Development of Primary and Secondary Immune Responses to Mouse Monoclonal Antibodies Used in the Diagnosis and Therapy of Malignant Neoplasms

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ABSTRACT

Human anti-mouse immunoglobulin immune responses were studied in ten patients, eight with ovarian cancer and two with grade IV gliomas, diagnosed and treated with radiolabeled (125I, 111In) murine monoclonal antibodies. It was found that serum from these patients before treatment and from 18 control healthy individuals contained detectable antibodies to antigenic determinants on the Fc but not the F(ab')2 region of mouse immunoglobulin. No change in this reactivity occurred after the initial (imaging) dose of monoclonal antibodies. However, repeated administration of mouse immunoglobulins for therapy resulted in an elevated immune response directed against determinants on both Fc and F(ab')2 regions of mouse immunoglobulin M as well as immunoglobulin G and showed a marked prozone effect in our enzyme linked immunosorbent assay system. None of the immunized patients developed a detectable antidiotypic response.

INTRODUCTION

Mouse monoclonal antibodies with specificity for tumor associated antigens are increasingly being used as vectors for imaging agents such as 125I and 111In and for therapeutic agents such as 131I (1–5). One problem with this treatment has been that the percentage of i.v. administered mouse monoclonal antibody reaching its target is very small (6, 7). More recently, antibody administration into body regions by intrapericardial, intrapleural, i.p. and i.a. infusion have been used. This regional administration has resulted in an increased percentage of the potential therapeutic radiolabeled mouse monoclonal antibody reaching its target (8–10). A second problem has been that a human anti-mouse immunoglobulin response develops after i.v. administration of murine monoclonal antibodies (11–14).

We report here that use of regional monoclonal antibody administration also generates a human anti-mouse immunoglobulin immune response in the recipient. This response has been studied in ten patients receiving regionally administered murine monoclonal antibodies.

MATERIALS AND METHODS

Patients

Histological confirmation of diagnosis and written consent from patients were obtained prior to administration of radiolabeled monoclonal antibodies. Eight patients participating in this study had stage III or IV ovarian carcinoma and 2 patients had grade IV glioma of brain (Table 1).

Antibody Protocols

For diagnostic studies patients received 250 µg i.v. of monoclonal antibody labeled with between 1 and 5 mCi (37–185 MBq) of 125I.

For therapy patients received between 5 and 15 mg of monoclonal antibody labeled with between 50 and 150 mCi (1.850–5.55 GBq) of 131I, injected either i.p. (ovarian carcinoma) or i.a. (glioma) (Table 1). Intervals between injections of antibody ranged from 2 weeks to 6 months.

Monoclonal Antibodies

HMFG1 and HMFG2 are both mouse IgG1 antibodies shown to bind to a large mucin-like molecule normally produced by the lactating breast, but also expressed by the majority (>90%) of ovarian, breast, and other carcinomas. Both antibodies are directed against oligosaccharide determinants (15, 16).

AUAI is a mouse IgG1 antibody which detects an antigen expressed by a wide range of adenocarcinomas, including approximately 75% of carcinomas of the ovary (17).

H17E2 is a mouse IgG1 antibody directed against placental alkaline phosphatase. This enzyme is expressed as a surface membrane antigen on many neoplasms, including 60–85% of ovarian carcinomas (18).

9A is a mouse IgG3 monoclonal antibody directed against epidermal growth factor receptor as expressed by the A431 cell line (19). It also cross-reacts with blood group A antigen. It binds to neoplastic tissues, including brain gliomas (10).

Measurement of Human Anti-Mouse Antibody

Hybridoma Targeting (Immunofluorescence)

Cytospins of the relevant hybridoma cells (i.e., those that secrete the monoclonal antibodies used in this study) were prepared using 3 × 10^4 cells/slide. Cytospins were fixed with methanol, washed in PBS, and incubated for 30 min at room temperature with 50 µl of a 1:100 dilution of patient's serum, after which they were washed for 15 min (3 × 5-min washes) in PBS. They were then incubated for 15 min at room temperature with a 1:40 dilution of a fluorescein conjugated sheep anti-human immunoglobulin reagent (Wellcome, Beckenham, Kent, United Kingdom). Slides were then washed as before, mounted in Hydramount (National Diagnostics, Somerville, NJ) and screened using a Leitz UV microscope equipped with epillumination optics. Controls consisted of PBS, serum from healthy controls, and serum from patients with neoplastic conditions identical to those of the patients under study, but receiving no monoclonal antibody therapy.

Enzyme Linked Immunosorbent Assay

Assay. Monoclonal antibodies were diluted to 5 µg/ml in bicarbonate buffer, pH 9.6 (coating buffer), and used to coat 96-well microtiter plates (Sterilin, Middlesex, United Kingdom) 100 µl/well (500 ng/well). The plates were incubated at 37°C overnight and then washed in PBS, pH 7.4, containing 0.05% Tween 20. Serial dilutions of each patient's serum together with control sera were made and the plates were incubated for 2 h at 37°C, washed three times in PBS plus 0.05% Tween (3 × 2-min washes), and then incubated for 1 h at 37°C with 50 µl of a 1:1000 dilution of a peroxidase conjugated goat anti-human immunoglobulin species specific reagent (Amersham International, United Kingdom). Alternatively, for IgM determination, a peroxidase conjugated rabbit anti-human µ chain specific reagent (Dako, Denmark) was used at this stage. Plates were then washed three times and...
incubated at room temperature with 100 μl of 2,2'-azino-di(3-ethylbenzthiazolinesulfonate) (Amersham), and the absorbance was determined in a Titerkit multispan plate reader (Flow, Irvine, Scotland) at 405 nm. Patients' sera were considered positive when their absorbance was greater than the mean + 2 SD of the absorbance for sera from 18 healthy control individuals (Fig. IA).

Absence of Nonspecific Binding to Plates (Control). To evaluate the absence of nonspecific binding, a 96-well microtiter plate was incubated at room temperature with 100 μl of coating buffer, in wells from Rows 1 to 6. Wells in Rows 7 to 12 had an equivalent volume of coating buffer placed in them, but containing no mouse monoclonal. Patient's serum was assayed in Rows 1 to 6 and duplicated in Rows 7 to 12. Binding did not occur on any wells in Rows 7 to 12, demonstrating that no nonspecific binding was occurring under identical experimental conditions to those used in wells with mouse monoclonal antibody.

Affinity Purification of Human IgG Anti-Mouse Immunoglobulin

The IgG fraction of the human anti-mouse immunoglobulin antibodies in serum from Patient 9 (Table 1) was affinity purified on a activated Bio-Gel column to which mouse immunoglobulin had been coupled. Serum (5 ml) was passed through and the column was reequilibrated with PBS, pH 7.4. Under these conditions the human IgG anti-mouse immunoglobulin will bind while the lower affinity human IgM anti-immunoglobulin will not (20). The bound IgG human anti-mouse immunoglobulin was eluted using 3 M magnesium chloride and gel electrophoresis using a 3–15% gradient gel. IgG but no IgM was present.

**RESULTS**

Human Anti-Mouse Immunoglobulin Response. Ten patients (8 with ovarian carcinoma, 2 with brain glioma) were studied. Five (4 ovarian carcinoma, 1 glioma; Table 1, Patients 1–5) showed no increase in anti-mouse immunoglobulin levels after both an initial injection of 250 μg for diagnostic studies and between 2 and 15 mg for therapeutic study, above preexisting anti-mouse immunoglobulin levels found in 18 healthy controls (Fig. 1A). Of the 5 that showed a response (Table 1, Patients 6–10), 2 (1 glioma, 1 ovarian carcinoma) developed a moderate response (Fig. 1B) and 3 (Patients 8, 9, and 10, all with ovarian carcinoma) developed a pronounced response following 2 therapeutic doses of 5 and 10 mg. This latter group had all previously developed a moderate “type 2” response after the first therapeutic dose. One (Patient 1) of the 5 patients who showed no increase above control levels had received 2 therapeutic doses of 5 and 10 mg. This latter group had all previously developed a moderate “type 2” response after the first therapeutic dose. One (Patient 1) of the 5 patients who showed no increase above control levels had received 2 therapeutic doses comparable to those received by the 3 patients who did show a pronounced response.

The two patients with grade IV gliomas showed marked differences in their response to the injected monoclonal antibodies. One (Patient 1), a 29-year-old man received 250 μg for diagnostic studies followed 1 week later by a 5-mg therapeutic dose. After 6 months he received a further 250 μg for diagnosis followed 1 week later by a 5-mg therapeutic dose. This patient did not develop an immune response above that demonstrated by healthy controls. The second patient (Patient 7), a 14-year-old girl, received 250 μg for diagnosis and 5 mg for therapy.
Fig. 1. A. Levels of preexisting anti-mouse immunoglobulin in the serum of 18 healthy control individuals (O) and two patients (■), each of whom had received two therapeutic administrations of radiolabeled mouse monoclonal antibodies (Table 1, Patients 8 and 9). B, three categories of response made by patients receiving murine monoclonals. Category 1 response is identical to that shown by healthy controls, Category 2 is a moderate response, and Category 3 is a pronounced response. C, change in levels of free circulating human anti-mouse immunoglobulin antibodies after administration of mouse monoclonal antibody. The initial reduction indicates immune complex formation.

She showed a moderate immune response. Three months later she received a further 250 μg diagnostic dose. Despite a negative skin test, she immediately produced symptoms typical of a type III hypersensitivity “serum sickness” reaction. An immune complex etiology for this reaction is indicated by the drop in free circulating human anti-mouse immunoglobulin in the 48 h following injection of murine monoclonal antibody (Fig. 1C). This monoclonal antibody treatment subsequently led to a boosting of the human anti-mouse immunoglobulin response (Fig. 1C). The binding of patients’ sera to immunoglobulin (same monoclonal as that used for therapy) on one half of the plate and F(ab’)2 fragments of the same antibody were assayed as described previously using the whole immunoglobulin and, unlike patient 7, a moderate response to the F(ab’)2 fragment.

Moderate anti-mouse immunoglobulin immune response contained antibodies that bound equally well to both the relevant (therapeutic) and the irrelevant (same isotype, IgG1, as therapeutic) monoclonal antibodies. These data suggest that the response is predominantly directed against antigenic determinants on the constant regions of mouse immunoglobulin and is not antiidiotypic. However, we cannot exclude the possibility that there is a small antiidiotypic component in the human anti-mouse immunoglobulin response that is below the “cutoff” level in our ELISA system.

Change in Response to Fc and F(ab’)2 with Further Therapy.

In order to determine the relative contributions in patients’ sera of antibodies to Fc and F(ab’)2, portions of the injected mouse immunoglobulin, ELISA plates were set up as follows. Wells in Rows 1–6 were coated with 500 ng of whole (IgG) relevant therapeutic monoclonal antibody, while wells in Rows 7–12 were coated with an equimolar amount of the F(ab’)2 fragments of the same immunoglobulin. The binding of patients’ sera to these different preparations could then be assayed and compared under identical experimental conditions. This assay was performed with serum from one patient who showed a moderate response (Patient 7) and one patient who showed a pronounced response (Patient 9) to mouse immunoglobulin, together with controls (Fig. 3). Patient 7 (2 diagnostic and 1 therapeutic treatment) showed an elevated response to the whole immunoglobulin but no significant response to the F(ab’)2 fragment, while Patient 9 who had received two injections of therapeutic antibodies showed a pronounced (prozone) response to the whole immunoglobulin and, unlike patient 7, a moderate response to the F(ab’)2 fragment.

In order to determine whether this was a progressive response, the experiment was repeated using sera from two patients who had pronounced anti-mouse immunoglobulin responses and had both been treated twice for therapy. Sera taken after the first and after the second therapy with monoclonal antibody were assayed as described previously using the whole immunoglobulin (same monoclonal as that used for therapy) on one half of the plate and F(ab’)2 fragments of the same immunoglobulin on the other half. The results for these two patients were identical in that although they both showed a moderate response to the whole immunoglobulin after their
HUMAN ANTI-MOUSE IMMUNOGLOBULIN RESPONSE

Fig. 3. Responses made by two patients, Patient 7 with a moderate (Category 2) response and Patient 9 with a pronounced (Category 3) response, to the whole immunoglobulin and the F(ab')2.

Fig. 4. Response made by the same patient after the first therapy with radiolabeled monoclonal antibodies and after the second therapy, to the whole and F(ab')2 fragments of the same murine monoclonal.

Fig. 5. Levels of total immunoglobulin and IgM human anti-mouse immunoglobulin detected in a patient's serum after a first therapeutic and second therapeutic injection of radiolabeled mouse monoclonal antibodies.

Fig. 6. Affinity purification of the IgG component from Patient 9's whole serum results in a loss of prozone effect when assayed against either whole mouse monoclonal IgG or the F(ab')2.

first therapy, they produced a minimal response to the F(ab')2 fraction (Fig. 4). However, after their second therapy, both patients produced an elevated response to the F(ab')2 fragment as well as to the whole immunoglobulin (Fig. 4) and the absolute amount of the IgM component of the human anti-mouse immunoglobulin antibody levels had doubled (Fig. 5).

Characterizing the Prozone Effect. The pronounced anti-
mouse immunoglobulin responses that developed after a second therapeutic injection of radiolabeled monoclonal antibody were typified by a marked prozone effect (Fig. 4). Since this response contained an increased IgM component, the contribution of this isotype to the prozone effect was studied further in Patient 9. To determine the effect on the prozone of removing the IgM from the serum, we tested the precolumn serum which had been shown to contain IgM (Fig. 5) and then tested both the precolumn serum and the affinity purified human IgG anti-mouse immunoglobulin, derived from the same patient.

As shown in Fig. 6, the precolumn serum (Patient 9, after second therapy) showed, as before (Fig. 4), a prozone effect against both whole and F(ab')2 fragment of the relevant monoclonal antibody. In contrast, affinity purified IgG from the same serum sample did not give a prozone to either whole mouse monoclonal IgG or the F(ab')2.

DISCUSSION

One of the major problems encountered when radiolabeled mouse monoclonal antibodies are used as in vivo diagnostic and therapeutic reagents is immunization of the patient to the injected mouse immunoglobulin. This results in the presence of circulating human anti-mouse immunoglobulin antibodies which, on repeated exposure to murine antibody, then lead to an immediate type III hypersensitivity reaction (serum sickness), due to the formation of immune complexes between human IgG anti-mouse immunoglobulin and the monoclonal antibodies. Such a reaction clearly precludes any further serotherapy. We have therefore studied the response of ten patients with ovarian carcinoma or glioma to injected mouse monoclonal antibodies in an attempt to clarify the factors involved in determining whether or not immunization occurs.

Preexisting human anti-mouse immunoglobulin antibodies were detected in the serum of all ten patients prior to any monoclonal antibody therapy and in the serum of 18 healthy controls. Similar antibodies have been demonstrated in melanoma and cutaneous T-cell lymphoma patients by Schroff et al. (14). We have found that the specificity of these preexisting human anti-mouse immunoglobulin antibodies is for antigenic determinants on the Fc rather than the F(ab')2 portion of mouse IgG. However, whether the original immunogen is an autoantigen (e.g., self-immunoglobulin or cross-reactive molecule) or is of extrinsic origin (e.g., immunoglobulin encountered from handling of pets or other animals, or cross-reactive pathogen) is currently unknown. The use of radiolabeled monoclonal
antibodies for imaging did not lead to any detectable increase in circulating anti-mouse immunoglobulin antibodies above this preexisting “background,” possibly because the dose used was too small (250 μg) to be immunogenic or because it was the first injection. In contrast, therapeutic doses (5–10 mg) did lead to further immunization in 50% of the patients in this study. Where increased anti-mouse immunoglobulin levels were detected after the first therapeutic treatment almost all antibody was still directed against the Fc portion [only minimal binding detectable to F(ab')2 fragments]. However, a subsequent therapeutic dose led to both qualitative and quantitative changes in the immune response to mouse immunoglobulin. Total amounts of human anti-mouse immunoglobulin increased, giving a prozone effect; this increase included a doubling of the IgM component. Moreover, at least some of these additional human IgM anti-mouse immunoglobulin antibodies are directed against antigenic determinants on the F(ab')2 portion of mouse immunoglobulin. Thus, injection of murine monoclonal antibodies may have a boosting effect upon the preexisting anti-mouse Fc response, leading to enhanced levels of IgG antibodies, while at the same time providing primary immunization to antigenic determinants on the F(ab')2 portion of the immunoglobulin molecule (apparently not detected by preexisting antibodies), resulting in increased IgM levels.

A prozone effect was characteristic of all ELISA tests on serum from patients with high levels of anti-mouse immunoglobulin antibodies. This probably resulted from an increase in monovalent (only one binding site per immunoglobulin molecule is occupied by antigen) binding of antibody to antigen, imposed by the high antibody concentration. This effect would be more extreme for the IgM component since IgM antibodies are generally of lower affinity (strength of monovalent binding to antigen) than IgG antibodies, and the difference in overall affinity of pentavalent versus monovalent monovalent binding to antigen is considerable (in the region of 10^6), whereas for IgG the difference in overall affinity between divalent versus monovalent is much less (approximately 10^3). The consequence of very low binding affinities is that such antibodies will be very easily washed off the antigen coated plate during the assay, hence giving spuriously low results at high antibody concentrations (the prozone). This is supported by the observation that removal of the IgM anti-mouse immunoglobulin antibodies from Patient 9’s serum also removed the prozone effect. However, IgM depletion would obviously also reduce the total anti-mouse immunoglobulin concentration which in itself would contribute to loss of the prozone. The practical importance of this phenomenon is clear; full titrations of a patient’s serum must be assayed in order to avoid the possibility of falsely low values for anti-mouse immunoglobulin activity at the higher serum concentrations.

Five of ten patients did not show elevated anti-mouse immunoglobulin levels after either one or two therapeutic doses of murine monoclonal antibody, while of the five who did, only one developed an immediate hypersensitivity response. The small drop in free circulating anti-mouse immunoglobulin antibodies observed in this patient after therapy is suggestive of an immune complex etiology, although further studies are necessary to establish this point. It would clearly be of considerable value if the immunological outcome of serotherapy could be predicted before the start of treatment; however, we have been unable to identify any consistent prognostic feature in the current group of patients. Furthermore, even the routine mouse immunoglobulin skin test has not been of predictive value, since the patient (Patient 7) who progressed to a type III hypersensitivity reaction did not show a positive skin test. However, this test primarily detects mast cell bound IgE mediated type I hypersensitivity (anaphylaxis) and so may be inappropriate for monitoring a disease that results from circulating IgG and IgM antibody-antigen complexes. The work presented in this paper is based upon data derived from ten patients receiving radiolabeled mouse monoclonal antibodies for tumor imaging and therapy. We are currently extending this study to cover a larger patient population to test the generality of our findings.

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