Anti-Chelate Antibodies after Intraperitoneal Yttrium-90-Labeled Monoclonal Antibody Immunoconjugates for Ovarian Cancer Therapy

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The development of stable chelating agents for metal isotopes (e.g., ^{90}Y) such as CITC-DTPA, a benzyl-analog of DTPA, allowed us to evaluate the efficacy of ^{90}Y-labeled HMFG1 MAb administered intraperitoneally in patients with ovarian cancer. Our previous studies of ^{90}Y-HMFG1 antibody, however, showed that all patients developed anti-chelate antibody responses (to the macrocycle benzyl-DOTA), resulting in clinical side effects in a significant percentage of this group. **Methods:** We evaluated the immunogenicity of CITC-DTPA (administered to 12 patients as ^{90}Y-HMFG1-CITC-DTPA after coupling it to HSA using solid-phase ELISA. **Results:** Eleven of 12 evaluable patients developed anti-CITC-DTPA antibodies. Five patients (~ 40%) developed hypersensitivity syndrome, most likely due to a type III immune reaction (serum sickness). Most patients had a low titer of pre-existing anti-chelate response which correlated positively with post-therapy response levels ($p=0.001$). IgM anti-CITC-DTPA antibodies developed 2 wk while IgG antibodies developed 3 wk after treatment. Western blot analysis of post-therapy sera revealed a reaction with HSA-CITC-DTPA (60 kDa band) and no reaction with HSA or HSA-DTPA, whereas pre-therapy sera of the same patients were negative to all antigens. **Conclusion:** CITC-DTPA is immunogenic in patients after intraperitoneal administration of ^{90}Y-CITC-DTPA labeled MAbs. Self-limiting clinical side effects consistent with a serum sickness-like immune reaction were observed in 5 of 12 patients.

Key Words: monoclonal antibodies; radioimmunotherapy; chelates; yttrium-90; ovarian cancer

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The in vivo instability of conventional metal chelating agents such as diethylenetriamine-pentaacetic acid (DTPA) for ^{90}Y in radioimmunotherapy leads to the generation of free isotope in the circulation, which is deposited in the bone matrix, thus causing dose-limiting bone marrow toxicity (*1*). Doses of 15–20 mCi of ^{90}Y-labeled antibody administered intraperitoneally do not appear adequate to eradicate even minimal residual disease in ovarian cancer. Administration of ethylenediamine-tetraacetic acid (EDTA) systemically after intraperitoneal instillation of ^{90}Y-labeled monoclonal antibodies (MAbs), which shift to free ^{90}Y to the urine, can partially circumvent the problem of bone marrow toxicity, thus allowing dose escalation of radioactivity to 20–25 mCi (*1*). Vriesendorp et al. (*2*) reported systemic administration of 30–40 mCi ^{90}Y-DTPA-labeled antiferritin antibodies that resulted in the development of profound myelosuppression, which was subsequently rescued by autologous marrow reinfusion.

The development of more stable bifunctional chelating agents such as (S)-4-{2,3-bis[bis(carboxymethyl)-aminoprolyl]isothyocynate} of DTPA (CITC-DTPA) (nonmacrocyclic), enabled us to evaluate the efficacy of intraperitoneal ^{90}Y-CITC-DTPA-labeled HMFG1 (15–34 mCi ^{90}Y) in a Phase I/II dose escalation trial in 12 patients with ovarian cancer.

Previous studies (*3,4*) have demonstrated the in vitro and in vivo stability of the macrocyclic bifunctional chelating agent (2-p-nitrobenzyl)-1,4,7,10-tetraazacyclododecane-N,N′,N″,N‴-tetraacetic acid (benzyl-DOTA) and have also documented increased immunologic toxicity, which was shown to be associated with the development of anti-chelate antibodies (*5*). Half of the patients receiving low intravenous doses of the benzyl-DOTA ^{111}In immunoconjugate (<1 mg) for radioimmunolocalization developed significant levels of anti-chelate antibodies in the serum, but there were no accompanying clinical syndromes (*5*).

MATERIALS AND METHODS

Patients

Twelve patients with epithelial ovarian cancer participated in a Phase I/II clinical trial of intraperitoneal administration of ^{90}Y-HMFG1-CITC-DTPA MAbs. The patients' stage, disease state at time of treatment, amount of MAb and administered isotope radioactivity are given in Table 1. The study was approved by the Institutional Review Board of Hammersmith Hospital, and written, informed consent was obtained from each patient before entry to the experimental protocol. The study patients were compared with a group of 22 ovarian cancer patients receiving intraperitoneal treatment with ^{90}Y-DTPA MAbs for any clinical evidence of immunological toxicity (*1*).

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TABLE 1
Clinical Details of Patients Studied with ^{90}Y-HMFG1-CITC-DTPA MAb after Intraperitoneal Radioimmunotherapy

Patient no.	FIGO stage at Dx	Disease state	MAb amount in (mg)	Activity (mCi)
1	III	>2 cm	HMFG1/25	15.9
2	III	Widespread <2 cm	HMFG1/25	15.4
3	IV	MRD	HMFG1/25	19.6
4	III	CR	HMFG1/25	25.6
5	Ic	CR	HMFG1/25	25.1
6	IIa	CR	HMFG1/25	24.5
7	IV	Widespread <2 cm	HMFG1/25	31.4
8	III	MRD	HMFG1/25	30.3
9	IIc	CR	HMFG1/25	30.2
10	III	Unassessable	HMFG1/25	29.6
11	IIc	CR	HMFG1/25	26.1
12	Ia (re)	Unassessable	HMFG1/25	25.3

Dx = diagnosis; CR = complete remission as assessed laparoscopically, serologically and by conventional radiology; re = recurrent; MRD = minimal residual disease. The label used in all patients was ^{90}Y-CITC-DTPA.

Monoclonal Antibody, Conjugation and Radiolabeling

HMFG1 is a murine IgG1 antibody which binds to an epitope of polymorphic epithelial mucin (PEM). It was originally raised against human milk fat globule membranes normally expressed by the lactating breast, but also expressed by the majority (> 90%) of ovarian and other carcinomas (*6*). HMFG1 was conjugated to the bifunctional chelating agent CITC-DTPA using the method of Meares et al. (*7*). HMFG1 in 0.1 *M* sodium phosphate buffer, pH 8.5, was reacted overnight at 4°C or for 2 hr at room temperature with a minimum threefold molar excess of CITC-DTPA. Similarly, human serum albumin (HSA) was conjugated to CITC-DTPA for use as an antigen in an enzyme-linked immunosorbent assay (ELISA) to detect anti-chelate antibodies. HMFG1-CITC-DTPA was purified by centrifugation through a 1-ml Sephadex G-50 column using 0.1 *M* ammonium acetate, pH 5.5, as elution buffer. Yttrium-90, a pure beta-emitting isotope was supplied in 0.04 *M* HCl. One-molar ammonium acetate was added to the $^{90}YCl_3$ at a final concentration of 0.2 *M* with respect to acetate, resulting in pH 5.5. The required amount of immunoconjugate was added to this and reacted for 15 min at room temperature. At this point, the reaction mixture was made 5 m*M* with respect to EDTA and then purified on a 20-ml Sephadex G-50 column and eluted with phosphate-buffered saline (PBS), pH 7.4. The protein-containing fractions were pooled and sterilized by 0.22-μm micropore filtration. The total dose of protein was made up to 25 mg using unconjugated MAb. The rationale for standardizing all doses of administered antibody to 25 mg was that toxicity might not be manifested until 50 mCi ^{90}Y had been administered. An antibody dose of 25 mg would be equivalent to a specific activity of 2 μCi/μg. Samples of the immunoconjugate preparations were tested for sterility and pyrogenicity before administration to patients. Fast-pressure liquid chromatography (FPLC) analysis of immunoconjugates before and after radiolabeling revealed the presence of minimally aggregated material <5% (*8*).

ELISA for Anti-CITC-DTPA and Human Anti-Mouse Antibodies

Ninety-six well, flat-bottomed, ELISA microtiter plates were coated with 2.5 μg/ml of HSA, HSA-DTPA and HSA-CITC-DTPA in bicarbonate buffer, pH 9.6, at 4°C for 18 hr. After washing excess antigen, serial dilutions of each patient's serum (tenfold) in PBS/0.05% Tween-20 were applied and incubated at 37°C for 2 hr in a humidified chamber. After three washes in PBS/0.05% Tween-20, the plates were incubated with a horse raddish, peroxidase-conjugated, species-specific sheep anti-human immunoglobulin second reagent at a 1:1000 dilution in PBS/0.05% Tween-20 (100 μl/well) for 1 hr at 37°C. For IgM and IgG determinations, peroxidase-conjugated rabbit anti-human μ- and γ-chain specific reagents were used, respectively, at 1:500 dilution in PBS/0.05% Tween-20. Finally, plates were washed three times in PBS/0.05% Tween-20 and incubated with 100 μl/well of 2,2'-azino-di(3-ethyl-benzthiazolinesulfonate) substrate at room temperature and the absorbance was determined at 405 nm in a multiscan plate reader. Previously analyzed sera from patients receiving benzyl-DOTA immunoconjugates and developing an anti-benzyl-DOTA immune response were tested for crossreactivity to CITC-DTPA; the same ELISA was used.

Intra-assay and interassay reproducibilities were determined for three different sera. For interassay determination, each sample was tested five times by intra-assay and the mean value was used for calculations of interassay results. ELISAs for human anti-mouse antibodies (HAMA) were carried out according to the standard method (*5*). Category 1 is a response not higher than pre-existing HAMA levels; Category 2 moderate response (significantly elevated titers > 500 absorbance units at 405 nm); and Category 3 strong response with a prozone phenomenon.

In order to examine the specificity of binding of anti-CITC-DTPA antibodies, inhibition experiments were carried out. Briefly, the sera of patients positive for anti-CITC-DTPA antibodies were pre-incubated overnight at 4°C with increasing concentrations of HSA-CITC-DTPA (0, 1, 10, 100 μg/ml) and then applied on the ELISA plates as previously described. Equal volumes of buffer (0.1 *M* sodium phosphate), where the coupling reaction of CITC-DTPA to HSA was carried out, were also incubated with the same sera to test for binding inhibition caused by buffer alone.

Western Blotting

An amount of 5–10 μg of HSA, HSA-CITC-DTPA or HSA-DTPA was separated on 10% sodium dodecylsulphate-polyacryl-

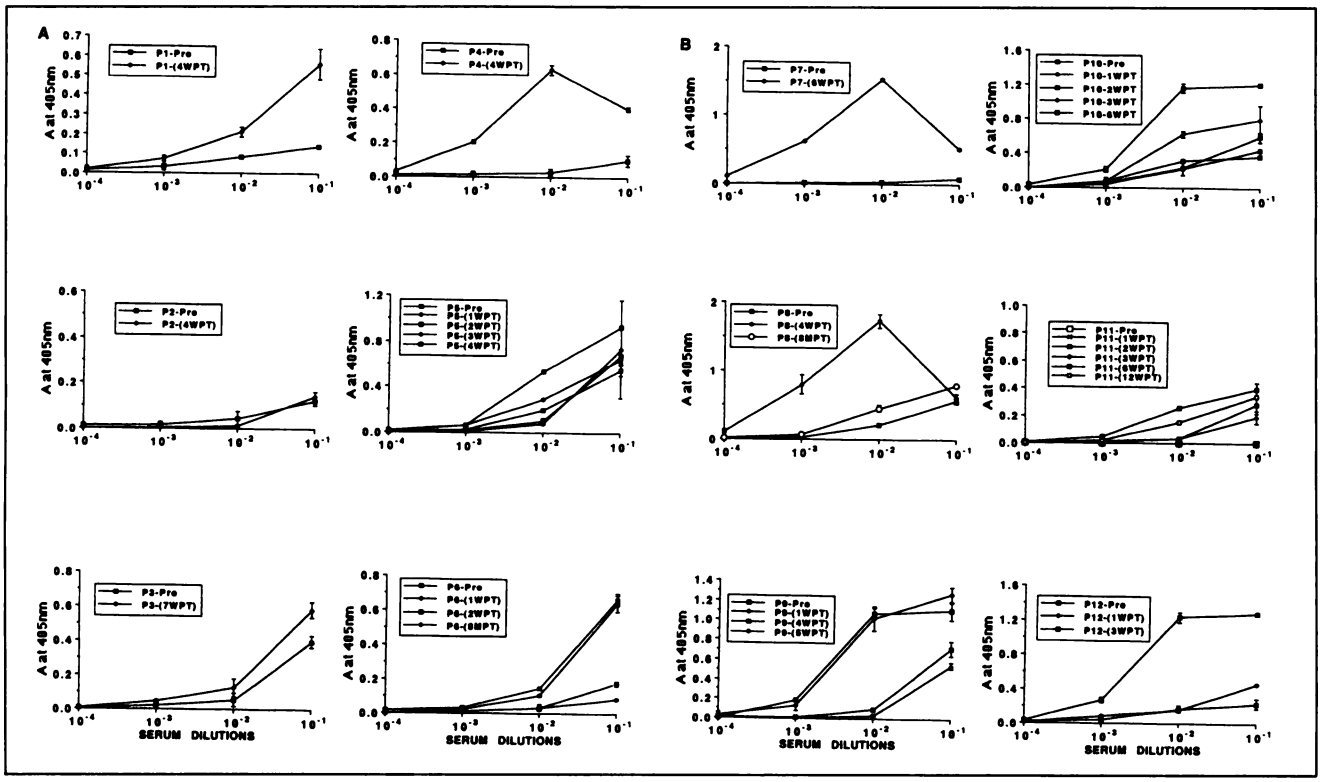

FIGURE 1. ELISA binding curves to CITC-DTPA pre- and post-therapy with intraperitoneal ^{90}Y-HMFG1-CITC-DTPA. All patients except P2 show statistically significant elevated anti-chelate antibody levels at least 2 wk post-therapy when compared to the pre-therapy levels. WPT = weeks post-therapy.

amide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose paper using the wet blot transfer technique described by Towbin et al. (*9*). The papers were then blocked for 10 min in tris buffered saline-Tween (TBST) buffer and for 30 min in TBST/1% bovine serum albumin (BSA) at room temperature, after which they were incubated with patient sera before or after CITC-DTPA-coupled HMFG1 MAb administration at 1:100 dilution overnight at 4°C. After three washes in TBST for 5 min each, the blots were incubated with an alkaline phosphatase-conjugated rabbit anti-human immunoglobulin second reagent at 1:1000 dilution in TBST for 30 min at room temperature under continuous rotation. After three washes, binding was detected by alkaline phosphatase substrate after adding 165 μl nitro-blue tetrazolium chloride (NBT) and 82.5 μl 5-bromo-4-chloro-3-indolyl-phosphate-p-toluidine salt (BCIP).

Statistical Analysis

For a positive result, post-therapy serum samples had to give statistically significant higher binding to CITC-DTPA than their corresponding pre-therapy samples. The pre- and post-therapy immune responses against CITC-DTPA were correlated using the Pearson correlation coefficient. The mean absorbance values (A at 405 nm) expressing binding of sera from different patients were compared using the paired Student's t-test. Continuous variables were analyzed for variance by calculating the F-ratio from ANOVA tables before performing intergroup comparisons with t-tests.

RESULTS

Development of Anti-CITC-DTPA Antibodies and HAMA

Eleven of twelve patients developed anti-chelate immune responses in comparison to the pre-existing anti-chelate antibody levels. Only Patient 2 did not develop elevated anti-CITC-DTPA antibody titers post-therapy (Fig. 1).

Clinical side effects were observed in five patients 10–14

TABLE 2
Spectrum of Clinical Reactions in Patients Receiving ^{90}Y-HMFG1-CITC-DTPA MAb Therapy

Patient no.	Clinical side effect
8	Polyarthralgias, cutaneous rash
9	Polyarthralgias, cutaneous rash, brachial plexopathy
11	Polyarthralgias, myalgias, brachial plexopathy
12	Polyarthralgias, myalgias, cutaneous rash
13*	Polyarthralgias, severe cutaneous rash.

*Patient 13 was not evaluated for anti-chelate antibodies because of lack of pre-therapy serum samples, but there was definite evidence for immunological toxicity.

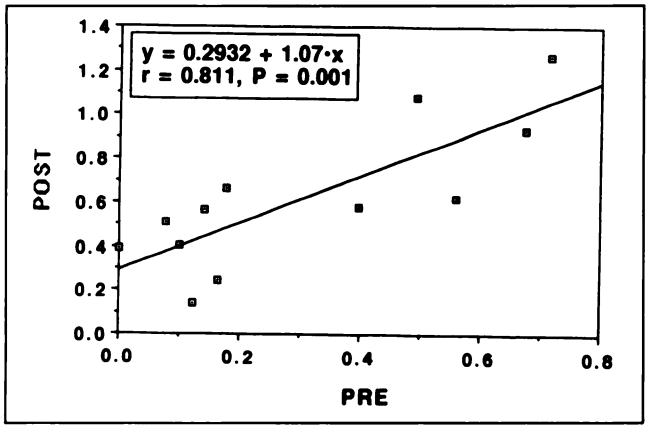

FIGURE 2. Correlation between pre- and post-therapy anti-CITC-DTPA antibody responses. A at 405 nm; absorbance at 405 nm.

days after ^{90}Y-CITC-DTPA immunoconjugate administration (Table 2). These consisted of a maculo-papular skin rash, polyarthralgias, myalgias and brachial plexopathy. An immune complex etiology for this reaction is indicated by a drop in serum complement component C3 and C4 levels 1–2 wk post-therapy, which is associated with an acquired reduction of CR1 complement receptor found on erythrocytes observed in two of our patients (data not shown). Elevated anti-CITC-DTPA antibody titers could be detected in serum as early as 2 wk post-treatment. It took 4 wks to detect elevated anti-chelate antibody titers in Patients 5 and 10. In contrast, a drop in anti-CITC-DTPA antibody titers could be detected 1 wk following ^{90}Y-HMFG1-CITC-DTPA administration (Fig. 1). A drop in anti-CITC-DTPA antibody titers in Patients 4, 6 and 8 was observed when studied long after MAb therapy: 12, 8, and 8 mo, respectively. Anti-CITC-DTPA antibody titers declined to pre-therapy levels in Patient 4 (Fig. 1).

A positive correlation was observed between the titers of pre-existing and maximum post-therapy anti-CITC-DTPA response; r = 0.811, p = 0.001 (Fig. 2). Inhibition of post-therapy serum binding to HSA-CITC-DTPA after pre-incubating the serum with HSA-CITC-DTPA correlated with the concentration of the chelate (Fig. 3). No inhibition was observed after incubating with HSA or buffer.

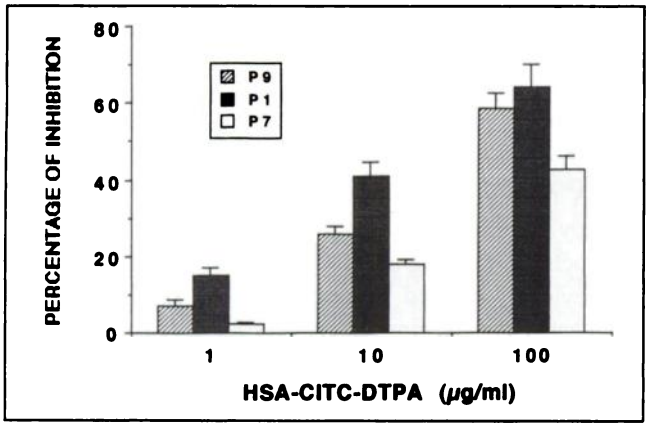

FIGURE 3. Binding inhibition of sera obtained from three patients who developed anti-CITC-DTPA antibodies after preincubation with increasing concentrations of soluble HSA-CITC-DTPA.

HAMA developed in all patients after ^{90}Y-HMFG1-CITC-DTPA administration. Seven patients developed a Category 2 and 5 developed category 3 (with prozone phenomenon) HAMA response. Patient 2 did not develop anti-CITC-DTPA response but was HAMA-positive.

In a previous study, none of our 22 patients with ovarian cancer receiving intraperitoneal MAb-DTPA-^{90}Y showed evidence of immunological toxicity despite the development of potent HAMA responses.

Development of IgM and IgG Anti-CITC-DTPA Responses. With the exception of Patients 1 and 9, all patients had very low or undetectable pre-existing IgG anti-chelate antibodies (Fig. 4). Most patients had predominantly IgM pre-existing anti-chelate antibodies, except Patients 7 and 11, who had undetectable pre-existing responses (Fig. 4). IgM antibodies increased as early as 2 wk post-therapy in Patients 5 and 6 and remained unchanged or minimally elevated 4, 6 and 7 wk in Patients 8, 7 and 1, respectively. IgG responses were elevated at least 3 wk post-therapy.

Cross-reactivity. Sera from patients binding to CITC-DTPA crossreacted with DTPA. Mean binding of patients' sera obtained pre-, post-1 wk and post-3 wk or more to CITC-DTPA was significantly higher than to DTPA: $p = 0.007$, $p = 0.026$ and $p \leq 0.0001$, respectively, at 10^{-1} dilution as well as $p = 0.017$, p = nonsignificant, and $p \leq 0.0001$, respectively at 10^{-2} dilution (Fig. 5).

A positive correlation was observed between the maximum post-therapy anti-CITC-DTPA and anti-DTPA serum reactivity ($r = 0.885$, $p \leq 0.0001$ at 10^{-1} titer, and $r = 0.861$, $p = 0.001$ at 10^{-2} titer). Sera from patients treated with ^{90}Y-HMFG1-benzyl-DOTA-^{90}Y and positive for anti-DOTA antibodies from our previous study (*5*) were tested for cross-reactivity with CITC-DTPA. Three of five Patients (1, 3, 4) had cross-reactive anti-CITC-DTPA antibodies (Fig. 6). On the other hand, sera from patients with anti-CITC-DTPA antibodies did not cross-react with DOTA (data not shown).

Western Blotting. Sera obtained from Patients 7 and 8 after intraperitoneal ^{90}Y-HMFG1-CITC-DTPA radioimmunotherapy were found to detect a Mr = 60 kDa band, which was consistent with the molecular weight of HSA (since the molecular weights of the chelates coupled to HSA are negligible compared to HSA only in the lanes where HSA-CITC-DTPA had run). There was no detectable reaction to HSA or HSA-DTPA. Pre-therapy sera of these patients did not bind to HSA-CITC-DTPA (Fig. 7).

DISCUSSION

This study shows that the chelating agent CITC-DTPA is immunogenic in patients and its immunogenicity is of clinical significance after one intraperitoneal administration of

study, however, did not cross-react with benzyl-DOTA. A theoretical possibility exists of repeating treatment in patients who received ^{90}Y-CITC-DTPA labeled MAbs with ^{90}Y-benzyl-DOTA labeled MAbs but not the reverse, which would carry the risk of secondary immune response to CITC-DTPA due to high levels of anti-CITC-DTPA cross-reactive antibodies. We do not know if DTPA is also immunogenic when coupled to antibodies because we have never tested this comprehensively. An acute post-treatment white blood cell count decrease on Day 7 followed by recovery prior to the radiation toxicity effect was documented in at least four of the six patients that manifested bone marrow toxicity (data not shown). Three of these patients also had clinical evidence of hypersensitivity syndrome. In another study, when HAMA was administered post-intraperitoneal injection of MAbs to enhance clearance of the radioactive antibody entering the systemic circulation and therefore reduce bone marrow toxicity, a drop in the white cell count was observed 20–30 min postinjection and preceded by the formation of immune complexes (*19*). In the present study, the drop in white cell counts was observed 1 wk post-treatment, a time when systemic immune complex formation had probably occurred as suggested by the parallel drop in anti-CITC-DTPA antibodies and clinical immune manifestations on Days 9–11. Increased lung margination as a result of C5a complement fragment binding to the cell surface of neutrophils and monocytes has been postulated as the underlying mechanism. Similar observations of systemic complement activation and C5a binding to neutrophils have been made in patients on hemodialysis and cardiopulmonary bypass (*20*). In the study of Vriesendorp et al. (*2*) a drop in white cells (mainly lymphocytes) was observed in some patients about 5–7 days after systemic infusion of the radioimmunoconjugate. This was observed much earlier than the expected myeloablative effect of 20–40 mCi of ^{90}Y-labeled antibody occurring 3–4 wk post-treatment. Although studies addressing benzyl-DTPA immunogenicity were not performed, many alternative explanations could be offered for this rapid lymphocyte drop, such as a radiation-induced effect or lymphocyte targeting by antiferritin antibodies.

The clinical syndrome observed in our patients was a type III immune complex-mediated reaction. The histologic lesion of immune complex disease is an acute inflammatory necrotizing angiitis with antigen-antibody complexes and complement deposited in the vessel wall (*21*). In the natural history of serum sickness, successive crops of new lesions appear over a 7–10-day period beginning 9–11 days after serum injection (*22*). This reflects successive precipitation of new immune complexes as antibodies to the different antigenic proteins present in crude serum make their appearance. When serum sickness follows the injection of a single antigen, the illness is monophasic. A rather unexpected observation in our patients with serum sickness was the development of brachial neuritis in two of them. There is reason to believe that immune complex-produced lesions in blood vessels may occur in the peripheral nervous system during serum sickness as a part of a generalized affliction. Characteristically the neuropathy of serum sickness, which is an uncommon complication of this syndrome, manifests itself as brachial neuropathy, and there is no correlation between the severity of serum sickness and the occurrence or severity of neurologic complications (*22*). Subclinical involvement of peripheral nerves in serum sickness is doubtless much more common than frank neuropathy (*22*). Passive (serotherapy) or active (vaccination) immunizations and viral infectious syndromes are well recognized causes of brachial neuropathy.

CONCLUSION

We have observed the development of humoral immune responses against the chelating agent CITC-DTPA in patients receiving intraperitoneal ^{90}Y-CITC-DTPA-labeled HMFG1 MAb for ovarian cancer therapy. This study extends our previous observations and experience using this drug, in which patients developed antibodies against the DOTA and serum sickness. These agents were employed as being more stable than conventional DTPA in chelating ^{90}Y and therefore could reduce bone marrow toxicity from free ^{90}Y deposited in the bone, thus allowing dose escalation of administered radioactivity. Doses of ^{90}Y (up to 30 mCi) were given with acceptable hematologic toxicity (*23*). Additionally, we previously showed that patients treated with ^{90}Y-HMFG1 labeled with ^{90}Y via stable chelating agents such as DOTA and CITC-DTPA developed more potent T-cell proliferative responses to the administered MAb as antigen when compared with patients treated with conventionally labeled MoAbs (e.g., ^{131}I and ^{90}Y-DTPA) (*24*). This finding strengthens the current hypothesis that an immunogenic hapten (chelate) enhances the immunogenicity of the carrier (MAb).

In our previous study, the macrocycle structure had been implicated for inducing immune responses. CITC-DTPA, an analog of benzyl-DTPA, does not have this, so we are studying the possibility of a benzene ring or a unique conformation of the CITC-DTPA molecule acting as an hapten. Analysis of in vivo immune complex formation and serum complement measurements are currently under investigation.

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