

A spectrum of monoclonal antibodies reactive with human mammary tumor cells

(breast cancer/tumor-associated antigens/radioimmunoassays/immunoperoxidase technique/breast tumor metastases)

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ABSTRACT Splenic lymphocytes of mice, immunized with membrane-enriched fractions of metastatic human mammary carcinoma tissues, were fused with the NS-1 non-immunoglobulin-secreting murine myeloma cell line. This resulted in the generation of hybridoma cultures secreting immunoglobulins reactive in solid-phase radioimmunoassays with extracts of metastatic mammary carcinoma cells from involved livers, but not with extracts of apparently normal human liver. As a result of further screening of immunoglobulin reactivities and double cloning of cultures, 11 monoclonal antibodies were chosen that demonstrated reactivities with human mammary tumor cells and not with apparently normal human tissues. These monoclonal antibodies could be placed into at least five major groups on the basis of their differential binding to the surface of various live human mammary tumor cells in culture, to extracts of mammary tumor tissues, or to tissue sections of mammary tumor cells studied by the immunoperoxidase technique. Whereas a spectrum of reactivities to mammary tumors was observed with the 11 monoclonal antibodies, no reactivity was observed to apparently normal cells of the following human tissues: breast, lymph node, lung, skin, testis, kidney, thymus, bone marrow, spleen, uterus, thyroid, intestine, liver, bladder, tonsils, stomach, prostate, and salivary gland. Several of the antibodies also demonstrated a "pancarcinoma" reactivity, showing binding to selected non-breast carcinomas. None of the monoclonal antibodies showed binding to purified ferritin or carcinoembryonic antigen. Monoclonal antibodies of all five major groups, however, demonstrated binding to human metastatic mammary carcinoma cells both in axillary lymph nodes and at distal sites.

Numerous investigators have reported the existence of antigens associated with human mammary tumors (1-9). These studies, all conducted with conventional hyperimmune polyclonal sera, however, were unfortunately hampered with regard to the heterogeneity of the antibody populations employed and the amount of specific immunoglobulin that could be generated. Since the advent of hybridoma technology (10), monoclonal antibodies of predefined specificity and virtually unlimited quantity may now be generated against a variety of antigenic determinants present on normal or neoplastic cells. The rationale of the studies reported here was to utilize extracts of human metastatic mammary tumor cells as immunogens in an attempt to generate and characterize monoclonal antibodies reactive with determinants that would be maintained on metastatic, as well as primary, human mammary carcinoma cells. Multiple assays using tumor cell extracts, tissue sections, and live cells in culture have been employed to reveal the diversity of the monoclonal antibodies generated.

MATERIALS AND METHODS

Immunizations. Membrane-enriched cell extracts were prepared from breast tumor metastases to the liver from two pa-

tients as well as from apparently normal liver as described (11). Part of the cell extract was repeatedly passed through columns of staphylococcal protein A-Sepharose (Pharmacia) to remove cell-associated immunoglobulin that would interfere with the solid-phase radioimmunoassays (RIAs). Four-week-old C57BL/6, BALB/c, and C3HfC57BL mice were immunized by intraperitoneal inoculation with 100 μ g of membrane-enriched fractions of either of two breast tumor metastases to the liver (termed Met 1 and Met 2; see Table 1) mixed with an equal volume of complete Freund's adjuvant. Fourteen and 28 days later, mice were boosted with an intraperitoneal inoculation of 100 μ g of the immunogen mixed with incomplete Freund's adjuvant. After an additional 14 days, 10 μ g of immunogen was administered intravenously. Spleens were removed for cell fusion 3-4 days later.

Hybridoma Methodology. Somatic cell hybrids were prepared by using the method of Herzenberg *et al.* (12) with some modifications (11). All hybridoma cell lines were cloned twice. All hybridoma supernatant fluids were assayed for antibody in solid-phase RIA by using 5- μ g cell extracts as described (11). Isotyping of antibodies was as described (11).

Live Cell Immunoassay. Cells were seeded into 96-well, flat-bottom tissue culture plates (Linbro), using the appropriate growth medium, at 5×10^4 cells per well and incubated at 37°C for 18-24 hr in a humidified incubator containing 5% CO₂. The remainder of the assay was as previously described for the solid-phase RIA (11) with the exception that wash buffer was RPMI 1640 medium in place of P_i/NaCl.

Immunoperoxidase Studies. Five-micrometer sections of formalin-fixed or frozen sections of tissue on slides were used as described (11).

RESULTS

Generation of Monoclonal Antibodies. Mice were immunized with membrane-enriched fractions of human metastatic mammary carcinoma cells from either of two involved livers (designated Met 1 and Met 2). Spleens of immunized mice were fused with NS-1 myeloma cells to generate 4250 primary hybridoma cultures. Supernatant fluids from these cultures were screened in solid-phase RIAs for the presence of immunoglobulin reactive with extracts of metastatic mammary tumor cells from involved livers and not reactive with similar extracts of apparently normal liver. Whereas many cultures demonstrated immunoglobulin reactive with all test antigens, 370 cultures contained immunoglobulin reactive only with the metastatic carcinoma cell extracts. After passage and double cloning of these cultures by endpoint dilution, the monoclonal immunoglobulins from 11 hybridoma cell lines were chosen for further study. The isotypes of all 11 antibodies were determined: 10 were IgGs of various subclasses and one was an IgM (Table 1).

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Abbreviations: P_i/NaCl, phosphate-buffered saline; RIA, radioimmunoassay; CEA, carcinoembryonic antigen.

Table 1. Reactivity of monoclonal antibodies in solid-phase RIAs

Monoclonal antibody	Isotype	Cell extracts*			Live cells†				
		Met 1	Met 2	Liver	Mammary carcinoma			Sarcoma‡	Normal§
					BT-20	MCF-7	ZR-75-1		
B6.2	IgG1	+++	++	Neg	++	+++	++	Neg	Neg
B14.2	IgG1	+++	++	Neg	+	++	+	Neg	Neg
B39.1	IgG1	+++	++	Neg	++	++	++	Neg	Neg
F64.5	IgG2a	+++	++	Neg	++	++	+	Neg	Neg
F25.2	IgG1	+++	++	Neg	+	+	+	Neg	Neg
B84.1	IgG1	+++	++	Neg	+	+	+	Neg	Neg
B38.1	IgG1	+	+	Neg	+++	++	+++	Neg	Neg
B50.4	IgG1	++	+	Neg	Neg	+	Neg	Neg	Neg
B50.1	IgG1	++	+	Neg	Neg	+	Neg	Neg	Neg
B25.2	IgM	Neg	+++	Neg	Neg	Neg	Neg	Neg	Neg
B72.3	IgG1	+++	Neg	Neg	Neg	Neg	Neg	Neg	Neg
W6/32	IgG2a	Neg	Neg	Neg	+	+	Neg	++	++
B139	IgG1	+++	+++	++	++	++	+	+++	++

* Solid-phase RIAs were as described in *Materials and Methods*. Neg, <500 cpm; +, 500–2000 cpm; ++, 2001–5000 cpm; +++, >5000 cpm.

† The live cell immunoassay was performed on human cells as described in *Materials and Methods*. Neg, <300 cpm; +, 300–1000 cpm; ++, 1001–2000 cpm; +++, >2000 cpm.

‡ Rhabdomyosarcoma (A204), fibrosarcoma (HT1080), and melanoma (A375).

§ Human cell lines were derived from apparently normal breast (Hs0584Bst), skin (Detroit 550), embryonic skin (Detroit 551), fetal lung (WI-38 and MRC-5), fetal testis (Hs0181Tes), fetal thymus (Hs0208Th), fetal bone marrow (Hs0074Bm), embryonic kidney (Flow 4000), fetal spleen (Hs0203Sp), and uterus (Hs0769Ut).

The primary screen for monoclonal antibodies reactive with human mammary carcinoma cells was a solid-phase RIA employing cell extracts of two breast tumor metastases (Met 1 and Met 2) and apparently normal human liver as test antigens. The 11 monoclonal antibodies could immediately be divided into three major groups on the basis of their differential reactivity to Met 1 vs. Met 2 (see Table 1 and Fig. 1). It is interesting to note that the immunogen used in the generation of monoclonal B72.3 was Met 1, while the immunogen used for the generation of monoclonal B25.2 was Met 2. All 11 antibodies were negative when tested against similar extracts from normal human liver, the A204 rhabdomyosarcoma cell line, the HBL100 cell line derived from cultures of human milk, the Mm5mt/c₁ mouse mammary tumor cell line, the C3H10T_{1/2} mouse fibroblast cell line, the CrFK feline kidney cell line, disrupted mouse mammary tumor virus and mouse leukemia virus, 60 ng of purified

carcinoembryonic antigen (CEA), and 1 μ g of ferritin (Table 1; Fig. 1). Two monoclonal antibodies were used as controls in all these studies: (i) W6/32, an anti-human histocompatibility antigen (13), and (ii) B139, which was generated against a human breast tumor metastasis, but which showed reactivity to all human cells tested. The solid-phase assays employed 5 μ g of tissue extract, but titration experiments showed that as little as 0.3 μ g of tissue extract could be used.

Live Cell RIAs. To further define the reactivities of the 11 monoclonal antibodies, and to determine if they bind cell surface antigens, each antibody was tested for binding to live cells in culture. Test cells included three established cell lines of human mammary carcinoma (BT-20, MCF-7, and ZR-75-1). The nine monoclonals grouped together on the basis of their binding to both metastatic cell extracts (Table 1) could now be separated into three different groups on the basis of their dif-

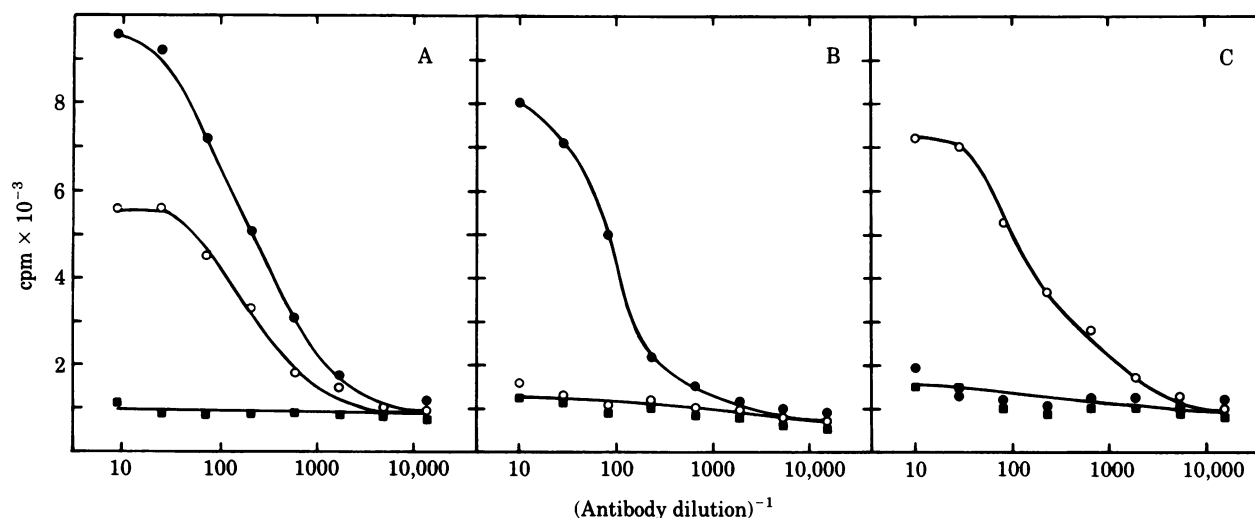


FIG. 1. Solid-phase RIAs for binding of monoclonal antibodies. Monoclonal antibodies B6.2 (A), B72.3 (B), and B25.2 (C) were titrated against cell extracts from breast tumor metastases to the liver, Met 1 (●) and Met 2 (○), as well as from normal liver and the following cell lines: NC37, A204, Mm5mt/c₁, C3H10T_{1/2} (■). The solid-phase RIAs were run with 5 μ g of extracts.

ferential binding to the surface of live cells in culture. Monoclonals B6.2, B14.2, B39.1, F64.5, F25.2, and B84.1 all bound the three mammary carcinoma lines. Monoclonal B38.1 bound not only to the surface of the three mammary carcinoma cells but also to the A549 lung carcinoma line, the A431 vulva epidermoid carcinoma line, and the KB oral epidermoid carcinoma line. This antibody, however, did not bind to sarcoma and melanoma cell lines tested. Monoclonals B50.4 and B50.1 constituted a third grouping in that they reacted only with the surface of the MCF-7 cell line and not BT-20 or ZR-75-1. None of the 11 monoclonal antibodies bound to any of the following cell lines derived from apparently normal human tissues; breast, uterus, skin, embryonic skin and kidney, and fetal lung, testis, thymus, bone marrow, and spleen (Table 1). Control monoclonals W6/32 and B139, however, did bind all of these cells (Table 1).

Immunoperoxidase Studies. To further define the range of reactivity of each of the 11 monoclonal antibodies, the immunoperoxidase technique on tissue sections was employed. As seen in Table 2, all the monoclonals reacted with mammary carcinoma cells of primary mammary carcinomas (both infiltrating ductal and lobular), but the percentage of primary mammary tumors that were reactive varied for the different monoclonals. In many of the positive primary and metastatic mammary carcinomas, not all tumor cells stained. In certain tumor masses, moreover, heterogeneity of tumor cell staining was observed in different areas of a tumor, and even within a given area (see Fig. 2B). A high degree of selective reactivity with mammary tumor cells, and not with apparently normal mammary epithelium, stroma, blood vessels, or lymphocytes of the breast was observed with all 11 monoclonal antibodies. This is exemplified with monoclonals B50.4 and B6.2 in Fig. 2A-E. A dark reddish-brown stain (the result of the immunoperoxidase reaction with the diaminobenzidine substrate) was observed only on mammary carcinoma cells, whereas only the light blue hematoxylin counterstain was observed on adjacent normal mammary epithelium, stroma, and lymphocytes. Occasionally, a few of the apparently "normal" mammary epithelial cells immediately adjacent to mammary carcinoma cell populations did stain weakly (Table 2). However, apparently normal mammary epithelial cells in distal areas of these same breasts (i.e., where no tumor was present) or from breasts of apparently normal patients were always negative (Table 2). This faint staining of some of the "normal" mammary epithelium immediately adjacent to tumor cells may therefore be the manifestation of antigens shed by

adjacent tumor cells, or may represent a "preneoplastic" population expressing the antigen being detected. The staining patterns of mammary carcinoma cells varied among the different monoclonals. This is exemplified in Fig. 2, where it can be seen that monoclonal B50.4 is reactive with mammary carcinoma cells displaying a dense focal staining, (Fig. 2A-C). Monoclonal B6.2, on the other hand, reacts with alternate sections of the same mammary carcinoma, displaying a more diffuse pattern. The monoclonal antibodies could also be distinguished from one another on the basis of which mammary tumors they reacted with; these experiments were carried out by allowing monoclonals to react with alternate tissue sections. Frozen sections of primary mammary tumors were also tested with some of the monoclonals; as expected from the surface binding experiments to live cells in culture (Table 1), the frozen sections revealed membrane staining. Several of the monoclonals also showed reactivity to selected non-breast carcinomas, such as carcinoma of the colon, thus demonstrating a "pancarcinoma" reactivity.

Experiments were then carried out to determine if the 11 monoclonal antibodies could detect mammary carcinoma cell populations at distal sites—i.e., in metastases. Because the monoclonals were all generated by using metastatic mammary carcinoma cells as antigen, it was not unexpected that the monoclonals all reacted, but with different degrees, to various metastases. Perhaps the most efficient of the monoclonals for this purpose was B6.2, which reacted with metastatic mammary carcinoma cells in lymph nodes of 6 of 7 patients, but did not react with uninvolved nodes of 8 patients (Table 2). As exemplified in Fig. 2F, none of the monoclonals reacted with normal lymphocytes or stroma from any involved or uninvolved nodes. The 11 monoclonals also varied in their ability to detect metastatic mammary carcinoma lesions in distal sites such as liver, uterus, and bone (Table 2). All 11 monoclonals were negative for reactivity with apparently normal tissues of the following organs: spleen, bone marrow, thyroid, intestine, lung, liver, bladder, tonsils, stomach, prostate, and salivary glands (Table 2).

DISCUSSION

The monoclonal antibodies described here can be placed into five major classes on the basis of their differential reactivities in solid-phase RIAs and live cell binding assays. Immunoperoxidase staining of tissue sections also revealed differences

Table 2. Reactivity of monoclonal antibodies, measured by using the immunoperoxidase technique

Monoclonal antibody	Mammary carcinoma	Mammary epithelium		Normal nodes	Metastatic nodes	Distal metastases	Normal tissues*
		Normal adjacent	Normal distal				
B6.2	23/31	6/22 [†]	0/3	0/8	6/7	3/6	0/24
B14.2	3/4	0/2	NT	0/6	3/6	2/3	0/11
B39.1	8/11	2/5	0/2	0/4	3/4	3/6	0/16
F64.5	2/4	0/2	NT	0/5	3/5	1/5	0/12
F25.2	3/4	1/3	0/1	0/2	1/1	1/1	0/8
B84.1	2/4	0/2	NT	0/2	0/2	1/5	0/12
B38.1	8/10	2/7	NT	0/4	1/2	2/2	0/13
B50.4	2/9	0/1	NT	0/4	2/4	1/5	0/17
B50.1	5/5	0/1	NT	0/2	0/2	2/6	0/15
B25.2	3/4	1/3	NT	0/2	1/1	1/1	0/7
B72.3	2/4	0/2	NT	0/3	1/3	0/4	0/19

All figures are immunoperoxidase-reactive samples/total samples tested. NT, not tested.

* Apparently normal thyroid, bone marrow, spleen, intestine, lung, liver, bladder, tonsils, kidney, stomach, prostate, salivary gland.

[†] If positive, approximately 2–10% of cells adjacent to tumor stain faintly, whereas distal mammary epithelium cells of the same breast are negative.

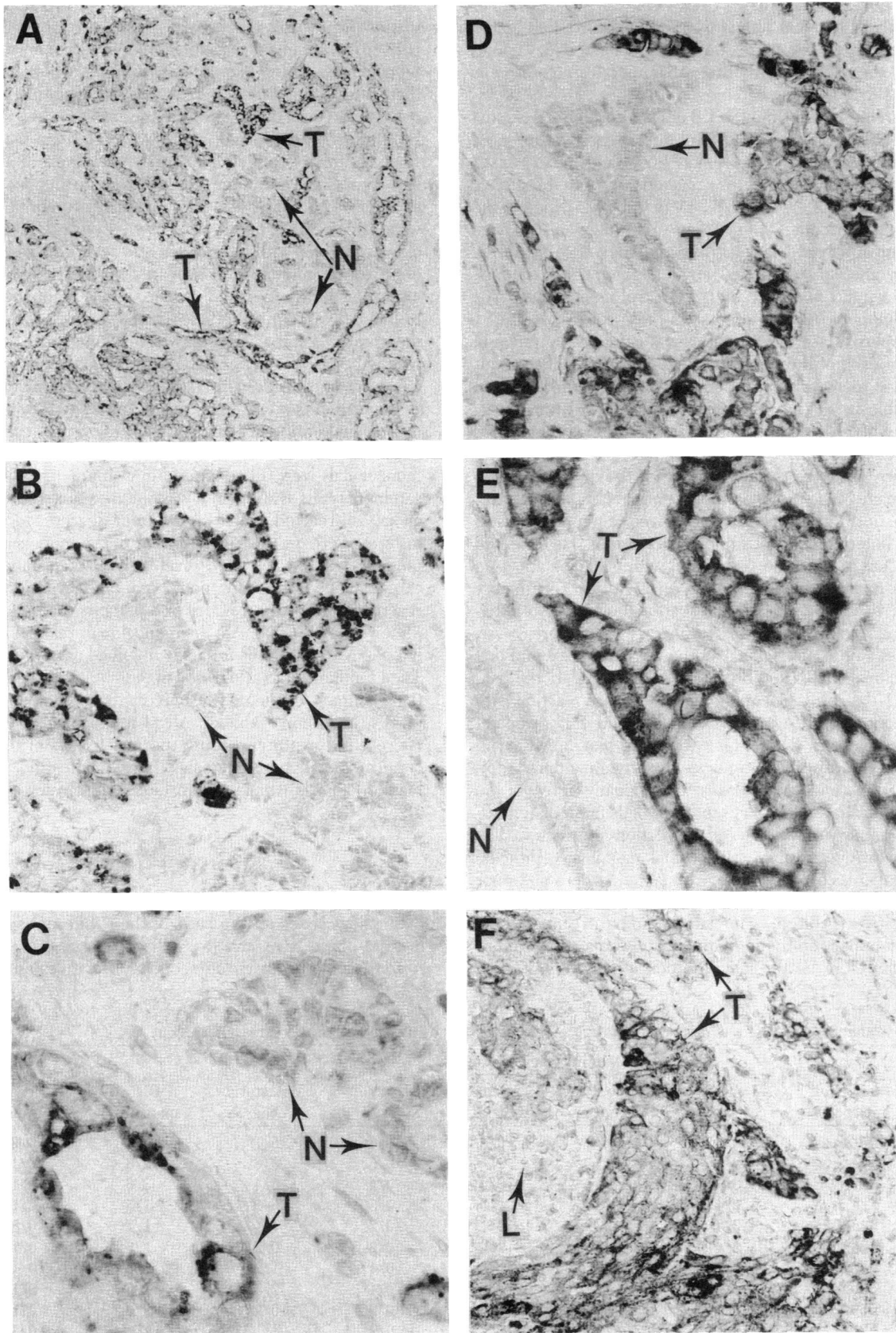


FIG. 2. Detection of binding of monoclonal antibodies to human mammary adenocarcinoma cells by using the immunoperoxidase technique. Tissue sections in A–E are from the same area of an infiltrating duct carcinoma. F shows an involved axillary lymph node of a patient with an infiltrating duct carcinoma. A, B, and C show tissues stained with monoclonal B50.4. D, E, and F show tissues stained with monoclonal B6.2. In A–E, note staining of mammary tumor cells (T) and absence of staining of apparently normal mammary epithelium (N) or stroma. Note the granular staining observed in A–C, as compared to the more diffuse staining in D and F. In F note the staining of mammary carcinoma cells (T) and the absence of staining of lymphocytes (L). (A, $\times 70$; B, $\times 290$; C, $\times 750$; D, $\times 290$; E, $\times 470$; F, $\times 290$.)

among monoclonals with respect to both the population of cells stained and the staining pattern (Fig. 2).

Whereas none of the monoclonal antibodies described here were reactive with (i) 11 apparently normal cell lines, (ii) sarcoma and melanoma cell lines, and (iii) tissue sections of more than a dozen types of apparently normal human organs, one can never rule out the possibility that any given monoclonal antibody will demonstrate either partial or complete crossreactivity with a particular cell type derived from a given adult or fetal tissue. The "pancarcinoma" activity of antibody B38.1 and some of the other monoclonals described requires further investigation.

Three points should be made concerning the immunoperoxidase studies described here: (i) different monoclonals react with different staining patterns of alternate tissue sections of the same tumor; (ii) monoclonals differed in the range of mammary tumors that showed reactivity; and (iii) a heterogeneity of staining of tumor cells was observed in many tumor masses. This heterogeneity has also been observed in staining of primary and metastatic murine mammary tumors (14) and in human mammary tumor tissues stained with human monoclonal antibody (15).

Because the live cell assays have demonstrated that at least nine of the monoclonals described here bind to the surface of human carcinoma cells preferentially, their potential clinical application for the immunodetection of primary and metastatic carcinoma lesions certainly merits consideration. It will also be of interest to determine if the presence or absence of any one or combination of the antigenic determinants recognized by the monoclonal antibodies described, on primary mammary tumor lesions or on involved nodes removed at mastectomy, are of any prognostic significance—i.e., do they reflect the degree of differentiation or malignancy of a given carcinoma cell population?

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