In Vivo Administration of Lymphocyte-Specific Monoclonal Antibodies in Nonhuman Primates: I. Effects of Anti-T11 Antibodies on the Circulating T Cell Pool

By Norman L. Letvin, Jerome Ritz, Louis J. Guida, Joanne M. Yetz, John M. Lambert, Ellis L. Reinherz, and Stuart F. Schlossman

MONOCLONAL antibodies are only beginning to find their place in the medical therapeutic armamentarium. While they have already proven to be useful adjuncts in treating episodes of renal transplant rejection and in the preparation of bone marrow for transplantation, their utility as primary immune modulators and as tools for cancer chemotherapy remains to be defined. The establishment of their use in such roles will require the systematic study of monoclonal antibodies in vivo.

We and others have demonstrated a remarkable conservation of cell surface antigens on bone marrow-derived elements of humans and those of a number of nonhuman primate species. This observation and the well-established physiologic similarities between humans and nonhuman primates suggest that nonhuman primates should provide an important experimental model for studying the uses of monoclonal antibodies as medical therapeutic agents. Initial exploitation of this model for such studies has recently been reported by others.

We have recently initiated studies using T lymphocyte-specific monoclonal antibodies as therapeutic agents in nonhuman primates. In the present series of experiments, we have characterized the alterations which three monoclonal antibodies reactive with T11, the sheep erythrocyte rosette (E rosette) receptor on T lymphocytes, cause on the circulating T lymphocyte pool when infused into rhesus monkeys. The experiments demonstrate that infusion of these monoclonal antibodies into animals can result in coating of circulating cells, modulation of the T11 antigen off the cell surface and a transient clearance of T cells from the circulation. Yet, the variation in the extent to which these effects were seen with these different antibodies indicates that extrapolating from studies of the in vivo use of one antibody to the use of another may be quite difficult. These studies clearly indicate the strengths of this nonhuman primate system for exploring the uses of monoclonal antilymphocyte antibodies as therapeutic agents. They, however, also demonstrate that differences may exist in the affinity of a particular antibody for homologous lymphocyte surface structures in humans and in a nonhuman primate species. These differences may make it difficult to predict the precise effects that the infusion of an antibody will cause in humans on the basis of alterations it induces in nonhuman primates.

MATERIALS AND METHODS

Animals. The monkeys used in this study were adult Macaca mulatta (rhesus) and ranged in weight from 5 to 10 kg. They were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources of the National Research Council.

Anti-T11 monoclonal antibodies. The production and characterization of the anti-T11 monoclonal antibodies used in these studies has been described previously. The antibody-producing hybridoma cells were grown in ascites in pristane-primed BALB/c mice. The IgG2 antibodies (anti-T11b, anti-T11c) were purified by diluting the ascites fluid with an equal volume of 10 mmol/L potassium phosphate buffer, pH 7.2, containing NaCl (145 mmol/L) (PBS), and passing them through protein A-Sepharose 4B-CL (Sigma, St Louis) columns as described by Ey et al. The columns were then washed with PBS, and the antibodies that bound to the column were eluted with 0.1 mol/L acetic acid containing NaCl (150 mmol/L). Antibody solutions were neutralized with NaHCO3 (0.1 vol of 1 mol/L solution) and then dialyzed against 10 mmol/L sodium phosphate buffer, pH 6.0, containing glycine (50 mmol/L) and NaN3 (0.4 mmol/L), in preparation for further purification by ion-exchange chromatography on a column (30 mL for 100 mg of protein) of carboxymethyl cellulose (Whatman, NJ, CMS2) equilibrated in the pH 6.0 buffer. The columns were developed with a gradient (15-column volumes) of 0 to 200 mmol/L NaCl in the same buffer. Purified antibodies were dialyzed against PBS and sterilized by filtration through Millex-GV filters (0.22 μm; Millipore, Bedford, Mass).

The IgG1 antibody (anti-T11c) did not bind to protein A. The ascites fluid was diluted with an equal volume of PBS and passed...
through a column of protein A-Sepharose 4B-CL to remove small amounts of murine IgG2 and IgG3. The antibody was precipitated from the eluate by adding (NH₄)₂SO₄ to 50% saturation. The precipitated protein was dissolved in PBS, dialyzed into the pH 6.0 buffer for purification by ion-exchange chromatography on carboxymethyl cellulose as described above, except that the column was developed with a gradient of 0 to 300 mmol/L NaCl. Antibody was then concentrated by ultrafiltration and further purified by gel filtration on a column of Sephacryl S-300 (Pharmacia, Piscataway, NJ) which was equilibrated with PBS. The purified antibody was sterilized by filtration through Millex-GV filters (0.22 µm).

The purity of all preparations of antibody was established by analysis by isoelectric focusing with using LKB (Rockville, Md) Ampholine PAG-plates (pH 3.5 to 9.5). All preparations were tested for the presence of endotoxin using the Limulus Amebocyte Lysate Test (Microbiological Associates, Walkersville, Md).

Staining and analysis of peripheral blood lymphocytes (PBLs). PBLs were prepared from heparinized venous blood of the macaques by density gradient centrifugation using a 9% Ficoll (Sigma)/34% sodium diatrizoate (Sterling Drug, New York) solution having a specific gravity of 1.076 g/mL. The cells were treated with 0.15 mol/L NH₄Cl to lyse erythrocytes and were washed with Hanks’ balanced salt solution containing 2.5% newborn calf serum. Aliquots of 10⁶ cells were incubated with monoclonal antibodies at 4 °C for 30 minutes. The antibodies included anti-T4, anti-T8, anti-T1, and anti-T2. The characterization of these antibodies has been described. This incubation in the mononuclear antibody was not included when the cells were being examined for cell-bound monoclonal antibody following an infusion. These cells were then washed twice with Hanks’ balanced salt solution/2.5% pooled human AB serum and incubated for 30 minutes at 4 °C with fluorescein isothiocyanate-conjugated goat anti-mouse Ig (GM-FITC) (Tago, Burlingame, Calif). Each sample was washed twice with PBS and the cells were then analyzed on a fluorescence-activated cell sorter (FACS I; Becton Dickinson).

Modulation of T11 antigen in vitro. Mononuclear cells were isolated from peripheral blood and adherent cells were removed by incubating 10⁴ cells per milliliter in 100-mm sterile plastic Petri dishes for one hour at 37 °C. After the addition of purified anti-T11 monoclonal antibodies at a final concentration of 0.01 mg/mL, aliquots of nonadherent cells were then cultured at a concentration of 1 x 10⁶ cells per milliliter for 48 hours at 37 °C. Control cells were incubated at 37 °C with a nonreactive monoclonal antibody (8A7) but were otherwise processed as T11-modulated cultures. Following this incubation, cells were washed twice with cold media and the presence of surface antibody was determined by incubation with GM-FITC for 30 minutes at 4 °C. Cultured cells were incubated for 30 minutes at 4 °C with each anti-T11 antibody (0.01 mg/mL) followed by two washes in cold media and further incubation with GM-FITC. Immediately after incubation with GM-FITC, cells were washed twice with PBS and fixed in 1% formaldehyde/PBS. Intensity of fluorescence was quantitatively analyzed using an EPICS C (Coulter Electronics, Hialeah, Fla) and the channel of mean fluorescence intensity (MFI) on a linear scale was determined. The percentage of antigenic modulation for each monoclonal anti-T11 antibody was determined from the following formula:

% modulation = \[ \frac{MFI \text{ test sample} - MFI \text{ negative control}}{MFI \text{ control anti-T11} - MFI \text{ negative control}} \times 100 \]

Protocol for anti-T11 infusions. Rhesus monkeys were sedated with ketamine throughout the infusion of the monoclonal antibodies. Antibodies were delivered intravenously by continuous infusion over a four-hour period in a 20-ml vol at 200-µg/kg or 2-mg/kg doses. Heparinized and citrated blood samples were drawn prior to and two hours after the initiation of each infusion, at 30 minutes, 4 hours, and 16 hours after the infusion and then daily thereafter until the distribution of lymphocyte subsets had returned to that seen prior to the initiation of the infusion. Serum samples from each drawing were frozen and stored for later analysis. PBLs were prepared from the heparinized blood for cell staining with monoclonal antibodies and complete blood cell counts including differentials were done on the citrated blood samples.

Measurement of anti-T11 in rhesus serum. Serial twofold dilutions of each serum aliquot were prepared and used to stain normal rhesus monkey and human PBLs. These stained samples were then developed with GM-FITC and examined on a FACS I. The titers of anti-T11 in the serum of an experimental animal at any time point is expressed as the lowest dilution of serum that can stain normal PBLs. These assays were frequently done using both rhesus monkey and human PBLs in parallel to assure that an inability to detect staining on PBLs could not be attributed simply to the low binding affinity of a monoclonal anti-T11 antibody to rhesus cells.

RESULTS

Characteristics of monoclonal anti-T11 antibodies chosen for study. A series of monoclonal antibodies that recognize the human T lymphocyte sheep erythrocyte rosette receptor were screened for reactivity with T lymphocytes of rhesus monkeys. Three antibodies of differing isotypes which exhibited good staining of rhesus T cells when facilitated with GM-FITC and examined by FACS analysis were selected for further study. Previous work with these three antibodies had shown that each blocks E rosette formation and cross-blocks the binding of the other anti-T11 monoclonal antibodies to sheep RBCs (Table 1).

To characterize further these antibodies, antibody-containing ascites was purified as described in Materials and Methods. The relative affinities of these purified monoclonal reagents for human and rhesus monkey T cells was then assessed by determining the binding of each antibody to these lymphocyte populations when used at limiting concentrations. As shown in Fig 1, anti-T11a has an intermediate affinity and anti-T11b has the least affinity for rhesus monkey T cells. In binding to human T cells, anti-T11b exhibits an intermediate affinity and anti-T11a exhibits the least affinity.

The capacity of each of these three monoclonal anti-T11 antibodies to modulate the T11 structure off the surface of lymphocytes in vitro was then determined. As shown in Fig 2, after 24- and 48-hour incubations of anti-T11c with rhesus monkey T lymphocytes, very little T11 antigen, either free or

<table>
<thead>
<tr>
<th>Table 1. Characteristics of Monoclonal Anti-T11 Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>T11a</td>
</tr>
<tr>
<td>Reactivity</td>
</tr>
<tr>
<td>Thymocytes</td>
</tr>
<tr>
<td>Resting T cells</td>
</tr>
<tr>
<td>Activated T cells</td>
</tr>
<tr>
<td>Molecule precipitated (kd)</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>Blocking of E rosette formation</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>Blocking of anti-T11 binding</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>Isotype</td>
</tr>
<tr>
<td>IgG1</td>
</tr>
</tbody>
</table>

Data from reference 15.
EFFECTS OF IN VIVO ADMINISTERED ANTI-T11

Fig 1. Relative affinities of monoclonal anti-T11 antibodies for human and monkey PBLs. Serial log dilutions of three anti-T11 monoclonal antibodies, purified as described in Materials and Methods, were made. These antibodies were used for staining rhesus monkey (A) and human (B) PBLs. Data are expressed as a percentage of the maximal fluorescence of PBLs as determined by FACS analysis, with monoclonal antibodies used at various concentrations.

bound to monoclonal anti-T11 antibody, remains on the surface of these cells. In contrast to that finding, such incubations of rhesus monkey T cells with anti-T11B results in almost no disappearance of T11 antigen from the surface of these lymphocytes. Incubation of rhesus monkey T cells with anti-T11A results in an intermediate degree of loss of T11 antigen from the membrane of the lymphocytes. This hierarchy in capacity of the antibodies to modulate the T11 antigen from the lymphocyte cell surface parallels the affinities of these antibodies for the T11 antigen in the rhesus monkey.

Infusions of monoclonal anti-T11 antibodies into rhesus monkeys. We then sought to determine what effects these three carefully characterized anti-T11 antibodies cause on circulating T lymphocytes in the monkey. Rhesus monkeys were sedated and infused over four hours with either 200 μg/kg or 2 mg/kg of endotoxin-free purified monoclonal antibody. The animals exhibited no untoward physiologic reactions to these infusions and never developed abnormal serum liver or renal function tests, even after as many as six such infusions. No individual animal was used for more than six infusions of antibodies. In no case did an animal that had received a number of prior infusions show a decrement in the expected response following the infusion which might be attributable to the presence of anti-mouse Ig in the circulation.

The rate at which these monoclonal antibodies were cleared from the serum of the monkeys did differ for each of the three antibodies. Anti-T11A, the IgG1 antibody, was cleared a bit more rapidly than the other two antibodies (Fig 3).

Fig 2. Relative capacities of monoclonal anti-T11 antibodies to modulate T11 in vitro. Nonadherent rhesus monkey PBLs were incubated in vitro with an excess of each anti-T11 antibody and at 24 hours and 48 hours after initiation of cultures the cells were stained with the same anti-T11 antibody, fixed, and analyzed on a FACS. The data are expressed as the percentage of modulation at those time points calculated according to the formula shown in Materials and Methods. □ Anti-T11A; ○ Anti-T11B; △ Anti-T11C.

Fig 3. Clearance of monoclonal anti-T11 antibodies from the serum of rhesus monkeys following infusion. Serial dilutions of serum samples taken from monkeys at regular time intervals following infusion were used to stain normal human (A) or rhesus monkey (B) PBLs. Titrations of samples from representative infusions at 0.2 mg/kg (A) and 2 mg/kg (B) are shown.
Effect of anti-T11 infusions on circulating T lymphocytes. Peripheral blood of the infused animals was assessed to determine the extent of binding of each antibody to circulating lymphocytes, the efficiency of clearance of circulating lymphocytes following infusion of each antibody, and whether each antibody causes modulation of the T11 antigen off the surface membrane of circulating lymphocytes in vivo.

Binding of an infused monoclonal antibody to circulating T lymphocytes was determined by immunofluorescence analysis of mononuclear cells isolated from peripheral blood taken from an animal at regular intervals following infusion using GM-FITC and by analyzing these cells by automated flow cytometry. Circulating lymphocytes coated with the monoclonal antibody stained with the fluoresceinated antimouse Ig. As shown in Fig 4, the degree and duration of binding of the infused antibodies differed significantly between the three anti-T11 antibodies studied. Anti-T11A, even at the high dose of 2 mg/kg, never showed evidence of lymphocyte coating in vivo. This observation is consistent with the relatively low affinity of the antibody for monkey T cells. The infusion of both anti-T11A and anti-T11C resulted in significant coating of circulating T cells. Anti-T11C coating was detectable following 2 mg/kg infusions for four days. Staining of PBLs from anti-T11A-infused animals was brighter and persisted longer than that seen in anti-T11C-infused animals, usually persisting until seven days following the infusions.

The three anti-T11 antibodies also differed in the efficiency with which they caused clearance of T lymphocytes from the circulation of the monkeys (Fig 5). The two IgG2 antibodies, anti-T11A and anti-T11C, caused a comparable transient clearance of T cells from the circulation of experimental animals following infusions of 2 mg/kg, as measured by the absolute number of circulating cells expressing T4, an independent marker of the majority of circulating T cells. This clearance was almost complete at four hours following conclusion of the infusion. The number of circulating T4-bearing cells returned to normal in these monkeys by 16 hours following infusion. The IgG1 antibody anti-T11A was considerably less efficient than the IgG2 antibodies at causing clearance of circulating T cells. By four hours following infusion, the absolute number of circulating T4-bearing cells was only one half the number seen prior to initiation of the infusions and, as in the case of the IgG2 antibodies, usually returned to normal by 16 hours following the infusions.

Finally, the three antibodies differed significantly from one another in the extent to which they induced modulation of the T11 antigen from the surface of circulating monkey T lymphocytes. Anti-T11A and anti-T11B caused almost no detectable modulation of T11. Yet, as shown in Fig 6, anti-T11C caused a selective long-standing loss of T11 expression on circulating T cells. By two hours following infusion of anti-T11C at 2 mg/kg, the absolute number of circulating T cells was dramatically diminished. By 16 hours following infusion, the number of circulating T cells had returned to pretreatment levels. At that time, cells expressing T12 and T4 were easily detectable. By two days later, a normal number of cells expressing these antigens was noted. However, it was not until eight days following the infusion that T11 was expressed by these circulating lymphocytes. T11 was thus re-expressed when anti-T11C was no longer detectable in the serum of the monkey.

DISCUSSION

These studies were initiated to provide a basis for developing strategies for using monoclonal antibodies as medical therapeutic agents in humans. A few cautionary points concerning the validity of extrapolating from these studies in monkeys to humans should be raised. As shown in Fig 1, the affinity of these particular monoclonal antibodies for the T11 structure on rhesus monkey and human lymphocytes differs. The affinity of a particular antibody for this structure may play an important part in determining its effects on circulating lymphocytes. Thus, the effect that one antibody may have in monkeys may, in fact, be quite different from its effect in humans. Furthermore, the experimental animals were under significant physiologic stress during these infusions due, in part, to prolonged anesthesia. Such stress may of itself have affected circulating lymphocyte distribution in the monkeys. To avoid the use of anesthesia and this potential problem, we have recently used specially constructed chairs to restrain experimental animals during the infusions.

IgG molecules have the longest survival time and lowest catabolic rate of all serum proteins. In humans, the total body pool of gamma globulin is less than one third that of albumin, yet its rate of synthesis is one fifth that of albumin.20 Most previous studies of the rate of antibody clearance in experimental animals have been done using homologous immunoglobulin. Several factors govern the rate at which IgG is catabolized. The single factor most directly related to its serum half-life appears to be the heavy-chain subclass of the IgG molecule. In a murine model system, Pollock et al20 showed that following initial equilibration between intra- and extravascular spaces, intravenously administered radiolabeled IgG of subclass 2b had a serum
EFFECTS OF IN VIVO ADMINISTERED ANTI-T11

Fig 5. Clearance of circulating T lymphocytes following infusions of monoclonal anti-T11 antibodies. The percentage of decrease in the absolute number of T4+ PBLs (absolute lymphocyte count \times the percentage of T4+ cells by FACS analysis) over time is shown following multiple 2-mg/kg infusions of the different anti-T11 antibodies. Each interconnected series of data points represents the results following a single infusion into a rhesus monkey.

half-life of approximately 100 hours, whereas IgG of subclasses 1 and 2a each had serum half-lives of 200 hours. Similar observations have been made by other investigators in murine systems.21 A second important factor affecting the rate of IgG catabolism is the total serum IgG level. Mice raised in a pathogen-free environment have very low serum IgG levels; however, the serum half-life of that IgG is considerably greater than normal. Conversely, the serum half-life of IgG in mice made hypergammaglobulinemic by implantation of myeloma cells is very short.22

The precise mechanism by which IgG is catabolized is not clearly understood. Intact IgG molecules are resistant to glomerular filtration. Using deletion mutants of rat Fc fragments of Ig, it has been shown that this resistance to renal filtration is related to a specific domain in the Fc fragment.23 Once an Ig molecule has undergone cleavage to Fab and Fc fragments, the Fab fragments are rapidly cleared by glomerular filtration.24 The Fc fragments, although similar in size to the Fab fragments, escape renal filtration and are gradually cleared, presumably by the various mononuclear-phagocyte cells in the liver, lung, and gastrointestinal tract.25

We have found that knowledge of the isotype of the infused monoclonal antibody did not enable us successfully to predict the fate of the antibody in vivo. Since previous studies have shown that IgG1 and IgG2a antibodies are not readily cleared while IgG2b antibodies are cleared efficiently,20,21 we might have predicted that anti-T111A and anti-T111B would be cleared slowly from the monkey circulation and anti-T111C would be cleared rapidly. What we in fact found was that anti-T111A, the IgG1 antibody, was cleared a bit more rapidly from the serum than the other two antibodies and the cells coated with anti-T111A were not efficiently cleared. In contrast to anti-T111A and anti-T111B, the IgG2b antibody anti-T111C remained in the circulation coating the lymphocytes for a prolonged period of time.

It is of note that clearance of circulating T11-bearing lymphocytes in the monkeys was an extremely transient phenomenon. While we found high titers of some of the antibodies in the serum of these animals for many days following the infusions, the clearance of T11-bearing cells from the circulation was always of only a few hours’ duration. We have seen a similar phenomenon in humans who have received infusions of the monoclonal antibody directed against the common acute lymphocytic leukemia antigen J5.26 The ability of circulating lymphocytes to “escape” from the effects of these antibodies does not appear to be explicable by any single mechanism. For example, antigenic modulation may well have played a role in this “escape” following the infusion of anti-T111C, but very little modulation was noted with the other antibodies.

These studies indicate the strengths of this system for exploring the uses of monoclonal antilymphocyte antibodies as therapeutic agents. Nonhuman primates should prove important in devising strategies for the use of these antibodies alone or conjugated to drugs and toxins for selectively eliminating specific cell populations. These studies also indicate the importance of devising these strategies on an empiric basis at this time.

ACKNOWLEDGMENT

The authors would like to thank David Leslie and Mary Kornacki for assistance in flow cytometric analysis, Susan Brodeur and Christine Murray for technical assistance, Wayne R. Aldrich for assistance in administration of the antibodies to the rhesus monkeys, and Bettye-Jean Roy for preparation of this manuscript.

Fig 6. In vivo T11 modulation following anti-T111C infusion. A rhesus monkey received a 2-mg/kg infusion of anti-T111C and PBLs were stained at regular intervals following completion of the infusion with anti-T11 and a pan-T cell antibody, anti-T12, which recognizes an independent T cell surface antigen.
REFERENCES

In vivo administration of lymphocyte-specific monoclonal antibodies in nonhuman primates: I. Effects of anti-T11 antibodies on the circulating T cell pool

NL Letvin, J Ritz, LJ Guida, JM Yetz, JM Lambert, EL Reinherz and SF Schlossman