ABSTRACT

The macrocyclic bifunctional chelating agent 2-(p-bromoacetamidobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (BAD), forms inert metal complexes ideal for radioimmunotherapy. Kosmas et al. (Cancer Res., 52: 904–911, 1992) found 2-iminiothiolane linker-(S)-BAD monoclonal antibody HMFG1 highly immunogenic in patients. We studied the immunogenicity of (S) and (R) enantiomers of 2-imminothiolane linker-BAD rabbit IgG, monoclonal antibody Lym-1, and Lym-1-2-imminothiolane linker-(S)-bromoacetamidobenzyl-EDTA in 15 rabbits. Five groups of each were given 0.1, 1.0, or 10 mg of 111In conjugate i.v., blood samples were taken daily for 14 days and biweekly for 70 days, and the plasma T1/2 was calculated. A drop in plasma 111In at 6–8 days coincided with the appearance of antibody on enzyme-linked immunosorbent assay. Specific anti-(S)-BAD, anti-(R)-BAD, anti-(S)-bromoacetamidobenzyl-EDTA, and anti-mouse IgG were measured. Rabbit IgG conjugates did not elicit an immune response. Mouse IgG conjugates were immunogenic on the first exposure, with both anti-1,4,7,10-tetraazacyclododecanetetraacetic acid and anti-mouse responses. Anti-1,4,7,10-tetraazacyclododecanetetraacetic acid N,N',N,N''-tetraacetic acid and anti-mouse response. Anti-1,4,7,10-tetraazacyclododecanetetraacetic acid N,N',N,N''-tetraacetic acid was specific for the (S) or (R) enantiomer, but cross-reaction appeared with reboosting. A second injection of the opposite enantiomer gave a response to that enantiomer. Lym-1 bromoacetamidobenzyl-EDTA produced anti-bromoacetamidobenzyl-EDTA and anti-mouse response.

INTRODUCTION

The macrocyclic bifunctional chelating agent BAD, incorporating DOTA, has been developed for use in radioimmunotherapy. The current dose-limiting factor in cancer therapy with radioimmunoconjugates is bone marrow irradiation, which is increased by loss of radiometals from unstable chelate conjugates to the bone marrow. Because DOTA forms highly stable, inert complexes with metals like yttrium, negligible metal loss occurs in vivo. For these reasons [18]DOTA conjugates of mAbs are ideal for radioimmunotherapy and human trials are now under way.

In a Phase I/II trial of i.p. therapy in ovarian cancer, Kosmas et al. (1) found the antibody-chelate conjugate [18]SY2-IT-(S)-BAD-mAb (HMFG1) highly immunogenic, with some patients developing serum sickness. To date, human anti-mouse antibody has been the only antibody response reported from the use of mAb conjugates in humans, but anti-chelate-conjugate responses have not been sought. This report of a severe reaction to the chelate moiety itself prompted us to study the immunogenicity of a variety of chelate conjugates in animals (2). Here we report the effects of chelate structure, protein carrier, amount of conjugate injected, and timing of administration to show that chelates behave like other haptons in eliciting a specific antibody response when injected as foreign protein conjugates. We also compared the antibody response with the mirror image structures (enantiomers) (S)-BAD and (R)-BAD.

MATERIALS AND METHODS

All reagents and solvents were the purest as were commercially available and were used without further purification, if not stated otherwise. Pure water (18MO/cm) was used throughout. Metal-free conditions were maintained during synthetic chemistry. All glass labware was washed with a mixed acid solution and rinsed thoroughly with pure water (3). All plastic labware was washed with 3 M HCl and rinsed thoroughly with pure water.

High Performance Liquid Chromatography. High performance liquid chromatography was carried out on a Rainin Rabbit HP system (Rainin Instruments, Woburn, MA). UV absorbance was measured at 214 nm using an ISCO Model UA-5 (ISCO, Inc., Lincoln, NE). The high performance liquid chromatography system was controlled and data were acquired using Rainin Dynamax software on a Macintosh SE/30 computer. Size exclusion chromatography was done using a SynChropak GPC 300 (4.6 x 250 mm) column operated at 0.3 ml/min using 0.1 M sodium phosphate, pH 7.2, with 1 mM EDTA.

Sephadex Gel Filtration (Centrifuged Column Method). A centrifuged Sephadex G 50–80 column method (4–6) was used to separate proteins from lower molecular weight compounds.

UV Spectrophotometry. Absorbance measurements at 280 nm were made on a Gilford Model 250 spectrophotometer using a 1-cm path length microcell. Absorances were measured on dilutions suitable to give absorbance readings of 0.1–1.0.

Radiation Counting. Gamma counting was done in a Beckman Model 310 counter with the appropriate energy windows set for 51Co. Thin layer chromatography plates containing radiolabeled materials were visualized with an AMBIS Radioanalytical Imaging System.

Reagents. Lym-1, an anti-B-cell lymphoma IgG2a mAb (7), was obtained from Damon Biotech (Needham Heights, MA). Rabbit IgG and 2-IT (Traut's reagent (8)) were purchased from Sigma Chemical Co. (St. Louis, Missouri). When IgG and Lym-1 were conjugated under identical experimental conditions, no significant variation in the chelate/mAb ratio was observed when a new bottle of 2-IT was used.

Conjugations. Conjugations were done as described by McCull et al. (9) in a two-step procedure using Traut's reagent (8), which reacts with amino groups to produce mercaptobutyrimidyl groups, followed by alkylation of the mercaptopurine sulfur with the bifunctional chelating agent BAD or BABE.

Macrocycles. (R)- and (S)-nitrobenzyl-DOTA were prepared according to the method of Renn and Meares (10). Reduction of each to the respective

Received 7/21/93; accepted 12/14/93.

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1 Supported by a Veterans Administration Merit Review grant (D. A. G.); National Cancer Institute Grants R01-CA 48282, R01-CA28343 (D. A. G.), and R01-CA16861 (C. F. M.); and United States Public Health Service Grants CA 28343, CA 48282 (D. A. G.), and CA 16861 (C. F. M.).

2 To whom requests for reprints should be addressed at Veterans Administration Medical Center, Department of Nuclear Medicine (115), 3801 Miranda Ave., Palo Alto, CA 94304.

3 The abbreviations used are: BAD, 2-(p-bromoacetamidobenzyl)-1,4,7,10-tetraazacyclododecanetetraacetic acid; DOTA, 1,4,7,10-tetraazacyclododecanetetraacetic acid N,N',N,N''-tetraacetic acid; mAb, monoclonal antibody; BABE, bromoacetamidobenzyl-EDTA; 2-IT, 2-iminiothiolane linker; KLH, keyhole limpet hemocyanin; Kf, human transferrin; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; ACA, anti-conjugate antibody.
p-amino compound was accomplished as described by McCall et al. (9). Conversion of the p-amino compounds to the p-bromoeetamido macrocycles BAD used a modification of the method of Mukkala et al. (11) and was done according to the method of McCall et al. (9).

**Lym-1-2-IT-BAD Conjugations.** The Lym-1 mAb solution (7.36 mg/ml) was prepared for conjugation with a centrifuged gel filtration column (6) with 0.1 M ammonium acetate solution, pH 7.0, as the column buffer. Excess BAD in aqueous solution and freshly prepared 100 mM 2-IT in triethanolamine hydrochloride, pH 8.7 (final approximate concentrations: Lym-1, 6 mg/ml; BAD, 2.0 mM; 2-IT, 1.1 mM) were added to the collected effluent (6.92 mg/ml) in order. The pH of the solution was adjusted to 9.0 and the solution was incubated at 37°C for 30 min. Excess BAD was removed and the conjugate was placed in 0.1 M ammonium acetate solution, pH 7.0, with a centrifuged gel filtration column. The concentration of protein-bound chelators available for metal binding was estimated as described by Moi et al. (12). The purified conjugates were stored at -70°C until use.

**IgG-2-IT-BAD/BABE Conjugations.** IgG (22 mg) was dissolved in 0.9 ml 0.1 M tetramethylammonium phosphate, pH 8.9, and an excess of a BAD (18–24 mM) or BABE (40 mM) solution and a freshly prepared solution of 100 mM 2-IT in triethanolamine hydrochloride, pH 8.7, was added to give the final approximate concentrations: IgG, 22 mg/ml; BAD, 2.0 mM; 2-IT, 1.1 mM. The pH of the solution was adjusted to 9.0 and the solution was incubated at 37°C for 30 min (for BABE: BABE, 4 mM; 2-IT, 2 mM, 22 h to obtain 9 chelates/mAb). Excess BAD/BABE was removed and the conjugate was placed in 0.1 M ammonium acetate solution, pH 7.0, with a centrifuged gel filtration column. The concentration of protein-bound chelators available for metal binding was estimated as described by Moi et al. (12). The purified conjugates were stored at -70°C until use.

- The synthetic route and the stereochemistry of (S) and (R) nitrobenzyl-DOTA and 2-IT-BABE are shown in Fig. 1. Conjugates of mouse mAb Lym-1 with (R)- and (S)-BAD, and also with (S)-BABE, were prepared by the method of McCall et al. (9).

**Radiolabeling Conjugates.** The antibody conjugates were radiolabeled in 0.1 M citrate (BABE) or 0.1 M ammonium acetate (DOTA) by adding the solution to dried purified 111In and incubating at room temperature for 15 min (6). Any unbound 111In was scavenged by adding EDTA and was removed by centrifugation in a Centricon filter followed by dilution in buffer.

**Rabbit Blood Clearance Studies.** Individual normal male rabbits weighing ~3 kg were given i.v. injections of either 0.1, 1.0, or 10 mg (equivalent to 2.3–233.0 mg for a 70-kg human) of 111In radiolabeled conjugate (1.8–4.5 chelates/molecule). In each experiment either the (S) or (R) enantiomer of rabbit IgG-BAD, mAb Lym-1-BAD, or Lym-1-(S)-BABE was given (Table 1). Heparinized blood samples were collected from an ear vein daily for 14 days and approximately biweekly for 70 days. Whole blood and plasma samples were counted and the percentage of the injected dose circulating in plasma calculated from the hematocrit and blood volume was estimated from body weight. Three months after the first i.v. injection of Lym-1 BAD, the 0.1-mg-treated rabbits were boosted with the same dose of the same conjugate and reboostered again 1 month later for a total of three doses. In the BABE boosting experiment the 10-mg-treated rabbit was given a 0.1-mg i.v. dose 4.7 months after the first injection.

In a “switch-boost” experiment 4.4 months after the first i.v. injection of Lym-1-(S)- or (R)-BAD, the 1.0-mg rabbits were boosted with an i.v. injection of the opposite enantiomer. This was done to see if it was possible to circumvent the immune response that occurred after reinjection of the same enantiomer. Blood samples were collected as before.

**Specificity of Antibody Response by ELISA.** Flat-bottomed 96-well microtiter plates were coated with 50 µl each (containing approximately 700 ng in PBS, pH 7.4) of hTr conjugate of 2-IT-(S)-BAD, 0.4 chelate/transferrin molecule, or 2-IT-(R)-BAD, 0.7 chelate/transferrin molecule, and incubated for 24 h at 37°C in a humidified chamber. To block nonspecific binding, 5% bovine serum albumin in PBS was added and the mixture was incubated for 1 h at 37°C. After the plates were washed, they were incubated with 50 µl of a 1:10 dilution of rabbit test serum for 2 h at 37°C. After three washes in PBS plus 0.05% Tween 80, the plates were incubated with 1 µl of mouse monoclonal anti-rabbit immunoglobulin alkaline phosphatase conjugate. The plates were then washed 3 times with PBS and incubated at 37°C with 50 µl of p-nitrophenyl phosphate, 1 mg/ml in 0.1 M glycine buffer containing 1 mM MgCl₂ and 1 mM ZnCl₂ (pH 10.4), and the absorbance was determined after ~30 min in a TiterTek Multiskan plate reader at 450 nm. A >0.20 (background ≤ 0.20) was considered positive. Mouse mAb 2D12-5 specific for Yttrium-DOTA (13) was used as a positive control.

For detection of specific anti-BABE antibody response, the ELISA was set up as described above except 50 µl (300 ng)well/transferrin-2-IT-BABE conjugate, 3.9 chelates/transferrin molecule was used on the plate as the antigen and mAb WC3A11 specific for 2-IT-BABE, also obtained in our laboratory by KLH immunization (14), was used as a positive control. For detection of specific rabbit anti-mouse antibody response by ELISA, plates were coated with 10 ng/well of mouse IgG. Polyclonal rabbit anti-mouse antibody was used as a positive control.

**Serial Dilution and Competitive Inhibition.** Sera with high absorbance on ELISA, after boosting or reboosting with the same enantiomer, were diluted serially to 10⁻¹, 10⁻², or 10⁻³, and absorbance was measured. For the inhibition studies, 50 µl containing 0, 1, 10, or 100 µg/ml of hTr-(2-IT-(S)- or (R)-BAD conjugate were added to 50 µl of sera, followed by incubation at 4°C overnight. Fifty µl of this incubate was used in the same ELISA assay and absorbance was recorded. The sera with high anti-BABE ELISA was inhibited in the same way with hTr-2-IT-BABE conjugate.

**Preparation of Murine Monoclonal Anti-Chelate Antibodies.** Since specificity for the metal ion was desired, the chelate hapten was filled with the desired stable element for immunization. The BABE conjugates contained cobalt, indium, and gallium; the BAD conjugates contained yttrium and gallium. BALB/c mice were immunized i.p. with 10 µg of the KLH-coupled antigen in complete Freund’s adjuvant. Two weeks later this was repeated in incomplete Freund’s adjuvant followed in another 2 weeks by an i.v. booster dose of the conjugate alone. The mice were screened for antibody production after 1 week by measuring the 24-h retention of radiolabeled hapten by whole body counting with a Picker dual probe scintillation counter. The mice with 20–50% retention at 24 h (compared with ~1–3% in control, nonimmunized mice) were hyperimmunized with 30 µg i.v. conjugate; 3 days later their spleen cells were fused with PDX3 AGS myeloma cells. Hybridomas were selected in hypoxanthine-aminopterin-thymidine medium. Clones obtained were screened by ELISA using hTr-2-IT-hapten as the solid phase antigen coated on microtiter plates.

**RESULTS**

**Rabbit Blood Clearance Studies.** The data points and bars plotted in Figs. 2–7 represent measurements taken from individual bleedings. A semilog plot of the disappearance half-life of rabbit IgG-2-IT-BAD conjugates is shown in Fig. 2. The disappearance followed a single exponential curve with no break over 13 days. The T₁/₂ (S) = 6.72 ± 1.24 (SD) days and (R) = 4.43 ± 0.21 days, which indicates that the two preparations were similar but not identical (P < 0.05). Blood disappearance of Lym-1 2-IT-BAD conjug-
of the six Lym-1 2-IT-BAD [0.1 mg (S), 1.0 mg (S), and 1.0 mg (R) = 1.98 _+ 0.15 days (not significantly different). Three of the six: 0.1 mg (S), 1.0 mg (S) and 1.0 mg (R), showed biexponential curves with a break at 6-8 days. The T\textsubscript{1/2} of the first phase of (S) = 1.85 _+ 0.19 days and that of (R) = 1.98 _+ 0.15 days (not significantly different); second phase T\textsubscript{1/2} = 0.75 _+ 0.15 days (P < 0.01).

Reinjection

7 0.1 x 3 Lym-1 2-IT-(S)-BAD 2.0/1 0 ND ND S+, R+ 1.85 _+ 0.19 0.83 _+ 0.09
8 1.00 Lym-1 2-IT-(S)-BABE 1.8/1 2 1.96 _+ 0.25 0.98 _+ 0.04 Negative
9 10.00 Lym-1 2-IT-(S)-BABE 1.8/1 2 (R) - 0.1 mg Lym-1 2-IT-(S)-BAD had anti-(R)-BAD antibody but none of the rabbits that were given injections of Lym-1 2-IT-(S)-BAD had anti-(R)-BAD antibody after one exposure (Fig. 6). The target antigen on plate was hTr-2IT-(S)-BAD. In this rabbit, a second injection of 0.1 mg Lym-1 2-IT-BABE, 4.7 months later, gave no detectable anti-2-IT-BABE on ELISA. Rabbits reboosted twice (a total of three injections) with the same enantiomer developed cross-reactivity to the opposite enantiomer (Table 1). After a second exposure to the same amount (1 mg) of the opposite enantiomer ("switch-boost" with Lym-1 2-IT-(S) or (R)-BAD reactivity developed to both enantiomers. Nine rabbits were studied for anti-mouse immune response. Rabbits that were given injections of 1.0 and 10 mg of immunoconjugates showed positive anti-mouse antibody.

Concentration and Specificity by Dilution and Inhibition. The Lym-1 (S) - (R)-BAD ELISA became negative at a dilution of 10^{-3} in all strongly positive sera tested. In the 0.1-mg (R) and (S) reboosting experiment, the ELISA was inhibited by a 100-µg/ml hTr

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**Table 1** Rabbit protocol: antibody half-life and specific immune response by ELISA

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Dose (mg)</th>
<th>Antibody</th>
<th>Conjugate</th>
<th>Chelate/antibody</th>
<th>% of aggregates</th>
<th>t_{1/2} α (days)</th>
<th>t_{1/2} β (days)</th>
<th>ELISA absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.10</td>
<td>R-IgG*</td>
<td>2-IT-(S)-BAD</td>
<td>2.5/1</td>
<td>ND</td>
<td>1, 2, 3</td>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>1.00</td>
<td>R-IgG*</td>
<td>2-IT-(S)-BAD</td>
<td>2.5/1</td>
<td>ND</td>
<td>(6.72 _+ 1.24)_b  no break</td>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>5.80</td>
<td>R-IgG*</td>
<td>2-IT-(S)-BAD</td>
<td>2.5/1</td>
<td>ND</td>
<td>4.5, 6</td>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>0.10</td>
<td>R-IgG</td>
<td>2-IT-(R)-BAD</td>
<td>4.5/1</td>
<td>ND</td>
<td>(4.43 _+ 0.21)_b  No break</td>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>1.00</td>
<td>R-IgG</td>
<td>2-IT-(R)-BAD</td>
<td>4.5/1</td>
<td>ND</td>
<td></td>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>6</td>
<td>10.00</td>
<td>R-IgG</td>
<td>2-IT-(R)-BAD</td>
<td>4.5/1</td>
<td>ND</td>
<td></td>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>7</td>
<td>0.10</td>
<td>Lym-1</td>
<td>2-IT-(S)-BAD</td>
<td>2.0/1</td>
<td>0</td>
<td>7.8, 9</td>
<td></td>
<td>S+, R-</td>
</tr>
<tr>
<td>8</td>
<td>1.00</td>
<td>Lym-1</td>
<td>2-IT-(S)-BAD</td>
<td>2.0/1</td>
<td>0</td>
<td>(1.85 _+ 0.19)_b  0.83 _+ 0.09</td>
<td>S+, R-</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>10.00</td>
<td>Lym-1</td>
<td>2-IT-(S)-BAD</td>
<td>2.0/1</td>
<td>0</td>
<td></td>
<td></td>
<td>S+, R-</td>
</tr>
<tr>
<td>10</td>
<td>0.10</td>
<td>Lym-1</td>
<td>2-IT-(R)-BAD</td>
<td>2.0/1</td>
<td>0</td>
<td>10, 11, 12</td>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>11</td>
<td>1.00</td>
<td>Lym-1</td>
<td>2-IT-(R)-BAD</td>
<td>2.0/1</td>
<td>0</td>
<td>1.98 _+ 0.15</td>
<td>0.6</td>
<td>R+, S-</td>
</tr>
<tr>
<td>12</td>
<td>10.00</td>
<td>Lym-1</td>
<td>2-IT-(R)-BAD</td>
<td>2.0/1</td>
<td>0</td>
<td></td>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>13</td>
<td>0.10</td>
<td>Lym-1</td>
<td>2-IT-(S)-BABE</td>
<td>1.8/1</td>
<td>2</td>
<td>13, 14, 15</td>
<td></td>
<td>S+ (to R)</td>
</tr>
<tr>
<td>14</td>
<td>1.00</td>
<td>Lym-1</td>
<td>2-IT-(S)-BABE</td>
<td>1.8/1</td>
<td>2</td>
<td>1.96 _+ 0.25</td>
<td>0.98 _+ 0.04</td>
<td>S+ (to R)</td>
</tr>
<tr>
<td>15</td>
<td>10.00</td>
<td>Lym-1</td>
<td>2-IT-(S)-BABE</td>
<td>1.8/1</td>
<td>2</td>
<td></td>
<td></td>
<td>S+ (to R)</td>
</tr>
</tbody>
</table>

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a R-IgG, rabbit polyclonal immunoglobulin; ND, not done; Lym-1, mouse monoclonal anti-lymphoma mAb; × 3, 3 times; × 1, once.
IMMUNOGENICITY OF mAb CONJUGATES

LYM-1-2IT-BABE

Fig. 4. Blood disappearance of Lym-1 2-IT-(S)-BABE. Two of the three curves (1.0 and 10 mg) were biexponential with a break at 6-8 days. The $T_1/2$ first phase = 1.96 ± 0.25 days; $T_2/2$ second phase = 0.9 ± 0.04 days ($P < 0.05$).

Fig. 5. ELISA specific for anti-(S)-BAD on all samples 0-70 days. Three rabbits that were given injections of Lym-1 2-IT-(S)-BAD had anti-(S)-BAD at days 6-8; rabbits that were given injections of Lym-1 2-IT-(R)-BAD produced no anti-(S)-BAD antibody after one exposure. O.D., Absorbance.

Fig. 6. ELISA specific for anti-(R)-BAD on all samples 0-70 days. Two of three rabbits that were given injections of Lym-1 2-IT-(R)-BAD had anti-(R)-BAD antibody at days 6-8; rabbits that were given injections of Lym-1 2-IT-(S)-BAD had no anti-(R)-BAD antibody after one exposure. O.D., Absorbance.

Fig. 7. Of the three rabbits that were given injections of Lym-1 2-IT-(S)-BABE, the one receiving 10 mg produced anti-BABE antibody. O.D., Absorbance.

conjugate of the same enantiomer [equivalent to $-0.86 \mu M$ (S) or (R) BAD]. In the positive switch boost sera the ELISA was inhibited specifically by the opposite (switch) enantiomer, which indicates a high concentration of specific antibody. The results were similar for the positive (10-mg rabbit) Lym-1 BABE sera.

Mouse Immune Response to KLH Conjugates. For the 24-h retention measurements, the hapten-chelate was radiolabeled with the same metal as used for immunization. The 24-h percentage dose retention of radiolabeled hapten is shown in Fig. 8. Control, nonimmunized mice retained only 1–3% at 24 h, the rest being rapidly excreted by the kidneys. The mean 24-h retention of BABE hapten is 27.5 ± 30% ($n = 16$) and BAD hapten retention is 86 ± 18.4% ($n = 15$) ($P < 0.0001$). The immune response to the BABE conjugates was lower and more variable than BAD conjugates.

DISCUSSION

The goal of this study was to define the immune response to enantiomerically related hapten-chelate conjugates of mAbs in animals, to devise strategies for avoiding it, and to consider the implications of the results for radioimmunotherapy and diagnosis using these conjugates. Human anti-mouse antibody is a universal finding in therapy with mAbs that severely limits their usefulness. However, the more serious reactions such as serum sickness have been rare and usually have occurred after large repeated doses in immunocompetent patients (15). While human anti-mouse antibody is well documented in human mAb conjugate therapy trials, anti-conjugate antibody is rarely measured. Such conjugates of small molecules or haptns with larger carrier molecules (mAb), are very efficient for generating a specific anti-hapten antibody response. This response is augmented when the carrier is foreign (e.g., KLH or mouse protein), when the conjugate/carrier ratio is high (>10/1), when the conjugate is injected i.p., and when the conjugate is coinjected with adjuvants or aggregated protein. We have used this technique very efficiently in another context to generate high affinity anti-hapten mAb WC3All specific for cobalt-2-IT-BABE (13) and mAb 2D12.5 specific for yttrium-DOTA (14). We are currently applying these mAbs in novel pretargeted immunoctigraphy and immunotherapy protocols in tumor mice (16). In this technique, injection of mAb, directly radiolabeled
by chelate conjugation, is replaced with the sequential administration of anti-hapten mAb, followed by radiolabeled hapten. The physical and temporal separation of mAb and radioactive chelate-hapten in pretargeting has many advantages, particularly in reducing normal tissue and marrow irradiation in radioimmunotherapy (13, 17), but it also greatly lowers the probability of an antibody response to the chelate hapten.

In a Phase I/II trial of i.p. therapy in ovarian cancer, Kosmas et al. (1) found the \[^{90}Y\]IT-BAD-mAb (HMFG1) conjugate highly immunogenic with some patients developing serum sickness. Interestingly, in this study, a high titer of human anti-hapten antibody was present, with reactivity restricted to the DOTA epitope and very little antilinker antibody. This was shown by low reactivity of DOTA-positive sera against anti-2-IT-benzoic acid or anti-BABE when they were used as the target antigen on the ELISA plate.

We have shown previously that the antibody response to a mAb chelate conjugate can be restricted to the chelate moiety or may also include the linker used to attach the chelate to the mAb. Our mouse anti-hapten mAb 2D12.5 raised against yttrium-2-IT-BAD was restricted to yttrium-DOTA (13), whereas the 2-IT-BABE linker made up part of the epitope recognized by mAb WC3A11, which were raised against KLH-2-IT-BABE (cobalt) (14).

Heavily substituted proteins are known to be more immunogenic than lightly modified ones. In our mouse immunization protocols the chelate/KLH ratio was >10/1. The highly immunogenic HMFG1 mAb conjugate used for i.p. therapy of ovarian cancer had an average degree of conjugation of 2.6–2.9 available DOTA groups/antibody. The mAb conjugates used in our rabbit experiments had a chelate/protein ratio of 1.8–4.5/1 (Table 1) and were also immunogenic when injected as foreign protein, such as mouse mAb in a rabbit.

Although Kosmas et al. (1) showed a positive correlation of injected dose to immune response, with a cutoff at 500 μg mAb, our data are not clear on this point. On the basis on this figure, these investigators cautiously suggest <250 μg and ≤1.3/1 antibody coupling ratios for safe clinical use. Our data showing absence of antigenicity for all amounts and coupling ratios injected (up to amounts equivalent to 233 mg for a 70-kg man and chelate/protein ratio of 4.5/1) when rabbit conjugates were used in rabbits underline the importance of the carrier and suggest that humanized mAb would be less antigenic in humans.

Thus, i.p. radioimmunotherapy for ovarian cancer with large amounts of mAb provides an ideal setting for eliciting an anti-hapten antibody response when aggregates are also present (1). Modifying one or other of the conditions that promote a response such as "humanizing" the carrier protein has the potential to reduce or abolish the anti-hapten response. Partial success with chimeric or "humanized" mAbs in reducing antigenicity has been reported, although anti-idiotypic antibody still occurs (18). Work on human monoclonal antibodies while promising is at a very early stage (19).

Johnson et al. (20) recently have considered the question of an ACA response in humans treated with genetically engineered "humanized" mAb drug conjugates. They chose to study the antigenic response to mouse mAb conjugates in rats as a suitable animal model for accurately simulating the effect of modifying a weakly immunogenic humanized mAb on its antigenicity in humans. Rats that were given injections of unmodified mouse mAb 225 IgG1 directed against human epidermal growth factor receptor failed to mount anti-mouse immune response, presumably due to their phylogenetic relatedness. In contrast, rats that were given injections of Vinca immunonjugates of mAb 225 IgG1 mounted a strong anticonjugate antibody response when four repeat doses of mAb conjugate were given i.p. In vivo inhibition studies showed the response was directed primarily against the linker portion of the molecule. The presence of ACA had profound effects on the serum pharmacokinetics, with the peak serum concentration inversely correlated with the level of reactivity of the ACA. This finding agrees with our demonstration of a break in the plasma disappearance curve at approximately 6–8 days with a sudden increase in the rate of disappearance of mAb (Figs. 3 and 4), coinciding with the appearance of antibody (Figs. 5 and 6).

Thus, one strategy used by these investigators to decrease the ACA response was to change the linker used to attach the drug to the antibody. Higher serum values were observed when a different linker was used with the same chelate. However, the peak serum value for the new linker was still slightly lower than that seen with unmodified mAb. This result corresponds to our finding of an incomplete escape from the anti-hapten chelate response in rabbits receiving subsequent injections of the opposite enantiomer. Nevertheless, the possibility of developing linker and chelate chemistry with low immunogenic potential was shown to be a feasible goal. Our finding in these experiments of an absent response after one i.v. injection of homologous mAb, even with immunogenic DOTA conjugates, also shows promise for optimized conditions that include the use of humanized and eventually human mAbs.

REFERENCES


Immunogenicity in Rabbits and Mice of an Antibody-Chelate Conjugate: Comparison of (S) and (R) Macrocyclic Enantiomers and an Acyclic Chelating Agent

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