Proc. Natl. Acad. Sci. USA
Vol. 76, No. 3, pp. 1438–1442, March 1979
Immunology

Colorectal carcinoma-specific antigen: Detection by means of monoclonal antibodies

(human cancer/hybridoma/anticolorectal antibodies)

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ABSTRACT **Fusion of P3 × 63 Ag8 mouse myeloma cells with splenocytes obtained from mice immunized with cells derived from human colorectal carcinomas resulted in the production of antibody-secreting hybridomas. Two hybridomas (1083-17 and 1116-56) and their clones secreted antibodies binding specifically to human colorectal carcinoma cells either grown in culture or obtained from patients, but did not bind to normal colonic mucosa or other normal and malignant human cells. The binding specificity was consistent in three assays: radioimmunoassay, mixed hemadsorption, and immunofluorescence. Adsorption of these antibodies to colorectal carcinoma cell lines totally eliminated their specific binding.**

Despite a large number of reports, there is no evidence available that tumor-specific antigens are present on cells of human colorectal carcinoma (1, 2). For instance, the carcinoembryonic antigen(s) present on colorectal carcinoma are also expressed by other human tumors and by normal tissues (1). In chemically induced colon carcinomas of the rat, on the other hand, there is evidence of common tumor-specific antigens (3–5).

In a previous publication (6), we reported the presence on human melanoma cells of tumor-specific antigens detected by monoclonal antibodies. The present paper extends these observations to antigen(s) expressed by human colorectal carcinomas either maintained in tissue culture or obtained directly from patients.

MATERIALS AND METHODS

Human Cell Lines. Human colorectal carcinoma cell lines SW 403, SW 480, SW 620, SW 707, SW 837, SW 948, Sw 1083, SW 1116, and SW 1222; human melanoma cell lines SW 690, SW 691, SW 1614, SW 1687, and SW 1694; and human astrocytoma cell lines SW 1088 and SW 1783 were kindly supplied by A. Leibovitz (7, 8). Other human cell lines included myeloma Sed (9), lymphoid line P3 HR-1 (10), and fibrosarcoma HT 1080, the last of which was obtained from C. M. Croce. Melanoma cell lines WM8 and WM9 and astrocytoma cell line ASTRO (3) MsBa (tumor obtained from J. T. Robertson, University of Tennessee Medical School, Memphis, TN) were established in our laboratory. Normal human cell lines were fetal fibroblasts Flow 4000 (kidney) and MRC-5 (lung) from Flow Laboratories (Rockville, MD) and WI38, Hff, and normal skin fibroblasts (6).

Human Cells Freshly Isolated from Patients. Histologically documented colorectal carcinomas with adjacent normal colonic mucosa were obtained during surgery for the treatment of patients (from E. Catalano, Hahnemann School of Medicine, Philadelphia, PA). Portions of tumor and normal colon tissue were extensively washed in Eagle's minimal essential medium. The samples were then freed of connective tissue and remaining necrotic areas and were processed as follows: Separate specimens of tumor and normal intestinal mucosa distal to the tumor were finely minced and washed vigorously with four changes of medium. After removal of all debris, small fragments of tissue were forced through a stainless steel mesh and resuspended in medium. The single cell suspension was then purified according to the method of Vose *et al.* (11). Briefly, the cell suspension was sedimented by centrifugation at 200 × *g* for 5 min and then purified on a discontinuous Ficoll/Isopaque gradient (50, 75, and 100%). The interphases and the bottom fraction were collected. The viability of purified cells, measured by dye exclusion, was in excess of 90%. The cells were washed with medium, adjusted to 1×10^7 cells/ml in radioimmunoassay (RIA) buffer (6), and used for testing.

Immune Spleen Cells. Five colorectal carcinoma cell lines were used for the immunization of mice (Table 1). BALB/c mice were immunized for a secondary response as described (6). For a primary response, BALB/c mice received a single intravenous injection of $1–2 \times 10^6$ colorectal carcinoma cells. Immunized mice were sacrificed 5–6 days later, and a spleen cell suspension was prepared as described (12).

Production of Hybridomas. The fusion of immune splenocytes with mouse myeloma P3 × 63 Ag8 cells was performed as described (12). Hybrids were selected according to established techniques in a medium containing hypoxanthine/aminopterin/thymidine (12, 13). Fused cells were seeded in wells of tissue culture plates (Linbro FB-16-24-Tc). Established hybridoma cultures were cloned by limiting dilutions and in soft agarose.

RIA. RIA was performed on live target cells at 5×10^5 cells per well, as described for melanoma cells (6).

Mixed Hemadsorption Assay (MHA). MHA was performed by the method of Espmark and Fagreus (14) as modified by Carey *et al.* (15). Antiserum against sheep erythrocytes was prepared in BALB/c mice. Goat anti-mouse IgG was purchased from Cappel Laboratories (Cochranville, PA). Tumor cells were prepared 1 day before the assay by the seeding of 10^3 cells per well (Falcon 30.40 microtest II plates). Cells were washed 20–24 hr later and incubated for 60 min at room temperature with dilutions of hybridoma supernatant. Unbound antibody was

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Abbreviations: RIA, radioimmunoassay; MHA, mixed hemadsorption; IF, immunofluorescence; ACRC, anti-colorectal carcinoma.

Table 1. Reactivity of hybridoma antibodies produced by fusion of P3 × 63 Ag8 cells with splenocytes from mice immunized with human colorectal carcinoma

Immunization of mice	Colorectal carcinoma cell lines used for immunization	Ratio of hybridomas showing reactivity*: With all human cells tested	With colorectal carcinoma cells only
Primary	SW 707	1/8	0/1
	SW 948	0/2	—
	SW 1083	8/24	1/8
	SW 1116	13/65	1/13
Secondary	SW 480	3/5	0/3

* Tested by RIA.

then washed out and 100 μl [0.2% (vol/vol) in phosphate-buffered saline] of the indicator system (sensitized sheep erythrocytes) was added. Plates were incubated for 60 min at room temperature and then agitated gently, washed, and examined by phase-contrast microscopy. Individual positive cells were scored in each well. A cell was considered to be binding antibody when 25% or more of its perimeter was covered by indicator cells. A well was scored negative if fewer than 2% of the cells bound indicator cells. Included in each experiment were control wells incubated with known positive and negative antibody. All samples were tested at least in duplicate.

Immunofluorescence (IF). Indirect IF was performed with dilutions of hybridoma-secreted antibodies and fluorescein isothiocyanate-tagged rabbit IgG directed against mouse IgG (Cappel Laboratories) at a 1:10 dilution.

Absorption Procedure. Dilutions of hybridoma antibodies that exhibited 50–70% maximal reactivity against the immunizing colorectal carcinoma cells were used. Diluted antibody was mixed with an equal volume of pelleted cells. Absorption was carried out on ice for 1 hr with frequent mixing. The absorbing cells were then removed by centrifugation and the supernatant was tested for binding to the appropriate target cells.

RESULTS

Characteristics of Hybridomas. In contrast to the results obtained with human melanomas (6), very few of the hybridomas secreted antibodies reactive with human cells. As shown in Table 1, of 104 hybridomas obtained by the fusion of splenocytes from immunized mice with cells of five colorectal carcinoma cell lines, only 25 showed binding in RIA to human cells. Of those, only two, 1083-17 and 1116-56, were found to be specific for colorectal carcinoma cells (see below). These specific hybridomas were obtained after primary immunization of the mice. Among hybridomas that secreted antibodies binding in RIA to all human cells, some, such as 480-1-4 or 480-4-12 (see Table 3), mediated complement-dependent lysis of all human target cells regardless of their origin.

Specific Immunoreactivity of Hybridoma Clones. Two or 3 weeks after fusion, when supernatants from established hybrid colonies showed binding to cells in RIA, the cultures were cloned and the progeny of individual clones were again assayed for reactivity of the secreted antibodies. Clones obtained from hybridomas 1083-17 and 1116-56 showed the same specificity for colorectal carcinoma cells as did the parental hybridoma cultures. As shown in Table 2, anti-colorectal carcinoma (ACRC) antibodies produced by clones 1083-17-1A and 1116-56-2 bound in RIA to cells of eight out of the nine colorectal carcinomas maintained in culture but did not bind to other human tumors of different origin or to normal human fibroblasts.

In general, ACRC antibodies produced by hybridoma clone 1083-17-1A showed binding to the target cells at higher dilutions than did those produced by hybridoma clone 1116-56-2 (Fig. 1). Cells of the colorectal carcinoma line SW 480 did not bind antibodies secreted by the two hybridomas and their clones. Negative results were also obtained with human melanoma cells and normal human fibroblasts at all dilutions tested.

Dilutions of ACRC antibodies secreted by clones 1083-17-1A and 1116-56-2 were tested for binding to SW 1083 and SW 1116 cells, respectively, in three different assays: RIA, MHA, and IF. As shown in Fig. 2, the results obtained in RIA and MHA were similar, whereas the dilution end point in IF was lower than those obtained in the other two assays.

When the two ACRC antibodies produced by clones 1083-17-1A and 1116-56-2 were tested in the three assays for binding to the other seven colorectal carcinomas, melanomas, and normal human fibroblasts (Table 3), they reacted only with colorectal carcinoma cells, with the exception of SW 480. For control purposes, melanoma cells and normal fibroblasts were also tested in the three assays for binding of anti-human antibodies secreted by two hybridoma clones, 480-1-4 and 480-4-12. The clones were shown to bind in RIA to all human cells tested.

Absorption of ACRC Antibodies. ACRC antibodies secreted by hybridoma cultures 1083-17 and 1116-56 lost their capacity to bind to the immunizing tumor cells (SW 1083 and SW 1116, respectively) when absorbed with any of the four colorectal carcinomas (Table 4). In contrast, none of the cells of human

Table 2. Reactivity of ACRC antibodies produced by clones of two hybridomas

Antibody secreted by clone	Ratio of human cell cultures* binding ACRC antibodies†							
	Colorectal carcinoma	Melanoma	Fibrosarcoma	Astrocytoma	Myeloma	Burkitt lymphoma	Buffy coat cells	Normal embryonic fibroblasts
1083-17-1A	8/9	0/7	0/1	0/2	0/1	0/1	0/4	0/4
1116-56-2	8/9	0/7	0/1	0/2	0/1	0/1	0/4	0/4

* Except for buffy coat cells, which were tested directly after collection.
† Tested by RIA.

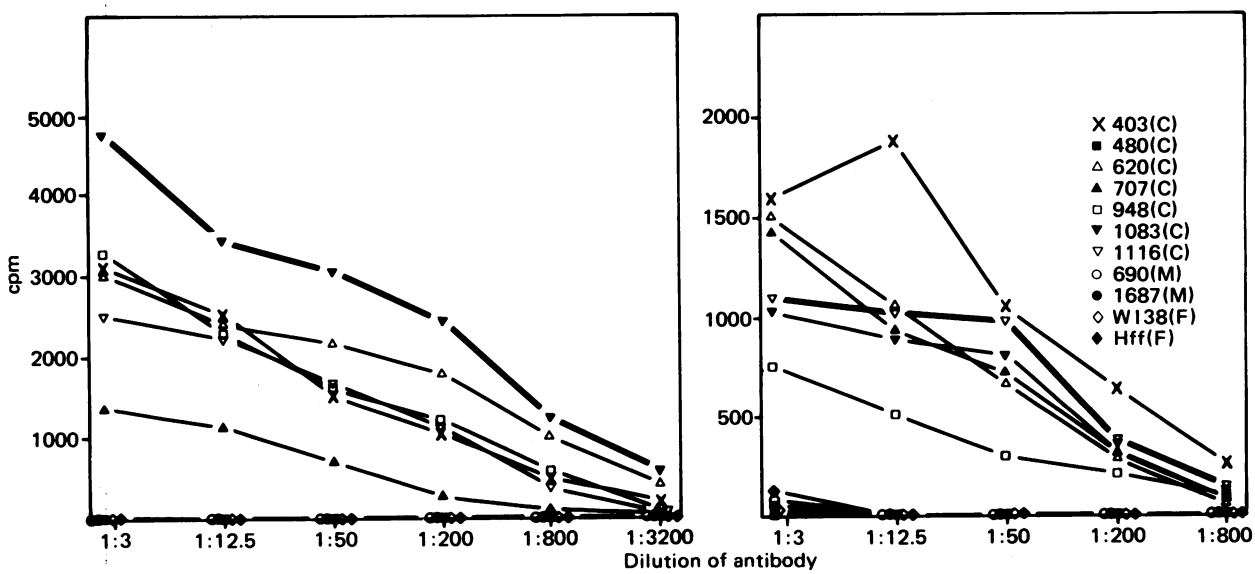

FIG. 1. Dilutions of ACRC antibodies produced by hybridomas 1083-17-1A (*Left*) and 1116-56-2 (*Right*) were tested by RIA for binding to human tumor or normal fibroblast cell lines. C, colorectal carcinoma; M, melanoma; F, normal human fibroblasts.

tumors other than colorectal carcinoma and none of the normal human cells (Table 4) absorbed binding activities from the ACRC antibody-secreting cultures. In addition, sheep erythrocytes, mouse IT-22 cells, and bacillus Clmette–Guérin did not reduce the binding capacity of the ACRC antibodies.

Binding of ACRC to Fresh Tumor Tissue. Colon carcinoma tissue and fragments of adjacent normal intestine were obtained from four patients operated on for the excision of a tumor. Only in two cases (WC 5155 and WC 5133) was it possible to secure both tumor and normal tissue from the same patient in a quantity sufficient for binding assays with dilutions of antibodies. From the third patient (WC ME20), only tumor cells were tested for the binding of antibodies diluted 1:2; from the fourth patient (WC LA21), because of the scarcity of viable tumor cells, only cells of normal intestine were assayed for binding. The results shown in Fig. 3 indicate that cells from the three colorectal carcinomas (confirmed by histological examination) but not cells from the normal intestinal tract bound hybridoma antibodies 1083-17. Antibodies of hybridoma 1116-56 were bound to cells of only two of the three colorectal carcinomas but did not react with normal cells.

DISCUSSION

The fusion of mouse myeloma cells with splenocytes from mice immunized with human colorectal carcinoma cells resulted in the production of a random assortment of hybridomas, many of which secreted antibodies that reacted with human cells regardless of their origin. Two hybridomas, obtained after primary immunization of mice, secreted antibodies that reacted with cells of eight out of nine human colorectal carcinomas but did not bind detectably to cells of other tumors or of normal colonic mucosa cells. These results differ from those obtained after the immunization of mice with human melanoma; in this case, only after secondary immunization were hybridomas produced that reacted specifically with human melanoma cells (6).

Differences in the extent of binding of the ACRC antibodies

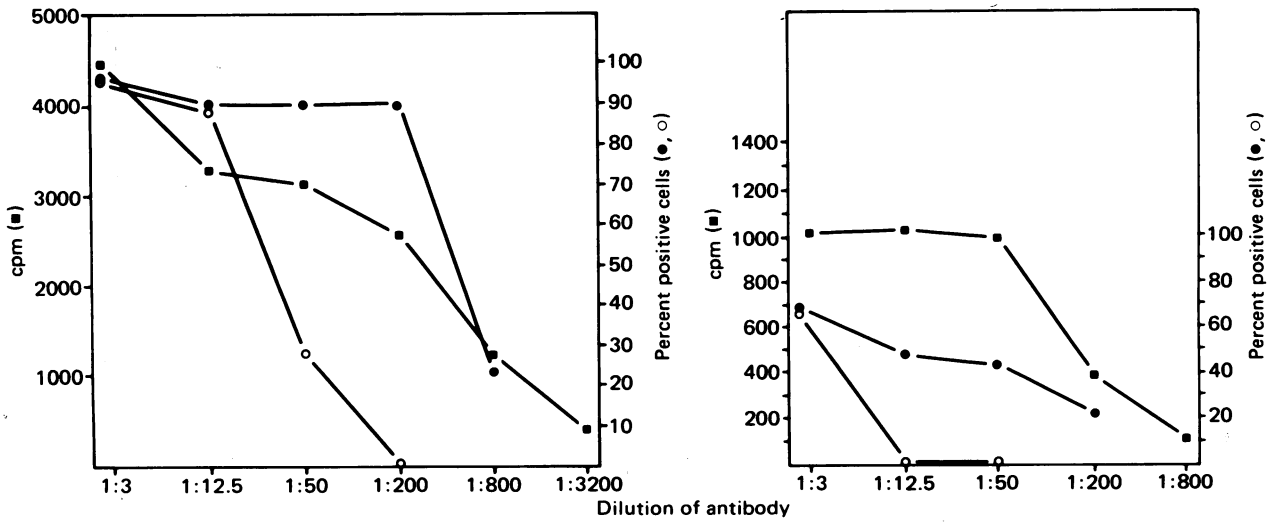

FIG. 2. Dilutions of ACRC antibodies produced by hybridoma clones 1083-17-1A (*Left*) and 1116-56-2 (*Right*) were tested, respectively, for binding to colorectal carcinoma cells SW 1083 (*Left*) and SW 1116 (*Right*) in three assays: ■, RIA; ●, MHA; ○, IF.

Table 3. Reactivity in three different assays of monoclonal ACRC antibodies against human cells

	Ratio of cultures binding antibodies by hybridoma:							
	1083-17-1A			1116-56-2			480-1-4	480-4-12
Target cell type	RIA	MHA	IF	RIA	MHA	IF	RIA	RIA
Colorectal carcinoma	8/9	7/8	4/5	8/9	7/8	3/5	6/6	6/6
Melanoma	0/8	0/8	0/2	0/8	0/8	0/2	5/5	6/6
Normal fibroblasts	0/4	0/4	0/2	0/4	0/4	0/2	3/3	3/3

in the RIA (expressed in cpm) may, as in the case of antimelanoma antibodies, reflect different affinities for the antigenic determinants, variations in the total number of determinants on the target cells, or differences in the distribution of the determinants. The presence of identical or crossreactive epitopes seems to be evident in all but one of nine tumor cell lines as determined by RIA. This was confirmed in two other assays (Table 3) and by the fact that absorption of the monoclonal antibodies by four different colorectal carcinomas totally eliminated the specific binding of ACRC antibodies (Table 4).

Identical or crossreactive antigenic determinants were detected on all three freshly isolated colon carcinomas by ACRC antibodies secreted by hybridoma 1083-17 and on two of the three by ACRC antibodies of hybridoma 1116-56. These results indicate that antigenic determinants recognized by monoclonal antibodies on tumor cells in culture are also recognized on tumor cells freshly isolated from patients. In the case of monoclonal antibodies against melanoma, we also found crossreactivity between the antigenic determinants detected on cells in culture and those found in 10 tumor cells isolated from patients (16).

The ACRC antibodies do not react with carcinoembryonic antigen (A. Leibovitz, personal communication) secreted by cells of colorectal carcinoma. Whether the ACRC antibodies are directed against a specific tumor antigen or against other antigenic determinants associated with colorectal carcinoma in man is difficult to say at present. Additional studies may elucidate this point. If this question is answered, the ACRC monoclonal antibodies may be of value in the classification of colorectal carcinomas by their antigenic determinants and may be applicable to immunodiagnosis and eventually to immunotherapy of one of the most common among the malignant tumors in man.

We thank members of the Wistar Institute staff, Dr. Walter Gerhard, Dr. Kenneth Mitchell, and Dr. Giovanni Rovera, for advice and help in the preparation of this manuscript. We also thank L. Vitek, I. Weiss-Wik, L. Augustine, and A. Safier for their expert technical assistance. This study was supported in part by U.S. Public Health Service Research Grants CA-10815 and CA-21124 from the National Cancer Institute and RR-05540 from the Division of Research Resources. This study was not funded by the National Large Bowel Cancer Project.

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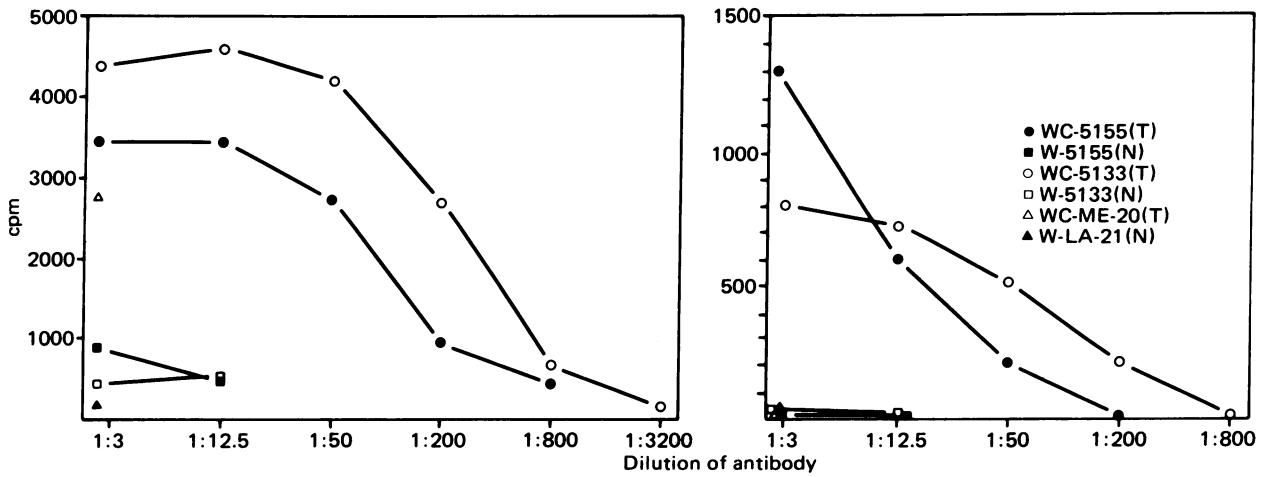

FIG. 3. Dilutions of ACRC antibodies secreted by hybridomas 1083-17 (*Left*) and 1116-56 (*Right*) tested by RIA for binding to cells of colon cancer (T) or cells of normal colonic mucosa (N) obtained from patients during surgery.

Table 4. Absorption of ACRC antibodies secreted by hybridoma cultures 1083-17 and 1116-56

Cells that absorbed ACRC antibodies	Cells unable to absorb ACRC antibodies		
	Human		
	Tumor cells	Normal cells	Other
Human colorectal carcinoma cells SW 403 SW 948 SW 1083 SW 1116	Melanoma SW 690 SW 691	Cultured fibroblasts Flow 4000 MRC5	Mouse fibroblasts IT-22
	Fibrosarcoma HT 1080	Suspension of brain cells	Sheep erythrocytes
	Astrocytoma SW 1088 SW 1783 Astro (3) MsBa	Erythrocytes of blood groups O, A, and B	BCG* microorganisms
	Leukemic spleen cells		

* Bacillus Calmette–Guérin.

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