Induction of in Vitro and in Vivo Antigenic Modulation by the Anti-Human T-Cell Monoclonal Antibody T101

Daniel L. Shawler,† Mary C. Miceli, Susan B. Wormsley, Ivor Royston, and Robert O. Dillman

ABSTRACT

Because of its implications for the therapeutic application of monoclonal antibodies, we have studied antigenic modulation in vitro and in vivo in patients receiving T101 monoclonal antibody. Incubation of normal peripheral blood T-cells, chronic lymphocytic leukemia cells, and cutaneous T-cell lymphoma cells with an excess of T101 at 37°C induced modulation of the T65 antigen. When assayed by indirect immunofluorescence, a change in cellular reactivity with T101 was seen after 1 hr. After 24 hr, normal T-cells showed a 94 ± 4% (S.D.) decrease in fluorescence, compared to an 82 ± 6% decrease for chronic lymphocytic leukemia cells and a 56 ± 4% decrease for cutaneous T-cell lymphoma cells. When T101 was removed from the culture, the cells reexpressed T65. Modulation was inhibited by cold temperatures, suggesting that it is energy dependent. Patients with chronic lymphocytic leukemia, cutaneous T-cell lymphoma, or T-cell lymphoma have received 24-hr infusions of 3 to 500 mg T101 in therapeutic trials. After infusion, in vivo binding of T101 was observed in 39 of 43 treatments not associated with endogenous host anti-T101 antibodies. T65-target cells were seen in 39 of 43 treatments associated with in vivo bound T101, suggesting that modulation had occurred. When cultured in vitro for 24 hr, these cells reexpressed T65. In vivo, reexpression of T65 occurred following disappearance of the serum T101 titer. The extent and duration of in vivo modulation were related to both the T101 dose and the tumor burden. These data suggest that the rapid rate of antigenic modulation may prevent potential target cell destruction by antibody-mediated cytotoxicity. However, if the process of modulation involves internalization of the antibody:antigen complex, it would be an advantage for the use of cytotoxic immunoconjugates.

INTRODUCTION

Passive therapy with monoclonal antibodies may produce an anticancer effect through a host antibody-mediated cytotoxicity mechanism, such as complement-mediated and antibody-dependent cell-mediated cytotoxicity, which requires the presence of whole antibody on the surface of the target cells (2). Antigenic modulation is an antibody-mediated decrease in the expression of target antigen (13, 14). The rapid occurrence of antigenic modulation during monoclonal antibody therapy could severely limit any antibody-mediated antitumor effects. Therapeutic clinical trials are being conducted with T101 (17) and anti-Leu 1 (6), murine monoclonal antibodies which recognize the T65 antigen, found on normal and neoplastic T-cells, and on CLL (3) cells (4, 5, 9–11). Several reports have appeared which comment on the occurrence of circulating antigen-negative target cells (i.e., cells which bear the target cell phenotype except for T65) during treatment, but no detailed analysis of T65 modulation has been reported. While the appearance of circulating T65 cells is consistent with antigenic modulation, the investigators have not provided rigorous evidence of antigenic modulation. An alternative explanation is that of immunoselection of T65−cells by selective elimination of T65+cells.

Because antibody-induced antigenic modulation is a possible obstacle to successful monoclonal antibody serotherapy, we have examined the kinetics of in vitro and in vivo T101-induced T65 modulation in normal T-cells, CLL cells, and CTCL cells and discussed the implications of these observations for passive or immunoconjugate monoclonal antibody therapy.

MATERIALS AND METHODS

Antibodies. T101 is a murine IgG monoclonal antibody reactive with a M, 65,000 to 67,000 antigen, termed T65, found on normal and neoplastic T-cells, thymocytes, and CLL cells (15). BA4 is a murine IgG monoclonal antibody reactive with the HLA-DR antigens found on normal B-cells, CLL cells, and activated T-cells (21). Anti-Leu 4 (a generous gift from Dr. Robert Evans of the Sloan-Kettering Institute) is a murine IgG monoclonal antibody reactive with a M, 20,000 to 30,000 antigen found on normal and neoplastic T-cells (8).

For indirect immunofluorescence, MFC11, a murine IgG myeloma protein (Litton Bionetics, Kensington, MD), was used as a negative control, and fluorescein-conjugated goat F(ab′)2 anti-mouse IgG (TAGO, Inc., Burlingame, CA) was used as the secondary antibody.

Immunofluorescence Staining. Ten ml of fresh heparinized venous blood were obtained from normal healthy donors and from patients with a clinical diagnosis of CLL or CTCL. Mononuclear cells isolated from these samples by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density centrifugation were used to study in vitro modulation. In addition, in vivo modulation was studied on mononuclear cells isolated from blood samples obtained from patients with CLL, CTCL, or T-cell lymphoma during i.v. T101 infusion given over 24 hr at doses of 3 to 500 mg T101. Indirect immunofluorescence was performed as described previously (3), and the percentage of cells reacting with each monoclonal antibody and the MIF were processed on a Model 50H Ortho Cytofluorograf equipped with a Model 2150 data processing system (Ortho Diagnostic Systems, Westwood, MA).

In Vitro Antigenic Modulation. Cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (Irvine Scientific, Irvine, CA) at a concentration of 10⁶ cells/ml at 37°C and 5% CO₂. A 100-μg/ml concentration of either T101 or MFC11 ascites was added to the cultures. At the appropriate time points, 10⁶ cells were removed and stained by indirect immunofluorescence with T101 or MFC11. Cell
numbers did not change during prolonged cultures, indicating that proliferation had not occurred. Cell viability, as determined by trypan blue exclusion, was 90 to 100%. Cells from the cultures containing T101 generated identical histograms when stained with T101 or MPC11, demonstrating that the culture was in antibody excess. The percentage of in vitro T101-induced modulation was calculated as a percentage of the MIF control by using the formula

\[
\text{Modulated MIF} - \text{background MIF} \\
\text{Control MIF} - \text{background MIF} \\
\times 100
\]

Reexpression of T65 was evaluated by incubating cells with T101 as described above, washing the cells 5 times to remove unbound T101, and culturing the cells without T101, at 10^6 cells/ml at 37°, 5% CO2. At appropriate time points, 10^6 cells were removed and stained by indirect immunofluorescence.

To determine the temperature dependence of modulation, cells were cultured for 1 hr on ice, at room temperature, or at 37° with an excess of T101 or MPC11, and were stained by indirect immunofluorescence.

**In Vivo Antigen Binding and Modulation.** To measure in vivo T101 binding, mononuclear cells obtained during and after infusion of T101 were stained directly with fluorescein-conjugated goat F(ab′)_2 anti-mouse IgG. The resulting histograms were compared with pretreatment cells stained indirectly with MPC11 or T101. The percentage of T101-induced in vivo modulation was calculated using the formulas

for CTCL patients:

\[
1 - \frac{\% \text{ of T101}^+ \text{ cells}}{\% \text{ of Leu 4}^+ \text{ cells}} \\
\times 100
\]

for CLL patients:

\[
1 - \frac{\% \text{ of T101}^+ \text{ cells}}{\% \text{ of HLA-DR}^+ \text{ cells}} \\
\times 100
\]

In order to examine the capacity of T65—malignant cells to synthesize T65, cells from pretreatment samples which expressed the malignant phenotype (except for T65) were incubated for 24 hr at 37°, 5% CO2, and were stained with T101 and other monoclonal antibodies.

**RESULTS**

The rates of T101-induced in vitro modulation for normal T-cells, CLL cells, and CTCL cells are shown in Chart 1. The results of experiments on 5 separate donors with each phenotype revealed significant differences among their rates of modulation. After the first hr of modulation, there was a 28 ± 3% decrease in fluorescence in normal T-cells, a 5 ± 8% decrease in fluorescence in CLL cells, and a 27 ± 10% increase in fluorescence in CTCL cells. After 24 hr of modulation, normal T-cells expressed 6 ± 4% of the original fluorescence, CLL cells expressed 18 ± 4% of the original fluorescence, and CTCL cells expressed 27 ± 6% of the original fluorescence. These experiments were performed in the presence of excess T101 (100 μg/ml). If less than saturating concentrations of T101 were used, incomplete modulation was observed (results not shown).

CLL cells and normal T-cells were incubated in an excess of T101 for 24 hr and were washed free of T101 in order to examine their ability to reexpress T65. Chart 2 shows the results of experiments performed on cells from 5 separate patients or normal donors. Seventy-two hr after the removal of T101 from culture, normal T-cells expressed 74 ± 3% of their original fluorescence, and CLL cells expressed 55 ± 4% of their original fluorescence. Table 1 shows the inhibitory effect of cold temperatures on T101-induced modulation that was seen in experiments performed on cells from 5 individual donors with CLL or normal donors.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Normal T-cells MIF (%) of control</th>
<th>CLL cells MIF (%) of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>2°</td>
<td>125.0 ± 7.0°</td>
<td>109.0 ± 9.1°</td>
</tr>
<tr>
<td>21</td>
<td>102.3 ± 9.0°</td>
<td>98.0 ± 1.0°</td>
</tr>
<tr>
<td>37</td>
<td>70.7 ± 10.6°</td>
<td>86.5 ± 2.9°</td>
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*Mean ± S.D. of 5 experiments.

Table 2 summarizes data obtained during 51 infusions of T101 in 7 patients with CTCL, 6 patients with CLL, and one patient with T-cell lymphoma. The infusions lasted 24 hr, and T101 doses ranged from 3 to 500 mg. To demonstrate in vivo binding
of T101, mononuclear cells obtained before and during infusion were stained with MPC11 or T101. Cells that had bound T101 during infusion demonstrated a higher fluorescence intensity when stained by MPC11, compared to pretreatment cells also stained with MPC11. Charts 3 and 4 illustrate representative examples in which CLL patients and a CTCL patient, respectively, demonstrated in vivo T101 binding during infusions of 100 mg T101. The identity between the histograms generated with MPC11 or T101 indicates that T101 had saturated the target cells in vivo in these examples.

In vivo T101 binding could not be detected during 8 infusions which were associated with the presence of endogenous host anti-T101 antibodies.\footnote{D. L. Shawler and R. O. Dillman, unpublished data.} In vivo binding of T101 could not be measured during 4 infusions of 10 mg T101 in a CLL patient with a lymphocyte count of 200,000/μl but was measurable in 2 of 2 infusions of 100 mg T101 in the same patient. In vivo, bound T101 was readily measurable during all infusions not associated with host anti-T101 antibodies in patients with CTCL and in the remaining CLL patients.

Chart 5 demonstrates in vivo T101-induced modulation in a CLL patient who received 100 mg of T101. Cells obtained prior to treatment were T101+ and BA4+, whereas cells obtained at the end of treatment were T101− and BA4+. After posttreatment, cells were incubated in vitro at 37°C for 24 hr, and they regained the T101+, BA4+ phenotype, demonstrating that they had the capability to express T85 and were not a clone of T65 cells selected during treatment. Examples of in vivo modulation can also be seen in Charts 3 and 4.

All 39 treatments associated with in vivo bound T101 demonstrated in vivo modulation. The relationship between T101 dose and modulation is shown in Chart 6. These data show that larger doses of T101 resulted in faster, greater, and more persistent modulation. Modulation has persisted for at least 9 days in a CLL patient who received 500 mg T101 and for at least 7 days in a CTCL patient who received 100 mg T101. In treatments with smaller T101 doses, modulation always disappeared by Day 7.

Serum levels of T101 obtained during treatment are shown in Chart 7. As with modulation, infusion of larger doses of T101 resulted in larger and more persistent concentrations of T101 in the serum. The disappearance of modulation always coincided with the disappearance of measurable serum T101.

The peripheral blood mononuclear cells of one CTCL patient consisted of 2 separate lymphocyte populations that were easily distinguished by right-angle light scatter on the cell sorter. Phenotypic analysis of the 2 populations demonstrated that one population was 84 ± 4% T101+, Leu 3+, and Leu 4+, while the other population was 27 ± 5% T101+, Leu 2+, and Leu 4+, and 38 ± 3% T101+, Leu 3+, and Leu 4+. Although we realize that there can be overlapping cell populations between the 2 regions, we believe that this suggests that the first population contains a homogeneous population of malignant cells bearing the helper phenotype, while the second contains phenotypically normal lymphocytes with a heterogeneous population consisting of both helper and suppressor-cytotoxic T-cells. This afforded a unique opportunity to compare the in vivo modulation of normal and malignant T-cells. The results of 4 infusions of 50 mg T101, shown in Chart 8, demonstrate that the population with the malignant phenotype modulated more slowly than did the population with the normal phenotype (p < 0.001 at 12 hr). These data were remarkably similar to the data obtained for in vitro modulation.

**DISCUSSION**

The data presented here provide the first detailed description and comparison of in vitro and in vivo antigenic modulation induced by a monoclonal antibody with clinical potential. Our results demonstrate that T101 induces in vitro T65 modulation in normal T-cells, CLL cells, and CTCL cells but that the rate of modulation differs for each. Normal T-cells modulate more rapidly than do CLL cells, which modulate more rapidly than do CTCL cells. After removal of T101, normal T-cells appear to reexpress T85 at a faster rate than do CLL cells. T65 modulation is inhibited by cold temperatures in both normal T-cells and CLL cells, suggesting that it is an energy-dependent phenomenon. More importantly, and with significant implications for the clinical use of T101 as well as other monoclonal antibodies, our data demonstrate that the in vivo use of T101 results in dose-dependent antigenic modulation of circulating target cells which persists
T101-INDUCED ANTIGENIC MODULATION

Chart 3. In vivo binding of T101 during infusion of 100 mg in a patient with CLL. A, background immunofluorescence on pretreatment lymphocytes stained with MPC11; B, reactivity of pretreatment lymphocytes with T101; C, immunofluorescence of lymphocytes obtained during infusion stained with MPC11; D, immunofluorescence of lymphocytes obtained during infusion with T101.

until T101 has been cleared from circulation.

The reason for the slower modulation kinetics of malignant cells, compared to normal T-cells, is unclear at this time. A number of other differences between CLL cells and normal lymphocytes have already been reported. CLL cells have lower levels of reactivity with T101 (20) and a lower density of surface immunoglobulin (17,19). The rate of capping of surface immunoglobulin (1) and the cellular motility (7) of CLL cells are considerably slower than those of normal B-cells, and CLL cells contain less actin than do normal B-cells (18). These data suggest there may be important cytoskeletal and membrane differences in CLL cells that result in slower modulation of surface proteins.

The results of our study are similar to data published with J5, a monoclonal antibody reactive with the common ALL antigen. J5 induced antigenic modulation in the ALL cell lines Laz 221 and NALM-1 and in primary cultures of ALL cells (12, 13). By using flow cytometry and indirect immunofluorescence, modulation was shown to be rapid, reversible, and temperature dependent with kinetics similar to T65 modulation. The similarities between T101-induced and J5-induced antigenic modulation suggest that a common mechanism for modulation of tumor-associated antigens may exist and that these results may therefore be useful in future work with other monoclonal antibodies.

The results described in our previous papers were primarily comprised of patients receiving short 15-min or 2-hr infusions of relatively low doses (1 to 10 mg) of T101 (4, 5). Our observation of in vivo antigenic modulation was preliminary in nature and was made in only the 3 patients receiving 24-hr infusions and...
Chart 4. In vivo binding of T101 during infusion of 100 mg in a patient with CTCL. A, background immunofluorescence on pretreatment lymphocytes stained with MPC11; B, reactivity of pretreatment lymphocytes with T101; C, immunofluorescence of lymphocytes obtained during infusion stained with MPC11; D, immunofluorescence of lymphocytes obtained during infusion with T101.

not in the patients receiving shorter infusions. The data we report here demonstrate that, with one exception described below, all 14 patients receiving 24-hr infusions at doses ranging from 10 to 500 mg T101 had evidence of antigenic modulation. In these patients, circulating target cells became T65- during infusion and remained negative until the serum T101 titer disappeared.

The appearance of T65- target cells in the circulation during infusion could be caused by either immunoselection by T101 or by antigenic modulation. Because this is an important distinction to make and it has important implications in the use of immuno-toxins and radioisotope-conjugated monoclonal antibodies, we designed an experiment to distinguish between modulation and immunoselection. We obtained T65- malignant cells from blood samples drawn during the sixth hr of 24-hr T101 infusions in patients with CLL and CTCL and incubated them in vitro for 24 hr at 37° in the absence of T101. Indirect immunofluorescence showed that these cells had regained the T65+ phenotype, indicating that the T65- population retained the ability to express T65. Because there was no in vitro proliferation or evidence of differentiation, this experiment confirmed that modulation and not just immunoselection had occurred and that the phenotype of the residual lymphocytes had not been permanently altered.

Of the 43 treatments not associated with endogenous host anti-T101 antibodies, 39 resulted in detectable in vivo T101 binding. The only exceptions were 4 infusions of 10 mg T101 in a CLL patient with a lymphocyte count of 200,000/µl. The same patient had measurable T101 binding during 2 separate 100-mg infusions, suggesting that the absence of measurable in
T101-INDUCED ANTIGENIC MODULATION

Chart 5. In vitro reexpression of T65 following T101-induced modulation in vivo. Peripheral mononuclear cells obtained before and after infusion of T101 in a patient with CLL were stained with T101 and BA4 (anti-HLA-DR) by indirect immunofluorescence. An aliquot of posttreatment cells was incubated 24 hr at 37° in T101-free medium before staining. A representative experiment is shown. Similar results were seen in 39 of 43 treatments (not including treatments associated with endogenous host anti-T101 antibodies).

Chart 6. Relationship between T101 dose and in vivo modulation in CTCL and CLL. The change in the percentage of T101+:Leu 4+ ratio for CTCL (A) and the percentage of T101+:HLA-DR+ ratio for CLL (B) is plotted versus time as 24-hr infusions of T101 at 3 different doses in patients with CTCL (A) and at 4 different doses in patients with CLL (B). The T101 doses used were: 10 mg (●), n = 6 for CTCL; 50 mg (○), n = 4 for CTCL; 100 mg (△), n = 8 for CTCL; and 500 mg (□), n = 1 for CLL. Bars, S.D.

vivo T101 binding resulted from antigen excess caused by a large circulating tumor burden.

All 43 treatments in which in vivo T101 binding was demonstrated were associated with some degree of in vivo antigen modulation, indicated by a decrease in the T101:HLA-DR or T101:Leu4 ratios during infusion. The extent and duration of modulation were related to the T101 dose and the tumor cell burden, and modulation persisted until T101 was cleared from circulation. These data show an important correlation between the antibody:antigen ratio and in vivo modulation and suggest that more frequent infusions of smaller T101 doses may result in less modulation.

Chart 7. Serum T101 levels in CLL and CTCL. The concentrations of serum T101 are shown as 24-hr infusions of T101 at 3 different doses in patients with CTCL (A) and at 4 different doses in patients with CLL (B). The T101 doses used were: 10 mg (●), n = 6 for CTCL; 50 mg (○), n = 4 for CTCL; 100 mg (△), n = 8 for CTCL; and 500 mg (□), n = 1 for CLL. Bars, S.D.

Other investigators have used anti-Leu 1 to treat CTCL patients and have reported results consistent with antigenic modulation, but they presented data in only a cursory manner (9–11). The first report was a preliminary study of a single patient receiving 6-hr infusions of 1 or 5 mg anti-Leu 1 (10). It described the appearance of Leu 1- cells after a 5-mg infusion, but not after 1-mg infusions. It also described anti-Leu 1-induced in vitro antigenic modulation of the patient’s circulating tumor cells. In a second preliminary study of another patient who received multiple 6-hr infusions of anti-Leu 1 in doses ranging from 1 to 20 mg (9), the investigators reported the transient appearance of Leu 1- target cells following each infusion. In a larger study involving 7 patients receiving 4- to 6-hr infusions of 250-g to 100-mg
The occurrence of antigenic modulation but did not present any data on the doses of anti-Leu 1 (11), the investigators mentioned the variable properties on the Model 50H Ortho Cytofluorograf. Points, mean of 4 infusions in one patient; bars, S.D.

Our data clearly demonstrate that T101 induces T65 modulation in vitro and also during the course of passive anticancer serotherapy. Modulation occurring during serotherapy inhibits target cell destruction, presumably because there is insufficient antibody on the cell surface to effect cell-mediated or complement-dependent cytotoxicity. Circulating cells optimized with a sufficient quantity of T101 are eliminated in the reticuloendothelial system, while cells below that threshold remain in circulation (5).

The mechanism of T101-induced T65 modulation is currently under study in our laboratory. Preliminary evidence suggests that it proceeds through the normally accepted mechanism of capping preceding endocytosis (16). If this is true, drugs which inhibit capping may be of possible clinical interest. More importantly, however, advantage could be made of modulation if the T101:T65 complex is internalized during modulation. Conjugates which covalently link T101 to toxins, such as ricin, or to drugs, such as Adriamycin, are most effective when the toxin or drug molecules are delivered into the cellular cytoplasm. The data obtained on T101-induced modulation suggest that such immunoconjugates are the next logical step in the use of monoclonal antibodies as therapeutic agents.

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