[CANCER RESEARCH 51, 4001–4007, August 1, 1991]

Therapy of Human Cervical Carcinoma with Monoclonal Antibody-*Pseudomonas* Exotoxin Conjugates[1]

Steve R. Roffler, Mu-Hsien Yu, Bing Mae Chen, Edward Tung, and Ming-Yang Yeh[2]

Institute of Biomedical Sciences, Academia Sinica [S. R. R., M-H. Y., B. M. C., E. T., M-Y. Y.], Cancer Research Laboratory, Department of Medical Research, Tri-Service General Hospital [M-H. Y., M-Y. Y.], and Department of Microbiology and Immunology, National Defense Medical Center [M-Y. Y.], Taipei, Taiwan, Republic of China

ABSTRACT

Pseudomonas **exotoxin A (PE) linked to the F(ab′)$_2$ fragment of 1H10, a murine monoclonal antibody recognizing a carbohydrate epitope of a glycoconjugate expressed on the surface of human cervical carcinoma tumor cells, was evaluated for *in vitro* and *in vivo* activity. PE can kill cells by ADP-ribosylating elongation factor 2 thus inhibiting protein synthesis. Disulfide- as well as thioether-linked immunotoxins (1H10-PE) killed cervical carcinoma cells *in vitro* and were 20–160 times more inhibitory to target than to control cells. Cell killing was antibody mediated as demonstrated by the reduction of 1H10-PE growth inhibition to target CaSki cells by free 1H10 F(ab′)$_2$. In addition, a control antibody immunotoxin was nontoxic to CaSki cells. Thioether-linked 1H10-PE administered either i.v. or i.p. suppressed the growth of established solid s.c. cervical carcinoma tumors xenografted in nude mice for over 30 days. Treatment with antibody alone or a control immunotoxin had no significant effect on tumor growth. Administration of immunotoxin i.p. was associated with less toxicity than administration i.v., but i.v. injections were more effective at suppressing the growth of established solid tumors.**

INTRODUCTION

Cervical carcinoma is one of the common lethal malignancies affecting women around the world. Despite the widespread use of cervical cytological screening programs, it is still a serious problem (1–3). Among black women in the United States between 1973 and 1977, the age-adjusted incidence of invasive and *in situ* cervical carcinoma was 87/100,000, over twice the rate among white women (4). In Taiwan, 49.9% of 23620 primary malignant tumors from females diagnosed by surgical pathological examinations from 1964 to 1983 were found to be carcinoma of the uterine cervix (5). In another study, the age-adjusted cancer mortality rate of cervical carcinoma in China between 1973 and 1975 was 10.0/100,000 females, ranking second behind stomach cancer at 10.2 deaths/100,000 (6). These high incidence and mortality rates suggest that improved methods of detection and treatment of cervical carcinoma are needed.

Immunotoxins, in which a protein toxin is chemically linked to a monoclonal antibody, represent a new class of therapeutic agents for treating a wide variety of tumors (7, 8). The efficacy of an immunotoxin depends on both antibody and toxin components. *Pseudomonas* exotoxin has been shown to form potent immunotoxins (9–12). PE[3] inhibits protein synthesis by catalyzing the transfer of the ADP-ribosyl moiety of NAD to elongation factor 2 (13, 14). PE was linked to Mab 1H10 F(ab′)$_2$ to form anti-cervical carcinoma immunotoxins. Mab 1H10 is a murine IgG3 monoclonal antibody which reacts with a carbohydrate epitope of a glycoconjugate expressed on the surface of several types of human carcinomas including human cervical tumors (15). Mab 1H10 reacts with several human tumor cell lines including CaSki and ME-180 cervical carcinoma, HT-29 and SW 1116 colon carcinoma, and RT 4 bladder carcinoma cells (15). Mab 1H10 was also shown to recognize 40% of human cervical and colon cancer tissues as well as some bladder, ovarian, lung, and stomach carcinoma tissues (15). No binding of Mab 1H10 to any of the normal human tissues and cells tested was found. Samples tested included cervix, ovary, breast, liver, colon, bladder, kidney, spleen, endometrium, lung, thyroid, cerebrum, esophagus, RBC, and lymphocytes (15).

In this report, we describe immunotoxins formed by linking *Pseudomonas* exotoxin to the F(ab′)$_2$ fragment of Mab 1H10 by disulfide as well as thioether bonds. We show that PE linked to Mab 1H10 F(ab′)$_2$ can specifically kill cervical carcinoma cells *in vitro*. We also show that 1H10-PE can suppress the growth of solid cervical carcinoma tumors growing s.c. in nude mice when administered either i.p. or i.v.

MATERIALS AND METHODS

Reagents. Trypsin was purchased from Gibco BRL, Grand Island, NY. G-25 and S-300 gels and 3-(2-pyridyldithio)propionic acid *N*-hydroxy succinimide ester were from Pharmacia LKB Biotechnology, Uppsala, Sweden, and succinimidyl-4-(*N*-maleimidomethyl)cyclohexane 1-carboxylate was purchased from Pierce Chemical Company, Rockford, IL. ACA 34 gel was obtained from IBF Biotechnics, Villeneuve-la-Garenee, France. Rabbit anti-mouse IgG was from Zymed Laboratories, Inc., San Francisco, CA, while all other immunochemicals as well as 2-iminothiolane and NAD$^+$ were obtained from Sigma Chemical Company, St. Louis, MO. Nicotinamide [*U*-^{14}C]adenine dinucleotide (220 mCi/mmol) was purchased from Amersham International Plc, Amersham, Buckinghamshire, England. [^{3}H]Leucine (50 Ci/mmol) and *Pseudomonas* exotoxin A were from ICN Biomedicals, Inc., Costa Mesa, CA.

Cell Lines. All cells used for immunotoxin testing were maintained in RPMI 1640 (Gibco) supplemented with 5% heat-inactivated fetal calf serum, 1000 units/ml penicillin, and 100 μg/ml streptomycin. Cells were routinely tested for *Mycoplasma* by a standard method (16). The CC7T cervical carcinoma cell line was provided by Dr. C. P. Hu, Veterans General Hospital, Taipei, Taiwan, Republic of China. The CaSki cervical carcinoma cell line was provided by Dr. R. A. Pattillo, Medical College of Wisconsin, Milwaukee, WI. H2669 melanoma cell line was provided by Drs. K. and I. Hellström, University of Washington, Seattle, WA. The TSGH 8302 cervical carcinoma cell line was developed in the Cancer Research Laboratory, Department of Medical Research, Tri-Service General Hospital, Taipei, Taiwan, Republic of China (17). All other cell lines were obtained from ATCC, Rockville, MD.

Production of Monoclonal Antibodies. Mab 1H10, a murine IgG3 antibody with specificity for several carcinoma types, has been described elsewhere (15). Mab 7T1.1 is also an IgG3 antibody but reacts with the

Received 2/5/91; accepted 5/22/91.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by National Science Council Grants 78-0418-B016-01H and 79-0418-B016-01, Department of Health and Academia Sinica, Republic of China.

[2] To whom requests for reprints should be addressed, at Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, Republic of China.

[3] The abbreviations used are: PE, *Pseudomonas* exotoxin A; Mab, monoclonal antibody; 1H10-PE, conjugate of 1H10 antibody F(ab′)$_2$ fragment with *Pseudomonas* exotoxin; 7T1.1-PE, conjugate of 7T1.1 antibody F(ab′)$_2$ fragment with *Pseudomonas* exotoxin; PBS, phosphate-buffered saline (0.14 M NaCl-2.7 mM KCl-1.5 mM KH_2PO_4-8.1 mM Na_2HPO_4); BSA, bovine serum albumin; IC_{50}, concentration of test sample causing 50% inhibition of cellular protein synthesis relative to control cells.

A blood group antigen. Mab HK-PEG-1 (ATTC CL189), an IgG3 antibody used in radioimaging experiments as a control, reacts with influenza virus. Monoclonal antibodies were produced in ascites of BALB/c mice or obtained from the culture supernatant of hybridomas grown in serum-free medium using an Opticell 5200R cell culture system. Antibodies were purified by protein A affinity chromatography.

Preparation of Immunotoxins. $F(ab')_2$ fragments of Mab 1H10 or Mab 7T1.1 were prepared as described previously (18). Undigested IgG was removed on Sepharose CL-4B-protein A while free pepsin and small peptides were removed by size exclusion chromatography on AcA 34 or Sephacryl 300 gel. PE was linked to Mab $F(ab')_2$ via a disulfide or thioether bond by following previously described methods (10, 19). An average of 3.5 3-(2-pyridyldithio)propionyl groups or maleimido groups were introduced with 3-(2-pyridyldithio)propionic acid *N*-hydroxy succinimide ester and succinimidyl-4-(*N*-maleimidomethyl)cyclohexane 1-carboxylate, respectively, into each $F(ab')_2$ as measured by methods described previously (20, 21). An average of 3.0 —SH groups were introduced with 2-iminothiolane into each PE when measured as described by FitzGerald (19). Immunotoxins were purified by ion exchange chromatography on a DEAE 5 PW high performance liquid chromatography column (Waters). Immunotoxin concentration was determined (22) using Mab 1H10 $F(ab')_2$ and BSA as standards. Purified immunotoxins were stored at −70°C in sterile PBS containing 1 mg/ml BSA or human serum albumin.

Immunotoxin Characterization. ADP ribosylation activities of PE and immunotoxins were measured *in vitro* (23). Incubation mixtures consisted of 435 μl 50 mM Tris, 1 mM EDTA (pH 8.0), 25 μl [^{14}C]NAD^+ (1 μCi/ml), 20 μl elongation factor 2-enriched wheat germ extract (24), and 10 μl sample pretreated with an equal volume of 8 M urea, 2% dithiothreitol, and 0.2 mg/ml BSA at 25°C for 15 min to activate PE. Trichloroacetic acid-precipitated radioactivity was counted with a Beckman LS 5801 scintillation counter. Immunotoxin antigen-binding activity was measured by enzyme-linked immunosorbent assay using whole CaSki cells coated on 96-well plates as antigen. Molecular weights of immunotoxins were estimated on nonreduced gradient (3–12.5%) sodium dodecyl sulfate gels after silver staining (25) from a linear regression curve of the log of the molecular weights of prestained standard proteins (Sigma) *versus* their fractional migration distance down the gel. The presence of PE and immunoglobulin in immunotoxins was assessed by immunoblotting electrophoresed samples after transfer to nitrocellulose paper. After blocking with 3% skim milk in PBS, PE was detected by serial incubation with rabbit polyclonal serum against PE and goat anti-rabbit alkaline phosphatase conjugate. Anti-PE rabbit serum was produced by modifying a previously described procedure (26). IgG was detected by incubation with goat anti-mouse IgG-$F(ab')_2$-specific biotin- and streptavidin-β-galactosidase conjugates. Immunobinding was visualized using the appropriate substrate solutions (27, 28).

***In Vitro* Growth Inhibition.** Cells (2×10^4/well) in 96-well plates were cultured overnight before addition of PE or immunotoxin for 8 h. [^{3}H]Leucine incorporation was measured 40 h later (29). All assays were performed in triplicate and 3 controls receiving only fresh medium were used at each sample concentration. Comparisons of the *in vitro* effect of immunotoxins on target and antigen-negative cells were quantified by calculating the sensitivity ratio and selectivity for each cell line. These factors are defined as

$$\text{Sensitivity ratio} = \frac{(IC_{50})_{PE}}{(IC_{50})_{1H10\text{-}PE}}$$

$$\text{Selectivity} = \frac{(\text{Sensitivity ratio})_{\text{target cells}}}{(\text{Sensitivity ratio})_{\text{antigen-negative cells}}}$$

The sensitivity ratio quantifies the relative growth inhibition of immunotoxin compared to free toxin for a particular cell line. A ratio >1 indicates that the immunotoxin is more inhibitory than free toxin to a particular cell line. Selectivity quantifies immunotoxin growth inhibition to target cells compared to antigen-negative control cells. A selectivity of 1 indicates no immunotoxin selectivity for target cells while a factor of 100 indicates that the immunotoxin is 100 times more toxic to target cells than control cells. These ratios minimize variations between assays and allow comparison of immunotoxin growth inhibition among cells with different *in vitro* sensitivities to free toxin. All calculations were based on the concentration of PE in immunotoxins.

***In Vivo* Localization of Mab 1H10.** Mab 1H10 and control antibody HK-PEG-1 $F(ab')_2$ fragments were labeled with ^{131}I to a specific activity of 5–6 μCi/μg by the chloramine-T method. Nude mice bearing well established CaSki or control H2669 xenografts in the right flank were fed Lugol solution for 2 days prior to antibody treatment. Radiolabeled Mab (100 μCi) was injected i.v. via the tail vein and mice were imaged with an Elscint Apex 400 gamma camera 96 h later.

Immunotoxin *in Vivo* Activity. Nude mice were given s.c. injections in the right flank of 10^7 exponentially growing CaSki cells on day 1 and randomly placed in groups of 5 mice. Treatment was delayed 4 to 14 days to allow the establishment of solid tumors. Mice received biweekly injections of immunotoxins or control substances as radioimaging studies showed maximum antibody localization in tumors after 72–96 h. Preliminary experiments indicated that the immunotoxin 50% lethal dose was about 1 μg. Doses used were one-fourth to one-half of the 50% lethal dose. Treatment schedules and doses are indicated in the figure legends. Injections i.v. were made via the tail vein using a volume of 50 μl sample in sterile PBS while i.p. injections were made in a volume of 100 μl. Tumor size and body weight were measured twice a week. Tumor volume was estimated by multiplying the product of the three tumor dimensions by 0.5 (30). Data were statistically analyzed with Student's *t* test.

RESULTS

Anti-Cervical Carcinoma Immunotoxins

Pseudomonas exotoxin A was covalently attached to $F(ab')_2$ fragments of Mabs 1H10 or 7T1.1 by a disulfide or thioether bond to form anti-cervical carcinoma immunotoxins (1H10-PE) or control immunotoxins (7T1.1-PE). Immunotoxins were separated from unconjugated antibody or PE by anion exchange high performance liquid chromatography. 1H10-PE appeared as a double band with average molecular weight of 176,000 on nonreduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1*A, Lane 1*). This molecular weight corresponds to a conjugate containing 1 molecule each of PE (M_r 66,000) and Mab 1H10 $F(ab')_2$ (M_r 110,000). The conjugate contained both PE and immunoglobulin as shown by immunoblots of 1H10-PE for IgG (Fig. 1*B, Lane 1*) and PE (Fig. 1*C, Lane 1*). Purified immunotoxin contained some free $F(ab')_2$ but appeared uncontaminated with free PE. The double band associated with the immunotoxin may be due to the heterogeneous molecular weight of the $F(ab')_2$ fragment used for coupling or to the generation of cryptic protein fragments during the coupling procedure.

Antigen-binding and ADP ribosylation activities of 1H10-PE were measured (Fig. 2). Although antibody immunoreactivity was decreased in the conjugates, significant activity was maintained in both disulfide- and thioether-linked immunotoxins (Fig. 2*A*). PE ADP ribosylation activity was also affected by conjugation to antibody but to a lesser extent in thioether-linked 1H10-PE; thioether-linked PE retained about 75% activity compared to 25% activity in disulfide-linked 1H10-PE (Fig. 2*B*).

1H10-PE *in Vitro* Activity

The *in vitro* activities of disulfide- and thioether-linked 1H10-PE were evaluated by assessing inhibition of protein synthesis in target (CaSki) and antigen-negative control cells (Fig. 3). Both conjugates inhibited growth of CaSki cervical carcinoma

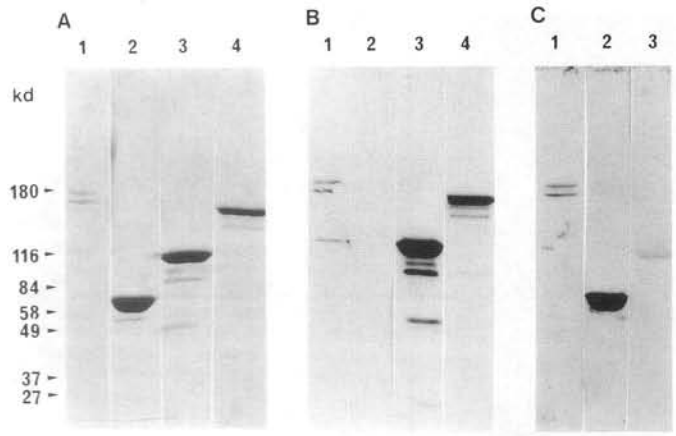

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis of 1H10-PE. Samples were electrophoresed on nonreduced gradient (3–12.5%) gels and silver stained (*A*) or transferred to nitrocellulose paper and immunoblotted for IgG (*B*) or *Pseudomonas* exotoxin (*C*). *Lane 1*, disulfide-linked 1H10-PE; *Lane 2*, PE; *Lane 3*, Mab 1H10 F(ab′)$_2$; *Lane 4*, Mab 1H10 IgG. *kd*, molecular weight in thousands.

cells with IC_{50}s of 28 and 18 ng/ml for disulfide- and thioether-linked immunotoxins based on the concentration of PE in immunotoxins. The conjugates were also more inhibitory to CaSki cells than free PE; the average IC_{50} of PE to CaSki cells was 150 ng/ml. Selectivity of 1H10-PE for CaSki cells was also estimated (Table 1). Selectivities took into account the different sensitivities of cells to PE which varied by a factor of 50–100-fold for different cell lines. 1H10-PE was less toxic than free PE to all antigen-negative cells tested (sensitivity ratio, <1). Disulfide-linked and thioether-linked 1H10-PE were 20–30 and 40–160 times more selective for CaSki cells, respectively. Mab 1H10 F(ab′)$_2$ alone did not affect cell growth at concentrations up to 200 μg/ml (data not shown).

1H10-PE *in Vitro* Specificity. The specificity of 1H10-PE for cervical carcinoma was confirmed by two additional experiments. In the first experiment, free Mab 1H10 F(ab′)$_2$ was added to CaSki cells to compete with disulfide-linked immunotoxin for antigen expressed on the surface of the cells. Addition of 50 μg of 1H10 F(ab′)$_2$ reduced the toxicity of 1H10-PE to CaSki cells about 10-fold (Fig. 4*A*). In the second experiment, PE was coupled via a disulfide bond to the F(ab′)$_2$ fragment of Mab 7T1.1 to form a control immunotoxin. Mab 7T1.1, a murine IgG3 Mab, is able to bind to CC7T cells but does not react with CaSki or H2669 cells. 7T1.1-PE was unable to kill CaSki or H2669 cells even though it was active against CC7T cells (Fig. 4*B*). The IC_{50} of the 7T1.1-PE was 6 ng/ml for CC7T cells compared to over 1000 ng/ml for CaSki and H2669 cells. These results indicate that 1H10-PE killing of CaSki cells was antibody mediated.

1H10-PE *in Vivo* Activity

Human cervical carcinoma cells (CaSki) were grown in the rear flank of nude mice as s.c. solid tumors to evaluate the efficacy of immunotoxin treatment *in vivo*. Immunotoxin or control treatments were initiated after 4–14 days to allow injected cells time to establish solid tumors. The ability of Mab 1H10 to localize in cervical carcinoma xenografts was first verified. ^{131}I-Labeled Mab 1H10 F(ab′)$_2$ clearly localized in established CaSki tumor xenografts (Fig. 5*B*). Localization was specific inasmuch as ^{131}I-labeled Mab 1H10 F(ab′)$_2$ did not localize in a control tumor xenograft (Fig. 5*C*) nor did a control antibody localize in CaSki xenografts (Fig. 5*D*).

Disulfide-linked 1H10-PE. Mice were treated with disulfide-linked 1H10-PE in the first two experiments. In a short term experiment, i.p. administered 1H10-PE suppressed the growth of solid cervical carcinoma tumors over the course of the experiment (Fig. 6*A*). The average tumor size in 1H10-PE-treated mice was 47 ± 9.8 (SE) mm^3 on day 23. Tumors in mice receiving control immunotoxin (7T1.1-PE) or BSA, on the other hand, continued to grow to average sizes of 165 ± 29 or 155 ± 80 mm^3 by day 23. In the second experiment, treatment was delayed for 14 days to allow the establishment of larger tumors (>100 mm^3). Administration i.v. of disulfide-linked 1H10-PE via the tail vein significantly retarded tumor growth compared with mice receiving BSA or Mab 1H10 F(ab′)$_2$ (Fig. 6*B*). On day 55, the average tumor size of 1H10-PE treated mice was 20 ± 12 mm^3, significantly smaller than the average tumor size (2320 ± 410 mm^3) in control mice ($P < 0.01$). The average V_t/V_c ratio determined from tumor volumes of treated and control mice was 0.009 on day 55. Administration of Mab 1H10 F(ab′)$_2$ alone affected tumor growth slightly as suggested by the appearance of necrosis in several tumors and a decrease in average tumor size after day 35. The average size of tumors in mice receiving antibody was 1360 ± 510 mm^3 on day 55, just significantly smaller than tumors in control mice ($P < 0.1$).

Thioether-linked 1H10-PE. The activity of thioether-linked 1H10-PE was also tested *in vivo*. Average tumor size in control mice receiving albumin increased throughout the experiment to an average volume of 1270 mm^3 on day 68 (Fig. 7). A total of 10 biweekly i.p. or i.v. administrations of thioether-linked

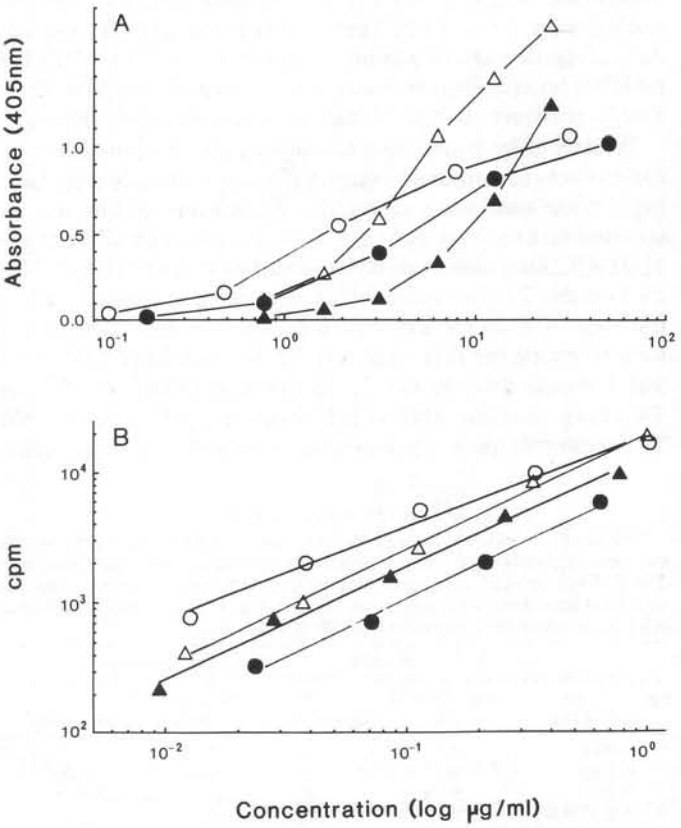

Fig. 2. Antibody and toxin activities of 1H10-PE. (*A*) Enzyme-linked immunosorbent assay measurement of antigen binding activities of disulfide (●)- or thioether (▲)-linked 1H10-PE compared to activities of Mab 1H10 F(ab′)$_2$ before conjugation (○, △). (*B*) Adenosine diphosphoribosyltransferase activities of disulfide (●)- or thioether (▲)-linked 1H10-PE compared to activities of PE before conjugation (○, △).

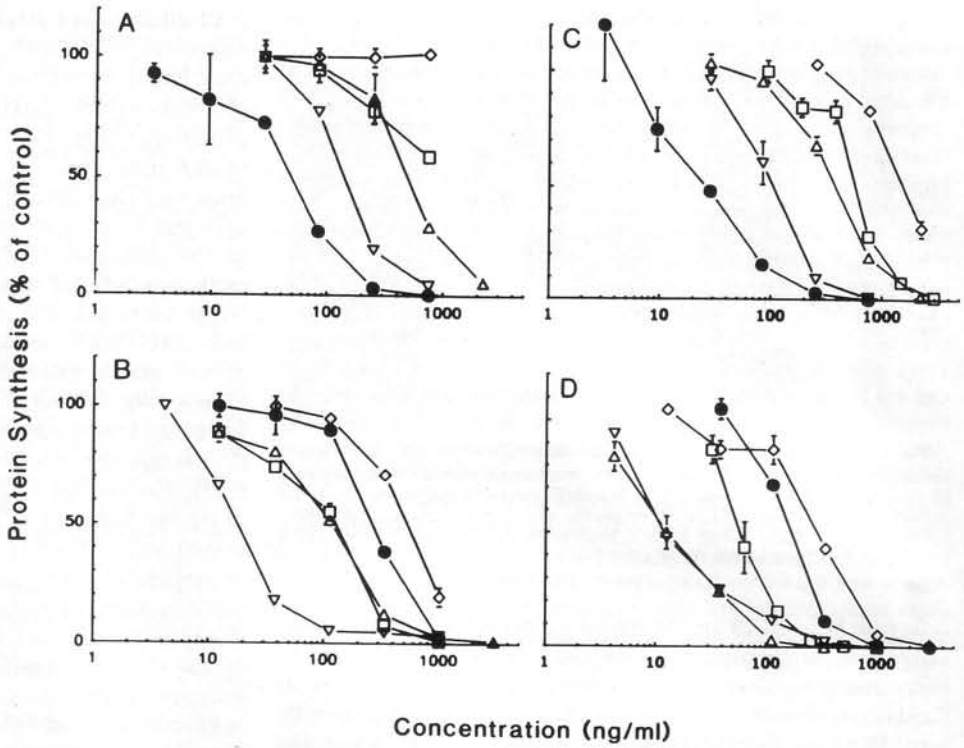

Fig. 3. *In vitro* growth inhibition of disulfide- and thioether-linked 1H10-PE. Cells were incubated with disulfide-linked 1H10-PE (*A*) or PE (*B*) or thioether-linked 1H10-PE (*C*) or PE (*D*) for 8 h, washed with PBS, and then incubated in fresh medium for 40 h. Cellular protein synthesis was assayed at hour 48 as described in "Materials and Methods." Antigen-positive CaSki cervical carcinoma cells (●), antigen-negative H2669 melanoma cells (△), 8302 cervical carcinoma cells (□), WISH fibroblasts (▽), and HEp 2 laryngeal carcinoma cells (◇) are shown. *Bars*, SE of triplicate determinations.

1H10-PE from days 5 to 36 significantly retarded tumor growth (Fig. 7). The average V_t/V_c ratio determined from tumor volumes in treated and control mice at the termination of the experiment was 0.19 for i.p.-treated mice and 0.05 for i.v.-treated mice ($P < 0.07$). Immunotoxin therapy was specific. Administration i.p. of a control immunotoxin (7T1.1-PE) had no effect on average tumor size (Fig. 7). In addition, Mab 1H10 F(ab′)$_2$ treatment had no significant effect on tumor growth.

Toxicity of Immunotoxin Treatment. Table 2 summarizes the toxic effects of immunoconjugate treatment of nude mice bearing cervical carcinoma xenografts. Significant toxicity was associated with both i.p. and i.v. administration of disulfide-linked 1H10-PE. One mouse receiving disulfide-linked 1H10-PE i.p. died on day 23 after receiving an accumulated dose of 2.85 μg immunotoxin. In the i.v.-treated group, two mice died shortly after receiving the first large dose of disulfide-linked 1H10-PE and 1 mouse died on day 31 (accumulated dose of 1.55 μg). Two mice receiving 1H10 F(ab′)$_2$ also died (days 44 and 50). The tumors of these mice exhibited necrosis and large reductions in tumor size (56–70%) before death. Although toxicity was also associated with multiple doses of thioether-linked 1H10-PE, toxic effects were less pronounced than in mice treated with disulfide-linked 1H10-PE. Two of five mice receiving multiple i.v. injections of 1H10-PE died within 3 days of the last administration of immunotoxin (accumulated dose of 1.6 μg). None of the mice receiving an accumulated dose of 3.9 μg 1H10-PE by i.p. injection died. The average weight of mice

Table 1 *In vitro selectivity of 1H10-PE*

The selectivity of disulfide- and thioether-linked 1H10-PE for CaSki cervical carcinoma cells relative to antigen-negative H2669 melanoma, WISH fibroblast, TSGH 8302 cervical carcinoma, and HEp 2 laryngeal carcinoma cells was calculated from data including results shown in Fig. 4. Sensitivities of individual cell lines to 1H10-PE compared to free PE are also shown.

	Disulfide		Thioether	
Cell line	Sensitivity[a] ratio	Selectivity[a]	Sensitivity ratio	Selectivity
CaSki	5.0		7.1	
H2269	0.22	23	0.045	160
WISH	0.18	28	0.11	65[b]
TSGH 8302	<0.18	>28	0.10	71
HEp 2	<0.33	>15	0.19	37[b]

[a] Sensitivity ratio and selectivity were calculated as described in "Materials and Methods" from interpolated IC_{50} values taken from graphs of protein synthesis inhibition *versus* 1H10-PE and PE concentration. Values are averages of two independent experiments unless indicated.

[b] Result from a single experiment.

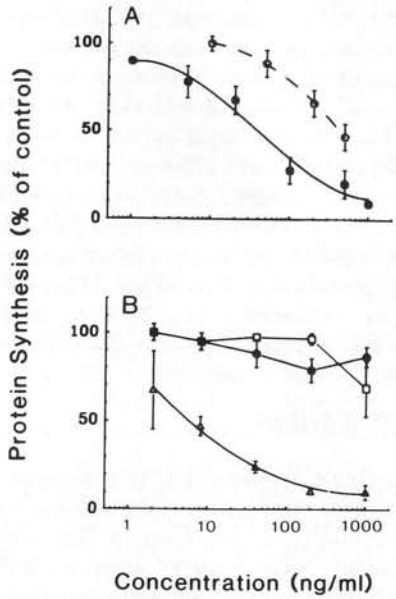

Fig. 4. 1H10-PE killing of CaSki cells is antibody mediated. (*A*) Adding Mab 1H10 F(ab′)$_2$ to CaSki cells decreased 1H10-PE growth inhibition. Plated CaSki cells were incubated with 1H10-PE for 1 h in the presence (○) or absence (●) of 50 μg/ml Mab 1H10 F(ab′)$_2$. Cellular protein synthesis was measured at hour 24 and compared to control cells. (*B*) 7T1.1-PE control immunotoxin was incubated with antigen-positive CC7T cells (△) or antigen-negative CaSki (●) or H2669 (□) cells for 1 h. Cells were subsequently incubated with fresh medium for 24 h before cellular protein synthesis was assayed. *Bars*, SE of triplicate assays.

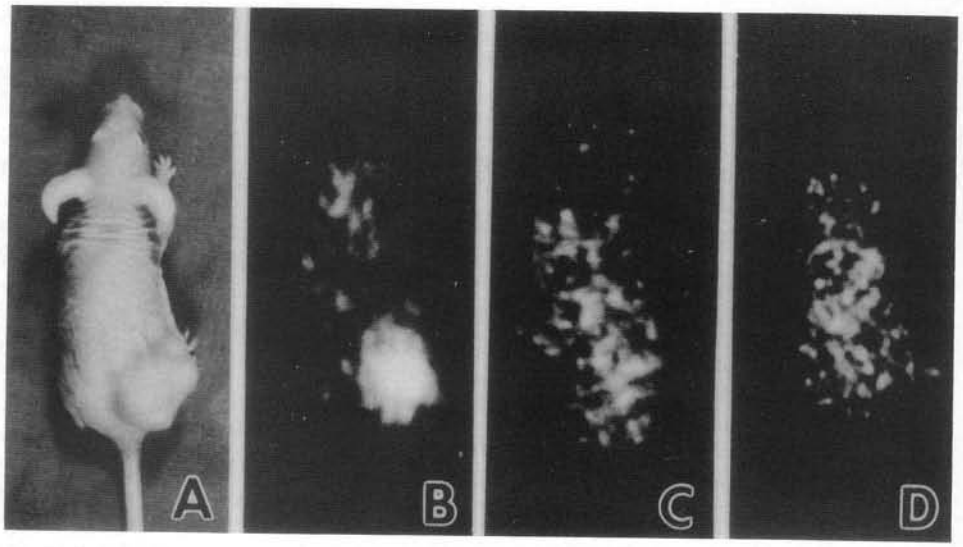

Fig. 5. *In vivo* localization of ^{131}I-labeled Mab 1H10. Athymic mice bearing established tumors were given i.v. injections of 100 μCi radiolabeled antibody. (*A*) Tumor xenografts were located in the right rear flank of mice. (*B*) Imaging of a CaSki xenograft with ^{131}I-labeled Mab 1H10 F(ab′)$_2$. (*C*) Imaging of a control H2669 melanoma xenograft with ^{131}I-labeled Mab 1H10 F(ab′)$_2$. (*D*) Imaging of a CaSki xenograft with ^{131}I-labeled control Mab HK-PEG-1 F(ab′)$_2$. All imaging was performed 96 h postinjection.

receiving 1H10-PE i.p., however, was 24.6 g on day 66 compared to 27.5 g for control mice, indicating some toxicity. One of the mice receiving control immunotoxin (7T1.1-PE) i.p. also died.

DISCUSSION

We have shown that 1H10-PE was an effective immunotoxin against cervical carcinoma *in vitro* and *in vivo*. *Pseudomonas* exotoxin was linked via a disulfide or thioether bond to the F(ab′)$_2$ fragment of Mab 1H10, an IgG3 antibody directed against an antigen present on the surface of cervical carcinoma cells but not on normal tissues or cells tested (15). Disulfide- and thioether-linked immunotoxins were about equally inhibitory to CaSki human cervical carcinoma cells *in vitro* with IC_{50} of 10^{-10} M. 1H10-PE potency was similar to anti-human breast carcinoma immunotoxins containing PE (10) and PE linked to antibody against the human transferrin receptor (9, 31). Both disulfide and thioether linked 1H10-PE in contact with CaSki cells for 8 h were able to totally inhibit protein synthesis measured at 48 h at a concentration of 4×10^{-9} M.

Thioether-linked immunotoxin appeared to be more selective than disulfide-linked 1H10-PE *in vitro*. Disulfide and thioether immunotoxins were 20–30 and 40–160 times more selective for CaSki cells than for antigen-negative cells, respectively. This result is similar to that reported by Morgan *et al.* (12) who found that linking PE to the Fab fragment of IgG via disulfide or thioether bonds resulted in equipotent immunotoxins but that thioether-linked Fab immunotoxins were about 200 times more toxic to antigen-positive cells than to antigen-negative cells while disulfide-linked Fab-PE was only about 1.7 times more selective for target cells. Although we could not show such a dramatic increase in selectivity for thioether-linked immunotoxins, thioether-linked 1H10-PE was consistently more specific than disulfide-linked 1H10-PE.

We also examined the *in vivo* efficacy of 1H10-PE. Most studies examining the effects of immunotoxins *in vivo* have focused on the i.p. therapy of tumors localized in the peritoneal cavity (11, 32–36). Although therapy of human tumors in mice with PE-containing immunotoxins is complicated by the extreme sensitivity of mouse cells to PE (37–40), we were interested in examining a model in which immunotoxins would have to pass through some of the anatomic barriers associated with solid tumors or established metastases before localizing at the tumor site. Immunotoxin therapy of s.c. tumors was therefore delayed for 4–14 days to allow the development of solid tumors with an established vascular network.

Using this model, we were able to demonstrate that therapeutic quantities of thioether-linked 1H10-PE could be delivered

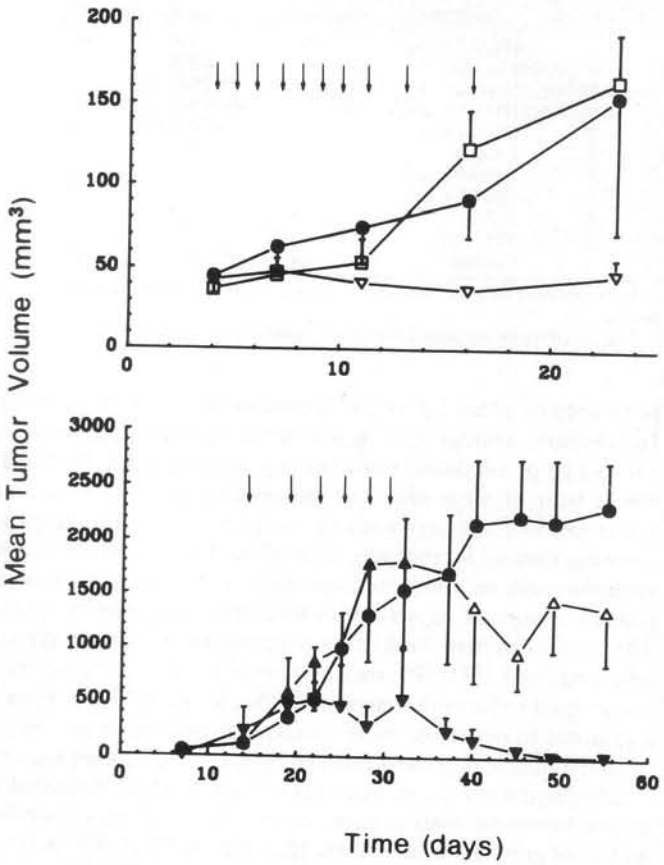

Fig. 6. Treatment of cervical carcinoma xenografts with disulfide-linked 1H10-PE. Athymic mice were given injections of 10^7 CaSki cells on day 1. (*A*) Groups of 5 mice received i.p. injections of 1H10-PE (▽), human serum albumin (●), or 7T1.1-PE control immunotoxin (□). Mice received doses of 200 ng on days 4–6; 300 ng on days 7–9; 450 ng on days 10, 11, and 13; and 300 ng on day 16. (*B*) Groups of 5 mice received i.v. injections of 1H10-PE (▼), BSA (●), or 1H10 F(ab′)$_2$ (△). Mice received 500 ng on day 14; 150 ng on days 19, 22, and 25; and 300 ng on days 28 and 30. Treatments are indicated by the *arrows*. Average tumor volumes and standard errors (*bars*) for each group are shown.

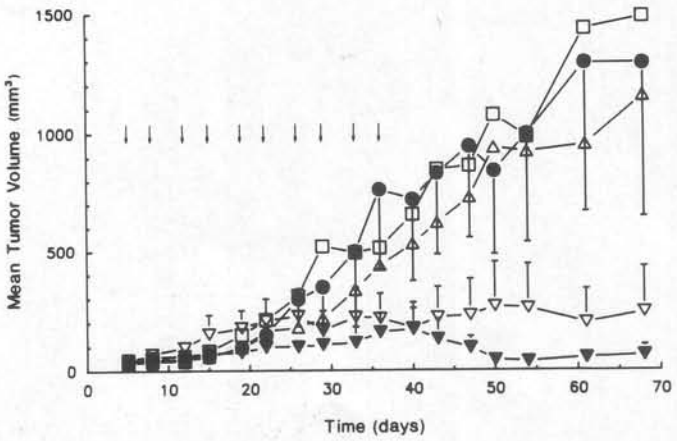

Fig. 7. Treatment of CaSki cervical carcinoma xenografts with thioether-linked 1H10-PE. Groups of 5 mice bearing s.c. solid CaSki tumors received i.v. injections of H10-PE (▼) (100 ng on days 5, 8, and 12; 150 ng on days 15, 19, and 22; 200 ng on days 26, 29, 33, and 36) or i.p. injections of 1H10-PE (▽), human serum albumin (●), 1H10 F(ab′)$_2$ (△), or 7T1.1-PE control immunotoxin (□) (250 ng on days 5, 8, and 12; 375 ng on days 15, 19, and 22; 500 ng on days 26, 29, 33, and 36) as indicated by the *arrows*. Average tumor volumes and standard errors (*bars*) are shown for each group.

Table 2 *Toxicity of in vivo immunoconjugate treatment*

The accumulated doses of immunotoxin or control substances at the time of death for mice receiving different therapies are shown.

Experiment	Treatment	Route of administration	Deaths/ group	Accumulated dose (μg)[a]
1	1H10-PE (ds)[b]	i.v.	3/5	0.5
1	F(ab′)$_2$	i.v.	2/5	1.55
1	Control	i.v.	0/5	1.55
2	1H10-PE (ds)	i.p.	1/5	2.85
2	7T1.1-PE	i.p.	0/5	2.85
2	Control	i.p.	0/5	2.85
3	1H10-PE (te)	i.v.	2/5	1.6
3	1H10-PE (te)	i.p.	0/5	3.9
3	F(ab′)$_2$	i.p.	0/5	3.9
3	7T1.1-PE	i.p.	1/5	3.9
3	Control	i.p.	0/5	3.9

[a] Accumulated dose at time of first death or total dose for groups with no deaths.
[b] ds, disulfide-linked immunotoxin; te, thioether-linked immunotoxin.

to tumors by either i.p. or i.v. administration of immunotoxin. In addition, average tumor size decreased (i.v. administered 1H10-PE) or remained constant (i.p. administered 1H10-PE) for at least 30 days after the termination of treatment. This result can be contrasted with the treatment of solid osteogenic sarcoma tumors by multiple injections of a ricin A chain-IgG immunotoxin in which tumors regrew at the same rate as control tumors 12 days after the termination of treatment (41). The more effective long term suppression of tumor growth resulting from 1H10-PE treatment may be due to the different toxins used to form the immunotoxins, to the ability of F(ab′)$_2$ fragments to penetrate more extensively into the tumor mass, or to the different growth characteristics of the tumors studied.

Administration i.v. of Mab 1H10 F(ab′)$_2$ alone appeared to induce tumor necrosis in some cases, but its suppressive effect on tumor growth was much less than that of 1H10-PE. Administration of 1H10 F(ab′)$_2$ i.p. had very little effect on tumor growth. Mab 1H10 F(ab′)$_2$ also had no effect on cell growth *in vitro*. Some toxicity was associated with F(ab′)$_2$ treatment of mice, but toxicity appeared to be related to large decreases in tumor size. The mechanism of toxicity is unknown and requires further work for elucidation.

We found that 1H10-PE was toxic to nude mice but that i.p. administrations were less toxic than i.v. injections of immunotoxin, paralleling the toxicity of PE in mice (26). Thioether-linked 1H10-PE was also less toxic than disulfide-linked 1H10-PE, indicating the disulfide-linked immunotoxin may be unstable *in vivo*. Morgan *et al.* (12) found that PE-containing conjugates constructed from intact antibody were less toxic to antigen-negative cells than were conjugates constructed using antibody fragments, suggesting that the Fc portion of IgG may block nonspecific binding of PE to normal cells. Our current efforts are focused on reducing the toxicity of 1H10-PE while maintaining its *in vivo* potency.

In summary, we have constructed new anti-cervical immunotoxins by linking PE to the F(ab′)$_2$ fragment of Mab 1H10 via disulfide as well as thioether bonds. 1H10-PE specifically killed human cervical carcinoma cells *in vitro* and suppressed the growth of solid s.c. tumors *in vivo*. We believe that 1H10-PE may help in the treatment of cervical carcinoma, especially for those cases with metastases. Mab 1H10 may also have potential for targeting other drugs, isotopes, or toxins to cervical carcinoma tumors due to its limited reaction with normal cells and tissues.

ACKNOWLEDGMENTS

We wish to thank M. L. Lee and M. R. Chen for expert technical assistance, Drs. J. Hwang and A. Wu for providing materials and advice, and Y. S. Lin for preparing the manuscript.

REFERENCES

1. Murphy, M. F., Campbell, M. J., and Goldblatt, P. O. Twenty years' screening for cancer of the uterine cervix in Great Britain, 1964–84: further evidence for its ineffectiveness. J Epidemiol. Commun. Health, *42:* 49–53, 1988.
2. Cox, B., and Skegg, D. C. Trends in cervical cancer in New Zealand. NZ Med. J., *99:* 795–798, 1986.
3. Dodgson, J., Walker, E. M., Hussein, K. A., Robertson, A. J., and Duncan, I. D. The increasing problem in Tayside of cervical cancer in younger women. Scot. Med. J., *34:* 403–405, 1989.
4. Swanson, G. M., Belle, S. H., and Young, J. L. U.S. trends in carcinoma of the cervix: incidence, mortality and survival. *In:* E. S. E. Hafez and J. P. Smith (eds.), Carcinoma of the Cervix: Biology and Diagnosis, pp. 1–9. The Hague: Martinus Nijhoff, 1983.
5. Yeh, S. Survey of cancer in Taiwan. *In:* E. H. Chang, J-K. Lin, and P. C. Huang (eds.), Proceedings of Molecular Biology of Neoplasia Symposium, pp. 23–31. Taipei, Taiwan: Academia Sinica, 1985.
6. King, H., and Locke, F. B. Selected indicators of current death status and major causes of death in the People's Republic of China: an historical perspective. *In:* J. L. Scherer (ed.), China, Facts and Figures Annual, pp. 376–422. Academic International Press, 1983.
7. Pirker, R. Immunotoxins against solid tumors. Cancer Res. Clin. Oncol., *114:* 385–393, 1988.
8. Blakey, D. C., Wawrzynczak, E. J., Wallace, P. M., and Thorpe, P. E. Antibody toxin conjugates: a perspective. Monoclonal Antibody Ther. Prog. Allergy, *45:* 50–90, 1988.
9. Pirker, R., FitzGerald, D. J. P., Hamilton, T. C., Ozols, R. F., Willingham, M. C., and Pastan, I. Anti-transferrin receptor antibody linked to *Pseudomonas* exotoxin as a model immunotoxin in human ovarian carcinoma cell lines. Cancer Res., *45:* 751–757, 1985.
10. Bjorn, M. J., Groetsema, G., and Scalapino, L. Antibody-*Pseudomonas* exotoxin A conjugates cytotoxic to human breast cancer cells *in vitro*. Cancer Res., *46:* 3262–3267, 1986.
11. FitzGerald, D. J., Bjorn, M. J., Ferris, R. J., Winkelhake, J. L., Frankel, A. E., Hamilton, T. C., Ozols, R. F., Willingham, M. C., and Pastan, I. Antitumor activity of an immunotoxin in a nude mouse model of human ovarian cancer. Cancer Res., *47:* 1407–1410, 1987.
12. Morgan, A. C., Sivan, G., Beaumier, P., McIntyre, R., Bjorn, M., and Abrams, P. G. Immunotoxins of *Pseudomonas* exotoxin A (PE): effect of linkage on conjugate yield, potency, selectivity and toxicity. Mol. Immunol., *27:* 273–282, 1990.
13. Iglewski, B. H., and Kabat, D. NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA, *72:* 2284–2288, 1975.
14. Iglewski, B. H., Liu, P. V., and Kabut, D. Mechanism of action of *Pseudomonas aeruginosa* exotoxin A: adenosine diphosphate-ribosylation of mam-

malian elongation factor 2 *in vitro* and *in vivo*. Infect. Immun., *15:* 138–144, 1977.
15. Roffler, S. R., Yu, M. H., Chen, M. R., Chen, S. C., Hsu, C. T., and Yeh, M. Y. New monoclonal antibody against human cervical carcinoma with diagnostic and therapeutic potential. Chinese J. Microbiol. Immunol., in press, 1991.
16. Russell, W. C., Newman, C., and Williamson, D. H. A simple cytochemical technique for demonstration of DNA in cells infected with mycoplasmas and viruses. Nature (Lond.), *253:* 461–462, 1975.
17. Yeh, M-Y., Yu, D-S., Chen, S-C., Lin, M-S., Chang, S-Y., Ma, C-P., and Han, S-H. Establishment and characterization of a human urinary bladder carcinoma cell line (TSGH-8302). J. Surg. Oncol., *37:* 177–184, 1988.
18. Lamoyi, E., and Nisonoff, A. Preparation of F(ab′)$_2$ fragments from mouse IgG of various subclasses. J. Immunol. Methods, *56:* 235–243, 1983.
19. FitzGerald, D. J. P. Construction of immunotoxins using *Pseudomonas* exotoxin A. Methods Enzymol., *151:* 139–145, 1987.
20. Carlsson, J., Drevin, H., and Axen, R. Protein thiolation and reversible protein-protein conjugation: *N*-succinimidyl 3-(2-pyridyldithio)propionate, a new heterobifunctional reagent. Biochem. J., *173:* 723–737, 1978.
21. Ishikawa, E., Hashida, S., Kohno, T., Kotani, T., and Ohtaki, S. Modification of monoclonal antibodies with enzymes, biotin, and fluorochromes and their applications. *In:* L. B. Schook (ed.), Monoclonal Antibody Production Techniques and Applications, pp. 113–137. New York: Marcel Dekker, 1987.
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265–275, 1951.
23. Collier, R. J., and Kandel, J. Structure and activity of diphtheria toxin. I. Thiol-dependent dissociation of a fraction of toxin into enzymatically active and inactive fragments. J. Biol. Chem., *246:* 1496–1503, 1971.
24. Chung, D. W., and Collier, R. J. Enzymatically active peptide from the adenosine diphosphate-ribosylating toxin of *Pseudomonas aeruginosa*. Infect. Immun., *16:* 832–841, 1977.
25. Morrissey, J. H. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal. Biochem., *117:* 307–310, 1981.
26. Iglewski, B. H., and Sadoff, J. C. Toxin inhibitors of protein synthesis: production, purification and assay of *Pseudomonas aeruginosa* exotoxin toxin A. Methods Enzymol., *60:* 780–793, 1979.
27. Ey, P. L., and Ashman, L. K. The use of alkaline phosphatase-conjugated anti-immunoglobulin with immunoblots for determining the specificity of monoclonal antibodies to protein mixtures. Methods Enzymol., *121:* 497–509, 1986.
28. Lee, N., Zhang, S. Q., and Testa, D. A rapid multicolor Western blot. J. Immunol. Methods, *106:* 27–30, 1988.
29. McIntosh, D. P. Antibody-toxin conjugates: a new approach to cancer therapy. *In:* A. Mizrahi and A. L. VanWezel (eds.), Advances in Biotechnological Processes, pp. 111–139. New York: Alan R. Liss, Inc., 1984.
30. Mayhew, E. G., Goldrosen, M. H., Vaage, J., and Rustum, Y. M. Effects of liposome-entrapped doxorubicin on liver metastases of mouse colon carcinomas 26 and 38. J. Natl. Cancer Inst., *78:* 707–713, 1987.
31. FitzGerald, D. J. P., Trowbridge, I. S., Pastan, I., and Willingham, M. C. Enhancement of toxicity of antitransferrin receptor antibody-*Pseudomonas* exotoxin conjugates by adenovirus. Proc. Natl. Acad. Sci. USA, *80:* 4134–4138, 1983.
32. Pearson, J. W., FitzGerald, D. J. P., Willingham, M. C., Wiltrout, R. H., Pastan, I., and Longo, D. L. Chemoimmunotoxin therapy against a human colon tumor (HT-29) xenografted into nude mice. Cancer Res., *49:* 3562–3567, 1989.
33. FitzGerald, D. J., Willingham, M. C., and Pastan, I. Antitumor effects of an immunotoxin made with *Pseudomonas* exotoxin in a nude mouse model of human ovarian cancer. Proc. Natl. Acad. Sci. USA, *83:* 6627–6630, 1986.
34. Griffin, T. W., Richardson, C., Houston, L. L., LePage, D., Bogden, A., and Raso, V. Antitumor activity of intraperitoneal immunotoxins in a nude mouse model of human malignant mesothelioma. Cancer Res., *47:* 4266–4270, 1987.
35. Willingham, M. C., FitzGerald, D. J., and Pastan, I. *Pseudomonas* exotoxin coupled to a monoclonal antibody against ovarian cancer inhibits the growth of human ovarian cancer cells in a mouse model. Proc. Natl. Acad. Sci. USA, *84:* 2474–2478, 1987.
36. Bjorn, M. J., and Groetsema, G. Immunotoxins to the murine transferrin receptor: intracavitary therapy of mice bearing syngeneic peritoneal tumors. Cancer Res., *47:* 6639–6645, 1987.
37. Middlebrook, J. L., and Dorland, R. B. Response of cultured cells to the exotoxins of *Pseudomonas aeruginosa* and *Corynebacterium diphtheriae*: differential cytotoxicity. Can. J. Microbiol., *23:* 183–189, 1977.
38. Vasil, M. L., and Iglewski, B. H. Comparative toxicities of diphtheria toxin and *Pseudomonas* exotoxin A. Evidence for different cell surface receptors. J. Gen. Microbiol., *108:* 333–337, 1978.
39. Pastan, I., Willingham, M. C., and FitzGerald, D. J. P. Immunotoxins. Cell, *47:* 641–648, 1986.
40. FitzGerald, D. J., Willingham, M. C., Cardarelli, C. O., Hamada, H., Tsuruo, T., Gottesman, M. M., and Pastan, I. A monoclonal antibody-*Pseudomonas* toxin conjugate that specifically kills multidrug-resistant cells. Proc. Natl. Acad. Sci. USA, *84:* 4288–4292, 1987.
41. Byers, V. S., Pimm, M. V., Scannon, P. J., Pawluczyk, I., and Baldwin, R. W. Inhibition of growth of human tumor xenografts in athymic mice treated with ricin toxin A chain-monoclonal antibody 791T/36 conjugates. Cancer Res., *47:* 5042–5046, 1987.