Two-Step Immunoscintigraphy for Non-Small-Cell Lung Cancer Staging Using a Bispecific Anti-CEA/Anti-Indium-DTPA Antibody and an Indium-111-Labeled DTPA Dimer

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Immunoscintigraphy (IS) using anti-CEA F(ab')$_2$ monoclonal antibody (MAb) is useful for improving mediastinal staging of nonsmall cell lung cancer (NSCLC), but the technique was limited because of an insufficient contrast between tumor and normal tissues. The aim of this study was to determine if the method could be improved by a two-step method which uses a bispecific anti-CEA/anti-di-DTPA antibody (Bs-MAb) and ^{111}In-labeled di-DTPA-tyrosyl-lysine bivalent hapten. **Methods:** Twelve patients were intravenously given a 30 min Bs-MAb infusion (0.1 mg/kg). Four days later, they were injected intravenously with 0.1 μg/kg hapten labeled with 185 MBq ^{111}In (5 mCi). Images were recorded immediately and 6 and 24 hr after hapten injection. A pharmacokinetic analysis was performed. Surgery was performed 3 days after ^{111}In-hapten injection, and samples of tumor and normal tissues were collected for immunohistochemical and biodistribution studies. IS results were classified as true-positive (TP), false-positive (FP), true-negative (TN) or false-negative (FN) according to the surgical data. **Results:** Primary tumors were visualized in nine patients. The contrast was excellent, generally higher than that obtained with direct labeling of anti-CEA. In the mediastinum, IS results were (after surgery) five TN, two TP and one FP. One case remains undetermined. The FP result was due to a Bs-MAb uptake in intrapulmonary lymph nodes. IS was in agreement with preoperative staging in six of these nine patients and discordant in three. **Conclusion:** Our study confirmed that the two-step method whith a bispecific antibody could greatly improve the performances of IS for lung cancer staging.

Key Words: non-small-cell lung carcinoma; staging; two-step immunoscintigraphy; carcino-embryonic antigen

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Mediastinal lymph node involvement is a very strong prognostic factor for non-small-cell lung carcinoma (NSCLC), which determines the potential for surgical resection. CT scanning is, at present, the best routinely available technique to assess this involvement, but it has some limitations: CT cannot depict neoplastic involvement of normal-sized lymph nodes. On the other hand, it retains as pathological some enlarged lymph nodes which sometimes are simply inflammatory.

PET imaging using [^{18}F]FDG are now recognized as an excellent method, but PET is not available in all nuclear medicine departments (*1*). We previously showed that immunoscintigraphy (IS) using anti-CEA F(ab')$_2$ monoclonal antibody (MAb) could provide useful information about mediastinal staging of NSCLC, but the technique was limited because of an insufficient contrast between tumor and normal tissues (*2*). This contrast could be improved by a two-step method, called the "affinity enhancement system" (AES), which uses a bispecific anti-CEA/anti-di-DTPA antibody (Bs-MAb) and ^{111}In-labeled di-DTPA-tyrosyl-lysine bivalent hapten (*3*). This method has been previously used with success for the detection of medullary thyroid carcinoma and colorectal tumors (*4,5*). In this study, we report our first results in NSCLC staging with AES in 12 patients classified as relevant for surgical treatment.

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MATERIALS AND METHODS

Patients

Twelve patients with NSCLC diagnosed by bronchoscopy were enrolled in the current study after giving written informed consent. Table 1 summarizes their relevant clinical characteristics. The protocol was approved by the local ethical commitee, according to the French Clinical Trial legislation.

Clinical TNM Staging

All patients underwent a complete metastasis workup including CT scan of the brain, chest and upper abdomen, chest radiograph and liver ultrasound, bone scintigraphy and fiber-optic bronchoscopy. All patients were classified according to the TNM classification and were considered as suitable cases for surgical treatment (*6*).

Nodes were considered as pathological when their diameter on CT scan exceeded 15 mm. Below this size, patients were classified as N0. The six patients who were classified as N1 or N2 had pathological lymph nodes the size of which never exceeded 35 millimeters. All patients had pulmonary function tests before surgery.

Bs-MAb Immunoscintigraphy

The production of (anti-CEA, anti-In-DTPA) bispecific Fab'-Fab antibody (Bs-MAb) and the synthesis of ^{111}In-labeled *N*-α-(In-DTPA)-tyrosyl-N-ε-(In-DTPA)-lysine (^{111}In-di(In-DTPA)-TL) have been previously described (*5*).

Patients were intravenously given a 30-min Bs-MAb infusion (0.1 mg/kg in 100 ml of NaCl 9 g/liter) 4 days before hapten injection. At day 0, they were injected intravenously with 0.1 μg/kg di-(In-DTPA)-tyrosyl-lysine labeled with 185 MBq ^{111}In (5 mCi) and diluted in 5 ml of 9 g/liter NaCl solution.

Image acquisition sequences were performed immediately and 6 and 24 hr after hapten injection. Immediately after injection, they consisted in two static thoracic views (posterior and anterior, 5 min each) and a 10-min tomographic acquisition of the thorax (64 steps); these early planar and tomographic views were considered as blood pool activity images. Six and 24 hr after injection, acquisitions consisted in a whole-body scan (8 cm/min) and a 40-min tomography (64 steps) of the thorax. All acquisitions were performed using a rectangular large field of view camera tuned to 173 and 247 keV energy peaks with 20% windows. At 24 hr,

TABLE 1
Bs-MAb Immunoscintigraphy Results Related to Clinical Staging, Immunohistochemistry and Final Diagnosis

Patient no.	Histology	CEA (ng/ml)	Initial staging T	N	M	Immuno-histochemistry	IS Results Primary tumor	Mediastinum	Final diagnosis	Biodistribution* Tumor*	Blood*	Tumor/ blood†	Urine recovery after 24 hr (%)	Ta (hr)
1	SCC	1.4	1	0	0	−	+	±(CL)	pT2N1	1.95	1.04	1.87	54.7	9.24
2	AC	4.2	2	0	0	++++	++++	−	pT2N0	4.66	0.83	5.61	38.9	11.00
3	AC/SCC	3.7	1	0	0	+++	−	−	pT3N0	3.70	1.20	3.08	37.6	14.44
4	SCC	1.7	2	2	0	+	+	±	pT2N1	6.69	2.12	3.15	64.9	14.44
5	LCC	8.1	2	2	0	++++	++++	−	pT2N0	7.30	1.80	4.05	25.7	12.38
6	SCC	3.3	2	2	0	−	−	−	pT4N1	2.06	1.21	1.70	41.7	18.73
7	SCC	2.6	1	0	0	±	±	−	pT1N0	nd	0.95	nd	(8.4)‡	21.00
8	SCC	2.1	2	0	0	−	+	−	pT1N1	0.20	0.96	0.21	72.3	13.59
9	AC	4.7	3	2	0	+	+	+	pT4N2	2.30	0.7	3.30	58.8	12.84
10	SCC	3.0	2	0	0	−	±	−	pT2N0	2.60	1.78	1.46	49.6	11.95
11	SCC	1.7	2	0	0	nd	+	+	pT2N2	5.67	3.96	1.43	nd	nd
12	SCC	1.0	2	0	0	++	−	−	pT2N0	nd	nd	nd	nd	nd

*.10^{-3} % injected activity/g

† ^{111}In-di-(In-DTPA)-TL uptake ratio at the time of surgery.

‡Urine collection was certainly not complete.

SCC = squamous-cell carcinoma; AC = adenocarcinoma; LCC = large-cell carcinoma; CEA = carcinoembryonic antigen; IS = immunoscintigraphy; nd = not done; Ta = half-life of the blood exponential decrease of ^{111}In-di(In-DTPA)-TL activity.

anatomical landmarks were obtained by injecting 185 MBq ^{99m}Tc-methylene diphosphonate (5 mCi) 2 hr before acquisitions (visualization of bone structures and bladder) and 74 MBq ^{99m}Tc-albumin (2 mCi) 5 min before tomography. Images of the anatomical structure were acquired at the same time as those of di-(In-DTPA)-tyrosyl-lysine distribution, using a 20% window centered on the photopeak of ^{99m}Tc (140 keV). Tomographic sections were reconstructed using a Wiener filter and a Sophy A computer interfaced with the Sophy camera.

All images were interpreted in a blinded manner, i.e., without knowledge of primary tumor localization, clinical staging results or CT scan results. Planar views were visualized on a grey scale and tomographic sections on a scale of 16 colors, both with 256 levels. In the tomographic mode, the criterion for considering an uptake area to be pathologic was its appearance in at least three successive sections in three section planes.

Pharmacokinetics Analysis

Blood samples were drawn 1, 3, 6, 10 and 24 hr after ^{111}In-di-(In-DTPA)-tyrosyl-lysine injection; urine was collected between 0 and 24 hr after hapten injection. Indium-111 radioactivity was determined in 1 ml of serum on each blood sample and urine pool. Exponential curves were fitted to individual plasma radioactivity data corrected from the physical decay of ^{111}In (as percentage of injected activity per milliliter) by nonlinear least square regression.

Surgical Procedure, Immunohistochemistry and Biodistribution Data

Surgery was performed 3 days after ^{111}In-di-(In-DTPA)-tyrosyl-lysine injection. The tumors were resected and biopsies of muscle, lymph nodes (suspect or not) and normal lung were taken; a blood sample was drawn during intervention. Biodistribution was determined by weighting and counting blood and biopsy samples collected during surgery. Surgical tumor samples were immunohistochemically stained for CEA using antibodies directed against CEA on paraffin sections. Endogenous peroxidase was blocked in ethanol-30% hydrogen peroxide for 10 min, followed by ethanol for 5 min. Sections were then washed thoroughly in tap water. Nonspecific protein staining was blocked using normal rabbit serum and human group AB serum in phosphate buffer saline. After incubation with the primary antibody, slides were exposed to the secondary biotinylated rabbit antimouse immunoglobulin, followed by the avidin-biotin complex (Dakopatts, Glostrup, Denmark) amplification system. The slides were treated with diaminobenzadine and hydrogen peroxide for 2 min and counterstained with hematoxylin. A control slide, in which normal mouse antibody was substituted for the primary antibody, was included. A minimum of 10% positive cells was required for the tumor to be classified overall as positive for CEA. Intensity of CEA expression was classified semiquantitively into five categories: (-), (+), (++), (+++) and (++++) corresponding respectively to < 10, ≥ 10 and < 20, ≥ 20 and < 50, ≥ 50 and < 80 and ≥ 80% of positive cells.

CEA Serum Levels and Human Anti-Mouse Antibodies

CEA serum levels before Bs-MAb injection were determined with the immunoradiometric kit RIA-GNOST CEA purchased from Behring, as described by the manufacturer. Human anti-Bs-MAb concentrations were assayed in serum the day of Bs-MAb injection and then on days 15, 30, 60 and 90 by a previously described one-step sandwich radioimmunoassay (*5*).

RESULTS

The 12 patients completed the study. No toxicity and no adverse reactions were observed.

The mean plasma CEA level was 3.5 ± 1.9 ng/mliter (range 1.4–8.1).

Before surgery, eight patients were considered as being free of mediastinal involvement; four patients were classified as N2. All patients were operated on. Surgical assessment confirmed preoperative staging in all but four cases. In three patients, mediastinal lymph nodes involvement was not confirmed, and in one case the patient was scored pN2 whereas he was initially N0.

IS Results

In three patients, primary tumors were not detected by IS; these patients were not considered for analysis of results obtained in the mediastinum. Immunoscintigraphy was negative for the mediastinum in all three cases (in agreement with the later surgical results) (Table 2). However, in the absence of uptake in the known tumor, it did not appear valid to consider

TABLE 2
Repartition of Patients According to IS Results and Surgical Findings in the Mediastinum

		IS					
	Primary tumor	+			−		
	Mediastinum	+	−	total	+	−	total
Surgical result	+	2	0	2	0	0	0
	−	1	5	6	0	3	3
For mediastinum	na	1	0	1	0	0	0
Total		4	5	9	0	3	3

these three cases as true negative regarding the mediastinum. It must be noted that immunohistochemistry performed after surgery was positive for CEA in two cases, and that in at least one of these patients, biodistribution seemed favorable (data are not available for the other patient). Both cases (Patients 3 and 12) represent false-negatives. The third patient had a tumor that was entirely negative for CEA by immunohistochemistry, and this patient can be considered as a true-negative from the immunological point of view.

Primary tumors were visualized in the nine other patients. The contrast was excellent, generally higher than that obtained with direct labeling of anti-CEA monoclonal F(ab')2 (Fig. 1). Semiquantitative evaluation in each patient showed a tumor-to-liver ratio greater than 1, which allowed tumor visualization without any saturation of the images. Tumors can be visualized from planar images, which can be done in exceptional cases with direct labeling of antibodies. This improved contrast is related to a decreased circulating activity compared to that obtained with directly labeled antibodies or even F(ab')2 fragments. However, the circulating activity is not nil, as shown by the visualization of activity of cardiac cavities and kidneys 24 hr after the injection.

Among these nine patients, IS was negative in the mediastinum in five cases, which were five true-negative (TN) after surgery and positive in four. Of these four positive cases, two were true-positives (TP) (Fig. 2), and one was a false-positive (FP) because of a Bs-MAb uptake in intrapulmonary lymph nodes, which are difficult to distinguish from hilar lymph nodes on scintigraphy scans. The last case remains undetermined, as IS showed a contro-lateral focus in the mediastinum which could not be evaluated by the surgeon during thoracotomy. In six of these nine patients, IS was in agreement with preoperative staging, i.e., four TN and two TP (staging was confirmed by surgery in all these six cases). In one patient (Patient 4), staged as N2, IS showed a low-contrast focus in the mediastinum, but surgically this patient was free of mediastinal involvement (pN1): So it was a FP case of both CT scan and Bs-MAb IS. In another patient (Patient 5), IS was negative in the mediastinum, although he was considered as N2; surgery showed the absence of mediastinal involvement (pN0). In the last case (Patient 11), IS was positive in the mediastinum although the patient was considered as N0, and surgery showed a mediastinal involvement.

Patient 2 is of particular interest. This patient was considered as operable on CT, despite N2 classification. A preoperative chest radiograph showed a contro-lateral image, which was suspect of malignancy. That fact, together with N2 stage, would have excluded surgery for this patient. However, the fact that IS was strongly positive in the tumor but otherwise negative

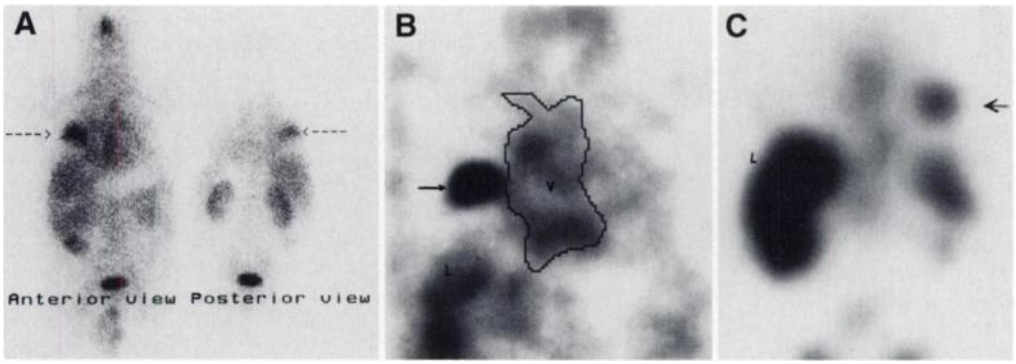

FIGURE 1. (A) Anterior and posterior planar views were obtained 24 hr after injecting ^{111}In-di-(In-DTPA)-tyrosyl-lysine in Patient 5. The tumor (shown by the arrows) is clearly visible. (B) Frontal tomographic section obtained in the same patient, which shows tumor uptake with excellent contrast (arrow), whereas liver (L) and vascular (V) activities were very low. (C) For comparative purposes, a frontal section obtained in a patient (not included in this study) who received the anti-CEA antibody as the F(ab')2 fragment directly labeled with ^{111}In. The tumor (arrow)/liver (L) ratio is clearly less favorable (it was a lung carcinoma strongly expressing CEA (>80% of the cells).

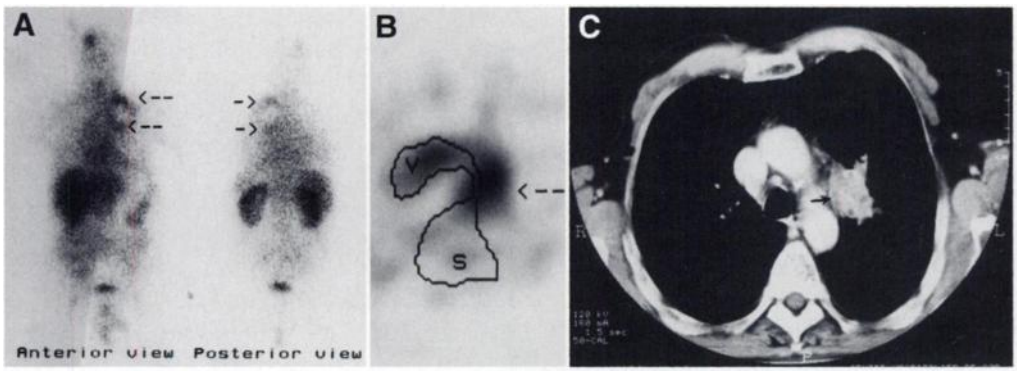

FIGURE 2. (A) Planar anterior view of the thorax obtained 24 hr after ^{111}In-di-(In-DTPA)-tyrosyl-lysine injection in Patient 9. The primary tumor is clearly visible in the top left portion of the lung (upper arrow); the mediastinal focus, which confirmed the N2 classification, was also clearly visible (lower arrow). (B) Transverse tomographic section obtained in the same patient through the mediastinal focus (arrow). Anatomical landmarks are represented (V = vascular activity; S = spine activity). (C) CT scan section obtained in the same patient approximately through the same plane as the scintigraphic section shown in Figure 2B. The adenopathies responsible for the uptake are visible (arrow); the complementarity of IS and CT is well-demonstrated in this patient.

incited the surgeon to operate on the patient. In fact, the physician did not find any mediastinal involvement or contralateral lesion (pT2N0).

Immunohistochemistry Results

Immunohistochemistry (IHC) was performed on 11 of the 12 patients and was positive for CEA in seven cases (64%). In six patients, we noticed a good agreement between anti-CEA expression in the tumor as demonstrated by immunohistochemistry and the intensity of the tumor activity in the scintigraphy. That was true for planar images as well as for tomographic sections. Two patients showed a very intense tumor uptake of the tracer (Patients 2 and 5) (Fig. 1). In these two patients, immunostaining for CEA was strongly positive on 100% of cells. However, results were less concordant in five patients. In patient 8, we observed a weak but significant uptake in the tumor, despite the fact that less than 10% of cells were positive for CEA on immunohistochemistry. In this patient, the tumor was small (1 cm) but was accompanied by a large pneumopathy, which could be responsible for a nonspecific uptake of Bs-MAb. In Patients 1 and 10, the tumor was also visualized by scintigraphy despite immunostaining for CEA being negative. In two other patients, despite positive immunohistochemistry, IS was negative in the primary tumor, as was previously mentioned (Patients 3 and 12).

Sensitivity and Specificity

The overall sensitivity of the method at the primary tumor level was 75% (9/12). However, taking into account the negativity of immunohistochemistry in one case, it was of 82% (9/11) (Table 2). Specificity could not be calculated as all the patients included in the study presented with a tumor (no negative case). At the mediastinum level, sensitivity and specificity were 66% (2/3) and 89% (8/9), respectively. However, the number of patients studied is low and such values must be regarded with caution.

Biodistribution

Biodistribution data were available in 10 patients (Table 1). Uptake by the tumor was rather low, compared with that observed in colorectal tumors (*5*), as were the tumor-to-blood ratios. However, comparing patients with negative and positive CEA-immunostaining, uptake was significantly higher in CEA positive tumors (5.22 ± 2.29 versus 2.22 ± 0.77% ID/kg, $p < 0.05$). There was no correlation between intensity of CEA-immunostaining and tracer uptake, but such a relationship may be implied from tumor-to-blood ratios (Fig. 3).

Pharmacokinetics

Pharmacokinetics data are summarized in Table 1 and Fig. 4. Mean half-life of radioactivity in blood was 13.96 ± 3.52 hr (range 9.24–21). Twenty-four-hour urine recovery of injected ^{111}In radioactivity ranged between 25.7 and 72.3% (Table 1). Interestingly, the greatest values were found in patients with lower CEA tumor expression (Fig. 3).

HAMA

Hama were detected in two patients (Patients 7 and 10). Values reached a maximum at day 30 in both cases (77.7 and 16.4 μg/ml, respectively) and returned to normal (<0.06 μg/ml) at day 90 and 60, respectively.

DISCUSSION

Immunoscintigraphy may be of interest for lung cancer staging, being complementary to morphological studies (CT) as we previously concluded (*2*). Several other studies have reached a similar conclusion (*7–11*).

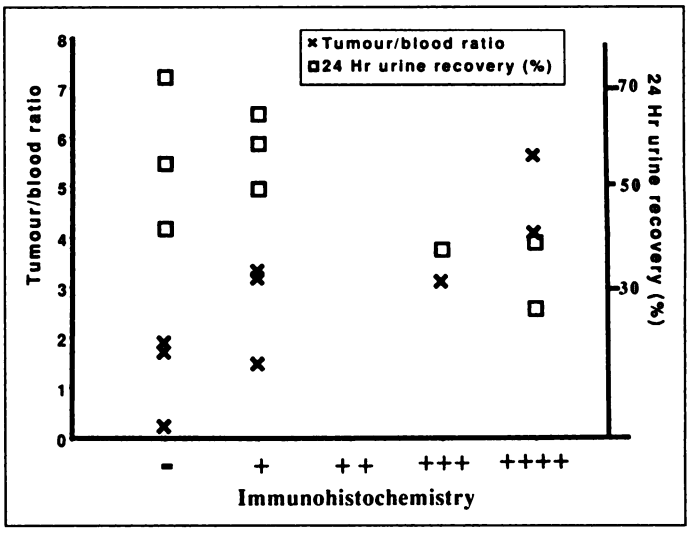

FIGURE 3. Tumor-to-blood ratios of radioactivity (biodistribution data) and 24 hr urine recovery according to the immunohistochemistry results for CEA positivity in nine patients.

Low tumor-to-nonspecific activity ratios are currently the major limitation of IS, especially in the thorax, because of the liver proximity and the presence of numerous vascular elements. Several solutions have been proposed to improve this ratio. Most interesting perspectives are pretargeting strategies that are based on the successive administration of "cold" antibody and radioactive label; the latter is injected in a second step, when the ratio of tumor-bound to nonspecific and circulating antibody is higher. It is retained preferentially in the tumor, as the availability of antibody for tracer binding in normal tissues has decreased. The avidin-biotin system has been widely explored (*12,13*) as well as bispecific antibodies. The aim of this study concerning a limited number of patients was to evaluate such a pretargeting approach, i.e., the AES technique (*3–5*) for the immunoscintigraphic study of mediastinum in NSCLC. To avoid surgical treatment delay, the same patients could not be investigated successively using directly labeled antibodies and the AES technique. Thus, the results will be discussed relatively to what we obtained in a previous study using the same antibody in the form of an ^{111}In-labeled $F(ab')_2$

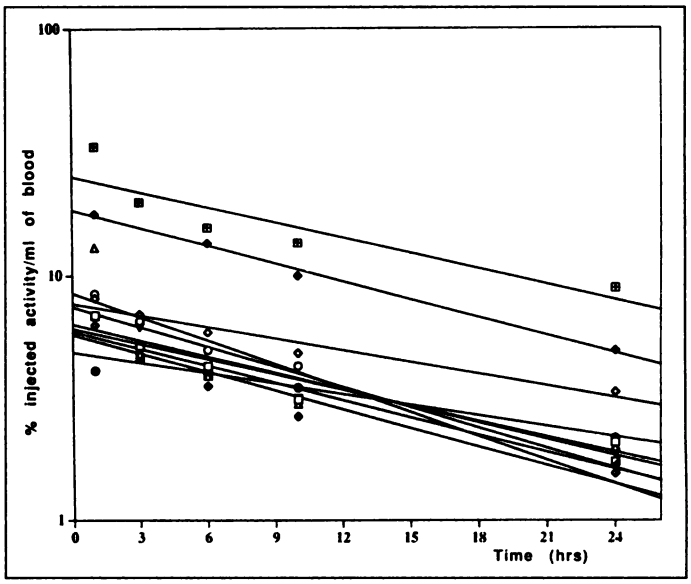

FIGURE 4. Blood pharmacokinetics of ^{111}In radioactivity in 10 patients (values $\times 10^3$).

fragment (*2*). Comparing with these previous data, Bs-MAb-mediated targeting of ^{111}In-bivalent hapten seems promising for at least two reasons.

The main advantage of the AES method is clearly the great improvement in contrast of images allowing easier delineation of tumors. Le Doussal et al. (*5*) have shown that the wider distribution and the accelerated clearance of the hapten (when compared to F(ab')2) led to a decrease in the radioactivity in normal tissues, followed by an increase in uptake ratio. Moreover, ^{111}In uptake by the liver was dramatically reduced. Our results are in agreement with these observations. When positive, the tumor activity was clearly visualized, without the need of image processing, while liver activity was rather low, especially in tomographic sections (Fig. 1). The hepatic activity that persisted on planar images was much lower than with directly labeled antibodies and was likely due to circulating activity. Furthermore, this sharper contrast was obtained within 24 hr following the injection of the radiolabeled hapten, whereas 72 hr were necessary to reach a sufficient contrast after the injection of directly labeled antibodies.

The other main advantage of the AES method relates to immunospecificity. It is obvious that a major limitation of immunoscintigraphy in NSCLC is the variability and weakness of CEA expression by these tumors (*14,15*). Four of the 11 patients in whom IHC was performed were totally CEA negative, and CEA expression was very low (<40%) in two other patients. Only in two cases (Patients 2 and 5) 100% of tumor cells strongly expressed CEA. In this context, the immunospecificity of AES seems good, better than those obtained with directly labeled antibodies. Indeed, we noted a relationship between tumor expression of CEA (as assessed by immunohistochemistry), tumor-to-blood ratios in biodistribution and tumor contrast in scintigraphic images. Although such data carry a relative value because of the low number of patients, they are in agreement with those obtained under other pathological conditions (*4,5*) and support the idea that AES technique is associated with a decrease in nonspecific uptake, i.e., not mediated by antigenic recognition. However, a nonspecific uptake is not totally eliminated as we have noticed in three cases an uptake in the tumor area, whereas IHC of the tumor was negative (Patients 1, 8 and 10). This might be explained in one case (Patient 8); in this patient the tumor volume was small and surrounded by an infectious pneumonia. A nonspecific uptake of the antibody or of di-DTPA-tyrosyl-lysine in the pneumonia area seems a likely explanation. Nonspecific uptake in the tumor itself is also a possibility but seems less likely because of the small size of the tumor. In Patients 1 and 10, it must be emphasized that the contrast was very moderate; furthermore, heterogeneity and large necrosis of the tumor (especially in Patient 10) make a false-negative by immunohistochemistry possible.

Renal activity was observed as in most clinical trials with the AES technique. Indeed, low molecular weight compounds such as peptides or small immunoglobulin fragments do accumulate nonspecifically in kidney glomeruli. This accumulation may be competed with by positively charged amino acids such as lysine (*16,17*). In this study, renal activity did not interfere with image interpretation.

Half-life of blood activity was quite similar in the 10 patients tested, and the mean value was closely similar to the value previously found by Le Doussal et al. (*5*) in patients with colorectal cancer. This parameter seems therefore constant and independent of other conditions, provided the serum CEA level is not strongly elevated. It must be noticed, although there is no obvious explanation, that the lower the CEA tumor expression, the greater the activity recovered in urine pool during the first 24 hr after radiolabeled hapten injection.

The low number of patients studied and the lack of negative cases did not allow us to conclude as to the sensitivity and specificity of the method. The results of this study are quite similar to those of Dosio et al. (*13*), which was conducted with a three-step avidin-biotin system and an anti-CEA antibody. In this study, primary tumor was detected in 8 of 10 cases (one with negative anti-CEA immunostaining); mediastinal metastases were detected in two of three patients.

It is interesting to consider Patient 5, in which IS contributed to the decision of surgery despite a doubt concerning a possible contra-lateral lesion. Moreover, it can be pointed out that, as in our previous study (*2*), surgery never found a mediastinal involvement when CT and IS were both negative in the mediastinal area.

CONCLUSION

Our results show that IS using the AES method is effective for mediastinal lymph nodes assessment in non-small-cell lung carcinoma, despite the limitation conferred by CEA which is a less interesting target in these tumors than in colorectal or medullary thyroid carcinomas. Furthermore, this system provides a better specificity and a better uptake ratio in tumor, which will probably improve the performances of IS in this application. Studies on larger patient groups are now necessary to establish the clinical usefulness of this approach.

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