Anti-Antibody Enhancement of Iodine-131 Anti-CEA Radioimmunodetection in Experimental and Clinical Studies

David M. Goldenberg, Robert M. Sharkey, and Edith Ford

Center for Molecular Medicine and Immunology and Department of Radiology, New Jersey Medical School, University of Medicine and Dentistry of New Jersey, Newark, New Jersey

Imaging of tumors with radiolabeled antibodies, especially when located in the blood-rich visceral organs, may be improved through administration of a second antibody directed against the primary tumor-associated antibody. In hamsters bearing a human colonic carcinoma xenograft producing carcinoembryonic antigen (CEA), we injected donkey anti-goat IgG 24 hr after administration of ^{131}I-labeled goat anti-CEA IgG and achieved enhanced tumor imaging 24–48 hr later, with a significant relative decrease of radioactivity in blood and all major organs except the spleen. In seven of nine patients, this method of anti-antibody clearance of nontargeted radioactive murine monoclonal antibodies revealed sites of cancer, including liver metastases. Characterization of radioactivity in the plasma before and after administration of the second antibody confirmed that complexes were quickly formed between primary and secondary antibodies, and imaging of the patients revealed a rapid uptake of radioactivity in the liver at 2 hr that dissipated within 24 hr. Radioactivity in the spleen gradually increased over time. The method of anti-antibody immunological enhancement of cancer imaging is feasible and may reveal tumor sites missed by conventional imaging.

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Since the first clinical study demonstrating that defined anticancer antibodies carrying iodine-131 (^{131}I) radioactivity can image tumors containing the appropriate antigen target, such as CEA (*1*), numerous reports with different antibodies and radiolabels have confirmed the general efficacy of this method for the noninvasive disclosure of known and occult cancers (reviewed in *2–4*). This method, called radioimmunodetection or RAID (*5,6*), has been found to require some form of background, nontarget radioactivity subtraction, such as using blood-pool and interstitial agents labeled with a second radionuclide of an energy that is different from that of the radionuclide conjugated to the tumor-locating antibody, when imaging is performed within 48 hr (*1,4,7*). The different pharmacokinetics and physical properties of the two radiopharmaceuticals can lead to a misinterpretation of the images, especially when using ^{131}I attached to the specific antibody, since radioiodine is also taken up by the thyroid, gastric mucosa, sometimes the intestinal mucosa, and is excreted through the urine, thereby showing radioactivity in the urinary bladder (*4,8*). However, we have achieved an accuracy of ~ 90% in disclosing sites of tumor (primary and metastatic) in colorectal cancer patients studied by RAID with subtraction (*9*).

In addition to compensating for nontarget radioactivity by dual-isotope subtraction techniques, the nontumor-bound antibody can be actively removed from the circulation and tissues by administration of a second, anti-antibody directed against the first, anti-cancer antibody bearing the imaging radionuclide. The immune complex formed is cleared from the blood by the reticuloendothelial system. We report here that administration of anti-antibody is a feasible approach for improved cancer RAID.

METHODS AND RESULTS

Adult female golden hamsters (*Mesocricetus auratus*) weighing 80–100 g were grafted in both cheek pouches with GW-39 human colonic carcinoma cells that produce copious quantities of CEA (*10*). After 7 days, when the cheek pouch

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For reprints contact: David M. Goldenberg, Center for Molecular Medicine and Immunology, 1 Bruce St., Newark, NJ 07103.

tumors weighed 0.21 ± 0.09 g, the hamsters were injected intracardially (IC) with 10 μg (0.15 mCi) of [^{131}I]anti-CEA IgG prepared in goats and affinity-purified as described previously (*11*). After radioiodination with ^{131}I by the chloramine-T method (*12*), the immunoreactivity of the antibody was found to be unaffected (70% by passage over a CEA-immunoadsorbent column). Twenty-four hours after injection of the primary antibody (PA), one group of five hamsters received 50, 100, 250, or 400 μg of affinity-purified donkey anti-goat (DAG) IgG IC, while another control group was not given the second antibody (SA). The SA doses were administered in ratios of 5, 10, 25, or 40:1, respectively, to the PA dose. The animals were imaged with a gamma camera* and killed at 4, 24, 48, and 72 hr after injection of SA. Imaging was done with the animals placed in a prone position on the face of a high-energy collimator collecting 30,000 counts/animal. Due to a high level of radioactivity released in the urine of animals given SA, we found it necessary to place lead shielding (3–4 mm) over the extreme lower portion of the animals to permit a greater portion of the count rate to be derived from the remaining torso.

The first question studied involved the ratio of SA:PA that is suitable for reducing the level of circulating PA radioactivity while maximizing tumor/blood ratios. Table 1 summarizes the percent injected dose per gram blood from 4 hr to 72 hr after the administration of the SA in comparison to animals that were not given the SA. The actual SA:PA ratio at the time the second antibody was administered as determined by the specific activity of the radiolabeled PA is also given. Our previous experience has shown that prior to SA, > 95% of the circulating radioactivity in the hamsters is native IgG. The amount of radioactivity in the blood was not appreciably changed at a SA:PA ratio of 5:1, but by increasing the amount of SA, a very rapid and significant decrease in the amount of circulating radioactive PA was observed. As shown in Table 2, at a SA:PA ratio of 25:1, significantly improved tumor/blood ratios were achieved already at 4 hr following SA application (or 28 hr after PA), while significant elevations in tumor/liver ratios were found at 24 hr and later after SA was given. As time progressed following SA application, the tumor/blood and tumor/liver ratios increased considerably, reaching 57.3:1 and 33.8:1 for each, respectively, at 72 hr post-SA injection. Although the 40:1 SA/PA ratio reduced blood radioactivity levels more than the 25:1 ratio, tumor/blood and tumor/liver ratios were not significantly different (data not shown). A comparison of the various observation times following SA application suggests that the time of 48 hr would provide the best imaging results. Figure 1 shows the imaging results of hamsters with or without SA application, indicating the advantage of the SA clearance method of RAID.

TABLE 2
Tumor/Nontumor Ratios Between SA-Treated and Control Hamsters Receiving Radiolabled Antitumor Antibody*

Hours post-SA	Tumor/Blood		Tumor/Liver	
	SA	Control	SA	Control
4	2.5 ± 0.7†	0.8 ± 0.3	2.4 ± 1.2	1.3 ± 0.7
24	6.5 ± 2.1†	0.9 ± 0.4	10.5 ± 3.7†	2.6 ± 1.0
48	42.5 ± 9.6†	1.2 ± 0.2	35.0 ± 15.1†	5.5 ± 1.9
72	57.3 ± 22.4†	2.1 ± 0.9	33.8 ± 8.3†	6.5 ± 2.0

* Values are means ± s.d., n = five animals, ten tumors.
† Values significantly higher than the control animals, with $p \leq 0.02$, as determined by a one-way analysis of variance with a one-tailed F-test.

On the basis of these encouraging experimental results, clinical trials with anti-antibody enhancement of RAID were undertaken. In place of goat anti-CEA PA, a murine monoclonal antibody against CEA, designated NP-3 (*13*), purified by protein A adsorption, was used with goat anti-mouse (GAM) IgG as the SA. The anti-CEA immunoreactivity of the murine antibody was unaltered at 95% after radioiodination, and gel filtration chromatography revealed that the radioiodinated preparation was over 95% monomeric IgG. The goat anti-mouse IgG antiserum (Pelfreeze) was purified by sequential passage over a human serum and a mouse IgG immunoadsorbent. After radiolabeling, it was found that 70% of the purified goat anti-mouse IgG bound to a murine IgG immunoadsorbent. The percent binding of this second antibody to its specific immunoadsorbent was similar to the percent binding of radiolabeled donkey anti-goat IgG to a goat IgG immunoadsorbent (data not shown).

After suitable quality control testing for sterility, pyrogenicity, and acute toxicity, and securing informed consent in accordance with our Institutional Review Board's guidelines, 15 patients with confirmed cancer were studied, of which nine proved to be evaluable because of adequate follow-up data

TABLE 1
Effect of Second Antibody/Primary Antibody (SA/PA) Dose Ratios on Clearance of PA

	SA/PA ratios				
	Control	5:1 (13:1)*	10:1 (27:1)	25:1 (67:1)	40:1 (107:1)
Hours post SA	Percent injected dose per gram blood				
4	2.0 ± 0.6†	1.2 ± 0.4	0.5 ± 0.2	0.3 ± 0.2	ND‡
24	1.2 ± 0.3	0.7 ± 0.2	0.4 ± 0.2	0.08 ± 0.04	0.08 ± 0.06
48	0.7 ± 0.2	0.6 ± 0.3	0.3 ± 0.1	0.05 ± 0.03	0.01 ± 0.01
72	0.5 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	0.03 ± 0.01	0.008 ± 0.005

* Values in parentheses are actual SA/PA ratios based on the average amount of PA in the blood when the SA was given.
† Mean ± s.d.
‡ ND (not determined).

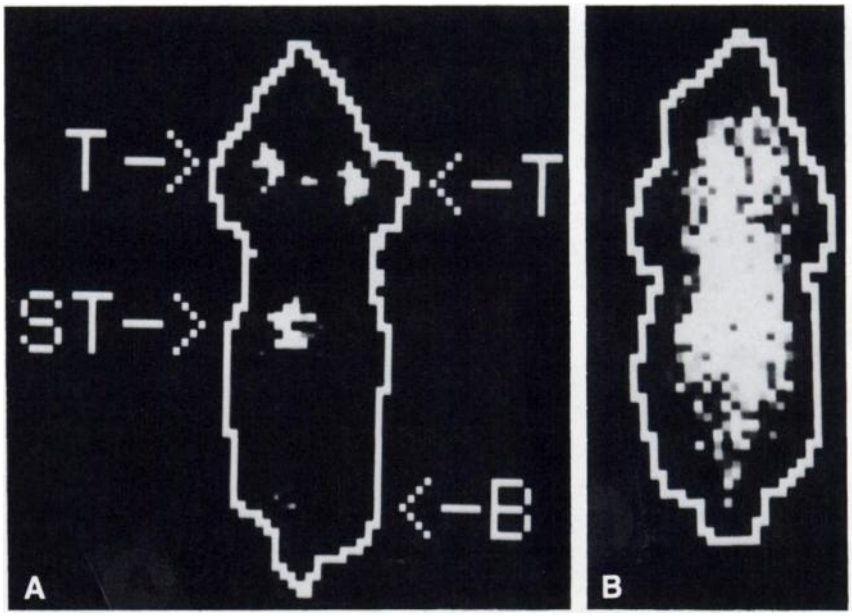

FIGURE 1
Imaging results in hamsters bearing human GW-39 tumors in both cheek pouches. The control animal in (B) did not receive an anti-antibody, but was imaged at the same time as was the hamster in (A), 24 hr after injection of the SA (or 48 hr after the PA application). A total of 30,000 counts were collected for each image and the images were then adjusted to an identical level of image intensification and background reduction. Tumors in each animal were 0.4–0.6 g.

being available at the time of this report. The patients received PA doses of 225–500 μg (3.5–5.4 mCi), followed 24–48 hr later with a SA dose of 1–5 mg IgG protein. All patients received Lugol's iodine and potassium perchlorate as previously described (*1*). The results are summarized in Table 3. The SA/PA ratio was calculated on the basis of circulating radioactivity measured at the time of the SA injection, while the % clearance PA was determined by comparing circulation radioactivity 24 hr after injection of SA to that at the time of the SA injection. Positive imaging results indicate correct disclosure of tumor metastasis, while negative results mean that known tumor(s) was missed. Patients were considered positive only when all known lesions were positive. There was rapid elimination of the PA in Patient No. 745 even before administering the anti-antibody, which may account for failure to image the tumor even when conventional dual-isotope subtraction RAID was used. Table 3 indicates that seven showed positive scan results with SA enhancement of RAID, whereas two failed. Interestingly, the two failures with SA-RAID were also false-negative results with conventional subtraction imaging using a similar radiolabeled primary antibody. In one patient (739) a colonic tumor metastasis found with SA imaging was not disclosed by our conventional RAID study using dual-isotope subtraction. In those patients in whom SA image enhancement was not observed, immune complexes between PA and SA did initially form, as revealed by gel filtration and affinity chromatography, but the ensuing blood-pool activity appeared to be less diminished compared with individuals in whom SA was effective. However, this preliminary observation needs to be studied further. It appears generally that sufficient SA needs to be administered to achieve a marked reduction of the radioiodinated PA. No untoward effects were noted in any of the patients studied. Plasma samples were taken from several patients before and after administration of the second antibody and characterized

TABLE 3
Clinical Results with [^{131}I]Anti-CEA Monoclonal Antibody NP-3 (PA) and Anti-Antibody (SA) RAID*

Patient no.	Primary cancer	Serum CEA (ng/ml)	Time SA (hr)	SA/PA	% Clearance of PA	Imaging results
708	Stomach	222.0	24	50	ND†	Pos.
716	Stomach	2.6	24	100	87	Pos.
723	Lung	9.9	24	62	94	Pos.
729	Rectum	326.0	24	133	ND	Pos.
736	Lung	36.0	48	24	21	Neg.
737	Colon	76.0	48	54	ND	Pos.
739	Colon	5.4	48	17	63	Pos.
742	Colon	24.0	24	102	ND	Pos.
745	Lung	24.7	48	100	71	Neg.

* The anti-antibody (SA) was administered at 24 or 48 hr after the PA injection.
† Not determined.

TABLE 4
Characterization of Radioactivity by Gel Filtration and Immunoaffinity Chromatography in Patients Given ^{131}I-NP-3 Followed 24 hr Later by Second Antibody

Patient no.	Sephacryl-200			% Immunoreactivity*			
	Void	IgG	Vi	CEA	GAM	GAH	DAG
^{131}I-NP-3†	0.6	98	0.4	96	98	0.5	0.7
708							
24 hr‡	90	4.0	6	52	72	0.2	0.4
26 hr	74	4.0	17	52	23	2.0	66
723							
24 hr	15	79	3	93	80	0.2	0.9
48 hr	11	0.3	82	21	6	ND	3.0

* Immunoadsorbents listed below were prepared by coupling CEA, goat anti-mouse IgG (GAM), goat anti-human Ig (GAH), or donkey anti-goat IgG (DAG) to either Affi-gel 10 or Sepharose 4B.
† Quality control analysis of typical radioiodinated NP-3 preparation prior to administration to patients.
‡ Time postinjection of ^{131}I-NP-3.

by gel filtration and immunoaffinity chromatography. Table 4 summarizes the characteristics of the blood radioactivity in two of the patients. Patient plasma was analyzed immediately prior to the administration of second antibody or at 2–24 hr after the second antibody by passage over a 1.6 cm × 90 cm Sephacryl-200 column. The percentage of total recovered activity was determined in three separate fractions, the voided fraction (molecular weight ≥ 300,000), native IgG fraction, and included volume (Vi; small molecular weight radioactivity). Plasma samples were also passed over immunoadsorbents and the percentage of total recovered activity bound to each adsorbent is given. In Patient 708, 90% of the radioactivity in the plasma 24 hr after administration of ^{131}I-NP-3 was voided by a S-200 column (molecular size ≥ 300,000), probably due to complexing with the high amount of CEA in the plasma (222 ng/ml). In other studies, we have found that this antibody quickly complexes with antigen in patient plasma (*14*). Although a reduction in immunoreactivity against CEA was found in comparison to the pre-injected NP-3, it is interesting that 52% of the radioactivity could still bind to a CEA-immunoadsorbent. Similar retention of immunoreactivity against CEA, despite the presence of a high percentage of high molecular weight radioactivity, was also seen in other patients (data not shown). These findings are consistent with our previous studies using polyclonal anti-CEA antibody (*15*). In Patient 708, there was also a reduction in the binding to the goat anti-human Ig (GAH)-immunoadsorbent. There was no evidence of human anti-mouse antibody (HAMA) since there was no binding of the radiolabeled NP-3 anti-CEA murine monoclonal antibody to the GAH-immunoadsorbent. Within 2 hr after administration of the second antibody (26 hr post-PA), there was a decrease in the voided fraction and a concomitant increase in the presence of small molecular weight radioactivity. Although the immunoreactivity against CEA was unaffected, the binding to GAM-immunoadsorbent was reduced by about threefold while the binding to DAG-immunoadsorbent increased to 66%. This suggests that the goat anti-mouse IgG second antibody complexed with ^{131}I-NP-3 and the immune complexes formed by this interaction were being more rapidly metabolized as evidenced by the increase in small molecular weight radioactivity. An increase in the metabolism of the radiolabeled NP-3 after administration of the second antibody is also suggested by the tremendous increase in small molecular weight radioactivity in the plasma of Patient 723 at 48 hr (24 hr post-SA). In addition, the data from this patient's plasma suggest that the second antibody can bind to the primary antibody even when the primary antibody is not complexed with antigen, altering the metabolism of the antibody.

In one patient, the kinetics of circulating radioactivity with and without SA was determined (Fig. 2), demonstrating the rapid fall of PA radioactivity after administration of SA. There was no HAMA activity detected in this patient in either study. This 38-yr-old white male had surgical removal of an adenocarcinoma at the gastro-esophageal junction in June, 1985. His blood CEA level showed a continuous rise from July. Radiological studies performed in September did not demonstrate unequivocally any metastatic disease, and the patient was referred for a RAID examination in December, 1985. Figures 3 and 4 show the results of the SA imaging study compared with radioactive PA without SA, in which metastatic foci in the patient's liver are seen only in the SA scan. This was then confirmed by a transmission computerized tomogram (CT) performed in January, 1986, as is shown in Figure 3E. A conventional radioantibody imaging study, using ^{99m}Tc subtraction (*1*), was performed on the same Patient 1 wk earlier, and abnormal radioactivity could only be seen in the region of the gastro-esophageal junction (Fig. 3F), not the liver. The extensive accumulation of radioactivity in the patient's spleen (Fig. 3D) indicates that this is one of the major organs of accumulation of the immune complexes formed in the blood. The liver is another organ primarily responsible for the clearance of immune complexes, but unlike the spleen, the liver showed a more rapid clearance of nonspecific ^{131}I radioactivity.

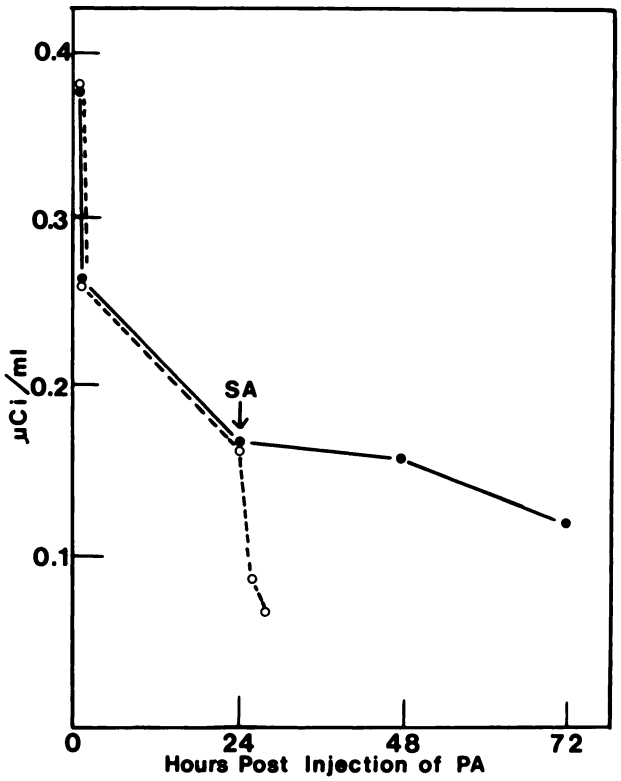

FIGURE 2
PA radioactivity clearance from the blood in Patient No. 708. Solid line shows blood radioactivity over 3 days, 1 wk before SA study was performed. In comparison, broken line indicates results obtained when anti-antibody (SA) was administered 24 hr after the PA. Blood radioactivity was determined at 2 and 4 hr after injection of the PA. Although percent clearance at 24 hr could not be determined, the graph indicates that rapid elimination of PA radioactivity was achieved.

DISCUSSION

Our experimental and clinical data suggest that immunological clearance of tumor-localizing radiolabeled antibody by anti-antibody can enhance tumor/blood and tumor/nontumor target ratios, thus permitting early imaging of cancer without the need of the dual

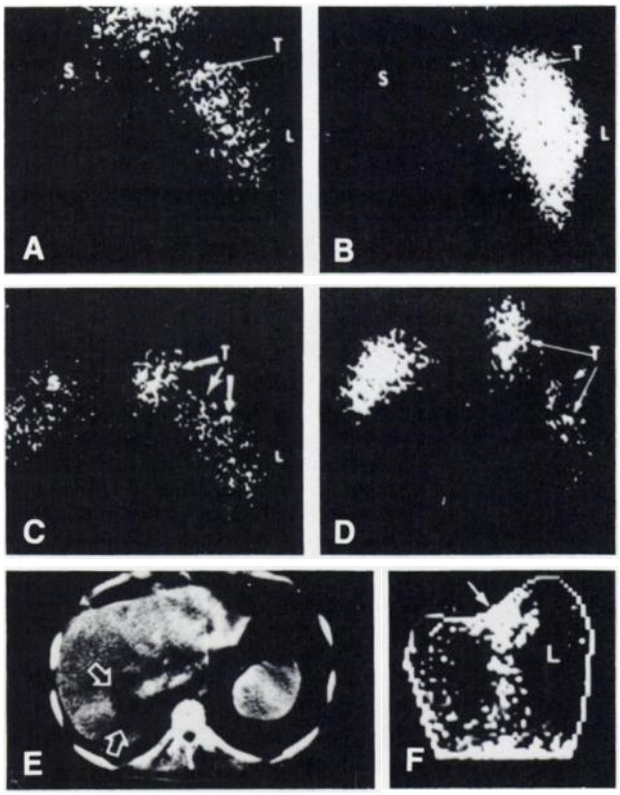

FIGURE 3
RAID imaging results in Patient No. 708 showing influence of anti-antibody administration. Posterior abdominal views showing diffuse liver radioactivity (L) before SA (A; 24 hr post-PA). Arrow (T) indicates area of increased activity in region of gastroesophageal junction. By 2 hr after the administration of SA (B), an increase in diffuse radioactivity in the liver is seen, but by 24 and 48 hr after the SA (C and D, respectively) the diffuse activity in the liver diminishes with the identification of two foci of increased radioactivity (T-arrows) in the liver. SA-RAID also shows increased radioactivity in spleen (s). Liver metastases were confirmed 4 mo later by CT scans, as shown by arrows in (E). (F) shows the only positive area observed in this same patient one week before the SA study, when a conventional radioimmunodetection-subtraction procedure with ^{131}I-NP-3 and technetium-99m human serum albumin and technetium-99m pertechnetate was performed. The subtracted posterior abdominal image is shown. A region of interest excluding the heart has been drawn. The area of intense radioactivity is in the region of the gastro-esophageal junction (arrow). No abnormal radioactivity is seen in the liver (L), in contrast to the SA images (C, D).

isotope subtraction method we developed with the introduction of the use of radiolabeled anti-cancer antibodies for tumor imaging (*1,4,7*). These findings thus agree with earlier animal studies (*16,17*), and support the view that such animal models may predict similar relationships in humans. Based upon these results, we believe that the use of liposome-entrapped second antibody, as originally suggested (*18*), does not appear to offer any advantage over use of free anti-antibody (*16,17*).

Both the hamster and human studies indicated that the only organ consistently showing increased accretion of ^{131}I radioactivity presumably due to radiolabeled SA/PA complexes was the spleen (Fig. 3). However, since this site is rarely involved with solid tumors, it does not present a problem in interpreting abdominal images. The initial diffuse radioactivity noted in the liver, which is another reticuloendothelial organ contributing to metabolism of antigen-antibody complexes, was not constant, and may be due to the dehalogenation known to occur at this site. Gel filtration and immunoaffinity chromatography of patient plasma revealed that a reduction of immunoreactivity with CEA was not found for the primary antibody after injection of the second, anti-antibody; a large portion of the circulating radioactivity could still bind to CEA even when complexes between the primary and secondary antibody were present. Complexation of the injected primary anti-CEA antibody with circulating CEA also contributed to rapid formation of low molecular size ^{131}I radioactivity. Another factor which could contribute to the liberation of low molecular size ^{131}I is the evocation of a HAMA response, and may indeed affect the ability of the primary antibody to bind to the antigen target, as well as the complexation of secondary to primary antibody. The enhanced liberation of low molecular radioactivity, presumably in the form of free ^{131}I, results in the usual uptake of radioactivity in the thyroid, gastric mucosa, and urinary bladder. Although there was enhanced activity in the stomach area in one case, this was at the site of a resected gastro-esophageal carcinoma, which was also disclosed by conventional subtraction RAID, and probably constituted tumor recurrence. This patient had liver metastases confirmed by transmission computed tomography. Whereas the anti-antibody study revealed these liver lesions, a conventional subtraction RAID scan or the pre-SA ^{131}I-antibody study failed to disclose these tumor sites. Further, in the two patients in whom SA scans failed to reveal tumor(s) known to be present, subtraction RAID studies with the same ^{131}I PA also failed to detect these lesions.

With ^{111}In-labeled antibodies, tumors outside of the liver may be imaged without subtraction after 3–5 days (*19,20*), but deep-seated tumors, especially near the liver and spleen, are seen only with difficulty using current methods of chelating ^{111}In to antibodies. The use of F(ab′)$_2$ fragments of IgG antibodies has been suggested as a means of improving images, since they do not bind specifically to Fc receptors of normal cells and because they clear from nontarget organs more rapidly than whole IgG does (*21*). This has been substantiated in animal (*22–25*) and less strikingly in clinical (*26–27*) studies. The use of single photon emission computed tomography may enhance our ability to image even deep-seated tumors without the use of computer-assisted subtraction, but the full advantage of this

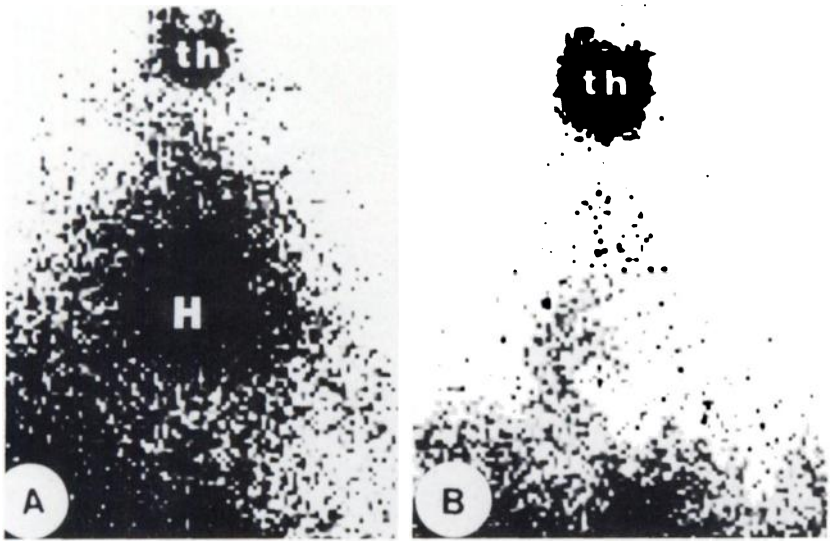

FIGURE 4
Anterior chest views before (A) and 24 hr after (B) anti-antibody injection, showing elimination of cardiac (H) radioactivity (th, thyroid). A total of 200,000 counts were collected for each image. Both images were adjusted identically for image intensity.

instrumentation and procedure needs further comparative evaluation.

We conclude from these initial studies that the method of anti-antibody immunological enhancement of cancer imaging is feasible, that it may reveal tumor sites missed by conventional subtraction RAID, and that it may also have application for antibody-mediated isotopic therapy by enhancing relative deposition of radioantibody in tumor. However, we appreciate that a number of important questions regarding this new imaging and potentially therapeutic antibody technology need to be addressed. Of major importance are the relationships between SA dose, time of SA administration, antigen-PA complexes or complexes of PA with HAMA in regulating clearance and target localization of PA by the anti-antibody. Patient variability in processing the complexes induced, as well as possible untoward effects resulting from circulating immune complexes, also need to be studied in more detail. Finally, we are interested in determining whether a SA made specifically against our imaging antibody, as compared with general anti-mouse IgG antibody, is more effective in enhancing target imaging, and whether this controlled clearance mechanism by SA has any advantage over the use of other forms of PA which are cleared rapidly, such as $F(ab')_2$ or Fab fragments. Experimental and clinical studies directed toward resolving some of these issues are in progress.

NOTE

* (Technicare Omega 500) Technicare, Solon, OH.

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