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THE SPATIAL DISTRIBUTION OF PARENTERALLY ADMINISTERED MONOCLONAL ANTIBODIES AGAINST CEA IN A HUMAN COLORECTAL TUMOUR XENOGRAFT

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Abstract

A recently developed experimental model consisting of athymic rats carrying human colonic tumours from the cell line LS 174 T in both hind legs was used. ^{125}I-labelled anti-carcinoembryonic (anti-CEA) monoclonal antibodies were injected either intra-arterially after a bolus injection of mannitol, or intra-peritoneally with or without mannitol. On the fourth day the rats were killed and pieces from the tumours and various organs were measured in a well scintillation counter. Tumour pieces were then submitted to autoradiography and immunohistochemistry for examination of the antibody distribution at the cellular level. In all examined tumours injected with anti-CEA antibodies, most of the antibodies were located in the periphery close to fibrovascular septa. It appears, in addition to the specificity of the antibody for the CEA, that the tumour vascular permeability and anatomy are of utmost importance for tumour targeting in this experimental model with the particular antibody used.

Key words: Colorectal cancer; xenograft, athymic rats, monoclonal CEA-antibodies, spatial distribution.

Both intact monoclonal antibodies (mabs) and fragments of mabs directed against human tumour-associated antigens can potentially be used as carriers of radionuclides for in vivo diagnosis of solid tumours. Many encouraging results have been reported about the use of radiolabelled mabs for tumour localization in experimental models (1-3) and in clinical situations (4-6). However, mabs that are highly specific in vitro are much less specific in vivo as evaluated by in vivo measurements and measurement of excised tissues (7).

Radiolabelled antibodies against tumour-associated antigens in tumours have usually been localized either by measuring the activity in removed tumour and normal tissue, or by external photoscanning. Only few studies have been reported in which the histological distribution of antibodies in tumours has been examined in conjunction with measurements of activity after administration of radiolabelled antibodies in vivo (5, 8). Hedin et al. have previously shown excellent tumour localization with an intact anti-CEA mab (mab 38S1) injected intraperitoneally in nude mice bearing human colonic tumour xenografts from the cell line LS 174 T (9). In a recently developed experimental model with nude rats carrying LS 174 T tumours, we investigated the pharmacokinetics of intra-arterially (i.a.) injected mabs of the same type as used by Hedin et al. (10, 11). In those studies it was found that i.a. infusion of mannitol prior to antibody injection increased the tumour uptake of antibodies (11).

The present investigation was undertaken to examine the tumour uptake and the histological distribution of the injected antibodies in the xenografts by different modes of antibody and mannitol administration. After measurements in a well counter pieces of the excised tumours were submitted to autoradiography and immunohistochemistry for localization of the anti-CEA antibodies. The immunohistochemical expression of CEA in the tumours was also studied.

Material and Methods

Tumour model. A tumour cell line, LS 174 T, from a moderately well differentiated colonic adenocarcinoma was used. The cells were grown in vitro in RPMI 1640

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supplemented with 10% fetal calf serum and penicillin-streptomycin (12). 10^7 cells suspended in 0.1 ml of medium were injected subcutaneously lateral to the knee in each hind leg of athymic nude male rats (LEW/MOL-rnu/rnu, Møllegaard, Denmark). Ten to 14 days after injection of the cells the tumours were palpable and measured between 0.5–1 cm in diameter.

Monoclonal antibodies. A monoclonal mouse anti-CEA antibody (mab 38S1) of the IgG1, kappa isotype (13), produced in vitro, was used. Purified antibody (14) was labelled with ^{125}I by the chloramine-T method (15). Before the injection into the rats, free iodine was separated from antibody-bound iodine by gel filtration on Sephadex G25M (Pharmacia AB, Sweden). The immunoreactivity of labelled antibody was checked by incubation in an excess quantity of CEA coupled to a solid phase. At equilibrium, the fraction of bound antibody was about 75%. As a control a monoclonal antibody of the IgG1, kappa isotype against thyroid stimulating hormone (anti-TSH; mab No. 79B), labelled with ^{125}I, was used.

Administration of antibodies. The operative techniques have been described previously (10). Briefly, 6 rats with a tumour in each hind leg were injected with 0.2 ml ^{125}I-labelled anti-CEA mabs (5 μg/ml, 50×10^6 counts per min (cpm)/ml) on one side into a branch of the femoral artery (Iam group). The mabs were injected immediately after a bolus injection of 1.5 ml of mannitol (150 mg/ml, 950 mosmol/kg) given in about 15 s into the i.a. inserted catheter. Convulsions were noted in the injected leg during injection of mannitol.

The same amount of anti-CEA mabs was given intraperitoneally (i.p.), either alone (Ipk group, 6 rats) or immediately after injection of 1.5 ml of mannitol (Ipm group, 6 rats).

On the fourth day after injection the rats were killed and pieces of the tumours and of various organs were weighed and the activity was determined with a well type gamma-counter. The uptake of anti-CEA mabs by various tissues was expressed as cpm/g tissue.

Tumours submitted to autoradiography and immunohistochemistry. After measurement in a well counter the tumours on the injected side in the Iam-group (n=6) and 8 tumours from the Ipk-group were cut into 2 pieces each. The 2 specimens of each tumour were then submitted to autoradiography and immunohistochemical staining procedures respectively.

As a control 3 rats were injected with radiolabelled irrelevant monoclonal antibodies (anti-TSH) i.p. Four days after injection these rats were killed and one tumour from each rat was excised and submitted to autoradiography.

The number of tumours submitted to autoradiography and immunohistochemistry in the groups treated in different ways is given in Table 1.

Autoradiography. Tumour pieces from rats injected with antibodies and from 2 'non-treated tumours' were fixed in Bouin's solution (15 ml of 1.2% picric acid, 5 ml of 40% formaldehyde, 1 ml of conc. glacial acetic acid). The fixed tissues were embedded in paraffin and serial-sectioned at 7 μm. After deparaffiniazation, these sections were dipped in Kodak NTB-2 emulsion and exposed for 2 weeks at 4°C. The autoradiographs were developed for 6 min in Kodak D-19 and fixed for 10 min in F-24. The emulsion base was allowed to dry overnight and the sections were lightly counterstained with haematoxylin and mounted. Preliminary experiments permitted establishment of the fixation and developing conditions that would give minimal background activity without appreciable loss of grain formation.

Table 1

Survey of the number of tumours submitted to autoradiography and immunohistochemistry in the groups treated in different ways.

Investigation method	Untreated	Anti-TSH antibodies	Anti-CEA antibodies	Total
Autoradiography	2	3	14	19
Immunohistochemistry	10	–	14	24
Total	12	3	28	43

Immunohistochemical procedures. The expression of CEA was studied in sections of all xenografts both from rats which had and had not received anti-CEA mabs. Modifications of the avidin-biotin peroxidase method (16) were employed with use of a commercial kit (Vectastain ABC Kit, Vector Laboratories, Burlingame, Calif. USA). Five-micrometre sections of frozen tissue blocks were placed on chromium-gelatin slides. The sections were then air-dried, fixed for 10 min in acetone at room temperature, and rinsed in PBS (0.01 M, pH 7.4). Non-specific background was reduced by incubation for 15 min with 1% bovine serum albumin. The primary antibody applied was the same anti-CEA mab that was used in the experiments (mabs 38S1). Biotinylated anti-mouse IgG served as secondary antibody (dilution 1/200 v/v; 30 min), and a peroxidase conjugated avidin-biotin complex as third step reagent (1/200v/v, 30 min). The slides were rinsed in PBS between the steps. Staining was developed in 3-amino-9-ethylcarbazole and 0.002% hydrogen peroxide (15 min). Mayers' haematoxylin was used for counterstaining (5 min). Primary antibody was regularly replaced on parallel sections by 1% bovine serum albumin in order to rule out non-specific staining, and positive controls were also included.

For the immunolocalization of anti-CEA mabs within xenografts from injected rats the same procedure was used as described above, except that the sections were not incubated with anti-CEA mabs, i.e. the injected anti-CEA mabs served as primary antibodies.

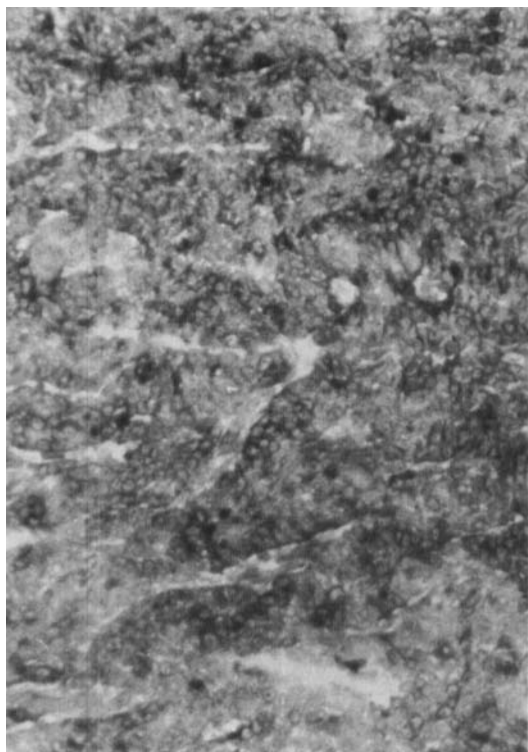

Fig. 1. Immunohistochemical staining for CEA in an 'untreated' tumour. Dark-stained (weakly or strongly) areas correspond to the presence of CEA. ×178.

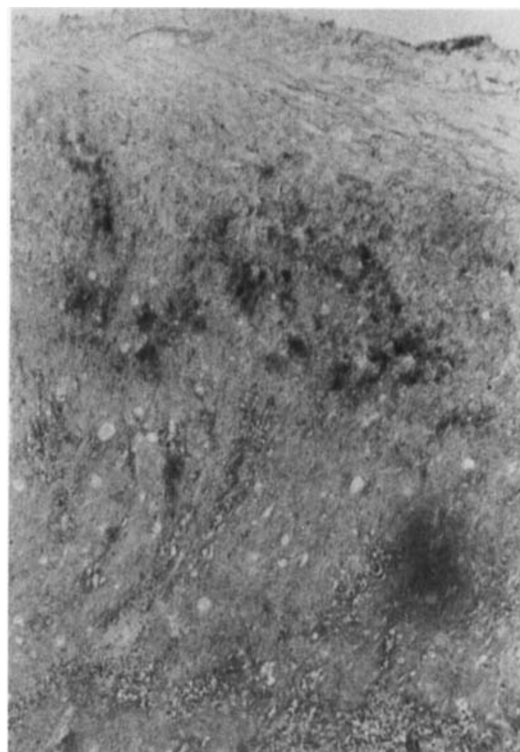

Fig. 2. Autoradiogram after administration of monoclonal anti-CEA antibodies i.p., showing accumulation of grains over the periphery of the tumour close to the capsule of connective tissue surrounding the tumour. ×59.

Sections of tumours from rats which had not received any antibodies were included as negative controls.

Results

Measurement of radioactivity. The mean ratios of uptake of anti-CEA mabs in tumours on the injected side to that in various tissues (Iam group), and in tumours on each side to that in various tissues (Ipk and Ipm groups) are given in Table 2. A significantly higher ratio was noted in the Iam group, except for tumour/muscle, tumour/thyroid and tumour/small intestine, than in the other 2 groups (Ipk and Ipm). No significant differences were seen between the ratios in the Ipk and Ipm groups.

Immunohistochemical expression of CEA. Virtually all cells in the tumours expressed CEA strongly, although negative as well as weakly stained cells occurred (Fig. 1). This was in contrast to the adjacent normal tissue which was negative. A positive reaction for CEA was seen mainly at the epithelial cell surface, but also within the cytoplasm. The cytoplasmatic staining was diffuse. Staining of secretion products in the glandular formations was occasionally observed.

Autoradiography. In each of the groups of rats injected with anti-CEA mabs over 200 sections of the tumours were studied in a light microscope. Antibody binding, defined as accumulation of grains, was present in all these sections and showed comparable patterns in all examined tumours injected with anti-CEA mabs. The different administration forms of anti-CEA mabs did not affect the gross features on the autoradiograms.

As a whole, most parts of the tumours, both necrotic and viable tumour tissue, and surrounding normal tissue displayed scattered grains with a density similar to those of the blank slide. The radioactivity in these parts was considered equal to the background level, i.e. no uptake of antibodies was observed.

Occasionally, a higher frequency of labelled anti-CEA antibodies was observed and there was a general tendency for these antibodies to be concentrated to the peripheral parts of the tumours (Fig. 2). Thus, clusters of grains were seen over viable tumour cells close to tiny fibrovascular septa (Fig. 3). On the other hand, there were other areas, morphologically very similar, which did not contain much radioactivity.

The precise site of antibody binding on individual tumour cells was difficult to assess on the autoradiograms. However, the grain clusters were projected over the tumour cells and not over the fibrovascular septa or the pseudolumen of the glandular formations.

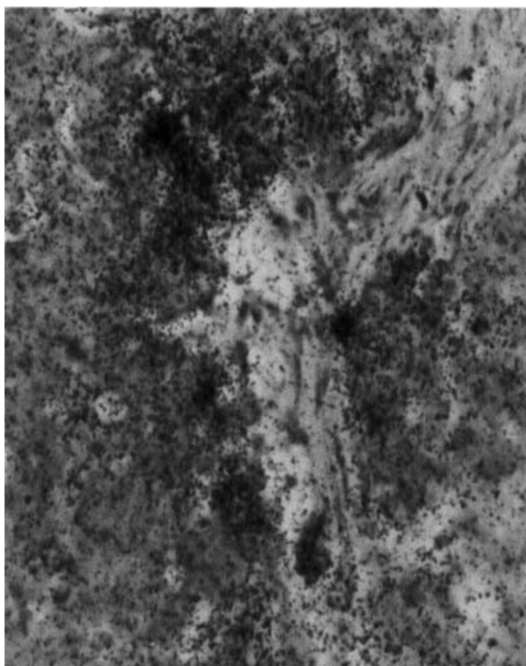

Fig. 3. Autoradiogram after i.a. administration of monoclonal anti-CEA antibodies and mannitol, showing clusters of grains over tumour cells close to a fibrous septum. ×416.

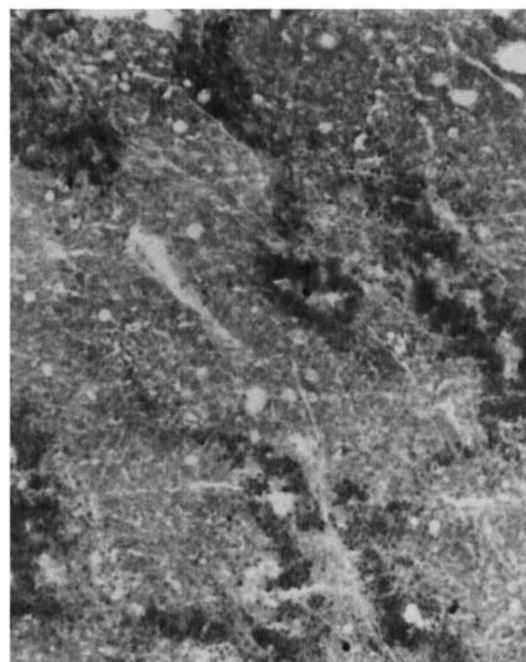

Fig. 4. Immunohistochemical staining for distribution of injected antibodies in a tumour injected i.a. with monoclonal anti-CEA antibodies and mannitol, showing antibody binding close to fibrous septa. Dark-stained areas correspond to the presence of antibodies. ×74.

Table 2

Ratios of activity (cpm/g tissue) in intra-arterially (Iam, n=6) and intraperitoneally injected tumours (T) (Ipk, n=12; Ipm, n=12) to that in other tissues. Mean values ±1 SD. An one-tailed unpaired Student's t-test is used to calculate the significance levels

T/other tissue	Anti-CEA antibodies i.p. (Ipk)	Anti-CEA antibodies+ mannitol i.p. (Ipm)	Anti-CEA antibodies+ mannitol i.a. (Iam)	Student's t-test (Iam/Ipk)
Muscle adjacent to tumour	55.4±18.5	48.7±23.7	57.8±28.5	NS
Spleen	13.6±5.5	11.8±3.6	18.6±4.4	0.01<p<0.05
Kidney	13.0±6.6	10.6±3.3	19.0±4.8	0.01<p<0.05
Liver	11.2±4.6	9.6±2.9	15.1±3.6	0.01<p<0.05
Thyroid	0.08±0.06	0.05±0.02	0.05±0.02	NS
Colon	31.5±11.0	30.5±12.9	47.5±7.8	0.01<p<0.001
Small intestine	28.6±10.2	21.2±9.6	40.7±20.7	NS
Gonad	15.3±5.1	14.2±4.0	32.7±10.4	p<0.001
Blood	1.5±0.5	1.3±0.4	2.5±0.6	p<0.001

No accumulation of grains was observed in the control tumours treated with unspecific antibodies. The untreated tumours showed a grain density at background level.

Immunohistochemical detection of anti-CEA mabs. The sections of xenografts from rats injected with anti-CEA mabs showed clearly defined areas with immunoreactivity (Fig. 4), while the controls were mainly negative (weak positive staining was occasionally seen in necrotic areas). The distribution of positive staining resembled the pattern found at autoradiography, i.e. positive foci close to fibrous septa were heterogeneously distributed over the sectioned tumour area. The cellular location of the antibodies was similar to that of the expression of CEA (membranous and cytoplasmatic).

Discussion

Measurements of radioactivity in various excised tissues did not show any difference between the 2 groups with i.p. injected mabs (Table 2). The ratios in these 2 groups were similar to those previously obtained with plain i.a. injection of mabs (10, 11). When mannitol was given as a bolus i.a. prior to antibody administration, the tumour/tissue ratios were slightly increased. This increase was less pronounced than when mannitol was infused i.a. prior to i.a. injection of anti-CEA antibodies in previous experiments (11).

Measurements in the present study together with those of previous investigations indicate that the form of administration—i.p., i.v. or i.a.—does not affect the tumour uptake of antibodies as compared with the uptake in other tissues in our experimental model.

Antigenic heterogeneity has proved to be a common property of most malignant tumours (17, 18). It constitutes a potential problem in the development and optimization of immunodiagnostic or immunotherapeutic procedures. This problem was minimized in the present experimental model by choosing the tumour cell line LS 174 T, which has proved to express and release high concentrations of CEA (12, 19). Immunohistochemical staining of sections from the xenografts revealed a homogenous expression of CEA, i.e. almost all tumour cells were positive for CEA. Despite the homogenous distribution of CEA within the tumours a heterogenous pattern of injected anti-CEA antibodies was found, both at autoradiography and at immunohistochemistry. Furthermore, with the antibody dose used in the present study only a minority of tumour cells was covered by injected antibodies. Apparently, the specificity of the antibody and the expression of CEA on the cells are not the only critical factors for tumour targeting in this experimental model. Several other possibilities must be considered.

Harwood et al. have shown in an experimental model that it is possible to increase the absolute tumour uptake of intact anti-CEA antibodies by increasing the antibody dose (20). However, they noted a concomitant decrease in the tumour to normal tissue ratios of uptake of mabs as they increased the antibody dose above the level used in the present study.

The importance of the vascularization of tumours for antibody targeting has been pointed out previously (2, 8, 21). In a previous study we investigated the angioarchitecture of the LS 174 T tumours and the histological relationship between vessels and tumour cells (22). The tumours were found to have an extensively vascularized periphery and an almost avascular necrotic centre. Vessels were present in septa of connective tissue surrounding adenomatous tumour nodules. By combining these results with the present of antibody distribution it seems that the injected antibodies accumulate adjacent to vessels. However, the observation that in practice all segregated antibodies in our tumours had this location does not mean that all vessels and fibrovascular septa were surrounded with antibodies. Morphologically very similar areas in the tumour tissue could display either a high concentration or total absence of antibodies. The reason for this heterogeneity is obscure.

One possible reason for the observation that antibodies were only adjacent to certain fibrovascular septa could be the non-homogeneous blood flow distribution in tumours (23). This explanation is supported by findings following vascular corrosion casting of LS 174 T tumours, of squeezed vessels and vessels with a number of blind endings indicating a high tumour pressure and vascular resistance predominantly in the centre of the tumours. On the other hand, other areas showed dilated vessels implying low vascular resistance (22). Another possible explanation might be that not all vessels are permeable to antibodies. Normally a molecule of IgG antibody size (mol wt 150 000) will not pass through the vascular wall, but tumour vessels are known to be more permeable to large proteins than other vessels (24).

Data on the actual site of antibody binding in experimental models using autoradiography as localization method are conflicting (2, 8, 25, 26). Antigens can be present within the cells, on the surface, or externally. Wolf et al. (27) found that the CEA antigen in LS 174 T cells was mostly attached to the outer cell membrane, which was supported by our present study. The in vivo binding of antibodies at the cellular level was similar to the expression of CEA, which supports the idea of specific binding between the antibody and the antigen.

Dehalogenation is known to be a problem when using radioisotopes of iodine. Epenetos et al. (5) reported that 30% of the activity of labelled antibodies was excreted as free iodine in the urine, and Ghose et al. (28) found that up to 90% of administered iodine was excreted. An index of in vivo dehalogenation can be calculated approximately by not suppressing the uptake of free iodine in the thyroid with potassium iodine (7). The activity (cpm/g tissue) of the unblocked thyroid in our experiments was far above that observed in the tumour (Table 2). Our results indicate that the intraperitoneal form of administration of antibodies does not increase the uptake of free iodine in the thyroid as suggested by Mann et al. (7).

The in vivo distribution of antibodies at the cellular level in our study indicates that an enormous increase in tumour uptake is needed to get almost all cells labelled with antibodies, as seen when the tumours are stained for CEA in vitro. As pointed out by other authors, there are many yet defined and undefined factors determining the tumour uptake of antibodies in vivo other than the specificity of the antibody for the antigen (5, 29–31). From the present investigation we conclude that the vascular permeability and vascular anatomy of tumours are of utmost importance for tumour targeting with intravascularly administered antibodies.

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