Therapy of Human Cervical Carcinoma with Monoclonal Antibody-\textit{Pseudomonas} Exotoxin Conjugates

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

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

\textit{Pseudomonas} exotoxin A (PE) linked to the F(ab')\textsubscript{2} fragment of 1H10, a murine monoclonal antibody recognizing a carbohydrate epitope 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')\textsubscript{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. \textit{Pseudomonas} exotoxin has been shown to form potent immunotoxins (9-12). PE\textsuperscript{-} 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')\textsubscript{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 \textit{Pseudomonas} exotoxin to the F(ab')\textsubscript{2} fragment of Mab 1H10 by disulfide as well as thioether bonds. We show that PE linked to Mab 1H10 F(ab')\textsubscript{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 IEF Biotechnics, Villeneuve-la-Garenne, France. Rabbit anti-mouse IgG was from Zymed Laboratories, Inc., San Francisco, CA, while all other immunochromatals as well as 2-iminohydantoin and NAD\textsuperscript{+} were obtained from Sigma Chemical Company, St. Louis, MO. Nicotinamide [\textsuperscript{14}C]adenine dinucleotide (220 mCi/mmol) was purchased from Amersham International Plc, Amersham, Buckinghamshire, England. [\textsuperscript{3}H]Leucine (50 Ci/ mmol) and \textit{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 \mu g/ml streptomycin. Cells were routinely tested for \textit{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...
A blood group antigen. Mab HK-PEG-1 (ATCC 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 AcaC 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 2.5 ± 0.3 (2-pyridyl-dithio)propionyl groups or maleimido groups were introduced with 3-(2-pyridyl-dithio)propionic acid N-hydrox 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-iminomutathione into each PE when measured as described by FritzGerald (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 [3H]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. [3H]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

\[
\text{Sensitivity ratio} = \frac{(I_{\text{CaSki}})_{\text{PE}}}{(I_{\text{CaSki}})_{\text{1H10-PE}}}
\]

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

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 Vitro Localization of Mab 1H10. Mab 1H10 and control antibody HK-PEG-1 F(ab')2 fragments were labeled with ^32P 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 radiolabeled 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 0 μl 110. Mice 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. 1A, Lane 1). This molecular weight corresponds to a conjugate containing 1 molecule each of PE (M, 66,000) and Mab 1H10 F(ab')2 (M, 110,000). The conjugate contained both PE and immunoglobulin as shown by immunooblots of 1H10-PE for IgG (Fig. 1B, Lane 1) and PE (Fig. 1C, 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. 24). 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. 2B).

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.
cells with IC_{50} 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. 4A). 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. 4B). 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. ^{125}I-Labeled Mab 1H10 F(ab')_2 clearly localized in established CaSki tumor xenografts (Fig. 5B). Localization was specific inasmuch as ^{125}I-labeled Mab 1H10 F(ab')_2 did not localize in a control tumor xenograft (Fig. 5C) nor did a control antibody localize in CaSki xenografts (Fig. 5D).

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. 6A). 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. 6B). 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/V 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

![Image](cancerres.aacrjournals.org)
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 \( V_V / V_c \) 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...
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Fig. 5. In vivo localization of 131I-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 131I-labeled Mab 1H10 F(ab’)2. (C) Imaging of a control H2669 melanoma xenograft with 131I-labeled Mab 1H10 F(ab’)2. (D) Imaging of a CaSki xenograft with 131I-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 IC50 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 (37–39), 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...
Mab 1H10 F(ab')2 also had no effect on cell growth in or to the different growth characteristics of the tumors studied. Administration i.v. of Mab 1H10 F(ab')2 alone appeared to fragments to penetrate more extensively into the tumor mass, to toxins used to form the immunotoxins, to the ability of F(ab')2 resulting from 1H10-PE treatment may be due to the different The more effective long term suppression of tumor growth result can be contrasted with the treatment of solid osteogenic sarcoma tumors by multiple injections of a ricin A chain-IgG for at least 30 days after the termination of treatment. This deaths.* ds. disulfide-linked immunotoxin; te. thioether-linked immunotoxin. 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. Mam 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.

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