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A monoclonal antibody with binding and inhibiting activity towards human pancreatic carcinoma cells

I. Immunohistological and immunochemical characterization of a murine monoclonal antibody selecting for well differentiated adenocarcinomas of the pancreas

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Summary. The murine monoclonal antibody (MAb) BW 494 was characterized in relation to its tissue specificity, the epitope recognized, in vitro and in vivo radiolocalization and its potential to mediate antibody dependent cellular cytotoxicity (ADCC) and complement mediated cytolysis (CMC). The MAb defined carbohydrate epitope located on a >200 k daltons glycoprotein was mainly expressed on the majority of well differentiated adenocarcinomas of the pancreas. Furthermore, the epitope is accessible to MAb BW 494 in vivo, allowing an enrichment of radioactive antibody at the tumor site in nude mice. Additionally, MAb BW 494 is able to use human peripheral blood lymphocytes as effector cells for ADCC reactions against appropriate tumor target cells in vitro. In contrast, the antibody does not mediate human or rabbit CMC.

Introduction

A variety of human pancreatic carcinoma associated antigens have been characterized using monoclonal antibodies (MAbs) combined with immunochemical methods [2, 33]. The antigenic determinant or epitopes recognized by these MAbs can be associated with glycolipids [21, 22] and/or glycoproteins [23], and can be carbohydrate or protein in nature [17]. Besides the chemical nature of a MAb-defined epitope its tissue distribution and accessibility for the corresponding MAb under in vivo conditions is important information especially in relation to MAb-guided immunoscintigraphy and radioimmunotherapy in the tumor patient.

In this publication we describe the tissue distribution, in vivo accessibility, and some molecular characteristics of an epitope detected by MAb BW 494 selective for well differentiated adenocarcinomas of the pancreas.

Materials and methods

Induction, screening, and purification of MAb BW 494. For the generation of MAb BW 494 the DE-TA colon carcinoma cell line (a gift from Dr. Vetterlein, University of Vienna) was used. Tissue culture cells (1×10^7) were removed from plastic tissue culture dishes using a rubber policeman and injected i.p. into Balb/c mice 4 times at weekly intervals. Then 2 days after the last immunization, spleen cells from the immunized mice were fused using the Sp2/0-Ag14 [30] mouse myeloma cell line as a fusion partner at a spleen cell to myeloma cell ratio of 5:1 essentially as described by Köhler et al. [19]. The supernatants of growing hybrids were tested for specific MAb production using an indirect immunofluorescence assay in Terasaki plates [5], cytofluorometric analysis [27], and histology combined with the indirect immunoperoxidase method on cryopreserved and formaldehyde-fixed paraffin-embedded tissue sections [13]. From 756 growing hybridomas 85 produced antibody binding to the DE-TA cell line. MAb BW 494 was selected from these hybrids, because of its selective reactivity with pancreatic carcinomas. Hybridoma cells (10^6) were injected i.p. into pathogen-free, pristane-primed [15] Balb/c mice. Ascites was harvested aseptically, delipidated with Lipoclean (Behringwerke, Marburg, FRG, No. 0SVW60) and purified by protein A-Sepharose chromatography according to Ey et al. [8].

Antibody dependent cell-mediated cytotoxicity and complement-mediated cytolysis. The capacity of MAb BW 494 to perform human K-cell-mediated tumor cell lysis, and human or rabbit complement-mediated lysis was evaluated according to the procedure described by Schulz et al. [29].

The percentage of specific cytolysis (^{51}Cr-release) was calculated according to the formula:

$$\frac{\text{experimental group release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100.$$

Radiolabeling of MAb BW 494 and radiolocalization experiments. Protein A-purified MAb or mouse immunoglobulin (MIg) were labeled according to Fraker et al. [10] up to a specific activity of 3 μCi ^{131}I/μg protein and used in radiolocalization experiments as described by Bosslet et al. [4].

Tumor tissue binding assay. The binding of radiolabeled MAb BW 494 and control MIgs to tumor fragments in vitro was evaluated as described by Rusckowski et al. [28]. Briefly, 1-mm^3 fragments of tumor xenotransplants were incubated in 96-well flat bottomed plates together with 1 μCi of ^{125}I-labeled MAb diluted in 200 μl of tissue culture medium for 24 h. After repeated washing of the tissue fragments the radioactivity bound and the dry weight of

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the tissue piece was determined, and the amount of MAb specifically bound was calculated in comparison to MIg or to MAbs reactive to *Mycoplasma pirum* antigens.

Periodic acid oxidation and neuraminidase treatment of tissue sections. Sections of tissues were treated with periodic acid or neuraminidase as originally described by Yachi et al. [34].

Radioimmunoprecipitation analysis. Cell ghosts were prepared by incubation of 2×10^8 trypsinized tissue culture cells for 10 min at 4 °C in 10 ml of a hypotonic buffer pH 8.1 (1 mM KCl, 1 mM $MgCl_2$, 10 mM trihydroxyaminomethane). After a 10 min centrifugation step at 500 *g* and removal of the supernatant, the cell pellet was quickly frozen in liquid nitrogen, then thawed in a water bath at 37 °C. The tube was centrifuged at 1800 *g* and 4 °C for 10 min and the supernatant and pellet harvested, and recentrifuged at 100000 *g* and 4 °C for 30 min. The remaining cytoplasmic or subcellular particle-containing fractions were either treated with lysis buffer (1 mM EDTA, 5 g/l Nonidet P-40, 5 g/l sodium deoxycholate, 5.8 g/l sodium chloride, 2 g/l phenylmethylsulfonylfluoride in 20 mM Tris-HCl, pH 7.5) or propagated without lysis buffer incubation for radioimmunoprecipitation essentially as described previously [3]. The fraction not treated with lysis buffer was washed during the radioimmunoprecipitation procedure using buffers lacking any detergents.

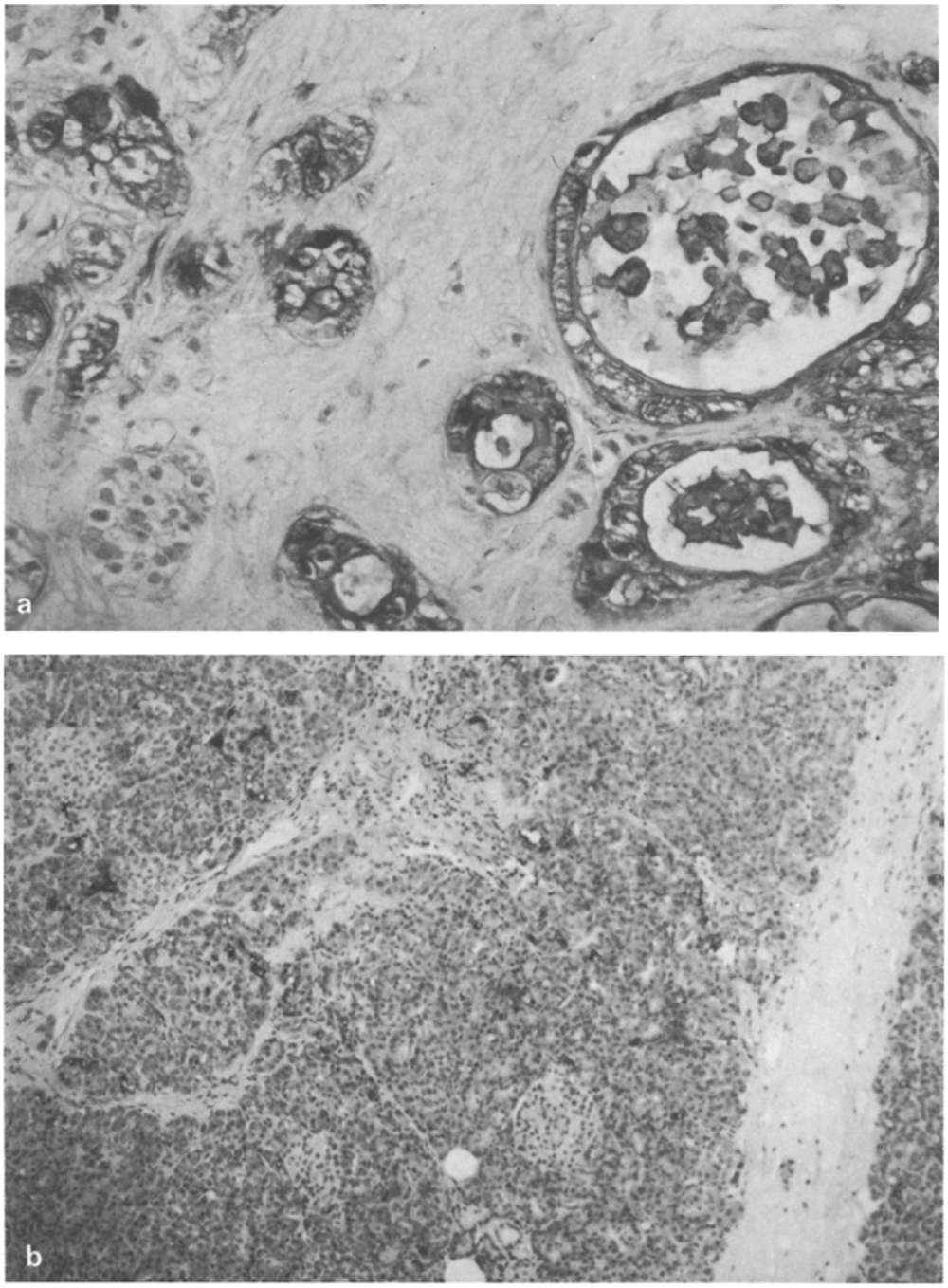

Fig. 1. Immunoperoxidase staining of formaldehyde-fixed, paraffin-embedded tissue sections from pancreatic carcinoma **(a)** and nonmalignant pancreatic tissue **(b)** using MAb BW 494 (counterstain: hematoxylin **(a)** 900×, **(b)** 360×)
MAb BW 494 preferentially stains pancreatic tumor cell membranes and material in the intercellular spaces and lumina (Fig. 1a). In normal pancreatic tissue MAb BW 494 reacts slightly with ductular epithelium (Fig. 1b)

Extraction and immunochemical visualization of glycolipids. Glycolipids were extracted from tumor tissue or tissue culture cells following the procedure described by Ledeen et al. [20]. Briefly, tissue fragments were extracted following the procedure originally described Folch et al. [9], modified according to Suzuki [31], followed by DEAE-Sephadex anion exchange chromatography and methanolic hydroxide treatment [35]. The dialyzed and lyophilized powder was solubilized in methanol and aliquots were chromatographed for analytical purposes on silica gel 60 HPTLC plates (Merck, Darmstadt, FRG) in a mixture of chloroform:methanol:H_2O (60:35:8). Thereafter, replicate chromatograms were dried thoroughly, cut into stripes and the glycolipids were either visualized using resorcinol-HCl reagent [32] or propagated for an immunobinding assay on thin layer chromatograms as described by Harpin et al. [11]. This method detects the binding of the MAb to the chromatographically separated glycolipids using an indirect immunoperoxidase technique in contrast to the original method [21] performed by autoradiography of an ^{125}I-labeled second antibody.

Results

Reactivity of MAb BW 494 with human tumor and normal tissues using the indirect immunoperoxidase method

A panel of cryopreserved and formaldehyde-fixed, paraffin-embedded human tissues was used to determine the distribution of the MAb BW 494-defined epitope in the human body. MAb BW 494 preferentially reacts with highly differentiated adenocarcinomas of the pancreas (11/14) and their metastases (3/4) as well as with the majority of colon carcinoma metastases (7/10). Figure 1 shows an example of an indirect immunoperoxidase stain on sections of a well differentiated human pancreatic adenocarcinoma (a) and normal pancreatic tissue (b). The tumor cells in Fig. 1a show a cytoplasmic and pronounced membrane staining. A significant reaction could also be seen in various extracellular lumina indicating that the antigen bearing the MAb BW 494-defined epitope is produced in large amounts in well differentiated pancreatic adenocarcinoma cells and secreted to the extracellular space. In normal pancreatic tissue sections (Fig 1b) MAb BW 494 only marginally bound to the inter- and intralobular ducts.

Furthermore, the MAb BW 494-defined epitope was weakly expressed in the goblet cells of normal human colon and on stomach mucosa (Table 1). None of the other normal human tissues tested (lung, liver, breast, kidney, spleen, lymph nodes, bone marrow, and peripheral blood cells) showed any significant binding.

Taken together, MAb BW 494 preferentially binds to highly differentiated adenocarcinomas of the pancreas and their metastases and to colon carcinoma metastases. The duct system of normal pancreas and the goblet cells of normal colon and stomach mucosa are weakly stained. Having established the tissue distribution of the MAb BW 494-defined epitope its chemical nature was investigated.

Molecular characteristics of the MAb BW 494-defined antigenic determinant

Comparative immunohistochemical investigations using human tumor xenografts from nude mice were performed to study the effect of various chemical treatments on the stability of the MAb BW 494-defined epitope. As treatment controls MAb R24 (a gift of Dr. Dippold, University of Mainz, FRG), reactive with the carbohydrate component of GD_3 [26] and MAb BW 431, reactive with a protein epitope on carcinoembryonic antigens [4] were included. MAb R24 intensively stained cryopreserved M21 and FM9 (a gift from Dr. R. Reisfeld, Scripps Clinic and Research

Table 1. (a) Survey of the immunoperoxidase staining pattern of MAb BW 494 on formaldehyde-fixed, paraffin-embedded human tumor thin sections

Tissue type	Grading type[a]	
	I/II	*III*
Pancreas tumors	Positive/total	Positive/total
Primary carcinomas	11/14	0/2
Liver metastases	3/4	0/5
Papillary carcinomas	1/2	
Cystadenomas	0/1	
Colon carcinomas		
Primary carcinomas	4/14	
Liver metastases	7/10	
Lung carcinomas	8[b]/18	
Mammary carcinomas	0/3	

[a] according to Klöppel et al. [18]
[b] a few cells/section positive

(b) Survey of the immunoperoxidase staining pattern of MAb BW 494 on formaldehyde-fixed, paraffin-embedded nonmalignant human tissue sections

Tissue type	Positive/total
Pancreas:	
Ducts of chronically inflamed pancreas	10/13
Normal endo- and exocrine pancreas	0/8
Colon:	
Goblet cells of mucosa luminal part	9/10
Mucosa adjacent to muscularis	3/10
Muscularis mucosae	0/7
Submucosa	0/7
Tunica muscularis	0/7
Stomach:	
Muscular system	0/2
Mucus producing cells	3/3
Miscellaneous organs:	
Lung	0/3
Liver	0/5
Breast	0/2
Kidney	0/5
Connective tissue	0/2
Muscular system	0/10
Lymphoid organs:	
Spleen[b]	0/2
Lymph node[b]	0/4
Bone marrow[b]	0/3
Peripheral blood leucocytes[a, b]	0/4
Erythrocytes (A, B, 0)	0/4

[a] Cytofluorometric analysis
[b] Cryopreserved materials

Table 2. Immunohistochemical staining of tissue sections

Human tumor xenograft tissue	After pretreatment with						
	MAb used	CP	FP	FP / PO	FP / NT	PO	NT
PaTuI	BW 494	++	++	–	++	n.d.	n.d.
PaTuII		+	+	–	+		
M 21	R 24	++	–	n.d.	n.d.	–	–
FM 9		++	–	n.d.	n.d.	–	–
CoCa4	BW 431	++	++	++	++	++	++
De-TA		+	+	+	+	+	+

Abbreviations:
CP = cryopreservation
FP = formaldehyde fixation and paraffin embedding
PO = periodic acid oxidation
NT = neuraminidase treatment

Foundation) xenograft tissue sections, whereas formaldehyde fixation and paraffin embedding, periodic acid oxidation, or neuraminidase treatment destroyed its reactivity in agreement with previously published data [6]. In contrast, the epitope detected by MAb BW 431 on the CoCa4 and DE-TA xenograft was resistant to all pretreatments performed, in agreement with previously published data [4] indicating that the chemicals applied showed the expected deteriorations of tissue determinants (Table 2). In contrast to these epitopes the antigenic determinant detected by MAb BW 494 in pancreatic carcinoma-derived human tumor xenografts (PaTuI, II a gift from Dr. M. v. Bülow, University of Mainz, FRG) was resistant to formaldehyde fixation and paraffin embedding or additional neuraminidase treatment, but sensitive to formaldehyde fixation and paraffin embedding followed by periodic acid oxidation. These data indicate that carbohydrates contribute to the epitope detected by MAb BW 494 on pancreatic carcinoma.

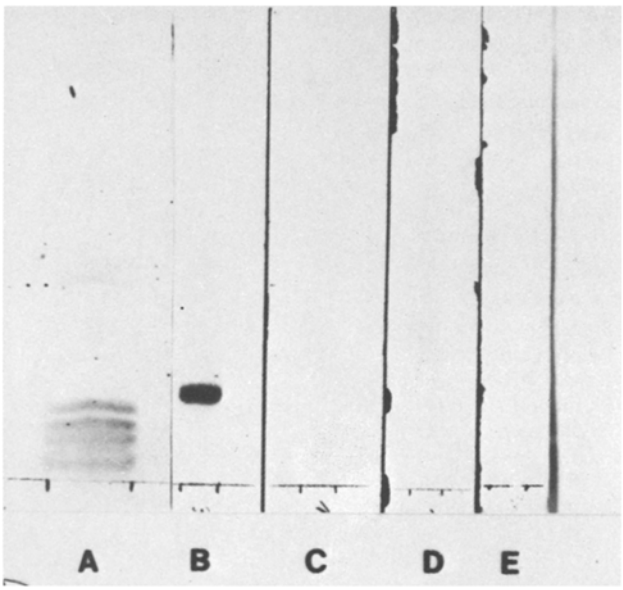

Fig. 2. Thin layer chromatography of glycolipids. *Lane A* represents glycolipids from bovine brain visualized with hot methanolic H_2SO_4. *Lane B* shows GD_3 detected in the M21 xenograft with MAb R24 using an indirect immunoperoxidase method. *Lane C* represents the same analysis using MAb BW 494 on the same xenograft tissue. Glycolipids from PaTuI *(lane D, E)* were treated identically as the glycolipids from M21

Molecular characterization of the antigen bearing the MAb BW 494-defined epitope

To clarify, whether the carbohydrate epitope detected by MAb BW 494 is located on glycolipids, glycoproteins, or both, we isolated glycolipids from the epitope positive PaTuI and from the M21 xenograft. After thin layer chromatography the separated glycolipids were reacted with MAbs BW 494 or R24 and visualized as described in *Materials and methods.* In Fig. 2 lane B, the GD_3 antigen is recognized by MAb R24 as a single band in the M21 glycolipid mixture. In contrast, MAb BW 494 did not bind to any of the glycolipid mixtures isolated, suggesting that the carbohydrate epitope detected by MAb BW 494 is not present on these glycolipids.

Thereafter, we performed a radioimmunoprecipitation analysis of water soluble extracts from cell ghosts of the PaTuI pancreatic carcinoma cell line and analyzed the immunoprecipitated material using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) combined with autoradiography as specified in *Materials and methods.* The autoradiography (Fig. 3) shows a high molecular weight band (lane B) (molecular weight >200 k daltons) immunoprecipitated by MAb BW 494 whereas R24 does not show any precipitate (lane A) confirming the specificity of the analysis.

The data indicate that MAb BW 494 recognizes a carbohydrate epitope located on a high molecular weight glycoprotein. Having performed this analysis we questioned whether the MAb BW 494 defined epitope is accessible to the antibody in vivo.

Bioaccessiblity studies in vitro and in vivo

For bioaccessibility studies purified protein from MAb BW 494 ($IgG_1-\kappa$, determined by double diffusion according to Ouchterlony [24] data not shown), and from MAb R24 or MAb BW 227 (reactive to *Mycoplasma pirum*) were radiolabeled with ^{131}I. The radiolabeled antibodies were reacted with tissue fragments from a human colon carcinoma xenograft (CoCa4) expressing the MAb BW 494-de-

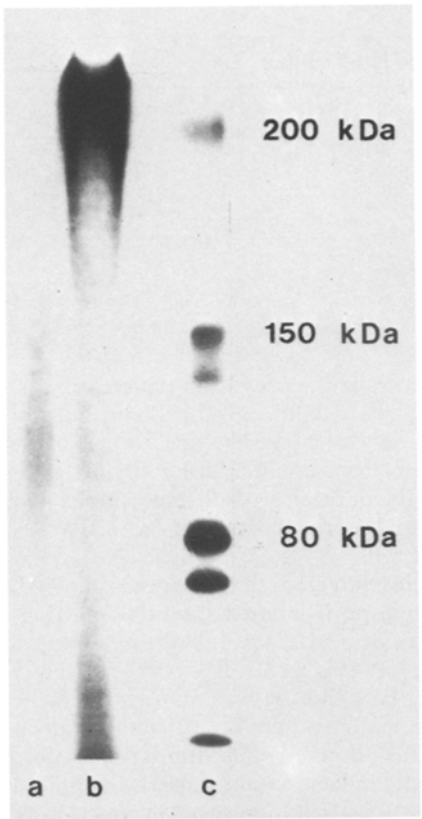

Fig. 3. Autoradiography of a 5%–15% SDS-polyacrylamide gel. Radiolabeled extracts from the PaTuI cell line were immunoprecipitated using MAb R24 *(lane a)* or MAb BW 494 *(lane b)* dissolved from the immunoadsorbents and electrophorized under reducing conditions together with molecular weight markers *(lane c)*

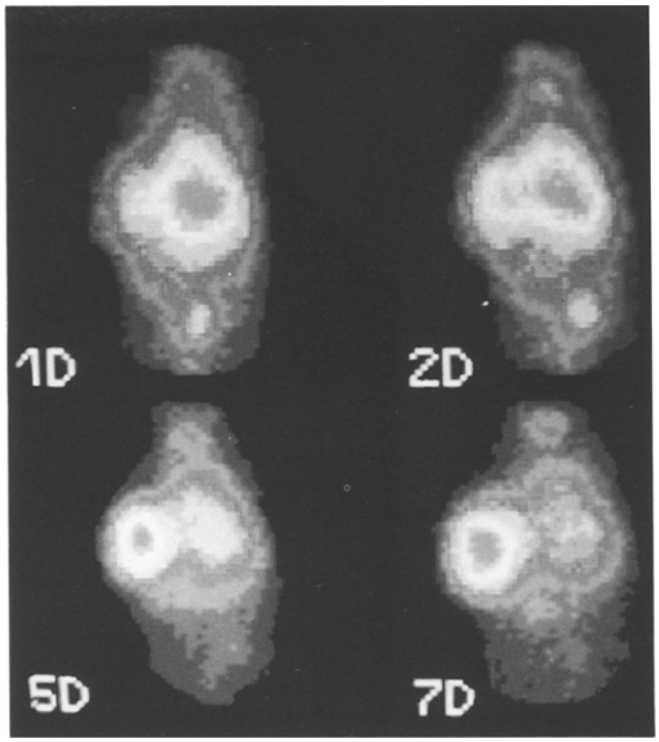

Fig. 4. Gamma camera picture of a CoCa4 colon carcinoma-bearing nude mouse scintigraphed from the back. Kinetics of the ^{131}I-labeled MAb BW 494 distribution (1, 2, 5, and 7 days) is given. Blue represents a low, yellow a medium and red a high radioactivity concentration in the corresponding area of the nude mouse

fined epitope and a small cell lung carcinoma xenograft (Oat-75) not expressing the epitope. The amount of radioactivity bound to the tumor fragments is given in Table 3.

The amount of radioactivity bound to the CoCa4 tumor fragments was >10 times (~12.000 versus ~1000) higher than the radioactivity nonspecifically associated with the CoCa4 tissue fragment via MAbs R24 and BW 227. No binding exceeding that of the control MAbs was detected on Oat-75 (epitope negative) tissue fragments (1307 versus 1212). Having performed these in vitro bioaccessibility studies we injected the ^{131}I-labeled MAb BW 494 i.v. in human tumor xenograft-bearing nude mice to establish its potential to radiolocalize in vivo.

Figure 4 shows the kinetics of the binding of MAb BW 494 to the CoCa4 xenograft in a nude mouse; 2 days after i.v. injection of 100 μCi of ^{131}I-labeled MAb BW 494 radioactivity accumulated at the tumor site (left part of the animal) and the tumor to blood ratio increased up to 7 days. In contrast, MAb BW 494 did not enrich at the tumor site in the Oat-75 system (data not shown). These experiments provided evidence for the ability of MAb BW 494 to localize human tumors in vivo.

Evaluation of antibody dependent cell-mediated cytotoxicity and complement-mediated cytolysis

Previously it was shown that MAb BW 494 is able to bind to human tumors in vivo. We were interested to find out if this antibody mediates cellular or humoral functions exceeding its capacity to bind to the target epitope at the tumor cell surface. In an antibody dependent cell-mediated cytotoxicity (ADCC) system using ^{51}Cr-labeled human tumor cells as targets and human peripheral blood lymphocytes as effector cells the potential of MAb BW 494 to specifically direct K-cell-mediated cytolysis to epitope positive tumor cells was evaluated.

Table 4 summarizes data from a representative experiment in which MAb R24 ($IgG_3-\kappa$) very efficiently lysed the GD_3-expressing SK-Mel-28 melanoma cell line (a gift from Dr. W. Dippold, Mainz, FRG), whereas the GD_3 ne-

Table 3. Tissue piece binding assay

Tissue pieces from	Input cpm			cpm bound		
	494	227	R24	494	227	R24
CoCa4	276000	239000	290000	*12470*	948	1214
Oat-75	265000	247000	281000	1307	1212	943

Table 4. ADCC system to investigate cell-mediated cytolysis

MAb	% Specific lysis on target			Binding to intact cells of		
	PaTuI	CoCa4	SkMel-28	PaTuI	DE-TA	SkMel-28
BW 431	3	5	6	+	++	–
BW 494	*20*	*17*	4	++	++	–
R 24	2	4	*45*	–	–	++

Effector:target cell ratio = 100:1
4 h ^{51}Cr-release assay

gative cell lines PaTuI and DE-TA were not destroyed. MAb BW 431 ($IgG_1-\kappa$) despite its potential to bind to the DE-TA cell line did not mediate any significant lysis. In contrast MAb BW 494 ($IgG_1-\kappa$) was able to kill the epitope expressing the PaTuI and DE-TA cell line at significant levels.

In addition the MAbs' potential to mediate complement-dependent tumor cell killing was evaluated using rabbit and human serum as complement sources. MAb R24 efficiently mediated complement-dependent cytotoxicity against the SK-Mel-28 tumor target, whereas the MAbs BW 431 or BW 494 did not show any significant lysis (data not shown).

Discussion

The experimental data presented describe some characteristics of the newly established MAb BW 494 and of the antigenic determinant detected. Using immunohistochemical methods, we showed that the epitope is mainly expressed on well differentiated adenocarcinomas of the pancreas. Cells of the pancreatic duct system and the mucin-producing goblet cells in the colon are weakly stained. This finding indicates that the MAb BW 494-defined antigenic determinant is located on a molecule which is produced in large amounts in pancreatic adenocarcinomas but less frequently in the goblet cells and the duct system of the normal pancreas. The epitope therefore defines a tissue differentiation antigen with a very restricted distribution in human tissues. Its absence from erythrocytes of different blood group donors distinguishes it from other gastrointestinal tract associated antigens of blood group nature [14]. The resistance of the epitope to formaldehyde fixation and paraffin embedding allowed an extensive evaluation of its distribution on a wide range of human tissue sections [7] (Table 1).

For the molecular characterization of the antigenic determinant detected by MAb BW 494 we chose extracts from human tumor xenografts or in vitro cultured epitope-expressing tumor cell lines. Using radioimmunoprecipitation combined with SDS-PAGE and autoradiography we showed that the epitope defined by MAb BW 494 on a human pancreatic carcinoma cell line is located on a >200 k dalton glycoprotein. The epitopes' sensitivity to periodic acid oxidation argues for its carbohydrate nature. Its resistance to neuraminidase treatment distinguishes it from the Ca 199 defined sialyl Lewis A structure which is sensitive to neuraminidase treatment [1].

Glycolipids isolated from epitope expressing human pancreatic carcinoma xenografts did not allow the binding of MAb BW 494 to their carbohydrate moieties, suggesting that the antigenic determinant is present in pancreatic carcinoma only on the >200 k dalton glycoprotein, but not on glycolipids. This analysis, however, does not exclude the possibility that the epitope could additionally be located on glycolipids or glycoproteins of different molecular weight and structure in other tissues binding MAb BW 494 (duct system or goblet cells).

Furthermore, we investigated the potential of MAb BW 494 to bind its epitope in human tumor tissue fragments in vitro. Compared to MAbs not detecting epitopes in the corresponding tissues, MAb BW 494 showed a >10-fold enrichment. This finding was confirmed in the nude mouse xenograft system where an efficient localization of radioactivity was observed 48 h after i.v. injection. This localization kinetic is comparable to data reported with MAbs of different specificity in other systems [25].

Because of the potential of MAb BW 494 to bind to human tumors in vivo, we investigated whether it is able to mediate functions exceeding that of binding [12]. Using an in vitro ADCC and complement-mediated cytolysis system, it was shown that the MAb BW 494 of $IgG_1-\kappa$ isotype mediates K-cell killing of human peripheral blood leucocytes but no complement-mediated killing with human or rabbit complement. The cytotoxicity in the ADCC system was less prominent than the lysis obtained with the MAb R24 of $IgG_3-\kappa$ isotype, but was significantly higher than the cytolysis induced by MAb BW 431 ($IgG_1-\kappa$).

Because of its tissue specificity, its binding capacity to human tumors in vivo, and the potential to mediate ADCC, MAb BW 494 might not only be a valuable reagent for immunoscintigraphy or radioimmunotherapy, but also for immunotherapy with cold untagged MAb as suggested in the melanoma system by Houghton et al. [16]. Clinical trials to evaluate the potential of MAb BW 494 to inhibit the growth of pancreatic carcinoma patients metastases after regional application in the common hepatic artery are in progress.

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