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Intrathecal administration of single-chain immunotoxin, LMB-7 [B3(Fv)-PE38], produces cures of carcinomatous meningitis in a rat model

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ABSTRACT **LMB-7 [B3(Fv)-PE38] is a single-chain immunotoxin constructed from the murine monoclonal antibody B3 and a truncated form of *Pseudomonas* exotoxin PE38. Antibody B3 recognizes a carbohydrate epitope found on solid tumors that frequently invade the intrathecal space and cause neoplastic meningitis. We tested the therapeutic value of intrathecally administered LMB-7 by using a model of human neoplastic meningitis in athymic rats. This model is representative of a clinical situation in that antibody B3 cross-reacts with a number of normal tissues that can be used to monitor potential systemic toxicity. Treatment was begun 3 days after A431 tumor implantation. Without treatment, the animals median survival was 10 days. Intrathecal administration of 10 μg of LMB-7 in 40 μl on days 3, 5, and 7 produced 4 of 10 and 8 of 10 long-term survivors (>170 days) in two experiments. Of the long-term survivors, 2 of 4 and 7 of 8 survivors had no microscopic evidence of tumor and were considered histologic cures. Lack of significant toxicity in the effective dose range and specificity make LMB-7 an excellent candidate for intrathecal treatment of neoplastic meningitis in humans.**

At one time neoplastic meningitis was thought to be a rare complication, but with improvements in systemic cancer treatment and an increased awareness of intrathecal complications, an increase in the number of cases of neoplastic meningitis has been seen. The intrathecal compartment provides a reservoir for tumor growth, most likely due to a failure of systematically administered chemotherapeutic agents to reach a therapeutic level in the cerebrospinal fluid (1). To overcome systemic delivery limitations, direct infusion into the intrathecal space has been used.

Monoclonal antibodies (mAbs) conjugated to specific radionuclides, drugs, and toxins are being actively investigated as therapeutic agents in the compartmental treatment of neoplastic meningitis. Immunotoxins are particularly appealing because they are not affected by tumor cell hypoxia as are some radiolabeled mAbs, and they are more efficient than mAb–drug conjugates (2). We have observed significant increases in survival by using a model of human neoplastic meningitis in athymic rats, in animals treated with LMB-1, an immunotoxin constructed with the intact IgG of mAb B3 and a truncated form of *Pseudomonas* exotoxin (D.D.B., G.E.A., R.E.M., H.S.F., H.E.F., L.H.P., J.H., and I.H.P., unpublished data). mAb B3 was chosen for this study because it reacts with many types of solid tumors including carcinomas of the colon, breast, lung, ovary, bladder, and stomach (3). Recombinant techniques have made it possible to isolate the antigen-binding variable regions of the light and heavy chains from a mAb and connect them with a flexible linker to form a single-chain antigen binding protein, termed sFv.

For this study, we have used a recombinant immunotoxin in which the sFv of mAb B3 is fused to PE38, an altered form of *Pseudomonas* exotoxin (4), to give LMB-7 or [B3(Fv)-PE38]. Previous studies have shown (5) that LMB-7 administered i.v. produced complete regression of large subcutaneous tumors arising from A431 epidermoid carcinoma cells and MCF-7 breast carcinoma cells. By using a human neoplastic meningitis model, we have now tested the therapeutic efficacy of this single-chain immunotoxin LMB-7. The nude rat is an excellent experimental model for this purpose because human cancer cells grow and produce meningitis and because some normal rat tissues such as stomach, lung, and pars intermedia of the pituitary express the Ley antigen, partially mimicking normal antigen distribution in humans. We report here that LMB-7 given intrathecally 3, 5, and 7 days after tumor implantation increases median survival from 10 days to >190 days, at which point the experiment was terminated.

MATERIALS AND METHODS

Animal Model. Subarachnoid catheters were implanted in athymic rats by using the method of Fuchs *et al.* (6). Neoplastic meningitis was initiated by injection of 5×10^6 tumor cells in phosphate-buffered saline through an indwelling subarachnoid catheter. The target cell line for these experiments is the human epidermoid carcinoma line A431, which homogenously expresses antigens with which mAb B3 reacts.

Treatment Studies. LMB-7 was prepared as described (7) at a final concentration of 0.25 mg/ml. In the initial activity studies, groups of 10 animals were treated with a dose (in 40 μl) of 10 μg of LMB-7 or an equal volume of saline on days 3, 5, and 7 after tumor inoculation. For the dose–response study, groups of 10 animals were treated with a dose (in 40 μl) of 2.5 μg, 5.0 μg, or 10 μg of LMB-7, saline, or the control sFv immunotoxin anti-Tac(Fv)-PE38 on days 3, 5, and 7 after tumor inoculation. At the time of treatment initiation, five animals were killed and their neuraxes were processed for histology to approximate the tumor burden at the time of treatment. The animals were followed with daily weight and neurologic checks until death. At the time of death, each animal was given a complete autopsy. The neuraxis and liver were processed for histology, along with any organs with gross pathology.

Toxicity Studies. Nontumor-bearing rats were given an intrathecal dose of LMB-7 (10 μg in 40 μl) every other day for a total of three doses, a dose schedule identical to that used in the treatment studies. The animals were followed for 50 days with daily weight and neurologic function tests.

Histology. The neuraxis was processed as described (6). Sections were taken from the brain at the level of the coronal

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Abbreviation: mAb, monoclonal antibody.

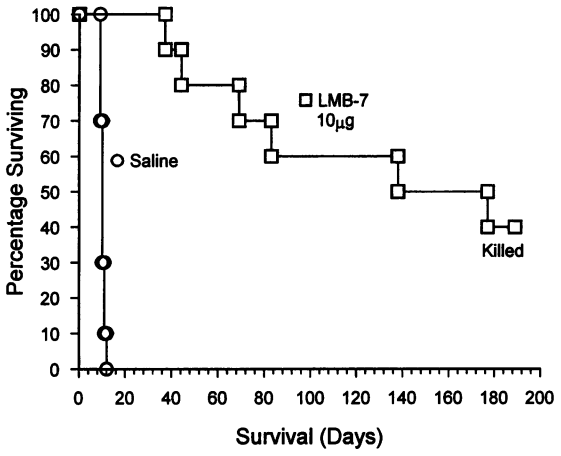

FIG. 1. Treatment of neoplastic meningitis from the human epidermoid carcinoma A431 in rats. The animals were treated with a dose (in 40 μl) of 10 μg of LMB-7 on days 3, 5, and 7 after tumor inoculation. LMB-7 increased median survival 1490% versus saline ($P < 0.001$).

suture and the pituitary gland and from the spinal cord at the cervical, thoracic, and lumbar levels and the cauda equina and stained with Luxol fast blue and with hematoxylin/eosin.

Statistical Methods. Survival cures were estimated for each treatment group by using the product-limit estimator of Kaplan and Meier (8). Comparison of these curves were conducted by using the log rank test (9).

RESULTS

To test the efficacy of LMB-7 on neoplastic meningitis, 5×10^6 A431 cells were introduced into each animal by using a preimplanted subarachnoid catheter. A431 cells were chosen for this study because they are derived from an epidermoid carcinoma that expresses large amounts of the B3 antigen. In cell culture, LMB-7 is very cytotoxic to A431 cells with an IC_{50} of 0.3–0.8 ng/ml. Furthermore, LMB-7 produces complete regressions of A431 cells growing as subcutaneous tumors in mice when three doses are administered intravenously, each at 0.063 mg/kg (1.25 μg per mouse) (6).

For the initial study, 10 μg of LMB-7 in 40 μl was administered to nude rats 3, 5, and 7 days after tumor implantation. The 10-μg dose was chosen because it produced no toxicity as manifested by death or loss of antigravity strength when administered to nontumor bearing rats; these animals were followed for 50 days before being sacrificed. The results of the first experiment in which LMB-7 (10 μg) or saline (diluent) were given to tumor bearing rats ($n = 10$) are shown in Fig. 1. All 10 rats that received saline on days 3, 5, and 7 died between days 7 and 12 (median, day 10), with a loss of antigravity strength preceding death (median, day 8.5). In contrast, the 10 rats treated with 10 μg of LMB-7 showed a dramatic response with the median survival extended to day 159. Furthermore, 4 of 10 rats survived to the end of the experiment at day 190. Autopsies showed that 2 of these long-term survivors were tumor-free, whereas 2 others had microscopic evidence of tumor in the cerebellum.

Next, a dose–response study was carried out by administering three doses of 10, 5, and 2.5 μg given 3, 5, and 7 days after tumor implantation (Fig. 2). Ten micrograms was the maximum dose that could be given since the protein concentration of LMB-7 was 0.25 mg/ml and a maximum volume of 40 μl could be administered. The control animals received either saline (the diluent for LMB-7) or anti-Tac(Fv)-PE38, a recombinant immunotoxin directed at the p55 subunit of the human interleukin 2 receptor, which is not present on A431 cells (10). As shown in Fig. 2, all three doses of LMB-7 produced significant antitumor effects. In this experiment, 10 μg produced an even more dramatic effect than in the first

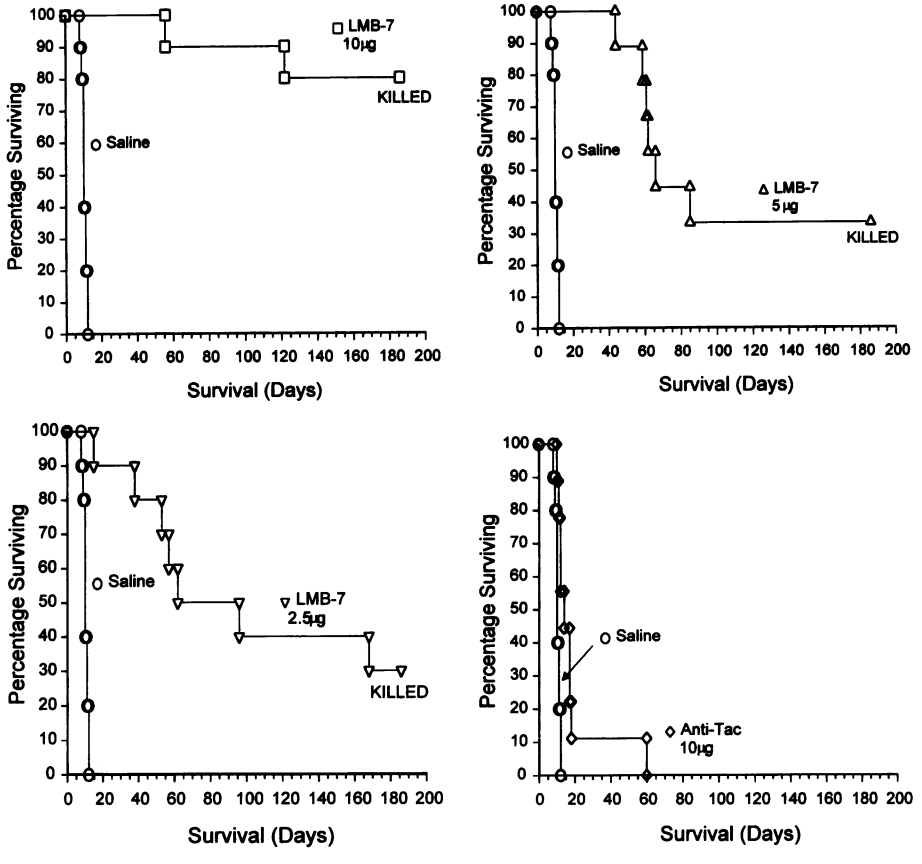

FIG. 2. Dose–response treatment of neoplastic meningitis from the human epidermoid carcinoma A431 in rats. The animals were treated with a dose (in 40 μl) of 10 μg (*Upper Left*), 5.0 μg (*Upper Right*), or 2.5 μg (*Lower Left*) of LMB-7 or 10 μg of anti-Tac(Fv)-PE38 (*Lower Right*) on days 3, 5, and 7 after tumor inoculation. Median survival was increased a minimum of 1807% (10 μg), 560% (5 μg), and 690% (2.5 μg) for the three LMB-7 dosage groups ($P < 0.001$ for all groups). Anti-Tac(Fv)-PE38 increased survival 40% compared with saline ($P = 0.032$).

experiment with 8 of 10 rats surviving until day 186, when the experiment was terminated. Only two animals died before day 186, one on day 56 and the other on day 122, and their deaths were preceded by loss of antigravity strength on days 39 and 77. By using the day the animals were sacrificed as the median survival, an estimate of the lower limit of median survival is 1807%. In the group treated with 5 μg of LMB-7, one animal died from an anesthesia death while receiving the third dose of immunotoxin. Six animals died between days 44 and 85 (median, 66 days), whereas the three other animals survived until the experiment was terminated. In the group treated with 2.5 μg of LMB-7 ($n = 10$), animals died between days 15 and 168 with three long-term survivors (median, 79 days). All the saline-treated animals were dead by day 12 (median survival, 10 days). In addition, a group of animals was treated with anti-Tac(Fv)-PE38, an immunotoxin directed at the p55 subunit of the interleukin 2 receptor (10). Anti-Tac(Fv)-PE38 is identical to LMB-7 except that the Fv portion binds to the p55 subunit of the interleukin 2 receptor, which is not expressed on A431 cells. When tested against cultured cells, anti-Tac(Fv)-PE38 is 500-fold less toxic to A431 cells than LMB-7 [B3(Fv)-PE38]. In the group treated with anti-Tac(Fv)-PE38, one animal died of anesthesia while receiving the second dose of immunotoxin ($n = 9$). The median survival of the other animals was 14 days with no long-term survivors.

When the median survival of the LMB-7-treated groups was compared with the saline controls, the percentage of increase was 1807% for 10 μg, 560% for 5.0 μg, and 690% for 2.5 μg. For the group treated with three 10-μg doses of LMB-7, the end of the experiment (day 186) was taken as the median day of survival. By the nonparametric log rank test, the survival of all LMB-7 treatment groups was statistically significant compared with saline controls ($P < 0.001$). The difference between the group treated with 10 μg of LMB-7 and the groups treated with 5 μg and 2.5 μg of LMB-7 was statistically significant ($P = 0.039$ and $P = 0.022$, respectively). No statistical significance was found in the difference between the groups treated with 5 μg and 2.5 μg of LMB-7.

Histologic analysis was performed on the neuraxes of all animals that died during an experiment. In almost all cases, a tumor was detected and was frequently accompanied by peripheral demyelination, edema, and hemorrhage. In a few animals, tumor was not detected but since all portions of the neuraxis were not sectioned we assume tumor was present in those locations. In most of the animals surviving to the end of the experiment (190 or 186 days), a tumor was not detected, but a tumor was present in a few of these animals. When given intravenously, the LD_{50} of LMB-7 is 7 μg per mouse (0.35 mg/kg) for three doses, and death is due to liver toxicity (L.H.P., unpublished data). No liver-related deaths were observed in mice given 10 μg per mouse for three doses by the intrathecal route. This is presumably because LMB-7 leaves the intrathecal space slowly and does not achieve high levels in the blood.

DISCUSSION

In this study, we have shown that neoplastic meningitis in athymic rats can be successfully treated with a recombinant single-chain immunotoxin (LMB-7) directed at an antigen present on many human cancers that metastasize to the brain but not present in normal human neural tissues. To carry out these studies, human epidermoid carcinoma cells were introduced into the intrathecal space and allowed to grow for 3 days before therapy was initiated. Untreated animals had a median survival of 10 days whereas in 8 of 10 animals in one experiment and 4 of 10 animals in another experiment that were treated with three doses of LMB-7 survived >180 days, when the experiment was terminated. The effect of LMB-7 [B3(Fv)-PE38] was specific since anti-Tac(Fv)-PE38, a recombinant immunotoxin directed at an antigen not present on the tumor, gave a very small increase in survival (median, 14 days).

The theoretical potential of specific immunotherapy with mAbs has been tempered by practical limitations. First, only a few tumor-specific surface antigens have been identified that can be used as specific tumor targets; one such antigen is the in-frame mutation of the epidermal growth factor receptor (11). A second obstacle to mAb therapy is poor delivery to the tumor. Poor delivery can be attributed to physiological barriers that exist within tumors: heterogeneous blood supply, elevated interstitial pressure, and large transport distances in the interstitium (12, 13). Numerous attempts have been made to alter the physiologic parameters of tumors to increase the delivery of macromolecules such as mAbs. Hyperthermia, radiation, and vasoactive drugs have been investigated as potential means of increasing blood flow (12, 14). To counteract the elevated interstitial pressure of tumors, osmotic agents that increase the vascular osmotic pressure have been studied (15). Another approach has been to increase the interstitial transport rate of molecules. This can be accomplished by using mAb fragments such as $F(ab')_2$, Fab, or small single-chain antigen-binding proteins that are produced by linking the variable regions of the light and heavy chains with a flexible linker (16, 17) or by a disulfide bond (18). A further way to approach the problems of the physiologic barriers encountered in tumors replaces commonly used systemic administrations with regional or compartmental delivery. Compartmental therapies with the mAbs that have been investigated include intraperitoneal injection, direct injection into cyst cavities within tumors, and intrathecal injection for the treatment of neoplastic meningitis (19, 20). Instilling mAbs directly into the intrathecal space can limit systemic exposure of noncentral nervous system tissues that share antigens with tumors targeted by the mAbs. This method can also eliminate the problem of physiologic barriers, leaving only rate of mAb diffusion as the major limiting factor in delivery of the therapeutic agent.

In this study, we investigated the therapeutic efficacy of the single-chain immunotoxin LMB-7 [B3(Fv)-PE38] in the treatment of human neoplastic meningitis in athymic rats. This model is directly applicable to the human because humans and rats share a subset of B3-positive normal tissues. The positive rat tissues include stomach, salivary glands, lungs, and pars intermedia of the pituitary (D.D.B. *et al.*, unpublished data). We have studied the treatment of neoplastic meningitis in athymic rats with LMB-1, a high molecular weight conventional immunotoxin. LMB-1 is composed of mAb B3 chemically linked to the PE38-engineered form of *Pseudomonas* exotoxin (D.D.B. *et al.*, unpublished data). In this model, three 200-μg doses of LMB-1 (same dose regimen as this study) produced an increase in median survival of 274% compared with saline controls. On a molar basis, the dose of LMB-1 (1.05 nmol) is approximately seven times that of LMB-7 (0.15 nmol), yet LMB-7 was much more effective. Several explanations are possible for the greater efficacy of the LMB-7 immunotoxin. One difference between LMB-1 and LMB-7 is their molecular size. LMB-1 is composed of an intact IgG chemically attached to a genetically engineered form of *Pseudomonas* toxin and has a molecular mass of ≈190 kDa. In contrast, LMB-7 is composed of only the heavy and light chain variable regions of mAb B3 fused to the same toxin and has a molecular mass of only 65 kDa. In tissue culture studies using the A431 cell line, both LMB-1 and LMB-7 have approximately equal activities (IC_{50} ≈10 pM). The primary reason for the greater efficacy of LMB-7 in the animal studies is most likely due to its smaller size relative to LMB-1. By using compartmental administration, we essentially bypass the limiting distribution factors associated with systemic administration. If no bulky disease exists at the time of treatment and, therefore, uniform flow of cerebrospinal fluid is present throughout the intrathecal

space, the limiting factor of therapy in these two models is diffusion. The formula for the amount of time required for molecular diffusion into tissue is $l^2/4D$, where l is distance of diffusion and D is diffusion coefficient, which is dependent on molecular size and shape (13). By using this equation, IgG would take ≈60 min to reach a distance of 100 μm, while $F(ab')_2$ (12) would take only 21 min to reach this distance. The size of the LMB-7 immunotoxin is on the order of that of $F(ab')_2$ fragments. In contrast, LMB-1 is considerably larger and more asymmetric than an IgG. Smaller molecules penetrate tumor tissue much faster than do larger ones and are, therefore, able to penetrate deeper into the multicellular layers of tumor.

The treatment of neoplastic meningitis can roughly be compared to the incubation of tumor spheroids bathed in solutions of mAbs. Sutherland *et al.* (21) studied the penetration of two different mAbs to carcinoembryonic antigen and the penetration of their $F(ab')_2$ and Fab fragments into tumor spheroids. Their studies showed that, for both of the intact anti-carcinoembryonic antigen mAbs, there was heterogeneous labeling from one to three cells deep after a 4-h incubation. Both $F(ab')_2$ and Fab fragments penetrated deeper than intact mAb, with Fab fragments penetrating deeper than $F(ab')_2$. Yokota *et al.* (22) studied the distribution of four immunoglobulin forms of the second generation mAb CC49, which reacts with the TAG-72 antigen, and found that, after 6 h, the IgG had penetrated the tumor to a depth of 40 μm, the Fab had penetrated to 70 μm, and the sFv had penetrated to 100 μm.

A second factor that must be considered is the influence of affinity on penetration (23). The K_d of LMB-7 is ≈1300 nM compared with 280 nM for LMB-1 (K. Webber, L.H.P., and I.H.P., unpublished data). Demignot *et al.* (24) suggested that the higher the affinity the greater the hindrance to penetration. The lower affinity of LMB-7 would, then, contribute to a deeper penetration and presumably, a greater reduction in the tumor mass. Baxter *et al.* (25) describes a two-pore model system that takes into account the influence of specific binding of mAb localization. This is supported by Milenic *et al.* (26) in the comparison of four immunoglobulin forms of the mAb CC49. The investigators found that the affinity of the Fab' and the sFv were 8-fold lower than the two dimeric forms, while the penetration of the sFv was greater than twice that of the intact IgG.

Increased therapeutic efficacy has been observed with other sFv immunotoxins as compared with their IgG chemical conjugate parent. The immunotoxin BR96 sFv-PE40 has shown the ability to totally regress subcutaneously growing L2987 lung carcinoma xenografts, whereas the IgG chemical conjugate could only keep the tumor size static (27). BR96 sFv-PE40 was found to inhibit *in vitro* protein synthesis four times better than the chemical IgG conjugate. In this instance, the greater efficacy is most likely due to a combination of immunotoxin size and increased ability to inhibit protein synthesis.

To our knowledge, there are currently no satisfactory methods of treating neoplastic meningitis due to the metastasis of solid tumors. Experiments using 4-hydroperoxycyclophosphamide, ACNU, melphalan, and ^{211}At-labeled antibody (81C6) (28–30) have produced increases in survival ranging from 41 to 111% in animals. Also, immunotoxins containing *Pseudomonas* exotoxin directed at a human small cell lung carcinoma have been evaluated and produced 35–40% increases in survival when given 1 day after tumor administration (31). These modest responses should be contrasted with the >1800% increase in survival noted in the current study using established tumors.

In summary, treatment with single-chain immunotoxin LMB-7 has resulted in long-term survival of rats with neoplastic meningitis produced by a human epidermoid carcinoma. These results suggest that this agent should be evaluated in humans with this disease.

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Note Added in Proof. Since submission of this manuscript, we have continued to evaluate the maximum tolerated dose of LMB-7 in non-tumor-bearing athymic rats. Groups of 12 non-tumor-bearing athymic rats were treated intrathecally with 40 μl of saline or saline containing 10, 15, and 20 μg of LMB-7 given every other day for a total of three doses. Animals treated with saline and 10 μg of LMB-7 showed weight gain and no loss of stepping and placing reflex or ability to climb an incline ramp. Toxicity was observed in the groups treated with 15 and 20 μg of LMB-7. Loss of stepping and placing reflex and ramp climbing ability was seen in 3 of 12 animals receiving 15 μg and 5 of 12 animals receiving 20 μg of LMB-7. In the 15-μg group, 3 of 12 animals died, and in the 20-μg group, 2 of 12 animals died. At 42 days, a predetermined group of 6 animals was killed from each dose level for investigation of acute toxicity. The remaining animals will be followed for a total of 180 days to determine chronic toxicity. Autopsy of the acute toxicity animals and of the 5 treated animals that died revealed no gross pathologic abnormalities, especially no neurotoxicity was observed. Histologic examination of the neuraxis and internal organs has not yet been done. Our data shows that a 10-μg intrathecal dose given three times every other day is the maximum tolerated dose that produces no toxicity in normal athymic rats.

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