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HUMAN IMMUNE RESPONSE TO MULTIPLE INJCTIONS OF MURINE MONOCLONAL IgG

DANIEL L. SHAWLER, RICHARD M. BARTHOLOMEW, LINDA M. SMITH, AND ROBERT O. DILLMAN

From the *Theodore Gildred Cancer Center, University of California, San Diego School of Medicine, La Jolla, CA; †Hybritech, Incorporated, San Diego, CA; and the *Division of Hematology/Oncology, Veterans Administration Medical Center, San Diego, CA.

Murine monoclonal antibody infusions in humans should induce a human anti-mouse immunoglobulin (mIgG) immune response, especially if multiple infusions over an extended period of time are necessary for therapeutic efficacy. We have administered multiple infusions of the murine monoclonal antibody T101 to patients with cutaneous T cell lymphoma (CTCL) or chronic lymphocytic leukemia (CLL). Of 10 CTCL patients, compared with zero of six CLL patients, developed antibodies to mIgG. In those CTCL patients who did not demonstrate anti-mIgG antibodies, we were unable to correlate the lack of response to any of a large number of clinical parameters. Anti-mIgG antibodies were of both the μ and γ isotypes and were detectable 14 days after the first infusion. Multiple infusions were associated with elevated titers. The anti-idiotypic portion of the anti-mIgG titer steadily increased with each infusion until eventually, in one patient receiving eight weekly infusions, well over one-half the serum anti-mIgG recognized only T101 and not four other murine IgG2AK antibodies tested. To increase our confidence in these findings, four separate assay systems were used to make these determinations. The identification of anti-idiotypic antibodies as the dominant species of the immune response to multiple infusions of murine monoclonal antibody has major implications for future work with monoclonal antibodies. Although it has been suggested that human monoclonal antibodies would obviate an immune response, our work implies that such antibodies might still induce anti-idiotypic antibodies if multiple infusions are administered.

Because most monoclonal antibodies today are derived from murine hybridomas, multiple infusions of these antibodies into humans would be expected to elicit an immune response. Preliminary investigations have substantiated that anti-mouse IgG (mIgG) antibodies can occur after infusions of monoclonal antibodies and have described their effect upon therapy (1–6).

We have given multiple infusions of the anti-human T cell monoclonal antibody T101 (7) to patients with cutaneous T cell lymphoma (CTCL) or chronic lymphocytic leukemia (CLL) (1, 8). In contrast to CLL, where none of the six patients had evidence of an immune response to T101, five of the 10 CTCL patients had measurable circulating anti-mIgG antibodies. By using several different serologic assays, we quantitated the immune response and characterized the T101 specificity of the antibodies involved. In this report, we present a detailed description of our observations and discuss implications for future clinical uses of monoclonal antibodies.

MATERIALS AND METHODS

Antibodies. T101 is a murine monoclonal IgG2AK antibody reactive with a 65,000 to 67,000 m.w. antigen found on normal and neoplastic T cells, thymocytes, and CLL cells (7). Other murine monoclonal IgG2AK antibodies used as irrelevant antibodies for in vitro experimentation were L22 (9), 9.2.27 (10), 96.5 (11), and ZME 018 (12). Monoclonal antibodies were purified by either protein A-affinity chromatography (13) or DEAE ion-exchange chromatography (14) as described.

Affinity-purified, horseradish peroxidase-conjugated goat IgG anti-human IgG (y-chain specific) and anti-human IgM [μ-chain specific] (TAGO, Inc., Burlingame, CA) were used at dilutions of 1/10,000 as secondary reagents in enzyme-linked immunosorbant assays (ELISA). Each reagent was absorbed against mlgG before use to remove cross-reactivity.

Therapy with T101. Multiple serum samples were obtained before and after T101 therapy in six patients with CLL and 10 patients with CTCL. The T101 dosage and unique patient numbers are shown in Table 1. Thirteen men and three women participated in the study, ranging in age from 22 to 82 yr, with a mean age of 61 ± 15 yr. The CLL patients had the characteristic membrane phenotype of CLL, which is both surface immunoglobulin-positive and T101+ (15). Of the 10 CTCL patients, eight had an excess population of T101+, Leu-3+ cells in their blood, whereas one patient had infiltration of such cells only in a skin biopsy, and the tenth patient had an increase in T101+, Leu-2+ cells in the blood. Patients had received prior therapy for their disease, but other than topical steroid preparation in CTCL, none had received antitumor therapy for at least 4 wk before T101 therapy.

Previous experiences with T101 have been published (1, 8, 16–18). In this study, patients received weekly i.v. infusions of T101 at doses of 10, 50, 100, or 500 mg/24 hr. In some cases, particularly at the larger doses, therapy was delayed because of the continued...
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Therapy with T101 monoclonal antibody. A summary of patients with T101 is shown in Table 1. Five of 10 CTCL patients, compared with zero of six CLL patients, developed anti-mIgG titers greater than their pretreatment titer. Elevated anti-mIgG titers were first apparent in the serum sample obtained 14 days after initiation of therapy. Circulating anti-mIgG antibodies were present at the time of infusion for 11 of 38 treatments in CTCL and zero of 18 in CLL. The presence of anti-mIgG antibodies before infusion was associated with decreased therapeutic effect as judged by lower serum T101 concentrations, decreased in vivo binding of T101 to circulating target cells, and decreased target cell destruction.

Table 1

<table>
<thead>
<tr>
<th>Disease</th>
<th>Patient Number (Unique Patient Number)</th>
<th>Number of Treatments x T101 Dose</th>
<th>Host Anti-mIgG Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTCL</td>
<td>1 (20) 4x10 mg 2 (51) 4x10 mg 3 (51) 4x10 mg 4 (60) 2x50 mg 5 (58) 3x50 mg 6 (36) 4x50 mg 7 (18) 4x100 mg 8 (14) 2x100 mg 9 (71) 2x500 mg 10 (70) 1x500 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLL</td>
<td>1 (8) 4x10 mg 2/27 1x10 mg 3 (45) 2x100 mg 4 (47) 1x100 mg 5 (68) 3x100 mg 6 (44) 1x500 mg</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Characterization of anti-mIgG immune response.* Human anti-mIgG antibodies were detected by solid-phase ELISA. The desired murine IgG2AK monoclonal antibody was immobilized on 96-well styrene U-bottom microtitration plates (Costar, Van Nuys, CA) at a concentration of 500 ng/well. The plates were washed one time with phosphate buffered saline (PBE) containing 0.3% swine skin gelatin (Sigma Chemical Co., St. Louis, MO) and then 50-μl serum samples diluted in PBS containing 10% fetal bovine serum (JMI, Kansas City, KS), 1% bovine serum albumin (Sigma), and 0.3% swine skin gelatin were added for 1 hr at 37°C. The plates were washed three times, and 50 μl of a 1/10,000 dilution of secondary antisera was added to each well for 1 hr at 37°C. The plates were washed three times, and 100 μl of a substrate solution composed of 0.4 mg/ml O-phenylene-diamine and 0.0065% H2O2 in 0.1 M citrate buffer, pH 5.0, was added to each well. The reaction was stopped after 30 min by the addition of 10 μl 2.5 N H2SO4. Plates were read at 495 nm on an Automatic ELISA Reader (Dynatech Instruments, Inc., Santa Monica, CA).

A standard curve to quantitate the anti-mIgG response was constructed by using human IgG anti-mIgG prepared by affinity chromatography (19) from a pool of sera obtained from two responding patients. The IgG fraction was separated from the IgM fraction by protein A-affinity chromatography (20) and by a Coomassie Blue protein determination assay with bovine γ-globulin as the standard (Bio-Rad, Richmond, CA). Similar results were obtained with both methods.

The proportion of anti-mIgG antibodies that could react only with T101 was determined by four separate methods. In the first, the T101 immobilized on the microtiter plate was replaced by other murine IgG2AK antibodies, and an ELISA was performed as described above. The second method was a competitive inhibition assay in which T101 or other murine IgG2AK antibodies were preincubated with serum samples at concentrations of 1 mg/ml for the IgG and 50 ng/ml for the anti-mIgG immunoglobulins before testing by ELISA. In the third method, T101 and other murine IgG2AK antibodies were coupled to CNBr-activated Sepharose 4B (Sigma) as described (21). Serum samples were diluted in RPMI media containing 10% fetal bovine serum and were incubated overnight at room temperature with the Sepharose adsorbents in a 200-fold excess over the anti-mIgG antibodies in the serum. The nonbound fraction was collected, and the bound antibodies were eluted with 0.1 M Tris glycine, pH 2.2, and were immediately neutralized. The fourth method employed hemagglutination as described above. The desired murine IgG2AK antibody was coupled to sheep red blood cells (SRBC) (Colorado Serum Co., Denver, CO) by using the chloroform technique (22). For coupling, the antibody was diluted in saline at a concentration of 1 mg/ml. Hemagglutination titers were done in microtiter plates by mixing 20 μl of a 25% antibody-SRBC suspension with 40 μl of the appropriately diluted serum samples. Negative controls included mixing the two different SRBC and mixing pretreatment normal serum with the antibody-control SRBC. Titers were described as the dilutions where agglutination was no longer visible.

*Results.*

Figure 1. Anti-mIgG antibodies in five patients with CTCL. Sera from patients with CTCL were tested by ELISA for IgG anti-mIgG reactivity. Concentrations of anti-mIgG were obtained by comparison with a standard curve with the use of affinity-purified human IgG anti-mIgG antibody. The T101 dose (mg) and the treatment schedule are indicated by arrows. The CTCL patients exhibiting anti-mIgG antibodies were patient 2 (A), patient 3 (B), patient 7 (C), patient 10 (D), and patient 9 (E).
found in CLL patients (1.99 ± 0.91 μg/ml), but normal human serum showed higher levels (3.86 ± 0.80 μg/ml).

A decrease in the concentration of anti-mlgG antibodies in serum obtained 7 days after the first infusion was noted both in those CTCL patients who later produced anti-mlgG antibodies (0.97 ± 0.69 μg/ml) and in the CLL patients (1.03 ± 0.11 μg/ml), but not in CTCL patients, who did not produce anti-mlgG antibodies (1.73 ± 0.60 μg/ml). Both competitive inhibition ELISA and immunoabsorption by T101-Sepharose failed to remove this reactivity from any serum.

T101-specific anti-T101 antibodies. A competitive inhibition ELISA, performed on immune serum from patient 7, shows that T101 competes for T101 binding sites more efficiently than irrelevant antibodies (Figure 2A), whereas the inverse is not true. T101 and irrelevant antibodies are equally competitive for binding sites on the irrelevant IgG2AK (Figure 2B).

Figure 3 shows that the T101-specific portion of the immune response, indicated by differences in T101 inhibition vs inhibition by irrelevant IgG2AK monoclonal antibodies, dramatically increases with the number of infusions. The difference was relatively low (5.4 to 22.6%) at the beginning of the immune response but eventually reached levels of greater than 50% after multiple infusions. It is important to note that identical results were obtained with either L22 or 9.2.27, two separate irrelevant IgG2AK antibodies. Both the IgM and IgG responses gave similar results in this assay.

Another method of detecting T101-specific antibodies involved adsorbing the antibodies on Sepharose conjugated to T101 or to three other irrelevant IgG2AK antibodies. Both the adsorbed fractions and the low pH eluates were collected and tested by ELISA. These procedures were performed on pretreatment serum from patient 3 and on serum obtained from the same patient 14 and 49 days after the first infusion. Previous results suggested that serum obtained on day 14 contained relatively little T101-specific reactivity, whereas serum obtained on day 49 contained significant T101-specific reactivity (see Figure 3B). As shown in Figure 4, pretreatment serum gave only background reactivity on plates containing immobilized T101 or 96.5. The T101 column was only slightly more efficient than the irrelevant IgG2AK columns in removing T101 reactivity from day 14 serum. In day 49 serum, however, the T101-Sepharose was able to remove the majority of the T101-specific reactivity, whereas the irrelevant IgG2AK-Sepharose adsorbants were much less effective, yielding levels equivalent to the FCS control. When the same serum was

![Figure 2](http://www.jimmunol.org/)

![Figure 3](http://www.jimmunol.org/)
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Distinguish between TlOl and other IgG2AK molecules, and can be
The anti-mIgG activity in the 7-wk serum, however, contains two subsets
first infusion showed a titer of 1:100 on TlOl-SRBC yet
contains immobilized TlOl
sorbed by other murine IgG2AK antibodies (A). The other subset does not
pretreatment serum from patient 3 was negative on both
TlOl-specific and that this portion of the response is against the variable region. We realize that
the lack of reactivity with only four irrelevant antibodies
to anti-idiotype is not reactive with the variable region. We realize that
the presence of immunoglobulin reactive
with TlOl but not with four other murine IgG2AK monoclonal antibodies
is significant because of future clinical applications of monoclonal antibodies
in general, and for the potential use of human monoclonal antibodies
in particular. Because the four irrelevant monoclonal antibodies are composed of the same heavy and light chains as TlOl, our data suggests that a portion of the response is against the variable region. We realize that
the lack of reactivity with only four irrelevant antibodies
does not rule out the possibility of differences in affinity binding or in cross-reactivity with other antibodies. This is why we also used four different methods to confirm our results. We feel that the consistency and reproducibility of our results suggest that the T101-specific response is anti-idiotype. This, however, is a functional
definition of anti-idiotype that does not make the finer
distinction between anti-idiotype that reacts with the antigen-binding site and anti-idiotype that reacts with other epitopes (23).

In each of the four responding patients who received multiple infusions of T101, the portion of the response that was T101 specific increased with the number of infusions. In two separate patients, more than 50% of the immune response to T101 was not reactive with other murine IgG2AK antibodies after multiple infusions. Significantly, the highest T101-specific response was achieved in the patient receiving the most infusions (eight). Other investigators have reported low levels of T101-specific antibodies in the immune sera of patients
and primates treated with murine monoclonal antibodies
(2, 3, 6, 24), but none have reported the high levels found
in our study. Nor did they rigorously confirm their results by testing with multiple irrelevant monoclonal antibodies
as we have done. Additionally, other investigators have not examined the effect of multiple infusions on the formation of monoclonal antibody-specific (anti-idiotype) antibodies.

Results similar to ours have been achieved in animals
immunized with xenogeneic immunoglobulin preparations. In mice immunized with a single human myeloma
IgG protein, 43% of the resulting hybridomas were reactive with the variable region of the antigen. In contrast, the same study showed that mice sequentially immunized with two human myeloma IgG proteins did not produce hybridomas secreting anti-variable region antibodies (25). Similar results were achieved in rabbits immunized with mouse myeloma proteins (26). In contrast, animals
immunized with polyclonal immunoglobulin preparations showed no reactivity with variable regions (27, 28).
Our findings are in complete agreement with these studies,
suggesting that multiple infusions of a single monoclonal antibody will result in a large specific response. It also suggests that infusion of two or more monoclonal antibodies may result in only anti-constant region antibodies.

Figure 4. Removal of anti-mIgG antibodies by adsorption. Pretreatment (nonimmune) serum, and serum obtained 2 wk and 7 wk after
initiation of therapy in patient 3 were adsorbed with FCS ( ), T101 ( ),
96.5 ( ), 9.2.27 ( ), and ZME 018 ( ) conjugated to Sepharose 4B, or were left unadsorbed ( ) and were tested by ELISA on microtiter plates containing immobilized T101 (A) or 96.5 (B). The pretreatment sample gives only background reactivity. The anti-mIgG reactivity in the 2-wk serum sample does not distinguish between T101 and other IgG2AK molecules. The anti-mIgG activity in the 7-wk serum, however, contains two subsets of antibodies. One subset reacts with T101 idiotype and cannot be adsorbed by other murine IgG2AK antibodies (A). The other subset does not distinguish between T101 and other IgG2AK molecules, and can be adsorbed with other murine IgG2AK antibodies as well as T101 (B).

The data reported in this paper demonstrate that a subset of CTCL patients (five of 10 patients in our study) developed an immune response to mIgG after infusions of murine monoclonal antibody T101. Conversely, none of the six CLL patients in our study developed such a response. When it was present, the immune response consisted of both IgM and IgG anti-mIgG antibodies. We have documented that a large portion of the response is T101-specific and that this portion of the response increases with the number of infusions.

To ensure that these data, which we were interpreting as the presence of significant amounts of T101-specific antibodies, were not artifacts of ELISA, we reconfirmed the T101-specific reactivity by using a hemagglutination assay. As shown in Table II, the agglutination titer of pretreatment serum from patient 3 was negative on both
T101- and 96.5-SRBC. Serum obtained 14 days after the first infusion showed a titer of 1:100 on T101-SRBC yet was still undetectable at the same dilution on 96.5-SRBC. Serum from day 49 showed agglutination at a dilution of 1/4000 on T101-SRBC compared with a dilution of 1/2500 on 96.5-SRBC.

DISCUSSION

The data reported in this paper demonstrate that a subset of CTCL patients (five of 10 patients in our study)
We were encouraged that many patients failed to produce anti-mlgG antibodies; in our study, only five of 10 CTCL patients and zero of six CLL patients developed an immune response to mlgG. In a study in which the anti-gastrointestinal adenocarcinoma antibody 1083-17-1A was used, eight of nine patients receiving doses of less than 200 mg monoclonal antibody developed an immune response compared with only one of nine patients receiving 366 to 1000 mg (6), and the investigators suggested that larger doses of monoclonal antibodies could tolerate patients to murine immunoglobulin. Our data do not confirm this finding, but significant differences between antibody reactivity and the tissue distribution of the antigens may account for this discrepancy.

A compilation of all available data on anti-mlgG immune responses (1-6, 23) shows that lower numbers of patients with lymphoid malignancies develop antibodies to murine IgG when compared with other patients. The rate of response for CLL and CTCL is 0% and 41%, respectively, compared with 89% for gastrointestinal adenocarcinoma (at lower doses of 1083-17-1A), 73% for renal allograft recipients, and 100% for normal chimpanzees. The 100% response of chimpanzees to anti-Leu-1 and the 100% response of other animals to xenogeneic immunoglobulin (25-28) suggests that healthy humans should also have a 100% response rate to murine monoclonal antibodies. The lower rate of response in cancer patients can possibly be attributed to the immunosuppressive state of their disease (29) as well as prior immunosuppressive therapy. CLL and CTCL patients, in particular, have been shown to be immunosuppressed (30, 31). Similarly, the response rate in renal allograft recipients can possibly be attributed to the immunosuppressive therapy accompanying the allograft (4).

We were able to consistently measure anti-mlgG reactivity in pretreatment CLL and CTCL serum, as well as normal serum. This led us to believe we could be measuring endogenous anti-mlgG reactivity. There was a decrease in this reactivity 7 days after the first infusion in CLL patients and in those CTCL patients who proceeded to mount an immune response to mlgG, but it was not statistically significant. The anti-mlgG reactivity found in normal serum was equivalent to the level of endogenous anti-human IgG (rheumatoid factor) also found in normal serum (32). Repeated attempts in which two separate techniques were used (adsorption and competitive inhibition) failed to remove any of the anti-mlgG reactivity, suggesting that the anti-mlgG levels measured were probably related to the sensitivity of the assay and were nothing more than background levels caused by nonspecific human immunoglobulin.

The production of anti-mlgG antibodies has significant implications for immunotherapy with monoclonal antibodies. In the five patients described here who developed anti-mlgG antibodies, and in a sixth patient previously described (1), the measurement of anti-mlgG antibodies was associated with a diminished clinical response. Once the anti-mlgG antibodies were present, the antibody-mediated decrease in the target cell population previously described (1, 8, 17) was measurably less in both extent and duration, even though some in vivo binding of T101 to circulating target cells was still demonstrable. The toxicity typically associated with the elimination of circulating target cells (fever, chills, and malaise) was no longer observed, and serum levels of T101 became negligible, even when higher doses were administered. We examined the relationship between development of anti-mlgG antibodies and a number of clinical parameters, including prior therapies, concurrent medications, in vitro mitogen responsiveness, serum immunoglobulin levels, circulating target cell concentrations, and performance status, but we could not find any correlation predictive of immune response to mlgG.

It has become increasingly evident that the production of anti-mlgG antibodies inhibits therapeutic efficacy of monoclonal antibodies and needs to be avoided. Many people hope that human monoclonal antibodies will be the key to preventing the immunogenicity associated with mlgG, but our finding of a significant portion of T101-specific antibodies in the response to mlgG suggests that the same phenomenon may also occur with human monoclonal antibodies. The use of immunoconjugates with toxin or drug molecules could possibly prevent an immune response by binding to and destroying the anti-mlgG-producing B cells as well as the target cells. This could be another potential advantage for the use of such immunoconjugates in monoclonal antibody therapy. Finally, we feel that monitoring patients for anti-mlgG responses is critical in properly determining the patient's therapeutic protocol, and we suggest the hemagglutination assay as a convenient, rapid method for screening patients just before treatment.

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