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Immunology

Anti-idiotype immunization of cancer patients: Modulation of the immune response

(anti-idiotypic antibodies/anti-tumor antibodies)

DOROTHEE HERLYN*†, MARTINE WETTENDORFF*, EKKEHARD SCHMOLL‡, DIMITRIOS ILIOPOULOS*, INGOLF SCHEDEL‡, URSULA DREIKHAUSEN‡, RUDOLPH RAAB‡, ALONZO H. ROSS*, HERBERT JAKSCHE§, MARIANNE SCRIBA§, AND HILARY KOPROWSKI*

*The Wistar Institute of Anatomy and Biology, 36th Street at Spruce, Philadelphia, PA 19104-4268; ‡Medizinische Hochschule, Hannover, Federal Republic of Germany; and §Sandoz Forschungsinstitut, Vienna, Austria

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ABSTRACT **Thirty patients with advanced colorectal carcinoma (CRC) were treated with alum-precipitated polyclonal goat anti-idiotypic antibodies (Ab2) to monoclonal anti-CRC antibody CO17-1A (Ab1) in doses between 0.5 and 4 mg per injection. All patients developed anti-anti-idiotypic antibodies (Ab3) with binding specificities on the surfaces of cultured tumor cells similar to the specificity of Ab1. Furthermore, the Ab3 competed with Ab1 for binding to CRC cells. Fractions of Ab3-containing sera obtained after elution of the serum immunoglobulins from CRC cells bound to purified tumor antigen and inhibited binding of Ab2 to Ab1. The Ab3, therefore, may share idiotopes with Ab1. Six patients showed partial clinical remission and seven patients showed arrest of metastases following immunotherapy. Four of the thirteen patients with measurable clinical responses had received Ab2 alone, whereas 9 patients had also received chemotherapy.**

We have previously suggested (1–3) the possible beneficial role of anti-idiotypic antibodies (Ab2) induced in patients with gastrointestinal tract cancer who improved clinically following treatment with murine monoclonal antibody (mAb) CO17-1A (Ab1) against colorectal carcinoma (CRC). Peripheral blood lymphocytes from these patients responded to *in vitro* stimulation with purified Ab2 by producing human antibodies that exhibit binding specificities similar to those of the administered mAb (4). Direct evidence for a beneficial effect of Ab2 in cancer patients would be the induction of anti-anti-idiotypic antibodies (Ab3) that bind to tumors in patients immunized with Ab2 and the demonstration that development of Ab3 correlates with clinical improvement of the disease.

We have previously described (1, 2, 5) goat polyclonal Ab2 to two different anti-CRC mAbs, Ab1 CO17-1A and Ab1 GA733. The two Ab1 have shown immunotherapeutic efficacy in preclinical and clinical trials (6–9). Each Ab2 functionally mimicked *in vitro* the tumor antigen defined by the corresponding Ab1 (1, 2, 5) and induced antigen-specific Ab3 after immunization across species barriers (5, 19).

In the present study, goat Ab2 to Ab1 CO17-1A was administered to patients with advanced CRC. We describe here the immune responses of the patients to the administered Ab2 and preliminary clinical observations.

MATERIALS AND METHODS

Selection and Treatment of Patients. Thirty CRC patients with advanced CRC (Dukes D stage) were enrolled in this phase of the trial. All patients had their primary tumor operatively removed. At the time the trial started, all had progressively growing liver metastases >2.0 cm in diameter, as revealed by computerized tomography. Fifteen of the 30 patients had undergone some form of unsuccessful chemotherapy, which was terminated at least 4 weeks before the Ab2 immunization. One of the 15 patients also received a course of radiotherapy ending 7 months before Ab2 treatment. Fourteen patients received no treatment between surgery and Ab2 administration. Alum-precipitated goat Ab2 to Ab1 CO17-1A (1, 10) was injected intradermally at weeks 0, 1, 2, and 5 in doses between 0.5 and 4 mg per injection. Between 4 and 8 patients were included in each dosage group. Eleven patients who did not respond clinically to the first Ab2 treatment course received a second course of Ab2 therapy consisting in the various patients of one to five injections of Ab2 given between 1.5 and 11 months after the first course of Ab2 therapy. Nine of the 11 patients received chemotherapy (5-fluorouracil and folic acid) simultaneously with the second course of immunotherapy.

Serum samples were obtained before therapy, two to four times during therapy, and every 2–5 weeks after the end of therapy for periods of up to 258 days. Because of possible interference of chemotherapy with the development of immunity after the second immunotherapy course, only serum samples obtained before the second course of immunotherapy were included in immunological studies. Clinical responses were categorized as: complete response (disappearance of metastases for >4 weeks); partial response [detectable decrease (>20%) in tumor metastases for >4 weeks)]; stable disease (no change in tumor size for >4 weeks); and progressive growth of the tumor. All clinical responses were objectively evaluated by sonography, computerized tomography, and conventional nuclear-radiographic methods; in addition, serial determination in serum of the tumor markers α-fetoprotein, carcinoembryonic antigen, and CA 19-9 antigen were performed using commercially available kits (Abbots, Centocor Kits).

Tumor Cells. All of the tumor cell lines shown in Fig. 1 have been described (11, 12).

CO17-1A Antigen. The antigen defined by mAb CO17-1A was isolated by immunoaffinity chromatography using mAb GA733, which binds to this antigen with significantly higher avidity than mAb CO17-1A, as described (13, 14), except that detergent was omitted from the elution buffer.

Antibodies. mAb CO17-1A and CO-29 to human CRC have been described (11, 15, 20). Ab2 to Ab1 CO17-1A were purified and precipitated with alum as described (1, 10). The Ab2 have been extensively characterized *in vitro* (1, 2) and *in vivo* in experimental animals (19).

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Abbreviations: Ab1, antibody(ies) to epitope; Ab2, anti-idiotypic antibody(ies); Ab3, anti-anti-idiotypic antibody(ies); CRC, colorectal carcinoma; mAb, monoclonal antibody(ies); MHA, mixed hemadsorption assay; SRBC, sheep erythrocyte(s).

†To whom reprint requests should be addressed.

Stimulation of Peripheral Blood Mononuclear Cells. Peripheral blood mononuclear cells were stimulated with either goat Ab2 or normal goat IgG (1–100 ng/ml) in a modified Mishell–Dutton culture as described in detail by us previously (1, 4).

Binding and Binding Inhibition Assays with Antibodies in Patients' Sera. Binding of human antibodies present in unfractionated pre- and posttherapy sera of patients to various tumor cells in culture was determined in mixed hemadsorption assay (MHA) using sheep erythrocytes (SRBC) coupled with $CrCl_3$ to rabbit anti-human IgG as indicator cells (16).

Binding of human antibodies to either CO17-1A antigen or Ab2 was tested in enzyme-linked immunosorbent assay (ELISA).

For detection of antibody binding to CO17-1A antigen, anti-CRC antibodies were isolated from patients' pre- and posttherapy sera by adsorption of 9 ml of sera diluted 1:3 in phosphate-buffered saline [PBS, g/liter: NaCl, 8.0; KCl, 0.20; KH_2PO_4, 0.20; Na_2HPO_4, 1.15; $CaCl_2$ (anhydrous), 0.10; $MgCl_2 \cdot 6H_2O$, 0.10] to glutaraldehyde-fixed CRC cells ($\approx 2 \times 10^7$ cells); this was followed by elution of bound antibody as described in detail elsewhere (5). Since eluates from posttherapy sera contained not only antibodies binding to goat Ab2 but also antibodies binding to normal goat IgG that had been bound nonspecifically to CRC cells, all eluates were preincubated with 300 μg of normal goat IgG per ml before their use in binding assays. Serum eluates were added to wells coated with CO17-1A antigen (10 μg/ml in 0.01 M bicarbonate buffer, pH 9.6). Wells were blocked with 1% bovine serum albumin in PBS, and antibody binding was detected with peroxidase-labeled goat anti-human IgG. Optical densities (OD) of the reaction mixtures were determined at 405 nm. A similar assay was used for detection of binding of eluates to Ab2 except that wells were coated with Ab2 instead of CO17-1A antigen.

For detection of binding of human serum antibodies to the administered Ab2, wells of microtiter plates were coated with goat anti-human IgG (10 μg/ml in 0.01 M bicarbonate buffer, pH 9.6). Sera preincubated with normal goat IgG, at a final concentration of 300 μg/ml to block human antibody binding to constant region determinants on the Ab2, were added to the wells. Biotinylated Ab2 (1 μg/ml) was then added to each well; this was followed by the addition of alkaline phosphatase-labeled avidin and substrate.

Inhibition of binding of Ab2 to Ab1 by human antibody was determined by preincubation of biotinylated Ab2 (2 μg/ml) with various concentrations of human sera or eluates for 30 min at 37°C before adding the mixtures to Ab1-coated wells (0.3 μg of Ab1 CO17-1A per ml in 0.01 M bicarbonate buffer, pH 9.6). Sera and eluates had been preincubated with normal goat IgG at 300 μg/ml (to minimize steric hindrance of the combining region of Ab2 by anti-isotypic and/or anti-allotypic antibodies present in the sera and eluates) before they were added to biotinylated Ab2.

The following control preparations were used in all ELISAs: patients' sera obtained before Ab2 therapy, sera from healthy individuals, eluates obtained from sera of patients before Ab2 therapy or from sera of healthy individuals.

Binding of Murine Antibody to Tumor Cells. Binding of Ab1 CO17-1A to tumor cells was tested in MHA using SRBC coated with mouse anti-SRBC antibody to which rabbit anti-mouse IgG had been bound (11).

RESULTS

Clinical Observation of the Patients.§ None of the 30 patients showed any systemic adverse effects after Ab2 immunization, except for 2 patients who showed short-lasting (<24 hr) febrile reactions after repeated injections of 4 mg of Ab2. Most of the patients developed local erythema surrounding the site of intradermal injection of Ab2. This reaction was more pronounced in the group of patients that had received larger (2 or 4 mg) doses of Ab2 and increased with the number of injections. Table 1 summarizes the clinical responses of the patients. Of 30 patients, 6 showed partial remission, and 7 patients showed arrest of previously progressing metastases; tumor masses increased in 17 patients. Five of the 6 patients with partial responses had received booster injections of Ab2 (1.5–11 months after the first course of immunization) in combination with chemotherapy. Of the 7 patients who showed stabilization of growth of metastases, 6 had received booster injections of Ab2 (3–9 months after the first immunization course), which were combined with chemotherapy in 4 patients.

The small number of patients in each dosage group prevents conclusions as to the relation between various Ab2 doses and clinical effects.

Presence of Ab3 in Patients' Sera. All of the 30 patients responded to Ab2 immunization with production of specific Ab3 binding to Ab2 in ELISA following adsorption to normal goat IgG. Average reciprocal antibody end-point dilutions ranged between ≈116 and ≈441 in the five groups of patients. However, there seemed to be no correlation between titer of Ab3 and dose of Ab2 administered. Ab3 comprised between 56% and 79% of the total anti-goat immunoglobulin antibody in various patients. None of the patients' sera obtained before Ab2 therapy showed binding to Ab2 even at serum dilutions as low as 1:30 (results not shown).

Binding of Patients' Antibodies to Tumor Cells. Since human antibodies to constant region determinants on goat IgG bound nonspecifically to CRC cells (see *Materials and Methods*), it was important to exclude participation of these antibodies in MHA with tumor cell targets. Therefore, various sera were tested for binding reactivities to CRC targets in MHA in the presence of normal goat IgG at 300 μg/ml, which efficiently bound human antibodies to goat IgG. Binding of human sera to CRC cells, however, was not affected by the presence of normal goat IgG (not shown).

In Table 2, 23 patients are grouped according to their binding reactivities to CRC cells as determined in MHA. Group 1 comprises 8 patients with no significant (<15% of cells reactive) reactivities in their pretherapy sera as compared to healthy individuals (≈5% of cells reactive) but high reactivities (>40% of cells reactive) in sera obtained after therapy. Patients of group 2 (n = 13) showed low but

Table 1. Clinical responses of colon carcinoma patients immunized with Ab2 against mAb CO17-1A

Ab2 dose per injection,* mg	Patient nos.	Total no. of patients	No. of patients showing clinical responses		
			PR	SD	Prog.
0.5	1–7, Br	8	1	1	6
1.0	8–12	5	1	1	3
1.5	13–18	6	4	2	0
2.0	19–22	4	0	0	4
4.0	26–29	7	0	3	4
	31–33				

PR = partial response; SD = stable disease; Prog. = progressive growth of tumor.

*A total of four injections was given at 0, 1, 2, and 5 weeks (first course of immunotherapy). Eleven patients (dose groups: 0.5 mg, 2 patients; 1 mg, 2 patients; 1.5 mg, 5 patients; 4 mg, 2 patients) received booster injections of Ab2 between 1.5 and 11 months after the first course. In 9 of the 11 patients, this was combined with chemotherapy.

§Detailed description of clinical responses of the patients will be published elsewhere.

Table 2. Summary of binding reactivities to CRC cells in sera of cancer patients immunized with Ab2 to mAb CO17-1A

Patient's group no.	No. of patients per group	Binding reactivities to CRC cells in sera obtained before and after Ab2 therapy	
		Before Ab2	After Ab2
1	8	Negative	Positive (high)
2	13	Positive (low)	Positive (high)
3	2	Positive (low)	Positive (low)

CRC cells SW948 or SW1116 adherent to wells of microtiter plates were incubated with various serum dilutions (1:5–1:45); this was followed by the addition of SRBC to which rabbit anti-human IgG had been coupled with $CrCl_3$. Patients' sera were scored positive if the percentage of CRC cells rosetted by SRBC was at least three times the value obtained with sera from healthy individuals, which bound ≈5% of the cells. Low positive = 15–40% of the cells reactive; high positive = >40% of the cells reactive.

significant (between 15% and 40% of cells reactive) reactivities in their pretherapy sera, which increased considerably (>40% of cells reactive) after Ab2 therapy. Antibody reactivities in sera of patients of group 3 (n = 2) were low (<15% of cells reactive), both in sera obtained before and after therapy. These reactivity patterns were independent of the dose of Ab2 administered (not shown).

Titers of anti-CRC antibodies in sera obtained after treatment ranged between reciprocal serum end dilutions of ≈45 and ≈405 in most patients (with one exception), independent of the Ab2 dose administered. One patient (no. 17) had a serum titer of ≈1215. This patient showed partial remission (≈75% decrease in tumor masses) lasting for >10 months following one course of immunotherapy with Ab2. The patient's peripheral blood mononuclear cells responded to *in vitro* stimulation by Ab2 with the production of human antibodies binding to CRC cells in MHA. No such antibody reactivities were found in supernatants of the patient's blood cells stimulated with normal goat serum (not shown). The duration of circulation of measurable amounts of anti-CRC antibody in sera varied considerably among patients, ranging between 6 and >258 days (not shown).

To investigate the specificity of the binding reaction of patients' sera to tumor cells, sera obtained from six patients after treatment with Ab2 were tested in MHA for binding to cultured cells of colorectal, pancreatic, or prostate cancer or melanoma. As shown in Fig. 1, serum from each patient showed high reactivity with cells of gastrointestinal cancer but not with cells of either prostate cancer or melanoma. Thus, the Ab3 bound to various tumor cells with specificities similar to that of Ab1.

To determine whether human Ab3 compete with Ab1 CO17-1A for binding to CRC cells, a competition MHA was performed. SW1116 CRC cells were first incubated with human sera diluted 1:2 in PBS; this was followed 1½ hr later by addition of a suboptimal concentration (1 μg/ml) of Ab1 CO17-1A, showing ≈30% of maximum binding of the mAb to CRC cells. Cell binding of the mAb was detected by SRBC coated with mouse anti-SRBC antibody to which rabbit anti-mouse IgG had been bound. Posttherapy sera from 5 of 14 patients completely inhibited binding of mAb CO17-1A to CRC cells. Posttreatment sera from 5 other patients partially (≈60%) inhibited this reaction and 4 sera displayed no inhibitory capacity. In contrast, none of the pretherapy sera derived from 7 of the 14 patients or normal sera from 3 healthy individuals inhibited binding of mAb CO17-1A to CRC cells and binding of control mAb CO-29 was not inhibited by any of the posttherapy sera. These results were confirmed with eluates derived from posttherapy sera of 9 patients (results not shown).

Patients' Antibody Binding to Isolated Tumor Antigen and Idiotope Analysis of Antibodies. Fractions of patients' sera eluted from CRC cells were assayed for binding to purified CO17-1A antigen in ELISA. Posttreatment eluates of nine patients and pretreatment eluates of four of these patients were used in the test (Fig. 2). For comparison, an eluate obtained with serum of a healthy individual was also included. As shown in Fig. 2*B*, all but one (no. 11) of the nine eluates obtained from posttreatment sera significantly bound (at least three times the binding of a healthy individual's serum eluate)

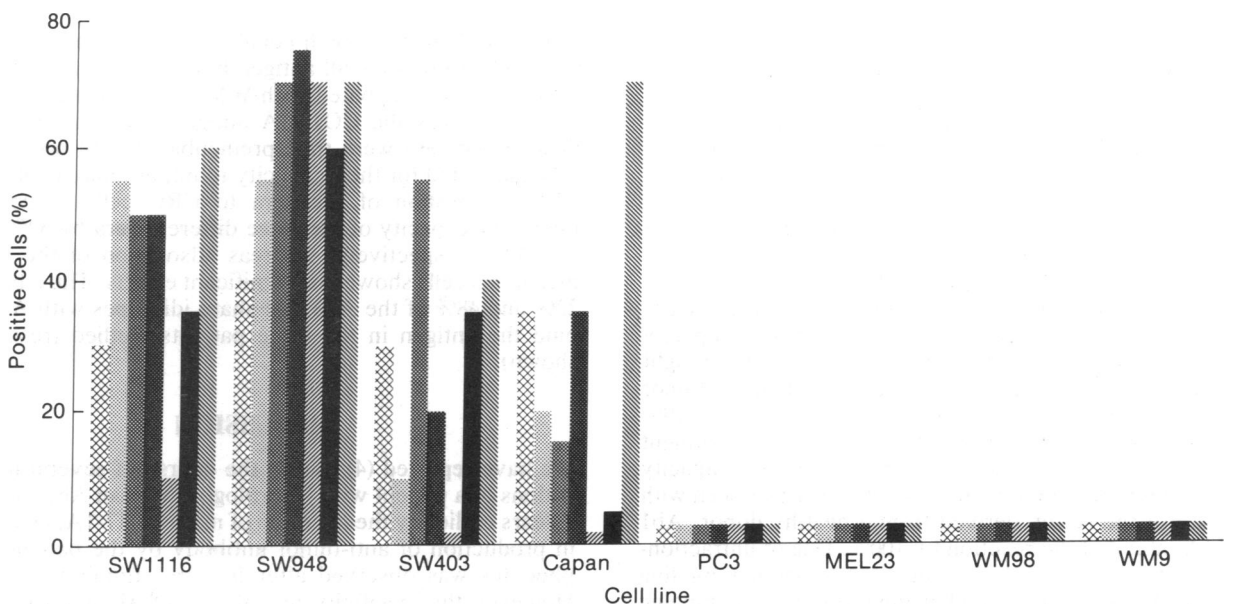

FIG. 1. Binding reactivities of patients' sera to tumor cells of various tissue origins in MHA. The MHA with human sera obtained from the various patients [first six columns (from the left) of each set; patient nos. 7, 5, 4, 6, 22, and 16] between 1 and 7 weeks after the end of Ab2 therapy was performed as described in the legend to Table 1. Binding of murine Ab1 CO17-1A (right-hand column of each set) was tested in MHA using SRBC coated with mouse anti-SRBC antibody to which rabbit anti-mouse IgG had been bound. Target cells used included SW1116, SW948, and SW403 CRC cells; Capan pancreatic carcinoma cells; PC3 prostate carcinoma cells; MEL23, WM98, and WM9 melanoma cells. All serum binding values are derived from serum dilutions between 1:15 and 1:45 and are corrected for values obtained with pretherapy sera.

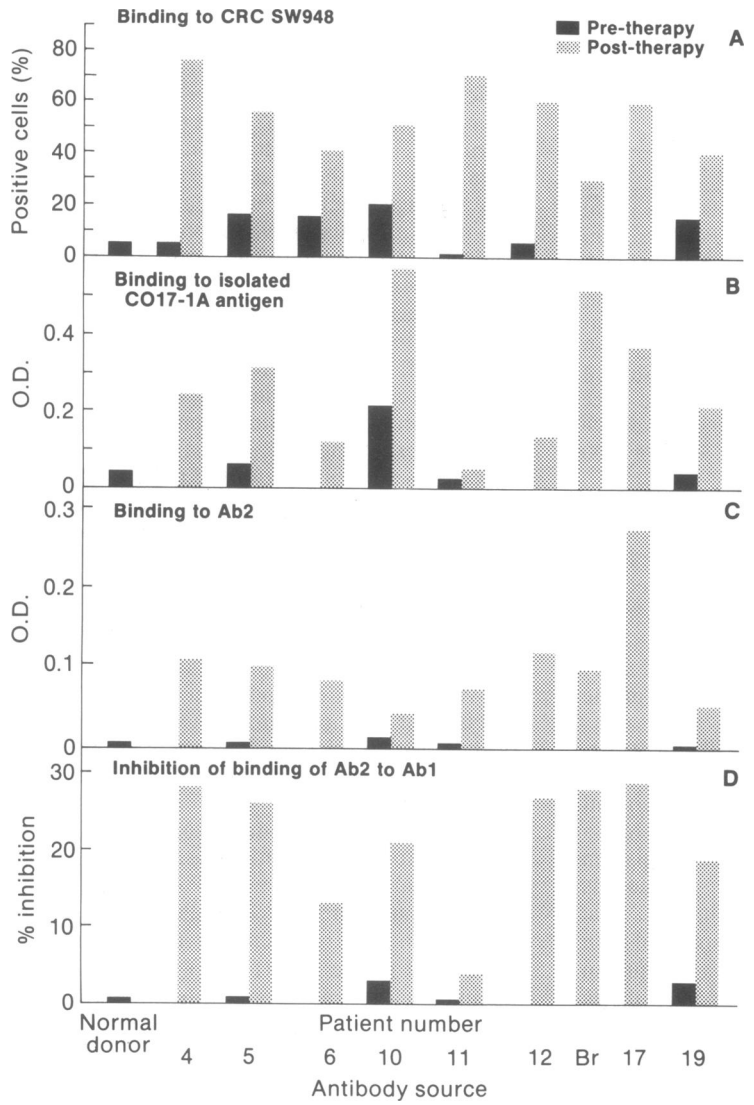

FIG. 2. Antigen-binding reactivities and idiotope analysis of human Ab3. (*A*) Binding reactivities of human sera (diluted 1:5) to SW948 CRC cells in MHA. (*B* and *C*) Binding of human antibodies, present in undiluted eluates obtained after adsorption of sera to SW948 CRC cells followed by elution of bound antibody, to isolated CO17-1A tumor antigen (*B*) or Ab2 (*C*). (*D*) Inhibition of binding of Ab1 to Ab2 by Ab3 present in the eluates. All determinations were done in ELISA and repeated at least once. Standard errors of duplicate determinations were usually <5%.

to isolated tumor antigen. Thus, in eight of nine patients, binding of eluates to isolated tumor antigen correlated with the reactivities of the corresponding sera screened for binding to CRC cells in MHA (Fig. 2 *A* and *B*). One of the four eluates (no. 10) derived from patients' pretherapy sera was found to bind to tumor antigen (Fig. 2*B*); the corresponding serum also bound to CRC cells (Fig. 2*A*). This serum therefore may contain human Ab1. All of the eluates that bound to antigen also bound significantly (more than three times the binding of eluates from sera of healthy donors) to Ab2 (Fig. 2*C*).

To test whether the antigen-binding Ab3 might share idiotopes with Ab1, all eluates were tested for their capacity to inhibit binding of Ab2 to Ab1 (Fig. 2*D*). All of the eight eluates obtained from posttherapy sera and binding tumor antigen as well as Ab2 inhibited this reaction (13–29% inhibition), whereas the eluates obtained from pretreatment sera of patients showed no or very low inhibitory capacity (0–3.4% inhibition). No inhibition whatsoever was seen with the eluate of the serum derived from a healthy donor. Ab1 CO17-1A inhibited the reaction by 100%. Use of unfractionated patients' sera instead of serum eluates to inhibit binding of Ab2 to Ab1 gave much higher inhibition values, ranging from 23% to 61% (mean, ≈43%) in the various sera. By comparison, the inhibitory capacity of the patients' control sera and healthy individuals' sera ranged between 0 and 15% (mean, ≈6%; results not shown).

To determine the percentage of antigen-binding Ab3 in patients' sera, sera from three patients (nos. 5, 10, and 17) were adsorbed twice with equal volumes of packed SW948 CRC cells to remove all antigen-binding Ab3. As a control, sera were also incubated with WM9 melanoma cells, which do not express the CO17-1A antigen. Both adsorbed and unadsorbed sera were then preincubated with normal goat IgG and tested for their capacity to inhibit binding of Ab2 to Ab1. Adsorption of the sera to CRC cells reduced the inhibitory capacity of the three different sera by 32%, 22%, and 48%, respectively, whereas adsorption of the sera to melanoma cells showed no significant effects. Thus, between 22% and 48% of the Ab3 that share idiotopes with Ab1 also bind the antigen in the three patients studied (results not shown).

DISCUSSION

We have reported (4) that in the course of several immunizations of a patient with heterologous human Ab2, preceded 2 years earlier by the injection of mAb CO17-1A, an increase in production of anti-tumor antibody by the patient's lymphocytes was observed after *in vitro* stimulation by Ab2. However, the specificity and efficacy of Ab2-induced effects were difficult to assess in this initial trial in light of the possible interference of the patient's autologous Ab2 produced against the administered Ab1.

In the present study, heterologous polyclonal goat Ab2 was administered to 30 cancer patients with advanced CRC. The Ab2 had been shown *in vitro* and in experimental animal

models to functionally mimic the gastrointestinal tract cancer antigen defined by anti-CRC mAb CO17-1A (1, 2, 19). In cancer patients, the Ab2 elicited the production of specific Ab3, which bound to various tumor cells with specificities similar to that of Ab1, competed with Ab1 for binding to CRC cells, bound to isolated tumor antigen, and may share idiotopes with Ab1. Note that all immunological studies were conducted with sera obtained before the second course of Ab2 therapy that was combined with chemotherapy in some patients. In one patient, the presence of specific Ab3 in serum was further confirmed by its production by lymphocytes stimulated *in vitro* with Ab2.

Elicitation of patients' immune responses by Ab2 immunization was independent of the dose of Ab2 administered. In preliminary trials with even larger doses of Ab2 (8 and 16 mg per injection), the titer of Ab3 did not increase further (results not shown).

Some patients clearly had produced low but significant amounts of surface-reactive anti-CRC antibodies before treatment with Ab2. In these patients, it is possible that priming of B cells by tumor cells greatly facilitated induction of antigen-specific Ab3 by Ab2 immunizations, although further studies are needed to prove this point.

Although the present trial was designed to evaluate the immune response of the patients to Ab2, favorable clinical responses were also observed in some patients. Whereas 9 of the 13 patients who improved clinically received chemotherapy in addition to Ab2, preventing conclusions about the benefit of Ab2 immunizations, Ab2 might well underlie the clinical improvement of the 4 remaining patients who received these antibodies alone. However, conclusions about the role of Ab2 in these 4 patients await further study, including the response of appropriate control patients.

In the present studies, we have shown that immunization of patients with Ab2 effectively elicits humoral anti-tumor immune responses that are highly specific. Future approaches to enhancing the effects of Ab2 immunization might include booster injections with tumor antigen, analogous to the studies reported in the hepatitis virus system (17). Our results have implications for cancer immunotherapy and also suggest a general applicability of Ab2 immunizations of humans in vaccination approaches to pathogens, especially in those cases in which the use of conventional vaccines presents difficulties (18).

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