Leukemic Cell Targeting and Therapy by Monoclonal Antibody in a Mouse Model System

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ABSTRACT

A monoclonal antibody prepared against the Rauscher virus envelope glycoprotein with a molecular weight of 70,000 targeted to neoplastic cells and cured the Rauscher leukemia virus-induced erythroleukemia in BALB/c mice. This antibody, 103A, specifically reacted with the Rauscher and Friend erythroleukemia viruses and erythroleukemic cells but did not react with other murine ecotropic or xenotropic viruses or feline leukemia virus or with normal spleen cells, thymocytes, or fibroblasts, as measured by radioimmunoassays. P3, a control antibody of the same immunoglobulin G subclass, did not bind to any of these cells or viruses. This specificity was maintained in vivo. 125I-Labeled 103A injected into mice targeted to leukemic spleen cells but not to normal cells. Mean uptake ratios of binding to leukemic over normal spleen cells ranged from greater than 70 at 7 hr after injection to less than 10 at 40 hr later. The control antibody showed no binding in vivo. A single small dose of 103A was able to cure leukemic mice as assayed by spleen focus formation on Day 8 or splenomegaly on Day 20. The 50% effective dose was 1.5 µg when injected 72 hr after onset of leukemia. The therapeutic potencies of drugs, toxins, and cytotoxic radioisotopes conjugated to antibodies can be quantitatively compared using the established dose-response curve. Such quantitative comparisons showed that 131I-labeled 103A immunoglobulin G was no more potent than unlabeled 103A immunoglobulin G in this system.

INTRODUCTION

An immunological approach to the diagnosis and treatment of neoplastic disease has been a promising goal for nearly a century (24, 28). Unfortunately, because of the difficulty in identifying tumor-associated proteins and in preparing tumor-specific antibodies, clinical success of such an approach has been only modest (2, 11, 16, 20, 25, 28). However, the recent development of the monoclonal antibody technique (17) makes possible the preparation of immunoglobulins of absolute tumor specificity. Such antibodies offer new potential for passive immunotherapy and radioimmunodiagnosis in vivo.

Monoclonal antibodies have been shown to be therapeutically effective against transplanted tumors in mouse model systems when injected in multiple large doses beginning immediately after tumor inoculation (5, 15, 39). Ballou et al. (3) have shown the uptake of radiolabeled antibody into large transplanted solid tumors by scanning scintimetry. These initial nonquantitative studies have encouraged a pharmacological examination of the diagnostic and therapeutic uses of monoclonal antibodies. We have used the Rauscher and Friend murine erythroleukemias as a model system to quantitate the targeting of radiolabeled monoclonal antibody to neoplastic cells in vivo and to determine precisely the doses of tumor-specific antibody required for therapeutic effects.

The RLV (29) and FLV (12) are 2 independently isolated murine virus complexes of nearly identical structure which cause an erythroleukemia characterized by rapid transformation of erythroid precursor cells, splenomegaly, and viremia. After virus infection of mice, neoplastic erythroblasts in the spleen are visible microscopically within a few days; macroscopic tumor foci are visible on the spleen surface after 6 to 8 days, and 10-fold increases in spleen size may occur in 2 weeks (1, 22, 26, 29, 32). The RLV and FLV envelope glycoprotein, gp70, is expressed in large amounts on the surface of virus-infected leukemic cells. The antigenic determinants displayed by the gp70 have a wide spectrum of specificities. Some are interspecies specific, present on viruses of other species; some are murine group specific; and some are virus type specific (35). Certain murine virus group-specific antigens are also expressed constitutively in small amounts on the cell surface of normal mouse tissues (19). Thus, only the monoclonal antibody technique would allow the isolation of a completely tumor-specific antibody, which would be unreactive with normal tissues.

From a library of monoclonal antibodies prepared against Rauscher virus gp70, one antibody targeted specifically to erythroleukemia cells in vitro and in vivo. Dose-response curves, generated to assess the therapeutic effects of a single dose of antibody or antibody derivatives on leukemic mice, indicated cures of the leukemia at extremely low doses of antibody when measured by spleen focus formation and splenomegaly.

MATERIALS AND METHODS

Antigen and Immunization. Rauscher murine virus gp70 was purified as described (36). Two 8-week-old C57BL/6 mice (The Jackson Laboratory, Bar Harbor, Maine) were immunized i.p. with 100 µg purified gp70 per injection. The mice received primary immunizations of protein emulsified with equal volumes of Freund's complete adjuvant and 4 additional immunizations of gp70 in Freund's incomplete adjuvant at 2-week intervals. The sixth and final injection of gp70 in PBS was given i.v. 3 days prior to fusion.

Cell Fusion, Cloning, and in Vivo Propagation. Spleen cells (2.5 x 10⁸) were hybridized with P3-NS1/1-Ag4-1 myeloma cells (8 x 10⁵) using polyethylene glycol and dimethyl sulfoxide, according to procedures described previously (34). Culture supernatant fluids were assayed for antibody activity by indirect radioimmunoassay (see below);
cloning of selected cultures was performed in soft agar. Selected clones were expanded in vivo to obtain antibody-containing ascites fluid by injecting 1 x 10⁷ hybridoma cells i.p. into BALB/c × C57BL/6 F₁ mice that had been primed with pristane.

Detection of Anti-Rauscher gp70 Antibody Production and Determination of Immunoglobulin Class and Subclass. Antibody production by hybridomas was measured by solid-phase radioimmunoassay in polystyrene microtiter wells (Falcon Plastics, Oxnard, Calif.) (21). Fifty µl, containing 5 µg of Rauscher virus protein in PBS with 0.02% NaN₃, were added to each well followed by incubation overnight at 37°C. The remaining protein-binding sites in the wells were then saturated by the addition of 200 µl of 5% fetal calf serum-0.01% Triton X-100-0.02% sodium azide in PBS (wash buffer), and the wells were incubated for 90 min more. The buffer was removed, and 50 µl of ascites fluid diluted in PBS were added, and the wells were then incubated for 60 min at 37°C. After 3 washes, bound antibody was quantitated by the addition of 10 ng ¹²⁵I-labeled goat anti-mouse immunoglobulin in 50 µl PBS, followed by incubation for 60 min at 37°C. Unbound antibody was again removed by washing. After incubation with 100 µl of 2 N NaOH for 15 min at 60°C, the solubilized proteins were transferred to glass tubes, and the radioactivity was measured in an LKB γ-counter.

Determination of Monoclonal Antibody Immunoglobulin Class. Immunoglobulin class was determined as described previously (21) using the solid-phase radioimmunoassay described above with the following modifications. Immunoglobulin class-specific antisera (goat IgG anti-mouse IgM, IgG1, IgG2A, IgG2B, IgG3, and IgA; Litton Biocentrics, Kensington, Md.) were added to the bound washed hybridoma antibodies (instead of ¹²⁵I-labeled goat IgG anti-mouse immunoglobulin), and incubated for 1 hr at 37°C. After 3 washings, ¹²⁵I-labeled pig IgG directed against goat IgG was then added, and the assay was processed as described.

Purification of Monoclonal Antibody, Preparation of Fab Fragments and Iodination. The immunoglobulins were purified from ascites fluid by affinity chromatography on protein-A Sepharose (Pharmacia Bioproducts), 5 to 7 weeks old, were infected with 250 µl of a 1:30 dilution of a 20% suspension of RVB3 (see below). Upon development of advanced splenomegaly (approximately 13 days), these mice and uninfected littermates were given i.p. injections of ¹²⁵I-labeled papain fragments or IgGs (6 µg at 2 µCi/µg). The spleens were removed 20 and 48 hr later, and the cells were isolated. Spleen cells were washed 3 times with 10 ml of PBS containing 0.2% bovine serum albumin and 0.02% NaN₃, were added to each well followed by incubation overnight at 37°C. The remaining protein-binding sites in the wells were then saturated by the addition of 200 µl of PBS containing 0.01% Triton X-100 and 0.02% sodium azide, and the wells were incubated for 60 min at 37°C. After 3 washings, ¹²⁵I-labeled pig IgG directed against goat IgG was then added, and the assay was processed as described.

Immunotheraphy. Mice were infected i.v. with approximately 20 spleen focus-forming units of RVB3 virus. Because of the variability among mice and virus batches, 3 groups of mice were inoculated, each with a slightly different dilution of virus, and only those groups of mice in which the control, untreated animals showed 15 to 30 foci at Day 8 were used. This range ensured significant numbers of foci without overloading the spleen with confluent foci. At various times before or after the infection, a single dose of purified monoclonal antibody or a derivative was injected in 0.5 ml PBS i.p. into each test mouse. Following treatment, disease severity was assessed by both focus formation and splenomegaly as described above.

RESULTS
Selection of an Erythroleukemia Virus-specific Monoclonal Antibody

A monoclonal antibody specific for the RLV and FLV erythroleukemia viruses was selected by screening antibodies of cloned hybridomas against type C viruses. Antibody 103A demonstrated near maximal binding with the erythroleukemia viruses at dilutions in excess of 1:20,000 and 50% binding at a dilution of 1:1000. No binding was observed with the Kirsten leukemia virus, Moloney leukemia virus, Gross leukemia virus, murine ecotropic viruses, the BALB, xenotropic virus 2 or feline leukemia virus.

Other cloned antibodies demonstrated murine group specificity and interspecies specificity and therefore were not suitable for targeting in vivo because of possible cross-reactivities with the constitutively expressed gp70.

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The isotype of antibody 103A was IgG₁. Therefore, the immunoglobulin produced by the P3-63/Ag8 cells (P3), which is also IgG₁, was used as a control in all of the experiments.

Cell-specific Binding of Monoclonal Antibody

The cell specificity of binding of ¹²⁵I-labeled Fab fragments was measured by direct binding on RLV-infected JLS-V9 cells, F4-6 erythroleukemia cells, RVB3 erythroleukemia spleen cells, normal BALB/c mouse spleen and thymus cells, and normal BALB/3T3 cells (Chart 1). Antibody 103A bound to RLV-infected fibroblasts and to both Friend and Rauscher erythroleukemia cells but did not bind to the normal BALB/c spleen cells, thymus cells, and BALB/3T3 fibroblasts. In contrast, the P3 antibody bound to none of the cells, even at high concentrations of cells and antibodies. Competition for binding of ¹²⁵I-103A fragments on RLV-infected cells with unlabeled Fab fragments demonstrated that binding was specific and saturable (Chart 2A). Additionally, binding of the fragments was cell concentration dependent (Chart 2B). Calculations based on the competition studies indicated that there were approximately 10⁶ antibody-binding sites per leukemic cell.

Targeting of Antibodies in Vivo

The 103A antibody specifically targeted to erythroleukemic cells in RLV-infected mice. When analyzed at 20 and 24 hr after injection, ¹²⁵I-103A papain fragments bound extensively to the erythroleukemic spleen cells but not to normal spleen cells (Chart 3). The P3 fragments did not bind to spleen cells of normal or leukemic mice.

The value of the uptake ratio (binding of antibody to leukemic spleen cells divided by binding to normal spleen cells) for 103A was time dependent. The uptake ratio of the intact 103A IgG was 66 ± 8 (S.E.) at 7 hr after antibody injection and 18.8 ± 2.4 at 20 hr after injection. At this time point, papain fragments of 103A showed a similar uptake ratio, 23.2 ± 4. At 48 hr after injection, the uptake ratio of the fragments was 8.4 ± 1. This continuous reduction in binding was probably due to virus budding from the spleen cells with displacement of the antibodies. The uptake ratios of P3, Fab, and IgG at 24 hr were 0.9 ± 0.1 and 1.9 ± 0.4, respectively, and these ratios did not change with time. Similar analysis of thymocytes from these mice demonstrated binding of the 103A antibody to the cells of leukemic but not normal mice. However, total binding to the thymocytes was 3 times lower than that to the spleen cells.

Therapeutic Effects of 103A

Selection of Virus Dose. The specific targeting of the 103A in vivo suggested that this antibody might be useful therapeutically against the Rauscher leukemia. The severity of erythroleukemia caused by a specific virus dose was analyzed to determine the appropriate virus inoculation, which would allow us to measure the therapeutic effects of antibody as a function of reductions in both spleen foci at 8 days after infection and splenomegaly at 20 days after infection. Groups of mice were inoculated i.v. with various dilutions of virus at Day 0. Spleen focus-forming assays were conducted on one-half of the mice at Day 8, and splenomegaly was assessed on the remainder at Day 20 (Chart 4). The spleen weight assay was much more sensitive, as has been reported (26), detecting virus diluted in excess of 1:60,000. Since no spleen foci were observed on Day 8 from virus diluted in excess of 1:4000, this splenomegaly found at high virus dilutions presumably resulted from secondary infections of the spleens by virus originally infecting other tissues. Secondary foci become visible on the spleen at Day 9 or later and are much smaller (1, 26).
Monoclonal Antibody Targeting and Therapy

Chart 3. Binding of 125I-labeled papain fragments to spleen cells in vivo at 20 and 24 hr after injection. O, P3; •, 103A. In order to compare results from different experiments, all data were normalized. One unit equals the mean of the binding of P3 to 10^6 normal spleen cells. This ranged from 250 to 1000 cpm/10^6 cells. Normal spleens contained 10^8 cells, and leukemic spleens contained 6 to 10 x 10^8 cells. All assays were conducted as described in "Materials and Methods."

Chart 4. Induction of spleen foci and leukemic splenomegaly by RVB3 virus in BALB/c mice. The assays were conducted as described in "Materials and Methods." Foci (O) were counted 8 days after virus inoculation. Spleen weight (C) was determined 20 days after virus inoculation. Abscissa, dilution of virus.

In order to obtain sufficient foci, virus dilutions of 1:200 to 1:400 were used in the therapeutic assays. This inoculation represented several hundred-fold more virus than was necessary to cause disease.

Selection of Antibody Dose Regimen. The half-life of the monoclonal IgGs, 103A, and P3 were determined by injecting 125I-labeled antibody i.v. and i.p. and measuring the amount of labeled antibody in the serum at various time points. The half-lives of both 103A and P3 IgG were approximately 5 days and were not affected by the route of injection. Since the spleen focus-forming assay is measured on Day 8 and the half-life of the immunoglobulins in vivo was 5 days, a single dose of antibody was chosen for use in therapy.

The appropriate time for injection of antibody was determined as follows. Mice were infected with approximately 20 focus-forming units of virus. At various times between 4 hr before virus inoculation and 6 days after the infection, a single 125-μg dose of monoclonal 103A5 was injected. This dose was effective in preventing at least 90% of the foci when injected within 3 days after virus infection, whereas antibody injected on Days 4 to 6 reduced focus formation only 40 to 60%. Similar therapeutic effects have been observed when using very large amounts of polyclonal sera prepared against the virus or its envelope (9, 30). Therefore, to obtain a complete dose-response curve ranging from no therapeutic effect to greater than 90% effectiveness, we chose to inject antibody at 72 hr after virus infection. Moreover, since the half-life of the Rauscher virus at 37°C is only 70 min (26), the effects of the antibody must be attributed to cytotoxic action against the cells and not to virus neutralization. Antibody present early after virus infection might simply act by neutralizing the inoculated virus. Spleen foci observed on Day 8 were all derived from the primary infection; therefore, antibody injected at 72 hr must act on these infected cells to be scored as therapeutic.

Dose-Response for 103A. The therapeutic effectiveness of antibody dose was measured by spleen focus assay and spleen weight assay (Chart 5). Foci and splenomegaly were expressed as the percentage of reduction from that found in control, untreated mice. P3 antibody had no effect. In contrast, approximately 1.5 μg of antibody 103A were effective in eliminating 50% of the expected foci and splenomegaly. There appeared to be a sharp threshold of effectiveness of the antibody when measured by splenomegaly. Doses above 1 μg were completely effective; lower doses were completely ineffective. Furthermore, at the 1-μg dose, 30% of the mice were almost completely cured and 70% remained highly leukemic, thus resulting in a wide standard error at this point.

Mice which had received a dose of 5 μg of antibody or

Chart 5. Dose-response curve for immunotherapy of Rauscher erythroleukemia in BALB/c mice. The assay was conducted as described in "Materials and Methods." P3 lgG (C) and 103A lgG (I) are shown. Points, means of 6 to 9 mice. Bars, S.E. Infected but untreated mice were used in each experiment as controls. In A, therapeutic response was measured as the percentage of reduction from control spleen foci. A 100% reduction was considered as no foci. A 0% reduction was 20 to 30 foci, depending on the experimental group. In B, therapeutic response was measured as the percentage of reduction from control spleen weight. A 100% reduction was a normal spleen, 100 to 150 mg. A 0% reduction was a 1- to 1.5-g spleen, depending on the experimental group.
greater showed no signs of disease on Day 20. However, on Day 8, some foci were found in mice which had received equivalent doses of antibody. The eradication of these final few foci can be attributed to either the cytotoxic action of 103A or host immune mechanisms. Since 103A F(ab')2 fragments showed no effect at similar doses, the Fc was required for cytotoxicity. Moreover, this experiment provided additional evidence that simple virus binding and subsequent neutralization was not responsible for the observed therapeutic effects.

**Therapeutic Effects of 131I-103A**

The dose-response curve can be used as a baseline to investigate whether drugs, toxins, or cytotoxic radioisotopes conjugated to the antibody can enhance its therapeutic effects. 131I is an isotope which is readily coupled to IgG without loss of biological activity; 131I-labeled polyclonal antibodies have been examined for their cytotoxicity in vivo (2, 13, 25). We investigated the therapeutic potency of 131I-labeled 103A IgG. Both 103A and P3 IgG were labeled to specific activities of 5 μCi/μg and tested in the same manner as the unlabeled antibodies. Doses ranged from 0.1 to 1.0 μg/mouse. 131I-Labeled 103A was no more potent than unlabeled 103A when measured by focus-forming and spleen weight assays.

**DISCUSSION**

The introduction of the techniques for preparing hybridoma antibodies (17) and the demonstration that tumor-specific monoclonal antibodies can be produced (18, 34, 39) has made the therapeutic and diagnostic use of immunoglobulins feasible. The Rauscher erythroleukemia of mice provides an excellent model system for diagnostic and therapeutic studies of monoclonal antibodies. The progression of the disease is rapid, and the pathology is well characterized. Two simple assays were available to quantitatively measure the course of the leukemia. One of the distinct features of this system is that the leukemia is a systemic, actual disease of mice and not simply a tumor transplanted at a convenient site.

Among several monoclonal antibodies that were prepared against the RLV gp70, clone 103A was selected for these studies because of its selective binding to the Rauscher and Friend viruses and complete lack of reactivity to other viruses. This selectivity suggested that the reactivity of the 103A might be erythroleukemic cell specific. This specificity was verified by direct cell-binding experiments, which demonstrated that the antibody or its papain digestion fragments reacted with the cell surface of Rauscher or Friend leukemia cells or RLV-infected fibroblasts but not with normal cells in vitro.

The high degree of specificity observed in vitro was also achieved in live animals. Uptake ratios of 103A binding to leukemic over normal cells were very high, approaching 100 in some mice. The total amount of 103A bound to the leukemic cells was probably much higher than was indicated by these observed ratios because the gp70 molecules were continuously being shed as the virus budded from the target cells.

At 7 hr, 3 to 5% of the injected radioiodinated antibody was found in the leukemic spleens. This binding decreased with time. In other studies, as high as 10% of radiometal chelate-conjugated 103A was found in leukemic spleens at 5 to 8 hr after antibody injection (31). However, only a fraction of the antibody should be expected to be bound in the spleens due to both the shedding of virus and the presence of large quantities of antigen on other infected tissues, such as the thymus and liver.

The tumor specificity indicated by such large uptake ratios suggested that nuclear imaging of the targeted spleens would be feasible. Polyclonal antitumor sera with much lower uptake ratios have been used to scan solid tumors (4, 10, 13, 14, 25). Gamma camera images were made of normal and leukemic mice at 6, 24, and 48 hr after injection of 131I-labeled papain fragments of antibodies 103A and P3. These experiments were only partially successful, because iodine on the bound immunoglobulin was rapidly metabolized resulting in images of only the spleen but also the stomach, bladder, and kidney. However, high-resolution gamma camera images of leukemic spleens have been obtained using an alternate labeling technique in which radiometal chelates were conjugated to the 103A IgG (31).

Under the conditions that we imposed, the antibody was extremely potent and able to cure the leukemia at very low doses. Although the mechanism of cytotoxicity is not known, 103A was not cytotoxic in vitro, with or without complement, suggesting that host cellular functions are involved in cytolysis. In a curative dose of 103A, the number of antibody molecules far exceeded the number of target cells. Theoretically, toxin-conjugated monoclonal antibodies could achieve single-hit cytotoxicity (6, 23), and certain radiometal chelate-conjugated monoclonal antibodies might be even more potent (31). Therefore, antibody conjugates with potencies many orders of magnitude higher can be quantitated using this system.

A derivatized 103A antibody, 131I labeled, showed no increase of potency over the unlabeled antibody. This may indicate that β-particle-emitting isotopes, which distribute their energy in a large 1- to 2-mm-diameter area, will not be useful for therapy of small tumor foci. Therapy of large tumor masses, however, has been reported (25). α-Particle-emitting isotopes with linear energy transfer orders of magnitude higher might be more useful in eradicating small tumors (7, 31), such as metastases.

In summary, these model studies demonstrated that a monoclonal antibody detecting a leukemic cell-specific antigen could cure a neoplastic disease of mice. The tumor-specific antigen that we have used is part of a protein which is nearly homologous to a normal mouse cell surface glycoprotein. By analogy, this suggests that diagnostic and therapeutic monoclonal antibodies can be produced that detect tumor-specific antigens or altered normal proteins on the surface of human tumor cells. Monoclonal antibodies against such antigens are specifically targetable and thus may be powerful drugs, either alone or derivatized with other cytotoxic agents.

**ACKNOWLEDGMENTS**

We thank Professor J. T. August for his enthusiastic support and critical reading of the manuscript; A. McMillan, L. Baker, and A. George for their excellent technical assistance; and L. Cumor for typing of the manuscript. We thank Dr. A. Colombatti for his advice on the focus-forming assays and Dr. J. Klein for 131I.

**REFERENCES**


— Unpublished observations.
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