin-conjugated HMFG1 IgG antibody intravenously. Several days later biotin (50 or 1000 μg), radiolabeled with ^{111}In, was also administered intravenously. Because of the possibility that tumor targeting might be achieved with labeled biotin alone, each patient was imaged on two occasions. On one occasion, the antibody had been administered 2–3 days earlier while on the second occasion the antibody had either not yet been administered or had been administered 2–3 wk earlier such that streptavidin binding would either be minimal or nonexistent.

MATERIALS AND METHODS

Streptavidin-Conjugated Antibody

The HMFG1 IgG antibody was obtained from Damon Laboratories, Medford, MA, in condition suitable for human administration. The streptavidin was obtained from Societa Prodotti Antibiotici, Milan, and was conjugated to the antibody under sterile conditions using biotin as a linker (*3*). Biotinylation of the antibody was accomplished with sulfosuccinimidyl 6-(biotinamide)hexanoate (Pierce Chemical Co., Rockford, IL). A three-fold molar excess of the biotinylating reagent was added to a 5 mg/ml solution of the antibody in 0.05 *M* bicarbonate buffer, pH 8.4 and allowed to react for 5 hr. The antibody was then added to a 2.5 molar excess of streptavidin in the bicarbonate buffer. After 30 min, the conjugated antibody was purified from unreacted streptavidin, biotin, HMFG1 and from dimeric and polymeric species by passage through a 50–200-cm long column of Sephadex G200 with 0.05 *M* bicarbonate buffer, pH 8.5 containing 0.9% saline. Selection of column fractions for pooling was on the basis of protein content determined both by size exclusion high-performance liquid chromatography (HPLC) and SDS polyacrylamide electrophoresis. The preparation was divided into sterile V-vials containing 1 mg of total protein. The final product was tested for sterility, apyrogenicity and for general safety using mice and guinea pigs.

The immunoreactivity of the conjugated antibody was compared to that of the unmodified antibody in a solid phase ELISA assay using purified milk fat globule as antigen (*14*). The assay was also performed on an antibody (H17E2) with no affinity for the antigen as a control.

The biotin-binding capacity of streptavidin conjugated HMFG1 was also determined by adding varying amounts of ^{111}In-biotin to a solution of the conjugated antibody in 0.05 *M* bicarbonate buffer, pH 8.4 and determining by Sephadex G50 chromatography the fraction of added radioactivity which bound to the higher molecular weight antibody.

The preparations were stored frozen and thawed just prior to use. After thawing, the contents of the vial were removed with 2.0 ml of 8.4% sodium bicarbonate buffer for the injection.

Radiolabeled Biotin

The biotin used in this investigation was covalently conjugated to DTPA and was obtained from commercial sources (Sigma Chemical Co., St. Louis, MO). Sterile and apyrogenic V-vials were prepared to contain 50 or 1000 μg of the biotin derivative in 0.05 *M* bicarbonate buffer for injection. These were stored frozen and only removed prior to use. After thawing, 2.0 mCi of ^{111}In (Amersham, UK) was added in 0.25 *M* acetate buffer. The content of the vial was then diluted to about 2.0 ml with 0.05 *M* bicarbonate buffer for injection. Quality assurance of the labeled biotin consisted of adding an aliquot of the preparation to an excess of avidin in bicarbonate buffer followed by size exclusion HPLC analysis to ensure that all the radioactivity was bound to avidin.

Using avidin-conjugated beads (Sigma Chemical Co., St. Louis, MO) in a competitive-binding assay, it was shown that the ^{111}In-labeled biotin competed equally with native biotin for immobilized avidin (Rusckowski M., unpublished observations).

Patients

All 10 patients enrolled in this study had documented squamous cell carcinoma of the lung. All had been treated with chemotherapy and most received external beam radiation therapy to the lung. Each patient had relapsed within 6 mo of this investigation. The contents of one vial of streptavidin conjugated HMFG1 was administered to each patient intravenously by slow (1 min) infusion. All patients received 2.0 mCi of ^{111}In-labeled biotin by bolus i.v. administration on two occasions. In three patients, the first biotin administration occurred 2–3 days after the administration of conjugated antibody while the second administration of biotin occurred 2–3 wk later. In the remaining seven patients, the first biotin administration was 3 wk before antibody administration and the second biotin administration was 2 days after antibody administration. Patients received either 50 μg (six patients) or

1000 μg of labeled biotin. Patients were imaged on a 40-cm field of view General Electric Co. Starcam 400A (Milwaukee, WI) camera fitted with a medium-energy collimator. Labeled biotin administration was performed under the camera so that a 30-min dynamic study of tumor, heart, kidneys, lungs, and liver could be obtained. Following this, whole-body anterior and posterior images as well as spot views were obtained at 1, 2, and 24 hr post-administration. Crude quantitation of radioactivity levels in liver and kidneys was obtained from the geometric mean counts in regions of interest about the organs in comparison to that of the whole body. Blood samples were taken from a peripheral vein remote from the infusion site at various times following administration of the conjugated antibody and labeled biotin. A complete urine collection was also obtained after each administration of labeled biotin.

Serum Studies

Blood samples were allowed to clot so that serum would be available for analysis. Aliquots of each collection were counted in a NaI(Tl) well counter against a sample of the injectate to measure clearance. These values were analyzed using the SAAM29 program (*15*) to estimate clearance half times. Blood samples were also collected in heparinized tubes at various times in the case of four of the patients so that the percentage of radioactivity on formed elements could be determined.

Human anti-streptavidin and human anti-HMFG1 (HAMA) responses were measured in five patients on serum collected before and 14 days after the injection of the conjugated antibody. A 96-well microtiter plate (Sterilin, UK) was coated with 500 ng/well of streptavidin (anti-streptavidin assay) or HMFG1 antibody (anti-HMFG1 assay) by applying the protein in a carbonated bicarbonate buffer at pH 9.6 and incubated at 37°C for 4 hr. After washing the plate three times with a washing buffer consisting of phosphate-buffered saline (PBS), pH 7.4 and 0.05% Tween 20 (Sigma Chemical Co.), patients' sera was applied at 1:10 dilutions. After 2 hr incubation at 37°C, the plate was again rinsed with washing buffer and peroxidase conjugated sheep species specific anti-human IgG antibody (1:500 dilution, Amersham, UK) was added. The plate was incubated for an additional 1 hr at 37°C, rinsed, and after addition of the substrate ABTS (Beringweeke, W. Germany) the absorbance of each well measured at 405 nm using a multiscan spectrophotometer (Titertek, Flow Laboratories, UK). A curve of absorbance versus dilution was plotted and the results expressed as the area under the ELISA serum titration curve (*16*).

In the case of the anti-HMFG1 (HAMA) response, an attempt was made to quantitate the response by comparison with a standard serum. The standard serum was obtained by separating HAMA by affinity chromatography from the sera of patients who had received multiple injections of murine antibodies.

Endogenous Biotin

As a naturally occurring vitamin, biotin is found in low concentration in all living cells and in serum. It has been estimated that biotin is present in human serum at a concentration of 0.5 ng/ml (*17*) and could, therefore, interfere with the binding of labeled biotin to its antibody target. This interference may be expected to result in a lowering or elimination of the ability of circulating streptavidin-conjugated antibody to bind labeled biotin. Thus, the ability of circulating streptavidin-conjugated antibody to bind ^{111}In-biotin was estimated by adding high-specific activity (125 mCi/mg) labeled biotin (~0.15 ng) to 40 μl aliquots of sera from four patients. Each serum sample was analyzed by SDS polyacrylamide gel electrophoresis in which the gels were divided into 3-mm slices and each slice counted in a NaI(Tl) well counter. The serum samples analyzed in this fashion were collected 10 min and 3 days post-antibody administration, immediately post-biotin administration (i.e., 3 days post-antibody administration) and 1 hr post-biotin administration.

RESULTS

Quality Assurance

All tests to which the injectates were subjected showed them to be safe for human administration. Both mice and guinea pigs were unaffected by administration of the conjugated antibody in the General Safety Test and both the conjugated antibody and biotin preparations were sterile and pyrogen-free. Sephadex G200 chromatography was used to purify the conjugated antibody and to establish that the product possessed a narrow molecular weight distribution centered around 210 K daltons as expected for the desired monomeric species. On average, each conjugated antibody molecule was found to be capable of binding 2.5 molecules of ^{111}In-biotin. In the ELISA of the conjugated HMFG1, native HMFG1 and irrelevant control antibody, the control antibody displayed essentially no ability to bind to antigens in patient sera, while the conjugated and native antibodies show similar binding abilities to demonstrate that conjugation did not impair immunoreactivity of the HMFG1 antibody.

Analysis of each radiolabeled biotin preparation by the addition of excess avidin to an aliquot followed by Sephadex G50 chromatography consistently showed the absence of labeled species other than ^{111}In-biotin.

Patients

Streptavidin-conjugated antibody and radiolabeled biotin were administered to 10 patients 43–77 (mean 65) yr of age. The antibody was administered once while the labeled biotin was administered on two occasions. No toxicity was evident at any time. Serum profiles (SMA 20) analysis run on sera of four patients obtained both immediately before and 2 wk after antibody and biotin administration showed no changes.

Localization of the radiolabel in lung cancer lesions was observed in eight patients both with and without the prior administration of conjugated antibody. Images of the remaining two patients were consistently negative. A blinded observer found no change in image quality with antibody administration in the case of five patients with positive images and an improvement in image quality (i.e., increased tumor and/or decreased background radioactivity levels) in the remaining three patients. Figure 1 presents anterior chest and upper abdomen images of one of the latter patients. This

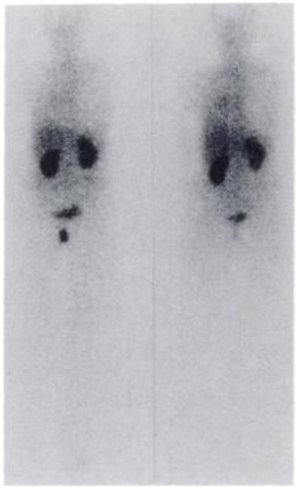

FIGURE 1
Anterior chest and upper abdomen images of the same patient obtained 2 hr after administration of labeled biotin and 2 days (right image) and 2 wk (left image) after administration of the streptavidin-conjugated antibody. The image on the left therefore represents a control study in which conjugated antibody was most likely not present at the time of biotin administration.

patient was diagnosed as having squamous cell carcinoma of the right lung 1.5 yr previously. She responded well to external beam radiation therapy but relapsed 6 mo prior to this study. Both images in the figure were obtained 2 hr post-administration of labeled biotin (50 μg). The image on the left was obtained 2 wk following administration of the antibody while the image on the right was obtained 2 days after the administration. Radioactivity in blood pool of the heart as well as uptake in the liver and spleen is essentially absent despite the early imaging time. Both kidneys (and bladder) are evident as the principal excretory organ for the labeled biotin (see below). Increased accumulation of label in the tumored lung in the right image is evident with respect to the control image on the left. The extent and location of tumor within this image correspond well with the location and extent of this patient's disease as evidenced from the chest X-ray presented in Figure 2.

At 24 hr post-administration, <1% of the injected radioactivity was localized in liver and ~2% in both kidneys. These values were similar whether or not the antibody was administered recently and whether 50 or 1000 μg of labeled biotin was administered.

Serum Studies

The serum clearance curve was adequately fit to two components with T1/2 α = 2.4 min (1.2 min s.d., n = 13), intercept 0.72 (0.13 s.d., n = 13) and T1/2β = 4.2 hr (1.8 hr s.d., n = 13), intercept 0.18 (0.06 s.d., n = 13). The volume of distribution at steady state was determined to be 1.20 liters (0.16 liters s.d., n = 13). There were no significant differences in these values when calculated separately for patients receiving the antibody recently with respect to those who either had not yet received the antibody or who received the antibody 2–3 wk previously. There were also no significant differences with the dose of labeled biotin administered.

The percentage of circulating radioactivity on formed

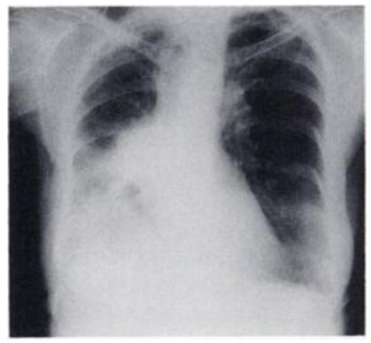

FIGURE 2
Recent chest X-ray of the patient whose images are shown in the preceding figure.

elements was determined in blood from four patients. Approximately 99% of the radioactivity in whole blood was found in the plasma fraction at all times.

Sera from five patients were analyzed for anti-streptavidin and anti-HMFG1 response in sera obtained before and 2 wk after the administration of conjugated antibody. Two patients showed no anti-streptavidin titer at either time. Two of the remaining patients showed less than a doubling of response while the remaining patient showed an increase of almost a factor of 10. Positive values for HAMA were obtained in the measurement of anti-HMFG1 in the preadministration serum samples from four patients, although at near-background levels (mean value 3.3 μg/ml). These values rose to a mean of 14 μg/ml in sera from these patients obtained at 14 days. The remaining patient also showed a preexisting anti-HMFG1 response but at a higher value of 27 μg/ml which rose to 48 μg/ml.

Figure 3 presents the results of counting SDS gel slices in an investigation of endogenous biotin effects on the biotin binding capacity of circulating streptavidin conjugated antibody. The top-most curve in the figure was obtained by analysis of a serum obtained 10 min post-administration of the conjugated antibody. The middle curve is the result of analyzing serum from the same patient obtained 3 days post-antibody administration. The bottom two curves are the result of ana-

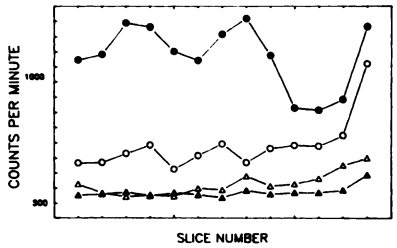

FIGURE 3
Distribution of radioactivity in SDS gels obtained by counting 3-mm gel slices. Upper curve (closed circles) is of serum obtained 10 min after antibody administration; middle curve (open circles), of sera obtained 3 days after antibody administration; bottom curves (open triangles) of serum obtained immediately after biotin administration, (closed triangles) of serum obtained 10 min after biotin administration.

lyzing sera obtained from the same patient immediately after the administration of labeled biotin (3 days post-antibody administration) and 10 min post-biotin administration. The decrease in counts immediately after with respect to immediately before the administration of labeled biotin is evidence that even after 3 days in circulation, streptavidin conjugated antibody was still able to bind biotin and therefore not yet saturated with endogenous biotin.

Two distinct peaks are apparent in the top curve and possible in the middle curve as well. Calibration of the gels used in this investigation show that the conjugated antibody is responsible for the first peak (slices 4–5) while free streptavidin may be responsible for the second (slices 8–9). Analysis by size exclusion HPLC of the conjugated HMFG1 preparation used in this investigation following saturation with ^{111}In-biotin shows a small peak (~20% of protein-bound radioactivity) with the retention time of free streptavidin. The presence of free streptavidin at low concentrations is occasionally observed following purification of the conjugated antibody by Sephadex G200. The increased intensity of a streptavidin peak relative to the antibody peak in the top gel may be attributable to a more rapid clearance rate of the antibody.

This assay was performed on sera from four patients. The counting rate of fractions comprising the first peak (i.e., due to labeled streptavidin-conjugated antibody) showed a decrease of 22, 18, 7, and 0% between serum samples obtained immediately before and immediately after the administration of biotin.

Urine Studies

Figure 4 shows the cumulative urinary radioactivity levels following administration of radiolabeled biotin in one patient. The results have been plotted separately for biotin administration 3 wk (upper curve) and 3 days after (lower curve) the administration of conjugated antibody. Urinary excretion is clearly reduced, as expected, when labeled biotin is administered soon after administration of the conjugated antibody. In both cases, however, excretion of radioactivity is rapid with ~50% of the injected radioactivity clearing in ~1 hr. The mean cumulative excretion of radioactivity over 24 hr in five patients having recently received the conjugated antibody was 71% (9% s.d., n = 5) in contrast to 83% (7% s.d., n = 5) in the same five patients without recent antibody administration. The differences are significant (Student's t-test, $p < 0.05$).

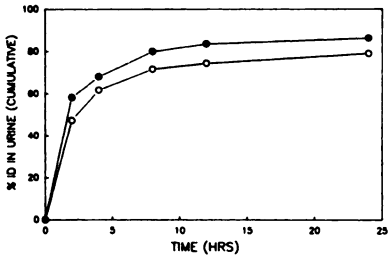

FIGURE 4
Cumulative urinary excretion of radioactivity from one patient plotted separately for each biotin administration. Top curve (closed circles) urinary excretion following biotin administration 3 wk after conjugated antibody administration. Bottom curve (open circles) urinary excretion following biotin administration 3 days after conjugated antibody administration.

Excess avidin was added to aliquots of the total collection of urine from each of four patients and the aliquot analyzed by Sephadex G50 chromatography to determine the percentage of radioactivity bound to avidin and therefore present in urine as labeled biotin. A mean value of 88% of the urine radioactivity was found in the void volume. This is in agreement with a value of 87% obtained in the same analysis of volunteer urine to which ^{111}In-biotin had been added.

DISCUSSION

This study, although preliminary, has succeeded in providing some encouraging results. First, both injectates appear to be nontoxic in humans at least at the doses considered herein. This conclusion is based not only on the absence of clinical manifestations following administration but also on an absence of changes in blood profiles. The conjugated antibody is, however, immunogenic as patients developed antibodies against both the streptavidin and the HMFG1 antibody. The fact that similar low doses of mouse antibody do not generally elicit an immune response (*12*) suggests that the conjugated antibody may be more immunogenic than either streptavidin or HMFG1 alone.

Second, our results suggest that endogenous biotin may be relatively unimportant to this application since the biotin binding capacity of circulating streptavidin-HMFG1 in three out of four patients was not saturated with endogenous biotin in 1–3 days. This was indicated by SDS electrophoresis of serum samples in which labeled biotin had been added in excess. Binding to high molecular weight species in these sera was observed relative to sera saturated with biotin.

Third, we have succeeded in reducing background radioactivity levels drastically in blood and in all organs other than kidneys. Blood radioactivity decreases initially with a short (2.4 min) half-life primarily through kidney clearance such that half of the injected activity was found in urine in ~1 hr. An estimate based on the geometric mean of anterior and posterior whole-body images showed that <1% of the administered dose was present in liver at 24 hr post-administration of labeled biotin while ~2% was present in kidneys. This represents at least an order of magnitude improvement in background levels for liver over the conventional approach using labeled antibodies and is even an improvement in kidney levels of about a factor of 5 (*19*). These low background levels may be partially responsible

for the positive images obtained even at 2 hr post-administration of the label. Another important reason for the positive images may be the specific binding of the labeled biotin to streptavidin at the tumor site. Specific binding is indicated by the decreased urinary clearance of radioactivity 2–3 days after antibody administration relative to that observed either before or 2–3 wk after antibody administration. Specific binding is also indicated by the improved targeting in three patients after the recent administration of conjugated antibody. The fact that targeting was not improved in all patients by this approach may reflect rapid internalization of the antibody such that biotin binding at the tumor site may have been significantly reduced. It is possible that increasing the antibody dose from the 1 mg administered may have mitigated this effect enough to permit increased localization in tumor. The use of 1000 μg of biotin rather than 50 μg, however, appears to have had no significant effect.

The positive images obtained in the majority of patients administered labeled biotin without the prior administration of conjugated antibody may reflect blood pool localization. However, it is also possible that the uptake may be due in part to localization of labeled biotin in tumor. We have recently obtained positive images of tumor growing subcutaneously in nude mice with ^{111}In-labeled biotin but not with labeled DTPA control. An affinity of this labeled biotin relative to other labeled biotin derivates in two tumor cell types growing in culture has also been observed (Rusckowski M, unpublished observations).

In conclusion, if these preliminary results are substantiated in a larger series of patients, especially with different antibodies and in patients with diverse tumor types, then a means will have been found to improve tumor to normal tissue radioactivity ratios. This will have implications for both the diagnosis and therapy of this disease. Furthermore, since favorable results are obtained with this technique within hours of administration, it will be possible to employ radionuclides such as technetium-99m and gallium-68 with physical half-lives now too short for many radioimmunolocalization studies.

ACKNOWLEDGMENTS

The authors wish to thank H. Bushe for the analysis of serum clearance curves, B. Dhokia for ELISA analysis of antibody immunoreactivity and B. Henderson for performing the imaging. This study was supported in part by the Imperial Cancer Research Fund and by Unipath UK (to AAE) and by the US NIH (CA 33029 to DJH). One of us (HPK) wishes to thank the Greek State Scholarship Fund for financial assistance.

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