Development of Humoral Immune Responses against a Macrocyclic Chelating Agent (DOTA) in Cancer Patients Receiving Radioimmunoconjugates for Imaging and Therapy

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ABSTRACT

The development of stable immunoconjugates by the advent of macrocyclic metal chelating agents (DOTA) has enabled us to study the ability of ¹¹¹In-DOTA-labeled monoclonal antibodies to detect tumor lesions in a pilot radioimmunolocalization study, as well as to evaluate the kinetics, toxicity, and efficacy of i.p. administered ⁹⁰Y-DOTA-labeled murine monoclonal antibody in a Phase I/II clinical trial of advanced ovarian cancer. The development of serum sickness-like reactions in three of six treated patients, in the absence of previous monoclonal antibody administration, led us to study the potential immunogenicity of the new chelate.

Six patients with ovarian cancer received 25 mg of HMFG1 monoclonal antibody coupled with ⁹⁰Y-DOTA (doses of radioactivity, 15 to 25 mCi), administered i.p. Eight patients with various malignant tumors received low doses (220 µg to 1 mg) of monoclonal antibodies, labeled with ¹¹¹In-DOTA, i.v. for imaging studies.

Using a solid-phase enzyme-linked immunosorbent assay method, the immunogenicity of DOTA was evaluated. Serial dilutions of patients' sera, before and after imaging or therapy with DOTA-coupled monoclonal antibodies, as well as sera from patients who did not receive DOTA-coupled antibody, were screened on enzyme-linked immunosorbent assay plates coated with human serum albumin (HSA), HSA-2-iminothiolane, and HSA-2-iminothiolane-benzyl-DOTA. All patients treated with i.p. monoclonal antibody developed anti-DOTA antibodies. Four of eight patients who received i.v. "imaging" doses of DOTA-coupled monoclonal antibody developed antibodies against DOTA. The levels of anti-DOTA response correlated with the amount of injected radioimmunoconjugate.

Tumor-associated radioactively labeled mAbs have been evaluated in a number of Phase I/II therapeutic clinical trials in different types of malignancies (1, 2). Studies from our group have focused on the efficacy of i.p. radioimmunotherapy of ovarian cancer with ¹³¹I- or ⁹⁰Y-labeled mAbs. Although ¹³¹I was the first isotope used in radioimmunotherapy, the emission of unwanted γ-radiation, its β-particles of low energy (3), and the potential decrease of its biological half-life in the tumor area by metabolic processes (4) make it less than ideal for antibody-guided radiotherapy. ⁹⁰Y has been suggested as an alternative isotope, as it is a pure β-emitter and not subject to tissue-mediated specific detachment from the antibody. Labeling of the mAb with ⁹⁰Y can be achieved by the use of bifunctional chelating agents, such as DTPA (5).

With both radiolabels, myelosuppression limits the mAb-associated radioactivity that can be safely administered. The myelosuppression following ¹³¹I-labeled antibody was predictable and directly correlated to the radiation dose the bone marrow received from circulating radiolabeled mAb. Activities of ¹³¹I up to 100 mCi could be administered with negligible myelotoxicity, but significant toxicity was observed at doses of 150 mCi (6). The myelosuppression observed with ⁹⁰Y-DTPA-labeled antibody did not correlate with the calculated radiation dose to the bone marrow from circulating radioimmunoconjugate (7). This is probably due to the instability of ⁹⁰Y chelation by DTPA, leading to in vivo release of the isotope. Since ⁹⁰Y is "bone-seeking" (8), it can deposit in the bone matrix, leading to non-specific irradiation of the bone marrow. Moderate to severe myelotoxicity was observed with administered activities, >15 mCi (7). This problem could be partially circumvented by the systemic administration of calcium disodium EDTA, which can chelate free ⁹⁰Y in the serum and increase its urinary excretion (7).

A better solution to the above-mentioned problems should be the clinical application of the new generation of more stable chelating agents (9), such as the macrocyclic bifunctional chelating agent DOTA. DOTA has been shown in previous in vitro studies (9, 10) and preclinical (11-13) studies to result in the production of stable radioimmunoconjugates. We carried out a Phase I/II trial of radioimmunotherapy using ⁹⁰Y-DOTA-HMFG1 mAb in patients with advanced ovarian cancer (14).

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2 To whom requests for reprints should be addressed, at ICRF Oncology Group, Department of Clinical Oncology, 3rd Floor MRC Bldg., Hammersmith Hospital, Du Cane Road, London W12 ONN, England.

INTRODUCTION

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With both radiolabels, myelosuppression limits the mAb-associated radioactivity that can be safely administered. The myelosuppression following ¹³¹I-labeled antibody was predictable and directly correlated to the radiation dose the bone marrow received from circulating radiolabeled mAb. Activities of ¹³¹I up to 100 mCi could be administered with negligible myelotoxicity, but significant toxicity was observed at doses of 150 mCi (6). The myelosuppression observed with ⁹⁰Y-DTPA-labeled antibody did not correlate with the calculated radiation dose to the bone marrow from circulating radioimmunoconjugate (7). This is probably due to the instability of ⁹⁰Y chelation by DTPA, leading to in vivo release of the isotope. Since ⁹⁰Y is "bone-seeking" (8), it can deposit in the bone matrix, leading to non-specific irradiation of the bone marrow. Moderate to severe myelotoxicity was observed with administered activities, >15 mCi (7). This problem could be partially circumvented by the systemic administration of calcium disodium EDTA, which can chelate free ⁹⁰Y in the serum and increase its urinary excretion (7).

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1¹¹¹In-labeled, DOTA-coupled murine and reshaped human antibodies

2 The abbreviations used are: mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; HSA, human serum albumin; 2IT, 2-iminothiolane; DTPA, diethylenetriaminepentaacetic acid; nitrobenzyl-DOTA, (2-p-nitrobenzyl)-1,4,7,10-tetraazaacycloodecane-N,N,N'-N''-N'''-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PLAP, placental alkaline phosphatase; IgG1, immunoglobulin G1; IgM, immunoglobulin M; BABE, β-bromoacetamidoethylenediamine; BABA, β-bromoacetamidobenzyl; BABE, (S)-2-(/>-bromoacetamidobenzyl)-EDTA; RSA, rabbit serum albumin; TBST, 10 mM Tris base:150 mM NaCl (pH 8.0):0.05% Tween 20; BSA, bovine serum albumin; HAMA, human anti-mouse antibody.
were also evaluated in pilot radioimmunolocalization studies (15). In none of the early clinical studies was the immunogenicity of DOTA addressed. Although the problem of immunogenicity of murine (16, 17) and chimeric (15) mAbs has been extensively studied by many groups, there are no data addressing the ability of antibody-coupled metal chelating agents to elicit immune responses in humans.

MATERIALS AND METHODS

Patient Details

Six patients with epithelial ovarian cancer aged between 50 and 72 yr received i.p. radioimmunotherapy with 90Y-DOTA-HMFG1 (Table 1). All patients were initially treated with cytoreductive surgery and 5 cycles of platinum-based chemotherapy. Treatment procedures have been previously described (1, 6, 7, 14).

The eight patients entering the immunoscintigraphy study with i.v. 111In-DOTA-labeled mAbs were aged between 16 and 65 yr and had undergone prior surgery and chemotherapy (Table 2). Patient 14 had previously been treated twice with mouse mAbs administered i.p. for recurrent ovarian cancer. Patient 5 had previously received 111In-DOTA-H17E2 for radioimmunoscintigraphy. Details of the administered mAb doses and radioactivity are shown in Tables 1 and 2.

These studies were approved by the Hammersmith Hospital Ethics Committee, and written informed consent was obtained from all patients.

Monoclonal Antibodies

HMFG1. This is a murine IgG1 antibody which binds to a mucin molecule normally produced by the lactating breast, but also expressed by the majority (>90%) of ovarian, breast, and other carcinomas (18).

H17E2. This is a murine IgG1 antibody directed against PLAP. This enzyme is expressed as a surface membrane antigen on many neoplasms, including 60 to 85% of ovarian carcinomas, as well as testicular germ cell tumors (19).

Hu2PLAP. This is a humanized mAb having the same specificity as the murine H17E2 mAb. It was produced by transplanting the genes of germ cell tumors (19).

Radiolabeling of Antibodies

Antibody HMFG1 was conjugated to the macrocycle DOTA using the one-step procedure of McCall et al. (21), which uses the linker 2-IT and the bifunctional chelating agent (S)-2-(p-bromoacetamidobenzyl)-DOTA. The average degree of conjugation was 2.6 to 2.9 available DOTA groups per HMFG1 antibody, with 3.0 to 5.2% aggregates (mainly antibody dimers) present in the conjugate. Six aliquots of the preparation were quickly frozen, packed in dry ice, and shipped by air from California to London. Antibodies H17E2 and Hu2PLAP were conjugated to DOTA using the two-step procedure of Moi et al. (22) including capping unreacted sulfhydryl groups with iodoacetamide. The average degree of conjugation was 1.3 and 1.0 available DOTA groups per H17E2 and Hu2PLAP antibody, respectively, with <16% aggregates (mainly antibody dimers) present in the conjugate.

Human serum albumin was conjugated with 2-iminothiolane and either (S)-2-(p-bromoacetamidobenzyl)-1,4,7,10-tetraazacyclododecane-N,N',N''-tetraacetic acid (3.6 chelators/albumin) (21) or HSA-BABA (HSA-benzoate) (3 benzoates/albumin), or HSA-BABE (HSA-benzyl-EDTA) (3.3 chelators/albumin), in order to investigate the nature of the immune response. HSA-2IT was prepared by capping the unreacted sulfhydryl groups using iodoacetamide immediately after their introduction. Each conjugate contained approximately 10% albumin dimers and higher aggregates.

Labeling with 90Y (A. E. R. E. Harwell, United Kingdom) and 111In (Amersham International, Amersham, United Kingdom) was carried out by applying standard procedures, as already described (21).

Immunocomplexes were assayed using purified antigen (HMFG for HMFG1 mAb conjugates and PLAP for H17E2 or Hu2PLAP conjugates). The immunoreactivity of HMFG1 conjugates was tested by ELISA on HMFG-coated plates and was comparable to that of the unconjugated HMFG1 antibody. The immunoreactivity of H17E2 and Hu2PLAP conjugates was tested by competition radioimmunoassay on PLAP-coated plates and was found to be >85% that of the unconjugated antibodies.

Table 1  Clinical details of patients studied for the development of anti-DOTA antibodies after i.p. radioimmunotherapy

<table>
<thead>
<tr>
<th>Patient</th>
<th>FIGO* stage at diagnosis</th>
<th>Disease state</th>
<th>mAb/amount</th>
<th>Label</th>
<th>Activity (mCi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>III</td>
<td>CR</td>
<td>HMFG1/25</td>
<td>DOTA-2IT-90Y</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>III</td>
<td>CR</td>
<td>HMFG1/25</td>
<td>DOTA-2IT-90Y</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>III</td>
<td>CR</td>
<td>HMFG1/25</td>
<td>DOTA-2IT-90Y</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>Ic + cancer of the endometrium</td>
<td>CR</td>
<td>HMFG1/25</td>
<td>DOTA-2IT-90Y</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>Ic+ nodes (&lt;1 cm)</td>
<td>Microscopic</td>
<td>HMFG1/25</td>
<td>DOTA-2IT-90Y</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>la recurred positive washings</td>
<td>Small nodules</td>
<td>HMFG1/25</td>
<td>DOTA-2IT-90Y</td>
<td>15</td>
</tr>
</tbody>
</table>

* FIGO, Federation Internationale de Gynecologie et d'Obstetrique.

** CR, complete remission as assessed laparoscopically, serologically, and by conventional radiology.

Table 2  Clinical details of patients studied for the development of anti-DOTA antibodies after i.v. radioimmunolocalisation

<table>
<thead>
<tr>
<th>Patient</th>
<th>Disease</th>
<th>FIGO stage</th>
<th>Extent of disease at scan</th>
<th>mAb/amount</th>
<th>Label</th>
<th>Activity (mCi)</th>
</tr>
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<tbody>
<tr>
<td>7</td>
<td>Ovarian cancer</td>
<td>Ia</td>
<td>Recurrent</td>
<td>Hu2PLAP/883</td>
<td>DOTA-2IT-111In</td>
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<tr>
<td>8</td>
<td>Testicular seminoma</td>
<td>III</td>
<td>CR</td>
<td>H17E2/500</td>
<td>DOTA-2IT-111In</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>Ovarian cancer</td>
<td>III</td>
<td>CR</td>
<td>Hu2PLAP/250</td>
<td>DOTA-2IT-111In</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>Ovarian cancer</td>
<td>III</td>
<td>CR</td>
<td>HMFG1/1</td>
<td>DOTA-2IT-111In</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>Breast cancer</td>
<td>IV</td>
<td>Malignant effusion</td>
<td>Hu2PLAP/765</td>
<td>DOTA-2IT-111In</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>Ovarian cancer</td>
<td>IIIc</td>
<td>CR</td>
<td>Hu2PLAP/765</td>
<td>DOTA-2IT-111In</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>Gastric cancer</td>
<td>IV</td>
<td>Liver metastases</td>
<td>Hu2PLAP/250</td>
<td>DOTA-2IT-111In</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>Ovarian cancer</td>
<td>IIIc</td>
<td>Ascites + s2-cm nodules</td>
<td>Hu2PLAP/220</td>
<td>DOTA-2IT-111In</td>
<td>1</td>
</tr>
</tbody>
</table>

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** CR, complete remission as assessed laparoscopically, serologically, and by conventional radiology.
ELISA for Anti-DOTA Antibodies

Flat-bottomed, 96-well microtiter plates were coated for 2 h at 37°C in a humidified chamber, or at 4°C for 18 h, with 2.5 μg/ml of HSA, HSA-2IT, and HSA-2IT-DOTA in bicarbonate buffer, pH 9.6. Control wells were treated with bicarbonate buffer alone in order to determine nonspecific binding of serum immunoglobulin to the plates. After washing the plates, serial dilutions of each patient's serum (10-fold) in PBS/0.05% Tween 20 were applied and incubated at 37°C for 2 h in a humidified chamber. After three washes in PBS/0.05% Tween 20, the plates were incubated with a peroxidase-conjugated, species-specific sheep anti-human immunoglobulin second reagent (Amersham International, United Kingdom) at 1:1000 dilution in PBS/0.05% Tween 20 (100 μl/well) for 1 h at 37°C. For IgM and IgG determinations, peroxidase-conjugated rabbit anti-human μ- and γ-chain-specific reagents (Dako, Copenhagen, Denmark) were used, respectively, at 1:500 dilution in PBS/0.05% Tween 20. Plates were then washed 3 times and incubated at room temperature with 100 μl/well of 2,2'-azino-di(3-ethyl-benzthiazolinesulfonate) (Amersham International), and the absorbance was determined in a Titertek multispan plate reader (Flow, Irvine, Scotland) at 405 nm. Antiserum against DOTA was raised after immunizing rabbits with DOTA coupled to RSA (gift from Dr. Dennis Allen, Unilever Research Labs., Bedford, United Kingdom) and was used as a positive control. In this case, a peroxidase-conjugated swine anti-rabbit immunoglobulin second reagent was applied (1:500 in PBS/0.05% Tween 20).

In the ELISA described above, each patient's serum was tested against (a) no antigen to determine any nonspecific binding on the plates, (b) HSA in order to determine if there is a preexisting response against HSA, (c) HSA-2IT to test for the development of antibodies against the 21T linker, and (d) HSA-2IT-DOTA to test the response against the macrocycle DOTA. Some control wells were incubated with PBS/0.05% Tween 20 instead of serum, after which only the second layer reagent was added, in order to determine any cross-reactivity between the above antigens and the second layer reagent. Intraassay and interassay reproducibilities were determined for four different sera. For interassay determination, each sample was tested 5 times by intraassay, and the mean value was used for calculations of interassay results.

In some experiments, in order to define the restriction of the immune response against DOTA, patients' sera were tested against HSA-2IT-BABE, HSA-2IT-BABA, and HSA-2IT-DOTA applying the same ELISA as already described.

Inhibition Experiments

The sera of patients positive for anti-DOTA antibodies were preincubated overnight at 4°C with increasing concentrations of HSA-2IT-DOTA (0, 1, 10, and 100 μg/ml) and then applied on the ELISA plates, as previously described. Equal volumes of buffer (0.1 M ammonium acetate), in which the coupling reaction of DOTA to HSA-2IT was performed, were also incubated with the same sera in order to test for inhibition of binding caused by buffer alone. In parallel experiments, sera from these patients were incubated with HSA and HSA-2IT at the same concentrations as with HSA-2IT-DOTA and subsequently tested by ELISA, as described above.

Western Blotting

Ten μg of HSA, HSA-2IT, and HSA-2IT-DOTA were separated on 7.5% SDS-PAGE and transferred onto nitrocellulose paper, using the wet blot transfer technique of Towbin et al. (23). The nitrocellulose papers were then blocked at room temperature for 10 min in TBST buffer and for 30 min in TBST/1% BSA, after which they were incubated with sera from patients before or after DOTA-coupled mAb administration, at 1:100 dilution in TBST at 4°C overnight. After three washes, for 5 min each in TBST, the blots were incubated with an alkaline-conjugated rabbit anti-human immunoglobulin second reagent, 1:1000 dilution in TBST, for 30 min at room temperature, under continuous rotation. After three washes in TBST, the reaction was detected by alkaline phosphatase substrate [165 μl of nitroblue tetrazolium chloride were added in 25 ml of substrate buffer (100 mM Tris base:100 mM NaCl:5 mM MgCl2, pH 9.5) and, after mixing 82.5 μl of 5-bromo-4-chloro-3-indoly-phosphate were added for color development]. The reaction was stopped after 1 to 5 min by transferring the blots to distilled water.

Purification of Anti-DOTA Immunoglobulin from Patients' Sera

One g of cyanogen bromide-activated Sepharose 6MB (Pharmacia LKB, Uppsala, Sweden) beads was swollen for 15 min and washed in a sintered glass funnel, using 200 ml of 1 mM HCl. HSA-2IT-DOTA was used as a ligand after dissolving in coupling buffer (0.1 M NaHCO3:0.5 M NaCl, pH 8.3) and then added to the swollen gel, which had been transferred to coupling buffer by brief washing with 20 ml of buffer per ml of gel, and the mixture was placed on a rotator overnight at 4°C. The ligand/gel coupling ratio was 2 mg of HSA-2IT-DOTA per ml of gel. After overnight incubation of ligand/gel, the mixture was washed 3 times using each time 0.1 M acetate buffer, pH 4.5, containing 0.5 M NaCl (10 ml/ml of gel) and coupling buffer (10 ml/ml of gel), in order to wash away excess noncovalently bound ligand. The remaining reactive groups were blocked by transferring the gel to coupling buffer containing 0.2 M glycine and by incubating with end-over-end mixing for 2 h at room temperature. Excess blocking agent was washed with coupling buffer, 10 ml/ml of gel. The HSA-2IT-DOTA-coupled beads were mixed with the patient's serum and placed on a rotator for 2 h at room temperature, after which the beads were packed into a suitable column and any unbound material was removed using PBS. When the PBS-eluted fractions had an absorbance of zero, at 280 nm, the bound material was eluted with 1 M propionic acid, which was neutralized after passing through the column with 1 M Tris buffer, pH 10. Fractions of 0.5 ml were collected until the protein peak had been eluted. The pooled protein was dialyzed overnight in PBS, pH 7.4, and analyzed by SDS-PAGE under nonreducing conditions and by ELISA for anti-DOTA immunoreactivity. A single Mr, 150,000 band was seen in a Coomassie-stained gel corresponding to human IgG. Another band of high molecular weight, not entering the gel, was seen, presumably corresponding to human IgM (data not shown).

Quantitation of Anti-DOTA Immunoglobulins in Patients' Sera

Sera from patients positive for binding to DOTA were applied to 96-well, flat-bottomed microtiter plates, coated with HSA-2IT and HSA-2IT-DOTA, at serial dilutions in parallel with standard concentrations of purified anti-DOTA immunoglobulin, and tested as in the already described ELISA for anti-DOTA antibodies. A standard curve was constructed after plotting the absorbance at 405 nm versus the log of anti-DOTA antibody concentration. The linear portion of the curve was used to calculate the amount of anti-DOTA antibodies in the serum. Serum dilutions giving absorbance values falling within the range of the linear portion of the curve were used in order to calculate the amount of anti-DOTA antibodies. Quantitation of anti-DOTA antibodies in the serum of these patients carries the same problems as the quantitation of any polyclonal immune response after an antigen challenge. Since every individual responds in a different way to an antigenic stimulus, by raising different amounts of high- and low-affinity antibodies, the purified standard antibody preparation should represent all the different affinities present. However, this is not always feasible.

ELISA for HAMA and Antiidiotypic Antibody

The ELISA methods used for measuring HAMA and antiidiotypic antibodies have been previously described (17). HAMA responses were subdivided into three categories: Category 1 is a response not higher than preexisting HAMA levels; Category 2 is a moderate response; and Category 3 is a strong response with a prozone phenomenon (17). HAMA levels in the serum were quantitated after purifying anti-mouse immunoglobulin from patients after one mAb therapy. HMFG1 antibody was coupled to cyanogen bromide-activated Sepharose 6MB beads. HAMA was purified and quantitated using the same methodology described for anti-DOTA antibodies.

For antiidiotypic antibody determination, the serum of each patient
was tested against the murine mAb which was administered for imaging or therapy and two other murine mAbs of the same isotype (IgG1) with the former, but of irrelevant specificity.

Statistical Analysis

ELISA positivity expressed as absorbance (at 405 nm) measured for sera tested in the same assay was correlated with the dose in µg of DOTA-coupled mAb, which was injected for immunoscintigraphy, using the Pearson correlation coefficient.

The mean values of absorbances (at 405 nm), expressing binding of sera from different patients, to HSA-benzyl-DOTA, HSA-benzyl-EDTA, and HSA-benzoate, tested in the same assay, as well as the mean percentages of inhibition with 1, 10, and 100 µg/ml, were compared using the unpaired Student t test. Continuous variables were analyzed for variance, by calculating the F ratio, applying analysis of variance tables, before performing intergroup comparisons by t test.

RESULTS

Development of Anti-DOTA Antibodies. All six patients receiving i.p. radioimmunotherapy with ⁹⁰Y-DOTA-HMFG1 developed anti-DOTA antibodies in their serum (Fig. 1).Patient 3 developed high titers of anti-DOTA antibodies with a marked prozone effect (Fig. 1). Patient 2 developed low titers of anti-DOTA antibodies. None of the sera obtained from these patients before mAb treatment contained antibodies against DOTA. Three patients (Nos. 1, 3, and 4) developed clinical and laboratory evidence of serum sickness-like reactions 10 to 14 days after radioimmunoconjugate administration, with widespread erythematous rashes with photosensitive distribution, generalized myalgias, and arthralgias which resolved completely after 3 to 4 wk. Serum complement C3 and C4 levels were decreased 2 wk after therapy and returned to normal after the third wk (not shown). In addition to these symptoms, Patient 3 developed a sixth cranial nerve palsy, thought to be due to vasa nervorum involvement from small vessel vasculitis. This reaction occurred in the absence of significant preexisting HAMA levels or previous exposure to murine antibodies. There were no pyrogens and <5% aggregates in the ⁹⁰Y-DOTA-HMFG1 preparations.

Four of eight patients who entered into the “imaging” study developed antibodies against DOTA (Fig. 2). In none of the positive sera was a prozone effect observed. Two of six patients receiving the reshaped human ¹¹¹In-DOTA-Hu2PLAP developed anti-DOTA antibodies. The two patients, who had previously received murine mAbs, ¹¹¹In-DOTA-H17E2 and -HMFG1, developed anti-DOTA antibodies. There were no detectable antibodies against DOTA in the sera of the above patients obtained before antibody injection. None of the patients undergoing diagnostic immunolocalization studies with DOTA-coupled antibodies developed side effects, such as serum sickness-like reaction, as observed in the therapy patients. The intra- and interassay variances were between 1.5 and 5%. No detectable anti-DOTA antibodies were found in a control group of patients that did not enter into the diagnostic or therapy protocols with DOTA-coupled mAbs. Results of a representative serum sample are shown for each patient in Figs. 1 and 2.

Anti-DOTA responses could be detected 10 to 14 days after immunonjugate administration. At the last follow-up (approximately 18 mo after therapy), patients' sera remain positive for anti-DOTA antibodies.

Sera were tested against HSA, HSA-2IT, and HSA-2IT-DOTA, and no detectable immune response against HSA or HSA-2IT was observed (data not shown). Results of quantitation of anti-DOTA immunoglobulins in the sera of these patients are shown in Table 3.

IgM and IgG Antibodies against DOTA. Patients developed both IgM and IgG anti-DOTA antibodies with variable titers,

![Graph](image-url)

**Table 3** Quantitation of HAMA and anti-DOTA immunoglobulin levels in patients' sera after ⁹⁰Y or ¹¹¹In-DOTA-mAb administration

<table>
<thead>
<tr>
<th>Patient</th>
<th>HAMA levels (µg/ml) in serum</th>
<th>anti-DOTA* immunoglobulin level (µg/ml) in serum</th>
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<td>1</td>
<td>2730</td>
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</tbody>
</table>

*Patients 9, 11, 13, and 14 did not develop anti-DOTA antibodies. Patient 14 had already high HAMA levels (4.84 mg/ml due to two previous treatments with mAbs). Patients 9, 11, and 13 had low levels of HAMA (0.74, 1.7, and 4.3 µg/ml, respectively) that were not higher than their preexisting HAMA levels. Statistical correlation between HAMA and anti-DOTA levels was significantly for those developing both responses (r = 0.79, P = 0.007).
ANTI-DOTA ANTIBODIES AFTER RADIOIMMUNOCONJUGATE ADMINISTRATION

depending on the time studied, after immunoconjugate administration (Fig. 3). Initial responses, 3 to 4 wk post-mAb administration, were mainly IgM with very low or undetectable IgG (Fig. 3, A and B). Responses, after 2 to 4 mo, contained high levels of IgG anti-DOTA antibodies with varying levels of IgM antibodies (Fig. 3). Patient 3 developed very high levels of IgG anti-DOTA antibodies 3 to 4 wk posttreatment (Fig. 3C) and had a prozone effect when tested with anti-human immunoglobulin second reagent (Fig. 1). Four mo after treatment the IgG titers remained high, but IgM titers were very low. Patient 10, undergoing immunolocalization studies with 111In-DOTA-HMFG1, developed only IgM anti-DOTA antibodies, persisting up to 10 mo after mAb administration. There were no detectable preexisting IgM or IgG anti-DOTA antibodies in any of these patients.

Correlation between the i.v. mAb Dose and ELISA Positivity for anti-DOTA Antibodies. The 8 patients entering the immunolocalization study received doses of i.v. mAb between 220 and 1000 µg and, therefore, varying absolute amounts of the chelating agent attached to it. Their post-mAb administration sera were tested in parallel in the same ELISA experiment. A strongly positive statistical correlation was found ($r = 0.889, P < 0.001$) between the mAb dose in µg and the absorbance at 405 nm (Fig. 4). One of these patients (Patient 11) receiving 765 µg of 111In-DOTA-Hu2PLAP was excluded from this analysis, since the only available serum sample, obtained 4 days postimaging, was negative and the patient was then lost to follow-up.

Restriction of the Immune Response against DOTA. In order to define to which part of the chelating agent the immune response was directed, patients’ sera were tested against HSA-benzyl-EDTA, HSA-benzyl-DOTA, and HSA coupled to bromoacetamidobenzoic acid via 2IT, since the chelating agent benzyl-DOTA consists of a benzyl ring attached to the macrocycle group. The response was found to be directed mainly against the macrocycle and very little against benzyl-EDTA and the benzoate group. Statistically significant differences were observed at all serum dilutions and were $P < 0.001, P < 0.01$, $P < 0.05$, and $P < 0.01$ at $10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$ titrations, respectively, between benzyl-DOTA and benzyl-EDTA, and $P < 0.0001, P < 0.01, P < 0.05$, and $P < 0.001$ at $10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$ titrations, respectively, between benzyl-DOTA and the benzoate group. There were no significant differences between binding to benzyl-EDTA and the benzyl group (Fig. 5).

Inhibition Experiments. Positive sera for anti-DOTA antibodies obtained from patients after i.p. therapy or i.v. imaging studies, after preincubation with increasing amounts of HSA-2IT-DOTA (0, 1, 10, and 100 µg/ml), showed a consistent dose-dependent inhibition of binding to HSA-2IT-DOTA (Fig. 6).
6A). Binding of sera from therapy patients was more difficult to inhibit as compared with the binding of sera from “imaging” patients (Fig. 6B). Inhibition with 100 µg/ml of HSA-DOTA was higher than with 10 µg/ml \( (P = 0.017) \) and 1 µg/ml \( (P = 0.0001) \) and 10 µg/ml inhibited more strongly than did 1 µg/ml \( (P = 0.028) \) (Fig. 6B). Preincubation of the sera with equal amounts of buffer only (0.1 N ammonium acetate) did not show any degree of inhibition of binding when compared with buffer-free serum. The percentage of inhibition by 100 µg/ml of HSA-2IT-DOTA correlated inversely with the serum levels of anti-DOTA antibodies \( (r = 0.792, P = 0.006) \).

Western Blotting. Sera from Patients 1 and 3, obtained after \(^{90}\text{Y}-\text{DOTA-HMFG1} \) treatment, were found to react with a \( M_{r} \) 60,000 band, consistent with the molecular weight of HSA, only in the lanes where HSA-2IT-DOTA had run (Fig. 7). Higher molecular weight bands represent some aggregates in the HSA-2IT-DOTA preparations (see also “Materials and Methods”). The pretherapy sera of these patients did not react with HSA-2IT-DOTA (Fig. 7). There was no binding to HSA or HSA-2IT.

Development of HAMA and Antiidiotypic Antibodies. All patients receiving i.p. therapy with \(^{90}\text{Y}-\text{DOTA-HMFG1} \) developed significant (Category 2) HAMA responses. Two of eight patients receiving i.v. \(^{111}\text{In}-\text{DOTA-labeled mAb} \) for imaging had significant HAMA responses. Patient 14, who had strong (Category 3) HAMA response due to the two previous i.p. treatments with radiolabeled mAbs, did not develop anti-DOTA antibodies. The other patient (Patient 7) with a HAMA response, due to previous murine mAb administration, developed anti-DOTA antibodies. Patients 8 and 10, who received murine mAbs for imaging, did not show any increase in their preexisting HAMA levels, which remained low (see also Table 3 for HAMA levels). Patients 9, 11, 12, and 13 who received the reshaped human antibody did not develop elevation of their preexisting HAMA, and only one of them (Patient 12) developed anti-DOTA antibodies (see Table 3).

None of the six patients who received the reshaped human \(^{111}\text{In}-\text{DOTA-Hu2PLAP} \) developed antiidiotypic antibodies or increasing titers of preexisting HAMA (rheumatoid factors) as measured by the previously described methods \( (24) \) (data not shown).

**DISCUSSION**

In the present study we evaluated the development of humoral immune responses against the macrocyclic chelating agent benzyl-DOTA, in patients receiving \(^{111}\text{In-} \) and \(^{90}\text{Y}-\text{labeled mAbs} \) for imaging or therapy. All patients (100%) receiving i.p. therapy with 25 mg of \(^{90}\text{Y}-\text{DOTA-HMFG1} \) developed anti-macrocycle antibodies posttreatment, and three patients had clinical evidence of serum sickness. Four of 8 patients (50%) receiving varying doses of murine or human \(^{111}\text{In}-\text{DOTA-labeled mAbs} \) for imaging studies developed antibodies against DOTA. From these results it is evident that the chelator on the mAb carrier protein is highly immunogenic. Supportive studies carried out in rabbits also show the development of anti-DOTA antibodies.
after immunization with RSA-DOTA.4

There are very limited clinical data addressing the immunogenicity of metal chelating agents when conjugated onto antibody for radioimmunodiagnosis and therapy. Various investigators have immunized mice with \(^{111}\)In-DTPA-BSA in order to generate antibodies against the \(^{111}\)In-DTPA complex and then synthesize specific mAbs with one part recognizing the chelate-isotope complex and the other part recognizing the tumor-associated antigen, allowing the achievement of better tumor:normal tissue ratios (25–27). Those data showed that small bifunctional metal chelating agents, such as DTPA, could be immunogenic in animals when coupled to proteins (BSA, RSA). In these cases the chelator could act as a hapten and the serum protein as the carrier molecule. With DTPA, high hapten:carrier molar coupling ratios were needed (19:1) to induce immunogenicity (28). There are no clinical data addressing the potential immunogenicity of chelating agents such as DTPA or DOTA. When DTPA-coupled mAbs are used for immunoscintigraphy or radioimmunotherapy, the molar ratio between DTPA and antibody molecule is 1.1 to 1:2:1 (29). This is much lower than when DTPA was coupled to a carrier protein in order to produce anti-DTPA mAbs in mice (28). In this study we found that the macrocycle benzyl-DOTA is highly immunogenic when coupled to mAbs by the methods used here, even at coupling ratios of only 1.0 to 2.9:1.

The development of IgG and IgM antibodies against DOTA follows the typical pattern of immune response against an antigen that generates T-cell memory. All patients except one developed initially (3 to 4 wk post-DOTA-mAb) IgG responses but low or absent IgM. The patient (Fig. 3C) who developed a strong IgG response 3 wk posttherapy also developed serious side effects 10 days post-\(^{90}\)Y-DOTA-HMFG1 i.p. administration (see “Results”). A possible explanation could be that this patient was previously sensitized to DOTA or an antigen sharing a cross-reactive epitope with DOTA. However, she had no previous exposure to DOTA-coupled mAbs, and her pretherapy serum was negative for anti-DOTA antibodies.

The clear dose-response relationship between the DOTA-mAb dose in \(\mu\)g and the positivity of the ELISA expressed as absorbance units (Fig. 4), in patients undergoing immunoscintigraphy, indicates that there is a cut-off point in the amount of DOTA in the immunoconjugate, above which the chelate on the antibody molecule becomes immunogenic. Our results suggest that, at doses of immunoconjugate above 500 \(\mu\)g, benzyl-DOTA becomes immunogenic. This finding is in agreement with previous studies of HAMA development in patients receiving low doses of murine mAbs, i.e., \(\leq\)1 mg, for imaging (17). None of the patients receiving single “imaging” doses of murine (HMFG1, H17E2) or humanized (Hu2PLAP) mAbs developed measurable HAMA, other than preexisting, or idiotypic responses in this or previous studies of our group. It is important to notice that both patients receiving 500 \(\mu\)g and 1 mg of murine mAbs H17E2 and HMFG1, respectively, developed measurable anti-macrocycle antibodies, and their preexisting HAMA remained low (Table 3). suggesting that the macrocycle was more immunogenic than its murine immunoglobulin carrier molecule in these two cases. However, further studies are required to determine the generality of this finding. Patients in the therapy group developed higher HAMA than anti-DOTA levels in the serum. A direct comparison between HAMA and anti-DOTA responses is not valid, since HAMA is almost always a secondary response, due to preexisting anti-mouse antibodies, and anti-DOTA antibodies were not detected before immunoconjugate administration. Two of six patients receiving benzyl-DOTA immunoconjugates of the “humanized” Hu2PLAP mAb developed anti-macrocycle antibodies. It is possible that the new chelate, when carried by a human or almost human protein (like Hu2PLAP mAb), is not so immunogenic and could be evaluated carefully in future therapy trials. Since we did not observe side effects in our immunolocalization studies using DOTA-coupled murine or reshaped human mAbs, we could suggest their application in such trials at doses of <250 \(\mu\)g and chelate:antibody coupling ratios of \(\leq\)1.3 to 1. In addition, the injection of up to 16% aggregated immunoconjugate in our immunolocalization study may be responsible for the development of anti-chelate antibodies. Aggregation of hapten-carrier molecules enhances immunogenicity, and the dose-response curve in patients receiving diagnostic doses can be extended to the therapeutic doses in regard to the amount of aggregated protein-hapten administered. The presence of 13\% mAb-21T-benzyl-DOTA dimers was also reported in a previous preclinical study (13). It was thought to be a result of the conjugation chemistry, applying the two-step procedure of Moi et al. (22), used for H17E2 or Hu2PLAP in our study. It is possible that non- or minimally aggregated material might substantially alter the degree of immunogenicity of the above conjugates. This could be achieved by applying the one-step procedure (21), as was done for the therapy trial, which was conducted after the imaging study.

Three patients receiving i.p. radioimmunotherapy with \(^{90}\)Y-DOTA-HMFG1 developed type III hypersensitivity serum sickness-like reactions. An immune complex etiology for this reaction is indicated by the drop of serum complement component C3 and C4 levels. In one of the patients high complement levels on RBC were found, resulting in an acquired reduction of CR1 complement receptor found on erythrocytes. Immune complex-mediated reactions have been described in patients receiving repeated administrations of murine mAbs (17, 30). In this case an elevation of the HAMA response is observed, mediated by repeated murine mAAb challenges. In the present study we observed this reaction after a single therapeutic dose of radioimmunoconjugate. Therefore it is unlikely that antibody-antibody complexes could cause the observed type III hypersensitivity reactions. At least two plausible explanations can be offered for this finding. (a) The immunoconjugate is highly immunogenic and its catabolism is altered, because of the presence of the 21T-bromoacetamidobenzyl-DOTA group. It could then persist longer in the circulation, leading to the development of immune complexes coupled with raised levels of anti-DOTA antibodies. (b) The macrocycle is found on small peptides generated from antibody catabolism in the liver. These small peptide-21T-bromoacetamidobenzyl-DOTA molecules could be released into the circulation over a prolonged period of time and complex with anti-DOTA antibodies. In one study (31), infusion of purified anti-mouse antibodies in patients undergoing i.p. radioimmunotherapy aimed to reduce bone marrow toxicity by removing the radiolabeled mAb getting into the systemic circulation (31). A detailed study of in vivo immune complex formation in these patients revealed similar findings to those shown in three patients developing side effects in the present study. However, the main difference was that none of the patients receiving purified HAMA developed signs and symptoms of small vessel vasculitis with skin rashes as observed in the three patients receiving \(^{90}\)Y-DOTA-HMFG1. Tissue deposition of circulating immune complexes depends mainly on

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their size and electric charge. In the above-mentioned study (31), antibody-antibody complexes are relatively large and are thought to be removed by macrophages in the liver and spleen. In the present study it is conceivable that the anti-DOTA antibody-HMFG1-DOTA complexes can deposit more easily in the subendothelium of small vessels. In this study, a marked systemic complement activation was observed, resulting from the in vivo immune complex formation. It is important to mention that patients developing side effects (Patients 1, 3, and 4) had the highest levels of anti-DOTA antibodies in the serum (518, 981, and 129 μg/ml, respectively).

In summary, the new chelating agent which contains the macrocycle DOTA proved to be immunogenic, under certain conditions, when coupled onto murine or even reshaped human mAbs and used for antibody-guided clinical imaging or therapy trials. This, unfortunately, can limit the clinical application of DOTA immunoconjugates. Therefore attempts to produce non-immunogenic, stable chelating agents are under way. Every new chelating agent should be tested for its potential immunogenicity in preclinical studies in mice and/or rabbits, as well as in clinical studies.

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Development of Humoral Immune Responses against a Macrocyclic Chelating Agent (DOTA) in Cancer Patients Receiving Radioimmunoconjugates for Imaging and Therapy

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