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Immunohistochemical monitoring of metastatic colorectal carcinoma in patients treated with monoclonal antibodies (MAb 17-1A)*

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Summary. A therapeutic trial using repeated doses of a mouse monoclonal antibody against the tumor-associated antigen (TAA) CO17-1A in metastatic colorectal carcinomas was carried out. Metastatic lesions sampled by repeated thick needle (1.2 mm) biopsies during therapy were examined immunohistochemically for the presence of various TAAs, mouse IgG, complement, and infiltrating leukocytes. The CO17-1A was consistently expressed in all cases along the basement membrane of tumor glands and could only be demonstrated on cryostat sections whereas the TAAs GICA19-9, GA73-3, and Br55-2 were also visualized in B_5-fixed paraffin-embedded biopsies. The CO17-1A and GA73-3 were predominantly present at the basal region in contrast to the GICA 19-9 and Br55-2 which were predominant at the luminal and the apical region of the tumor glands. Antigenic modulation was not seen either after 24–72 h or during prolonged treatment. In all cases the infused mouse IgG was detected, from 24 h after infusion up to 6–8 weeks, mainly along the basal region of tumor glands. In 13/14 posttreatment biopsies, complement factor C3 was found at the same sites as mouse IgG. In 6 out of 9 posttreatment biopsies an increase in mononuclear cells (monocytes, natural killer (NK) cells and/or T cells) was observed. Monocytes were close to the tumor cells whereas NK cells and T cells were predominantly scattered in the stroma.

Introduction

Tumor cells might be distinguished from their nonmalignant counterparts by increased expression of tumor-associated antigens (TAAs). Many TAAs are however also found on normal cells during various stages of differentiation, notably on fetal cells. On normal, mature cells some TAAs may be detected with highly sensitive methods only. These TAAs often become highly expressed on malignant cells [20]. Such antigenic structures are useful as diagnostic markers as well as potential targets for immunotherapy.

On the cell surface of colorectal carcinomas several TAAs have previously been identified, such as CO17-1A, GICA19-9, GA73-3, and Br55-2 and have also been chemically characterized [13, 16, 22, 31]. Mouse monoclonal antibodies (MAb) have been raised against these antigenic structures [14]. One of them, MAb 17-1A (IgG_{2A}) has been used for immunotherapy in a large number of patients with colorectal and pancreatic carcinomas. Objective responses and even complete remissions have been observed [5, 8, 24, 25, 26, 28, 30, 32].

The mechanisms responsible for tumor regression after immunotherapy with MAb are not fully understood. Monocyte/macrophage and/or natural killer (NK) cell-mediated antibody-dependent cellular cytotoxicity (ADCC) has been suggested as an important effector function [1, 11, 12, 23, 29]. It is not known whether complement-mediated cytolysis also has a role in the in vivo destruction of solid tumors.

To evaluate the therapeutic efficacy of MAb 17-1A in metastatic colorectal carcinomas we have initiated a phase II study. In the present report we describe the immunohistopathology of metastases from patients treated with MAb together with isolated autologous mononuclear cells (MNC) armed with MAb. As autologous MNC might be regarded as potential ADCC effector cells, it has been suggested that isolation of autologous MNC by leukapheresis and incubation in vitro with MAb before infusion, would increase the number of cytotoxic cells in the tumor lesions (Douillard personal communication 1987). The study focused on the evaluation of the binding of infused MAb to the tumor cells, antigenic modulation, infiltration of the tumor by MNC, and local deposition of complement during immunotherapy. Such information should be of value in the development of future treatment protocols with MAb. A detailed description of the clinical effects of MAb therapy have been reported elsewhere [18, 19].

Materials and methods

A total of 16 patients with metastatic colorectal cancers initially treated with surgery entered the study. The clinical characteristics of the patients are shown in Table 1.

Three different treatment protocols of MAb 17-1A were used. During the observation period only MAb therapy was given. The protocol for the first 10 patients (nos. 1–10) has been described in detail elsewhere [8]. In short, a total dose of 1000 mg MAb 17-1A was given; initially 400 mg and then 200 mg a further three times with about 6-week intervals. The antibody was incubated in vitro with

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Table 1. Patient characteristics

Patient no.	Sex/age (years)	Site of primary tumor	Dukes modified classification	Time from primary surgery to therapy (months)	Site of metastasis
1	F/37	Rectum	C2	24	Liver, lung, pelvis
2	F/69	Rectum	B3	25	Liver, lung, colostomy
3	M/70	Rectum	C3	9	Liver, lung, pelvis, colostomy
4	M/32	1. Cecum	A	1	Mesenteric root
		2. Transverse colon	C3		
5	F/55	Colon	D	1	Liver
6	F/48	Rectum	D	3	Liver
7	M/75	Colon	C2	13	Liver
8	F/81	Colon	C2	75	Liver
9	F/65	Colon	B1	24	Lung, pelvis
10	M/69	Colon	C3	18	Pelvis
11	F/69	Colon	C3	2	Iliac nodes
12	F/73	Colon	D	5	Liver
13	M/44	Colon	B3	12	Liver, pelvis, abdominal wall
14	F/60	Colon	C2	16	Peritoneum, liver
15	F/66	1. Rectum	B3	11	Liver
		2. Rectum	B3	11	Pelvis
16	M/68	Colon	D	3	Liver

autologous MNC and thereafter infused in 225 ml balanced salt solution with 1% human albumin. The other 5 patients (nos. 11–15) received a total dose of 3.6 g MAb scheduled as follows: 400 mg on days 0, 2, and 5 (total dose 1.2 g) repeated twice at 3-week interval. The first infusion (day 0) was given together with autologous MNC as described before and the following infusions (days 2 and 5) in 250 ml physiological saline. Patient no. 16 received 4.8 g of MAb in total given in 200 mg doses every 2nd day for 24 days. The first infusion was given together with autologous MNC and all the others in physiological saline.

Biopsy technique. Biopsies were taken prior to and 24–72 h after an infusion of MAb and after checking the patient's hemostasis. At the third treatment course, patient no. 13 was also biopsed during therapy (day 3) in addition to the pre- and posttreatment (day 6) biopsies. Usually a liver metastasis was selected for biopsy using a dynamic ultrasound sector scanner (Technicare, Autofocus 3.5 or 5 MHz) to determine the route of puncture. Under ultrasonic guidance, an aspiration biopsy using a fine needle (0.5–0.9 mm diameter/20–25 G) was taken from the marginal part of the metastasis. The smears were studied immediately for the presence of tumor cells. The aspiration biopsy was repeated until representative material was found. The direction was thus determined for the final thick needle biopsy which was performed using an automatic biopsy system (Biopty-cut, Radiplast Comp., Uppsala, Sweden) with an 18 G (1.2 mm diameter) tru-cut needle (Radiplast Comp., Uppsala, Sweden). The tissue core, 17 mm long, was immediately placed in cold physiological saline.

Processing of the biopsy material. In all patients, the primary tumor and the metastases or the local recurrence (Patient no. 10) were analyzed for the presence of TAA. Representative pretreatment needle biopsies were obtained from 10 of 16 patients. In 5 patients (nos. 2, 3, 9, 11, and 15) pretreatment needle biopsy material was not available. These patients were selected for treatment with MAb 17-1A on the basis of the presence of the antigen GA73-3, which is another epitope on the same molecule as CO17-1A [22]. One part of the biopsy was fixed in formal sublimate (B5) for routine histopathology and immunohistochemistry on paraffin sections. The rest of the material was snap frozen in liquid nitrogen and stored at −70° C. Cryostat sections (8 to 10 μm) were cut and dried overnight at +4° C. The sections were then fixed in −20° C acetone for 10 min.

Monoclonal antibodies. The MAbs used for immunohistochemistry are shown in Table 2.

Table 2. Monoclonal antibodies used for immunohistochemical staining

MAb	Dilution	Relevant specificity
CO-171A[a]	1 : 25	Gastrointestinal cancer
GICA19-9A and B[a]	1 : 10	Gastrointestinal cancer
GA73-3[a]	1 : 10	Gastrointestinal cancer
Br55-2[a]	1 : 200	Gastrointestinal cancer
PKK1[b]	1 : 25	Cytokeratin (GP40–55)
Leu-2a[c] (CD8)	1 : 40	Cytotoxic/suppressor T cells
Leu-3a[c] (CD4)	1 : 40	Helper/inducer T cells
Leu-4a[c] (CD3)	1 : 40	Pan T cells
Leu-7[c]	1 : 60	T and natural killer (NK) cell subsets
Leu-11b[c] (CD16)	1 : 20	NK cells and neutrophils
Leu-M1[c] (CD15)	1 : 40	Monocytes, granulocytes
Leu-M2[c]	1 : 40	Monocytes, platelets
Leu-M3[c] (CD14)	1 : 10	Monocytes, macrophages
Leu-M5[c] (CD11c)	1 : 25	Monocytes, macrophages

[a] Courtesy of Prof. H. Koprowski and Z. Steplewski, Wistar Institute, Philadelphia, USA
[b] Lab System, Helsinki, Finland
[c] Becton Dickinson, Mountain View, Calif., USA

Immunohistochemistry. The MAb binding to cryosections was demonstrated by the peroxidase anti-peroxidase (PAP) method with rabbit anti-mouse serum and mouse-PAP complex (Dakopatts, Copenhagen, Denmark) [17]. Alternatively, a double indirect alkaline phosphatase (AP) method with AP conjugated rabbit anti-mouse Ig and AP conjugated swine anti-rabbit Ig (Dakopatts, Copenhagen, Denmark) was used [21]. All conjugates were absorbed with normal human Ig to minimize cross-reactivity with human Ig.

Fixed cryostat sections were incubated with the antibodies in a moist chamber for 30 min at room temp. Between incubations the sections were washed in Tris-buffered saline (TBS) for 10 min. The marker enzymes were visualized using diaminobenzidine (Sigma, St. Louis, Mo., USA) for the PAP and the substrate kit Vector Red (Vector Lab., Burlingame, Ca., USA) for the AP. Sections were then rinsed in TBS, counterstained with hematoxylin and mounted either in Eukitt (O. Kindler, Freiburg, FRG) (PAP method) or gum syrup (AP method). Immunohistochemical staining of formalin- or B5-fixed paraffin-embedded tissue was performed after pretreatment with pronase by the ABC method [15] using an ABC kit from Vector.

The presence of mouse IgG was detected by staining with enzyme conjugated anti-mouse Igs. Immunofluorescence staining for complement factor 3 (C3) was performed with a fluorescein isothiocyanate-conjugated sheep anti-serum against C3 (Wellcome, Bekenham, Kent, UK) and the sections examined in a Zeiss epifluorescence microscope.

Evaluation of immunohistochemical staining. The frequency of tumor cells positive for TAAs was counted in 10 high power fields (HPF = ×40) of nonnecrotic tumor tissue. Similarly, evaluation of leukocytes infiltrating the tumor was made in areas without obvious necrosis. The immune phenotypes of the MNC were estimated by counting cells stained by the various MAbs in 10 HPF. Only cells identified as nontumor cells were counted when cross-reacting leukocyte antibodies were evaluated. Giemsa-stained frozen sections were used to estimate total number of MNC infiltrates.

To assess the changes in the number of various MNC infiltrating the tumor, an infiltration index i.e., a ratio between post- and pretreatment biopsies was calculated as follows:

$$\text{infiltration index} = \frac{\text{mean nos. of MNC/HPF after treatment}}{\text{mean nos. of MNC/HPF before treatment}}$$

In patient no. 13, a corresponding ratio for MNC infiltration during treatment (days 3 and 6 respectively) was also calculated. A significant change in the number of MNC between the two biopsies was arbitrarily chosen as a ratio >3.0 or <0.3.

Criteria for response. A clinical complete response was defined as complete disappearance of all clinical, radiological, and biochemical evidence of tumor disease. A partial response was attained when there was a decrease of at least 50% in the product of two perpendicular diameters of all measurable disease manifestations and a $>50\%$ decrease in the serum concentration of carcinoembryonic antigen (CEA), CA19-9, and CA50. A minor response was defined as a decrease in the product of two perpendicular diameters of at least one tumor lesion with 25%–50%, and/or $>50\%$ decrease in the serum concentration of CEA, CA19-9, and/or CA50 and no increase ($>25\%$) in any lesion. Stable disease was defined as no significant change ($<25\%>$) in the size of all measurable lesions and no significant change ($<50\%>$) in the serum concentration of CEA, CA19-9, and CA50 for at least 3 months. Progressive disease was defined as an increase of $>25\%$ in the size of at least one measurable lesion and $>50\%$ increase in the serum concentration of CEA, CA19-9, and/or CA50.

Results

Histopathology

Of the patients, 6 had moderately and 8 had poorly differentiated adenocarcinomas, and 2 patients (nos. 4 and 15) had primary tumors at two sites (Table 3). The guidelines of Dukes and Bussey [6] were followed in classifying the differentiation of the tumor. Considering the current belief of in vivo selection of increasingly malignant clones, grading was based on the worst area and not on an overall appraisal. The glands at the advancing front of a tumor often appeared somewhat disorganized, especially when there was an associated inflammatory infiltrate. Such elements were not downgraded. As noted by Dukes and Bussey, principal emphasis was placed on cellular arrangement as opposed to cellular differentiation proper.

The metastases were in the majority of cases surrounded by vascularized fibrotic stroma and areas of necrosis were seen in the tumor. A small increase in the proportion of necrotic tissue was scored in 5 out of 11 evaluable posttreatment biopsies as compared to the pretreatment biopsies. In 4 biopsies no change was scored while in 2 a decrease in the proportion of necrotic area was noted.

Immunohistopathology

Table 3 shows the estimated frequency of tumor cells positive for the various MAbs and their staining intensity in the primary tumor and the first pretreatment biopsy of a metastatic lesion or local recurrence (patient no. 10). The staining patterns of the various MAbs in cryostat sections and in paraffin-embedded material are presented in Table 4. In paraffin sections stained with MAb 73-3, a moderate to strong diffuse cytoplasmic staining with infranuclear accentuation was seen. On occasions a strong supranuclear dot-like activity and a moderate to weak basement membrane-like pattern was also seen. Extracellular basophilic mucin was not stained by MAb 73-3. With MAbs 19-9 and 55-2 strong apical staining was noted. Extracellular basophilic mucin was always positive with the MAb 19-9 whereas the MAb 55-2 gave a variable reaction to mucin in paraffin-embedded material. In contrast intraluminal eosinophilic material was always positive for MAb 19-9 and MAb 55-2. With MAb 55-2 an occasional moderate to weak basement membrane-like staining was also noted. With MAb 19-9 and 55-2, the cells of the poorly differentiated areas showed a stronger and more diffuse staining as compared to those of better differentiated parts of the tumor.

In cryostat sections, MAbs 17-1A and 73-3 stained prominently the basal region of the tumor cells which in

Table 3. Proportion (%) of tumor cells stained by MAbs against tumor-associated antigens (TAAs) and cytokeratin and their staining intensiry (+/+++) in the pretreatment specimen

Patient no.	Differentiation of the primary tumor	Specimen for immunohistology	MAbs				
			17-1A	73-3	19-9	55-2	Cytokeratin
1	Moderate	Primary tumor[a]	NT	>90%+/++	10%+++	70%+++	NT
		Liver biopsy[b]	>90%+++	>90%+++	20%+++	>90%+++	>90%+++
2	Moderate	Primary tumor[a]	NT	60%(+)/+++	70%+++	80%++/+++	NT
3	Poor	Primary tumor[a]	NT	>90%++	>90%+++	>90%+++	NT
4	1. Well	Primary tumor[a] 1.	NT	>90%++	<10%+++	50%+++	NT
	2. Poor	2.	NT	>90%++/+++	30%+++	80%++/+++	NT
		Lymph node[b]	>90%+++	>90%+++	<1%(+)	<1%++	>90%+++
5	Poor	Primary tumor[a]	NT	>90%++/+++	25%+++	35%++/+++	NT
		Liver biopsy[b]	>90%+++	>90%+++	50%+++	>90%++	>90%+
6	Poor	Primary tumor[a]	NT	>90%+++	50%+++	>90%++/+++	NT
		Liver biopsy[b]	>90%+++	75%+++	25%+++	>90%+++	>90%+++
7	Moderate	Primary tumor[a]	NT	>90%++	20%+++	20%+++	NT
		Liver biopsy[b]	>90%+++	>90%+++	35%+	>90%+++	>90%+++
8	Poor	Primary tumor[a]	NT	>90%++/+++	10%+++	>90%+++	NT
		Liver biopsy[b]	>90%+++	>90%+++	<1%(+)	>90%+++	>90%+++
9	Moderate	Primary tumor[a]	NT	>90%++/+++	<1%+++	>90%(+)/+++	NT
10	Moderate	Primary tumor[a]	NT	>90%++/+++	>90%++/+++	>90%+/+++	NT
		Local recurrance[b]	>90%(+)	>90%+++	>90%+++	>90%++	>90%+++
11	Poor	Primary tumor[a]	NT	70%++/+++	>90%+++	60%++/+++	NT
12	Poor	Primary tumor[a]	NT	>90%+++	70%++/+++	90%++/+++	NT
		Liver biopsy[b]	>90%+++	>90%+++	>90%+++	>90%+++	>90%+++
13	Moderate	Primary tumor[a]	NT	>90%++	<10%+++	85%+++	NT
		Cutaneous biopsy[b]	>90%+++	>90%+++	>90%+++	>90%+++	>90%+++
14	Poor	Primary tumor[a]	NT	>90%(+)/+++	>90%+++	>90%++/+++	NT
		Peritoneal biopsy[a]	>90%+++	>90%+++	>90%+++	>90%+++	>90%+++
15	1. Poor	Primary tumor[a] 1.	NT	>90%+/+++	80%++/+++	80%++/+++	NT
	2. Moderate	2.	NT	>90%+/++	70%+++	70%+++	NT
16	Poor	Primary tumor[a]	NT	80%(+)/+++	<10%+++	>90%++/+++	NT
		Liver biopsy[b]	>90%+/+++	>90%+++	<1%+	>90%+++	>90%+++

NT, not tested
Staining intensity: (+), weak; +, mild; ++,moderate; +++,strong
[a] Paraffin-embedded material; [b] Cryostat material

Table 4. Staining pattern of the various TAA MAbs in paraffin and cryostat sections

MAb	Paraffin section	Cryostat section
17-1A	– Negative	– *BM-like* – Diffuse cytoplasmic
73-3	– *Diffuse cytoplasmic* – Supranuclear dot like – Infranuclear – BM-like	– *BM-like* – Diffuse cytoplasmic
19-9	– *Apical* – *Intraluminally secreted eosinophilic material* – Diffuse cytoplasmic	– *Apical* – *Intralumnially secreted eosinophilic material* – Diffuse cytoplasmic
55-2	– *Apical* – *Intraluminal.y secreted eosinophilic material* – Diffuse cytoplasmic – BM-like	– *Apical* – *Intralumnially secreted eosinophilic material* – Diffuse cytoplasmic – BM-like

BM, Basement membrane
Italics denote the most prominent staining pattern

tumor glands often appeared as a continuous basement membrane-like pattern (Figs. 1 and 2). In addition, moderate to strong diffuse cytoplasmic staining was also seen. However, no enhanced staining was seen in the apical region of the cytoplasm. The secreted intraluminal eosinophilic material was unstained. In contrast MAbs 19-9 and 55-2 both preferentially stained the apical regions of tumor glands and intraluminally secreted eosinophilic material. MAb 19-9 also gave weak cytoplasmic staining in some tumor cells leaving others virtually negative. MAb 55-2 in contrast stained the cytoplasm of most tumor cells with moderate intensity in addition to the strong apical staining. Occasionally basement membrane-like staining was also seen with MAb 55-2 (Figs. 3 and 4). The liver cells were unstained by any of the four MAbs.

In 11 out of the 16 cases, where the primary tumor was compared to the metastasis or the local recurrence (patient no. 10, Table 3), there was no difference in expression of the antigen GA73-3, while in 4 cases (patient nos. 4, 5, 7, and 13) a difference in the percentage of tumor cells expressing the antigen Br55-2 or GICA 19-9 was recorded.

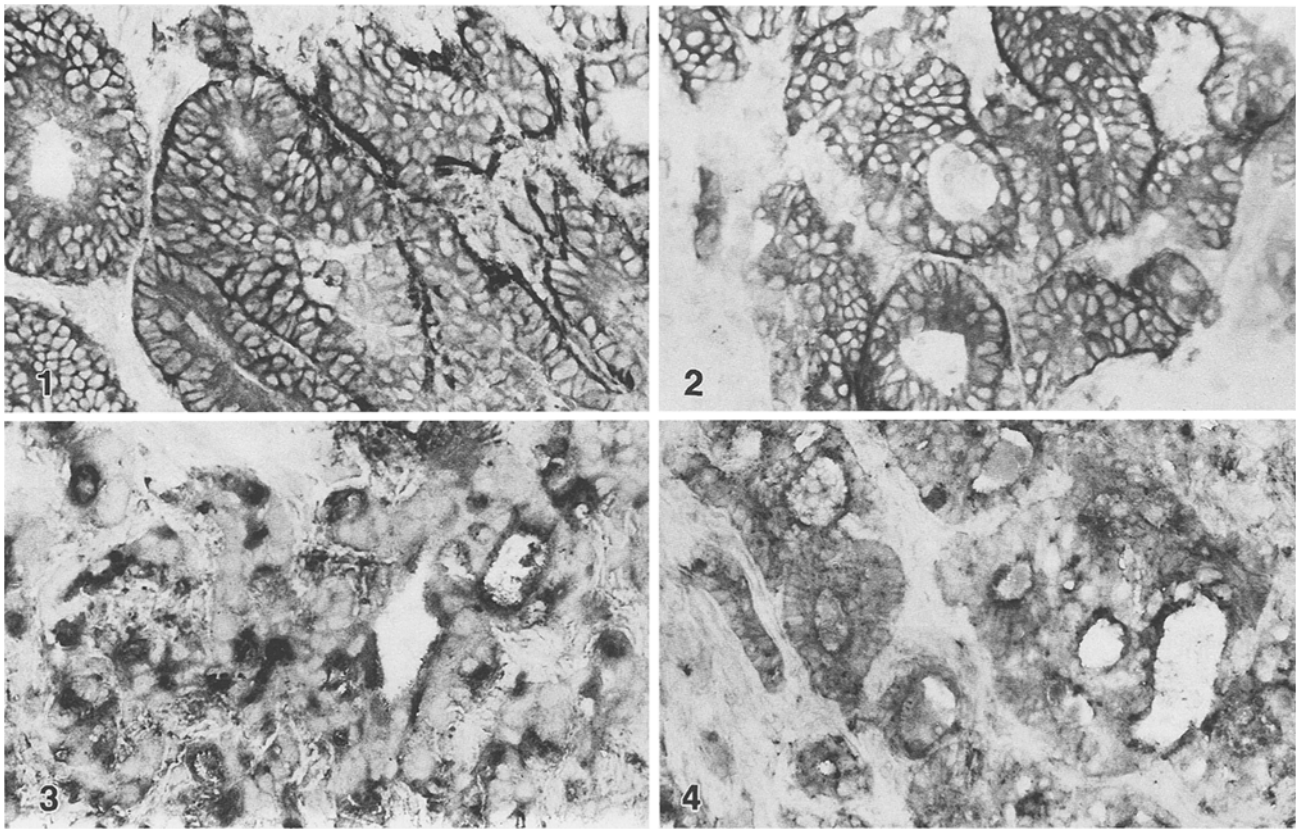


Fig. 1. Immunostaining of a cryostat section of liver biopsy with monoclonal antibody (MAb) 17-1A by the peroxidase anti-peroxidase (PAP) method. All tumor cells showed diffuse cytoplasmic staining. Note the characteristic basement membrane-like staining of the tumor glands (×380)

Fig. 2. Immunostaining of a cryostat section from a liver metastasis with MAb 73-3 (PAP). Note the similarity in staining to MAb 17-1A (see Fig. 1) (×380)

Fig. 3. Immunostaining of a cryostat section from a liver metastasis with MAb 19-9 (alkaline phosphatase, AP). The apical region of the tumor cells and the intraluminally secreted material were strongly stained. In some cells strong cytoplasmic staining was also seen (×390)

Fig. 4. Immunostaining of a cryostat section from a liver metastasis with MAb 55-2 (AP). The cytoplasm of all tumor cells was diffusely stained. In addition, stronger reactivity was seen in the apical region of some cells. Some luminal material was also stained (×290)

This change could be due to variability in expression of the antigens and/or sampling errors inherent in the biopsy technique. However, in no case was a complete loss of antigenic expression seen.

Immunohistopathological monitoring during MAb therapy

On nine occasions in patient nos. 6, 7, 8, 13, and 16 representative pairs of pre- and posttreatment biopsies were obtained. The staining intensity and the percentage of viable tumor cells reacting with the various MAbs to TAAs did not differ significantly between the two biopsies (data not shown). No signs of antigenic modulation were observed, either after 24–72 h or after repeated infusions.

Staining for mouse Ig in the first pretreatment biopsy was negative in all cases. After therapy, mouse Ig could be seen most prominently along the basal region of the tumor glands (Fig. 5A, B) though occasionally a stromal deposition was seen. The infused mouse Ig could be seen notably in all posttreatment biopsies and also in 6/8 subsequent biopsies obtained just before repeated infusion 6–8 weeks later. The intensity of the staining was however very weak in the latter biopsies.

Using MAbs identifying T cells, NK cells, and monocytes/macrophages the number and type of infiltrating MNC was analyzed at 77 instances and an infiltrating index calculated (Table 5). Detailed identification of MNC subpopulations was restricted by the limited amount of representative material available. It was also influenced by the cross-reactivity of Leu-M1 and sometimes also of Leu-11b antibodies with tumor cells. In total, 9 evaluable pairs were collected. Comparing the pre- and posttreatment biopsies, 5 pairs had an increase in cells expressing monocyte-related antigens; in 1 pair a decrease was seen. In 2 pairs there was an increase in NK cells and in 2 other pairs an increase in T cells. Of the 5 pairs with an increase in monocytes, 2 also had an increase in T cells and in 1 of the pairs both monocytes and NK cells were increased. Taken together, in 6 out of 9 evaluable pairs of biopsies there were indications of an increase in MNC. In 2 paired

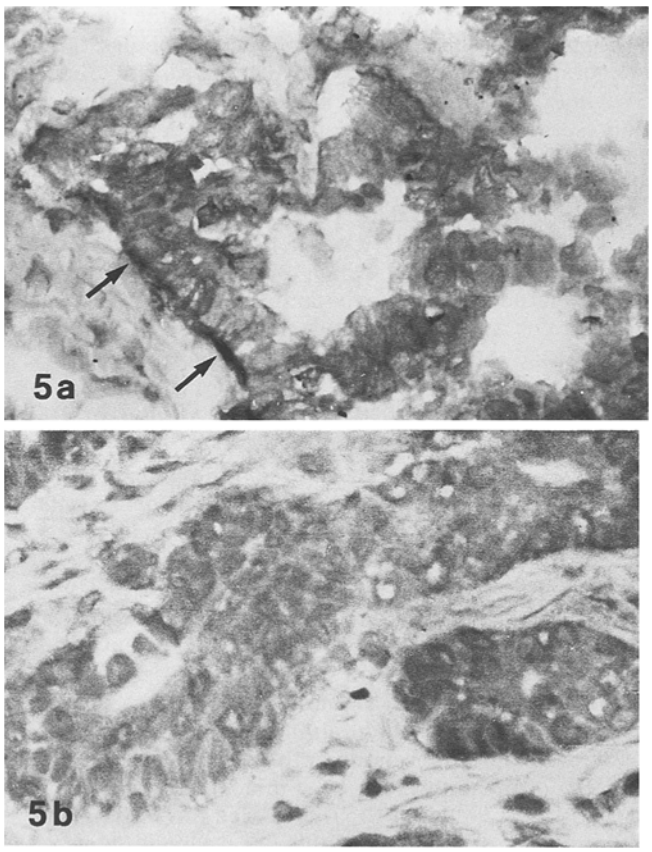


Fig. 5 A, B. Immunostaining of a cryostat section from a liver metastasis demonstrating the localization of infused mouse IgG seen mainly along the basal region of the tumor glands (*arrows*) **(A)** (PAP) (×560). The corresponding pretreatment biopsy was negative **(B)** (PAP) (×320)

biopsies, the numbers of MNC remained unchanged while only in 1 pair a marginal decrease in Leu-M2$^+$ cells was scored. T cells and NK cells were predominantly scattered in the stroma (Fig. 6). The majority of Leu-M1$^+$ and Leu-M3$^+$ cells (Fig. 7) and especially Leu-M5$^+$ cells were close to the tumor cells.

In the first pretreatment biopsy focal staining for C3 was occasionally noted, localized to leukocytes in the stroma. However, C3 was clearly detectable, associated with the basal region of the tumor glands in 13/14 posttreatment biopsies and in 3/6 subsequent biopsies 6 weeks later just before the next infusion (Fig. 8).

Relation to clinical response

Of the 16 patients, 1 (no. 4) achieved a clinical complete remission, 2 patients (nos. 3, 8) had a minor response, and 1 patient (no. 10) had stable disease for 5 months. Evaluable paired pre-and postinfusion biopsies were obtained in 5 of the 16 patients. Changes in the MNC infiltration pattern, in the proportion of necrotic tissue area, and the relation to clinical response are shown in Table 5. However, in patient nos. 3, 4, and 10 no paired biopsies were available.

Discussion

As no effective therapy exists for disseminated colorectal carcinoma, a search for new therapeutic modalities is warranted. Systemic administration of MAb directed against TAA might offer a new approach. Such MAb can be used unconjugated or as carriers of cytotoxic substances. The rationale of using naked antibody is based on the assumption that activation of various immunological mechanisms may take place locally and destroy the tumors.

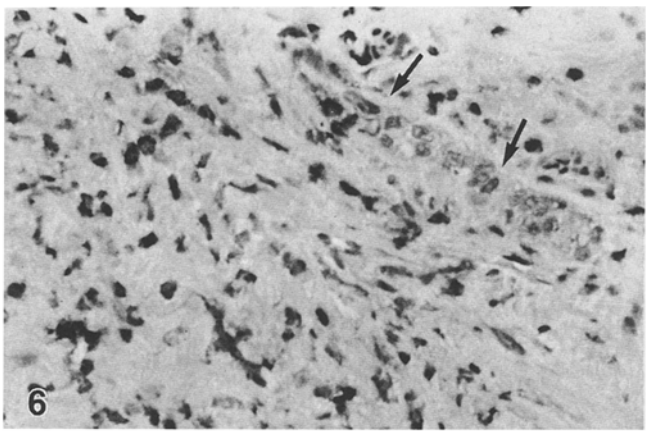
In the development of this therapeutic system, the need became apparent for a method allowing characterization of the metastatic tumor lesions before and during therapy at the tissue and cellular level. Thus, a biopsy technique was developed which met the requirements for immunohistopathological analyses. In the present report the applicability of this technique to monitor in vivo tumor changes after infusion of MAb 17-1A is described.

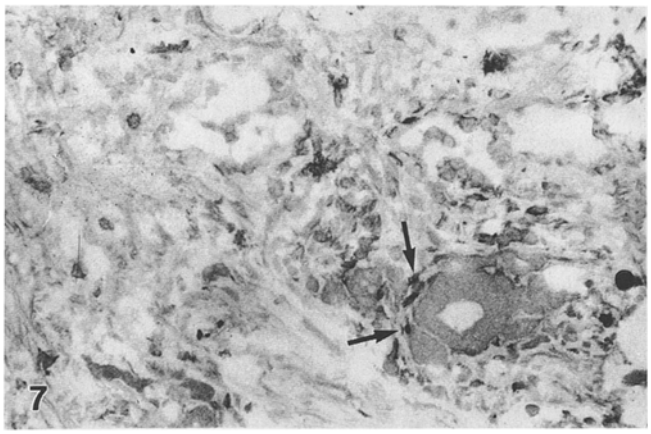
Cryostat sections, fixed in acetone allowed excellent staining for all antigens tested (CO17-1A, GICA19-9A and B, GA73-3, Br55-2). The antigen CO17-1A could not be detected by us in paraffin sections as also reported by Shen et al. [27]. GA73-3, which is a different epitope on the same molecule as CO17-1A [22] was however, well-visualized in paraffin-embedded material. As the expression of CO17-1A and GA73-3 correlated closely in cryosections, staining for GA73-3 could be used as a marker for the presence of the CO17-1A antigen. Thus, in cases where only formalin-fixed paraffin-embedded material is available, the detection of GA73-3 could be used for the selection of

Table 5. Changes between the pre- and posttreatment tumor biopsy (see *Material and methods*) in mononuclear cell subpopulations and in the proportion of necrotic tissue after infusion of MAb 17-1A and the relation to clinical response

Patient no.	Treatment course	Mononuclear cell subpopulations									Changes in necrotic tissue area	Clinical response
		Leu-4$^+$	Leu-3$^+$	Leu-2$^+$	Leu-M$_1^+$	Leu-M$_2^+$	Leu-M$_3^+$	Leu-M$_5^+$	Leu-7$^+$	Leu-11b$^+$		
6	2	8$^\$$	0.6	0.4	14$^\$$	3	0.6	0.7	3	0.8	No change	NR
	3	0.3	0.7	0.9	0.6	1.2	1.0	0.8	1.0	0.6	No change	
7	1	0.5	0.8	0.6	0.7	0.2$^\$$	0.7	0.7	1.0	0.3	Increase	
	2	2.3	0.7	1.0	2.0	1.7	1.5	1.0	1.0	1.0	Increase	NR
	3	1	NE	NE	NE	NE	0.5	9$^\$$	1	1.5	No change	
8	1	0.5	0.8	0.8	0.5	4.5$^\$$	3.7$^\$$	0.8	12$^\$$	8$^\$$	Increase	MR
13	3 Day 2	0.5	0.8	0.3	2	15$^\$$	0.5	1.5	1	2	Increase	
	3 Day 6	2.4	2	2.3	1.4	1	1.2	2	1	15$^\$$	No change	NR
16	1	5$^\$$	4.4$^\$$	20$^\$$	2	1	0.6	3.3$^\$$	1.5	0.3	Decrease	NR

$, a ratio >3.0 or <0.3 was regarded as a significant change; NE, not examined; NR, no response; MR, minor response





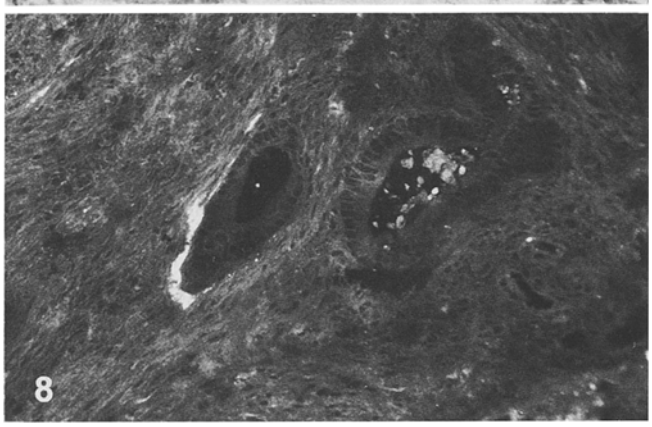


Fig. 6. Immunostaining of a cryostat section from a liver metastasis with Leu-11 showing cells scattered in the stroma of the tumor. Tumor gland indicated by *arrows* (AP) (×240)

Fig. 7. Liver metastasis immunostained with MAb Leu-M3 showing staining of scattered cells in the stroma and elongated cells close to the tumor glands (*arrows*) (AP) (×153)

Fig. 8. Immunofluorescence staining of a cryostat section of liver metastasis with an antiserum against complement factor 3 (C3). Deposition of C3 can be seen focally along the basal region of the tumor glands. In addition, leukocyte-associated staining was seen in the stroma (×222)

patients for immunotherapy with MAb 17-1A. The intense staining with MAb 17-1A and 73-3 of the basal region of the tumor glands and the basement membrane-like structure around the tumor glands suggested that these antigens are mainly exposed on the basal surface of the tumor cells and thus accessable to bind circulating antibodies. Surface membrane staining with MAb 17-1A was also obtained on SW948 (a human colorectal cancer cell line) by immunofluorescence of viable cells. Isolated single cells from collagenase-digested tumor biopsies stained in cell suspension also showed the surface expression of CO17-1A and GA73-3 (unpublished data).

The antigen GICA19-9 was constantly seen in extracellularly secreted material, which is compatible with its presence in serum. Likewise, the antigen Br55-2 was demonstrated to be a secreted product and has also been detected in serum (Z. Steplewski, personal communication 1987). In contrast, CO17-1A and GA73-3 were not associated with secreted products and are not found in serum (H. Koprowski, personal communication 1987).

There was a difference in the staining pattern of MAb 73-3 between paraffin and cryosections. The better cellular preservation in formalin-fixed tissue was probably the reason for the supranuclear dot-like staining seen in paraffin sections. This staining was seen in an area corresponding to the Golgi zone. The predominant basement membrane-like pattern seen in cryosections was less frequent and less intensely stained in paraffin sections. This alteration could be due to the effects of formalin fixation since there was variability related to the duration of fixation.

A prerequisite for immunotherapy with MAb is the presence of the antigen on most tumor cells. The antigens CO17-1A, GA73-3, and Br55-2 fulfilled this criterion both with respect to the primary tumor as well as to metastatic lesions in the liver, skin, and lymph nodes and also in autopsy material from the lung and adrenal gland suggesting a stable association of these antigens with colorectal cancers (data not shown). However, antigen-negative metastases have been described [24]. Assuming that these antigens do not totally overlap, a cocktail of the corresponding antibodies would increase the number of cells binding MAbs and a high concentration of bound antibody on the tumor cells may facilitate tumor cell lysis. However, on the basis of our results, MAb 19-9 seems less suitable for immunotherapy.

In all examined biopsies, the infused MAb 17-1A could be demonstrated most prominently along the basal region of the tumor glands where the antigenic expression was high. Of interest was the finding of mouse Ig (MAb 17-1A) in some cases, 6 to 8 weeks after the last infusion although no circulating mouse Ig could be detected in our patients 2 weeks after an infusion (to be published). Evidently, the tumor bound MAb 17-1A was metabolized or shed slowly. In a previous study no tumor cell-bound mouse Ig was detected after 3 weeks [27]. However, the patients in that study had received a lower amount of MAb 17-1A than our patients. The biological significance of bound MAb for a long time is not known but may be of therapeutic benefit.

The median survival time from diagnosis of metastasis in colorectal carcinoma varies between 4 and 9 months in untreated patients and 8 and 14 months in patients receiving cytostatic therapy [2, 3, 4, 7, 10]. In a large clinical trial of patients with metastatic colorectal carcinoma who received a single infusion of 500 mg MAb 17-1A, the median survival time was about 14 months. As only few patients showed an objective response the result was interpretated as a growth retardation of the tumor (Douillard, personal

communication 1987). Such a tumor-static effect could be the result of a prolonged blockage by MAb of the antigen CO17-1A, since this antigen may act as a receptor for calcium transport over the cell membrane (H. Koprowski, personal communication 1987). Furthermore, our observations imply that no major modulation of CO17-1A takes place during immunotherapy, which strengthens the rationale for the use of MAb 17-1A for therapy.

The mechanisms for the destruction of solid tumors in vivo by MAb are not fully understood. From various in vivo and in vitro experimental systems of solid tumors, macrophages/monocytes and NK cells seem to have important effector functions. In the paired biopsy analyses where we had the possibility of addressing this question, a significant increase in potentially cytotoxic cells was seen in 6/9 pairs of biopsies. In addition, in 2 of the 5 pairs where an increase in tumor associated monocytes was seen, there was also an increase in T cells. The role, if any, these T lymphocytes might have in the interaction between the host and the tumor is not known. These may be unprimed cytotoxic T lymphocytes. An increase in T cells following immunotherapy with MAb was also reported by Garcia et al. [9]. Furthermore higher doses of MAb may induce more pronounced infiltration of MNC (unpublished observations). However, there is presently no proof that these cells originate from the infused isolated MNC given together with the antibody. This question is under study.

Based on experimental studies, it has been claimed that C3-mediated lysis does not contribute to any major extent to tumor cell lysis in vivo [28]. Deposition of C3 in most posttreatment biopsies at the same sites where the highest concentration of the antigen CO17-1A and mouse Ig was found, may indicate a role for C3.

In summary, we described an immunohistochemical approach to evaluate and monitor the effect of therapy with MAb 17-1A on metastases in patients with colorectal carcinomas. After infusion, MAb reached the relevant target structure. Some objective evidence was obtained that after infusion there was an increase in potentially cytotoxic cells, deposition of C3 and an increase in tumor necrosis.

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