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Immunohistochemical phenotyping of human solid tumors with monoclonal antibodies in devising biotherapeutic strategies

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Summary. A panel of 14 monoclonal antibodies (MoAbs) (4 raised against breast cancer, 6 against colon cancer and 4 against melanoma) were used to phenotype frozen sections of tumor biopsies obtained from 110 patients, by avidin-biotin-peroxidase complex techniques. We observed heterogeneity of antigen expression among the multiple metastatic lesions of single patients, as well as among tumor lesions from different patients with similar tumor histotypes. A wide range of cross-reactivity of anti-(breast-carcinoma) and anti-(colon-carcinoma) MoAbs with other carcinoma histotypes and limited reactivity with melanoma and sarcoma was detected. Some of our anti-melanoma MoAbs were also found to cross-react with selected carcinomas. Nine of the 14 MoAbs most reactive with carcinomas of diverse histotypes have been identified. A mixture or 'cocktail' of different MoAbs could be selected for each individual patient in order to achieve binding of MoAbs with most, if not 100% of tumor cells. This study illustrates the approach that we have taken to individualize the cocktail of MoAbs for the development of patient-specific therapeutic immunoconjugates.

Introduction

The advent of hybridoma technology for generation of monoclonal antibodies (MoAbs) has resulted in major advances in developing reagents and methods for tumor detection, localization and therapy. Early claims of tumor specificity of MoAbs, such as one 'specific' for all carcinomas by recognition of a single antigen known as Ca [3], have been discounted after extensive immunohistochemical evaluation of the MoAb reactivity with a wide range of normal human tissues [29, 37]. However, MoAbs having strong binding with malignant cells and having minimal or restricted binding with certain normal adult tissues have proven to be useful for radioimaging of melanoma [8, 19] and carcinomas of the breast [12, 38], colon and rectum [2, 10, 12, 28]. Targeting of tumor cells by MoAbs with or without therapeutic agents has been demonstrated in melanoma [16, 34, 40, 44, 46], gastrointestinal malignancy [12, 17] and leukemia/lymphoma [21, 27].

Immunohistochemical phenotyping of tumor biopsies at varying stages of malignant progression of various tumor types with the use of a panel of MoAbs has confirmed the existence of antigenic heterogeneity [9, 13, 31]. Information generated from such phenotyping has contributed to a number of important areas: early detection of micrometastasis, discrimination between benign and malignant lesions, prognostic indication, and better understanding of the origin and histogenesis of neoplasms. From the therapeutic standpoint, although 'perfect' antibodies as targeting reagents for all or certain types of human solid tumors have not been identified, a mixture of two or more MoAbs could recognize different antigens or different antigenic determinants on the same antigen, expressed by the patient tumor [9, 18, 25, 50]. Enhanced binding by combinations of such MoAbs with tumor tissues or cell lines has been reported [5, 18, 50]. In this communication, we describe our experience in phenotyping a variety of human solid tumors by immunohistochemical techniques using a panel of monoclonal antibodies. This study illustrates the heterogeneity of cancer, the method to individualize the 'cocktail' of MoAbs, and the use of these antibodies in the development of therapeutic immunoconjugates [35].

Materials and methods

Tumors and normal tissues. Biopsies were from cancer patients who entered our Monoclonal Antibody/Immunoconjugate Program at Biotherapeutics Inc., Franklin, Tenn. In several patients multiple specimens and portions from each specimen were available. These fresh biopsies were from primary tumors, their metastases and subsequent recurrences producing over 126 specimens and 110 stainable samples. A portion of each tumor specimen was cut and used in routine pathological examination at the hospitals or clinics where tissues were procured. The remaining tumor specimens were frozen in OCT compound (polyvinyl alcohol, benzalkonium chloride, polyethylene glycol, d. H_2O; Miles Labs, Elkhart, Ind) at $-70°$ C before use. In most instances a portion of the tumor tissue was dispersed for tissue culture and implantation in nude mice.

Human peripheral blood samples were obtained from cancer patients and healthy volunteers. Histologically normal tissues (lung, heart, breast, liver, kidney, colon, skin) were from autopsy specimens of patients who died of non-malignant diseases. Additional normal skin and breast tissues were from surgical specimens obtained from foreskin circumcisions and breast reductions. These specimens

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were obtained through Dr. S. Kincaid of Williamson Medical Center, Franklin, Tenn and through Dr. W. H. West, Baptist Hospital, Memphis, Tenn. Some of the normal tissues used in this study were obtained from Dr. W. E. Grizzle, Southern Division of the Cooperative Human Tissue Network based at the University of Alabama, Birmingham, Ala. These tissues were cut and embedded in OCT as described above for tumor biopsies.

Monoclonal antibodies. Monoclonal antibodies (MoAbs) were derived from murine hybridomas obtained by immunizing mice with preparations of different tumor types. Their sources and derivations are listed in Table 1. The MoAbs generated at Biotherapeutics are designated BT- as prefixes and are described elsewhere [5]. Those MoAbs acquired from other institutes are prefixed with BA-. The hybridomas secreting MoAbs BA-Br-1 (B38.1) and BA-Br-5 (B72.3), originally developed by the Schlom group [15], were obtained from American Type Culture Collection, Rockville, Md. The generation and some properties of MoAbs BA-Br-3 (BTMA8), BA-Me-4 (140.72) and BA-Me-5 (140.240) have been reported elsewhere [22, 24, 25]. IgG1 and IgG2 MoAbs were purified by the caprylic acid method [32, 42] from ascites fluid harvested from BALB/c mice. IgG3 MoAbs were purified by protein A chromatography using the Pierce binding and elution buffers according to manufacturer's instructions [32].

Immunohistochemical procedures. The avidin-biotin-peroxidase complex (ABC) method was used (Vectastain ABC kit, Vector Laboratories, Burlingame, Calif). Sections of frozen tissues 5 μm thick, embedded in OCT, were cut and placed on gelatin-coated slides. The tissue sections were fixed with cold acetone, and incubated with normal horse serum for 15 min to reduce non-specific binding of the horse anti-(mouse IgG) antiserum. Purified murine MoAbs (see below) at the concentration of 5 μg/ml were used as primary antibodies to incubate with the sections for 1 h. The slides were washed with phosphate-buffered saline (PBS) pH 7.4, and incubated with biotinylated horse anti (mouse IgG) antiserum for 30 min. Endogenous peroxidase in tissue was blocked by reaction with methanol and hydrogen peroxide for 15 min. The sections were then incubated with a preformed complex of avidin-biotinylated-horseradish-peroxidase for 1 h. Staining was developed by the oxidation of 3-amino-9-ethylcarbazol and 0.002% hydrogen peroxide for 20 min and counterstained in Gill's hematoxylin (Fisher Scientific, Norcross, Ga) for 20 min. Positive (anti-HLA-A,B,C MoAb W6/32 and anti-epithelial membrane antigen MoAb) and negative (non-specific murine IgG and PBS) antibody controls were included in each test. One or two sections from the frozen tissue block were routinely stained with hematoxylin and eosin for histopathological examination prior to immunohistochemical typing. Poorly preserved tumor tissue sections or tissue sections containing no tumor cells were not used for immunohistochemical analysis. All results were evaluated independently by two observers. The reactivity patterns scored independently were consistent in most cases. Intensity of staining (reddish-brown color) was scored as negative (−) or positive (from weak: +, to very strong: 4+). A designation of ± was given to those tumor tissues which stained mostly negative with few tumor areas positive.

Qualitative microabsorption. The qualitative absorption analysis was performed as previously described [25] except that the assay used in this study was the ABC immunohistochemistry against appropriate tumor tissues. Briefly, the appropriately diluted [22] MoAb was absorbed with an equal volume of packed homogenate prepared from fresh tissue material (centrifuged at 3000 *g* for 3 min in a microcentrifuge) at room temperature for 1 h. The absorb-

Table 1. Monoclonal antibodies used in the present study

MoAb	Isotype	Immunogen	Antigen structure	Reference
BA-Br-1	IgG1	Membrane extract of breast carcinoma tissue	ND[a]	[15]
BA-Br-3	IgG1	Membrane extract of breast carcinoma cell line CAMA-1	>300 kDa glycoprotein	[22]
BA-Br-5	IgG1	Membrane extract of breast carcinoma tissue	220–400 kDa glycoprotein	[15]
BT-Br-6	IgG1	Dispersed cells from breast carcinoma tissue	ND	Unpublished
BT-Co-1[b]	IgG3	Dispersed cells from colon carcinoma grown as xenografts in nude mice	29 kDa + 31 kDa protein	Unpublished
BT-Co-2	IgG3		ND	Unpublished
BT-Co-3	IgG3		ND	Unpublished
BT-Co-4	IgG3		ND	Unpublished
BT-Co-5[b]	IgG3	Dispersed cells from colon carcinoma grown as xenografts in nude mice	29 kDa + 31 kDa protein	Unpublished
BT-Co-6	IgG1		ND	Unpublished
BA-Me-4	IgG1	Melanoma cell line CaCL 78-1	95–150 kDa glycoprotein (s)	[24]
BA-Me-5	IgG2a	Melanoma cell line CaCL 78-1	p97-like (97 kDa) glycoprotein	[22, 25]
BT-Me-7	IgG1	Melanoma cell line BUR	110 kDa	[5]
BT-Me-8	IgG1	Melanoma cell line BUR	110 kDa + 40 kDa	[5]

[a] Not yet defined, although attempts were made to determine the molecular mass of antigen involved
[b] Based on epitope blocking and indirect immunoprecipitation experiments, BT-Co-1 and BT-Co-5 recognized different epitopes residing on the same or extremely similar molecules

ed antibody was separated from the tissue homogenate by centrifugation, as above, and the unabsorbed antibody was titrated against previously known reactive tumor tissues.

Flow cytometric analysis. Heparinized whole blood from a normal donor was centrifuged at 800 *g* for 20 min at 4° C. Platelets were obtained from the plasma layer and washed twice in PBS containing 2% fetal calf serum by centrifugation at 1800 *g* for 20 min. A mixture of lymphocytes, monocytes and granulocytes was recovered from the buffy coat layer and washed in PBS containing 2% fetal calf serum twice by centrifugation at 400 *g* for 5 min. The cells were dispensed into 96-well Falcon microtest plates (3×10^5/well for nucleated cells; 5×10^5/well for platelets). The plates were centrifuged at 400 *g* to pellet the cells. Monoclonal antibody samples (5 μg/ml) were added (100 μl/well), and incubated for 1 h at room temperature. Cells were pelleted, supernatants were removed, and cells were washed in PBS + 2% fetal calf serum three times, pelleting between each wash. Fluorescinated goat-(anti mouse Ig) antiserum (Coulter Immunology, Hialeah, Fla) at an optimized concentration was added (100 μl/well), and incubated for 45 min at 4° C. Cells were pelleted, supernatants were removed, and cells were washed twice as above, pelleting between each wash. Non-specific mouse myeloma IgG of a specific isotype (5 μg/ml) and PBS were used as negative controls. MoAb W6/32 (anti HLA-A,B,C monomorphic determinant) was used as a positive control. Cell fluorescence, in terms of the percentage of positive cells and peak mean channel, was analyzed by flow cytometry (Cytofluorograph IIS, Ortho Diagnostic System Inc., Westwood, Mass). Platelets, as well as buffy coat analysis for lymphocytes, monocytes, and granulocytes were gated from forward angle and 90° light scatter patterns, and analysed independently for fluorescence.

Results

Reactivity of MoAbs with normal adult tissues

The results of testing 14 MoAbs on various adult tissues are summarized in Table 2. Most normal tissues were non-reactive. Exceptions were sweat glands, liver and kidney tubules, which were reactive with most of the MoAbs. The positive liver reactivity seemed to be restricted to the cytoplasm of hepatocytes and to bile ducts. One of the MoAbs (BT-Br-6) reacted weakly with skin epidermal cells.

Testing of MoAbs for reactivity with autologous (patient from whom MoAbs were derived) and allogeneic normal peripheral blood cells indicated that they were negative, with the exception of BA-Me-4, which gave positive reactivity with polymorphonuclear cells. As determined by absorption studies, none of the MoAbs reacted with erythrocytes and platelets, separately prepared from 3/3 healthy allogeneic adult individuals. Testing of nine MoAbs (BT-Br-6; BT-Co-1,-2,-3,-4,-5,-6; BT-Me-7,-8) with autologous peripheral blood cell components revealed negative reactivities. The observed absence or presence of cross-reactivity with various blood components was confirmed by flow cytometry.

Patterns of reactivity

The 14 MoAbs were used to phenotype large numbers of different tumor tissues. The distribution of antigens detected on tumor tissue sections of primary and metastatic origins by MoAbs fell into several categories of staining patterns using the ABC method. The staining patterns varied depending on which target tissue was tested and on which MoAb was employed (Fig. 1). For example, in the majority of breast tumors tested with MoAb Ba-Br-1, intense but rather homogeneous staining was seen on the membrane and cytoplasm of tumor cells. In similar tissue sections BT-Co-5 or BA-Br-5 gave a 'patchwork' staining pattern, positive within given tumor areas, leaving adjacent negative tumor areas virtually unstained. The third type of reactivity pattern was noted to be scattered reactive tumor cells in a 'sea' of non-reactive tumor cells (focal binding).

Anti-(breast carcinoma) MoAbs

Four anti-(breast carcinoma) MoAbs (BA-Br-1, BA-Br-3, BA-Br-5, BT-Br-6) were tested on 110 tumor tissues from patients with various tumor histotypes (Tables 3 and 4). BA-Br-1 reacted with 100% of the following carcinomas: breast, colon, lung, prostate and pancreas. Most of the carcinoma types other than those previously specified were also reactive with these four MoAbs in varying degrees, although the number of cases in each category was relatively small. BA-Br-1 and BA-Br-5 did not react with any of the melanoma and sarcoma tissues, while BA-Br-3 and BT-Br-6 reacted with approximately 38% of the melanoma and 55% of the sarcomas tested.

BA-Br-3 reacted very broadly with carcinoma tissues of diverse histotypes: 91% of breast carcinoma, 87% of colon carcinomas, 91% lung carcinomas and 50% of prostate carcinomas. Furthermore, BA-Br-3 reacted sporadically with some melanomas and sarcomas with frequencies of 33% and 64% respectively.

BA-Br-5 reacted with only a few selected cases of carcinomas (11% of breast, 40% of colon, 55% of lung, and 25% of prostate), but did not react with any of the melanoma or sarcoma tissues tested.

Fewer tumor tissues were phenotyped by BT-Br-6 (70 cases) than BA-Br-1, BA-Br-3, and BA-Br-5. MoAb BT-Br-6 reacted with 45% of breast carcinomas, 67% of colon carcinomas, 57% of lung carcinomas and 0% of prostate carcinomas. In contrast to MoAbs BA-Br-1 and BA-Br-3, BT-Br-6 showed much broader reactivity to non-carcinoma tumors including melanoma (43%) and sarcoma (45%).

Anti-(colon carcinoma) MoAbs

Six anti-(colon carcinoma) MoAbs (BT-Co-1, BT-Co-2, BT-Co-3, BT-Co-4, BT-Co-5, BT-Co-6) were tested on 102 specimens from patients with diverse tumor histotypes (Tables 3 and 5). The first five MoAbs exhibited similar binding patterns: 80–87% of colon carcinomas, 63–68% of breast carcinomas, 70–90% of lung carcinomas, and 50–100% of prostate carcinomas, were positive. They also showed some reactivity with other carcinoma tissue types (59–68%). Melanoma tissues were generally non-reactive, whereas 10–20% of sarcomas gave positive reactivity with these MoAbs.

The MoAb BT-Co-6 was not tested with as many tumor tissues as the first five MoAbs in this series. It reacted with 100% of colon, lung, and prostate carcinomas. It also reacted with 100% of other carcinoma histotypes: squamous cell carcinoma, salivary gland and hepatoma. Fur-

Table 2. Reactivity of MoAbs with normal adult tissues based on the ABC immunohistochemical technique

MoAb	Reactivity[a]											
	Lymphocytes	PMN[b]	RBC[c]	Platelets[c]	Lung	Heart	Breast	Liver[d]	Kidney	Colon	Skin	Tumor/stroma[e]
BA-Br-1	–	–	–	–	–	–	–	±[f]	+T[g]	3+MC[i] gl. epith.[j]	–epid.[m]	+/–
								–	–G[h]	–MC lp.[k]	+sw. gl.[n]	
										–SMC[l]		
BA-Br-3	–	–	–	–	±[o]	–	+	±[f]	+T	–	–epid.	+/–
									–G		+sw. gl.	
BA-Br-5	–	–	–	–	–	–	–	–	–	–	–	+/–
BT-Br-6	–	–	–	–	–	–	–	+	+T	–	+epid.	+/–
									–G		–sw. gl.	
BT-Co-1	–	–	–	–	+	–	–	+,2+	+T	+MC gl. epith.	–	+/–
									–G	–MC lp.		
										–SMC		
BT-Co-2	–	–	–	–	+	–	–	+,2+	+T	+MC gl. epith.	–	+/–
									–G	–MC lp.		
										–SMC		
BT-Co-3	–	–	–	–	–	–	–	+,2+	+T	+MC gl. epith.	–	+/–
									–G	–MC lp.		
										–SMC		
BT-Co-4	–	–	–	–	–	–	–	+	+T	+MC gl. epith.	–	+/–
									–G	–MC lp.		
										–SMC		
BT-Co-5	–	–	–	–	–	–	–	+	+T	+MC gl. epith.	–	+/–
									–G	–MC lp.		
										–SMC		
BT-Co-6	–	–	–	–	–	–	–	–	+T	2+MC gl. epith.	–epid.	+/–
									–G	–MC lp.	+sw. gl.	
										–SMC		
BA-Me-4	–	+	–	–	–	–	–	+	+T	–	–epid.	+/–
									–G		+sw. gl.	
BA-Me-5	–	–	–	–	–	–	–	+	+T	–	–epid.	+/–
									–G		+sw. gl.	
BT-Me-7	–	–	–	–	+	–	–	+	+T	–MC gl. epith.	–epid.	+/–
									–G	+MC lp.	+sw. gl.	
										–SMC		
BT-Me-8	–	–	–	–	+	–	–	+	+T	–MC gl. epith.	–epid.	+/–
									–G	+MC lp.	+sw. gl.	
										–SMC		

[a] Results are expressed by: –, negative; +, weakly positive; 2+, moderately positive; 3+, strongly positive; 4+, very strongly positive; ±, mostly negative with few areas positive

[b] PMN: polymorphonuclear cells

[c] RBC (erythrocytes) and platelets were tested indirectly by absorption studies (see Materials and Methods)

[d] Reactivity was seen in the cytoplasm of hepatocytes with those MoAbs giving ±, + and 2+ reactivities

[e] Stroma refers to 'normal' connective tissue in area of or adjacent to tumor in same organ

[f] Hepatocytes were mostly negative with occasional positivity. In addition, bile ducts were positive with BA-Br-1 and BA-Br-3

[g] T, tubules; [h] G, glomeruli; [i] MC, mucosa; [j] gl. epith., gland epithelium; [k] lp., lamina propia; [l] SMC, submucosa; [m] epid., epidermis; [n] sw. gl., sweat glands; [o] some of alveolar epithelia were stained with the intensity of + to 2 t

thermore, MoAb BT-Co-6 was reactive with 89% of breast carcinomas, but failed to react with melanoma and sarcoma tissues.

Anti-melanoma MoAbs

Four anti-melanoma MoAbs (BA-Me-4, BA-Me-5, BT-Me-7 and BT-Me-8) were evaluated on 107 tumor tissues of different histotypes (Tables 3 and 6). BA-Me-4 reacted with 78% of melanomas, 56% of breast carcinomas, 93% of colon carcinomas, 55% of lung carcinomas, 50% prostate carcinomas, and 36% of sarcomas. BA-Me-5 reacted with 29% of melanomas, 28% of breast carcinomas, 30% of colon carcinomas, 0% of lung carcinomas, and 56% of sarcomas. BT-Me-7 reacted with 88% of melanoma tumors, 67% of breast carcinomas, 64% of colon carcinomas, 44% of lung carcinomas, and 55% of sarcomas. BT-Me-8 reacted with 100% of melanomas, 71% of breast carcinomas, 55% of colon carcinomas, 63% of lung carcinomas and 64% of sarcomas.

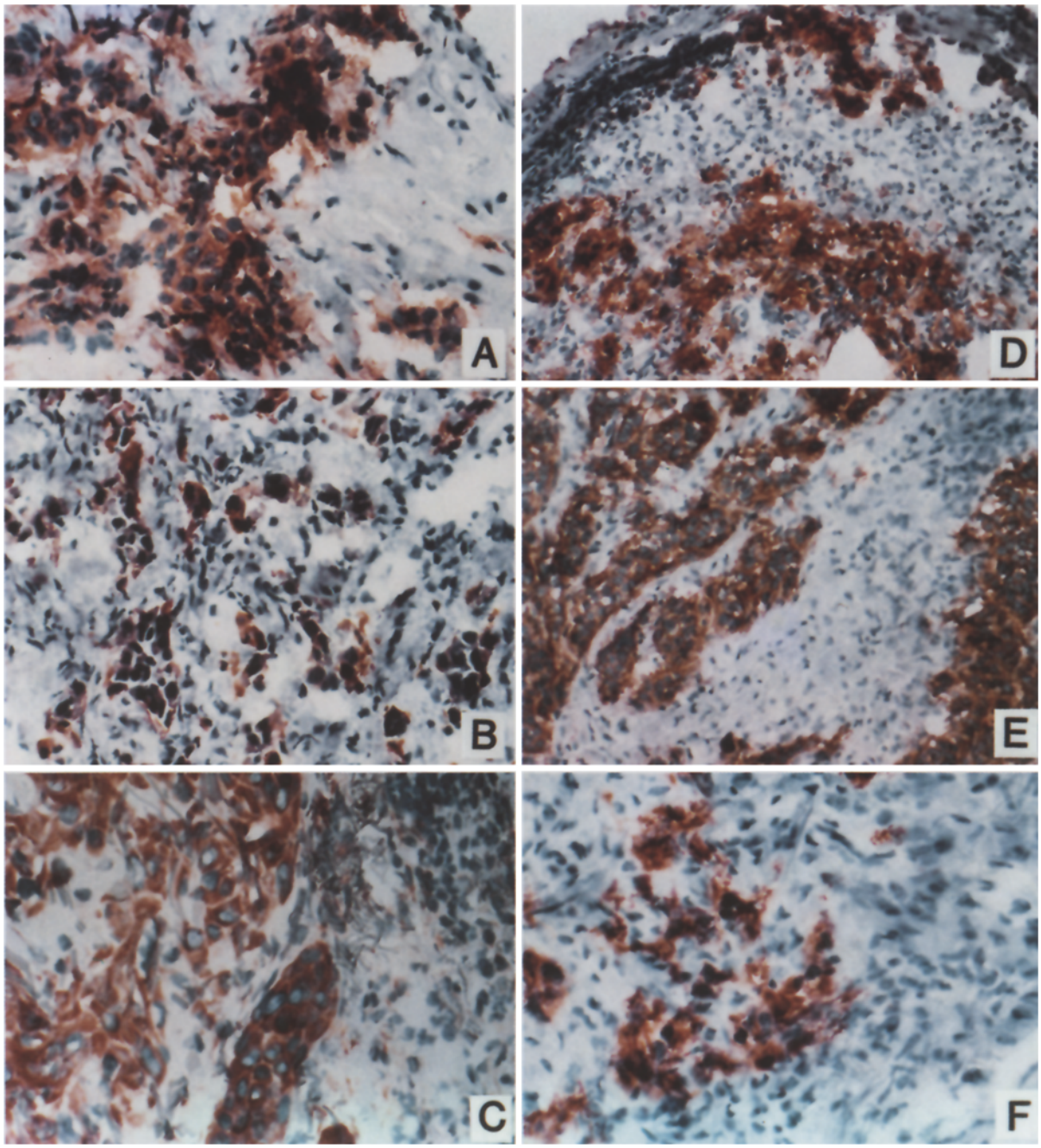

Fig. 1. Indirect immunoperoxidase staining of sections of various human tumor tissues with anti-tumor MoAbs, counterstained with hematoxylin. **A** Breast adenocarcinoma metastatic to axillary lymph node (pt.HAH), reacted with MoAb BA-Br-3, ×400. Packets of positive-staining epithelial cells (5–7 cells thick) are observed within the lymph node stroma. Cytoplasmic staining is reddish-brown and coursely granular. Cytoplasmic limits are not seen. **B** Breast carcinoma metastatic to cervical lymph node (pt.REU), reacted with MoAb BT-Co-5, ×400. Positive-staining epithelial cells are observed in a random pattern in stroma adjacent to the capsule. Cytoplasmic staining is reddish-brown in color and was smooth to finely granular in pattern. Occasional cells form clusters of 2–4. Other unstained tumor cells are observed in the same field. This may represent different stages of differentiation. **C** Adnexal microcystic carcinoma of the face (pt.GOL), reacted with MoAb BT-Br-6, ×400. Positive-staining cells form islands that extend into adjacent skin tissue. All staining activity is limited to the cytoplasm, and is reddish-brown in color. These cells have sharply defined cytoplasmic limits, and staining is intense. Most tumor cells are round to oval, but several are stellate in shape. **D** Primary colon adenocarcinoma (pt. RIE), reacted with MoAb BA-Me-4, ×200. Positive carcinoma cells stained reddish-brown in color and imparted a coarsely granular appearance to the cytoplasm. Positive cells transect the wall of the colon from the mucosa to the mesentaries. Some tumor cells are present within submucosal lymphatics (upper middle of the frame). **E** Primary colon carcinoma (pt.LAV), reacted with MoAb BT-Co-6, ×100. Some positive-staining cells form large rows of 3–5 cells thick, and when observed on cross-section formed microacini with discernable cytoplasmic limits. **F** Primary prostate carcinoma (pt.CLE), reacted with MoAb BA-Br-1, ×400. Positive-staining carcinoma cells are interspersed between muscle bundles and stained reddish-brown in color in the cytoplasm. Staining is finely granular and cytoplasmic limits are not always discernable (Figure reduced to 90%)

Table 3. Survey of immunohistochemical reactivity of a panel of MoAbs on cryostat malignant tissue sections of diverse histotypes

Tumor primary histotype	Anti-(breast carcinoma) MoAb				Anti-(colon carcinoma) MoAb						Anti-melanoma/MoAbs			
	BA-Br-1	BA-Br-3	BA-Br-5	BT-Br-6	BT-Co-1	BT-Co-2	BT-Co-3	BT-Co-4	BT-Co-5	BT-Co-6	BA-Me-4	BA-Me-5	BT-Me-7	BT-Me-8
Breast	35/35[a]	32/35	4/35	9/20	21/31	20/31	19/30	19/30	20/30	16/18	19/34	5/18	14/21	15/21
Colon	15/15	13/15	6/15	6/9	13/15	12/15	13/15	12/15	12/15	8/8	14/15	3/10	7/11	6/11
Lung adeno carcinoma	11/11	10/11	6/11	4/7	9/10	9/10	8/10	7/10	9/10	7/7	6/11	0/5	4/9	5/8
Prostate	4/4	2/4	1/4	0/1	4/4	4/4	3/4	2/4	3/3	1/1	2/4	NT	1/1	1/1
Pancreas	2/2	1/2	0/2	1/1	2/2	2/2	2/2	2/2	2/2	1/1	2/2	NT	1/1	1/1
Islet cell	1/1	0/1	0/1	NT[b]	0/1	0/1	0/1	0/1	0/1	NT	0/1	NT	NT	NT
Squamous cell[c]	4/5	5/5	3/5	3/5	2/5	4/5	3/5	3/5	2/5	4/4	3/5	1/3	5/5	4/5
Stomach	1/2	1/2	1/2	NT	0/2	0/2	0/2	0/2	0/2	0/2	0/2	NT	0/1	0/1
Ovarian	3/4	3/4	1/4	2/3	2/4	2/4	2/4	2/4	2/4	2/3	1/3	0/3	2/3	3/3
Tongue	1/1	1/1	0/1	NT	NT	NT	NT	NT	NT	NT	1/1	NT	NT	NT
Bile duct	1/1	1/1	0/1	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Adnexal	1/1	1/1	1/1	NT	0/1	0/1	0/1	1/1	1/1	NT	0/1	NT	1/1	1/1
Cervical	1/1	1/1	0/1	NT	1/1	1/1	1/1	1/1	1/1	NT	0/1	NT	1/1	1/1
Salivary gland	1/1	1/1	1/1	0/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	NT	1/1	1/1
Parotid	2/2	0/2	0/2	1/2	1/2	1/2	1/2	1/2	1/2	2/2	2/2	0/1	1/2	1/2
Renal	0/2	2/2	0/2	1/1	2/2	1/2	1/2	2/2	2/2	0/1	0/2	0/1	1/1	1/1
Hepatoma	1/1	1/1	0/1	0/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	0/1	0/1
Bladder	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	0/1	1/1	0/1	1/1	1/1
Melanoma	0/9	3/9	0/9	3/7	0/9	0/9	0/9	0/9	0/9	0/5	7/9	2/7	7/8	8/8
Leiomyosarcoma	1/5	4/5	0/5	4/5	2/5	2/5	2/5	1/5	1/5	0/5	1/5	2/5	2/5	3/5
Osteosarcoma	0/2	1/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	1/2	1/1	2/2	2/2
Chondrosarcoma	0/1	0/1	0/1	0/1	NT	NT	NT	NT	NT	NT	1/1	NT	0/1	0/1
Ewing sarcoma	0/1	1/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	NT	0/1	0/1	1/1	1/1
Liposarcoma	0/1	1/1	0/1	1/1	0/1	0/1	0/1	0/1	0/1	0/1	1/1	NT	1/1	1/1
Angiosarcoma	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Total (%)	86/110 (78)	86/110 (78)	25/110 (23)	36/70 (51)	62/102 (61)	61/102 (60)	58/101 (57)	56/101 (55)	59/100 (59)	44/64 (69)	64/107 (60)	15/58 (26)	53/79 (67)	56/78 (72)

[a] No. of specimens positive/no. of specimens tested; [b] not tested

[c] This group consist of five squamous cell carcinomas: tonsil (1), tongue (1), mouth (1), pharynx (1), mediastinum (1)

Table 4. Frequency of cross-reactivity of anti-(breast carcinoma) MoAbs with other tumor types

Parameter	MoAb			
	BA-Br-1	BA-Br-3	BA-Br-5	BT-Br-6
Total no. cases	110	110	110	70
Total no. positive	86	86	25	36
% Pos. vs breast ca.[a]	100	91	11	45
% Pos. vs colon ca.	100	87	40	67
% Pos. vs lung ca.	100	91	55	57
% Pos. vs prostate ca.	100	50	25	0
% Pos. vs other ca.	83	75	33	57
% Pos. vs all carcinomas	87	80	26	52
% Pos. vs melanoma	0	33	0	43
% Pos. vs sarcomas	0	64	0	45
% Pos. vs all tumors	79	78	23	51

[a] Pos., positive; ca., carcinoma

Table 5. Frequency of cross-reactivity of anti-(colon carcinoma) MoAbs with other tumor types

Parameter	MoAb					
	BT--Co-1	BT--Co-2	BT--Co-3	BT--Co-4	BT--Co-5	BT--Co-6
Total no. cases	102	102	101	101	100	64
Total no. positive	62	61	58	56	59	44
% Pos. vs colon ca.[a]	87	80	87	80	80	100
% Pos. vs breast ca.	68	65	63	63	67	89
% Pos. vs lung ca.	90	90	80	70	90	100
% Pos. vs prostate ca.	100	100	75	50	100	100
% Pos. vs other ca.	59	64	59	68	64	85
% Pos. vs all carcinomas	66	65	62	61	65	83
% Pos. vs melanoma	0	0	0	0	0	0
% Pos. vs all sarcomas	20	20	20	10	10	0
% Pos. vs all tumors	61	60	57	55	58	71

[a] Pos., positive; ca., carcinoma

Table 6. Frequency of cross-reactivity of anti-melanoma MoAbs with other tumor types

Parameter	MoAb			
	BA-Me-4	BA-Me-5	BT-Me-7	BT-Me-8
Total no. cases	107	58	79	78
Total no. positive	64	15	53	56
% Pos. vs melanoma	78	29	88	100
% Pos. vs breast ca.[a]	56	28	67	71
% Pos. vs colon ca.	93	30	64	55
% Pos. vs lung ca.	55	0	44	63
% Pos. vs prostate ca.	50	NT[b]	100	100
% Pos. vs other ca.	55	11	82	82
% Pos. vs all carcinomas	62	22	71	74
% Pos. vs all sarcomas	36	56	55	64
% Pos. vs all tumors	60	25	66	72

[a] Pos., positive; ca., carcinoma
[b] Not tested

Micro- and macroheterogeneity

Table 7 shows an example of data on reactivity patterns of multiple metastatic lesions from a single patient (BUR) with malignant melanoma. The results, based on 14 MoAbs tested, indicate that the antigenic phenotype of five lymph node metastases and one brain metastasis biopsied over an 18-month period did not vary greatly. The two notable differences are that the MoAbs BT-Br-6 and BA-Me-5 recognized antigens that were not expressed on the earlier biopsies, but were present in the subsequent metastatic lesions.

Table 8 presents examples of our phenotyping results on eight patients (five with breast carcinoma and three colon carcinoma) illustrating heterogeneous expression of the various antigens among tumor biopsies obtained from eight different patients (macroheterogeneity). From these typing results we were able to select 2–5 MoAbs which were most reactive with each individual patient's tumor.

Table 7. Immunohistological typing of metastatic lesions removed from patient BUR at different times. Tissue-culture cells derived from one of the lymph node (LN) metastases were also evaluated

MoAb	LN 1 occipital 01/23/86	LN 2 mediastinal 05/05/86	LN 3 cervical 04/27/86	LN 4 supra-clavicular 06/15/87	LN 5 femoral 06/15/87	Brain 09/28/87	Tissue-culture cells derived from LN 1
BA-Me-4	4+[a]	2+	2+	4+	4+	4+	4+
BA-Me-5	−	−	−	+	2+	2+	−
BT-Me-7	4+	4+	4+	4+	4+	4+	±
BT-Me-8	4+	3+	4+	4+	4+	4+	2+
BA-Br-1	−	−	−	−	−	−	−
BA-Br-3	−	−	−	+	+	+	−
BA-Br-5	−	−	−	−	−	−	−
BT-Br-6	−	4+	4+	4+	4+	2+	−
BT-Co-1	−	−	−	−	−	−	−
BT-Co-2	−	−	−	−	−	−	−
BT-Co-3	−	−	−	−	−	−	−
BT-Co-4	−	−	−	−	−	−	−
BT-Co-5	−	−	−	−	−	−	−
BT-Co-6	−	−	−	−	−	−	−

[a] Results are expressed by: −, negative; +, weakly positive; 2+, moderately positive; 3+, strongly positive; 4+, very strongly positive; ±, mostly negative with few tumor areas positive

Table 8. Heterogeneous expression of antigens in tumor biopsies from different patients as revealed by immunohistochemical typing with a panel of anti-tumor MoAbs

	Breast carcinoma of patient					Colorectal carcinoma of patient		
MoAb	BLO	JAK	RUB	PAR	GIB	GOO	JOH	KLO
Control								
W6/32[a]	3+[c]	4+	2+	4+	4+	+	4+	3+
Mouse IgG	–	–	–	–	–	–	–	–
PBS[b]	–	–	–	–	–	–	–	–
Anti-tumor								
BA-Br-1	+	2+	4+	+	4+	4+	4+	3+
BA-Br-3	3+	–	3+	3+	3+	4+	±	–
BA-Br-5	–	–	–	–	–	–	3+	–
BT-Br-6	–	–	4+	+	–	+	2+	+
BT-Co-1	–	2+	3+	–	2+	–	+	+
BT-Co-2	–	2+	3+	±	2+	–	+	2+
BT-Co-3	–	3+	2+	±	+	–	±	2+
BT-Co-4	2+	3+	2+	2+	+	–	±	+
BT-Co-5	+	3+	4+	+	+	–	±	+
BT-Co-6	+	3+	2+	+	4+	4+	4+	3+
BA-Me-4	3+	–	–	2+	+	3+	4+	3+
BA-Me-5	–	–	–	±	–	–	–	–
BT-Me-7	–	–	+	±	2+	–	+	+
BT-Me-8	–	–	+	±	2+	–	2+	+

[a] Anti-HLA class 1 antigens, reactive with both tumor and stroma areas of thin sections prepared from frozen tissue blocks of the eight patients listed

[b] Phosphate-buffered saline

[c] See the footnote under Table 7 for explanation of scoring results. Positivity (+ – 4+) refers to tumor reactivity

These observations stress the existence of such micro- and macroheterogeneity, as well as individuality of the expression of antigens among metastatic lesions and the importance of having a panel of MoAbs for tumor-typing purposes.

Discussion

Immunohistochemical detection of tumor cells in tissue sections using MoAbs has recently received considerable attention (for review, see [41]). During the past decade, many human tumor-associated antigens and unique epitopes have been identified by MoAbs. Immunohistochemistry and application of MoAbs have revealed that some of these antigens display a restricted distribution in normal adult tissues and, therefore, may represent appropriate markers for tumor radioimaging and useful targets for immunotherapy.

Tumor heterogeneity in terms of morphological, functional and antigenic characteristics has been described previously in many animal and human tumor systems [4, 7, 11, 14, 15, 30, 31, 48, 50]. Antigenic heterogeneity of tumors may allow antigen-negative populations to escape targeting and subsequent destruction in patients when single-antibody-guided therapy is applied [4, 14, 33, 47, 50]. Heterogeneous expression of tumor-associated antigens may result from genetic variation [1, 36, 49], cell-cycle-dependent expression [20, 26], antigenic modulation [39, 43, 45], and other epigenetic factors [44]. The tumor heterogeneity seen in these studies draws attention to the need to understand and approach the same issue for therapy. We feel that antigenic heterogeneity, which demonstrates that each cancer is unique, calls for the design of therapy based on antibody recognition in each patient [4, 14, 33, 36].

Using a panel of 14 MoAbs, we have demonstrated a wide range of cross-reactivity of anti-(breast carcinoma) and anti-(colon carcinoma) MoAbs with various other carcinoma histotypes. This observation is not unique, since it has been documented in the literature that more than twenty well-characterized MoAbs raised against various carcinomas have revealed a pancarcinoma type of reactivity, with the exception of MoAbs to prostatic tissue antigen. Some of our anti-melanoma MoAbs have also shown cross-reactivity with selected carcinomas. Among the 14 MoAbs, the 9 most reactive with carcinomas of diverse histotypes can be ranked in terms of the extent of carcinoma cross-reactivity in the following order: BA-Br-1 = BA-Br-3 > BT-Me-8 > BT-Co-6 = BT-Me-7 > BT-Co-1 > BT-Co-2 = BA-Me-4 > BT-Co-5. Although BA-Br-1 and BT-Co-6 were raised differently against breast tumor membrane extract and colon tumor cells prepared from a nude mouse xenograft, respectively, there is strong similarity between them. Both reacted highly with carcinoma tissues (over 83%) but were non-reactive with tumors of non-epithelial origin, i.e., melanoma and sarcoma thus far tested. Therefore, BA-Br-1 and BT-Co-6 may be qualified for a pancarcinoma specificity. It is of interest that certain anti-(breast carcinoma) MoAbs, including BA-Br-3 and BA-Br-5, reacted more frequently with prostate carcinoma than breast carcinoma. Similarly, some anti-(colon carcinoma) MoAbs, such as BT-Co-1, BT-Co-2 and BT-Co-5, reacted more frequently with prostatic carcinoma than colon carcinoma.

From the reactivity patterns of MoAbs obtained with patient biopsy tissues, a single MoAb rarely reacted with 100% cancer cells with uniform intensity at a specific site. From both the tumor radioimmunolocalization and biotherapeutic standpoints, there is a need to examine biopsy tissues with a panel of MoAbs by immunohistochemical techniques to select a mixture or cocktail of different MoAbs which are most reactive with the patients' tumors. It is obvious from our own studies in a series of primary and metastatic lesions obtained simultaneously or over a period of time, that appropriate cocktails or combinations of MoAbs can be selected for clinical use.

While the immunohistochemical phenotyping of tumor tissues of a given patient with a panel of MoAbs is an important step for selections of appropriate MoAbs for antibody-guided therapy, these selected MoAbs may not all act on viable tumor synergistically in terms of binding activity. Our recent studies by flow cytometric analysis indicate that certain combinations of MoAbs may give quantitatively less binding activity, such that the percentage of reactive cells and/or the intensity of reactivity per cell were found to decrease as compared with each single MoAb [5]. Thus, flow cytometric analysis of the immunohistochemically selected MoAbs against patient tumor cells is of great value. For those patients whose monodispersed viable tumor cells are not readily available or whose tumor cells have not been grown in vitro as short-term or long-term cultures, flow cytometric studies with the patient tumor cells will not be possible. However, if an available allogeneic tumor cell line of a similar histotype

behaves in a like fashion with regard to immunohistochemical binding (when compared with the patient biopsy) in response to exposure to various combinations of reactive MoAbs, we may be able to use the appropriate reference cell lines as an approximate in place of patient tumor cells and select the best combination of MoAbs by flow cytometric assessment. Experiments are underway to test this hypothesis.

We have utilized the phenotyping results presented herein to select two to five MoAbs as cocktails for each of 23 cancer patients treated through the Biological Therapy Institute. These patients have undergone one or two cycles of infusion with selected MoAbs conjugated to doxorubicin. The results of conjugation, preclinical, and clinical studies are reported elsewhere [35].

What is the acceptable level of an anti-tumor MoAb in terms of normal tissue cross-reactivity for tumor radioimaging and therapeutic uses? This question has been a matter of great concern. It has been shown that certain normal tissues reactive with anti-tumor MoAbs may be protected and unavailable to in vivo administered MoAbs because of their anatomical sites and the catabolic rate of the MoAbs in that particular tissue site [6]. We have observed no kidney toxicity in cancer patients in whom doxorubicin-conjugated MoAbs, known to cross-react with tubules but not with glomeruli of the kidney, were administered in milligram to gram quantities [35]. Furthermore, even though several of these antibodies react with the cytoplasm of normal hepatocytes and sweat glands, no clinical toxicities associated with these reactivities have been observed [35].

We continue to broaden our panel of MoAbs for tumor typing. Over 50 antibodies have been examined and 20 are now used routinely in our current typing panel. Immunoconjugates with doxorubicin, daunorubicin and mitomycin C are being prepared for further preclinical and clinical studies in our patient-specific drug immunoconjugate therapy program.

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