Lack of Radioimmunodetection and Complications Associated with Monoclonal Anticarcinoembryonic Antigen Antibody Cross-Reactivity with an Antigen on Circulating Cells


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ABSTRACT

Characterization of several high-affinity murine monoclonal anticarcinoembryonic antigen (CEA) antibodies suggested good specificity except for cross-reactivity with an antigen on granulocytes and erythrocytes which was different from the previously described normal cross-reacting antigen of granulocytes. In vivo studies in athymic mice using an indium conjugate of an anti-CEA monoclonal antibody (MoAb) revealed excellent specific uptake in colorectal carcinoma xenografts. Studies were conducted in humans to determine the limitations produced by the cross-reactivity with granulocytes and erythrocytes. Patients with metastatic colorectal cancer received 3 to 6 mg of anti-CEA MoAb over 10 min or 2 hr. In five of six trials, the MoAb infusion was associated with a 40 to 90% decrease in circulating granulocytes and systemic toxicity including fever, rigors, and emesis. One patient had no change in cell count and had no toxicity. Radionuclide scans with 111In-anti-CEA MoAb showed marked uptake in the spleen when cells were eliminated, and in the liver, especially when pretreatment CEA levels were high. Metastatic tumor sites failed to concentrate the isotope. This study emphasizes the potential limitations for radioimmunodetection and/or radioimmunotherapy imposed by reactivity with circulating cells, and suggests that certain toxic reactions associated with MoAb infusions are related to destruction of circulating cells rather than allergic reactions to mouse protein. It also emphasizes how variables such as dose and binding affinity of antibody, radioisotope used, and assessment at different observation points can obscure lack of antibody specificity.

INTRODUCTION

The potential utility of MoAbs as in vivo diagnostic and therapeutic agents in cancer has been widely publicized (4, 24). There are a rationale and supportive preliminary data for using MoAbs alone in passive serotherapy, or conjugated to radioisotopes, cellular toxins, or chemotherapeutic agents either in vitro or in vivo (5, 14, 27, 38). The potential utility of MoAbs in the passive serotherapy of human cancer has been described recently (8, 26, 27). The use of antibody-isotope conjugates for radioimmunodetection has been demonstrated in animals and humans (14). There also have been claims of therapeutic efficacy using 125I conjugates of antiferritin antisera therapeutically in humans (29). In the setting of passive serotherapy, absolute tumor specificity may not be a necessity, depending on the distribution of the particular target antigen. Thus, investigators have observed some clinical benefit in both animals and humans who received MoAbs directed against tumor-associated, but not tumor-specific, antigens (1, 8, 27). However, for effective diagnostic or clinical use of radiolabeled MoAbs, the degree of tumor specificity may be more crucial.

In this paper, we report certain problems encountered when we attempted to perform radioimaging in human subjects with anti-CEA-MoAbs conjugated to 111In. Animal models of human colorectal carcinoma xenografts in athymic mice had previously been used to demonstrate the in vivo tumor specificity of such conjugates (15, 16, 37). In one study, extraction ratios of 23% per g of tumor were achieved by 72 hr with 111In anti-CEA (37). This was 15 to 20 times the amount of 125I-anti-CEA acquired via the tumor. Thus, this study and others have suggested superiorium of indium over radioiodine in terms of isotope-conjugate stability and percentage of tumor uptake of injected dose (15, 16, 32, 34, 37). Unfortunately, in humans, cross-reactivity of this antibody with an antigen on the surface of granulocytes, and probably erythrocytes, prevented successful imaging. The problem encountered was due to the absorption of antibody by circulating granulocytes, erythrocytes, and free CEA antigen resulting in subsequent clearance by the RE system. This problem persisted in the face of rapid or slow infusion, and following efforts to clear circulating cells and CEA with unconjugated antibody.

This study emphasizes the necessity for careful screening of antibodies with potential in vivo use in humans against all circulating cells and emphasizes the limitations of animal models in the testing of antibody tumor specificity. The results support the previous observations suggesting the RE system as the major site of clearance of circulating cells which bind MoAbs. In conjunction with other MoAb studies, these results support the notion that the majority of toxicity accompanying such therapy is associated with removal of circulating target cells, rather than allergic phenomena. We also discuss how variables such as dose, tumor affinity, radioisotope, and differences in observation time points may obscure a lack of antibody specificity in vivo.
R. O. Dillman et al.

MATERIALS AND METHODS

The 5 patients selected for study had a diagnosis of metastatic colorectal carcinoma with a wide range of CEA levels (Table 1). None of the patients had received chemotherapy or radiation therapy within 8 weeks of administration of the anti-CEA antibody. All of the patients were ambulatory at the time of study and asymptomatic from their metastases. Informed consent was obtained as per the guidelines of the Investigational Review Board for Protection of Human Subjects of the University of California San Diego School of Medicine and the San Diego VA Medical Center.

Anti-CEA Antibody. The anti-CEA antibodies used in these studies were 065 and 326, IgG1 murine MoAbs produced by Hybritech, Inc. (La Jolla, CA). The antibody-producing hybridomas were derived by fusing NS-1 myeloma cells with spleen cells from BALB/c mice which had been immunized with purified CEA using variations of techniques described previously (33). The antibody was purified from mouse ascites by salt fractionation and isoelectric focusing, and had an affinity of $57 \times 10^6$ liters/mol by Scatchard analysis. Total immunoglobulin was 87% of total protein by absorbance measurements at 280 nm using an extinction coefficient of $E_{\text{cm}}^\text{ moc=y}=14$. Specific IgG1 isotope antibody was 79% of total protein as determined by incubation of $125^\text{I}-$labeled antibody with excess adsorbent-bound CEA. Purity and clonality were confirmed using the acrylamide gradient gel technique of Orstein and Davis (3, 30) by comparison to commercial murine IgG. Prior to clinical administration, the antibody was passed through a 0.22-$\mu$m filter and tested for pyrogen and sterility.

Testing on frozen tissue sections of human colon, colorectal carcinoma, lung and bronchogenic carcinoma, and stomach and stomach carcinoma revealed marked reactivity with colorectal carcinomas, less reactivity with gastric and lung cancers, and minimal to no reactivity with the nonmalignant tissues using indirect immunofluorescence techniques as described previously (39). Mouse immunoglobulin was used as a negative control. Bright immunofluorescence was also seen when the technique was applied to biopsies of various human colorectal carcinoma xenografts grown in athymic mice.

No reactivity was observed when the MoAbs were tested against purified normal cross-reacting antigen (2, 17). This is an antigen which has shown cross-reactivity with anti-CEA antisera and is found primarily on mature granulocytes. However, indirect immunofluorescence assays revealed MoAb reactivity with granulocytes and erythrocytes (18). Additional work revealed that the anti-CEA reacted predominantly with mature PMN leukocytes and less so with earlier granulocytes. Precipitation of the anti-CEA with excess normal cross-reacting antigen failed to block anti-CEA staining of colon cancer cells or granulocytes, while precipitation with CEA totally inhibited such binding (18).

Indium-labeled Anti-CEA. The purified anti-CEA MoAbs described above were labeled with $111^\text{In}$ using a modification of the bifunctional chelation techniques described by Halpern et al. (16) and Krejcarek and Tucker (19). This method incorporates 80 to 95% of the $111^\text{In}$ onto the protein without altering its immunoreactivity, which was sustained at 70% or greater for all MoAbs administered. Labeling efficiency was such that 1 to 4.0 mCi were bound per mg of protein. Preparations were again proven sterile and pyrogen-free prior to administration.

Athymic Mouse Studies. Testing of the anti-CEA indium conjugates in athymic mice bearing human colorectal carcinoma xenografts demonstrated stability of the conjugate and specific uptake of the isotope in human colon cancer tissue as opposed to human melanoma tissue or normal mouse tissue. Details of these animal studies have been reported elsewhere (16, 37).

Antibody Administration. The anti-CEA MoAbs were administered i.v. in either 10-ml 0.9% NaCl solution (saline) over 10 min, or in 120-ml saline over 2 hr. Three patients were treated simultaneously with 3, 3, and 6 mg of unlabeled anti-CEA by rapid infusion on one occasion. Subsequently, one additional patient and one of the first 3 patients were treated with 3 and 5 mg of indium-labeled MoAb administered over 2 hr. Another patient received 2.5 mg of unlabeled MoAb over 1 hr immediately followed by 2.5 mg of labeled MoAb over 1 hr. Patients were carefully followed by a clinical research nurse who was constantly in attendance to monitor vital signs during and following each infusion. Patients were subsequently monitored weekly with CBC and tests of renal and hepatic function and/or injury. Nuclear medicine scans were performed over the anterior and posterior head, chest, abdomen, and pelvis at the end of the infusion, and 4, 24, 48, and 72 hr after the infusion. Imaging was performed on a GE 4041 gamma-camera using the 173- and 247-keV photopeaks.

Peripheral Blood Tests. Blood was obtained before the start of each infusion and then at 0.25, 0.5, 1, 2, 4, 8, 24, and 144 hr after the start of each treatment for CBC, differential, platelet count, and plasma and/or serum CEA level. The CBCs were performed using a Coulter Counter and review of 100 leukocytes by light microscopy. Plasma CEA was measured using the Roche radioimmunoassay kit, and serum CEA was measured using the Abbott radioimmunoassay kit and a Hybritech tandem monoclonal antibody assay. Blood chemistry was obtained prior to treatment and weekly thereafter for 1 month. CBC and CEA were also monitored weekly for 1 month after treatment. Total granulocyte cell counts per $\mu$l were obtained by multiplying the percentage of granulocytes by the number of WBC per $\mu$l.

RESULTS

Table 1 summarizes the individual patients and their CEA levels and toxicities. The effect of the anti-CEA infusions on the circulating PMN and juvenile granulocyte counts is illustrated in Charts 1 and 2 for both the rapid and 2-hr infusions, respectively. The toxicities which were observed are also noted in the figures and included fever, rigors, nausea, and vomiting. With the rapid infusion, all 3 patients had some toxicity. Patient 1, who had a very low circulating CEA (21 to 92 ng/ml), had violent rigors and fever which began within 1 hr of receiving the antibody. The patient’s granulocyte count fell from 10,400/$\mu$l to a low of 1,540/$\mu$l, with 1,100 mature PMNs/$\mu$l and 440 juvenile forms/$\mu$l. By 4 hr after treatment, his symptoms had resolved, and his granulocyte count had stabilized at pretreatment levels.

Patient 2 had a CEA of only 60 to 108 ng/ml. Following rapid infusion of 3 mg of anti-CEA, his granulocyte count decreased from 4900 to a nadir of 1800/$\mu$l within 1 hr of giving the antibody. About 2 hr after treatment, he developed fever and chills, although they were much less severe than in the first patient. These symptoms resolved within 2 hr. By 24 hr later, his granulocyte counts had also returned to pretreatment levels.

Patient 3 had a CEA of 800 to 1767 ng/ml; he received 6 mg of anti-CEA over 15 min. In spite of the higher dose, this patient had much less change in his granulocyte count (8500/$\mu$l pretreatment to a nadir of 6750/$\mu$l 2 hr after therapy) and had no toxicity other than a temperature elevation to 38.2°C.
In Vivo Complications with Anti-CEA MoAb

Pre 0'/4 HOURS AFTER ANTI-CEA

Chart 1. Effect on circulating granulocytes after 3 to 6 mg of anti-CEA MoAb 065 were given i.v. over 10 min to 3 different patients. Top line, total granulocyte number; II, number of PMN sites; III, number of juvenile forms (bands) which, when added to the PMN cell count, yields the total granulocyte count. Onset and duration of toxicity are shown.

None of these patients had delayed complications of this therapy. The circulating CEA levels were reduced but returned to pretreatment levels in a pattern similar to the effect on granulocytes (Chart 3). Thus, by 24 hr, the CEA had returned to pretreatment levels in all cases. The assay used measured both free and complexed CEA. The results are consistent with the hypothesis that immune complexes were being formed and removed, presumably in the RE system.

In addition to these rapid infusions, 3 patients received 3 to 5 mg of the indium-conjugated anti-CEA MoAb as a 2-hr infusion as summarized in Table 1 and Chart 2. Patient 1B (the same as the first patient treated by rapid infusion) again received 3 mg of total antibody dose. His pretreatment CEA was only 21 to 45 ng/ml. His granulocyte count dropped from 7900/µl to a nadir of 1100 at 2 hr after terminating the infusion. He again had fever, rigors, and sweats which began 1.5 hr into the infusion, and which resolved 4.5 hr later. By that time, his granulocyte count had returned to pretreatment levels. Radionuclide scans showed nonspecific uptake in the liver and failed to illuminate metastatic disease in the liver or pelvis. In fact, areas of liver metastases had decreased uptake; the overall picture was similar to a technetium liver spleen scan, but with marked spleen radioactivity (Fig. 1).

Patient 4 received 5 mg of the radiolabeled conjugate over 2 hr. His pretreatment CEA was 840 to 2305 ng/ml. He had no change in his granulocyte count and no toxicity during or following the antibody administration. Radionuclide scans failed to show uptake in hepatic metastases which were readily demonstrable by a liver technetium scan and, once again, the area of metastases had diminished uptake of the isotope (Fig. 2) rather than the desired increase. Splenic uptake was minimal in this case, presumably because circulating cells were not removed.

Patient 5 had a CEA of 930 to 2340 ng/ml. In this patient, 2.5 mg of unlabeled anti-CEA were given over the first hr to clear the circulating cells and free CEA; 2.5 mg of indium-conjugated...
anti-CEA were given over the second hr. In contrast to the other patients with high CEAs, who had little change in granulocytes and little toxicity, this patient had both. His granulocytes dropped from 5000/μl to a nadir of 500/μl 30 min after the infusion ended. This was associated with nausea, emesis, chills, and a temperature of 37.6°C, which started as the infusion finished and persisted for 1 hr. Body scans again revealed uptake in the RE system and failed to show increased uptake in known metastases in the liver. Again, a large area of decreased uptake corresponded to a metastatic lesion seen in a liver scan (Fig. 3). None of these patients had any delayed complications during 1 to 4 months of follow-up after the infusions. Despite the in vitro reactivity with erythrocytes, no change in hematocrit or RBC number was detected in any of the patients.

**DISCUSSION**

This study addresses several potential obstacles to successful use of murine MoAb immunoconjugates as diagnostic or therapeutic agents in humans. These include cross-reactivity with circulating cells, the presence of free target antigen, and the toxicities or side effects associated with such infusions. First, any MoAbs with potential human clinical application must be rigorously tested for reactivity with normal antigens, particularly those on circulating cells. As shown here, lack of reactivity with known cross-reactive antigens is not sufficient to assure lack of binding to such cells and tissues. Circulating elements are immediately exposed to the MoAb at the highest obtainable concentrations following i.v. administration. For a blood volume of 5000 ml, following a 5-mg MoAb infusion, serum levels as high as 1 μg could be achieved. Cells opsonized with MoAb are rapidly removed from the circulation by the RE system, particularly the liver and spleen (8, 25). Using radiolabeled autologous lymphocytic leukemia cells, we and others previously showed that such cells are permanently removed from the circulation rather than merely being sequestered, since return of circulating counts to pretreatment levels was not associated with a return of radiolabeled cells into the circulation (8, 25). This study is consistent with that observation in that a large portion of cells which entered the circulation after maximal granulocyte suppression were juvenile forms rather than mature PMN cells. The binding and removal of the targeted circulating cells seemed to be specific inasmuch as no other circulating cells were depleted in this study, although the vast pool of erythrocytes would make detection of subtle decline difficult. Only T-lymphocytes were eliminated with an anti-T-cell MoAb in earlier studies (8, 13). In all cases, the serum CEA rose again rapidly following completion of the infusion in the same manner as the granulocytes.

A second theoretical obstacle to in vivo use of certain murine MoAbs is the presence of circulating antigen. Although uptake of labeled antibodies into colorectal carcinomas has been demonstrated in athymic mice with human xenografts (16, 22) and in human subjects with colorectal carcinomas (9, 10, 21, 23, 35), even in the presence of high circulating CEA, it is clear that such free circulating antigen does bind a portion of infused antibody (31). However, inasmuch as free CEA is usually found only in ng/ml quantities, even in colorectal cancer patients, infusion of mg quantities of MoAb should readily exceed the blocking capacity of the free antigen. In our study, all patients showed a drop in CEA following antibody infusion as compared to pretreatment values. The complexes formed may well have been cleared in the RE system adding to the problem of nonspecific imaging. Immune complexes of this size are probably removed primarily in the liver (13). In all cases, the serum CEA rose again rapidly following completion of the infusion in the same manner as the granulocytes.

A third issue is the toxicity associated with MoAb infusions. Five of 6 treatments were associated with unpleasant, albeit not severe, side effects, particularly the fever, chills, and malaise. It may be noteworthy that we have seen no toxicity in melanoma patients who received 2-hr infusions of an antimalanoma MoAb which apparently does not react with circulating cells (36). On the other hand, 9 of 12 patients treated with anti-human-T-cell MoAb have had toxicities ranging from urticaria and fever to anaphylactoid reactions (6–8). These data suggest that much of the acute toxicity associated with infusions of murine MoAbs is associated with removal of circulating target cells. In fact, in patients with significant levels of blocking factors, such as endogenous antitumor antibodies, when no decreases in circulating cells occurred, such reactions were not observed (6). In the patients described herein, granulocytes, and perhaps erythrocytes, were the unintended target cells while, in the T-cell lymphoproliferative disorders, the T-lymphocytes were the target. The one patient who had no decrease in circulating cells also had no toxicity. With regard to infusion rate, it is interesting to note that one patient had the same toxicities regardless of whether the MoAb was given over 15 min or 2 hr.

Another issue regarding toxicity concerns the distribution of the radioisotope in vivo. The binding of antibody to circulating antigen in cells results in a large portion of infused labeled antibody in the liver and spleen. If a therapeutic isotope-conjugate were to be used, this would have important implications for the radiodosimetry and toxicity to these organs.

As illustrated by Figs. 1 to 3, these anti-CEA-indium conjugates were not effective in radioimmunodetection in this study. In the patient who had a low CEA level and marked removal of granulocytes, there was striking uptake in the spleen and a lesser but generalized uptake in the liver except in the area of metastases (Fig. 1). In the patient who had no elimination of circulating cells, there was very little splenic uptake but, again, there was substantial liver uptake except in the areas of metastatic disease (Fig. 2). In the patient who had a high CEA level and removal of circulating granulocytes, there was marked diffuse uptake in both the liver and the spleen with diminished radioactivity in a left hepatic lobe metastatic mass (Fig. 3). These findings are consistent with the generally held concept that large immune complexes, such as IgG-opsonized cells, are removed primarily in the spleen, while smaller complexes and immunoglobulin are more likely to localize in the liver (13). The removal of opsonized cells is apparently Fc receptor dependent (13).

Several groups of investigators have reported successful radioimmunodetection of colorectal cancer in humans (9–12, 20, 21, 23, 35). All used iodine-conjugated antibody preparations, and all infused less than 1 mg of immunoreactive antibody. There are several possible explanations for their successes as opposed to our failure.

1. They could have had more specific antibody. Certainly those investigators who absorbed their anti-CEA antisera with spleen cells and other sources of circulating cells may have had a specific antisera which lacked cross-reactivity with granulo-
cytes and erythrocytes (9, 19, 21).

2. It is certainly conceivable that some investigators may have anti-CEA MoAbs which also cross-react with circulating cells, yet still have achieved tumor uptake of such agents. For example, anti-CEA MoAbs which also cross-react with circulating cells, granulocytes, and erythrocytes (9, 19, 21).

3. Other investigators may also have succeeded in tumor imaging with anti-CEA despite cross-reactivity with circulating granulocytes, because of the low affinity of the antibody preparations used or the low doses of immunoreactive antibodies infused. Both the 365 and 326 anti-CEA antibodies used in this study were of high affinity, and doses of 3 to 6 mg of high immunoreactivity were given. Conceivably, lower-affinity MoAbs might dissociate more readily from immune complexes or circulating cell surfaces.

Based on our observations, it seems imperative to rigorously exclude binding to circulating cells of all types for MoAbs with potential human application inasmuch as such reactivity may pose substantial problems for potential in vivo diagnostic or therapeutic application. This may require careful testing of erythrocytes of various blood groups in view of the complex glycoprotein antigens on their surfaces which may be shared with certain tissues in addition to leukocytes and/or lymphocytes. Investigation into other radioisotopes for diagnostic and therapeutic use as MoAb conjugates should continue also. For radioimmunodetection and possible radioimmunotherapy, it is important to clarify whether lack of specificity can be disguised by variables such as antibody affinity, dose administered, or the stability of a given isotope conjugate before concluding that MoAbs directed against a particular antigen may have adequate specificity for in vivo diagnostic use as immunocojuagates.
Human T cell antigens defined by monoclonal antibodies: the 65,000 dalton antigen of T cells (T65) is also found on chronic lymphocytic leukemia cells bearing surface immunoglobulin. J. Immunol., 125: 725–731, 1980.


Fig. 1. 48-hr scintillation scan after 2-hr infusion of 3 mg of anti-CEA MoAb 326 in a patient who had an 83% decrease in circulating granulocytes and an initial CEA of 21 ng/ml. Liver metastases appear as decreased rather than increased uptake, and other sites of disease in pelvis fail to illuminate. Marked splenic uptake is evident.

Fig. 2. 48-hr scintillation body scan after 2-hr infusion of 5 mg of anti-CEA MoAb 326 in a patient who had no change in granulocyte count and an initial CEA of 840 ng/ml. Liver metastases again had decreased rather than increased uptake. Little splenic uptake is noted.

Fig. 3. 48-hr scintillation scan after 1-hr infusion of unlabeled anti-CEA MoAb 326 and a 1-hr infusion of **Indium-labeled anti-CEA MoAb 326 in a patient who had a 90% decrease in granulocyte count and an initial CEA of 930 ng/ml. Marked splenic uptake is again noted as well as a large "cold" area in the left lobe of the liver.
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