Toxicity and Immunogenicity of Monoclonal Antimelanoma Antibody-Ricin A Chain Immunotoxin in Rats

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ABSTRACT

This study was performed to assess the subacute toxicity and immunogenicity in rats of XOMAZYME-MEL, an antimelanoma monoclonal antibody-ricin A chain immunotoxin. Female Sprague-Dawley rats received 14 consecutive daily i.v. injections of XOMAZYME-MEL at doses of 5 mg/kg/day, 1 mg/kg/day, or normal saline. Animals from each dose group were sacrificed on days 8, 15, and 22. The low dose of immunotoxin was well tolerated and produced only minimal signs of toxicity. Side effects in animals receiving the high dose of immunotoxin consisted of transient weight loss, peripheral edema, leukocytosis, hy- poalbuminemia, and mildly elevated liver function tests. Histological findings in these animals included cytoplasmic vacuolization of hepatocytes, focal myocardial and skeletal muscle degeneration, and renal deposits of proteinaceous casts. The administration of immunotoxin resulted in the appearance of anti-mouse and antiricin A chain immunoglobulin binding activity in the sera of treated animals. This study documents the systemic effect of the multiple-dose administration of a ricin A chain immunotoxin in rats.

INTRODUCTION

Recent advances in protein chemistry (1, 2) and hybridoma technology (3) have led to the development of immunotoxins consisting of monoclonal antibodies conjugated to RIP2 such as ricin A chain. These hybrid molecules are designed to incor- porate the binding specificity of antibodies with the cytotoxicity of a RIP to selectively destroy malignant or other disease-producing cells while minimizing the toxicity to normal cells and tissues. Over the past decade, a number of immunotoxins have been reported to specifically kill targeted cells in both in vitro and in vivo experimental models (4–9). Nonspecific tox- icity is anticipated following the systemic administration of immunotoxins; however it will likely be less than that produced by most conventional chemotherapeutic agents.

The A chain of ricin is one RIP which is a frequent compo- nent in immunotoxins. Intact ricin consists of two polypeptides, an A chain and a B chain, each with a molecular weight of about 30,000 (1). The A chain of ricin reacts enzymatically with EF-2 binding sites on 60S ribosomal subunits causing inhibition of protein synthesis and cell death. The B chain of ricin is a lectin moiety that binds to lactose and galactose and facilitates the entry of A chain into virtually all types of eukaryotic cells. Separated from B chain, ricin A chain is poorly internalized and is at least 1,500 times less toxic to cells than whole ricin (1). Therefore, an immunotoxin incorporating ricin A chain should be relatively less toxic than whole ricin to nonphagocytic cells lacking the specific surface antigens recognized by the targeting antibody.

Since preparations for clinical trials of immunotoxins are underway at several centers, a thorough evaluation of the potential systemic toxicity of immunotoxins is becoming increasingly important. This study was performed to assess the sub- acute toxicity and immunogenicity of daily i.v. doses of an antimelanoma monoclonal antibody-ricin A chain immuno- toxin in rats.

MATERIALS AND METHODS

Animals. Female Sprague-Dawley rats (Simonsen Laboratories, Gil- roy, CA) aged 10–12 weeks and weighing 150–200 g were used. Animals were acclimated for 1 week prior to the start of experiments, identified by ear tags, and housed individually. Pelleted rat feed (Purina Certified Rat Chow;Ralston Purina, Richmond, IN) and water were provided ad libitum. All rats were weighed each morning before dosing and before sacrifice.

Test Article. For the generation of the hybridoma, the hybridization, cloning, and recloning were performed according to the procedure described by Köhler and Milstein (3) with minor modifications (10). BALB/c mice were immunized with cultured human melanoma cells. Spleen cells were harvested from immunized mice and fused with the 8-azaguanine-resistant, nonsecreting murine myeloma line P3X63-Ag8 in the presence of polyethylene glycol. Hybridomas secreting antibodies with the appropriate specificity were subcloned twice by limiting dilu- tion using BALB/c splenocytes as feeder cells.

The monoclonal antibody is an IgG2a subclass that reacts with melanoma-associated antigens having molecular weights of 220,000 and over 500,000 daltons. It is produced from murine ascites and purified by using a Staphylococcal protein A column with an elution of pH 3.5. The purified antibody is free of contamination by murine viruses, polynucleotides, or other immunoglobulin classes or sub- stances.

XMMME-001-RTA (XOMAZYME-MEL; produced by XOMA Corporation, Berkeley, CA) consists of the murine antimelanoma monoclonal antibody conjugated to RTA. The ricin A chain is purified by affinity chromatography using an anti-B chain column. The conjugation technique has been described in detail elsewhere (11). Briefly, the antibody is activated with N-succinimidyl-3-(2-pyridyldithio)propionate followed by addition of affinity-purified ricin A chain which has been reduced with dithiothreital. The immunotoxin is then purified by gel chromatography. For the product used in these studies, there was an average of 2.3 RTA molecules conjugated to each antibody. There was no detectable free RTA and 6.3%-free IgG was observed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The conjuga- tion was free of other contaminants. It is provided in sterile, pyrogen- free formulation at a concentration of 1.0 mg/ml in 0.9% phosphate buffered saline, pH 7.0.

Immunotoxin binding to normal human and rat tissues was evaluated using the indirect immunoperoxidase technique. Studies were performed by Dr. Clive Taylor, Department of Pathology, University of Southern California. In these studies, frozen sections of untreated human tissues and untreated rat tissues were incubated with immuno- toxins and then tested with either peroxidase-conjugated goat anti- mouse antibody or peroxidase-conjugated goat antiricin A chain anti- body. Sites of localization of the immunoperoxidase were revealed by the use of 3-amino-9-ethylcarbazole as the chromogen. The immuno- toxin was previously titrated using human melanoma to demonstrate reactivity and to determine the appropriate test doses for normal tissue. The immunotoxin was reactive with all metastatic melanomas tested and with human nevus cells as well. On normal human tissue sections,
there was minimal staining in all normal tissues tested, except for vascular endothelium, in which the reactivity appeared to be cytoplasmic. On the rat tissue sections, evaluation of antibody localization with the goat anti-mouse antibody reagent was difficult because of a high degree of background reactivity with native rat immunoglobulin. However, background reactivity was less marked when tissues were stained with goat antirat A chain reagent. With this procedure, the pattern of reactivity in rat tissues was similar to that observed in human tissues, with no reactivity in muscle, eye, lymph node, spleen, or liver, except for vascular endothelium. Trace reactivity was observed in rat brain and kidney sections. Therefore, it appeared that XMMME-001-RTA immunotoxin has a similar pattern of cross-reactivity in normal human and rat tissues.

Study Design. Dosages used in this subacute study were determined by the results of a multiple-dose range-finding study. In the current study, animals were randomly assigned to one of three dose groups; 12 animals each for the control and low dose groups, and 15 animals for the high dose group. All animals received a daily i.v. injection via tail vein for 14 consecutive days. The high dose group received a dose of 5.0 mg/kg/day of immunotoxin; the low dose group received a dose of 1.0 mg/kg/day; and the control group received 5 cc/kg/day of normal saline. Animals were dosed in the morning under light ether anesthesia, and the first day of dosing was considered day one of the study. Four animals from each dose group were sacrificed on day 8 (after 7 doses), on day 15 (after 14 doses), and on day 22 (after 14 doses and 8 days of recovery). Three animals from the high dose group were sacrificed on day 3 of the study to evaluate early toxic changes. The rats were anesthetized with ether for blood collection and exsanguination prior to terminal necropsy.

Clinical Observations. Daily observations included body weight, estimates of food and water consumption, and any notable clinical or behavioral changes.

Laboratory Studies. Blood samples were obtained from all animals by cardiac puncture performed under ether anesthesia. Studies performed included complete blood count and chemistry panel including determination of sodium, potassium, chloride, carbon dioxide, calcium, alkaline phosphatase, BUN, creatinine, glucose, serum glutamyl oxalacetic transaminase, serum glutamyl pyruvyl transaminase, uric acid, total bilirubin, cholesterol, total protein, albumin, globulin, creatinine phosphokinase, gamma glutamyl transpeptidase, and lactate dehydrogenase. These studies were performed by Veterinary Reference Laboratories, San Leandro, CA.

Pathological Studies. The tissues sampled at necropsy included mesenteric lymph nodes, stomach, duodenum, pancreas, jejunum, ileum, cecum, colon, spleen, kidney, adrenals, liver, lung, heart, quadriceps, femur, eye, and brain. All specimens were preserved in 10% buffered formalin. Paraffin sections were stained with hematoxylin and eosin and examined by light microscopy. Histopathological studies were performed by Dr. Dee O. N. Taylor, Kensington, CA.

Immunostains. Enzyme-linked immunooassay was used to determine the presence of rat anti-mouse and rat antirat A chain antibodies in the serum of treated and control animals. In these assays, 96-well microtiter plates were coated with either 50 μL XMMME-001 antibody (4 μg/well) or 50 μL ricin A chain (2 μg/well), incubated overnight at 4°C, and then thoroughly washed. Serum samples from the test animals were first diluted 1:100 in 1% bovine serum albumin in phosphate buffered saline and then serially diluted for titration of binding activity. Sample dilutions were added to wells in triplicate and incubated overnight at 4°C. Wells were washed thoroughly, and to each well was added 50 μL of alkaline phosphatase-conjugated affinity-purified goat anti-rat IgG previously absorbed with murine immunoglobulin (Cappel Laboratories, Malvern, PA). A 1-h incubation was followed by thorough washing with phosphate buffered saline at 37°C. Substrate (50 μL/well of 1 mg/ml p-nitrophenolphosphate [Sigma Chemical Company, St. Louis, MO] in 10% diethanolamine buffer) was added to each well and incubated for 1 h at room temperature. Binding activity was measured at dilution levels ranging from 1:10 to 1:10,000. Results were read on a Microelisa Autoreader at 405 nm and reported as optical density values. Binding activity at the 1:100 dilution was selected to provide a standard of comparison between samples.

Statistical Analyses. All statistical comparisons were made using Student's independent groups t test.

RESULTS

Clinical Observations. No mortality occurred in any of the dosing groups. The control group gained weight steadily throughout the observation period. By contrast, animals receiving the low dose of immunotoxin maintained a steady weight until day 14 (the last day of treatment), and therefore their average weight was significantly less than controls. Weight gain was subsequently observed in this group. Animals receiving high doses of immunotoxin lost weight steadily until day 10 of the dosing period, at which time an increase in weight was observed that continued throughout the remaining treatment and observation period (Fig 1). All animals remained active and appeared healthy, except for weight loss, during the first 7 days of treatment. Edema in the rear footpads appeared during the second week of treatment in all of the animals in the high dose group and in one of eight animals in the low dose group. Animals receiving the high dose of immunotoxin also demonstrated lethargy, rough fur coats, nasal or lacrimal discharges, and unsteady gaits. All clinical abnormalities remitted by the 14th day of observation.

Laboratory Studies. Transient changes in hematological parameters were observed in the treatment groups. The WBC increased to a mean of 19,500/mm³ in the high dose group and to 14,800/mm³ in the low dose group on day 8 as compared to a mean WBC of 9,000/mm³ in the control group. In the high dose group the leukocytosis was associated with a significant increase in percentage of neutrophils (61 versus 28%) and a decrease in percentage of lymphocytes (38 versus 68%) on day 4 when compared to control animals. There was no difference in WBC or differential between control and treatment groups on days 15 and 22 of the study. No significant changes were noted in hemoglobin, hematocrit, or RBC indices at any time point during the study.

All observed changes in serum chemistry values were mild and transient, and were observed only in animals receiving the high dose of immunotoxin (Table 1). No significant changes in chemistry values were observed in the animals receiving the low dose of immunotoxin. Mild abnormalities in liver function were noted in the high dose group as manifested by increases in serum glutamyl oxalacetic transaminase and serum glutamyl

\[ \text{Control} \quad \begin{array}{c} 1 \text{ mg/kg/day} \quad 5 \text{ mg/kg/day} \\ \end{array} \]

\[ \begin{array}{c} \text{Body Weight (g)} \\ \text{Day of Study} \end{array} \]

Fig. 1. Body weight (in grams) of rats receiving daily i.v. injections of XMMME-001-RTA immunotoxin or normal saline control for 14 consecutive days. All standard deviations were less than ±7 g.

\[ ^{4} \text{S. Harkonen, unpublished data.} \]
creatinine without an associated elevation in BUN was noted. Early decreases in serum albumin concentration were observed. Transient, pyruvyl transaminase values without an associated increase in lactate dehydrogenase or bilirubin. There was a slight increase in alkaline phosphatase at one time point only (day 15) in the animals receiving the high dose of immunotoxin. Transient, early decreases in serum albumin concentration were observed (Table 1). There was an associated fall in serum total protein in the high dose group and in one of the animals in the control group. Mild foci of inflammation in the high dose group as compared to the 1-mg/kg dose group. On day 22, however, the level of antibody response was equivalent in both dose groups. Neither an anti-mouse nor antiricin A chain binding level significantly above background was detected in the control group at any time during the study.

**DISCUSSION**

In this study we determined the subacute toxicity of high and low doses of a monoclonal antimelanoma antibody-ricin A chain conjugate in rats and defined a dosing regimen which appears to be safe. Administration of 1 mg/kg/day of immunotoxin for 14 consecutive days was well tolerated and resulted in minimal evidence of systemic toxicity. Administration of 5 mg/kg/day resulted in no mortality, but several changes in clinical, laboratory, and histopathological parameters were observed. These changes included weight loss, peripheral edema, lethargy, leukocytosis, hypoalbuminemia, and mild elevation of liver function tests. Histological findings included cytoplasmic vacuolization of hepatocytes, focal myocardial and skeletal muscle fiber degeneration, and renal deposits of proteinaceous casts. The abnormalities, in general, were transient and reversible even in the face of continued administration of immunotoxin.

Toxicity following the systemic administration of immunotoxins could occur by one or more mechanisms. First, there could be specific toxicity mediated by binding of the antibody component of the immunotoxin to normal tissues. No tumor-specific antigen has yet been identified, and all known antibodies to tumor-associated antigens cross-react to some degree with normal cells and tissues. Therefore, the binding activity and degree of cross-reactivity of the antibody will determine part of the systemic toxicity induced by immunotoxins. Another potential mechanism of immunotoxin toxicity is nonspecific uptake of antibody-toxin conjugates by cells of the RES, mediated either by antibody binding to Fc receptors or by A chain uptake of antibody-toxin conjugates by cells of the RES, mediated either by antibody binding to Fc receptors or by A chain binding to carbohydrate receptors. Finally, free ricin A chain, either recycled from lysed cells or dissociated from the antibody-toxin conjugate, may also produce nonspecific toxicity.

Much of the toxicity observed in this study can probably be attributed to the nonspecific activity of the immunotoxin. The binding activity with normal rat tissues, as demonstrated by in vitro immunoperoxidase studies, was limited to vascular endothelium suggesting that the observed in vivo effects were not due to targeted activity. And no histopathological changes in vascular endothelium were observed, suggesting that this reactivity in frozen sections was cytoplasmic in nature, and not physiologically significant. Moreover, we have conducted preclinical evaluations of two additional immunotoxins consisting of an anti-pan T lymphocyte monoclonal antibody-ricin A chain conjugate and an anticolorectal carcinoma antibody-ricin A chain conjugate, and the observed side effects were similar to those reported in the present study.

The fall in serum albumin could be due to one of several mechanisms. Decreased hepatic synthesis of albumin as a result of ribosomal inactivation is possible, but seems unlikely to be...
Fig. 2. Photomicrographs of tissues obtained on day 15 from Sprague-Dawley rats that received high doses (5 mg/kg/day) of immunotoxin for 14 days (x 400) demonstrating (A) degeneration and fibrosis of myocardial tissue; (B) scattered degeneration of striated muscle fibers with infiltration of mononuclear cells; (C) proteinaceous casts in proximal renal tubules; and (D) vacuolization of hepatocytes.

the exclusive etiology. Other possible mechanisms of hypoalbuminemia include increased rate of excretion from the body, increased volume of distribution due to increased transcapillary passage of albumin, or a combination of these factors. Focal degenerative lesions in myocardial and skeletal muscle have been observed by other investigators following ricin A chain immunotoxin treatment, and appear to be the result of nonspecific immunotoxin toxicity (12). Leukocytosis is a finding observed following the administration of ricin (13) as well as with other ricin A chain immunotoxins. Therefore, it too appears to be a nonspecific result of immunotoxin administration.

Hepatocellular injury following administration of ricin and ricin A chain has been well documented (12, 13). The hepatocellular injury observed in this study was mild and reversible, being maximal 15 days after the initiation of immunotoxin therapy. The observation of cytoplasmic vacuolization of hepatocytes could be an early manifestation of chemically induced liver injury (14). No intestinal lesions were observed in any animals in the present study, although duodenitis is observed in animals treated with high doses of ricin A chain (15). Decreased oral intake of food and water due to lethargy is a likely explanation for the observed weight loss, but the etiology for this finding requires further investigation.

Kupffer cells and sinusoidal endothelial cells have receptors for the high mannose type carbohydrate chain glycoproteins that are present on the A chain of ricin (7). These receptors are

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jugates to the RES. Thus, one might have predicted significant toxicity to cells or tissues of these tissues. However, in this study, no such toxicity was seen.

Renal toxicity was mild and was limited to the high dose group. Pathological lesions consisted of tubular casts located in the renal medulla. There was an isolated elevation of the serum creatinine on day 3 of treatment that was not associated with a change in BUN, so the significance of this finding is unclear. In other studies, renal tubular lesions were the dominant histopathological finding in mice that received LD₅₀ doses of an antilymphocyte antibody ricin A chain immunotoxin, and in mice that received LD₅₀ doses of unconjugated, purified ricin A chain.

The immunotoxin used in this study was immunogenic in rats. Significant levels of both anti-mouse and antiricin A chain antibodies developed in the high dose and low dose treatment groups. Although the immunogenicity of antibody ricin A chain conjugates has not been previously reported, the development of antiricin antibodies following the administration of unmodified murine monoclonal antibodies is well recognized. Anti-mouse immune responses have been associated with altered pharmacodynamics and pharmacokinetics of antibody-ricin A chain conjugates. In mice that received LD₅₀ doses of an antilymphocyte antibody ricin A chain immunotoxin, and in mice that received LD₅₀ doses of unconjugated, purified ricin A chain.

In conclusion, the subacute administration of high doses (5 mg/kg/day) of XMMME-001-RTA immunotoxin produces a number of toxic manifestations in rats. Most of these effects can be attributed to the generalized, nonspecific effects of immunotoxin. Since most of the observed toxicity is probably related to the toxicity of the ricin A chain moiety in the immunotoxin, it might be expected to occur following the administration of high doses of all ricin A chain immunotoxins. On the other hand, lower doses of XMMME-001-RTA (1 mg/kg/day) were well tolerated and resulted in minimal evidence of systemic toxicity. It is possible that additional toxic effects might be seen in human trials if the antibody cross-reacts with normal, nontargeted tissue. XMMME-001-RTA immunotoxin was also immunogenic in rats, and the development of antimouse and antiricin A chain antibodies was temporally associated with the reversal of several toxic effects. Several studies comparing various strategies to abrogate this immune response are currently underway. The results of this present study will help in the design and interpretation of clinical trials of immunotoxins.

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