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Immunohistochemical Antigenic Expression and *in Vivo* Tumor Uptake of Monoclonal Antibodies with Specificity for Tumors of the Gastrointestinal Tract

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ABSTRACT

Two monoclonal antibodies with specificity for carcinoembryonic antigen and Ca 19-9 gastrointestinal tract tumor associated antigens were infused after iodination with ^{125}I and ^{131}I, respectively, in six patients 3 days and in one patient 4 days before radical surgery for colon or rectal carcinoma. Biopsy specimens from tumor, normal colon, fat, muscle, and skin along with a blood sample were excised at surgery and counting was performed for γ emission. Fragments were then studied by two independent pathologists for immunohistochemical expression of corresponding antigens using the avidin-biotin peroxidase complex. A correlation study was thereafter performed between the amount of antibody bound *in vivo*, expressed as the percentage of injected dose per gram of tissue and the quantitative expression of tumor associated antigens, taking into account both the percentage of cells expressing the antigen and intensity of staining.

For this limited number of patients a good correlation was found between amount of targeted antibodies and amount of expressed antigens. For carcinoembryonic antigen, *r* values were 0.69 and 0.90 for each pathologist (with an *r* value of interobserver correlation of 0.74); for Ca 19-9, values of 0.78 and 0.84 were obtained for each observer, with an interobserver *r* value of 0.97. Based on this limited study, it may be assumed that the possibility of imaging a given tumor is in part correlated to intensity of antigenic expression at the tumor site; other parameters, like tumor vascularization and blood flow for instance, are, however, to be considered for accessibility of antibodies to corresponding antigens.

INTRODUCTION

Monoclonal antibodies with tumor cell specificity have allowed identification and better characterization of tumor associated antigens. Their clinical usefulness is presently being investigated in diagnostic as well as therapeutic fields. Tumor imaging is one of the areas where the utilization of radiolabeled antibodies has been of interest (1–3) and pharmacokinetic studies demonstrate the rationale for infusing iodinated monoclonal antibodies and recording images with a γ camera in order to visualize primary tumors, recurrences, or metastases (4). Tumor uptake of monoclonal antibodies are shown to be specific for antigenic expression, as expressed by a localization index (5) based on the concomitant infusion of tumor specific and irrelevant monoclonal antibodies. Tumor detection through immunoscintigraphy is therefore dependent upon the expression of tumor associated antigens. Immunohistochemical studies of tumors with monoclonal antibodies show that tumor antigen expression is heterogeneous among tumors of the same type and within a given tumor from cell to cell (6).

In order to study the correlation between tumor associated antigen expression and tumor uptake of the corresponding antibodies, a study was undertaken comparing the immunohistochemical expression of tumor antigens and the tumor uptake of *in vivo* infused radiolabeled monoclonal antibodies in patients with colorectal carcinomas.

PATIENTS, MATERIALS AND METHODS

Patients. Seven patients with colon or rectal carcinoma were included in this study. Patients' characteristics are displayed in Table 1.

Monoclonal Antibody Labeling and Infusion. Two monoclonal antibodies with specificity for gastrointestinal tract adenocarcinoma were used. Anti-CEA[2] $F(ab')_2$ monoclonal antibody was iodinated as previously described (1) using the iodogen method. Specific activity of labeling ranged from 0.2 to 3 mCi/mg, and patients received 0.38 to 3.8 mg with a total activity of 0.60 to 1.5 mCi of ^{125}I anti-CEA $F(ab')_2$ fragments. Ca 19-9 has more recently been described (7) as tumor antigen associated with gastrointestinal tract adenocarcinomas. Labeling of 19-9 $F(ab')_2$ fragments was performed with the iodogen technique using ^{131}I. Specific activity was 0.575 to 3.3 mCi/mg; patients received 0.65 to 3.3 mg of $F(ab')_2$ fragments, with a total activity of 0.65 to 2 mCi. Monoclonal antibodies were infused as a mixture of anti-CEA and 19-9 diluted in 100 ml of saline over a 15- to 20-min period through a peripheral vein.

Surgical Procedure and Sample Counting, Results. Surgery was performed 3 days after infusion for patients 1 to 6, and after 4 days for patient 7. Radical surgery (hemicolectomy or abdominoperineal amputation) was performed as initially scheduled. Three tumor biopsy specimens were excised along with normal colon, fat, parietal muscle, and skin samples. Normal liver biopsies were obtained in 2 cases (patients 2 and 7). Surgical specimens were weighed and immediately counted for ^{125}I and ^{131}I γ emission before being fixed in 10% formalin. Results were expressed as a percentage of the injected dose per gram of tumor, as already described (4). The mean of the three tumor specimens was calculated for each patient.

Immunohistochemical Study and Results. The immunohistochemical tumor studies were performed using immunoperoxidase with the avidin-biotin-peroxidase complex, as previously described (8). Briefly, formalin fixed biopsy samples were embedded in paraffin and sections were obtained (5 to 6 μm thick). Sections were deparaffinized and rehydrated in toluene and graded alcohols. Endogenous peroxidase was blocked by incubation with methanol and H_2O_2 0.3%. After washing in phosphate buffered saline, slides were incubated for 20 min with normal horse serum and sequentially washed and incubated for 60 min with anti-CEA or 19-9 monoclonal antibodies (10 μ/ml), biotinylated anti-mouse antibodies, and avidin-biotin-peroxidase complex (Vector laboratories). Each step was followed by washing in phosphate buffered saline. Sections were finally allowed to react with aminoethyl carbazol and H_2O_2 before being counterstained with hematoxylin. Results were expressed as a percentage of stained tissues per total tissue of the section. Labeling intensity per cell was graded from + to ++++. In order to take into account both the number of cells stained and the intensity of staining the final result was expressed as the percentage of stained cells corrected for staining intensity as follows: for +, the percentage was divided by 2; for ++, it was divided by 1.5; for +++, it was multiplied by 1.5, for ++++, it was multiplied by 2. Two independent observer pathologists scored the slides from all specimens in a double blind fashion.

Statistical Analysis. Statistical correlation between antigenic expression and monoclonal antibody tumor uptake was evaluated for each

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[2] The abbreviation used is: CEA, carcinoembryonic antigen.

antigen by each observer using a Pearson test. Comparison of evaluation between observers 1 and 2 was performed using the T1 test of Bartko and Fleiss.

RESULTS

Anti-CEA Monoclonal Antibody. *In vivo* biodistribution of ^{125}I anti-CEA monoclonal antibody expressed as a percentage of injected dose per gram of tissue and tumor/tissue ratios are shown on Table 2. Anti-CEA antibody preferentially accumulates at the tumor site as compared with normal tissues. The amount of antibody, however, varies from patient to patient at the tumor site and in the normal tissues as reflected by the SE ($927 \times 10^{-5} \pm 433$ (SE) for tumor; $327 \times 10^{-5} \pm 151$ for normal colon). Tumor to normal colon ratio has a mean of 2.8 ± 0.9, with a range of 1.6 to 4.2.

Table 1 *Patients' characteristics*

Patient no.	Tumor site	Astler-Coller classification	Time delay between infusion and surgery (days)
1	Rectum	B_2	3
2	Rectum	B_2	3
3	Left colon	B_2	3
4	Rectum	B_1	3
5	Left colon	B_2	3
6	Rectum	C_2	3
7	Left colon	B_2	4

Table 2 *In vivo infusion of iodinated monoclonal antibody*

	% injected dose/g of tissue ($\times 10^{-5}$)			
	CEA	Tumor/tissue ratio	Ca 19-9	Tumor/tissue ratio
Tumor	927 ± 433[a]		273 ± 202	
Normal colon	327 ± 151	2.8 ± 0.9	110 ± 40	2.5 ± 1.5
Liver	64 ± 12	14.5	90 ± 57	3
Fat	52 ± 13	17.8	43 ± 47	6.3
Muscle	80 ± 13	11.6	49 ± 15	5.5
Skin	140 ± 69	6.6	102 ± 52	2.7

[a] Mean ± SE.

Immunohistochemical expression (Table 3), combining both the number of stained cells and intensity ranged from 30 to 60 among the patients, with some heterogeneity found for a given patient between the 3 tumor samples studied. Patient 7 also showed reactivity with normal colon mucosa, comparable to autologous tumor. Monoclonal antibodies *in vivo* targeted to tumor cells, as reflected by the iodine counting procedure were not detectable with the immunohistochemical technique presently used.

Monoclonal Antibody 19-9. Biodistribution of 19-9 monoclonal antibodies, similar to CEA, showed antibody uptake at the tumor site as compared to normal tissues. Here again, and comparable to anti-CEA antibody accumulation, variability was noticed among the patients with a mean of 273×10^{-5}% of injected dose/g at the tumor site and a SE of 202×10^{-5}. Tumor

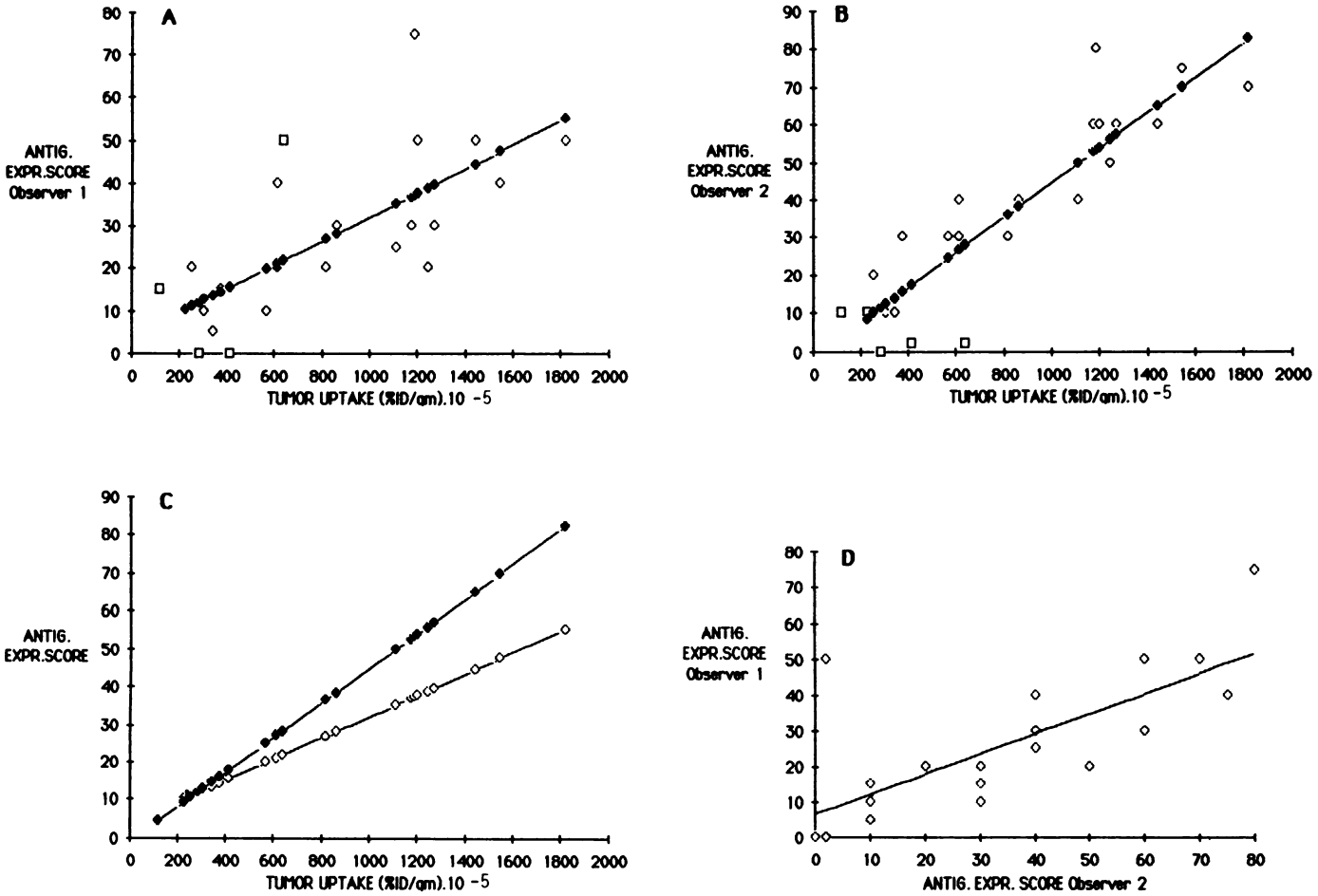

Fig. 1. *A*, correlation study between antigenic expression (*ANTIG. EXPR.*) of CEA in immunohistochemical study (*ordinate*) and antibody tumor uptake (*abscissa*) when slides were evaluated by observer 1. ○, tumor; □, normal colon; ●, trend of CEA. $r = 0.69$; $P = 0.001$. *B*, correlation study between antigenic expression of CEA in immunohistochemical study (*ordinate*) and antibody tumor uptake (*abscissa*) when slides were evaluated by observer 2. ○, tumor; □, normal colon; ●, trend of CEA. $r = 0.90$; $P = 0.001$. *C*, comparison of correlation study for observer 1 (○) and observer 2 (●) when anti-CEA monoclonal antibody was used. *D*, correlation of immunohistochemical analysis results with CEA for observer 1 *versus* observer 2. $r = 0.74$; $P = 0.001$. *ID*, injected dose.

Table 3 *Immunohistochemical study scores*

Patient no.	Type of tissue	CEA	Ca19-9
1	Tumor	60 ± 10[a]	0
	Normal colon	0	0
2	Tumor	43 ± 25	46 ± 15
	Normal colon	ND	ND
3	Tumor	50 ± 10	43 ± 15
	Normal colon	5	0
4	Tumor	30 ± 10	21 ± 7
	Normal colon	0	<5
5	Tumor	33 ± 5	48 ± 12
	Normal colon	5	10
6	Tumor	40 ± 26	10 ± 0
	Normal colon	5	<5
7	Tumor	36 ± 15	20 ± 10
	Normal colon	30	5

[a] Mean of 3 biopsy specimens.

to normal colon ratio was 2.5 ± 1.5 with a range of 1.6 to 5.4; interestingly, patient 1 did not bind 19-9 preferentially to normal colon, with low values at both sites (tumor, 65×10^{-5}%) and tumor to normal colon ratio of 0.65 (this patient was not included in the calculation of the mean percentage of injected dose).

Immunohistochemical expression of Ca 19-9 antigen (Table 3) showed heterogeneity in antigenic expression with values ranging from 0 to 48 among the 7 patients and variability between the different biopsy specimens of the tumor for a given patient as indicated by the SE. Normal colon was generally only faintly stained, as compared to autologous carcinoma. Correlating with the *in vivo* biodistribution study, patient 1 was completely negative for Ca 19-9 expression, both at the tumor site and normal colon mucosa.

For Ca 19-9 as for CEA, *in vivo* bound monoclonal antibodies were not detectable.

Statistical Correlation Analysis. The Pearson test was used to study the correlation between monoclonal antibody tumor uptake and antigenic expression independently for observers 1 and 2.

As shown in Fig. 1, *A* and *B*, where analysis was done by observers 1 (*A*) and 2 (*B*), a very good correlation was obtained between antigenic expression and tumor uptake of anti-CEA monoclonal antibodies with an r of 0.69 for observer 1 and 0.9 for observer 2. Normal colon always showed low tumor uptake and immunohistochemical score, except in one case where observer 1 scored a normal colon specimen to 50, when observer 2 scored it <5. Fig. 1*C* compares the 2 trends of correlation according to observers 1 and 2; in both instances P was <0.001. A T1 test of Bartko and Fleiss was performed to analyze the results obtained, respectively, by each observer for CEA. A good correlation was found between both pathologists with r = 0.74 and P <0.001 (Fig. 1*D*). In all cases, however, observer 2 had a tendency to overscore as compared to observer 1.

Similar methods were used to study the correlation of Ca 19-

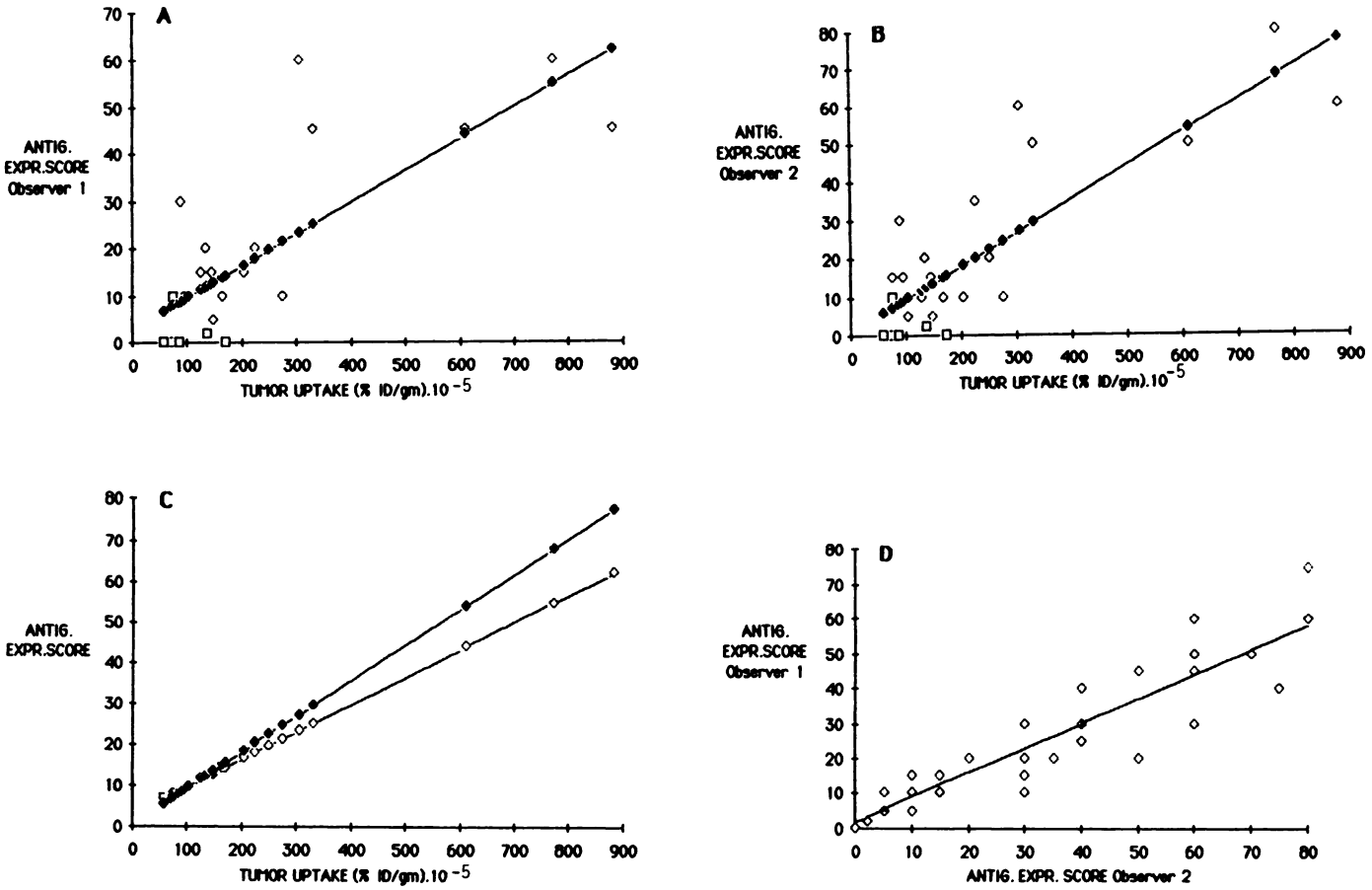

Fig. 2. *A*, correlation study between antigenic expression (*ANTIG. EXPR.*) of Ca 19-9 in immunohistochemical study (*ordinate*) and antibody tumor uptake (*abscissa*) when slides were evaluated by observer 1. ○, tumor; □, normal colon; ●, trend of 19-9. r = 0.78; P = 0.001. *B*, correlation study between antigenic expression of Ca 19-9 in immunohistochemical study (*ordinate*) and antibody tumor uptake (*abscissa*) when slides were evaluated by observer 2. ○, tumor; □, normal colon; ●, trend of 19-9. r = 0.84; P = 0.001. *C*, comparison of correlation study for observer 1 (○) and observer 2 (●) when anti 19-9 monoclonal antibody was used. *D*, correlation of immunohistochemical analysis results with 19-9 antibody for observer 1 *versus* observer 2. r = 0.97; P < 0.001. *ID*, injected dose.

9 expression and 19-9 antibody uptake. As displayed in Fig. 2, a very good correlation was noticed between immunohistochemical score and tumor uptake ($r = 0.78$ and 0.84 for observers 1 and 2, respectively, with $P < 0.001$). In all cases normal colon mucosa had low antibody uptake and low score at immunohistochemical study. Fig. 2*C* shows the 2 trends obtained with each observer. The T1 test of Bartko and Fleiss revealed excellent correlation between both pathologists (Fig. 2*D*) with $r = 0.97$ and $P < 0.001$.

DISCUSSION

Immunoimaging of tumors with monoclonal antibodies is one of the promising areas of clinical oncology where the use of radiolabeled antibodies may allow disclosure of primary tumors, recurrences, or metastases. False negative results, however, have sometimes been observed, and one of the reasons for these failures may rely in part on the absence or too low antigenic expression at the tumor site. Tumors are known to be heterogenous from a pathological point of view, and this has clearly been confirmed by monoclonal antibody defined antigen analysis; in addition, numerous tumor associated antigens have been disclosed using the hybridoma technology, and other well-known tumor molecules have been dissected in several epitopes thanks to monoclonal antibodies (9–11).

Successful radioimmunoimaging of tumors with radiolabeled antibodies depends on different parameters including pharmacokinetics of infused antibodies, accessibility to the target cell through vascularization, and antigenic expression of the tumor. In the present study, 2 antibodies with well-characterized specificity for gastrointestinal tract adenocarcinomas were labeled with radioactive iodine and infused into patients with colorectal carcinomas. Antibody targeting to the tumor *in vivo* was evaluated 3 to 4 days after infusion by measuring the radioactivity of tumor and normal tissue samples obtained during surgery; in addition, tumor and normal tissue biopsies were assayed immunohistochemically for antigenic expression. Among the limited number of patients studied, 7 of 7 expressed CEA antigens and showed preferential tumor uptake of anti-CEA monoclonal antibodies, whereas 6 of 7 displayed Ca 19-9 antigens and showed accumulation of 19-9 antibodies at the tumor site; interestingly, a single patient had very low counts for ^{131}I 19-9, as compared to normal tissues, and accordingly was totally negative for antigenic expression of Ca 19-9. Correlation studies between amount of antibody uptake at the tumor site and antigenic expression proved to be excellent, independent of the pathlogist's subjective assessment; high antibody uptake after *in vivo* infusion reflected high antigenic expression at the tumor site and *vice versa*. The present study therefore shows that tumor imaging through radiolabeled monoclonal antibodies is dependent in part upon tumor cell expression of tumor associated antigens, among several other parameters. The absence of antigenic expression on a given tumor might be responsible for failure of detection with immunoscintigraphy. In order to decrease the false negative results due to absence of antigens, cocktails of radiolabeled antibodies might be used and an average profile of antigenic expression for a given tumor type should be defined by studying a panel of monoclonal antibodies on a large series of this given tumor type. Parameters other than antigenic expression, however, will interact with the ability to image a tumor with radiolabels; vascularization, tumor necrosis, and blood flow at the tumor level may impair the accessibility of tumor cells for antibodies. In the present study the good correlation between antigenic expression and antibody uptake was probably favored by good tumor vascularization in this limited number of patients.

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