17. Mejean, C., Pons, F., Benyamin, Y. & Roustan, C. Antigenic probes locate binding sites for the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase, aldolase and phosphofructokinase on the actin monomer in microfilaments. *Biochem. J.* **264**, 671–677 (1989).
18. Somers, M., Engelborghs, Y. & Baert, J. Analysis of the binding of glyceraldehyde-3-phosphate dehydrogenase to microtubules, the mechanism of bundle formation and the linkage effect. *Eur. J. Biochem.* **193**, 437–444 (1990).
19. Schulze, H. *et al.* Rat brain glyceraldehyde-3-phosphate dehydrogenase interacts with the recombinant cytoplasmic domain of Alzheimer's beta-amyloid precursor protein. *J. Neurochem.* **60**, 1915–1922 (1993).
20. Perutz, M.F., Johnson, T., Suzuki, M. & Finch, J.T. Glutamine repeats as polar zippers: Their possible role in inherited neurodegenerative diseases. *Proc. Natl. Acad. Sci. USA* **91**, 5355–5358 (1994).
21. Stott, K., Blackburn, J.M., Butler, P.J.G. & Perutz, M. Incorporation of glutamine repeats makes protein oligomerize: Implications for neurodegenerative diseases. *Proc. Natl. Acad. Sci. USA* **92**, 6509–6513 (1995).
22. Roses, A.D. Alzheimer's disease as a model of molecular gerontology. *J. Natl. Inst. Health Res.* **7**, 51–57 (1995).
23. Trottier, Y. *et al.* Cellular localization of the Huntington's disease protein and discrimination of the normal and mutated form. *Nature Genet.* **10**, 104–110 (1995).
24. Sharp, A.H. *et al.* Widespread expression of Huntington's disease gene (IT15) protein product. *Neuron* **14**, 1065–1074 (1995).
25. DiFiglia, M. *et al.* Huntingtin is a cytoplasmic protein associated with vesicles in human and rat brain neurons. *Neuron* **14**, 1075–1081 (1995).
26. van Tuinen, E. & Riezman, H. Immunolocalization of glyceraldehyde-3-phosphate dehydrogenase, hexokinase, and carboxypeptidase Y in yeast cells at the ultrastructural level. *J. Histochem. Cytochem.* **35**, 327–333 (1987).
27. Minaschek, G., Groschel-Stewart, U., Blum, S. & Bereiter-Hahn, J. Microcompartmentation of glycolytic enzymes in cultured cells. *Eur. J. Cell Biol.* **58**, 418–428 (1992).
28. Ronai, Z. Glycolytic enzymes as DNA binding proteins. *Int. J. Biochem.* **25**, 1073–1076 (1993).
29. Morgenegg, G. *et al.* Glyceraldehyde-3-phosphate dehydrogenase is a nonhistone protein and a possible activator of transcription in neurons. *J. Neurochem.* **47**, 54–62 (1986).
30. Knull, H.R. & Fillmore, S.J. Glycolytic enzyme levels in synaptosomes. *Comp. Biochem. Physiol.* [B] **81**, 349–351 (1985).
31. Hoogeveen, A.T. *et al.* Characterization and localization of the Huntington disease gene product. *Hum. Mol. Genet.* **2**, 2069–2073 (1993).
32. Servadio, A. *et al.* Expression analysis of the ataxin-1 protein in tissues from normal and spinocerebellar ataxia type 1 individuals. *Nature Genet.* **10**, 94–98 (1995).

Treatment of advanced solid tumors with immunotoxin LMB-1: An antibody linked to *Pseudomonas* exotoxin

LEE H. PAI[1], ROBERT WITTES[2], ANN SETSER[2], MARK C. WILLINGHAM[3] & IRA PASTAN[1]

[1]*Laboratory of Molecular Biology and* [2]*Medicine Branch, National Cancer Institute, National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892, USA*
[3]*Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, South Carolina 29425, USA*

Immunotoxin LMB-1 is composed of monoclonal antibody B3 chemically linked to PE38, a genetically engineered form of *Pseudomonas* exotoxin. B3 recognizes a carbohydrate antigen (Le^y) present on many human solid tumors[1]. LMB-1 has excellent antitumor activity in nude mice bearing Le^y-positive tumors[2]. We conducted a phase I study of 38 patients with solid tumors who failed conventional therapy and whose tumors expressed the Le^y antigen. Objective antitumor activity was observed in 5 patients, 18 had stable disease, 15 progressed. A complete remission was observed in a patient with metastatic breast cancer to supraclavicular nodes. A greater than 75% tumor reduction and resolution of all clinical symptoms lasting for more than six months was observed in a colon cancer patient with extensive retroperitoneal and cervical metastasis. Three patients (two colon, one breast cancer) had minor responses. The maximum tolerated dose of LMB-1 is 75 μg/kg given intravenously three times every other day. The major toxicity is vascular leak syndrome manifested by hypoalbuminemia, fluid retention, hypotension and, in one case, pulmonary edema. Although immunotoxins have been evaluated in clinical studies for more than two decades, this is the first report of antitumor activity in epithelial tumors.

Immunotoxins are potent cell-killing agents composed of monoclonal antibodies conjugated to protein toxins made by bacteria or plants. Ricin, saporin, *Diphtheria* toxin and *Pseudomonas* exotoxin (PE) have been widely used for this purpose[3]. Immunotoxins have been shown to be active against lymphomas[4,5], but trials targeting solid tumors have been unsuccessful and have been associated with severe toxicities induced by the lack of specificity of the antibody or by the toxin moiety[3,6]. Since epithelial tumors make up more than 80% of all adult cancers and cures are rare once they have metastasized, the development of new therapeutic approaches for solid tumors is necessary.

Our laboratory has focused on immunotoxins made with *Pseudomonas* exotoxin A (PE). It is a 66-kD protein and kills cells by adenosine 5'-diphosphate (ADP) ribosylation and inactivation of elongation factor 2, which causes the arrest of protein synthesis and cell death. PE is composed of three major structural domains each with a different function[7,8]. Domain Ia is responsible for cell recognition, domain II for translocation across the cell membrane, and domain III for the ADP-ribosylation activity of the toxin. The major toxic effect of PE and immunotoxins containing PE is liver necrosis, a process initiated by the binding of domain Ia to hepatocytes.

To decrease this nonspecific toxicity, domain Ia (amino acids 1–252) of PE was removed by recombinant DNA technology. The resulting molecule, PE40, was much less toxic to the liver[9]. Subsequent deletion of amino acids 365–384 resulted in an even smaller molecule, PE38, that also retains full ADP-ribosylation activity, and yet is 0.5% as toxic to mice as native PE (ref. 10). To facilitate chemical coupling to antibodies, a small peptide containing a lysine residue was placed at the amino end of PE38 to make LysPE38.

The monoclonal antibody B3 (IgG1κ) recognizes a carbohydrate antigen in the Le^y family that is abundant on the surface of many human solid tumors and is present on only a few normal tissues[1]. Because of its very high reactivity with cancers, monoclonal antibody B3 was used to make the immunotoxin LMB-1 (B3-LysPE38) by chemically coupling monoclonal antibody B3 to LysPE38. LMB-1 was tested on cell lines and found to be very cytotoxic to cancer cells expressing the Le^y antigen. Subsequently LMB-1 was found to produce complete regressions of human tumor xenografts growing in immunodeficient mice[2]. Because of its promising activity in preclinical models, we initiated a phase I trial of LMB-1 in cancer patients at the Medicine

Table 1 Toxicities

LMB-1 (mg/kg)	Vascular leak syndrome - grade 0	1	2	3	4	Patients (*n*)
10		4				4
15	2	1				3
20	1	2				3
25	1	3	2			6
30		3				3
45	2	1				3
60	1	2	3			6
75		3	3			6
90		1		2		3
100					1	1
Total (%)	7 (20)	19 (54)	8 (23)	2 (4)	1 (2)	38

Branch, National Cancer Institute, in July 1993.

To date, 38 patients with advanced solid tumors have been treated (16 male, 22 female) with a mean age of 47 (range 30–70). Of these, 26 patients had colorectal cancer, 8 breast cancer, 1 cancer of the esophagus, 1 cancer of the stomach, 1 ovarian cancer and 1 cancer of the ampulla of Vater. Patients received doses ranging from 10 to 100 μg/kg (10, 15, 20, 25, 30, 45, 60, 75, 90 and 100 μg/kg). Three to six patients were treated at each dose level. The starting dose was 100 μg/kg (1/30th of the dose lethal to 10% of the mice tested (LD_{10})). This was subsequently reduced to 10 μg/kg because of grade 4 toxicity observed in one patient.

The major side effect of LMB-1 was found to be vascular leak syndrome (VLS), manifested by hypoalbuminemia, fluid retention and peripheral edema. At doses higher than 75 μg/kg, transient postural hypotension and scanty urination (oliguria) that did not require fluids or pressor agents were observed in some patients. Pulmonary edema and severe hypotension occurred in one patient who received 100 μg/kg. The severity of the VLS and the number of patients treated at each dose level are shown in Table 1. Other less frequent and well-tolerated side effects include "flulike" symptoms, fever, malaise, skin rash, headache and nonspecific EKG changes. All drug-related side effects occurred during the week of therapy and resolved within two weeks. Although normal tissues such as mucosal surface of the stomach, trachea and bladder, exocrine glands of the pancreas and the colloid of the thyroid gland do express Le^y antigen[1], no drug-related side effects on these organs were observed. The maximum tolerated dose (MTD) of LMB-1 was defined as 75 μg/kg three times every other day.

Antitumor activity was observed in 5 patients, 18 patients had stable disease, 15 patients progressed. A complete remission lasting two months was observed in a 40-year-old female with metastatic breast cancer to the supraclavicular lymph nodes. This patient received two cycles of LMB-1 at 15 μg/kg. Shrinkage of supraclavicular node was observed five to seven days after the first dose of LMB-1. A greater than 75% tumor reduction was observed in a 50-year-old male with extensive metastatic colon cancer to the abdomen and supraclavicular lymph nodes. Tumor shrinkage was observed after one single dose of LMB-1 at 90 μg/kg. Because this patient did not develop antibodies, he received three additional cycles of LMB-1 at a 50% dose (45 μg/kg) (Fig. 1). The dose was reduced because of grade 3 toxicity after cycle 1. Computed tomography (CT) scan of the abdomen showed that the tumor continued to decrease in size after each cycle. This patient has been followed without evidence of disease progression for seven months. Before therapy, the patient complained of chronic diarrhea and abdominal pain, which required therapy with acetaminophen plus codeine. Symptoms resolved completely after treatment. Minor responses (<25% decrease in tumor size) were observed in three additional patients who received 10, 75 and 90 μg/kg. One colon cancer patient had shrinkage of pulmonary nodules lasting for up to nine months. Minor responses were observed in two other patients, one colon cancer patient with transient decrease of an inguinal mass lasting for less than four weeks and a breast cancer patient with adrenal metastasis had less than 50% tumor reduction lasting more than two months. In all cases, tumor shrinkage was observed within the first month after treatment. Stable patients who could not be retreated because they developed antibodies against LMB-1 were

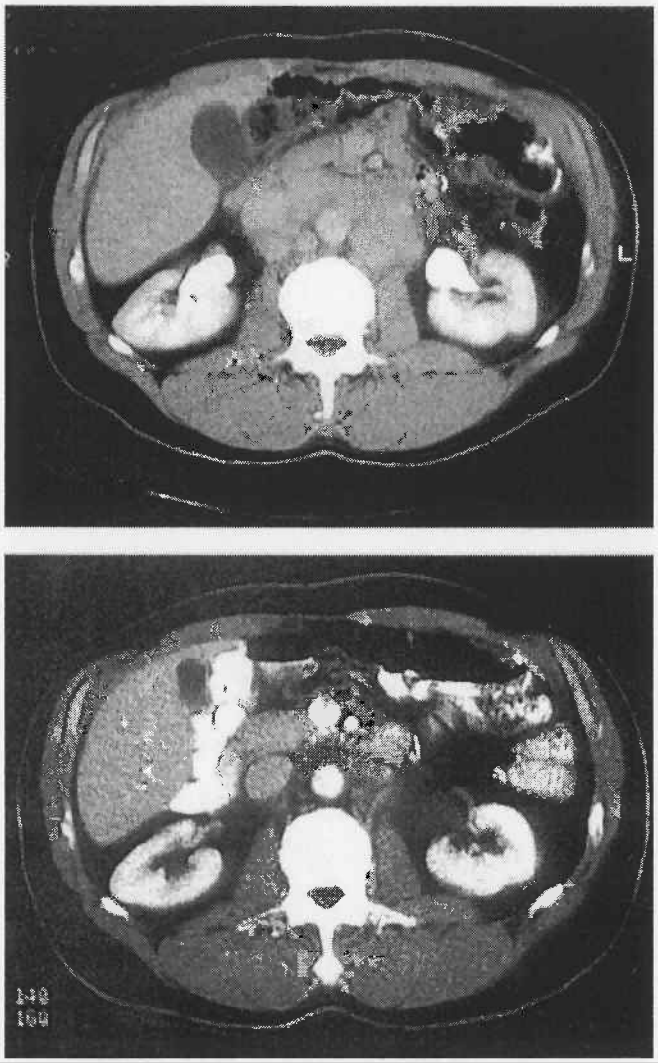

Fig. 1 Abdominal computed tomogram of colon cancer patient with abdominal metastasis. Upper scan shows extensive retroperitoneal adenopathy before therapy. Lower scan followed two cycles of LMB-1 and shows marked tumor shrinkage. This patient received two additional cycles of LMB-1 with continuous shrinkage of the tumor (>6 months).

followed until disease progression. The median time to disease progression was three months (range two to four months).

Of 38 patients, 33 (90%) developed neutralizing antibodies against LMB-1 three weeks after one cycle of treatment. Three patients received two cycles of LMB-1 (10, 15, 60 μg/kg) and one patient received four cycles (90, 45 μg/kg). Enzyme-linked immunosorbent assays (ELISA) indicated that all patients (38/38) developed antibody titers against PE38 and 33/38 had antibody titers against monoclonal antibody B3. We conclude that although all patients formed antibodies against the toxin moiety, these antibodies have no neutralizing effect against the LMB-1 in 15% of the cases. Based on the five responses observed in this trial, we plan phase 2 studies for colon, breast and other Le^y-containing malignancies.

Our data clearly show that immunotoxin LMB-1 has antitumor activity in heavily pretreated cancer patients. By using recombinant DNA technology we have selected a mutant form of PE that is less toxic to patients. Although immunotoxin therapy has been investigated for two decades, this is the first time that objective antitumor activity against metastatic colon and breast cancers has been documented. At the MTD, side effects of LMB-1 were well tolerated and transient. The major side effect, VLS, is secondary to targeting of LMB-1 to antigen-positive endothelial cells[11].

Immunotoxins are foreign proteins and highly immunogenic. Antibodies can generally be detected 7 to 14 days after the initial exposure. These antibodies neutralize the activity of the immunotoxin, precluding a multiple course therapy. Strategies presently being explored to overcome this problem are to identify and mutate highly immunogenic regions of the toxin[12] or to modify the toxin chemically with polyethylene glycol, as has been done for L-asparaginase[13,14]. Concomitant use of LMB-1 and immunosuppressive agents such as cyclophosphamide, cyclosporine, or high-dose corticosteroids needs to be explored.

It is not clear why some tumors are sensitive to LMB-1 and others are not. There are several factors that could contribute. One is that the MTD in humans (75 μg/kg three times) is less than the dose that regularly causes tumor regression in animal models[2]. If higher doses could be given to humans, more responses might be observed. Other possibilities are differences in tumor penetration due to variations in vascular permeability, differences in interstitial pressures in tumors[15], and differences among the cell surface glycoproteins to which Le^y is attached. The latter is important because LMB-1 must bind to glycoproteins that are internalized for it to kill cells. The majority of the patients treated in our study had large bulky tumors into which antibodies and immunotoxins enter slowly.

Several strategies are being pursued to increase the responses to LMB-1. One is to give more doses. A second is to treat patients with smaller tumors. A third is to produce a smaller immunotoxin that can penetrate into tumors more effectively. Genetic engineering has been used to make LMB-7 (B3(Fv)-PE38) which is a small single-chain counterpart of LMB-1 with a molecular mass of 65 kD. It is composed of the Fv portion of monoclonal antibody B3 fused to the toxin[16]. This agent is more active than LMB-1 in animal models. A phase I trial using this second generation recombinant immunotoxin is now in progress at the National Cancer Institute, National Institutes of Health.

Methods

Patients eligible for this study have a histologic diagnosis of a malignant solid tumor and have exhausted the standard therapeutic options for their disease, or have a malignant disease for which no established therapy exists. The tumors must express the B3 antigen on the surface of >30% of the cells. The patients must not have neutralizing antibodies to LMB-1, must be 18 years or older, have a Karnofsky performance status of >80%, have adequate bone marrow (absolute granulocyte count ≥2,000/mm^3, platelet count ≥100,000/mm^3), liver (normal bilirubin, aspartate aminotransferase (SGOT) and alanine aminotransferase (SGPT) <2.5 × normal), and kidney (creatinine level ≤1.5 mg/dl) function. Patients had no chemotherapy or radiation therapy within four weeks of entry on to this study (eight weeks for drugs with delayed toxicity such as nitrosoureas or mitomycin). All patients have given written informed consent in accordance with Federal and institutional guidelines.

Before therapy, a medical history, physical examination, complete blood cell (CBC) count, biochemical profile, urinalysis, electrocardiogram, chest X-ray and scans required for tumor measurement were obtained. Patients were monitored weekly with CBC, biochemical profile and serum antibodies against LMB-1 for four weeks. Toxicity was reported using the Common Toxicity Criteria, Clinical Trials Evaluation Program, National Cancer Institute. For vascular leak syndrome the following grading was used: grade 1, reduced serum albumin and fluid retention (A); grade 2, (A) + reduced blood pressure (BP) not requiring therapy and lasting for <48 h; grade 3, (A) + reduced BP for >48 h, requiring fluid replacement; dyspnea on normal activity; grade 4, (A) + reduced BP that required pressor agents; dyspnea at rest. The MTD is defined as one dose level below that which causes unacceptable toxicity in patients on this study. If one patient experienced grade 3 or 4 toxicity at a specified dose level, additional patients were treated at that level until a total of six patients completed one full cycle at the suspect level. If two of six patients experienced nonhematologic grade 3 or grade 4 toxicity, the suspect level then becomes the level of unacceptable toxicity, and the MTD is determined to be the level below that.

Patients were restaged with noninvasive techniques at one month after each cycle, and those with progressive disease removed from the study. A complete response was defined as the disappearance of all clinical evidence of tumor for a minimum of four weeks. Partial response was defined as a decrease of 50% or more in the sum of the products of all diameters of measured lesions for a minimum of four weeks without the appearance of any new lesions. Minimal response was defined as a decrease of less than 50% in any tumor and/or any response lasting for less than four weeks. Stable disease was defined as a steady state. Progression was defined as the unequivocal increase of at least 25% in the size of any measurable lesion or appearance of new lesions.

Treatment plan. LMB-1 was constructed by Inland Labs, Houston, Texas from monoclonal antibody (mAb) B3 provided by Verax Co. (Lebanon, New Hampshire) and NLysPE38 purified at National Institutes of Health. LMB-1 in phosphate-buffered saline (PBS) is supplied as a sterile solution, 1 mg/ml in 5-ml vials containing 5 mg of LMB-1. The appropriate dose of LMB-1 was diluted in 100 ml of normal saline containing 0.2% human serum albumin before therapy. LMB-1 was administered over 30 min by intravenous infusion on days 1, 3 and 5. All patients received a 0.1-mg test dose 30 min before the treatment dose. The second and third doses of each cycle were delayed or withheld if any measure of toxicity was grade 2 or greater on the scheduled day of administration. Patients with responses or stable disease were eligible for additional cycles of therapy at 28-day intervals provided they had not developed anti-LMB-1 antibodies. For grade 3 or 4 toxicity, the dose was decreased by 50% in the subsequent cycles. Stable patients who developed antibodies were

followed at two-month intervals until disease progression.

B3 antigen expression was evaluated by immunohistochemistry using sections derived from paraffin blocks as described[17]. Sections were treated by boiling using TUF antigen retrieval solution (Kreatech), incubated in a blocking solution (4 µg/ml goat globulin, 0.1% saponin, PBS), followed by 10 µg/ml of mAb B3, affinity-purified goat-anti-mouse IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, Pennsylvania), detected using diaminobenzidine substrate solution and counterstained with hematoxylin before postfixation in 1% osmium tetroxide. Well-preserved tissues generally showed homogeneous reactivity throughout the surface of the section, whereas heavily overfixed tissues frequently only showed significant reactivity at the marginal zone on the surface of the tissue block. Homogeneity of tumor cell reactivity in those cases was evaluated only in these marginal regions. Specific B3 reactivity appeared as a cell surface and Golgi region pattern. Controls included deletion of the B3 antibody, or the use of DO-1 and anti-p53 monoclonal antibody (Novocastra Laboratories) which reacts only with nuclear p53, and not in a cell surface pattern.

Immunogenicity of LMB-1. Antibodies against LMB-1 were assessed weekly for four weeks then bimonthly using ELISA and serum neutralization assays. For the ELISA, 96-well microtiter plates were coated with PE38 or mAb B3 and blocked with gelatin (3%). Serum samples were added in dilutions beginning at 1/10, followed by peroxidase-conjugated AffiniPure goat anti-monkey and developed with ABTS. Samples were scored as positive when the optical density was at least twice the background. For the serum neutralization assay, LMB-1 was added to serum samples at 5 and 25 ng/ml and incubated at 37 °C for 15 min. The activity of LMB-1 was assayed by incubating the samples with MCF-7 cells and measuring its ability to inhibit protein synthesis. Cells were seeded at 1.5×10^4 cells per milliliter in 96-well plates, 24 h before the addition of serum containing LMB-1, incubated at 37 °C for 24 h, and then assayed for incorporation of [^{3}H]leucine. A standard curve with LMB-1 was used to determine mean inhibitory concentration (IC_{50}). A serum sample was considered negative for antibodies against LMB-1 when the cytotoxic activity of LMB-1 was not neutralized when incubated with sera, and positive or strongly positive when the serum sample neutralized the cytotoxic activity of 5 ng/ml and 25 ng/ml of LMB-1, respectively.

Acknowledgments

We thank D. FitzGerald, M. Gallo and Y. Shiloach for helping to produce LMB-1. We thank A. Rutherford for technical assistance and B. Goldspiel from the Pharmacy Department of the Clinical Center, National Institutes of Health, for his support.

RECEIVED 20 NOVEMBER 1995; ACCEPTED 31 JANUARY 1996

1. Pastan, I. *et al.* Characterization of monoclonal antibodies B1 and B3 that react with mucinous adenocarcinomas. *Cancer Res.* 51, 3781–3187 (1991).
2. Pai, L.H. & Pastan, I. Immunotoxin therapy for cancer. *JAMA* 269, 78 (1993).
3. Pai, L.H. & Pastan, I. Monoclonal antibodies in cancer therapy: Immunotoxins and recombinant toxins. in *Biologic Therapy of Cancer* 2nd edn. (eds DeVita, V.T., Hellman, S. & Rosenberg, S.A.) 521–533 (Lippincott, Philadelphia, 1995).
4. Vitetta, E.S. *et al.* Phase I immunotoxin trial in patients with B cell lymphoma. *Cancer Res.* 51, 4052–4058 (1991).
5. French, R.R., Hamblin, T.J., Bell, A.J., Tutt, A.L. & Glennie, M. Treatment of B-cell lymphomas with combination of bispecific antibodies and saporin. *Lancet* 346, 223–224 (1995).
6. Gould, B.J. *et al.* Phase I study of an anti-breast cancer immunotoxin by continuous infusion: Report of a target toxic effect not predicted by animal studies. *J. Natl. Cancer Inst.* 81, 775–781 (1989).
7. Allured,V.S. *et al.* Structure of exotoxin A of *Pseudomonas aeruginosa* at 3.0 Angstrom. *Proc. Natl. Acad. Sci. USA* 83, 1320–1324 (1986).
8. Hwang, J., FitzGerald, D.J., Adhya, S. & Pastan, I. Functional domains of *Pseudomonas* exotoxin identified by deletion analysis of the gene expressed in *E. coli*. *Cell* 48, 129–136 (1987).
9. Kondo, T., FitzGerald, D., Chaudhary, V.K., Adhya, S. & Pastan, I. Activity of immunotoxins constructed with modified *Pseudomonas* exotoxin A lacking the cell recognition domain. *J. Biol. Chem.* 263, 9470–9475 (1988).
10. Pastan, I.H., Pai, L.H., Brinkmann, U. & FitzGerald, D.J. Recombinant toxins: New therapeutic agents for cancer. *Ann. N.Y. Acad. Sci.* 758, 345–354 (1995).
11. Kuan, C.-T., Pai, L.H. & Pastan, I. Immunotoxins containing *Pseudomonas* exotoxin targeting Le^y damage human endothelial cells in an antibody specific mode: Relevance to vascular leak syndrome. *Clin. Cancer Res.* (in the press).
12. Roscoe, D.M. *et al.* Primate antibody response to immunotoxins: Serological and computer-aided analysis of epitopes on a truncated form of *Pseudomonas* exotoxin. *Infect. Immun.* 62, 5055–5065 (1994).
13. Wang, Q.-c., Pai, L.H., Debinski, W., FitzGerald, D.J. & Pastan, I. Preparation of polyethylene glycol modified forms of TGFα-PE40 that are cytotoxic and have a markedly prolonged survival in the circulation of mice. *Cancer Res.* 53, 4588–4594 (1993).
14. Yoshimoto, T. *et al.* Characterization of polyethylene glycol-modified L-asparaginase from *Escherichia coli* and its application to therapy of leukemia. *Jpn. J. Cancer Res.* 77, 1264–1270 (1986).
15. Jain, R.K. & Baxter, L.T. Mechanisms of heterogeneous distribution of monoclonal antibodies and other macromolecules in tumors: Significance of elevated interstitial pressure. *Cancer Res.* 48, 7022–7032 (1988).
16. Brinkmann, U., Pai, L.H., FitzGerald, D.J., Willingham, M.C. & Pastan, I. B3(Fv)-PE38KDEL, a single-chain immunotoxin that causes complete regression of a human carcinoma in mice. *Proc. Natl. Acad. Sci. USA* 88, 8616–8620 (1991).
17. Willingham, M. Immunohistochemical methods: Useful and informative tools for screening of hybridomas in the evaluation of antigen expression. *Focus* 12, 62–67 (1990).