LOCALIZATION OF HUMAN BREAST-CARCINOMA XENOGRAFTS USING ANTIBODIES TO CARCINOEMBRYONIC ANTIGEN

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Summary.—Affinity-purified antibodies to carcinoembryonic antigen (CEA) have been injected into immune-suppressed mice bearing xenografts of human breast tumours. It has been shown that the antibodies localized in the tumours but not in normal tissues. The degree of tumour localization correlates with the amount of tumour CEA, and is unaffected by levels of circulating CEA or CEA/anti-CEA immune complexes.

THE CONCEPT of injecting anti-tumour-cell antibodies to localize neoplasms was first introduced in experimental systems by Pressman (1957). Since then, success has been achieved with human tumours using antibodies to the carcinoembryonic antigen (CEA) (Gold & Freedman, 1965). It has been demonstrated that such radio-labelled antibodies localized preferentially in human colonic neoplasms xenografted into animals (Primus et al., 1973; Mach et al., 1974). More recently, localization of anti-CEA antibodies has been attained in patients, but only when the blood-pool background was subtracted by a computerized technique (Goldenberg et al., 1978; Dykes et al., 1980; Mach et al., 1980). Although some workers have achieved almost complete success with this method, others have expressed doubts as to its present suitability for routine clinical use (Mach et al., 1980).

Many breast carcinomas produce CEA, the level of circulating CEA being stage-dependent (Laurence et al., 1972; Coombes et al., 1980; Tormey & Waalkes, 1978). The need for early detection of systemic metastases and metastatic involvement of inaccessible internal mammary lymph nodes has prompted us to examine the localizing potential of anti-CEA in human breast carcinomas. The establishment of xenografts of human breast carcinomas in immune-suppressed mice (Bailey et al., 1980) has enabled us to examine different tumours and thus investigate the kinetics of tumour localization and its relationship to the CEA content of the tumour before starting clinical studies.

MATERIALS AND METHODS

Animals and tumours.—Four-week-old, female CBA/lac mice, weighing on average 20 g, were immune suppressed by thymectomy and total-body irradiation (6 gray) preceded by i.p. injection of cytosine arabinoside (200 mg/kg) (Steel et al., 1978). Three different xenograft lines of human breast carcinomas were used (HX99, HX106 and HX104) (Bailey et al., 1981). These have been shown to maintain their characteristic human morphology, chromosome number and tumour-marker expression throughout passaging. HX104 was used at Passage 6, HX99 at Passage 7 and 8 and HX106 at Passage 8. The tumours were implanted s.c. bilaterally in the flanks 3–5 weeks before use.

The antibody and iodine labelling.—The CEA used as immunogen and for coupling of Sepharose 4B (see below) was isolated from hepatic metastases of a human colonic carcin-

oma and purified to satisfy the criteria pre-
viously described (Westwood & Thomas,
1975; Westwood, 1978). Anti-CEA sera were
raised by monthly s.c. injections of a goat
with 100 μg of CEA, emulsified with Freund's
adjuvant. The first 2 CEA injections were in
complete adjuvant. Regular bleeds were
taken after the 4th injection, and the goat
was exsanguinated 2 weeks after the 7th
injection. The γ-globulin fraction of the
immune serum was obtained and the solution,
in phosphate-buffered saline (PBS) (0·15M,
pH 7) was passed over a column comprising
CEA (15 mg) covalently bound to Sepharose
4B (10 ml). The column was washed with
0·1M PBS to remove all unbound protein, and
anti-CEA antibodies were recovered from the
column using 6M guanidine HCl in 0·1M PBS.
The guanidine HCl was removed by dialysis.

The affinity-purified goat anti-CEA anti-
bodies were labelled with ^{125}I, using chlor-
amine-T (Greenwood *et al.*, 1963). Typically,
an activity of 1 μCi/μg was achieved. IgG
from a non-immunized goat was labelled with
[131]I by the same method and admixed with
the specific antibody before injection. Column
chromatography (Sephacryl S-300) of the
radiolabelled proteins demonstrated the
absence of aggregated material in the void
volume.

Radiolocalization.—Simultaneous i.v. in-
jection of ∼15 μg each of ^{125}I-anti-CEA and
goat [131]I IgG was given to tumour-bearing
and tumour-free animals. At intervals of
between 1 and 96 h after injection, the
animals were exsanguinated and tumours and
organs (salivary gland, thyroid, heart, lungs,
liver, spleen, stomach, kidney and intestine)
were removed. After weighing each tissue,
radioactivity was measured in an L.K.B. 1280
ultra-gamma counter. Gel-filtration chroma-
tography of the plasma of the injected ani-
mals at death was performed to demonstrate
circulating CEA/anti-CEA immune com-
plexes.

The results were expressed in the form of a
Localization Index, which is the ratio of
specific (^{125}I) to nonspecific (^{131}I) antibody
in normal tissues or tumour, divided by the
same ratio in blood. This is similar to the
localization ratio of Primus *et al.* (1973) and
the specificity index of Mach *et al.* (1974)
except that the former authors made a com-
parison with the ratio in the injected solution
and the latter with liver. We chose blood,
because this gives the closest comparison to

an external photoscan of a patient when the
activity in the blood pool is subtracted
(Goldenberg *et al.*, 1978; Dykes *et al.*, 1980).

*Demonstration of CEA in tumours and
plasma.*—CEA was demonstrated at the histo-
logical level in conventionally prepared tissue
sections, using methods and reagents de-
scribed previously (Heyderman & Neville,
1976; Ormerod, 1978). In addition, the CEA
content of xenografted tumours and in the
plasmas of animals was measured by the
radioimmunoassay methods of Laurence *et al.*
(1972). Each tumour was homogenized at
0°C in 0·9% saline. 2M perchloric acid was
then added to each homogenate and the mix-
ture was left at 0°C for 30 min. Precipitated
protein was removed by centrifugation and
the supernatant was well dialysed with dis-
tilled water at 4°C. The radioimmunoassay
was then performed.

Chromatography of plasma samples.—For
the purpose of detecting CEA/anti-CEA com-
plexes, each sample of plasma was applied to
a column of Sephacryl S-300 (0·9 × 60 cm) and
eluted with 0·1M phosphate buffer (pH 7·5)
at a rate of 5 ml/h. 0·5ml fractions were
collected and the radioactivity of each
fraction (^{125}I and ^{131}I) was measured using
an LKB 1280 ultra-gamma counter.

RESULTS

Two to three weeks after tumour im-
plantation (range of tumour wet wt
36–98 mg; mean 67 ± 6 mg), the mice were
injected i.v. with equal amounts by weight
of ^{125}I-labelled anti-CEA and ^{131}I-labelled
normal γ-globulin. At each sampling
time after injection at least 3 mice,
carrying a total of 3–6 tumours, were
exsanguinated and tumours and organs
removed for estimation of the radioacti-
vity. Altogether there were 10 experi-
ments, using 211 animals carrying collec-
tively 328 tumours. In addition, 2 non-
tumour-bearing animals, injected with the
same amounts of antibodies, were ex-
sanguinated at each sampling time. Table
I shows typical results for one of the
tumours (HX99) 24 h after injection. It
can be seen that the tumour was the only
tissue which showed specific localization
of the anti-CEA antibodies. This tumour
was the only 1 of the 3 to show a high

TABLE I.—*Specific* (^{125}I-*anti-CEA*) *and non-specific* (^{131}I-*normal*) *Ig activity in tissues 24 h after injection of antibodies. Values of radioactivity are mean ± s.e. in 4 animals* (HX 99). *The localization index is calculated from the values in the first two columns*

Tissue	^{125}I-anti-CEA ct/min/mg*	^{131}I-normal Ig ct/min/mg*	LI†
Tumour	293 ± 39	196 ± 28	3·7
Blood	222 ± 22	553 ± 66	1·0
Liver	65 ± 12	120 ± 29	1·3
Thyroid gland	58 ± 8	131 ± 27	1·1
Salivary gland	63 ± 19	123 ± 19	1·3
Stomach	49 ± 17	118 ± 20	1·0
Intestine	51 ± 12	126 ± 18	1·0
Heart	48 ± 7	117 ± 23	1·0
Lung	47 ± 16	106 ± 18	1·1
Spleen	36 ± 8	98 ± 16	1·0
Kidney	28 ± 11	92 ± 12	0·8

* Wet weight.

† Localization index:

$$\text{tissue or tumour } \frac{^{125}I}{^{131}I} \text{ divided by blood } \frac{^{125}I}{^{131}I}.$$
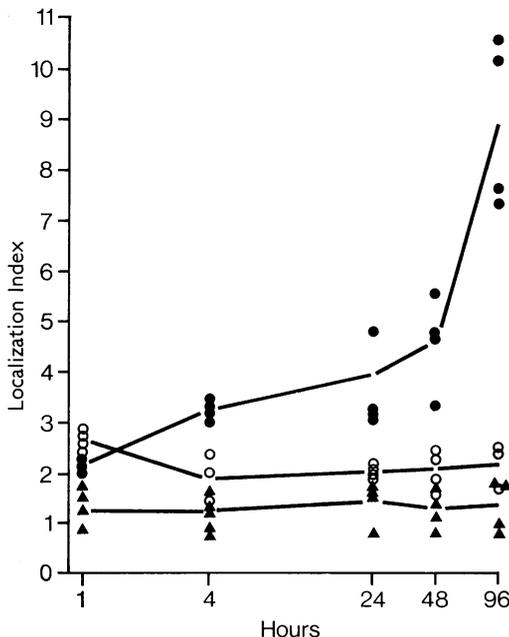

FIG. 1.—Localization index in 3 different human breast adenocarcinoma xenografts. For definition of localization index, see text. ●, HX99 (CEA + +, see Table II); ○, HX-104 (CEA ±); ▲, HX106 (CEA +).

TABLE II.—*Relation between tumour localization, immunoperoxidase staining and direct CEA estimation in 3 breast adenocarcinoma xenografts. Values are mean ± s.e.* (6 *tumours from each line*), (*the difference in LI between* HX106 *and* HX104 *was not significant*)

Tumour	Tumour CEA (µg/g)	Plasma CEA (ng/ml)	Maximum LI*	CEA stain†
HX 99	14·0 ± 4·0	377 ± 59	9·0 ± 1·3	+ +
HX 106	1·1 ± 0·3	463 ± 26	1·5 ± 0·6	+
HX 104	0·6 ± 0·01	257 ± 34	2·0 ± 0·8	±

* I.e. at 96 h after injection.

† The strength of the immunoperoxidase stain for CEA on an arbitrary scale.

degree of specific localization (Fig. 1); HX106 and HX104 showed little or no localization of the anti-CEA. Table II compares the maximum localization index (at 96 h after injection) for each tumour, the levels of CEA circulating in the plasma of the mice, the CEA content of each tumour and the result of immunohisto-chemical stain for CEA. It is of interest to note that the amount of CEA circulating in the plasma did not correlate with the amount of CEA stored in the tumour. With this series of 3 tumour lines, the degree of localization of radiolabelled anti-CEA correlated with the amount of CEA in the tumour.

When the tumours were allowed to grow to a larger size (mean weight 172 ± 23 mg) by delaying injection of the radiolabelled antibodies until 5 weeks after implantation, a lower localization index was obtained at all times after injection (Fig. 2). A similar result was obtained with human colonic tumours xenografted in the hamsters by Primus et al. (1973); also Mach et al. (1974) showed lower specificity indices in more necrotic tumours. Lowering the amount of antibody injected into the mice also lowered the localization index (Fig. 2).

To investigate methods of increasing the activity localized in the tumour, we gave a second injection of radiolabelled anti-CEA and normal Ig 24 h after the first injection; some of the mice received
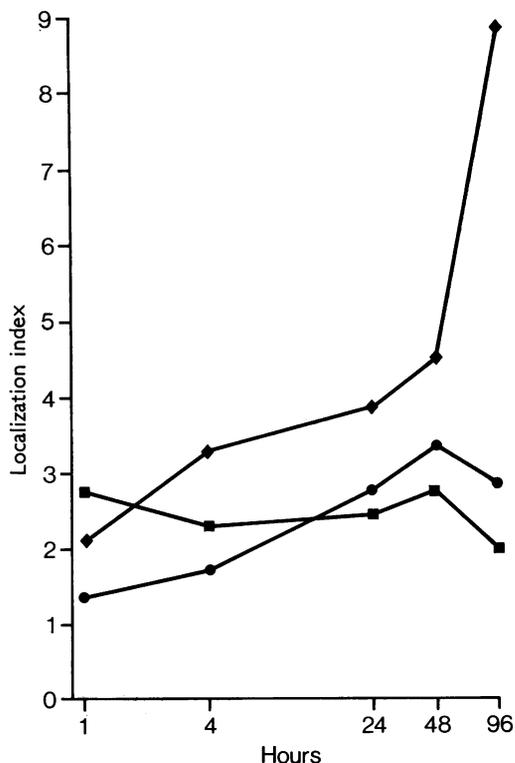
FIG. 2.—Localization index in one human breast-adenocarcinoma xenograft (HX99) at different tumour weights and after different amounts of anti-CEA injected. The localization index decreases when larger tumours are used, and when lower amounts of antibody are injected. Each line represents a separate experiment. 4–6 tumours at each time point of each experiment. ◆, Weight = 63 ± 13 mg; antibody injected = 15 μg. ■, Weight = 69 ± 11 mg; antibody injected = 3·5 μg. ●, Weight = 172 ± 23 mg; antibody injected = 15 μg. (For clarity s.e. values have been omitted; the scatter of the points was as in Fig. 1.)

a 3rd injection on the 3rd day. These manoeuvres increased the activity in each tumour but at the expense of the localization index (Fig. 3).

When samples of plasma were passed over Sephacryl columns, the bulk of the radioactivity was eluted in a position corresponding to the mol. wt of IgG (Fig. 4). Tumour-bearing mice showed an increased amount of labelled specific antibody eluting in the void volume of the column. This peak was absent from the profile of the ^{131}I-labelled normal Ig and
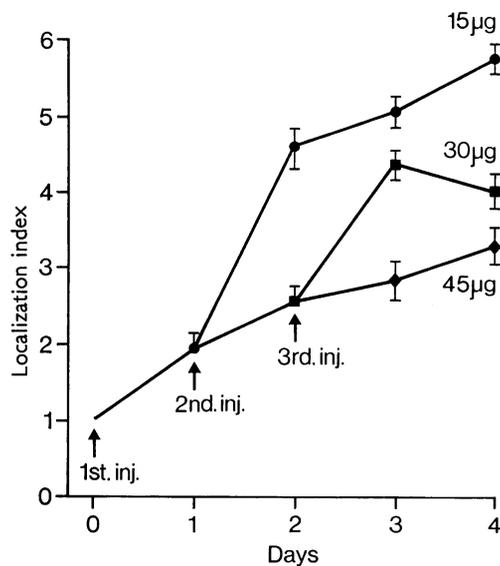
3(a)
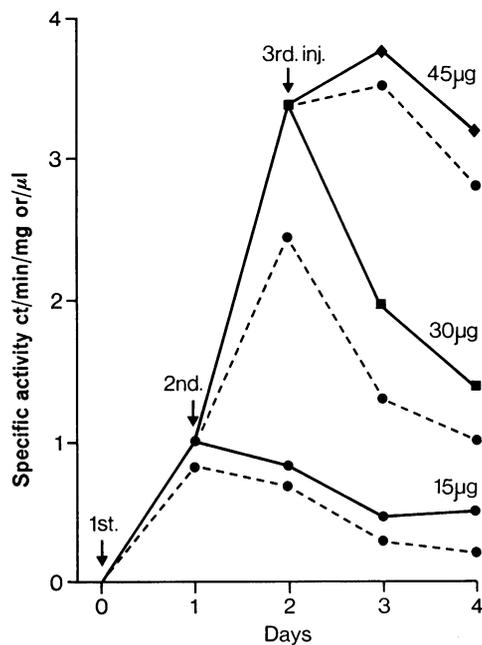
3(b)

FIG. 3.—(a) Localization index after 15 μg, 30 μg and 45 μg of antibody injected. Arrows indicate the timing of the injections. (b) Specific activity (^{125}I-anti-CEA) in tumour (—) and blood (---) after 15 μg, 30 μg and 45 μg of antibody injection. Arrows indicate the timing of the injections.
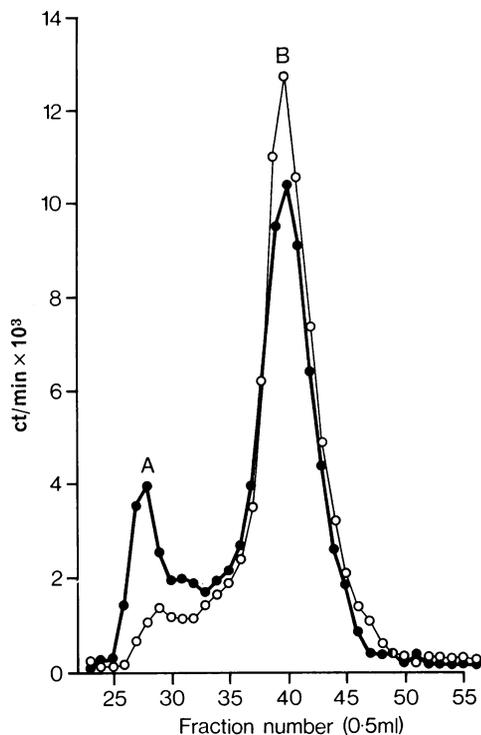
FIG. 4.—Column chromatography of plasma after injection of antibody. A marks the position of the voided material which we assume represents CEA/antibody complexes. B marks the position of the labelled IgG. ●, Tumour-bearing animal; ○, Tumour-free animal.

FIG. 5.—Upper graph: ^{125}I-anti-CEA clearance from blood after injection, as measured by gamma counter. Lower graph: Immune complex clearance from blood after injection, as measured by column chromatography. (HX99 (●) showed high localization. HX106 (○) showed no localization.)

was greatly reduced in the profile of specific antibody obtained from the plasma of mice without tumours. We assume, although we did not prove it, that this voided material represents CEA–anti-CEA immune complexes. The peak of ^{131}I-labelled normal Ig was normalized to coincide with the ^{125}I peak and subtracted from it. The remaining ^{125}I activity was the voided material. We used this subtraction method to estimate the amount of radioactive material eluted as immune complex, and thereby followed the clearance of these complexes from the blood; there was no significant difference in the rate of clearance of immune complexes between mice bearing HX99 tumours (which gave a high localization index) and those bearing HX106 tumours
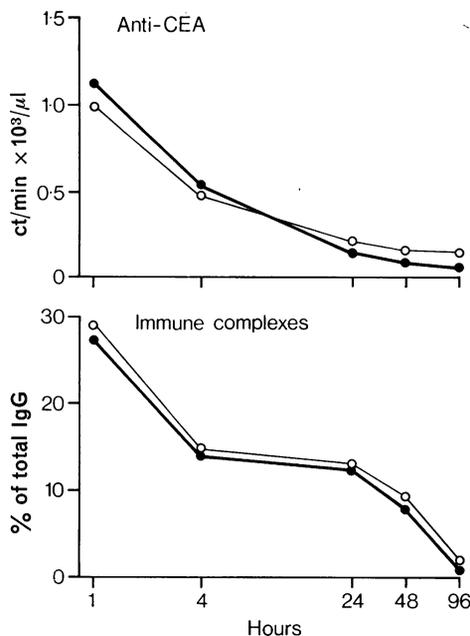
(which gave no specific localization) (Fig. 5).

We observed that, in both tumour-bearing and tumour-free mice, the specific (anti-CEA) IgG was cleared more rapidly in the first hour after injection than the nonspecific Ig. The specific ^{125}I-labelled IgG also contained a small amount of aggregated material (Fig. 4) which was absent from the ^{131}I-labelled nonspecific Ig. It is possible that the anti-CEA antibodies were more susceptible to damage during iodination, and that this resulted in an initial difference in behaviour between the two types of IgG.

To ascertain whether immune complexes of CEA and anti-CEA might localize in tumours through their content of histiocytes, we took C57 BL mice carrying a murine fibrosarcoma (FS6) (Mantovani et al., 1977), which has a macrophage population of 25%, and administered both

^{125}I-labelled anti-CEA with CEA at equivalence and ^{131}I-labelled goat Ig. No specific localization in the tumour was detected.

DISCUSSION

Other workers have successfully demonstrated the localization of specific anti-CEA antibodies in human colonic carcinoma xenografted either into hamsters (Primus *et al.*, 1973) or into nude mice (Mach *et al.*, 1974). We have expanded this work by showing that anti-CEA antibodies will localize in human breast carcinomas.

The quantity of CEA circulating in the plasma of these mice will depend on the rate of synthesis and release of CEA by the tumour, and also on its rate of clearance. Our results suggest that the degree of localization of specific antibodies is dependent on the amount of CEA stored in the tumour, and that the amount of circulating CEA is irrelevant. The latter observation accords with the clinical experience recorded by Goldenberg and his co-workers (Goldenberg *et al.*, 1978; Nagell *et al.*, 1980; Primus *et al.*, 1980). This is important since it indicates that plasma CEA levels cannot be used to select patients for the application of this technique. The amount of CEA in the tumour as estimated either by an immunohistochemical stain or by extraction and subsequent radioimmunoassay may be a more reliable guide.

The results accord with the hypothesis that the anti-CEA antibodies are localized in the tumour by reaction with CEA at that site. In relation to this, we have shown by autoradiography that monoclonal antibodies raised to cell-surface antigens of human teratoma xenografts localize in close association to viable tumour cells after *in vivo* administration of the antibodies (Moshakis *et al.*, 1981).

Many tumours contain large numbers of histiocytes, and it could be visualized that specific antibodies would be localized in tumours, owing to uptake of immune complexes by the histiocytes. Although we looked for evidence to support this alternate hypothesis we found none. The levels of circulating immune complexes were similar in mice bearing HX99 tumours and those bearing HX106, yet one tumour showed a high degree of localization of specific antibody, whilst the other had none. Furthermore, anti-CEA alone and anti-CEA/CEA complexes failed to localize in a murine fibrosarcoma which is known to have a high percentage of macrophages in its cell population.

Although in this system, using these particular tumours, the localization index increased with time, the total amount of radioactivity in the tumours decreased. The optimal time at which to use an external radioscan in patients is balanced between the two factors. The other point to note is that at all times, apart from the tumour, the most radioactive tissue was the blood. This emphasizes the need for a technique to subtract the activity in the blood pool.

Having established the usefulness of this model system in relation to the well-known antigen CEA, we are now in a position to extend this work to other less well studied antigens and to patients. Experiments have started using affinity-purified antibodies to the epithelial membrane antigen (EMA) expressed by human breast carcinomas (Heyderman *et al.*, 1979; Sloane & Ormerod, 1981) and to teratomas, through the use of monoclonal antibodies (Moshakis *et al.*, 1981). We hope that this latter approach can, in time, be extended to the study of human breast carcinomas.

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