Monoclonal Antibody C242–*Pseudomonas* Exotoxin A

A Specific and Potent Immunotoxin with Antitumor Activity on a Human Colon Cancer Xenograft in Nude Mice

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Abstract

Two immunotoxins were constructed by chemically coupling the monoclonal antibody C242 to *Pseudomonas* exotoxin A (PE) or a modified form, NlysPE40, that lacks the cell binding domain of PE. Monoclonal antibody C242 recognizes a specific sialylated carbohydrate epitope on a high molecular weight membrane glycoprotein present on cells of human colon, pancreatic, and cervical cancers. C242-PE and C242-NlysPE40 were very cytotoxic for cells expressing this antigen with 50% inhibition of protein synthesis occurring on Colo205 cells at 0.2 ng/ml (0.9 pM) for C242-PE and 6.0 ng/ml (31 pM) for C242-NlysPE40. The two immunotoxins also exhibited a strong antitumor effect on a human colon cancer xenograft grown in nude mice. The specificity and potency of these two C242 immunotoxins warrant their further development for the treatment of cancer. (*J. Clin. Invest.* 1992. 90:405–411.) Key words: colon cancer • immunotoxins • *Pseudomonas* exotoxin A • solid tumor therapy

Introduction

A number of human cancers, such as those in the colon and pancreas, are very resistant to presently available methods of treatment. In the search for an alternative or supplemental therapy more than a decade of research has revived a simple idea of specific targeting of toxic agents to cells causing disease (1). One of the possible ways to accomplish this goal is to find an "identification marker" on cancer cells in the form of a unique antigen not shared with normal tissues. By means of hybridoma monoclonal antibody technology, this antigen could be used to produce an antibody directed specifically against cells expressing the marker (2). Then, the monoclonal antibody coupled, for example, to a bacterial toxin would selectively bind to the cancer cells and deliver the lethal toxin. This concept has been reviewed in several recent articles (1, 3, 4). However, for this therapy to be successful there are several problems to be solved. One is to produce an antibody fulfilling the minimum criteria of specificity. Other problems revolve around the toxin portion of the immunotoxin, namely, for the toxin portion to have low nonspecific toxicity and low immunogenicity (5, 6).

We have been working on developing immunotoxins based on *Pseudomonas* exotoxin A,[1] (PE) as reported in Pastan and FitzGerald (4). PE is a 66-kD single-chain protein composed of three structural and functional domains: I, II, and III (5, 7). Domain I contains the cell receptor-binding sequence, domain II takes part in translocation and proteolytic cleavage of the toxin, and domain III catalyzes the ADP-ribosylation and inactivation of elongation factor 2 (4). To construct an immunotoxin, PE can be used either as the native molecule or in a truncated form, called PE40. PE40 lacks domain I, the cell-binding domain, but retains all the functions of domains II and III (8, 9). The efficacy of immunotoxins made with PE or PE40 has been demonstrated in vitro on cultured cells as well as in mice bearing intraperitoneal and subcutaneous tumors (9–11).

Recently, the monoclonal antibody C242 that reacts with human colon carcinoma cells was isolated.[2] The C242-reactive antigen is a carbohydrate attached to membrane proteins.[3] We were attracted by this antibody because of the strong and fairly uniform reactivity it displayed with > 70% of human colon cancers (our unpublished observation) as well as with pancreatic and cervical cancer samples (12, 13).[2] C242 exhibits very little and weak cross-reactivity with vital normal human and monkey tissues. This makes C242 an excellent candidate for targeting toxins to human cancer. We report here that C242-PE and C242-PE40 are very active and specific immunotoxins which show strong antitumor activities on a solid tumor growing subcutaneously in nude mice.

Methods

Bacterial strains and plasmids. Plasmid pMS8, encoding a derivative of PE40, NlysPE40, containing an extra lysine at the amino end, was propagated in the HB101 strain of *Escherichia coli* and expressed in strain BL21 (λDE3) as described (14). pMS8 was prepared by ligating

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1. *Abbreviations used in this paper:* HSA, human serum albumin; PE, *Pseudomonas* exotoxin A.

2. Lindholm, L., C. Johansson, O. Nilsson, G. Lindholm, D. Baeckstrom, B. Karlsson, and B. Engaras. New human carcinoma-associated antigens defined by monoclonal antibodies obtained after immunization with colon adenocarcinoma cell lines. Manuscript in preparation.

3. Baeckstrom, D., G. C. Hansson, O. Nilsson, C. Johansson, S. J. Gendler, and L. Lindholm. A membrane bound and a secreted mucin-type glycoprotein carrying the carcinoma-associated sialyl-Lewis^a epitope show distinct core protein structures. Manuscript submitted for publication.

an oligonucleotide duplex to the plasmid pVC8 f(+)T (14) linearized with Nde I restriction endonuclease. The sequence of the linker was as follows:

5′ TATGCTGCAGGGTACCAAGCT 3′

3′ ACGACGTCCCATGGTTCGAAT 5′

A variant of NlysPE40, NlysPE38, which has amino acids 365–380 in domain Ib of PE deleted, was also used where indicated (15).

Expression and purification of NlysPE40. BL21 (λDE3) were transformed with plasmid pMS8 and grown in a fermentor (ML-4100, New Brunswick Scientific Co., Inc., Edison, NJ) in 10 liter of Super-Broth with 100 μg/ml ampicillin under conditions to be described elsewhere. Cells were allowed to grow until the absorbance at 650 nm had reached an OD of 2.0 at which point isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM. Cells were harvested 90 min later and centrifuged to give a bacterial pellet. Then, the bacteria were first hyper-, and subsequently, hypoosmotically shocked in order to prepare the periplasm in which NlysPE40 is predominantly found. NlysPE40 was purified by using a fast protein liquid chromatography (FPLC) system (Pharmacia Inc., Piscataway, NJ). Periplasm containing NlysPE40 in 20 mM Tris-HCl, pH 7.4 (usually 100 ml) was loaded on an 8 ml Q-Sepharose column (Pharmacia Inc.) and proteins bound to the column were eluted with a linear gradient of NaCl. Fractions enriched in NlysPE40 (verified by SDS-PAGE) were eluted usually with 0.2–0.3 M NaCl and pooled. The pool of the toxin was further diluted 1:5 or 1:10 in 20 mM Tris, pH 7.4, and loaded on a Mono-Q column (10/10, Pharmacia Inc.). Again, a linear gradient of NaCl was applied to elute NlysPE40. Fractions containing a monomer of NlysPE40 were pooled and concentrated on Centricon 10 (model YM/10, Amicon Corp., Danvers, MA) membranes to a volume < 5 ml. This preparation was injected onto a sizing column, TSK250 (60 × 2.25 cm, Bio-Rad Laboratories, Richmond, CA), equilibrated with 0.1 M NaH_2PO_4, pH 7.0, in 1 mM EDTA. NlysPE40 was then purified to near the homogeneity under isocratic elution conditions. PE was obtained from Swiss Serum Co., Berne, Switzerland.

Construction of immunoconjugates. To prepare immunotoxins, we used a protein modification reagent and a heterobifunctional crosslinker to form a thioether linkage between the two proteins. 2-iminothiolane-HCl (IT) (16) was used to provide C242 with sulfhydryl groups and succinimidyl 4-(*N*-maleimidomethyl) cyclohexan 1-carboxylate (SMCC) (17) served to provide the toxin with maleimides ready to react with thiols present on the derivatized antibody. C242 exhibited 0.8, 1.6, and 2.4 thiols per mole when subjected to 5-, 10-, and 25-fold molar excess of IT, respectively. This reaction was performed in 0.1 M NaH_2PO_4/1 mM EDTA, pH 8.0, in 1 mM β-2-mercaptoethanol at 37°C for 30 min. The number of sulfhydryl groups was determined using Ellman's reagent (18). Derivatized antibody was separated from the reactants by gel filtration on Sephadex G-25 (PD10 column, Pharmacia Inc.) equilibrated with 0.1 M NaH_2PO_4/5 mM EDTA, pH 6.0, C242 with 1.6 thiols per mole gave the highest yield of conjugate.

PE and NlysPE40 were derivatized with threefold molar excess of SMCC in 0.1 M NaH_2PO_4, pH 7.0, during a 1-h incubation at room temperature. Toxins were separated from the crosslinker on a PD10 column. Derivatized C242 and PE or NlysPE40 were mixed together in a 1:3 molar ratio and left at room temperature for an overnight incubation. To block unreacted groups, iodoacetamide was added in a final concentration of 1 mM for 20 min at room temperature. Then, the immunotoxins were purified by the two-step liquid chromatography, as previously described (9). As a negative control, we used monoclonal antibody MOPC-21 (Sigma Chemical Co., St. Louis, MO), which has no known reactivity with mouse or human tissues, and monoclonal antibody B3 (11), which does not react with Colo205 cells. They are both IgG1κ as is MAb C242. Both MOPC-21 and B3 were conjugated to PE or NlysPE40 by the method described above.

Protein synthesis inhibition and cytotoxicity assays. C242-PE immunotoxins were tested on Colo205, HT29, KATO III, DU145, and A431 cells and their activities determined by measuring inhibition of [^{3}H]leucine incorporation into cells (5). ID_{50} indicated the concentration of immunotoxin at which the isotope incorporation fell by 50% when compared to nontreated cells. For the cytotoxicity assay, the cells were plated at 2×10^4 per well in 24-well plates 24 h before addition of immunotoxin. The cells were incubated for 3, 4, and 7 d and viable cells were identified by methylene-blue staining (5).

Binding studies. We attempted to determine the binding ability of C242 immunotoxins as compared to the antibody alone. To this end, we labeled C242 with ^{125}I (New England Nuclear, Boston, MA) using a lactoperoxidase method, exactly as described (19). C242 (8 μg) was mixed with 0.5 mCi of the isotope. Labeled antibody was separated from unreacted isotope on a PD10 column. The fractions collected from gel filtration were enriched in human serum albumin (HSA) to 0.2% (wt/vol) final concentration. We estimated the binding ability of labeled C242 according to a previously described protocol (20), with slight modification. Binding assays were done by a standard saturation and displacement curves analysis. Briefly, Colo205 cells were seeded at 2×10^5 cells per well in a 24-well tissue culture plates 24 h before the experiment. The plates were placed on ice and cells were washed with ice-cold PBS without Ca^{++}, Mg^{++} in 0.2% BSA. Then, increasing concentrations of C242 or C242 immunotoxin were added to cells, and incubated for 30 min before the addition of fixed amount of ^{125}I-C242 for 1.5 h. After incubation, the cells were washed twice and lysed with 0.1N NaOH, and the radioactivity was counted.

Treatment of nude mice bearing human colorectal cancer. Colo205 cells (3×10^6 per mouse) were injected subcutaneously on day 0 into female nude mice. Tumors developed in all injected animals and the tumor size reached around 5 × 4 mm on days 7–8. The mice started to receive immunotoxins on either day 1 or day 8. Each treatment group was composed of four to six mice. Tumors were measured with a caliper every fourth day and the formula for tumor volume calculation was as previously reported (9).

Tumors from several treated and nontreated animals were removed from the mice either for immunoperoxidase histochemistry or for preparation of the primary culture of Colo205 cells derived from tumors. In addition, some animals bearing large tumors (> 450 mm^3) were bled and their sera were checked for the presence of inhibitory substances by incubating serum samples with different immunotoxin concentrations and then testing the mixture on Colo205 cells and measuring [^{3}H]leucine incorporation. Blood was withdrawn from the orbital vein under methoxyflurane anesthesia.

Results

In the present investigation, we used PE and PE40 to make chemical conjugates with the anti-human colorectal cancer monoclonal antibody, C242. The form of PE40 used in this study, termed NlysPE40, has 11 amino acids (MLEGT*K*LMAEE; Fig. 1) preceding Gly^{253} of PE; domain II of PE begins at amino acid 253. This sequence was confirmed by the amino acid analysis using repeated cycles of the Edman degradation reaction. The lysine residue introduced at the amino terminus of PE40 is believed to facilitate coupling to antibody (9). We have chosen this form of PE40 because expression levels are higher than with another lysPE40 version (9), and because it is found primarily in the periplasm, easing the process of toxin purification. In addition, immunotoxins made with NlysPE40 and MAbs HB21 (see below) or C242 exhibited similar cytotoxic activities as immunotoxins made with the previously described form of lysPE40 (9).

Cytotoxicity of C242 immunotoxins. To evaluate the cytotoxic activities of C242-PE and C242-NlysPE40 we tested various human cancer cell lines with different amounts of the C242-reactive antigen on their surface (Table I). We compared these activities with that of an anti-human transferrin receptor

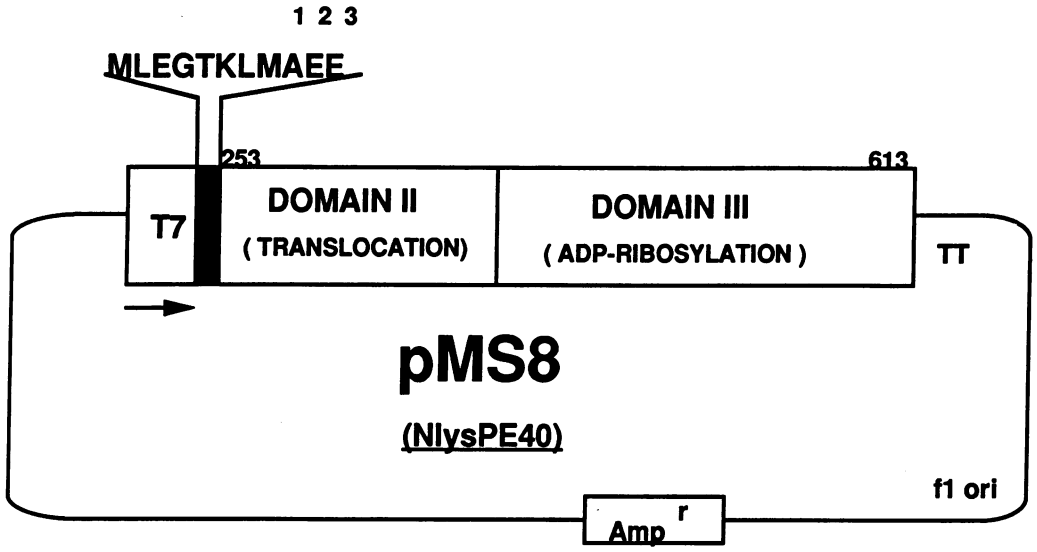

Figure 1. Plasmid pMS8 which encodes NlysPE40. DNA encoding the toxin was cloned into the vector with an ampicillin resistance gene (*Amp*^{r}) and an f1 origin of replication (*f1 ori*). The toxin gene is under the control of the T7 phage promoter. A transcription terminator (*TT*) is placed after the open reading frame. Translation starts with methionine separated by seven amino acids (including one lysine residue) from sequences found in PE. The numbers indicate positions of amino acids in PE.

MAb (HB21) conjugated to PE toxins (9). Colo205 cells, which uniformly express the C242 antigen, were very responsive to C242 immunotoxins; the ID_{50} for C242-PE was found to be 0.2 ng/ml and for C242-NlysPE40 it was 6.0 ng/ml. These values are comparable to those obtained with immunotoxins made by using the MAb HB21 that reacts with the human transferrin receptor. C242 antigen density and uniformity, as measured by immunofluorescence, was less on several other tested cell lines, such as HT29 (Table I), KATO III, and DU145 (not shown), and absent on ovarian (OVCAR-3, OVCAR-4, 1847, 2780), breast (MCF-7, SKOV-3), cervical (KB), and gastric (AGS) carcinoma cell lines. This antigen was also completely absent on human epidermoid carcinoma A431 cells (Table I). The relative amount of the C242-reactive antigen on the cell surface was related to the susceptibility of these cells to treatment with C242 immunotoxins, i.e., the greater amount of antigen, the greater response to C242-PE or C242-NlysPE40. A431 cells were not killed by C242 immunotoxins, however, they were very responsive to HB21 conjugates, as expected (9). These results demonstrate that C242 immunotoxins specifically target cells which express the appropriate antigen. To determine if exposure to C242 immunotoxin results not only in the inhibition of protein synthesis but also in cell death, we performed a cytotoxicity assay. C242-NlysPE40 added to Colo205 cells killed 50% of these cells at a concentration of 0.5 ng/ml and > 95% of cells at a concentration of 5.0 ng/ml after 3 d of incubation.

To ascertain further the specificity of C242 conjugates on cells expressing the C242 antigen, we employed the competition assays illustrated in Fig. 2 *A*. C242 MAb alone did not have any effect on the protein synthesis of Colo205 cells at concentrations up to 50 μg/ml. However, C242, added at a 500-fold excess was able to block completely the cytotoxic effect of C242-NlysPE40 and to diminish it by 50% when added at a 50-fold excess. On the other hand, C242 did not interfere with the cytotoxic effect of HB21-NlysPE40. Colo205 cells were also subjected to treatment with B3-NlysPE38 conjugates with or without MAb C242 (Fig. 2 *B*). B3 immunotoxins did not affect protein synthesis at concentrations up to 1 μg/ml, whether or not C242 was present. These results indicate a highly specific action of the C242 conjugates.

We also measured to what extent the affinity of the C242 MAb for the C242-reactive antigen had been changed when chemically attached to PE. Binding efficiencies of C242, C242 modified with IT and C242-NlysPE40 to Colo205 cells were established in a competition assay with ^{125}I-C242. C242 displaced the labeled antibody with an IC_{50} of 0.8×10^{-8} M. C242 antibodies which had thiol groups introduced into the molecule demonstrated a similar competitiveness to the nonderivatized antibody (data not shown). This suggests that the antibody binding region was not altered by reaction with the thiolating agent which is the first step in the production of immunotoxin. However, C242-NlysPE40 competed for the binding of ^{125}I-C242 less efficiently than the antibody alone

Table I. Activity of C242 and HB21 Chemical Conjugates with PE Toxins on Various Human Cancer Cell Lines

Tumor	Cell line	ID_{50} C242-PE	ID_{50} C242-NlysPE40	ID_{50} HB21-PE	ID_{50} HB21-NlysPE40	C242 antigen
		ng/ml				*% of cells*
Colon	Colo205	0.2	6	0.03	4	100% homogeneous
Colon	HT29	38.0	ND	0.06	ND	50% heterogeneous
Adenocarcinoma	A431	>1,000	>1,000	0.02	0.8	negative

ND, not determined.

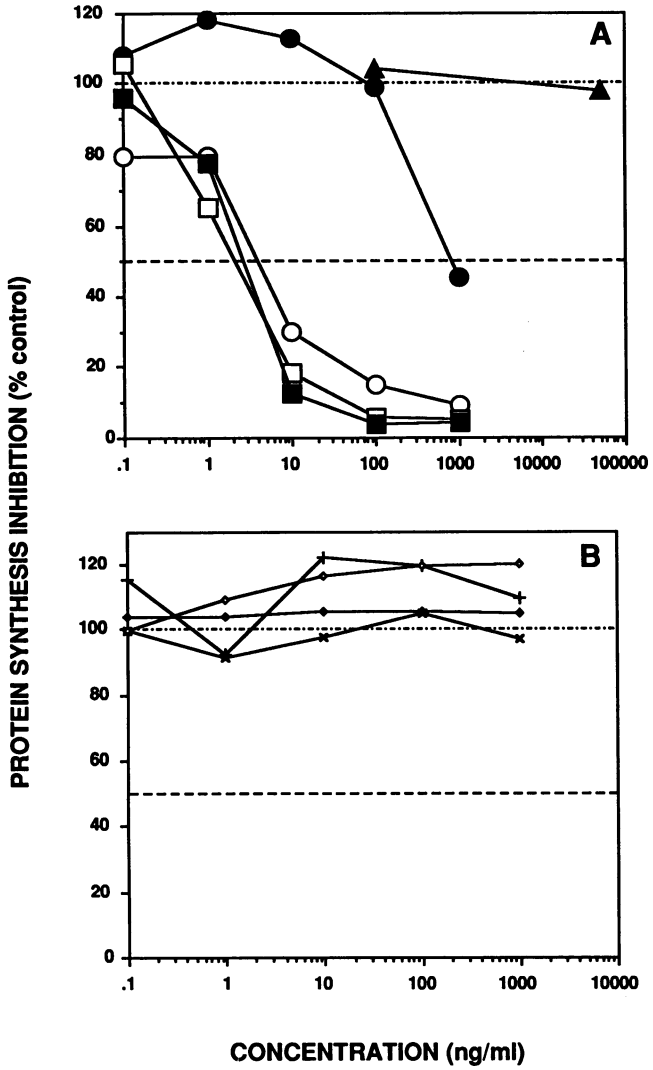

Figure 2. Inhibition of protein synthesis in Colo205 cells by C242-NlysPE40, HB21-NlysPE40 (*A*) and B3-lysPE38, B3-NlysPE38 (*B*). C242-NlysPE40 at various concentrations was added in the absence (○) or presence (●) of excess C242 (50 μg/ml) for 20 h at 37°C. The same was done with HB21-NlysPE40 added without (□) or with (■) C242 at 50 μg/ml, B3-lysPE38 without (◇) or with (◆) C242, and B3-NlysPE38 without (+) or with (×) C242. C242 alone (▲) was also added to the assay. Isotope incorporation was measured as described in the methods section. The interrupted line shows 50% of [^{3}H]leucine incorporation and the line with alternating dots and dashes represents isotope incorporation in the control.

with an IC_{50} threefold higher than that for C242 (Fig. 3). Thus, C242-NlysPE40 has decreased binding to the C242 antigen when compared to the antibody alone.

Antitumor activity of C242-PE and C242-NlysPE40. MAb C242 conjugated to different forms of PE was shown to be specific and very active at killing cancer cells in culture. To determine whether C242 immunotoxins are also active in vivo, we tested these compounds for their ability to inhibit the growth of Colo205 subcutaneous xenografts in nude mice. Colo205 tumors are relatively fast growing tumors and on days 7–8 they reach a size of 5 × 4 mm in planar dimension (Fig. 4). In the first study we administered C242-NlysPE40 at a dose of 20 μg per mouse intraperitoneally (i.p.) 1, 3, 5, and 7 d after tumor implantation (Fig. 4 *A*). This treatment markedly inhibited the growth of Colo205 tumors, however, the difference between treated and nontreated animals was on the border of significance at the end of observation, owing to the unusually high variability of the results. Next, we delayed treatment until solid tumors developed. As seen in Fig. 4, *B* and *C*, the treatment was initiated on day eight after tumor implantation and consisted of four i.p. injections of different amounts of conjugate every second day. C242-NlysPE40 (Fig. 4 *B*) and C242-PE (Fig. 4 *C*) were very effective at slowing the growth of Colo205 tumors. C242-NlysPE40 exhibited a striking effect at 20 μg per mouse per day i.p., and the tumors in the immunotoxin treated animals were significantly smaller ($P < 0.001$; unpaired Student's *t* test) than in the vehicle treated group on day 24. B3-NlysPE38 administered similarly (Fig. 4 *B*) did not have any effect on tumor growth. Increasing the dose to 50 μg of C242-NlysPE40 per mouse did not result in a better response (not shown). C242-PE at a dose of 0.5 μg per mouse effectively inhibited tumor growth and on day 24 the immunotoxin-treated mice had significantly smaller tumors ($P < 0.01$) than the C242 Mab alone treated group. This was the highest dose tolerated by the tumor-bearing mice; one animal out of five died because of the treatment. The LD_{50} for C242-PE was established to be 0.5 μg per mouse and 125 μg for C242-NlysPE40 in non-tumor-bearing Balb/c mice. The effect of C242-PE treatment was dose dependent because 0.375 (Fig. 4 *C*), 0.250, and 0.125 μg of C242-PE (not shown) had significantly lower antitumor activities than 0.5 μg. In control experiments we prepared an immunotoxin with MAb MOPC-21, an isotype-matched antibody that does not react with the tumor or mouse tissue. Neither C242 nor MOPC-21-PE had any effect on the growth of Colo205 tumors (Fig. 4 *D*). These results demonstrate that C242-PE and C242-NlysPE40 are very effective and specific antitumor agents.

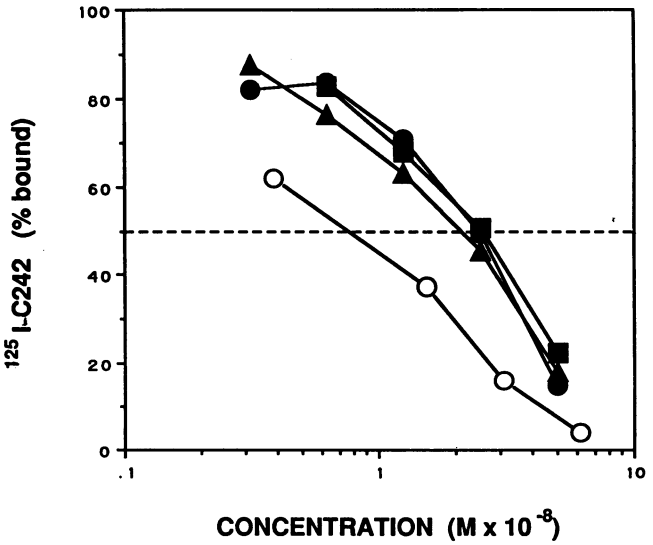

Figure 3. The ability of C242 (○) and C242-NlysPE40 to displace the binding of ^{125}I labeled C242 to Colo205 cells. C242 with either 0.8 (■), 1.6 (●), or 2.4 (▲) mol of thiol per mol of antibody was conjugated to NlysPE40 and added to cells at various concentrations. ^{125}I-C242 had a specific activity of 20 μCi/μg and 30,000 cpm of bindable C242 was added into the assay. Each point represents the average of four determinations.

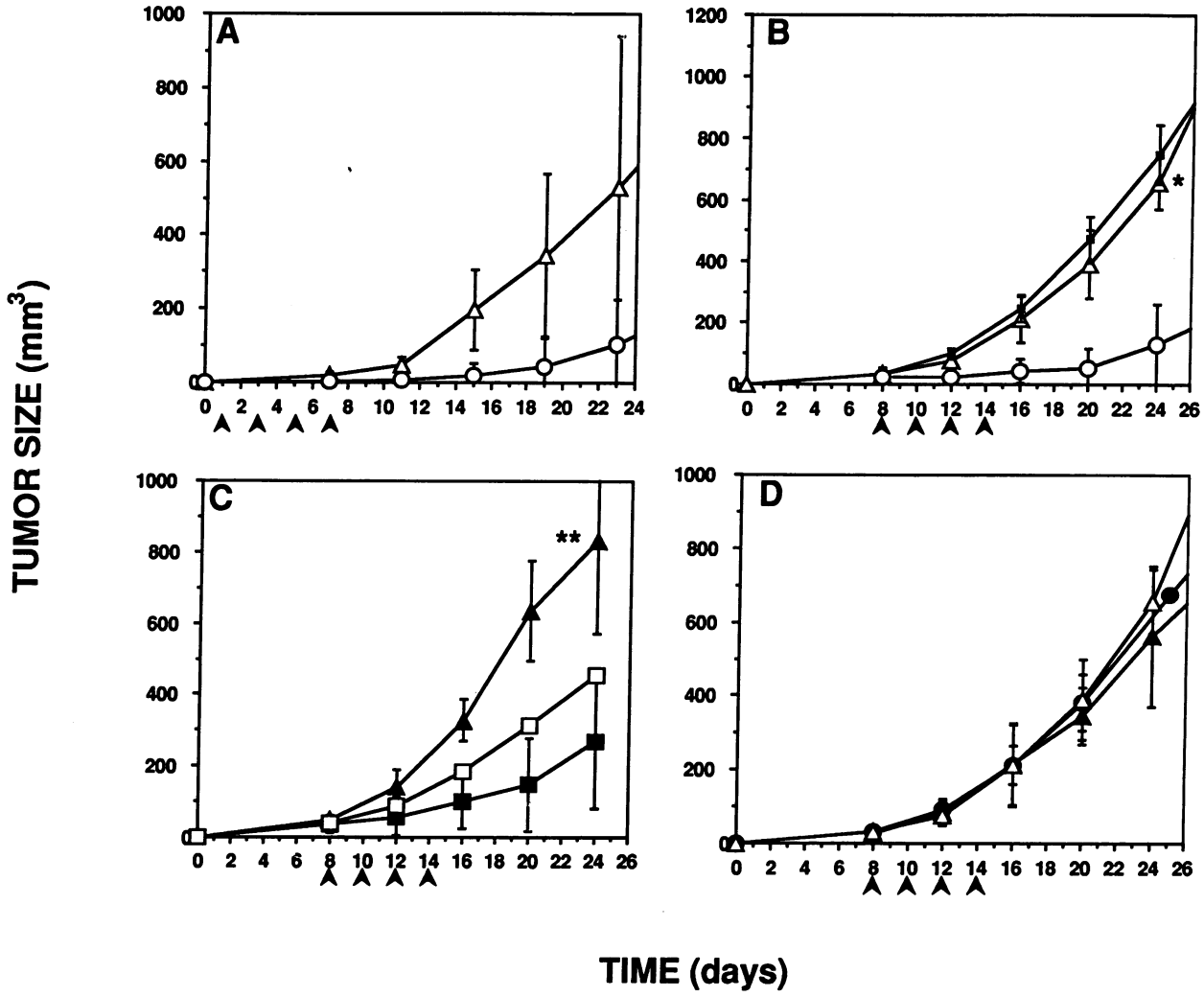

Figure 4. Antitumor effect of C242-NlysPE40 and C242-PE on Colo205 xenografts in nude mice. Mice were injected with 3×10^6 cells s.c. and treated i.p. on days indicated by arrows. (*A,B*) Mice received the vehicle (△) or 20 μg of C242-NlysPE40 (○), or B3-NlysPE38 (*). (*C*) Mice were given 0.375 μg of C242-PE (□), 0.5 μg of C242-PE (■), or vehicle (▲). (*D*) Mice were given no treatment (△) or 37.5 μg of C242 antibody alone (▲) or MOPC-21-PE (●). Vertical bars in A, B, C and D indicate standard deviations of individual points. Statistically significant difference between: C242-NlysPE40 and the vehicle-treated group, $*P < 0.001$; C242 and C242-PE 0.5 μg, $**P < 0.01$.

Although the antitumor effect of C242 conjugates was very significant, it did not cause a complete regression of tumors, as was previously observed using immunotoxins that react with A431 tumors (9, 11). To understand this discrepancy, we performed several control experiments. We treated mice bearing Colo205 tumors with HB21-PE immunotoxin which has a higher cytotoxic activity in culture on Colo205 cells than C242-PE (Table I), to see whether it would exert a better antitumor effect. We found that HB21-PE did not evoke a complete regression of Colo205 tumors (data not shown). Thus, the failure to cause complete regression of Colo205 tumors was not unique to C242 immunotoxins. We also took into account the fact that the C242 antigen is a shed antigen in mice xenografted with Colo205 cells.[2] Therefore, it was important to establish if there was an abundant amount of circulating C242 antigen blocking the action of immunotoxin. The results of an experiment that addressed at this possibility are shown in Table II. Various concentrations of C242-NlysPE40 were incubated with sera of mice bearing large Colo205 tumors (> 450 mm^3). Then, the preincubated toxin was added at various concentrations to Colo205 cells and a protein synthesis inhibition assay was performed. There was no significant inhibition of immunotoxin activity by preincubation with mouse sera. Thus, it seems very unlikely that the blood of mice with Colo205 tumors contains sufficient C242 antigen to neutralize C242 immunotoxins.

To determine whether Colo205 cells which form the tumor become C242 antigen-negative, we removed tumors from the same mice from which the blood was withdrawn for the neutralizing study and verified the expression of the C242 antigen by immunoperoxidase histochemistry. C242 antigen as well as transferrin receptors were strongly and uniformly localized in these tumors (data not shown). To determine if the tumor cells had become resistant to the immunotoxins by a mechanism different from antigen depletion, we prepared primary cultures from treated tumors and subjected these cells to treatment with immunotoxins. The results of this experiment are presented in Fig. 5. C242 and HB21 immunotoxins had approximately the same ID_{50}'s on the reisolated tumor cells (Fig. 5) as on the original cell line that produced the tumors (Table I). The last

two experiments indicate that Colo205 cells do not change their characteristics when grown in animals with regard to C242 antigen expression and susceptibility to the toxic action of C242-NlysPE40.

Discussion

Specificity and potency of C242 immunotoxins. In the present study we employed C242 MAb which showed high specificity towards human colon cancer.[2] To prepare immunotoxins, C242 was conjugated either with PE or NlysPE40, a recombinant form of PE lacking the cell-binding domain I. Both immunotoxins exhibited high cytotoxic activities against cultured cell lines but only against those bearing the appropriate antigen. We also demonstrated that the action of C242-NlysPE40 immunotoxin was competitively inhibited by excess unconjugated C242, further underlining the specificity of C242 immunotoxin targeting.

Antitumor activity of C242-PE and C242-NlysPE40. We were able to treat nude mice bearing human colon cancer xenografts with very similar doses of C242 immunotoxins as with other immunotoxins used in our laboratory (9, 11). Using these doses (0.5 μg for C242-PE and 20 μg for C242-NlysPE40) we achieved a significant degree of antitumor activity by i.p. administration. C242 immunotoxins were active when they were administered both 1 and 8 d after tumor implantation when the tumors had grown to form a detectable solid tumor. The injections of immunotoxins were repeated four times every other day which was well tolerated by the animals. However, we did not observe complete tumor regression as seen previously with B3-PE or B3-lysPE40 (11), and several factors may account for this result.

Colo205 tumor model in mice. In previous studies we have found that increasing the dose of an immunotoxin increases the antitumor activity (9, 11). Unexpectedly, in this study increasing the dose of C242-NlysPE40 from 20 to 50 μg per dose did not result in an increase in activity. We ruled out the possibility that the human cells had lost their response to C242-NlysPE40 by isolating cells from treated tumors and showing both that they had surface antigen and responded normally to C242-NlysPE40 when put back into culture. Because C242 is known to be a shed antigen we looked for blocking activity in the blood of tumor bearing mice and could not detect any. Currently, we favor the idea that there is a poor penetration of immunotoxins into Colo205 tumors, perhaps owing to the relatively poor vascularization of this tumor (21). Unfortunately, the Colo205 cell line is the one yet identified model that reacts uniformly with the C242 and grows as a tumor in mice. We tested many other carcinoma cell lines for the presence of the C242-reactive antigen, but this search did not reveal any suitable one for our studies.

Table II. Failure of Serum from Tumor-bearing Mice to Block the Cytotoxic Action of C242-NlysPE40 on Colo205 cells

Mouse no.	Tumor size	Protein synthesis inhibition assay (a)	(b)	(c)
	mm^3		% control	
1	979	31	9	89
2	486	49	8	79
3	749	42	11	88
4	806	36	5	98
5	560	—	9	113
6	946	38	8	111
HSA		40	15	123

(*a*) 5 ng of immunotoxin incubated in 98% serum—6.25 ng/ml added to assay. (*b*) 50 ng of immunotoxin incubated in 95% serum—62.5 ng/ml added to assay. (*c*) mouse serum without immunotoxin—20 μl/ml added to assay.

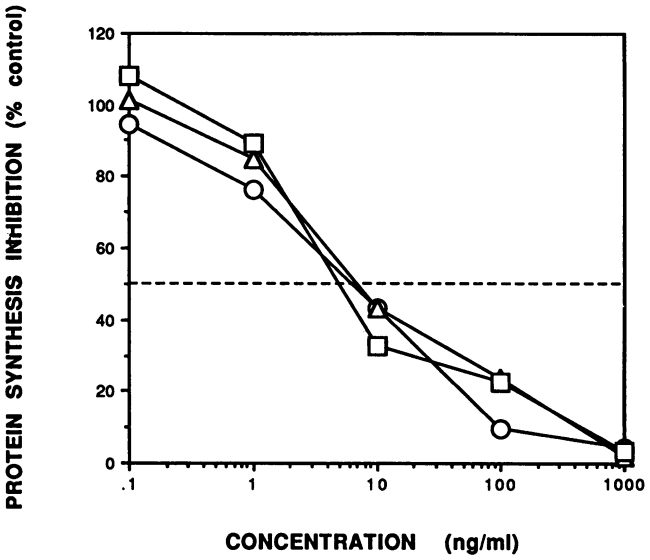

Figure 5. Inhibition of protein synthesis in cells isolated from Colo205 tumor. C242-NlysPE40 (□), C242-lysPE40 (9) (○), and HB21-NlysPE40 (△) were added at various concentrations for 20 h at 37°C. The interrupted line indicates 50% incorporation of [^{3}H]leucine.

Binding efficiency of C242-NlysPE40. Conjugation of C242 to NlysPE40 results in a threefold loss of affinity towards the antigen. This is probably due to some type of steric effect caused by the toxin being attached close to the antigen binding site, since derivatization of the MAb without coupling it to NlysPE40 had no effect on binding. It would be desirable to look for other methods of attaching PE40 or PE to C242 which would not compromise the efficiency of binding to its antigen. This would probably render immunotoxins considerably more active.

Summary. C242 MAb specifically recognizes an antigen which is uniformly present on certain types of human cancer, but only on a limited number of established cancer cell lines and normal human tissues. C242 immunotoxins caused a very significant retardation of tumor growth in nude mice and warrant further evaluation for the treatment of cancer.

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References

1. Pastan, I., M. C. Willingham, and D. J. FitzGerald. 1986. Immunotoxins. *Cell.* 47:641–648.

2. Koprowski, H., Z. Steplewski, D. Herlyn, and M. Herlyn. 1978. Study of antibodies against human melanoma produced by somatic cell hybrids. *Proc. Natl. Acad. Sci. USA.* 75:3405–3409.

3. Vitetta, E. S., R. J. Fulton, R. D. May, M. Till and J. M. Uhr. 1987. Redesigning nature's poisons to create anti-tumor reagents. *Science (Wash. DC).* 238:1098–1104.

4. Pastan, I., and D. FitzGerald. 1989. *Pseudomonas* exotoxin: chimeric toxins. *J. Biol. Chem.* 264:15157–15160.

5. Hwang, J., D. J. FitzGerald, S. Adhya, and I. Pastan. 1987. Functional domains of *Pseudomonas* exotoxin identified by deletion analysis of the gene expressed in *E. coli. Cell.* 48:129–136.

6. Pai, L., D. J. FitzGerald, M. Tepper, B. Schacter, G. Spitalny, and I. Pastan. 1990. Inhibition of antibody response to *Pseudomonas* exotoxin (PE) and an immunotoxin containing *Pseudomonas* exotoxin by 15-deoxyspergulain in mice. *Cancer Res.* 50:7750–7753.

7. Allured, V. S., R. J. Collier, S. F. Carroll, and D. B. McKay. 1986. Structure of exotoxin A of *Pseudomonas aeruginosa* at 3.0 Angstrom resolution. *Proc. Natl. Acad. Sci. USA.* 83:1320–1324.

8. Kondo, T., D. FitzGerald, V. K. Chaudhary, S. Adhya, and I. Pastan. 1988. Activity of immunotoxins constructed with modified *Pseudomonas* exotoxin A lacking the cell recognition domain. *J. Biol. Chem.* 263:9470–9475.

9. Batra, J. K., Y. Jinno, V. K. Chaudhary, T. Kondo, M. C. Willingham, D. J. FitzGerald, and I. Pastan. 1989. Antitumor activity in mice of an immunotoxin made with anti-transferrin receptor and a recombinant form of *Pseudomonas* exotoxin. *Proc. Natl. Acad. Sci. USA.* 86:8545–8549.

10. Pearson, J. W., D. J. P. FitzGerald, M. C. Willingham, R. H. Wiltrout, I. Pastan, and D. L. Longo. 1989. Chemoimmunotoxin therapy against a human colon tumor (HT-29) xenografted into nude mice. *Cancer Res.* 49:3562–3567.

11. Pai, L., D. J. FitzGerald, M. C. Willingham, and I. Pastan. 1991. Anti-tumor activities of immunotoxins made of monoclonal antibody B3 and various forms of *Pseudomonas* exotoxin. *Proc. Natl. Acad. Sci. USA.* 88:3358–3362.

12. Haglund, C., J. Lindgren, P. J. Roberts, P. Kuusela, and S. Nordling. 1989. Tissue expression of the tumor associated antigen CA 242 in benign and malignant pancreatic lesions. *Br. J. Cancer.* 60:845–851.

13. Ouyang, Q., M. Vilien, B. Ravn Jul, L. G. Larsen, and V. Binder. 1987. CEA and carbohydrate antigens in normal and neoplastic colon mucosa. *Acta Pathol. Microbiol. Immunol. Scan. Sect. A.* 95:177–183.

14. Chaudhary, V. K., Y.-H. Xu, D. J. P. FitzGerald, S. Adhya, and I. Pastan. 1988. Role of domain II of *Pseudomonas* exotoxin in the secretion of proteins into the periplasm and medium by *E. coli. Proc. Natl. Acad. Sci. USA.* 85:2939–2943.

15. Siegall, C. B., V. K. Chaudhary, D. J. FitzGerald, and I. Pastan. 1989. Functional analysis of domains II, Ib and III of *Pseudomonas* exotoxin. *J. Biol. Chem.* 264:14256–14261.

16. Traut, R. R., A. Bollen, T.-T. Sun, J. W. B. Hershey, J. Sundberg, and L. R. Pierce. 1973. Methyl 4-mercaptobutyrimidate as a cleavable cross-linking reagent and its application to the *Escherichia coli* 30S ribosome. *Biochemistry.* 12:3266–3273.

17. Yoshitake, S., Y. Yamada, E. Ishikawa, and R. Masseyoff. 1979. Conjugation of glucose oxidase from *Aspergillus niger* and rabbit antibodies using N-hydroxysuccinimide ester of N-(4-carboxycyclohexylmethyl)-maleimide. *Eur. J. Biochem.* 101:395–399.

18. Ellman, G. L. 1959. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* 82:70–77.

19. Murthy, K. K., G. Thibault, E. L. Schiffrin, R. Garcia, L. Chartier, J. Gutkowska, J. Genest, and M. Cantin. 1986. Disappearance of atrial natriuretic factor from circulation in the rat. *Peptides.* 7:241–246.

20. Lindmo, T., E. Boven, F. Cutitta, J. Fedorko, and P. A. Bunn, Jr. 1984. Determination of the immunoreactive fraction of radiolabeled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. *J. Immunol. Methods.* 72:77–89.

21. Kennel, S. J., R. Falcioni, and J. M. Wesley. 1991. Microdistribution of specific rat monoclonal antibodies to mouse tissues and human tumor xenograft. *Cancer Res.* 51:1529–1536.