

# Eradication of large colon tumor xenografts by targeted delivery of maytansinoids

(immunoconjugate/colon cancer xenografts)

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**ABSTRACT** The maytansinoid drug DM1 is 100- to 1000-fold more cytotoxic than anticancer drugs that are currently in clinical use. The immunoconjugate C242-DM1 was prepared by conjugating DM1 to the monoclonal antibody C242, which recognizes a mucin-type glycoprotein expressed to various extents by human colorectal cancers. C242-DM1 was found to be highly cytotoxic toward cultured colon cancer cells in an antigen-specific manner and showed remarkable anti-tumor efficacy *in vivo*. C242-DM1 cured mice bearing subcutaneous COLO 205 human colon tumor xenografts (tumor size at time of treatment 65–130 mm<sup>3</sup>), at doses that showed very little toxicity and were well below the maximum tolerated dose. C242-DM1 could even effect complete regressions or cures in animals with large (260- to 500-mm<sup>3</sup>) COLO 205 tumor xenografts. Further, C242-DM1 induced complete regressions of subcutaneous LoVo and HT-29 colon tumor xenografts that express the target antigen in a heterogeneous manner. C242-DM1 represents a new generation of immunoconjugates that may yet fulfill the promise of effective cancer therapy through antibody targeting of cytotoxic agents.

Colorectal cancer is one of the most common malignancies and is among the leading causes of death from cancer. Surgical resection is the primary treatment modality for these tumors, but about half of all patients will die of disseminated disease (1). Because of the high incidence and poor prognosis of patients with metastatic disease, successful treatment of colorectal cancer requires effective systemic therapy in addition to surgery, either as adjuvant treatment to surgery or for primary treatment of those 25% of all patients for whom surgery alone cannot achieve a complete response (2). Unfortunately, the conventional systemic treatment options for colon cancer, including radiation therapy, chemotherapy, and immunotherapy, have limited efficacy (3, 4). To date, 5-fluorouracil (5-FU) has served as the standard cytostatic drug for adjuvant therapy after surgery. However, the overall response rate to 5-FU is less than 25%, and the treatment has not significantly improved patient survival (1–3). Although the improved regimen of 5-FU plus levamisole in the adjuvant setting has proven to be more effective in patients with stage II and III colorectal cancers, the estimated reduction in the mortality rate is still less than 30% (2, 5). Thus, there is an urgent clinical need for new agents with greater efficacy.

Conventional chemotherapeutic agents are limited in their therapeutic effectiveness by severe side effects due to their poor selectivity for tumors. The development of monoclonal antibodies against specific tumor antigens made it possible to think of enhancing the selectivity of anticancer drugs by a targeted delivery approach. However, several such reported

attempts using monoclonal antibodies and the anticancer drugs doxorubicin (6), methotrexate (7), and *Vinca* alkaloids (8), have been largely unsuccessful. These antibody-drug conjugates were only moderately potent and usually less cytotoxic than the corresponding unconjugated drugs. In fact, antigen-specific cytotoxicity toward cultured tumor cells was rarely demonstrated (6–8). *In vivo* therapeutic effects with these conjugates in tumor xenograft animal models were, in general, observed only when the treatments were commenced before the tumors were well established (8) or when exceedingly large doses (up to 90 mg/kg, drug equivalent dose) were used (6). It is, therefore, not surprising that in human clinical trials, no significant antitumor effects were observed with these agents (9, 10). Indeed, the peak circulating serum concentrations of conjugates were only in the same range as their *in vitro* IC<sub>50</sub> values and, thus, capable of eliminating at best only about 50% of tumor cells.

These observations have led us (11, 12) and others (13, 14) to conclude that the previous attempts at delivering therapeutic doses of cytotoxic drugs via monoclonal antibodies have met with little success in clinical trials because of inappropriate choices of drug. We concluded that immunoconjugates must be composed of drugs possessing much higher potency than the clinically used anticancer agents if therapeutic levels of conjugate at the tumor sites are to be achieved in patients. We have recently described antibody conjugates with CC-1065 analogs and with maytansinoids that are 100- to 1000-fold more cytotoxic than the chemotherapeutic agents doxorubicin, methotrexate, and *Vinca* alkaloids (11, 12). Herein, we report the results of preclinical efficacy tests with C242-DM1, a maytansinoid drug (DM1) linked to the monoclonal antibody C242 directed against human colorectal cancer.

## MATERIALS AND METHODS

**Preparation of C242-DM1 Conjugate.** Ansamitocin P-3 (compound 1) provided by Takeda (Osaka) was converted to the disulfide-containing maytansinoid DM1 (compound 2) (Fig. 1) as described (15). The C242 antibody, a murine IgG1 (16), was provided by Pharmacia. C242-DM1 (compound 3) was prepared as described (12). The conjugate was purified by gel filtration through a column of Sephacryl S300 and the peak corresponding to monomeric conjugate (>80% overall yield) was collected. The final conjugate contained on the average four DM1 molecules linked per antibody molecule.

**Specific Affinity of C242-DM1.** The specific binding affinity of C242-DM1 conjugate and C242 antibody to CanAg-positive COLO 205 cell membranes was determined by a binding assay as described (17). Samples of C242-DM1 or C242 at various concentrations (10<sup>-12</sup> to 10<sup>-9</sup> M) were incubated for 18 h at

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Abbreviations: 5-FU, 5-fluorouracil; DM1, maytansinoid drug; MTD, maximum tolerated dose.

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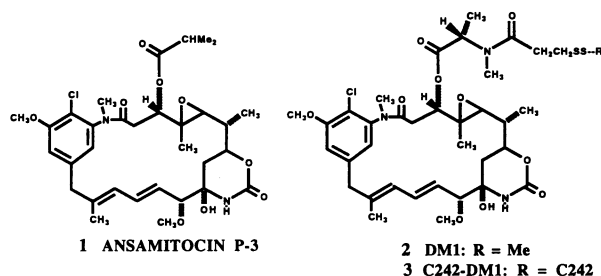


FIG. 1. Structural representation of Ansamitocin P-3 (compound 1), DM1 (compound 2), and C242-DM1 (compound 3).

ambient temperature with COLO 205 cell membranes immobilized in 96-well plates. The membranes were then washed and the amount of bound conjugate or antibody was determined using a  $\text{Eu}^{3+}$ -labeled anti-mouse IgG. Results are plotted as concentration of conjugate or antibody versus relative fluorescence.

**In Vitro Cytotoxicity of C242-DM1 Conjugate.** The cytotoxicity of C242-DM1 was measured on antigen-positive human colon carcinoma cell lines COLO 205 [American Type Culture Collection (ATCC) CCL 222], LoVo (ATCC CCL 229), and HT-29 (ATCC HTB 38) and on the antigen-negative human melanoma cell line A-375 (ATCC CRL 1619) in a clonogenic assay. Cells were plated in 96-well tissue culture plates with each plate containing a fixed number of cells (ranging from 3 to 10,000 cells per well) in 0.2 ml of DMEM containing 20% fetal calf serum. Immunoconjugate at varying concentrations ( $4 \times 10^{-11}$  to  $4 \times 10^{-8}$  M) was added and the cells were maintained in a humidified atmosphere at 37°C and 6%  $\text{CO}_2$  for 18–21 days. In some experiments, the cells were incubated with C242-DM1 for 24 h and then washed, and the medium was replaced with fresh medium without drug. Colonies were then counted and the plating efficiency was determined. Surviving fractions of cells were then calculated as the ratio of the plating efficiency of the treated sample and the plating efficiency of the control.

**Immunohistochemical Studies.** Tumor tissues excised from either humans or mice were frozen in O.C.T. embedding medium (Miles), sectioned, and treated with biotinylated-C242 antibody. The bound antibody was detected using the avidin-biotin immunoperoxidase technique as described (18).

**In Vivo Tumor Growth Assays.** Female CB-17 SCID mice, 6–7 weeks of age, were obtained from Massachusetts General Hospital. The human colon cancer cell lines COLO 205, LoVo, and HT-29 were maintained as adherent cultures in DMEM containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 6%  $\text{CO}_2$ /94% air. Each mouse was inoculated subcutaneously at the right flank with tumor cells ( $2 \times 10^6$  to  $1 \times 10^7$  cells in different experiments) in 0.1 ml of medium. Treatments were started on days 7–9 after tumor inoculation, when the tumor sizes reached from 65 to 500  $\text{mm}^3$ , depending on the experiment. The therapeutic agents were administered intravenously to groups of 7–10 mice. Tumor size was measured weekly in two dimensions using a caliper, and the volume was expressed in  $\text{mm}^3$  using the formula:  $V = 0.5a \times b^2$ , where  $a$  and  $b$  are the long and short diameters of the tumor, respectively.

**Measurement of Concentration of C242-DM1 in Serum.** A group of eight CD1 mice obtained from Charles River Breeding Laboratories were injected with C242-DM1 at a dose of 300  $\mu\text{g}$  per kg per day $^\ddagger$  for five consecutive days. Blood (0.1 ml) was withdrawn from the retroorbital sinus once per day from each mouse, either at 1 h (four mice) or at 24 h (four mice) after injection of the conjugate. C242-DM1 was determined by an ELISA using a murine monoclonal IgG2a anti-DM1 antibody

$^\dagger$ All concentrations used *in vitro* refer to conjugated DM1.

$^\ddagger$ All doses used *in vivo* refer to conjugated DM1. A DM1 dose of 1  $\mu\text{g}$  corresponds to 54  $\mu\text{g}$  of C242-DM1 conjugate.

(developed at ImmunoGen) to capture the C242-DM1. The amount of bound conjugate was then quantified by detection of the C242 antibody using IgG1-specific goat anti-mouse IgG-alkaline phosphatase/*p*-nitrophenyl phosphate as described (17).

**Immunostaining of Cells.** Cells grown on coverslips were fixed with 2% paraformaldehyde, permeabilized in methanol at  $-20^\circ\text{C}$ , and stained with C242 antibody for fluorescence microscopy as described (19). A similar protocol was used for flow cytometry (Becton-Dickinson FACScan), except that cells were trypsinized, stained live without fixation, and then fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS).

**Magnetic Bead Depletion.** Cells were harvested with trypsin, counted, incubated with C242 antibody, and washed. Cells were then mixed with magnetic beads (Dynabeads M-450, goat anti-mouse IgG coated, DYNAL, Oslo) at a beads/cells ratio of 5:1 and incubated for 30 min with rocking at 4°C. Beads plus adhering cells were magnetically removed, and an equal number of fresh beads were added for a second cycle. The remaining cells were analyzed by flow cytometry.

## RESULTS

**Evaluation of C242-DM1 for Specificity, Cytotoxicity, and Selectivity.** The delivery agent of C242-DM1, the C242 antibody, recognizes a sialidase-sensitive carbohydrate epitope on the CanAg antigen, a mucin-type glycoprotein expressed to various degrees by all human colorectal cancers (20–22). C242 has only minimal cross-reactivity with normal tissues (21, 22). C242-DM1 was prepared in a manner similar to that described for other maytansinoid conjugates (12) (Fig. 1). The conjugate contains, on the average, four covalently linked DM1 molecules per antibody molecule. In a binding assay, C242-DM1 binds as well as unconjugated C242 to the CanAg antigen expressed on COLO 205 cell membranes (Fig. 2A), indicating

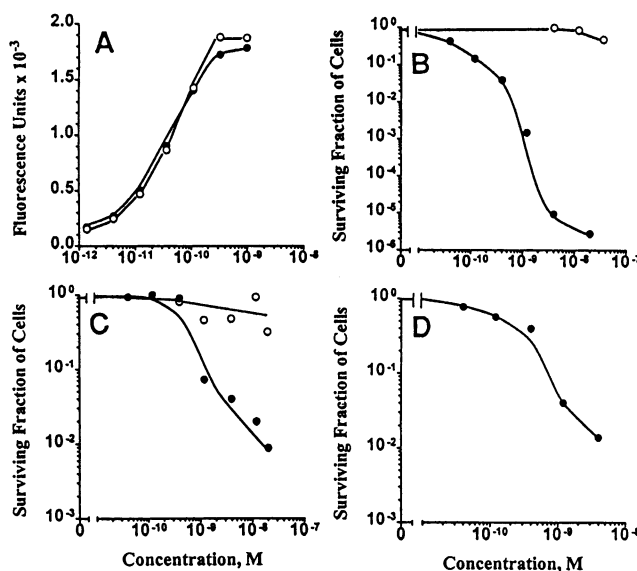


FIG. 2. Evaluation of binding and cytotoxicity of C242-DM1. (A) Binding affinity of C242-DM1. The specific affinity of conjugated C242 ( $\bullet$ ) for the CanAg antigen expressed on COLO 205 cell membranes is compared with that of C242 ( $\circ$ ). (B) *In vitro* cytotoxicity and selectivity of C242-DM1. The *in vitro* cytotoxicity of C242-DM1 for antigen-positive human colon carcinoma COLO 205 cells ( $\bullet$ ) and antigen-negative human melanoma A-375 cells ( $\circ$ ) was measured in a clonogenic assay. (C) *In vitro* cytotoxicity and specificity of C242-DM1 for antigen-positive colon carcinoma COLO 205 cells in the absence ( $\bullet$ ) or presence ( $\circ$ ) of  $1 \times 10^{-6}$  M C242 antibody. Cells were incubated with immunoconjugate for 24 h in this experiment. (D) *In vitro* cytotoxicity of C242-DM1 for the human colon cancer cell line LoVo, which expresses the CanAg antigen heterogeneously.

that the conjugation of DM1 does not diminish the binding avidity of C242. The cytotoxic potency and selectivity of C242-DM1 was assayed with the antigen-positive COLO 205 cell line and the antigen-negative A-375 melanoma cell line (Fig. 2B); both cell lines were equally sensitive to free DM1 ( $IC_{50} = 4 \times 10^{-11}$  M). C242-DM1 was found to kill COLO 205 cells with an  $IC_{50}$  value of  $3.2 \times 10^{-11}$  M (23.5 pg/ml), and treatment of cells with a concentration of  $4.5 \times 10^{-9}$  M (3.3 ng/ml) left a surviving fraction of less than  $1 \times 10^{-5}$  (>99.999% of cells killed, detection limit of the assay). In contrast, C242-DM1 was 1100-fold less cytotoxic for the antigen-negative A-375 cells ( $IC_{50} = 3.6 \times 10^{-8}$  M; 26.5 ng/ml), demonstrating that cell killing was selective for the antigen-positive colon cell line (Fig. 2B). COLO 205 cells were killed even after a 24-h exposure to C242-DM1, with an  $IC_{50}$  value of  $6 \times 10^{-10}$  M (Fig. 2C). Furthermore, a large excess of free C242 antibody greatly diminished the cytotoxicity of the conjugate toward the target cells (Fig. 2C), further demonstrating that the cytotoxic effect was dependent on specific binding through the antibody component of the conjugate.

The COLO 205 cell line cultured *in vitro* expresses the target antigen homogeneously on all cells (22). We also evaluated the cytotoxic potency of C242-DM1 against two colon tumor cell lines, LoVo and HT-29, which express the CanAg antigen heterogeneously on only 20–30% of their cells when grown *in vitro*, as judged by indirect immunofluorescence analysis of C242 binding using flow cytometry (data not shown). In spite of this low expression, treatment of these cells with C242-DM1 could eliminate 99% of the cells at a concentration of  $4 \times 10^{-9}$  M (shown in Fig. 2D for the LoVo cell line).

**Immunohistochemical Analysis of Tumor Xenografts and Human Colon Tumor Samples.** The three human colon tumor cell lines, COLO 205, LoVo and HT-29, were grown subcutaneously in SCID mice to test the *in vivo* therapeutic efficacy of C242-DM1. The particular cell lines were chosen because their antigen expression, when grown *in vivo*, was in the range of that seen by immunohistochemical examination of human colon tumor specimens from 20 patients. COLO 205 tumor xenografts excised from mice on day 7 after tumor inoculation exhibited, on immunohistochemical analysis, uniform staining of the CanAg antigen (Fig. 3A) in a manner similar to that of the section of a human colon tumor biopsy representative of 6/20 specimens shown in Fig. 3B. Tumor xenografts established with LoVo cells expressed the antigen heterogeneously at all time points. The staining pattern of a section taken on day 7 after tumor inoculation was classified as moderately heterogeneous (Fig. 3C) and resembled the staining pattern of the typical (10/20 specimens) human colon tumor biopsy shown in Fig. 3D. The third human colon tumor xenograft model established with HT-29 cells showed very heterogeneous staining for antigen, with many cells being antigen-negative (Fig. 3E), again in a fashion similar to that seen in some biopsies (4/20) of human colon tumors (Fig. 3F).

**Antitumor Efficacy of C242-DM1.** In the first therapy experiment (Fig. 4A), animals bearing COLO 205 tumors were treated with five daily injections of C242-DM1 at a dose of 300  $\mu$ g per kg per day, with an equivalent dose of the isotype-matched conjugate N901-DM1 that does not bind to COLO 205 cells, or with a mixture of corresponding amounts of C242 antibody (16 mg per kg per day) and unconjugated DM1 (300  $\mu$ g per kg per day). Treatment with C242-DM1 completely

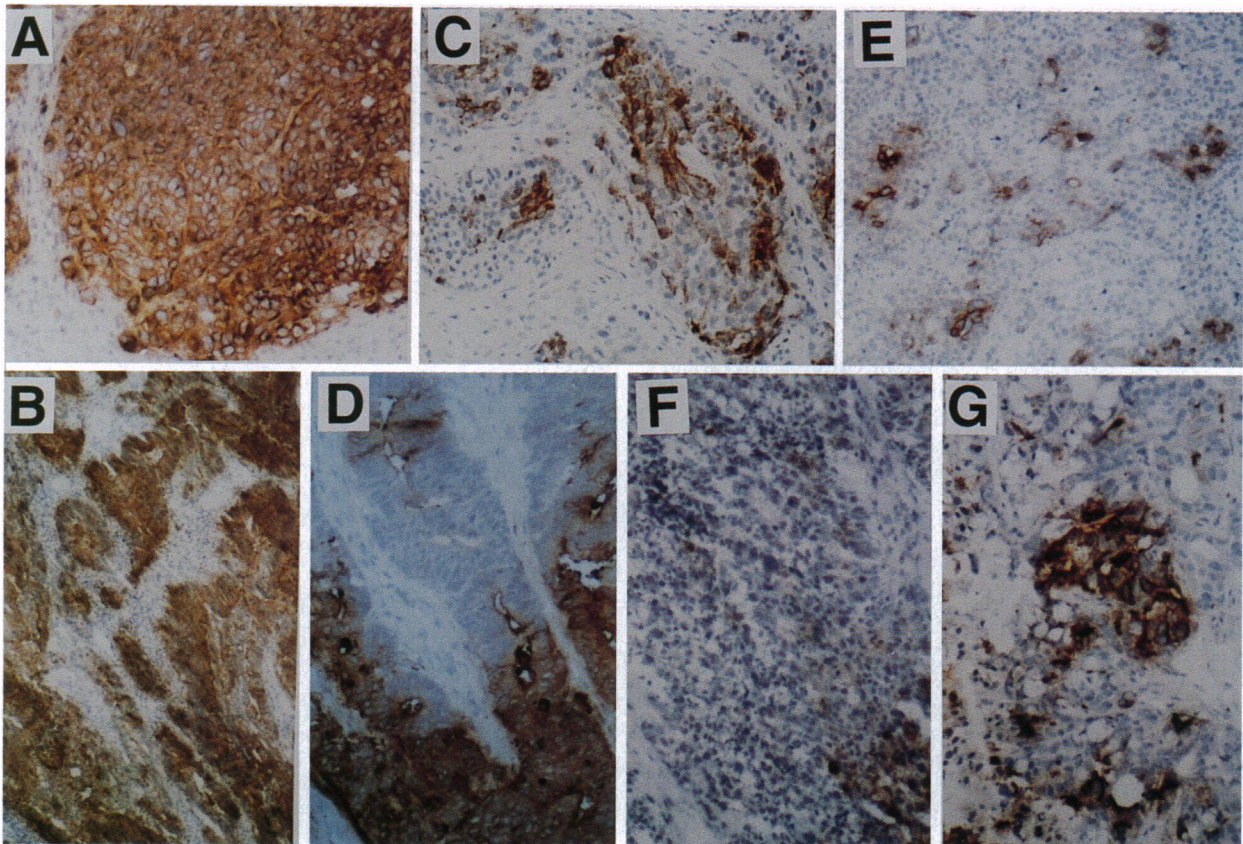


FIG. 3. Immunohistochemical analysis of tumor xenografts excised on day 7 after tumor inoculation and comparison with human colon tumor biopsies. (A) A COLO 205 xenograft. (B) A human colon tumor biopsy with homogeneous expression of antigen. (C) A LoVo xenograft. (D) A human colon tumor biopsy with moderately heterogeneous expression of antigen. (E) An HT-29 xenograft. (F) A human colon tumor biopsy with very heterogeneous expression of antigen. (G) A relapsed LoVo xenograft removed on day 91 from a mouse that received one course of C242-DM1 treatment.

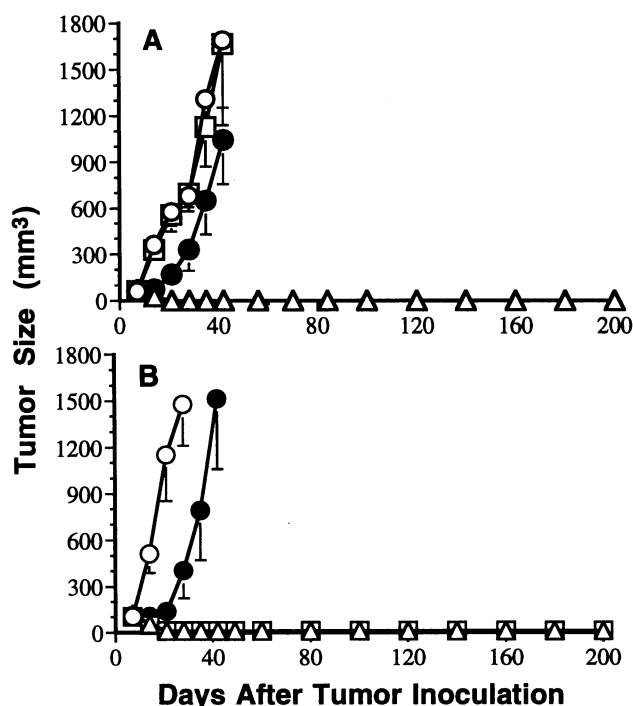


FIG. 4. Antitumor activity of C242-DM1 conjugate in SCID mice bearing COLO 205 human colon tumor xenografts. Each mouse was inoculated with  $2 \times 10^6$  COLO 205 cells. The treatments were given from day 7 to day 11 after tumor inoculation (average tumor size = 65–100 mm<sup>3</sup>). (A) Antigen-specific antitumor activity of C242-DM1. The antitumor activity of C242-DM1 (300 µg per kg per day for 5 days) ( $\Delta$ ) was compared with that of PBS (0.2 ml per mouse per day for 5 days) ( $\circ$ ), a mixture of C242 (16 mg per kg per day for 5 days) plus free DM1 (300 µg per kg per day for 5 days) ( $\square$ ) or a nonbinding conjugate, N901-DM1 (300 µg per kg per day for 5 days) ( $\bullet$ ). (B) Dose dependence of antitumor activity of C242-DM1. Tumor-bearing animals were treated with PBS (0.2 ml per mouse per day for 5 days) ( $\circ$ ), C242-DM1 (150 µg per kg per day for 5 days) ( $\bullet$ ), C242-DM1 (225 µg per kg per day for 5 days) ( $\Delta$ ), or C242-DM1 (300 µg per kg per day for 5 days) ( $\square$ ).

eliminated any measurable tumors within 2 weeks of the initiation of therapy, and all eight animals were tumor-free for 200 days (duration of the experiment). Furthermore, toxic side effects were minimal at this dose as judged by the absence of body weight loss. The dose of C242-DM1 used in this experiment was below its maximum tolerated dose (MTD), which was defined for these experiments as the highest dose that could be administered to tumor-bearing mice without causing drug-related deaths (MTD = 380 µg per kg per day for five consecutive days). In contrast, very little antitumor activity was observed in animals treated with nontargeted conjugate or with the mixture of antibody and free DM1 (Fig. 4A). Thus, the DM1 moiety is a potent therapeutic agent against colon cancers *in vivo* when targeted to the tumors as a conjugate with the C242 antibody and shows high antitumor efficacy at doses that cause little toxicity.

The circulating serum concentrations of C242-DM1 were determined in CD1 mice by ELISA. One hour after each injection (five daily injections of 300 µg per kg per day), the concentration of C242-DM1 was about 1.8 µM, equivalent to DM1 at 1.3 µg/ml. After 24 h, the serum concentration was about 0.26 µM, which is still 58-fold higher than the concentration required to kill >99.999% cells *in vitro*.

Next, the dose-response effect of C242-DM1 in the COLO 205 xenograft model was evaluated. Animals were treated with C242-DM1 at doses ranging from 150 to 300 µg per kg per day for 5 days (Fig. 4B). C242-DM1 eliminated tumors in all animals at a daily dose as low as 225 µg per kg per day when

given for 5 consecutive days, which is 59% of the MTD. Even at the lowest dose tested (150 µg per kg per day for 5 days), a significant delay in tumor growth was observed.

These results encouraged us to evaluate the therapeutic efficacy of C242-DM1 in mice bearing larger (average size, 260 mm<sup>3</sup>) subcutaneous COLO 205 xenografts (Fig. 5A). Animals received two courses of 5-day treatment with C242-DM1 or, for comparison, treatment with 5-FU, the standard chemotherapeutic drug used for the treatment of colorectal cancer. C242-DM1 again cured all animals rendering them tumor-free for greater than 200 days (duration of the experiment). This therapeutic effect on large tumors is especially remarkable in view of the finding that 5-FU at its MTD (15 mg per kg per day for 5 days) only slightly (by about 5 days) delayed the tumor growth. We extended this study to even larger tumors. A group of animals bearing the largest COLO 205 tumor xenografts tested (average size 500 mm<sup>3</sup>) was treated with one course of C242-DM1 at a dose of 300 µg per kg per day for 5 days (Fig. 5B). Complete tumor regressions were achieved in all animals. In six out of eight animals, the complete response lasted 7 weeks. In the remaining two animals, no signs of tumor could be detected when the experiment was terminated on day 120 after tumor inoculation (representing more than 17 tumor size doubling times *in vivo*).

The COLO 205 cell line, both cultured *in vitro* and grown as tumor xenografts, expresses the target antigen homogeneously on all cells (Fig. 3A). We then evaluated the antitumor activity of C242-DM1 against established colon tumor xenografts from the LoVo and HT-29 cell lines that express the CanAg antigen heterogeneously on only 20–30% of their cells when grown *in*

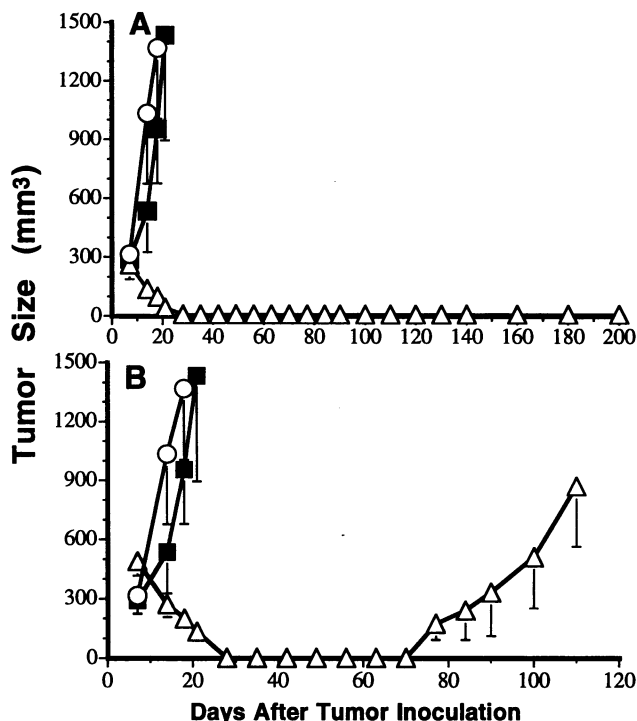


FIG. 5. Antitumor activity of C242-DM1 against large COLO 205 tumors. Each mouse was inoculated with  $5 \times 10^6$  COLO 205 cells and treatments were started on day 7 after tumor inoculation. (A) Efficacy in treatment of large COLO 205 xenografts (mean tumor size = 260 mm<sup>3</sup>). Tumor-bearing animals were treated with PBS (0.2 ml per mouse per day for 5 days) ( $\circ$ ), 5-FU (15 mg per kg per day for 5 days) ( $\blacksquare$ ), or two courses of C242-DM1 (300 µg per kg per day for 10 days; days 7–11 and days 14–18) ( $\Delta$ ). (B) Efficacy in treatment of very large COLO 205 xenografts (mean tumor size = 500 mm<sup>3</sup>). Tumor-bearing animals were treated with PBS (0.2 ml per mouse per day for 5 days) ( $\circ$ ), 5-FU (15 mg per kg per day for 5 days) ( $\blacksquare$ ), or one course of C242-DM1 (300 µg per kg per day for 5 days, days 7–11) ( $\Delta$ ).

*in vitro* or, as shown in Fig. 3 (C and E), *in vivo*. Animals bearing LoVo tumor xenografts were treated with either one or two courses of C242-DM1 (300  $\mu\text{g}$  per kg per day for 5 days). Two additional groups of tumor-bearing animals were treated with either a mixture of C242 antibody (16 mg per kg per day for 5 days) and unconjugated DM1 (300  $\mu\text{g}$  per kg per day for 5 days) or with 5-FU at its MTD (15 mg per kg per day for 5 days). Remarkably, complete tumor regressions lasting 5 weeks were observed in all animals treated with one course of C242-DM1 (Fig. 6A). The LoVo tumors from mice that were treated with C242-DM1 and relapsed after the period of complete regressions were evaluated for antigen expression. A section of the tumor excised on day 91 exhibited similar heterogeneous staining with C242 (Fig. 3G) as was seen prior to therapy (day 7, Fig. 3C). The period of complete regression could be prolonged to 9 weeks by a second course of treatment with C242-DM1 initiated 21 days after the start of the first course (Fig. 6A). No indication of toxic side effects as assessed by body weight loss was observed for either treatment protocol. In contrast, tumors in animals that were treated with the mixture of antibody and DM1, or with 5-FU, grew rapidly to large sizes (Fig. 6A). Similar effects were obtained in the tumor model with HT-29, classified as expressing the CanAg antigen very heterogeneously (see Fig. 3E). C242-DM1 (375  $\mu\text{g}$  per kg per day for 5 days) induced complete tumor regressions lasting 4 weeks (Fig. 6B). These results demonstrate that C242-DM1 is an effective therapeutic agent against colorectal cancer x-

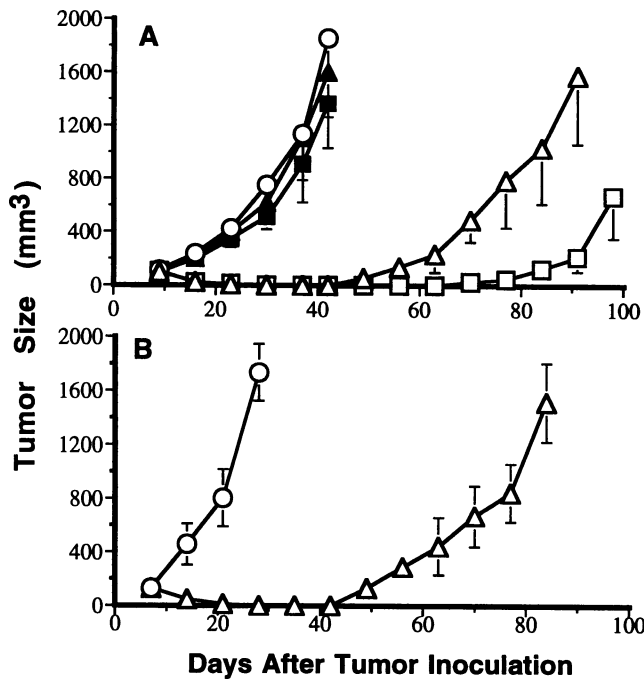


Fig. 6. Antitumor activity of C242-DM1 in SCID mice bearing human colon tumor xenografts that express the antigen heterogeneously. (A) Antitumor activity of C242-DM1 against LoVo tumor xenografts (mean tumor size = 103 mm<sup>3</sup>). Each mouse was inoculated with  $5 \times 10^6$  LoVo cells, and treatments were started on day 9 after tumor inoculation. Tumor-bearing mice were treated with PBS (0.2 ml per mouse per day for 5 days) (○), 5-FU (15 mg per kg per day for 5 days) (■), a mixture of C242 (16 mg per kg per day for 5 days) plus DM1 (300  $\mu\text{g}$  per kg per day for 5 days) (▲), one course of C242-DM1 (300  $\mu\text{g}$  per kg per day for 5 days) (△), or two courses of C242-DM1 (300  $\mu\text{g}$  per kg per day for 10 days, days 9–13 and days 30–34) (□). (B) Antitumor activity of C242-DM1 against HT-29 tumor xenografts (mean tumor size = 130 mm<sup>3</sup>). Each mouse was inoculated with  $1 \times 10^7$  HT-29 cells and treatments were given from days 7 to 11 after tumor inoculation. The tumor-bearing mice were treated with PBS (0.2 ml per mouse per day for 5 days) (○) or C242-DM1 (375  $\mu\text{g}$  per kg per day for 5 days) (△).

nografts including those that express the antigen heterogeneously and causes little toxicity even after two courses of treatment.

**Relationship Between CanAg Expression and Colony Formation in Colon Cancer Cell Lines.** The rather unexpected *in vivo* results that C242-DM1 induced complete regressions of tumors in which 70–80% of the cells do not express detectable amounts of the CanAg antigen led us to evaluate whether it was possible *in vitro* to generate homogeneous antigen-positive and antigen-negative sublines from cell lines that express the antigen heterogeneously. Repeated attempts to subclone the LoVo and HT-29 cell lines to select sublines that were completely antigen-negative (by staining with C242) were unsuccessful; no CanAg-negative subclones grew in a total of 210 subclones. In fact, most subclones expressed more antigen (about 60% positive cells) than the parental cell lines (20–30% positive cells). In another attempt, an HT-29 cell culture was first depleted of antigen-positive cells using magnetic beads coated with the C242 antibody and the remaining cells, which had a very low level of CanAg expression (<1%, by flow cytometry analysis of C242 binding), were subcloned. Initial screening of subconfluent cultures by immunofluorescence showed that many clones expressed very low levels of CanAg (about 70% of 79 clones had <10% antigen-positive cells). However, as the colonies were expanded, CanAg expression on some sublines again increased to levels greater than in the parental line or else the colonies died out (only 34 sublines could be expanded sufficiently for flow cytometry analysis). These results suggest that there is a direct relationship *in vitro* between the ability to form colonies and CanAg expression and may also provide an explanation for the experimental observations in the LoVo and HT-29 tumor xenograft models whereby the elimination of antigen-positive cells *in vivo* by C242-DM1 may cause the collapse of the entire tumor in animals.

## DISCUSSION

C242-DM1 represents a new generation of immunoconjugates that may yet realize the potential of effective cancer therapy through antibody targeting of cytotoxic agents. The conjugate is highly cytotoxic *in vitro* in an antigen-dependent and tumor-cell-selective manner and produced long-term cures of mice bearing human colon tumor COLO 205 xenografts at doses that caused little toxicity. Cures were even obtained at doses that were well below the MTD of the conjugate. Importantly, the circulating serum concentrations of C242-DM1 (1.8  $\mu\text{M}$  at 1 h after injection of 300  $\mu\text{g}/\text{kg}$ ) were about 380-fold higher than that required to kill greater than 99.999% of target cells *in vitro*. C242-DM1 was capable of curing mice bearing very large COLO 205 tumors, even those that were 500 mm<sup>3</sup> at the start of treatment. In contrast, 5-FU, the standard chemotherapeutic drug used for the treatment of colorectal cancer, showed very little therapeutic benefit against the same tumors.

In many human colorectal tumor biopsies, it was demonstrated that CanAg, the target antigen for C242, is expressed in a heterogeneous manner. C242-DM1 showed remarkable antitumor activity in tumor models derived from the LoVo and HT-29 colon cancer cell lines that express the antigen on only 20–30% of the cells. The immunoconjugate induced complete regressions of such tumors lasting 4–5 weeks. Tumors harvested from regrowing LoVo xenografts after this period of complete regression exhibited heterogeneous staining similar to that seen prior to treatment (see Fig. 3 C and G), suggesting that the phenotype of the regrowing tumor xenografts was unchanged. Indeed, the period of complete regression could be prolonged by a second course of treatment that was well tolerated by the mice, suggesting that using multiple cycles of this immunoconjugate for treatment of colorectal cancer may be a feasible clinical regimen, with the potential for totally eradicating even those tumors that show heterogeneous expression for the CanAg antigen. The similarity of antigen

expression and tumor morphology between the xenografts and the human tumor biopsies suggests to us that antitumor activity of C242-DM1 exhibited in these models may well predict potent antitumor activity in the clinical situation.

The *in vitro* experiments suggest that CanAg antigen-negative cells do not grow (for long) in the absence of antigen-positive cells, which could provide a mechanism by which C242-DM1 could induce complete regressions of tumors that express the CanAg antigen heterogeneously. Secretion of cytokines essential for the survival of CanAg-negative cells by the CanAg-positive cells could explain these results, although initial experiments indicated that conditioned medium did not support growth of CanAg-negative colonies derived from the HT-29 or LoVo cell lines. Other possible mechanisms may be (i) all cell lineages go through a period of transient CanAg expression during which they are susceptible to the action of C242-DM1; (ii) a bystander effect, that is, antigen-positive cells may concentrate sufficient C242-DM1 at the tumor site to cause killing of neighboring antigen-negative cells either directly or by release of DM1 from the conjugate concentrated inside antigen-positive tumor cells, followed by diffusion of DM1 into neighboring cells via gap junctions; and (iii) recruitment of host immune mechanisms upon killing of CanAg-positive cells, including activated macrophages and natural killer cells (23). Also, it cannot be ruled out that CanAg-negative cells actually express antigen at low density, below the sensitivity of detection by flow cytometry or immunofluorescence microscopy but sufficient to mediate cell death using this potent immunoconjugate.

C242-DM1 exhibits a degree of potency and selectivity *in vitro* and *in vivo* that is superior to that of other immunoconjugates against colorectal cancer described thus far (6–8, 24–26). C242 has been linked to other cytotoxic agents, such as ricin A chain (24) or *Pseudomonas* exotoxin (25, 26). The ricin-A-chain conjugates showed very limited efficacy in the COLO 205 model (inducing a tumor growth delay of 16 days) and no significant efficacy in tumor models that express the CanAg antigen heterogeneously (24). The *Pseudomonas* exotoxin conjugates also showed little efficacy in the mouse models (25, 26). In one case where a “cure” was reported, the follow-up period was short (only 4 weeks), and the dose used was toxic and left only one survivor in the group (24). Of the other antibody-drug conjugates developed against colorectal cancer (6–8), cures were reported only in the case of BR96-Dox (6), a conjugate of the antibody BR96 with doxorubicin. However, 100% cures were only achieved in mice with BR96-Dox at its MTD (a doxorubicin dose of 20 mg per kg per day for 3 days). C242-DM1 cured all tumor-bearing mice at doses that are well below its MTD and that are 53-fold lower than those of BR96-Dox. This is an important result since the clinical MTDs of toxin- or drug-conjugates in humans are, in general, up to 10-fold less than those determined in animal studies, resulting in the inability of achieving therapeutically effective concentrations of conjugates in patients in the clinical studies to date (9, 10). Thus, from the preclinical data, C242-DM1 stands out as a promising new candidate for clinical evaluation against colorectal cancer. C242-DM1 made with a humanized antibody (27) will allow the treatment of patients with several courses of targeted chemotherapy with the potential for substantial clinical benefit.

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