Efficacy of Compartmental Administration of Immunotoxin LMB-1 (B3-LysPE38) in a Rat Model of Carcinomatous Meningitis

Darell D. Bigner,² Gary E. Archer, Roger E. McLendon, Henry S. Friedman, Herbert E. Fuchs, Lee H. Pai, James E. Herndon II, and Ira H. Pastan

Department of Pathology [D. D. B., G. E. A., R. E. M., H. S. F., H. E. F.], Preuss Laboratory for Brain Tumor Research [H. S. F., D. D. B.] and Community and Family Medicine, Division of Biometry [J. E. H.], Duke University Medical Center, Durham, North Carolina 27710, and the Laboratory of Molecular Biology, Division of Cancer Biology, Diagnosis, and Centers, National Cancer Institute, NIH, Bethesda, Maryland 20892 [L. H. P., I. H. P.]

ABSTRACT

LMB-1 (B3-LysPE38) is an immunotoxin composed of the tumor-reactive monoclonal antibody B3 and a genetically engineered form of Pseudomonas exotoxin. Monoclonal antibody B3 reacts with a carbohydrate epitope that is found on a number of solid tumors (e.g., breast, ovarian, and lung carcinomas) that frequently invade the intrathecal space, causing neoplastic meningitis. The Pseudomonas exotoxin has been engineered to remove the binding domain to eliminate nonspecific binding. A model of human neoplastic meningitis using rats bearing the human epidermoid carcinoma A431 was used for therapeutic studies of immunotoxin LMB-1. Therapy was initiated 3 days after injection of the tumor cells, which was one of three median survival time of untreated rats. A single intrathecal injection of 40 μg increased median survival from 9 days with saline injection to 16 days (78%, P < 0.001), and a single dose of 200 μg increased median survival to 25 days (188%, P < 0.001). Two doses of 40 or 200 μg given on days 3, 6, and 8 significantly increased the median survival of 9.5 days associated with saline injection to 40.5 days (326% increase) and 33.0 days (247% increase), respectively, with two long-term survivors (191-day survival) in each treatment group. LMB-1 had no therapeutic effect on the treatment of two B3 antigen-negative neoplastic meningitis models. Treatment of the antigen-positive A431 neoplastic meningitis with B3 alone or a nonspecific monoclonal, MOPC, coupled to the engineered Pseudomonas exotoxin produced no survival effects. Nontumor-bearing athymic rats showed no toxicity with a single dose of either 40 μg or 200 μg, or 3 doses of 40 μg. However, when they were given three doses of 200 μg, these rats showed weight loss and loss of neurological function, and two of eight animals died. These studies indicate that, in the range of the most therapeutically effective dosage, the immunotoxin LMB-1 is tolerated in the intrathecal space and should be considered for human intrathecal trials.

INTRODUCTION

In spite of aggressive treatment, patients with neoplastic meningitis have a median survival of only 2–3 months (1, 2). Neoplastic meningitis can result from a direct seeding of the neuraxis by primary brain tumors such as medulloblastoma, or it may result from hematogenous dissemination of systemic solid tumors such as gastrointestinal neoplasms. At one time neoplastic meningitis was thought to be a rare complication. With today’s improved treatment of systemic solid tumors, however, the incidence of neoplastic meningitis is an increasing complication of breast and lung adenocarcinomas and melanomas, as well as a direct result of primary central nervous system tumors. The most likely cause of central nervous system relapse of solid tumors resulting in neoplastic meningitis is failure of tumoricidal agents, administered systemically, to achieve a therapeutic level in the i.t. space (3). One of the recognized methods for increasing the level of chemotherapeutic agents in the cerebrospinal fluid is compartmental administration into the i.t. space. Only a few chemotherapeutic agents, such as methotrexate, cytarabine, thiopeta, 4-hydroperoxycyclophosphamide, and melphalan, have been approved for i.t. administration, and the latter two only for Phase I trials (4–7). Drug resistance and tumor cell heterogeneity can limit the effectiveness of chemotherapy agents, particularly if an agent is used as a single-modality approach.

A new class of specific cytotoxic reagents, immunotoxins, constructed by altering the receptor specificity of naturally occurring protein toxins, are actively being investigated for the treatment of malignancies. The first immunotoxins were constructed with peptide hormones. With the advent of MAAb technology, immunotoxins were constructed with MAbs to define the target specificity. The toxins most commonly used are plant bacterial toxins that inhibit protein synthesis. Compartmental administration into the i.t. space for the treatment of neoplastic meningitis was first investigated by Zovickian and Youle (8). Compartmental administration avoids complications associated with systemic delivery, and relatively high local concentrations of immunotoxin can be achieved (9). Immunotoxins composed of various targeting proteins and toxins have been used in compartmental therapy of neoplastic meningitis animal models of lymphoma and melanoma and for direct injection in treatment of central nervous system tumors (8, 10–14).

The immunotoxin in this study is composed of the MAB B3 and the Pseudomonas exotoxin. B3 reacts with Le⁢a⁡ and several

¹ The abbreviations used are: i.t., intrathecal; MAB, monoclonal antibody; FACS, fluorescence-activated cell sorting; ZO, zinc option.
closely related carbohydrate antigens found in carcinomas of the breast, colon, stomach, esophagus, ovary, and lung (15). An inherent property of protein toxins is their ability to bind to cells and translocate to the cytosol where they can inhibit protein synthesis. Therefore, the design of an immunotoxin must limit such nonspecific binding. *Pseudomonas* exotoxin in LMB-1 (also known as B3-LysPE38) has been engineered to remove the cell binding domain to eliminate nonspecific binding (16). An athymic rat with an indwelling i.t. catheter was used to establish antigen-negative and antigen-positive models of human epidermoid carcinoma in the i.t. space and to investigate the specificity, effect of dose escalation and dose scheduling of i.t. LMB-1 on animal survival. The rat model is an excellent representation of human neoplastic meningitis. Not only does it simulate disseminated neoplastic i.t. disease and allow multiple-dose treatment, but the rat and human share a number of normal tissues that are positive for the B3 antigen, and the model may therefore be more sensitive to toxic side effects than are animal models with no normal tissue cross-reactivity. In this study, the most effective dosage of the LMB-1 immunotoxin (40 µg X 3) produced no toxic side effects while increasing median survival in a dose-dependent manner as compared with saline-treated controls. Furthermore, multiple dosing produced a 20% long-term survival.

**MATERIALS AND METHODS**

**Animal Model**

Subarachnoid catheters were implanted using the method of Fuchs et al. (17). In brief, female athymic rats (190–240 g) were maintained in the Duke University Cancer Center Isolation Facility according to procedures approved by the Duke Institutional Review Board and were anesthetized using i.p. injection of ketamine (55 mg/ml)/xylazine (9 mg/ml) at a rate of 1 ml/kg. Each animal was placed in a stereotactic frame with tilt adapter (David Kopf Instruments, Tujunga, CA), the neck was flexed at a 90° angle, and a PE-10 catheter (Intramedic; Clay Adams, Franklin Lakes, NJ) with a 5–0 stainless steel wire stylet was inserted into the subarachnoid space and passed along the posterior aspect of the spinal cord to the lumbar region (8.5 cm). The catheter was occluded using a small piece of 2–0 stainless steel wire. The animals were allowed to recover for 7 to 10 days. Only animals showing normal motor and sensory function were used in these experiments.

Neoplastic meningitis was initiated by inoculation of tumor cells through the indwelling subarachnoid catheter. The animals were anesthetized by light halothane anesthesia, the 2–0 wire stylet was removed, and a 3-fold molar excess of 2-imidothiolane hydrochloride at 37°C for 30 min. The protein was separated from unreacted cross-linker on a PD10 column. B3 at a concentration of 5–10 mg/ml in 0.2 M phosphate buffer (pH 8.0) with 1 mM EDTA was reacted with a 3-fold molar excess of 2-imidothiolane hydrochloride at 37°C for 1 h. Modified protein was purified with a PD10 column. Activated MAb and LysPE38 were mixed in equal molar amounts and incubated at room temperature for 16 to 20 h. LMB-1 was purified by successive chromatography on MonoQ and TSK-250 columns (16).

**Tumor Cell Lines**

The antigen-positive cell line A431 was originally obtained from George Todaro and has a homogeneous expression of the Leα antigen to which MAb B3 reacts. The number of MAb B3 antigenic sites per cell has been estimated to be 1 X 10^4. The antigen-negative cell line A431n is a clone of A431 (CRL1555: American Type Culture Collection, Rockville, MD). In vitro the LMB-1 has failed to show any cell killing with the A431n cell line. D341 Med is a human medulloblastoma line that was established at Duke University and grows as a suspension; it is unreactive with MAb B3 by FACS analysis. The cell lines were maintained in Riechter’s ZO medium supplemented with FCS of either 5% (A431, A431n) or 10% (D341 Med) concentration. A431 and A431n were removed from culture flasks with 0.125% trypsin and 0.02% EDTA and were washed three times in Dulbecco’s PBS; the concentration was adjusted to 1.25 X 10^6 cells/ml. D341 Med cells were decanted and washed three times in Dulbecco’s PBS, and the concentration was adjusted to 1.25 X 10^6 cells/ml. For each cell line, 40 µl cell suspension were used for animal inoculation.

**Toxicity Studies**

For toxicity studies, groups of 10 nontumor-bearing athymic female rats were given a single i.t injection of 0.472, 4.72, 47.2, and 200 µg LMB-1 or saline. For multiple-dose toxicity, nontumor-bearing athymic female rats were given 40 µg X 3, 200 µg X 3, or saline. The animals were followed up with daily weight and neurological checks for 6 weeks, after which the animals were killed and a complete autopsy was performed. The neuraxis was sectioned for histopathology as outlined below.

**Treatment Studies**

**Single-Dose Treatment of the Antigen-positive Cell Line.** Three days after inoculation with A431 cells, groups of 10 animals were treated with a single injection of either 40 µg LMB-1, 40 µg MOPC-LysPE38, 40 µg unconjugated MAB B3 in 40 µl, or saline. MOPC-LysPE38 is a nonspecific immunotoxin that uses MOPC-21 (Sigma Chemical Co., St. Louis, MO), an isotype-matched IgG1 MAb with no known reactivity, cou-

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4 I. H. Pastan, unpublished results.
The survival benefits of dose escalation and multiple dosing were studied in two experiments with the antigen-positive cell line. Following initiation of neoplastic meningitis with 5 × 10⁶ A431 cells, groups of 10 animals were treated in experiment 1 with either a single injection of 40 μg LMB-1 on day 3; two injections of 40 μg LMB-1 on days 3 and 6; or a single injection of 200 μg LMB-1 on day 3; or saline. In experiment 2, following tumor inoculation, the treatment groups were given one injection per day for 3 days (on days 3, 6, and 8 of 40 μg LMB-1, 200 μg LMB-1, or saline).

**Antigen-negative Cell Line**

Groups of 10 animals having neoplastic meningitis initiated with A431n, a cell line that does not express MAb B3 antigen in *vivo*, were treated with single injections of either 40 μg LMB-1, 40 μg MOPC-LysPE38 (nonspecific MAb), 40 μg unconjugated MAb B3, or saline 8 days after inoculation. Groups of animals having neoplastic meningitis initiated with D341 Med, the FACS-negative cell line, were treated with three 40-μg doses of LMB-1 or saline 8 days after inoculation. The animals were followed up with daily weight and neurological checks until death. Each animal was then given a complete autopsy, and the neuraxis and liver were processed for histology.

**Immunohistochemistry**

Normal rat and human tissues and neuraxis tissue from athymic rats bearing A431 and A431n i.t. xenografts were harvested at autopsy and immediately frozen in liquid nitrogen; tissue samples did not generally exceed 10 mm in the longest dimension. Samples were stored at −70°C until sectioning. Twenty-four to 48 h before assay, 5-6-μm sections were cut, allowed to air dry, and fixed for 30 s in −20°C acetone. Immunohistochemistry was performed as previously described (18). In brief, rehydrated sections were blocked with 10% normal goat serum, sequentially exposed to the appropriate primary reagents, and developed with the Zymed biotinylated antibody-streptavidin-diaminobenzidene system (19). Primary reagents included B3 (10 and 5 μg/ml), isotype control murine IgG1 (10 and 5 μg/ml), dilution buffer, an assay-positive control, and a polyvalent murine anti-human cell control that reacts with rat membrane proteins. Slides were counterstained with hematoxylin, mounted, and scored for reactivity by a minimum of two observers. Positivity was evaluated with respect to nuclear, cytoplasmic, and/or membranous localization of bound reagents.

**FACS**

A431 and A431n were removed from tissue culture flasks with 0.02% EDTA, washed twice in ZO medium, and counted, and the concentration was adjusted to 2 × 10⁶ cells/ml. Non-specific binding was blocked by 15-min incubation in 0.5 ml 15% normal goat serum in ZO medium on ice. The cells were pelleted and incubated on ice with 0.5 ml MAb B3 or nonreactive IgG1 control for 45 min. After being washed twice in 0.02% EDTA-ZO with 10% FCS, the cells were then incubated for 30 min on ice in the dark with 0.25 ml fluoresceininated goat antimouse IgG. The pellets were washed as described above and resuspended in 1 ml ZO with 1% FCS. Analysis was done on the FacSort (Becton Dickinson, Mansfield, MA).

**Histology**

The entire neuraxis was removed and fixed in buffered neutral formalin for 7 to 10 days and then decalcified in a decalcifying solution (Scientific Products, Baxter, Charlotte, NC). Six representative sections of the neuraxis were submitted from each animal. The sections were taken from the brain at the level of the coronal suture and the pituitary gland and from the spinal cord at the cervical, thoracic, and lumbar levels and the cauda equina. Sections were embedded in paraffin, and 6-μm sections were stained with Luxol fast blue and with hematoxylin and eosin and examined by light microscopy.

**Statistical Analysis**

Survival curves were estimated for each treatment group using the product-limit estimator of Kaplan and Meier (20). The survival time for animals that were killed prior to their natural death are considered censored at the time of sacrifice. Comparisons of survival curves were conducted using the log rank test (21).

**RESULTS**

**Neoplastic Meningitis Models.** Three models of neoplastic meningitis were established to study the therapeutic effects of the LMB-1 immunotoxin. Model 1 used an A431 human epidermoid carcinoma cell line, which reacts with MAb B3 from which the LMB-1 immunotoxin was derived, as the antigen-positive target. Model 2 used the American Type Culture Collection line A431n, which reacts with B3 in *vitro* by FACS analysis but does not react with B3 in *vivo* by immunohistochemistry. Model 3 used the human medulloblastoma cell line D341 Med, which does not react with B3 in *vitro* by FACS analysis. The antigen-positive model, established by injecting 5 × 10⁶ cells of the parent A431 cell line, developed a progressive quadriparesis with loss of ramp climbing ability by days 6-8, and death occurred by days 7-10. The antigen-negative model was initiated with 5 × 10⁶ A431n cells and developed a progressive quadriparesis with loss of ramp climbing ability by days 8-43 and death by days 11-57. The human medulloblastoma model D341 Med was initiated with 5 × 10⁶ cells and developed a progressive quadriparesis with loss of ramp climbing ability by days 17-21; death occurred by days 18-23. Median survival for the three models was days 9, 29, and 20, respectively. The histopathology produced by the antigen-positive A431 cell line revealed tumor in every section of all animals. For the antigen-negative A431n cell line, all animals had tumor except one; this animal appeared unhealthy and died very early. There was no histological evidence of infection in
the neuraxis; however, the possibility that the animal died from systemic complications cannot be ruled out. In the antigen-positive model of neoplastic meningitis, histological examination of the untreated animals revealed evidence of hemorrhage (3/13), necrosis (3/13), edema (2/13), and fibrosis (1/13). These histological abnormalities were not necessarily associated with each other. Examination of the untreated antigen-negative model revealed evidence of hemorrhage in 1 of 10 animals and edema in 4 of 10 animals, with evidence of arachnoid fibrosis in 5 of 10 animals. There was no correlation between any of the histological findings. Histological examination of the untreated animals with D341 Med neoplastic meningitis revealed tumor in seven of eight animals. In the animals with tumor, one had accompanying hemorrhage and three had evidence of edema. The hemorrhage, necrosis, and edema in both models may have been associated with and directly caused by tumor involvement.

**Single-Dose Treatment of Antigen-positive Cell Line.**

A neoplastic meningitis model established with the antigen-positive A431 cell line was treated on day 3 with 40 μg LMB-1 (n = 10), and survival was compared with the control groups receiving 40 μg unconjugated MAb B3 (n = 10), 40 μg non-reactive immunotoxin conjugate MOPC-LysPE38 (n = 9), and saline (n = 10). The treatment with LMB-1 significantly increased the median survival (P < 0.001) compared with all three control groups. The median survival for each of the groups was LMB-1, 18.0 days; unconjugated MAB B3, 10.0 days; MOPC-LysPE38, 10.0 days; and saline, 10.5 days. The loss of the stepping and placing reflex and antigravity strength preceded death by approximately 2 days. Clinically and histologically, there was no evidence of toxicity. Histological findings were hemorrhage, necrosis, and spinal edema, which were scattered throughout the neuraxis. There was no evidence of peripheral demyelination in any of the animals. Tumor was found in all animals that died.

**Comparison of Dose Escalation and Multiple Dosing.**

The next two experiments compared the therapeutic efficacy of multiple dosing and dose escalation of LMB-1 with a saline control group in the antigen-positive cell line. In experiment 1, single doses of 40 μg (n = 10) and 200 μg (n = 9) LMB-1 were given on day 3 after tumor inoculation, and multiple doses of 40 μg (n = 10) LMB-1 were given on days 3 and 6. All LMB-1 treatment groups exhibited a statistically significant increase in median survival as compared with the saline control group (P < 0.001 for all groups, Fig. 1). Tumor-bearing animals treated with a single dose of 40 μg LMB-1 had an increase in median survival to 16 days (78% increase, P < 0.001) as compared with saline-treated animals whose median survival was 9 days, and the LMB-1 group produced two long-term survivors. Animals that received two doses of 40 μg LMB-1 had an increased median survival to 27.33 days (204% increase, P < 0.001), and there was one long-term survivor in this group. A single dose of 200 μg LMB-1 increased median survival to 25 days (178% increase, P < 0.001), with no long-term survivors. There was no statistically significant difference in the median survival between the groups treated with a single dose of 200 μg and those treated with two doses of 40 μg LMB-1.

Experiment 2 compared three doses each of 40 μg (n = 9) and 200 μg (n = 10) with a saline control group (n = 10). Tumor verification animals killed at the initiation of treatment showed the presence of tumor in all six standard histological sections (Fig. 2). Rats treated with a total of three doses, given on days 3, 6, and 8, of 40 μg LMB-1 had a median survival of 40.5 days, and those treated with three doses of 200 μg LMB-1 had a median survival of 33.0 days. The percentage of increase in median survival compared with rats receiving only saline was 326% and 247%, respectively (P < 0.001). There were two long-term survivors in each group (Fig. 3). Statistically, there was no difference in median survival between the two LMB-1 treatment groups. Histological examination of the animals treated with a single 40-μg dose of LMB-1 showed tumor in the neuraxis of all of the animals that died (8/10). The two animals that survived until the end of the experiment showed no evidence of tumor and no histological abnormalities (Fig. 4). The animals that died as a result of tumor had histological findings of hemorrhage (6/8), necrosis (4/8), and spinal edema (3/8). Of the animals treated with a single dose of 200 μg LMB-1, nine of nine showed presence of tumor, one of nine showed hemorrhage, and four of nine showed spinal edema. There was no evidence of necrosis. In the group treated with two doses of 40 μg LMB-1, all animals that died had evidence of tumor (9/10), 5/9 showed hemorrhage and necrosis, and 4/9 exhibited signs of spinal edema. All animals in the saline control group showed tumor throughout the neuraxis; hemorrhage was found in 4/10, necrosis in 4/10, and spinal edema in 2/10. None of the animals in this experiment showed any evidence of peripheral demyelination.

In the dose escalation experiments where animals were treated with three doses of 40 μg LMB-1, four of seven of the animals that died had histological evidence of tumor. Of the three animals that died and had no evidence of tumor, all exhibited characteristic signs of tumor progression, namely, weight loss and loss of neurological function. None of the
Fig. 2  Sections of tumor verification animal that was killed at day 3 following tumor inoculation for assessment of tumor burden at the initiation of LMB-1 therapy. A, focus of tumor located above the optic nerves (arrow). B, level of the cerebellum on which the tumor is ventrally located (arrow). At the cervical (C), thoracic (D), lumbosacral (E), and sacral (F) levels, tumor is found in a diffuse subarachnoid location surrounding the spinal cord (arrows). G, high-power micrograph of tumor cells in sacral cord; note mitotic figure (arrow). All sections were stained with hematoxylin and eosin-Luxol fast blue. A and B, ×11.5; C–F, ×25; G, ×400.
animals had any histological abnormalities other than one animal, which had arachnoid fibrosis. Of the animals that died, one had evidence of hemorrhage, necrosis, and peripheral demyelination. Two other animals had peripheral demyelination. None of the animals that survived the experimental period of 191 days had any evidence of histological abnormalities. In the group of animals treated with three doses of 200 µg LMB-1, four of the animals that died showed no evidence of tumor. Two of these animals did have signs of spinal edema, while the other two animals had evidence of hemorrhage, necrosis, and spinal edema. The failure to find tumor may be due to sampling error. Of the animals that died with evidence of tumor, one had evidence of hemorrhage, necrosis, and spinal edema; one had spinal edema alone; and one had peripheral demyelination and spinal edema. None of the animals that survived for the duration of the experiment had any histological abnormalities. In the saline control group, all animals had evidence of tumor. Other histological findings included one animal with spinal edema, one with hemorrhage, and one with hemorrhage and necrosis.

**Treatment of the Antigen-negative Cell Line.** A neoplastic meningitis model established with the antigen-negative A431 cell line was treated on day 8 with 40 µg each of LMB-1 (n = 9), unconjugated MAb B3 (n = 10), and nonreactive immunotoxin conjugate MOPC-LysPE38 (n = 10). The survival of these groups was compared with a saline control group (n = 10). The median survival for each of the groups was LMB-1, 14.5 days (P = 0.223); unconjugated B3, 31.0 days (P = 0.122); MOPC-LysPE38, 24.0 days (P = 0.269); and saline, 17.0 days. There was no significant difference in median survival among any of the groups. On first glance, the nearly 80% increase in median survival of the MOPC-LysPE38-treated group would appear to be significant. Because of a large variability in the survival curve estimates, however, this seemingly large increase in median survival is not statistically significant. No clinical evidence of toxicity in any of the groups was seen. Of the LMB-1-treated animals receiving 40 µg, all animals that died had evidence of tumor. Other histological findings in this group included one animal with spinal edema and arachnoiditis and three animals with hemorrhage. Of the group of animals treated with unconjugated B3, all animals that died had evidence of tumor (8/10). Two animals had evidence of hemorrhage and necrosis, two had only evidence of hemorrhage, one had hemorrhage of spinal edema, and one had only edema. The two animals that lived had no histological abnormalities. In the group treated with MOPC-LysPE38, one of nine animals that died did not show any signs of tumor and had no other histological findings. Of the animals that died with evidence of tumor, one had evidence of spinal edema only, two had hemorrhage only, one had necrosis only, and two had evidence of hemorrhage and necrosis. The one animal that survived the study period showed only arachnoid fibrosis. In the saline control group, all animals that died had evidence of tumor (8/10), 1 had only hemorrhage, 1 only necrosis, 1 necrosis and spinal edema, and 1 hemorrhage and spinal edema. No histological abnormalities were found in the two animals that survived.

To complete the therapeutic specificity studies, we used cell line D341 Med, which is negative for B3 reactivity by FACS analysis, as a therapeutic target. A group of nine animals was treated with 40 µg LMB-1 on days 8, 11, and 13 after tumor inoculation, and they were compared with a group of eight animals receiving saline only. The median survival for both groups was 20 days, indicating no therapeutic effect of LMB-1.

**LMB-1 Toxicity in Nontumor-bearing Rats.** To test the neurotoxicity of LMB-1, we gave the immunotoxin to nontumor-bearing rats in the following dosages: a single dose of 0.472 (n = 10), 4.72 (n = 9), 47.2 (n = 10), or 200 µg (n = 9); 3 doses of 40 (n = 10) or 200 µg (n = 8); or saline (n = 10). Two single-dose saline and one 0.472-µg animal showed transient loss of ramp climbing ability. Once neurological function returned, these three animals thrived until termination at 77 days. One animal from each of the LMB-1 4.72-µg, 42.7-µg, and saline groups died. None of the animals that died had any loss of neurological function at any time during the course of the experiment. Both animals given LMB-1 immunotoxin had an infection at the site of the catheter placement and most likely died from a secondary infection. There was no histological evidence in the neuraxis of peripheral demyelination, hemorrhage, necrosis, or edema in the animals that showed a transient loss of neurological function and the animals that died. None of the animals that were terminated at the end of the experimental period showed any histological evidence of toxicity in the neuraxis or the liver. Animals treated with a single dose of 200 µg or 3 doses of 40 µg LMB-1 showed no symptoms of toxicity. All animals gained weight as did the saline controls. In the group treated with 3 doses of 200 µg LMB-1, two of eight died, and the four of six that remained were neurologically impaired.

Histological examination of the neuraxis of the animals treated with 3 doses of 40 µg showed, as the only findings, two of nine animals with mild hydrocephalus. In the group of animals treated with a single dose of 200 µg LMB-1, three of nine had mild pericatheter demyelination and four of nine had mild nerve root demyelination. In the animals treated with saline, 2 of 10 had mild hydrocephalus, 3 of 10 had pericatheter demyelination, and 1 of 10 had pericatheter demyelination. None of the animals treated with a single dose of 200 µg, 3 doses of 40 µg LMB-1, or saline had any loss of neurological function through-
Fig. 4  Histological sections taken from an animal that was treated with three doses of 200 μg LMB-1 and survived the experimental period of 191 days. The sections were taken from approximately the same levels as in Fig. 2. There was no evidence of residual tumor. With the exception of deformation and reaction associated with the catheter, no histopathological abnormalities were noted in these asymptomatic animals. All sections were stained with hematoxylin and eosin-Luxol fast blue. A and B, × 11.5; C–F, ×25.

out the experimental period. In the group of animals treated with 3 doses of 200 μg LMB-1, the one animal that had no neurological symptoms had no histological evidence of pathology. The two animals that died had evidence of spinal cord demyelination and edema, and the five animals that survived but had a loss of neurological function also had spinal cord demyelination. The focal pericatheter demyelination seen in the groups treated with a single dose of 200 μg LMB-1 or saline was histologically different from the demyelination observed in the group treated with 3 doses of 200 μg LMB-1. The pericatheter
demyelination was mild, focal, and characterized by myelin-laden macrophages clustered only around the catheter. The demyelination found in the animals treated with two doses of 200 μg LMB-1 was associated with demyelination of the long fiber tracts distal to the catheter tip. The pericatheter demyelination does not appear to be associated with any toxic effects of the LMB-1 immunotoxin since a similar number of saline-treated, animals. Tumor was found in 96% of all of the 174 sections taken from the saline control rats. This suggests that this pan-

dimensional fluid pathway, de novo or acquired drug resistance, and inability of available systemic chemotherapeutic agents or MAbs to cross the blood-brain barrier, resulting in a subtherapeutic level of drug in the subarachnoid space. To overcome the lack of chemotherapeutic penetration into the subarachnoid space, investigators have used direct infusion into the i.t. space either through the lumbar cistern or an indwelling ventricular reservoir. At present, only methotrexate, cytarabine, thiopeta, 4-hydroperoxycycbophosphamide, and melphan have been used for i.t. treatment (5–7, 23). Most of these agents have a limited activity against the tumors that most frequently cause neoplastic meningitis or, as seen with methotrexate, can cause a leukoencephalopathy that can be clinically devastating (24).

An approach that takes advantage of the specificity of MAbs to target cancer cells is conjugating them with chemotherapy drugs or toxins. MAb toxin conjugates have several advantages as compared with MAB drug conjugates. First, immunotoxins are not affected by tumor cell hypoxia, a major factor that may be responsible for some chemotherapeutic and radiotherapeutic resistance. Second, immunotoxins are extremely potent and may require that only one toxin molecule reach the cytosol to kill a single tumor cell. Most chemotherapeutic drugs often require >10^4–10^5 molecules/cell to cause cell death (25).

The model used to study the therapeutic effects of LMB-1 has several salient features. First, it mimics disseminated i.t. neoplastic growth. Animals killed at the initiation of treatment showed disseminated tumor from the base of the brain to the tip of the cauda equina (Fig. 2). The even distribution of the tumor is further illustrated by looking at the untreated saline control animals. Tumor was found in 96% of all of the 174 sections taken from the saline control rats. This suggests that this particular model grows tumor evenly throughout the neuraxis. If

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⁵ From Pastan et al. (5).
⁶ B3 reacts with cells of the anterior pituitary that are cuboidal and have clear cytoplasm and round regular nuclei.
⁷ ND, not determined.

**DISCUSSION**

The tumor reactivity spectrum of MAb B3, which forms the immunotoxin LMB-1, correlates well with the types of solid tumors that frequently cause disseminated neoplastic meningitis. Clinically, the incidence of neoplastic meningitis is on the rise. A review of several clinical studies reveals that the most common solid tumors to disseminate to the leptomeninges are breast, lung, and gastrointestinal cancers, which occur with an incidence ranging from 4 to 15% (3, 22). By immunohistochemistry, the tumor tissue reactivity of MAb B3 is 75% of gastric, 66% of breast, 55% of ovarian, and 30% of lung carcinomas. The increased findings of clinical leptomeningeal tumor spread are attributed to three factors: increased success in the treatment of primary tumors resulting in longer patient survival; failure of systemic chemotherapy to reach the subarachnoid space; and increased awareness of the problem of leptomeningial relapse in patients with solid tumors.

Some of the many problems associated with the treatment of neoplastic meningitis are compartmentalization of the cerebrospinal fluid pathway, de novo or acquired drug resistance, and inability of available systemic chemotherapeutic agents or MAbs to cross the blood-brain barrier, resulting in a subtherapeutic level of drug in the subarachnoid space. To overcome the lack of chemotherapeutic penetration into the subarachnoid space, investigators have used direct infusion into the i.t. space either through the lumbar cistern or an indwelling ventricular reservoir. At present, only methotrexate, cytarabine, thiopeta, 4-hydroperoxycycbophosphamide, and melphan have been used for i.t. treatment (5–7, 23). Most of these agents have a limited activity against the tumors that most frequently cause neoplastic meningitis or, as seen with methotrexate, can cause a leukoencephalopathy that can be clinically devastating (24).

An approach that takes advantage of the specificity of MAbs to target cancer cells is conjugating them with chemotherapy drugs or toxins. MAb toxin conjugates have several advantages as compared with MAB drug conjugates. First, immunotoxins are not affected by tumor cell hypoxia, a major factor that may be responsible for some chemotherapeutic and radiotherapeutic resistance. Second, immunotoxins are extremely potent and may require that only one toxin molecule reach the cytosol to kill a single tumor cell. Most chemotherapeutic drugs often require >10^4–10^5 molecules/cell to cause cell death (25).

The model used to study the therapeutic effects of LMB-1 has several salient features. First, it mimics disseminated i.t. neoplastic growth. Animals killed at the initiation of treatment showed disseminated tumor from the base of the brain to the tip of the cauda equina (Fig. 2). The even distribution of the tumor is further illustrated by looking at the untreated saline control animals. Tumor was found in 96% of all of the 174 sections taken from the saline control rats. This suggests that this particular model grows tumor evenly throughout the neuraxis. If
uneven distribution of tumor was occurring, more animals would evidence tumor in only one or two sections. The even growth and distribution of tumor was investigated by Fuchs et al. (17) when they found that a 40-μl volume gave better tumor dispersal than a 20-μl volume. The tumor was confined to the neauraxis and did not initiate a systemic disease. Second, an indwelling catheter allows for reliable multiple-dose treatment and an examination of the best possible dosing regimen. Finally, the rat has a subset of normal tissues that express the Le' antigen, which allows an examination of both therapeutic effectiveness and normal organ toxicity. Immunohistochemistry of normal rat tissues show that MAb B3 reacts with stomach, salivary gland, lung, and pituitary. Although this is only a subset of the B3-positive normal human tissues, it can still provide a useful insight into possible normal tissue cross-reactivity. Many of the in vivo models used to study the treatment of human cancers with MAbs lack this type of normal tissue cross-reactivity.

We investigated the effect of dosage on survival. The therapeutic efficacy of the LMB-1 immunotoxin was enhanced by giving multiple doses. Single doses of 40 μg and 200 μg LMB-1 were both effective in increasing median survival compared with a saline-treated control, 78% and 178%, respectively, whereas single doses of unconjugated MAb B3 (40 μg) and nonspecific MOPC-LysPE38 (40 μg) had no effect on survival. Multiple-dose regimenz not only showed a further increase in median survival, but, more importantly, produced a 6-month disease-free survival for up to 20% of the animals when three doses of 40 μg were given. Friedman et al. (26), using a similar model of neoplastic meningitis with the human rhabdomyosarcoma TE-671, compared equivalent amounts of melphalan given as a single dose and in a multiple-dose regimen. The multiple-dose regimen was statistically better than a single equivalent injection (P = 0.004). These data suggest that multiple dosing is an important factor in determining long-term survival and should be taken into consideration when Phase I clinical trials are designed.

The specificity of LMB-1 was demonstrated by three experiments. In two separate experiments, a Le' antigen-negative neoplastic meningitis was established, and LMB-1 showed no survival effect compared with saline controls. In a second experiment, the antigen-positive neoplastic meningitis was treated with a nonspecific immunotoxin MOPC-LysPE38, and again no therapeutic effect was observed.

Because a number of the rats treated with three doses of 200 μg died without evidence of tumor, LMB-1 toxicity was investigated in nontumor-bearing rats. No toxicity was observed in rats given a single dose of 200 μg or three doses of 40 μg LMB-1. Significant toxicity was seen, however, in those animals treated with three doses of 200 μg LMB-1. This may account for the failure to find tumor in some of the animals and may explain the slightly lower median survival of rats treated with three doses of 200 μg compared with those treated with three doses of 40 μg LMB-1. In normal rat tissues that are Le' antigen-positive (stomach, salivary gland, and lung), no gross toxicity was observed from animals treated at all dosage regimens. Histopathological analysis of the pituitary glands revealed no difference among the animals treated with LMB-1 and the saline controls with regard to pituitary parenchymal hemorrhage and necrosis. There was no evidence in any animals of toxicity in the liver or gastric mucosa, a site of Pseudomonas exotoxin-associated toxicity seen in mice and monkeys, respectively, when treated with systemic LMB-1 (27). Examination of the central nervous system of the long-term survivors found no tumor or histological features indicative of neurotoxicity such as hemorrhage, necrosis, peripheral demyelination, or edema (Fig. 4). In a review of immunotoxin treatment of solid tumors, Hertler and Frankel (28) found a common systemic toxicity associated with treatment: a vascular leak syndrome that included hypoalbuminemia, edema, and weight gain. In our study, there was no clinical evidence of any vascular leak syndrome, and the surviving animals did gain weight within the limits of a growing rat.

The doses of LMB-1 that were given i.t. to the rats exceeded the LD50 (treated animals) as determined in the mouse and monkey (27). The toxicity studies of monkeys used systemic administration of the immunotoxin, while our studies used compartmental administration to better target the therapy. Pai et al. (16) determined that the in vivo half-life of LMB-1 was 4 h. The cerebrospinal volume of a rat is estimated to be 400 μl, and the bulk flow is calculated to be 2.0 μl/min (29). Using these calculations, the turnover of cerebrospinal fluid would be 3–4 h. Muraszko et al. (30) estimates that, for the immunotoxin, 454A12-rRA, a MAb to the transferrin receptor coupled to recombinant ricin A chain toxin, the disappearance of immunotoxin in the cerebrospinal fluid is a combination of bulk flow and loss to brain tissue. The time for clearance of the immunotoxin LMB-1 from the cerebrospinal fluid by bulk flow and diffusion into the brain, coupled with the short half-life of LMB-1, may limit the systemic exposure and explain why the unusually large dose of immunotoxin is tolerated when given i.t.

Three other reports of immunotoxin treatment of neoplastic meningitis in experimental animals need to be considered. Zoviczian and Youle (8) treated the guinea pig B-cell lymphoma L2C with the anti-idiotype MAb M6 conjugated to intact ricin. These authors report an increase of 5 days, or 33% increase in the median survival of animals treated with the specific immunotoxin as compared with animals treated with nonspecific immunotoxin. The survival of the immunotoxin-treated animals is clouded due to the fact that L2C tumor also causes a systemic disease from which the animals die. Urch et al. (11) modified the model by controlling the systemic disease with systemic cyclophosphamide treatment. The immunotoxin used in these experiments was the anti-idiotype MAb anti-Id-1 conjugated to the toxin saporin. The immunotoxin-treated group in this study survived for 100 days with no sign of tumor, whereas the controls had a median survival of only 28 days.

Myklebust et al. (10) established a model of neoplastic meningitis by injecting small cell lung carcinoma cells into the cisterna magna of the rat. When this model was treated with Pseudomonas exotoxin A conjugated to the anticarcinoma antibodies MOC-31 and Nrlu10, the symptom-free latency was increased by 35–46%. Transferrin conjugated with Pseudomonas exotoxin A had no effect in vivo.

The LMB-1 immunotoxin substantially improved median survival up to 326% in the multiple-dose immunotoxin-treated groups, and produced up to 20% tumor-free survival for 191 days in three different experiments compared with saline-treated
animals. Our 20% cure rate may have been due to the large tumor burden at the start of therapy. In the study by Urch et al. (11), therapy was initiated 1 day following tumor inoculation, whereas our therapy was not initiated until the third day following tumor inoculation. A comparison of the median survival rates of the controls for the two models showed our model to have approximately one third the median survival of the L2C lymphoma when treatment was initiated 2 days later. This suggests that our model may have a greater tumor burden and is a closer approximation of the human condition with neoplastic meningitis. The models of LOX-treated melanoma and small cell lung carcinoma described by Myklebust et al. (10) exhibited no toxicity to these tissues. The present study indicates that the immunotoxin LMB-1 deserves serious attention for the treatment of carcinomatous meningitis in patients.

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Efficacy of compartmental administration of immunotoxin LMB-1 (B3-LysPE38) in a rat model of carcinomatous meningitis.


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