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Eradication of large colon tumor xenografts by targeted delivery of maytansinoids

(immunoconjugate/colon cancer xenografts)

CHANGNIAN LIU*, B. MITRA TADAYONI, LIZABETH A. BOURRET, KRISTIN M. MATTOCKS, SUSAN M. DERR, WAYNE C. WIDDISON, NANCY L. KEDERSHA, PAMELA D. ARINIELLO, VICTOR S. GOLDMACHER, JOHN M. LAMBERT, WALTER A. BLÄTTLER, AND RAVI V. J. CHARI

ImmunoGen, Inc., 148 Sidney Street, Cambridge, MA 02139-4239

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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 antitumor efficacy *in vivo*. C242-DM1 cured mice bearing subcutaneous COLO 205 human colon tumor xenografts (tumor size at time of treatment 65–130 mm^3), 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^3) 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$_{50}$ values and, thus, capable of eliminating at best only about 50% of tumor cells.

These observations have led us (11, 12) and others (13) 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 3) (Fig. 1) as described (15). The C242 antibody, a murine IgG$_1$ (16), was provided by Pharmacia. C242-DM1 (compound 4) 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^{-12} to 10^{-9} M) were incubated for 18

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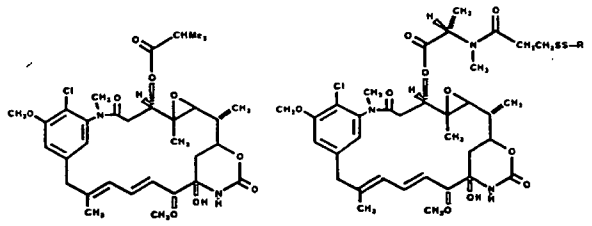
Abbreviations: 5-FU, 5-fluorouracil; DM1, maytansinoid drug; MTD, maximum tolerated dose.

*To whom reprint requests should be addressed.



1 ANSAMITOCIN P-3

2 DM1: R = Me
3 C242-DM1: R = C242

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 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 (4×10^{-11} to 4×10^{-8} M†) was added and the cells were maintained in a humidified atmosphere at 37°C and 6% 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% CO_2/94% air. Each mouse was inoculated subcutaneously at the right flank with tumor cells (2×10^6 to 1×10^7 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 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 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 μg per kg per day‡ 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 (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°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. 2*A*), indicating

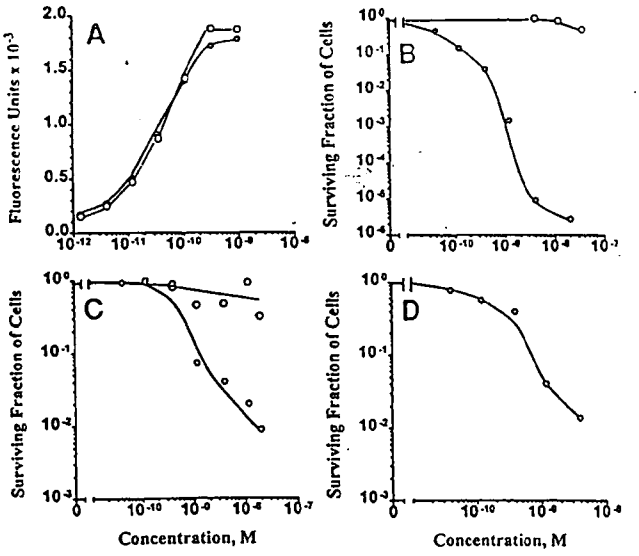


FIG. 2. Evaluation of binding and cytotoxicity of C242-DM1. (*A*) Binding affinity of C242-DM1. The specific affinity of conjugated C242 (○) for the CanAg antigen expressed on COLO 205 cell membranes is compared with that of C242 (○). (*B*) *In vitro* cytotoxicity and selectivity of C242-DM1. The *in vitro* cytotoxicity of C242-DM1 for antigen-positive human colon carcinoma COLO 205 cells (○) and antigen-negative human melanoma A-375 cells (○) 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 (○) or presence (○) of 1×10^{-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.

†All concentrations used *in vitro* refer to conjugated DM1.
‡All doses used *in vivo* refer to conjugated DM1. A DM1 dose of 1 μg corresponds to 54 μg of C242-DM1 conjugate.

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. 2*B*); 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 3.2×10^{-11} M (23.5 pg/ml), and treatment of cells with a concentration of 4.5×10^{-9} M (3.3 ng/ml) left a surviving fraction of less than 1×10^{-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. 2*B*). COLO 205 cells were killed even after a 24-h exposure to C242-DM1, with an IC_{50} value of 6×10^{-10} M (Fig. 2*C*). Furthermore, a large excess of free C242 antibody greatly diminished the cytotoxicity of the conjugate toward the target cells (Fig. 2*C*), 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 4×10^{-9} M (shown in Fig. 2*D* 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. 3*A*) in a manner similar to that of the section of a human colon tumor biopsy representative of 6/20 specimens shown in Fig. 3*B*. 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. 3*C*) and resembled the staining pattern of the typical (10/20 specimens) human colon tumor biopsy shown in Fig. 3*D*. 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. 3*E*), again in a fashion similar to that seen in some biopsies (4/20) of human colon tumors (Fig. 3*F*).

Antitumor Efficacy of C242-DM1. In the first therapy experiment (Fig. 4*A*), animals bearing COLO 205 tumors were treated with five daily injections of C242-DM1 at a dose of 300 μg 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 μg per kg per day). Treatment with C242-DM1 completely

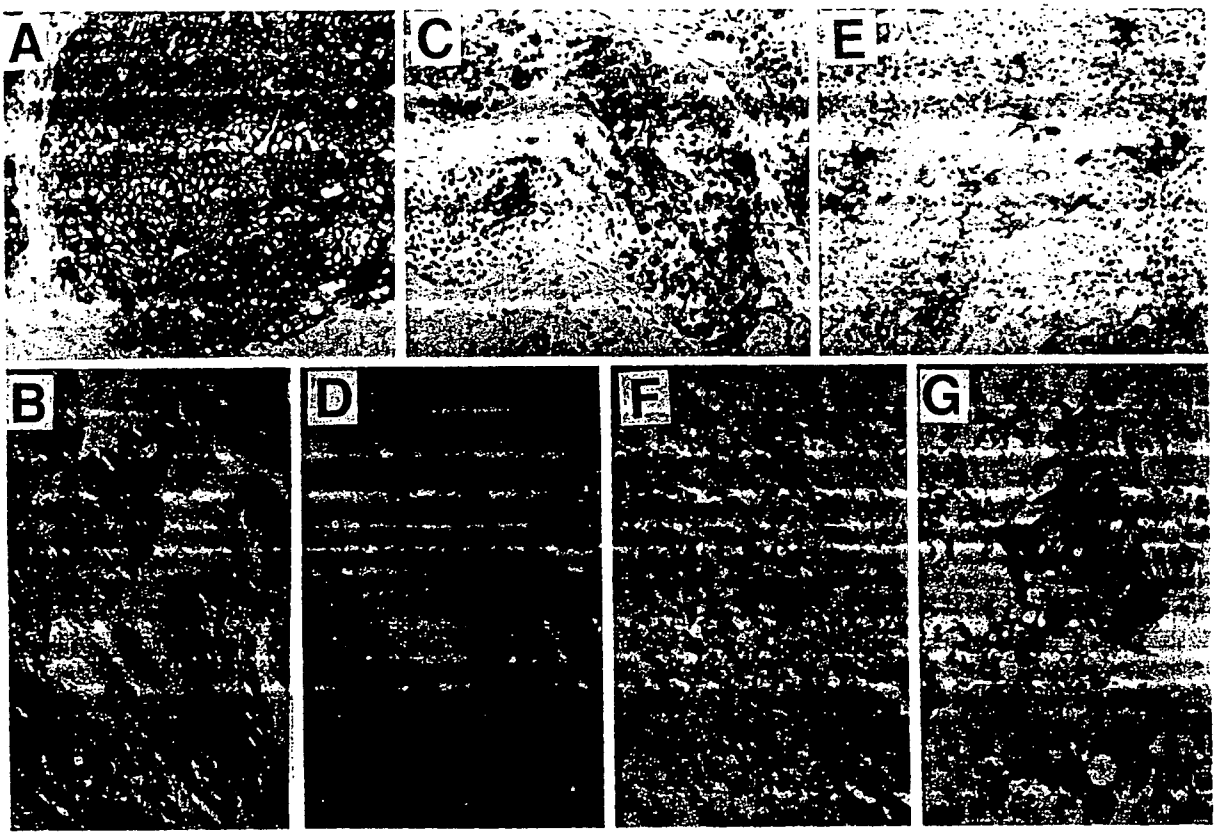


FIG. 3. Immunohistochemical analysis of tumor xenografts excised on day 7 after tumor inoculation and comparison with human colon tumor biopsies. (*A*) A COLO 205 xenograft. (*B*) A human colon tumor biopsy with homogeneous expression of antigen. (*C*) A LoVo xenograft. (*D*) A human colon tumor biopsy 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.

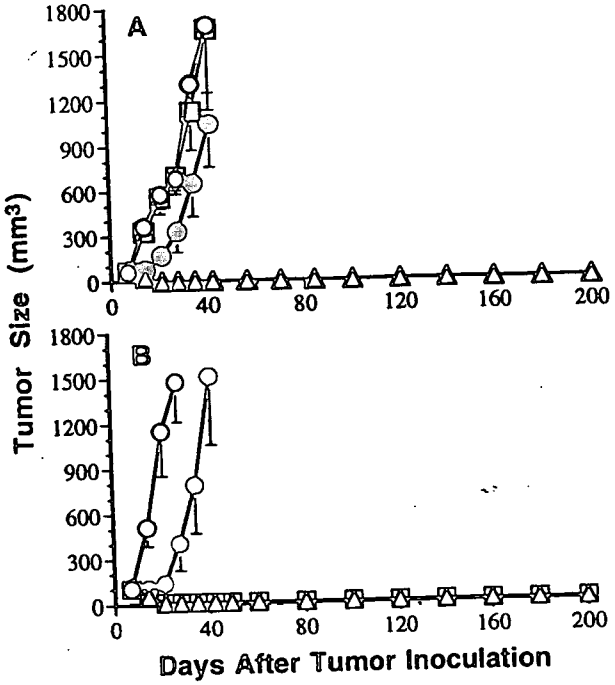


FIG. 4. Antitumor activity of C242-DM1 conjugate in SCID mice bearing COLO 205 human colon tumor xenografts. Each mouse was inoculated with 2×10^6 COLO 205 cells. The treatments were given from day 7 to day 11 after tumor inoculation (average tumor size = 65–100 mm³). (*A*) Antigen-specific antitumor activity of C242-DM1. The antitumor activity of C242-DM1 (300 μg per kg per day for 5 days) (△) was compared with that of PBS (0.2 ml per mouse per day for 5 days) (○), 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) (□) or a nonbinding conjugate, N901-DM1 (300 μg per kg per day for 5 days) (○). (*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) (○), C242-DM1 (150 μg per kg per day for 5 days) (○), C242-DM1 (225 μg per kg per day for 5 days) (△), or C242-DM1 (300 μg per kg per day for 5 days) (□).

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. 4*A*). 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. 4*B*). 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 mm³) subcutaneous COLO 205 xenografts (Fig. 5*A*). 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 mm³) was treated with one course of C242-DM1 at a dose of 300 μg per kg per day for 5 days (Fig. 5*B*). 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. 3*A*). 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*

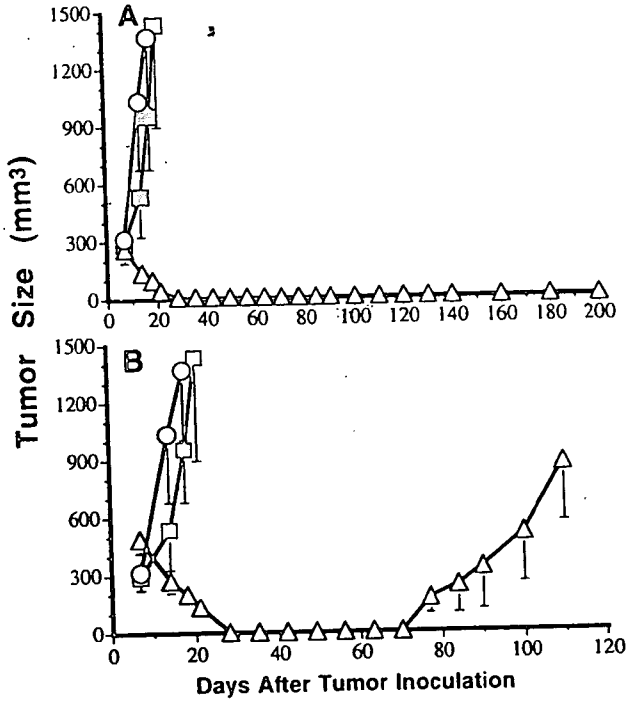


FIG. 5. Antitumor activity of C242-DM1 against large COLO 205 tumors. Each mouse was inoculated with 5×10^6 COLO 205 cells and treatments were started on day 7 after tumor inoculation. (*A*) Efficacy in treatment of large COLO 205 xenografts (mean tumor size = 260 mm³). Tumor-bearing animals were treated with PBS (0.2 ml per mouse per day for 5 days) (○), 5-FU (15 mg per kg per day for 5 days) (□), or two courses of C242-DM1 (300 μg per kg per day for 10 days; days 7–11 and days 14–18) (△). (*B*) Efficacy in treatment of very large COLO 205 xenografts (mean tumor size = 500 mm³). Tumor-bearing animals 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) (□), or one course of C242-DM1 (300 μg per kg per day for 5 days, days 7–11) (△).

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