Antiidiotype Antibodies in Cancer Patients Receiving Monoclonal Antibody to Carcinoembryonic Antigen

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ABSTRACT

The initial 10 patients of a Phase I clinical trial involving multiple injections of murine monoclonal anti-carcinoembryonic antigen (CEA) antibody, NP-2, were studied for the presence in their sera of antiidiotype antibody. Most patients had advanced gastrointestinal adenocarcinoma and received 1 mg/m² monoclonal antibody three times weekly, or once a week, resulting in five to 13 injections over 12 to 240 days. Antiidiotype antibody was detected with a blocking radioimmunoassay using [125I]NP-2(Fab')₂ binding to CEA-coated microwells and [125I]NP-4-F(ab')₂ as a control antibody. Five out of 10 patients demonstrated 65–96% inhibition of NP-2 binding at 1/20 dilution of serum compared to NP-2 binding in the presence of pretreatment sera. The inhibitory activity was preserved after adsorption over a polyclonal mouse IgG immunoadsorbent whereas exposure to a NP-2 affinity column completely depleted the activity. Specificity testing, including the blocking effect of patient sera on the control antibody NP-4, and interference by the possible presence of circulating NP-2, circulating CEA, and human anti-CEA activity, confirmed that the inhibition observed was specific for NP-2 and was caused by an agent with CEA-like characteristics. Longitudinal studies demonstrated that elevated titers of antiidiotype antibody appeared later in the course of immunization than did antibodies against mouse immunoglobulin. These studies indicate that patients can be sensitized to the idiotype (anti-combining site and/or combining site-related) of monoclonal antibodies to CEA following multiple infusions.

INTRODUCTION

The idiotype network hypothesis (1, 2) and the accumulating knowledge about anti-Id³ interactions in human immunoregulation (3, 4) have opened new perspectives for cancer therapy. Interest has emerged to investigate a possible role for MoAbs in modulating this self-regulatory system towards an enhanced immune response. Antitumor effects of MoAbs and their complementary anti-Id interactions have been demonstrated in animal studies (5, 6). Evidence for a beneficial role of MoAbs in the treatment of cancer patients is more limited (7–12). Sears et al. (10) reported clinical improvement and tumor regression in several patients treated with anticolorectal MoAb.

In other studies, antibodies to CEA have been utilized for diagnostic imaging and radioimmunotherapy (13–16). As part of an overall program investigating the therapeutic effectiveness of CEA MoAbs and related immunon conjugates, we initiated a Phase I clinical trial involving multiple injections of a CEA MoAb, NP-2 (15, 17). This study was established in an effort to elicit an anti-Id antibody response, and hopefully, anti-anti-Id antibody (Ab₃). The present report describes the development of an assay for the detection of anti-Id antibodies against a CEA MoAb, as well as our initial observations on the appearance of these antibodies in immunized cancer patients.

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3 Abbreviations used: Id, idiotype; CEA, carcinoembryonic antigen; EIA, enzyme immunoassay; HAMA, human anti-mouse immunoglobulin antibody; MoAb, monoclonal antibody; PBS, 0.01 M PO₄ (pH 7.2) plus 0.15 M NaCl.

MATERIALS AND METHODS

Patients and Treatment Schedule. As part of a Phase I clinical serotherapy trial, 10 patients with metastatic adenocarcinoma (four of colorectal, two of pancreatic, and one each of gastric, breast, lung and unknown primary) who had failed conventional treatment with chemotherapy or radiation, or were beyond primary therapy at presentation, were treated with multiple infusions of a murine anti-CEA MoAb. Nine patients received 1 mg/m² MoAb diluted in 500 ml of 5% dextrose solution over 30 min, three times a week, and one patient only once a week. Patients received five to 13 infusions in a period of 12–240 days. The amount of total antibody per patient ranged from 13 to 31 mg. Therapy was discontinued when there was evidence of progression by increasing tumor size or rising serum CEA levels. None of the patients examined experienced untoward complications as a consequence of MoAb administration.

EIA for CEA. The concentration of CEA in the plasma sample was determined by a sandwich EIA using two monoclonal antibodies against CEA. A microtiter plate sensitized with MoAb NP-1 at 10 µg/ml in PBS was washed with PBS containing 0.05% Tween-20. Plasma samples (100 µl) or standards were added to wells containing 100 µl of buffer composed of PBS with 10% heat-inactivated horse serum and 1% mouse serum. To avoid false-positive elevations in CEA titers caused by HAMA, heat extraction of plasma specimens was carried out according to a modification of the procedure developed by Kim et al. (18). Briefly, 1.0 ml of 0.2% sodium acetate buffer, pH 5.0, was added to 0.5 ml plasma. The tubes were vortexed and then incubated for 15 min at 90°C prior to centrifugation at 1200 x g for 10 min. Heating plasma samples in this fashion eliminates HAMA without affecting CEA titers (19). The supernatants were collected and stored at -20°C until testing.

Ortho Tri-level Ligand Assay Control Sera (Ortho Diagnostics, Inc., Raritan, NJ), containing different concentrations of CEA served as the standards. After a 90-min incubation at 37°C, the plates were washed and 200 µl of peroxidase-conjugated MoAb NP-3 diluted in PBS-Tween were added to the wells. After 30 min at 37°C, the plates were washed and 200 µl of substrate solution prepared from o-phenylenediamine dihydrochloride tablets (Pittman-Moore, Amwell, NJ) were added and allowed to develop in the dark for 30 min at room temperature. The reaction was terminated with 50 µl of 4 N H₂SO₄ and the plates were read at 490 nm. The concentration in the samples was determined by reference to the Tri-level Control Sera standards.

EIA for HAMA. The antibody response of patients to mouse IgG was measured by EIA. Mouse IgG (Pelfreez Biologicals, Rogers, AR), NP-2 IgG, or NP-2 F(ab')2 at 10 µg/ml in PBS were adsorbed overnight at 4°C to the wells of microtiter plates (Dynatech Laboratories, Alexandria, VA). Dilutions of plasma were incubated in the wells at 37°C for 1 h, washed, and then reacted with peroxidase-conjugated mouse anti-human IgG (Jackson Immunoresearch, Avondale, PA). The wells then received a substrate solution of 0.0125% H₂O₂ and 0.0044 M o-phenylenediamine dihydrochloride (Sigma Chemical Co., St. Louis, MO) in 0.1 M phosphate citrate buffer, pH 5.0. This was allowed to develop for 15 min before 4 N H₂SO₄ was added and the plates read at 490 nm. The reciprocal dilution of plasma that gave an absorbance reading equal to 1.0 in the assay was referred to as the HAMA titer.

Immunoadsorbents. Plasma samples (0.3 ml) were applied to 4 ml immunoadsorbent columns of Affigel 10 (Bio-Rad, Richmond, CA) containing mouse IgG or NP-2 IgG preconjugated with PBS. Mouse IgG was coupled at 5 mg/ml gel, while NP-2 IgG was coupled at 10 mg/ml gel according to the manufacturer's instructions. The samples were incubated on the columns for 120 min, then flushed with buffer until the reading equal to 1.0 in the assay was referred to as the HAMA titer.

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the unadsorbed fraction was eluted as monitored at A₂₈₀. The bound fraction was eluted with 0.1 M glycine-HCl, pH 2.8, immediately dialyzed against PBS, and then concentrated to 1.0 ml.

Anti-CEA Immunoassay. The presence of circulating NP-2 antibody or human anti-CEA antibody in patient plasma was examined by utilizing CEA sensitized microtiter plates and peroxidase-conjugated goat anti-mouse IgG or mouse anti-human IgG (heavy and light chain), respectively. Assay conditions were identical to that used in the HAMA immunoassay.

CEA MoAbs. The development and characterization of murine NP anti-CEA MoAbs are described elsewhere (17). The NP-1, -2, -3, and -4 antibodies used in various aspects of the present study all have an IgG₁ isotype and recognize separate epitopes on the CEA molecule. These antibodies were purified from mouse ascites fluid by chromatography over protein A-Sepharose CL-4B (Pharmacia, Piscatway, NJ). F(ab')₂ antibody fragments were a generous gift from Dr. Dan Shochat, Immunomedics, Newark, NJ.

Anti-Id Immunoassay. A blocking assay was established to detect anti-Id antibody by measuring the ability of patient plasma to interfere with the binding of radiiodinated F(ab')₂ to antigen-coated microwells. Purified CEA at 4 ng/well in PBS was adsorbed overnight at 4°C to the wells of microtiter plates (Dynatech). CEA was purified from liver metastases of a colonic adenocarcinoma as previously described (20). The microwells were then postcoated with 1.0% human sera albumin in PBS for 1 h at 37°C. Dilutions of plasma, usually beginning at 1:10 dilution, were made in PBS containing 10% normal human serum that was prescreened for the absence of HAMA by EIA.

Following the addition of diluted patient plasma to the wells, 125I-labeled NP-2 F(ab')₂ or NP-4 F(ab')₂, about 0.1 μCi of each, diluted in PBS containing 1% human serum albumin was added and the plates were then incubated at 37°C for 1.5 h. Antibody fragments were radioiodinated to a specific activity of 15 μCi/μg by the chloramine-T procedure (21). When added to the wells in the presence of 10% normal human serum diluent only, approximately 5-10% of the radioiodinated antibody bound to CEA-coated wells as contrasted to a nonspecific binding of 0.1% to wells without antigen but sensitized with 1.0% human serum albumin. After incubation, the wells were washed three times with PBS containing 0.05% Tween-20 and the wells were then cut and measured for radioactivity. Interference with antibody binding to antigen by posttherapy plasma samples was computed by comparison to antibody binding in other wells in the presence of a 1:10 dilution of the same patient's pretherapy plasma specimen. The latter antibody binding level was usually within 10% of that obtained with antibody in the presence of the 10% normal human serum diluent.

RESULTS

HAMA Response. Of 10 patients studied after therapy, seven showed a three- to 15,000-fold increase of HAMA levels over pretherapy levels or titers in 10 healthy controls (Fig. 1). Three others showed no response or remained within the ± SD of healthy individuals. Except for Patient 6, who only received five injections, there appears to be no relationship between HAMA titers and treatment schedule.

Anti-Id Response. Sera of the 10 patients selected were screened for the presence of antibodies directed against idiotype determinants of NP-2. Three showed low inhibitory activity (less than 25% inhibition of NP-2 binding) in the blocking radioimmunoassay (Table 1). Titration of five others demonstrated higher levels of blocking activity as shown in Fig. 2. In five out of seven patients showing inhibitory activity greater than 25%, the blocking effect on NP-2 binding was more than 6- to 32-fold higher than the effect seen with the control MoAb (NP-4) (Table 1). In one patient (patient 1), the activity was only 1.7 times control, and one patient (patient 7) showed greater inhibition of binding of the control MoAb.

Immunoadsorption of Inhibitory Activity. To determine the extent that HAMA possibly contributed to the blocking effect of patient sera in the radioimmunoassay, the five sera with high blocking activity were processed over an affinity column conjugated with polyclonal mouse IgG. Over 96% efficacy in reduction of HAMA titers was observed (Table 2). Retesting of the unadsorbed fraction in the blocking assay demonstrated variable retention of the inhibitory activity for NP-2 binding (Fig. 2). While there was a substantial loss in the NP-2 blocking activity of the serum from Patient 2, the decline in blocking activity was much less evident in the processed sera from the other four patients. There was no loss of blocking activity in Patient 1, whereas the processed sera from the other three patients were 25 to 50% less active. Inasmuch as the blocking activity for NP-4, which also shares the same isotype with NP-2, was completely abolished in all cases (Fig. 2), this shows that blocking activity with specificity for NP-2 exists in these serum specimens.

Three sera having been processed over the polyclonal mouse IgG immunoadsorbent (Patients 1, 3, and 8) were further exposed to an NP-2 affinity column. Over 96% reduction of anti-NP-2 titers, as measured by EIA, was observed (Table 2). Blocking activity in the radioimmunoassay was absent in the unadsorbed fraction recovered from the NP-2 immunoadsorbent in all three sera, indicating reversal of the inhibition observed previously with the unadsorbed fraction after the first column. The possibility that this was due to nonspecific adsorption to the NP-2 column was excluded by the fact that a previous
obtained from the NP-2 immunoadsorbent using NP-2 IgG as antigen target in its first immunoadsorbent) was compared to that of the unadsorbed fraction (post) and the HAMA titer of the preadsorption specimen (pre = post specimen from the immunoadsorbent was determined in comparison to the titer of the unadsorbed fraction (post) corresponded to a serum concentration of 5000 ng/ml, was low. Whether this was caused by denaturation of antibody during the eluting process requires further study.

Circulating CEA as Blocking Agent. The serum concentrations of whole NP-2 on [125I]NP-2-F(ab')2 (O) binding to CEA demonstrates that blocking is attributable to an antigen-like substance with specificity for NP-2. The absence of blocking activity in the unadsorbed fraction of the NP-2 column demonstrates that blocking is attributable to an antigen-like substance with specificity for NP-2. The recovery of the blocking activity in the eluate from the NP-2 immunoadsorbent was then exposed to an immunoadsorbent containing NP-2 IgG, and the HAMA titer of the preadsorption specimen (pre = post specimen from the first immunoadsorbent) was compared to that of the unadsorbed fraction (post) obtained from the NP-2 immunoadsorbent using NP-2 IgG as antigen target in EIA.

Table 2 Influence on HAMA titers by exposure of patient plasma to immunoadsorbents containing mouse immunoglobulin

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Polyclonal mouse IgG</th>
<th>NP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>1</td>
<td>133,538*</td>
<td>255</td>
</tr>
<tr>
<td>2</td>
<td>159,457</td>
<td>2,214</td>
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<tr>
<td>3</td>
<td>1,664</td>
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<tr>
<td>8</td>
<td>4,676</td>
<td>184</td>
</tr>
<tr>
<td>10</td>
<td>33,928</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* Reciprocal dilution of plasma giving an adsorbance equal to 1.0 in EIA.

ND, not done.

second passage over the polyclonal mouse IgG column had not significantly altered the inhibiting activity present.

The absence of blocking activity in the unadsorbed fraction of the NP-2 column demonstrates that blocking is attributable to an antigen-like substance with specificity for NP-2. The recovery of the blocking activity in the eluate from the NP-2 column was low. Whether this was caused by denaturation of antibody during the eluting process requires further study.

Circulating CEA as Blocking Agent. The serum concentrations required to cause interference by circulating CEA with NP-2 in our blocking assay were determined by adding increasing concentrations of purified CEA in 10% normal human serum to the assay. A concentration of over 50 ng/well, which corresponds to a serum concentration of 5000 ng/ml, was necessary to achieve 10% blocking of NP-2 binding to CEA (Fig. 3A). In addition, examination of blood specimens obtained at initiation of therapy did not reveal any appreciable inhibitory activity (data not shown). In Patient 7 a moderate inhibitory activity could be attributed to the high CEA plasma concentration and/or HAMA.

Circulating Mouse Anti-CEA Antibody. No circulating NP-2 antibody was detected in all but one patient. Patient 2 demonstrated circulating mouse anti-CEA activity at a titer that only partially was explained by increased nonspecific binding to the plate. The extent to which this could account for the blocking in the radioimmunoassay was assessed by using dilutions of whole NP-2 in the blocking assay. No inhibition was seen with increasing NP-2 concentrations up to an equivalent of a serum concentration of 0.5 μg/ml. Thirty-eight % of inhibition occurred at 5 μg/ml, which is a serum level 5-fold higher than the maximal serum level immediately after injection of the antibody (Fig. 3B).

Human Anti-CEA Antibodies. The possibility that human anti-CEA antibody could contribute to the inhibition observed was tested with an EIA using CEA-coated microwells and
peroxidase-labeled mouse anti-human IgG. Low reactivity was only observed in one patient and this was eliminated after polyclonal mouse IgG adsorption.

Sequential Analysis of Anti-Id Response. In one patient (Patient 3) whose treatment extended over a period of 200 days, serial unprocessed specimens at a 1:10 dilution were analyzed for the presence of blocking activity. Fig. 4A demonstrates appearance of elevated blocking activity only after a latent period following the first eight injections. Upon rechallenge with the 9th injection on Day 86, a persistent blocking activity was observed. Titration of the inhibitory activity on Days 114, 142, and 172 showed that 30% inhibition was present at dilutions of 1:30, 1:30, and 1:200, respectively, which indicates rising titers to be present at the plateau of the inhibition curve. Fig. 4B illustrates the HAMA response over the same time period. Antibodies against polyclonal mouse IgG and whole NP-2 appear clearly with a peak on Day 21, which was 5 days after the last injection of the initial series of eight injections, and was followed by a gradual decline after cessation of immunization. Anti-whole NP-2 antibodies and anti-NP-2 F(ab')2 antibodies reached higher titers after rechallenge when polyclonal anti-mouse Ig titers tended to remain moderate. The difference between the anti-NP-2 (intact and fragments) and polyclonal anti-mouse IgG titers probably reflects the contribution of the anti-Id to the anti-NP-2 titers.

**DISCUSSION**

In the evaluation of the initial 10 patients of a Phase I trial involving multiple infusions of the CEA MoAb, NP-2, an antiidiotype immune response was detected. Five out of seven patients who developed high HAMA titers demonstrated blocking activity to NP-2 as shown in an inhibition of NP-2-F(ab')2 binding to CEA in a solid-phase radioimmunoassay. This blocking activity was specific for NP-2 when tested with a control anti-CEA MoAb, NP-4, which recognizes another epitope on CEA, and it was also not attributable to the effects of circulating CEA or NP-2. The blocking activity was removed by NP-2 affinity column adsorption but not by passage of patient sera over a polyclonal mouse IgG affinity column. Since there is still a possibility that factors other than anti-Ig may affect the inhibition assay results, isolation of anti-Id is in progress.

Herlyn *et al.* (22), in their CO17-1A MoAb trial, reported that anti-Id antibodies comprised 21–80% of the total HAMA in four patients, and Ab2 reacting with the antigen combining site consisted of 20–68.5% of the total Ab2 (in four patients). In our five patients, the blocking activity showed evidence of anti-combining site specificity, but we do not know whether this was due to an internal-image anti-Id (β type) or due to blocking caused by an α-type anti-Id, or both (23).

Our patients received much smaller doses of MoAb, ranging from 1.6 to 2.2 mg/infusion, whereas in the study by Herlyn *et al.* (22), doses of 5–771 mg/infusion were chosen for those patients receiving two to eight treatments (10, 24). The duration of immunization was less than 4 months, while we had the opportunity to extend our treatment to greater than 200 days in two out of 10 initial patients.

A quantitative analysis of the anti-Id response in four patients by Herlyn *et al.* (24) revealed 2.8–42 μg of anti-Id IgG/ml of blood as isolated by immunoadsorbent techniques. We have not yet isolated and further characterized the anti-Id in our system because of small amounts of antibody eluted from the column. Estimations of the anti-Id quantities causing the blocking effect observed in our radioimmunoassay gave a similar range (0.1–44 μg/ml) in our five patients. These estimations were extrapolated from the quantities of NP-2 required to cause inhibition of homologous antibody, and thus, only provide a crude assessment of blocking anti-Id human antibodies. Furthermore, as suggested by longitudinal studies in one patient, the dosage schedule utilized in the present study seemed to favor the heightened appearance of anti-Id antibodies only after several infusions were given, and perhaps also requiring a certain latent period. Additional examination of other patients and sensitization schedules should help define protocols for optimal generation of anti-Id antibodies.

The clinical antitumor response claimed in 37 out of 142 patients in the CO17-1A trial seemed to show correlation to the presence of an Ab2 response (in 65% of patients who responded) (22). Among the 10 initial patients of our trial, no clinical response was detected, but the patient population is too small to arrive at firm conclusions at this time. Great controversy has centered around the issue of immunogenicity of CEA in humans, and it has been debated extensively with studies in support of (25, 26) and against this possibility (27, 28). Whether it is biologically feasible for a xenogeneic MoAb to act across species lines and induce an anti-Id that will carry the internal image of the tumor antigen and induce Ab3 formation as described by Herlyn *et al.* (29) remains to be investigated for the CEA system. Means of identifying murine MoAbs with appropriate specificities and other factors associated with the production of human Ab3 antibodies with antitumor activities are presently unknown. Our initial assays did not demonstrate Ab3 to CEA, but refinement of assay sensitivity and/or approach may be needed as well as more aggressive or prolonged immunization treatment schedules. Moreover, whether the breaking of tolerance to CEA is beneficial for the patient regarding tumor suppression cannot be predicted on the basis...
of the limited data yet available. Nonetheless, modulation of the idiotypic network may prove to be a potent and relatively safe method of cancer therapy. As reported here, the demonstration of anti-id antibodies to CEA in patients treated with MoAbs to this antigen is the first step in determining the feasibility of this immunotherapy concept using CEA as the tumor-associated antigenic target.

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REFERENCES

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