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Therapeutic Effect of Ansamitocin Targeted to Tumor by a Bispecific Monoclonal Antibody

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We have constructed a murine hybrid hybridoma that secretes a bispecific monoclonal antibody (mAb) by fusing a hybridoma secreting an anti-ansamitocins mAb with a hybridoma secreting an anti-human transferrin receptor (TfR) mAb that binds to human A431 epidermoid carcinoma cells. The bispecific mAb, reactive to both ansamitocins and TfR, was purified by a combination of hydrophobic column chromatography and hydroxyapatite high-performance liquid chromatography, and evaluated in *in vivo* experiments using human tumor cell-implanted nude mice. Ansamitocin P-3 targeted through one of the antigen combining sites of the bispecific mAb was potentially more effective in suppressing the growth of established A431 tumor xenografts implanted on nude mice than unconjugated ansamitocin P-3 or the immunoconjugate of ansamitocin P-3 and monospecific anti-ansamitocins antibody, and the targeted ansamitocin P-3 finally eradicated the tumor mass. The bispecific mAb also played an important role in reducing such undesirable side-effects of ansamitocin P-3 as the loss of body weight, the damage to liver functions and the decrease in the number of white blood cells.

Key words: Hybrid hybridoma — Bispecific antibody — Ansamitocin P-3 — Human transferrin receptor

Ansamitocins were discovered as a novel class of maytansinoids produced in the fermentation broth of *Nocardia* species[1] and showed high anti-tumor activity in *in vitro* and *in vivo* experiments.[2–6] However, they had to be withdrawn from clinical studies because of their potent toxicity.

Bispecific antibodies have been used in enzyme-linked immunosorbent assays (ELISA) and in immunohistochemistry,[7–9] and have also been applied to the targeting of tumor cells for attack by toxin molecules or effector T cells.[10–13] As for chemotherapeutic agents, Corvalan *et al.* developed a bispecific antibody with specificity for both carcinoembryonic antigen (CEA) and vinca alkaloids, allowing the antibody to be specifically located on CEA-expressing cells with the ability to concentrate circulating vinblastine around the tumor mass.[14–16] Tsukada *et al.* also reported a bispecific antibody which targeted adriamycin to hepatoma cells, prolonging the survival of tumor-bearing rats.[17] Nitta *et al.* have done a clinical trial using lymphokine-activated killer cells targeted to malignant glioma by a bispecific antibody that was prepared by chemical conjugation.[18]

Recently we have developed several hybrid hybridomas secreting bispecific antibodies which can be used in an ELISA for human lymphotoxin,[19] in tumor-targeting of *Pseudomonas aeruginosa* exotoxin A[20] and in fibrin clot-targeting of tissue-plasminogen activator[21] or pro-urokinase.[22] We applied this bispecific antibody technique to eradicate cancer cells by use of the potent antitumor activity of ansamitocins while reducing their systemic toxicity. Here we describe the construction of a murine hybrid hybridoma secreting a bispecific monoclonal antibody (mAb) that recognizes both ansamitocins and human transferrin receptor (TfR) expressed on tumor cells, and its selective anti-tumor activity in nude mice xenografted with human epidermoid carcinoma A431 cells.

MATERIALS AND METHODS

Materials Ansamitocin P-3 and ansamitocin S-3 were purified from a fermentation product of *Nocardia* species,[1] and two related compounds TAC-580 and TAC-582 were chemically synthesized from ansamitocin P-3. Their structures are shown in Fig. 1.

Conjugates TAC-580 containing a free carboxyl group was conjugated with bovine serum albumin (BSA) using water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide[23] and used as the immunogen for producing anti-ansamitocins antibodies. TAC-582 containing a free amino group was maleimidated with N-(γ-maleimidobutyryloxy)succinimide and coupled to human serum albumin (HSA) dithiopyridylated with N-succinimidyl-3-(2-pyridyldithio)propionate. TAC-582-HSA conjugate obtained was used as the solid-phase antigen in an ELISA. Ansamitocin S-3 containing a sugar moiety was

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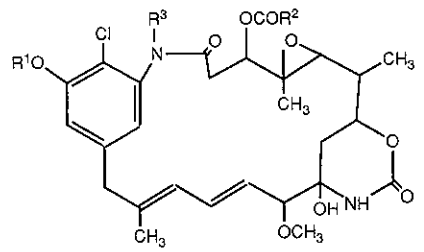


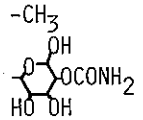
Ansamitocin P-3	$-CH_3$	$-CH(CH_3)_2$	$-CH_3$
TAC-580	$-CH_2COOH$	$-CH(CH_3)_2$	$-CH_3$
TAC-582	$-CH_3$	$-CH(NH_2)-C_6H_5$	$-CH_3$
Ansamitocin S-3	$-CH_3$	$-CH(CH_3)_2$	

Fig. 1. Chemical structure of ansamitocins and related compounds.

oxidized with sodium periodate and coupled with BSA. After reduction with sodium borohydride, ansamitocin S-3-BSA conjugate was labeled with biotinyl-N-hydroxysuccinimide ester and used in an ELISA.

Fusion procedures Splenocytes were harvested from BALB/c mice immunized with TAC-580-BSA conjugates or TfR preparations isolated from human placenta,[24] and were fused with mouse myeloma P3-X63-Ag8-U1 cells using polyethylene glycol (PEG).[25] Fused cells were selected in a hypoxanthine-aminopterin-thymidine medium, and their culture supernatants were screened by an ELISA for anti-ansamitocins or anti-TfR antibody. Positive hybridomas were cloned by a limiting dilution method, and the hybridomas AS6-44 and 22C6 secreting anti-ansamitocins (IgG1, κ) and anti-TfR (IgG1, λ) antibodies, respectively, were obtained. Further fusion and selection to produce hybrid hybridomas were carried out on the AS6-44 hybridoma and the 22C6 hybridoma using a fluorescence-activated cell sorter (FACStar IV, Becton Dickinson) according to the method described by Karawajew *et al.*[26] Briefly, the hybridomas AS6-44 and 22C6 were labeled with fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate, respectively, and fused with PEG. The heterofluorescent cells were sorted out and cultured as described before.[21] The culture supernatants of hybrid hybridomas were screened by an ELISA for a bispecific mAb, and one of the positive hybrid hybridomas, ATF1-170 was cloned by a limiting dilution method.

Screening assays for hybridomas Two ELISA systems and one fluorescence immunoassay (FIA) were adopted for screening hybridomas secreting anti-ansamitocins, anti-TfR and bispecific antibodies. In an anti-ansamitocins assay, samples were added to TAC-582-HSA conjugate-coated microplates, followed by incubation at 37°C for one hour. After washing of the plates, bound anti-ansamitocins antibody was detected with horseradish peroxidase (HRP)-labeled anti-mouse IgG antibody, followed by an enzyme reaction using 6 m*M* hydrogen peroxide and 40 m*M* *o*-phenylenediamine in 0.1 *M* citrate buffer (pH 5.5) as substrates.[27] In a bispecific mAb assay, TAC-582-HSA conjugate-coated plates were used as solid-phase antigen and biotinylated anti-mouse immunoglobulin λ chain-specific antibody as labeled antibody. Bound bispecific mAb was detected with HRP-labeled avidin, followed by the enzyme reaction as described above.

In an FIA for the measurement of anti-TfR antibody, samples were added to human erythroleukemia K562 cells,[28] followed by incubation at 37°C for one hour. After washing of the cells, bound anti-TfR antibody was detected with FITC-labeled anti-mouse IgG antibody by using a fluorometer (Baxter FCA-VIP).

Purification of bispecific antibody Hybrid hybridoma cells, ATF1-170, were grown as ascitic fluids in hybrid nude mice (Jcl:AF-*nu*), and the ascites were directly applied to an ether-Toyopearl column (Toyo Soda) employing a linear gradient of ammonium sulfate (ranging from 1.3 *M* to 0 *M* in 0.1 *M* phosphate buffer, pH 7.0) for elution.[29] Immunoglobulin fractions containing bispecific mAb activity were further subjected to hydroxyapatite high-performance liquid chromatography (HPLC) (Mitsui Toatsu) employing a linear gradient of potassium phosphate (ranging from 10 m*M* to 300 m*M*, pH 6.8) for elution.[21] Elution was monitored by measuring the absorbance at 280 nm.

***In vitro* cytotoxicity assay** Human K562 cells (2.0×10^4 cells/well) were plated in a sterile tissue culture plate with 24 flat-bottomed wells (Flow Laboratories) and sample drugs were added to the wells. After incubation at 37°C for 4 days, cell growth was judged by counting the cell number with a Coulter counter (TCA Microcell Counter CL-108).

In an alternative method, the target cells were plated in microplates with 96 flat-bottomed wells and incubated in the presence of drugs at 37°C for 4 days. The number of viable cells was then measured by a conventional colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.).[30]

***In vivo* anti-tumor assay** Human epidermoid carcinoma A431 cells (1.0×10^6 cells/mouse) were subcutaneously inoculated into hybrid nude mice (Jcl:AF-*nu*), and then

ansamitocin P-3 either alone or together with antibodies was injected intravenously. Tumor volumes were estimated at frequent intervals by using the following equation based on two perpendicular diameters of the products.[31]

$$\text{Tumor volume (cm}^3\text{)} = \text{length} \times (\text{width})^2 \times 0.5$$

Systemic toxicity Ansamitocin P-3 was injected intravenously into BALB/c mice and the change of their body weight was measured. The numbers of white blood cells and platelets, and the serum level of glutamic oxaloacetic transaminase (GOT) in blood samples were also determined using a Coulter counter (Sysmex CC-180A) and a test kit (Wako), respectively.

Pharmacokinetic studies Pharmacokinetics of ansamitocin P-3, bispecific mAb and the conjugate of ansamitocin P-3 and bispecific mAb were studied in BALB/c mice. Ansamitocin P-3 in plasma specimens of mice intravenously injected with ansamitocin P-3 at the dose of 50 μg/mouse, was determined by an ELISA employing competitive binding of ansamitocin P-3 in plasma and biotinylated ansamitocin S-3 to anti-ansamitocins antibody-coated microplates. Bispecific mAb in plasma specimens of mice intravenously injected with the mAb at the dose of 1.5 mg/mouse was determined by the anti-ansamitocins ELISA described aove. The immunoconjugate of ansamitocin P-3 and bispecific mAb in plasma specimens of mice intravenously injected with the conjugate at the dose of 10 μg/mouse as ansamitocin P-3 was assayed for cytotoxicity against K562 cells. The plasma levels of these three drugs were analyzed with a computer program based on the Simplex method.[32]

RESULTS

Preparation of the bispecific antibody A bispecific mAb-secreting hybrid hybridoma ATF1-170 was constructed by fusing a hybridoma AS6-44 secreting an IgG1 antibody against ansamitocins with a hybridoma 22C6 secreting an IgG1 antibody against human TfR. Growing tetraoma cells were screened for their ability to secrete a bispecific mAb composed of two different light chains, as described in "Materials and Methods."

A bispecific mAb ATF1-170 was purified from mouse ascites by two consecutive chromatographies employing ether-Toyopearl and hydroxyapatite columns. Mouse ascites gave several IgG peaks when they were directly applied to hydroxyapatite HPLC (Fig. 2a). Only in the middle peak was bispecific antibody activity detectable, but the isolated product was contaminated with monospecific antibodies against ansamitocins and TfR. Prefractionation of ascites on a hydrophobic column using ether-Toyopearl followed by hydroxyapatite HPLC, however, gave almost complete separation of a bispecific

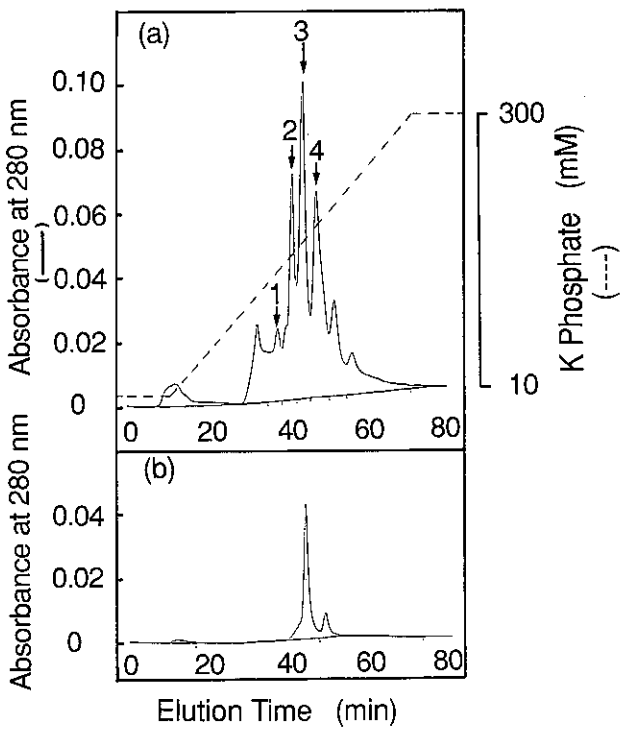


Fig. 2. Hydroxyapatite HPLC of immunoglobulins produced by the hybrid hybridoma ATF1-170. (a) Ascitic fluid was analyzed by hydroxyapatite HPLC and each fraction was assayed for the activities of bispecific, anti-ansamitocin and anti-transferrin receptor antibodies. 1 and 2, anti-ansamitocin antibody; 3, bispecific antibody; 4, anti-transferrin receptor antibody. (b) Ascitic fluid was fractionated on an ether-Toyopearl column and then the fraction with bispecific antibody activity was subjected to hydroxyapatite HPLC. The HPLC procedures were carried out at a flow rate of 1 ml/min with a linear gradient of 10–300 m*M* potassium phosphate buffer (pH 6.8), employing hydroxyapatite HPLC column HCA (Mitsui Toatsu, 20×250 mm). The column effluent was monitored by measuring the absorbance at 280 nm.

mAb from other parental and hybrid antibodies (Fig. 2b). This purified bispecific antibody preparation demonstrated antibody activities in anti-ansamitocins and bispecific mAb ELISA, and in an anti-TfR FIA using K562 cells (data not shown).

Neutralization of ansamitocin P-3 by antibodies Ansamitocin P-3 demonstrated a highly potent cytotoxic activity in an *in vitro* test against K562 cells, but its toxicity was significantly reduced in the presence of monospecific mAb AS6-44 or bispecific mAb ATF1-170 (Table I). Ansamitocin P-3 also exerted a lethal effect upon BALB/c mice with an LD_{50} of 30–50 μg/mouse, causing a significant loss of body weight, decreases of white blood cells and platelets, and increased GOT,

together with diarrhea and rough hair (Fig. 3). The immunoconjugate of ansamitocin P-3 and anti-ansamitocins mAb, however, demonstrated little or no toxic effect upon mice in terms of body weight and white blood cells, or liver damage, when injected at the dose of 30 μg/mouse, a level at which ansamitocin P-3 itself would cause severe side effects (Fig. 3).

Therapeutic effect of the immunoconjugates Ansamitocin P-3 was incubated with monospecific mAb AS6-44 and bispecific mAb ATF1-170 and then intravenously injected at the dose of 10 μg/mouse (as ansamitocin P-3), on the 2nd, 5th and 7th days after cell inoculation, into nude mice xenografted with human A431 carcinoma cells (Fig. 4). The mice receiving ansamitocin P-3 alone demonstrated a marked reduction in tumor size and the tumor mass apparently disappeared on the 21st day after tumor implantation in two out of five animals. But on the 30th day recurrent tumor growth was observed in all the mice of this group. One out of six mice given ansamitocin P-3 alone died on the 9th day after tumor implantation because of the systemic toxicity of the drug and the other five mice demonstrated a significant weight loss (Fig. 5). The mice receiving ansamitocin P-3 together with the bispecific mAb, however, developed no tumor mass and the trace finally disappeared by two weeks after inoculation without loss of body weight. No reoccurrence was observed by the 30th day after inoculation (Fig. 6). On the other hand, the monospecific anti-ansamitocins mAb AS6-44 did not enhance the antitumor activity of ansamitocin P-3, but blocked it completely. The tumor volumes of the mice given the immunoconjugate of ansamitocin P-3 and AS6-44 were similar to those in control mice dosed with phosphate-buffered saline instead of drugs, although the mice showed little weight loss.

Table I. Neutralization of Ansamitocin P-3 Cytotoxicity by Antibodies

Ansamitocin P-3 (pg/ml)	% Cytotoxicity against K562 cells[a]		
	Antibody free	AS6-44[b]	ATF1-170[c]
10	11	0	7
30	54	7	19
100	95	95	92

a) Numbers of K562 cell were determined by a Coulter counter.
b) Monospecific anti-ansamitocins antibody (20 ng/ml).
c) Bispecific anti-ansamitocins/anti-human transferrin receptor antibody (20 ng/ml).

Pharmacokinetics of the immunoconjugate Prolongation of the half-lives of ansamitocin P-3 by immunoconjugation with the antibody was investigated by measuring the plasma levels of ansamitocin P-3 or its antibody conjugate after intravenous injection into mice. The results were plotted on semilogarithmic paper and fitted with a sum of two exponential terms by graphical curve peeling, then the exponents describing the half-lives of the drugs were calculated. Table II summarizes the half-

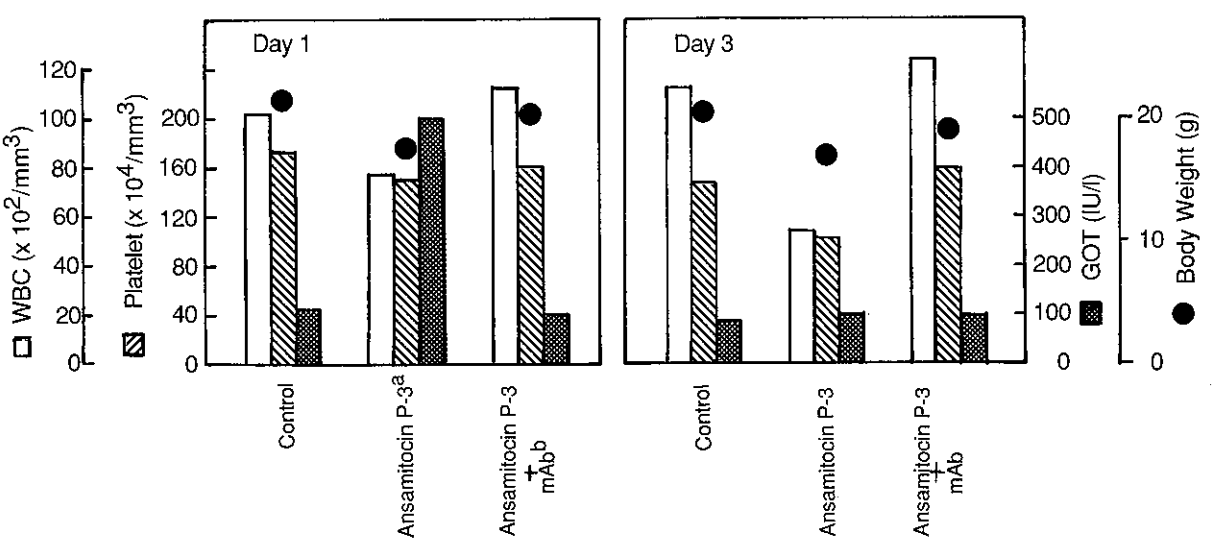


Fig. 3. Effects of anti-ansamitocins antibody (mAb) upon ansamitocin P-3 toxicity in BALB/c mice. Toxicity was evaluated by measuring body weights (●), the numbers of white blood cells (□), the numbers of platelets (▨) and the serum values of glutamic oxaloacetic transaminase (GOT) (⊠). a: ansamitocin P-3 30 μg, b: mAb 4.5 mg.

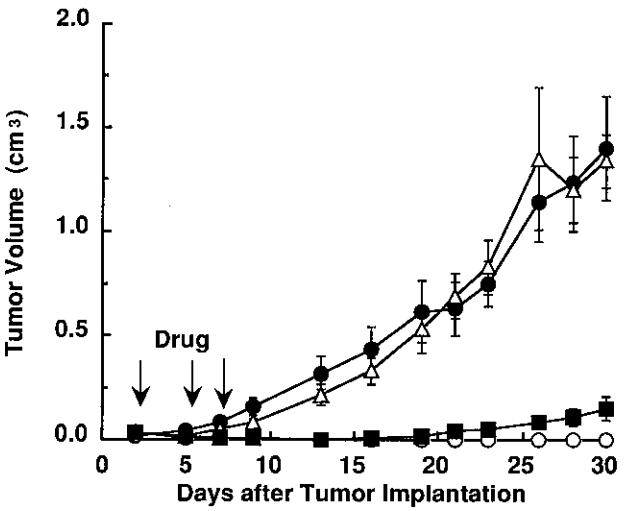


Fig. 4. Anti-tumor effects of ansamitocin P-3 conjugated with antibodies. Results are means (±SE) of tumor volumes from groups of 6 nude mice bearing the A431 human tumor xenograft; control (●), ansamitocin P-3 (10 μg, ■), ansamitocin P-3 (10 μg)+monospecific mAb (AS6-44, 1.5 mg) (△) and ansamitocin P-3 (10 μg)+bispecific mAb (ATF1-170, 1.5 mg) (○). Ansamitocin P-3 or the conjugates with AS6-44 and ATF1-170 antibodies was intravenously dosed three times on the 2nd, 5th, and 7th days after tumor implantation.

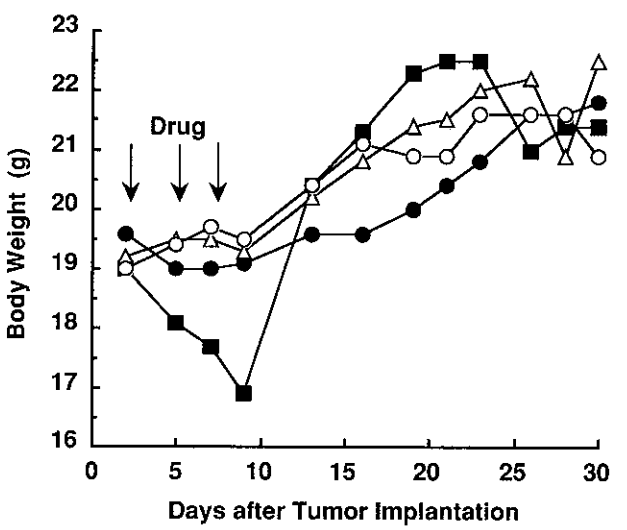


Fig. 5. Toxic effects of ansamitocin P-3 and the conjugates with antibodies upon tumor-bearing nude mice. Changes in their body weight are shown; control (●), ansamitocin P-3 (■), ansamitocin P-3+AS6-44 (△) and ansamitocin P-3+ ATF1-170 (○). Experimental details were the same as in Fig. 4.

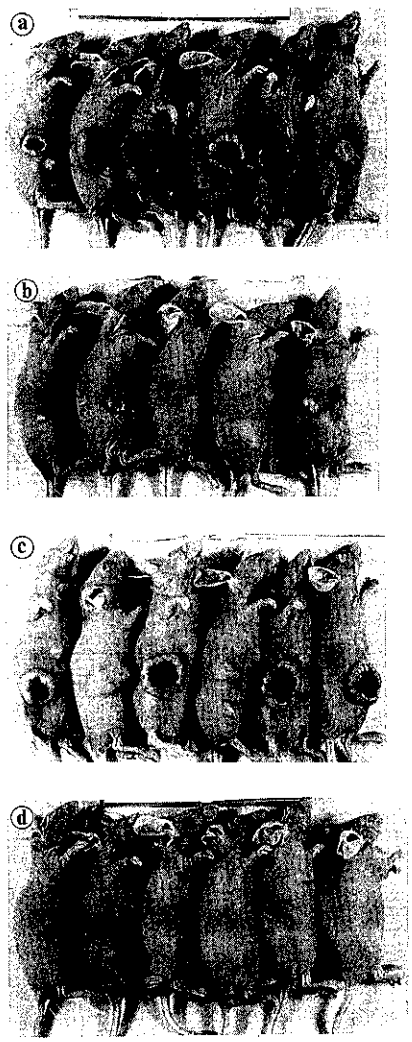


Fig. 6. Whole-body photographs of human tumor-bearing nude mice on the 30th day after tumor implantation. Experimental details were the same as in Fig. 4. a) Control, tumor volume 1.40±0.25 cm^3, b) ansamitocin P-3, tumor volume 0.15±0.06 cm^3, c) ansamitocin P-3+AS6-44, tumor volume 1.34±0.13 cm^3, d) ansamitocin P-3+ATF1-170, tumor volume 0 cm^3.

lives of ansamitocin P-3, bispecific mAb and the conjugate. Ansamitocin P-3 rapidly disappeared from blood following intravenous injection of ansamitocin P-3 alone with half-lives of 12.4 and 159 s in the α- and β-phases, respectively. Ansamitocin P-3 immunoconjugated with bispecific mAb, however, was found to have approximately 300-fold longer half-lives than unconjugated ansamitocin P-3 in both the α- and β-phases. The half-life in the α-phase was almost equal to that of the bispecific

Table II. Plasma Half-lives of Ansamitocin P-3, ATF1-170, and Ansamitocin P-3/ATF1-170

Sample	Plasma half-life[a]	
	α-Phase	β-Phase
Ansamitocin P-3	12.4 s	159.0 s
ATF1-170[b]	1.4 h	113.0 h
Ansamitocin P-3/ATF1-170	1.1 h	13.5 h

a) Plasma clearance curves were analyzed biphasically.
b) Bispecific anti-ansamitocins/anti-human transferrin receptor antibody.

antibody itself, although there was a marked difference in the β-phase.

DISCUSSION

Many researchers have covalently linked chemotherapeutic agents to anti-cancer antibodies and successfully applied them to selective tumor killing.[33-35] Such chemical conjugates, however, were usually much less potent *in vivo* than the parental free drugs, because the drugs can hardly be released in the free and pharmacologically most active form. Corvalan *et al.* reported that a bispecific mAb recognizing both CEA and vinca alkaloids was able to deliver the drugs to tumor sites while retaining them in an active form. The immunochemical conjugate was significantly effective in suppressing tumor growth of established tumor xenografts.[15, 16] We applied this technique to targeting ansamitocins with a highly potent cytotoxicity which is 10- to 100-times higher than that of vinca alkaloids and other clinically useful drugs.[36]

We have been able to prepare a bispecific mAb recognizing both ansamitocins and human TfR, and have observed the specific targeting of ansamitocins to tumor xenografts implanted subcutaneously on nude mice by the bispecific antibody. Two methods have been reported for the preparation of a bispecific antibody; one is chemical conjugation and the other is a hybrid hybridoma technique. The former method allows easy and rapid preparation of a bispecific antibody, but the product would usually be heterogeneous and often in a polymerized form.[37] The latter cell fusion method, however, gives a homogenous preparation in a large quantity, although there would be some difficulties in purifying a bispecific antibody from immunoglobulin mixtures secreted by a hybrid hybridoma.[19] A hybrid hybridoma stably secreting a bispecific mAb was easily constructed by the method of Karawajew *et al.* using a double-fluoresceinating technique.[26] The hybrid hybridoma, ATF1-170, thus obtained seemed to produce mainly three kinds of immunoglobulin species: one of them was the relevant bispecific mAb and the others were the parental mAbs. The bispecific mAb was isolated from other immunoglobulins by a combination of hydrophobic column chromatography and hydroxyapatite HPLC. As shown in Fig. 2b, the hybrid column chromatography was found to be useful for the isolation of the bispecific mAb from the parental and other hybrid immunoglobulins, and its purity reached approximately 78% after a single chromatographic procedure. The bispecific mAb-containing fraction was further purified by hydroxyapatite HPLC for use in *in vitro* and *in vivo* experiments.

The bispecific mAb neutralized the highly potent toxicity of ansamitocin P-3 not only in an *in vitro* cytotoxicity test using human K562 cells as the target but also in an *in vivo* experiment using BALB/c mice dosed with the antibody conjugate. There was little apparent toxicity, on injection of the immunoconjugate, even at a dose equivalent to an LD_{50} dose of unconjugated ansamitocin P-3. Conjugation of ansamitocin P-3 with the antibody might block the entry of the drug into cells.

The TfR on human cells is thought to have an essential role in iron transport across the cell membrane and is selectively expressed on proliferating cells.[38-40] In some cases, TfR can be used as a marker to distinguish between tumor cells and normal tissues.[41-43] Here we employed human epidermoid carcinoma A431 cells highly expressing TfR as the target and investigated the therapeutic effect of ansamitocin P-3 targeted to the established tumor xenografts by the bispecific mAb. Three successive doses of the drug immunoconjugated with the bispecific mAb seemed completely to eradicate the tumor mass, while the administration of the drug alone did not prevent reoccurrence of tumor growth. Moreover, the immunoconjugate did not induce systemic toxicity such as that observed in experimental animals given the unconjugated drug. On the other hand, the immunoconjugate of ansamitocin P-3 and the monospecific anti-ansamitocins mAb showed no therapeutic effect upon tumor-implanted nude mice, although the systemic toxicity was not seen. The anti-ansamitocins monospecific mAb or the bispecific mAb is likely to block the active site of ansamitocin P-3 and to inhibit its toxicity in test animals. As for the tumor eradication by the immunoconjugate of ansamitocin P-3 and the bispecific mAb, it is probable that the bispecific mAb delivered the drug to tumor sites, where the conjugate was endocytosed by tumor cells, followed by the release of the drug in a free and active form. The prolonged half-lives of ansamitocin P-3 immunoconjugated with the antibody might also contribute to the increased therapeutic effect. Biodistribution of ansamitocin P-3 and the bispecific antibody was not investigated in this study, but specific killing of target cells in cell cultures and regression of solid tumors growing as subcutaneous xenografts

by a chemical conjugate of a toxin and an anti-TfR antibody have been reported.[44,45] Moreover, our *in vivo* results demonstrated a remarkable difference in therapeutic effects between the parental anti-ansamitocins mAb and the bispecific mAb.

Further studies of this bispecific mAb will require care, because TfR is obviously not a tumor-specific antigen but is expressed in some normal hematopoietic cells. However, the present technique employing the immunoconjugate of ansamitocins and a bispecific antibody could be applied to an anti-tumor antibody with sufficient specificity and should then be of practical therapeutic importance.

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REFERENCES

1) Higashide, E., Asai, M., Ootsu, K., Tanida, S., Kozai, Y., Hasegawa, T., Kishi, T., Sugino, Y. and Yoneda, M. Ansamitocin, a group of novel maytansinoid antibiotics with antitumor properties from *Nocardia*. *Nature*, **270**, 721–722 (1977).
2) Ootsu, K., Kozai, Y., Takeuchi, M., Ikeyama, S., Igarashi, K., Tsukamoto, K., Sugino, Y., Tashiro, T., Tsukagoshi, S. and Sakurai, Y. Effects of new antimitotic antibiotic, ansamitocins, on the growth of murine tumors *in vivo* and on the assembly of microtubules *in vitro*. *Cancer Res.*, **40**, 1707–1717 (1980).
3) Tanida, S., Hasegawa, T. and Higashide, E. A simple method for determining antitubulinic activities of ansamitocins and related compounds using a cilia regeneration system with decililated *Tetrahymena*. *Agric. Biol. Chem.*, **44**, 1847–1853 (1980).
4) Kawai, A., Akimoto, H., Hashimoto, N. and Nomura, H. Chemical modification of ansamitocins. I. Synthesis and properties of 4,5-deoxymaytansinoids. *Chem. Pharm. Bull.*, **32**, 2194–2199 (1984).
5) Akimoto, H., Kawai, A., Hashimoto, N. and Nomura, H. Chemical modification of ansamitocins. II. Synthesis of 3-epimaytansinoids via 3-maytansinones. *Chem. Pharm. Bull.*, **32**, 2565–2570 (1984).
6) Kawai, A., Akimoto, H., Kozai, Y., Ootsu, K., Tanida, S., Hashimoto, H. and Nomura, H. Chemical modification of ansamitocins. III. Synthesis and biological effects of 3-acyl ester of maytansinol. *Chem. Pharm. Bull.*, **32**, 3441–3451 (1984).
7) Milstein, C. and Cuello, A. C. Hybrid hybridoma and their use in immunohistochemistry. *Nature*, **305**, 537–540 (1983).
8) Suresh, M. R., Cuello, A. C. and Milstein, C. Advantages of bispecific hybridomas in one-step immunocytochemistry and immunoassays. *Proc. Natl. Acad. Sci. USA*, **83**, 7989–7993 (1986).
9) Karawajew, L., Behrsing, O., Kaiser, G. and Micheel, B. Production and ELISA application of bispecific monoclonal antibodies agaist fluorescein isothiocyanate (FITC) and horseradish peroxidase (HRP). *J. Immunol. Methods*, **111**, 95–99 (1988).
10) Staerz, U. D., Kanagawa, O. and Bevan, M. J. Hybrid antibodies can target sites for attack by T cells. *Nature*, **314**, 628–631 (1985).
11) Lanzavecchia, A. and Scheidegger, D. The use of hybrid hybridomas to target human cytotoxic T lymphocytes. *Eur. J. Immunol.*, **17**, 105–111 (1987).
12) Clark, M. and Waldmann, H. T-Cell killing of target cells induced by hybrid antibodies: comparison of two bispecific monoclonal antibodies. *J. Natl. Cancer Inst.*, **79**, 1393–1401 (1987).
13) Fanger, M. F., Segal, D. M. and Romet-Lemonne, J.-L. Bispecific antibodies and targeted cellular cytotoxicity. *Immunol. Today*, **12**, 52–54 (1991).
14) Corvalan, J. R. F. and Smith, W. Construction and characterisation of a hybrid-hybrid monoclonal antibody recognizing both carcinoembryonic antigen (CEA) and vinca alkaloids. *Cancer Immunol. Immunother.*, **24**, 127–132 (1987).
15) Corvalan, J. R. F., Smith, W., Gore, V. A., Brandon, D. R. and Ryde, P. J. Increased therapeutic effect of vinca alkaloids targeted to tumor by a hybrid-hybrid monoclonal antibody. *Cancer Immunol. Immunother.*, **24**, 138–143 (1987).
16) Corvalan, J. R. F., Smith, W. and Gore, V. A. Tumor therapy with vinca alkaloids targeted by a hybrid-hybrid monoclonal antibody recognising both CEA and vinca alkaloids. *Int. J. Cancer: Suppl.*, **2**, 22–25 (1988).
17) Tsukada, Y., Ohkawa, K., Hibi, N., Tsuzuki, K., Oguma, K. and Satoh, H. The effect of bispecific monoclonal antibody recognizing both hepatoma-specific membrane glycoprotein and anthracycline drugs on the metastatic growth of hepatoma AH66. *Cancer Biochem. Biophys.*, **10**, 247–256 (1989).
18) Nitta, T., Sato, K., Yagita, H., Okumura, K. and Ishii, S. Preliminary trial of specific targeting therapy against malignant glioma. *Lancet*, **335**, 368–371 (1990).
19) Tada, H., Toyoda, Y. and Iwasa, S. Bispecific antibody-producing hybrid hybridoma and its use in one-step immunoassays for human lymphotoxin. *Hybridoma*, **8**, 73–83 (1989).
20) Honda, S., Ichimori, Y. and Iwasa, S. A human hybrid

hybridoma producing a bispecific monoclonal antibody that can target tumor cells for attack by *Pseudomonas aeruginosa* exotoxin A. *Cytotechnology,* **4,** 59–68 (1990).
21) Kurokawa, T., Iwasa, S. and Kakinuma, A. Enhanced fibrinolysis by a bispecific monoclonal antibody reactive to fibrin and tissue plasminogen activator. *Bio/Technology,* **7,** 1163–1167 (1989).
22) Kurokawa, T., Iwasa, S., Kakinuma, A., Stassen, J.-M., Lijnen, H. R. and Collen, D. Enhancement of clot lysis *in vitro* and *in vivo* with a bispecific monoclonal antibody directed against human fibrin and against urokinase-type plasminogen activator. *Thromb. Haemostasis,* **66,** 684–693 (1991).
23) Iwasa, S., Kondo, K., Miya, T. and Takeda, K. Enzyme immunoassay of β-adrenergic agent using β-galactosidase as label. *Immunopharmacology,* **1,** 3–12 (1978).
24) Seligman, P. A., Schleiche, R. B. and Allen, R. H. Isolation and characterization of the transferrin receptor from human placenta. *J. Biol. Chem.,* **254,** 9943–9946 (1979).
25) Gefter, M. L., Margulies, D. H. and Scharff, M. D. A simple method for polyethylene glycol-promoted hybridization of mouse myeloma cells. *Somatic Cell Genet.,* **3,** 231–236 (1977).
26) Karawajew, L., Micheel, B., Behrsing, O. and Gaestel, M. Bispecific antibody-producing hybrid hybridomas selected by a fluorescence activated cell sorter. *J. Immunol. Methods,* **96,** 265–270 (1987).
27) Iwasa, S., Konishi, E., Kondo, K., Suzuki, T., Akaza, H. and Niijima, T. Selective cytotoxicity of drug-monoclonal antibody conjugates against murine bladder tumor cells. *Chem. Pharm. Bull.,* **35,** 1128–1137 (1987).
28) Klausner, R. D., Ashwell, G., Remswonde, J., Harford, J. B. and Bridges, K. R. Binding of apotransferrin cycle. *Proc. Natl. Acad. Sci. USA,* **80,** 2263–2266 (1983).
29) Sakihama, N., Kitagawa, Y., Kitazume, Y. and Shin, M. Application of Toyopearls to purification of *Azotobacter* flavodoxin. *Agric. Biol. Chem.,* **47,** 2917–2919 (1983).
30) Tada, H., Shiho, O., Kuroshima, K., Koyama, M. and Tsukamoto, K. An improved colorimetric assay for interleukin 2. *J. Immunol. Methods,* **93,** 157–165 (1986).
31) Geran, R. I., Greenberg, N. H., Macdonald, M. M., Schumacher, A. M. and Abbott, B. J. Protocols for screening chemical agents and natural products against animal tumors and other biological systems (third edition). *Cancer Chemother. Rep., Part 3,* **3,** 1–61 (1972).
32) Yamaoka, K., Tanigawara, Y. and Uno, T. A pharmacokinetic analysis program (Multi) for microcomputer. *J. Pharmacobio-Dyn.,* **4,** 879–885 (1989).
33) Blair, A. H. and Ghose, T. I. Linkage of cytotoxic agents to immunoglobulin. *J. Immunol. Methods,* **59,** 129–143 (1983).
34) Ohkawa, K., Hibi, N. and Tsukada, Y. Evaluation of a conjugate of purified antibodies against human AFP-dextran-daunorubicin to human AFP-producing yolk sac tumor cell lines. *Cancer Immunol. Immunother.,* **22,** 81–86 (1986).
35) Shin, L. B., Sharkey, R. M., Primus, F. J. and Goldenberf, D. M. Site-specific linkage of methotrexate to monoclonal antibodies using an intermediate carrier. *Int. J. Cancer,* **41,** 832–839 (1988).
36) Chari, R. V., Martell, B. A., Gross, J. L., Cook, S. B., Shah, S. A., Blattler, W. A., Mckenzie, S. J. and Goldmacher, V. S. Immunoconjugates containing novel maytansinoids: promising anticancer drugs. *Cancer Res.,* **52,** 127–131 (1992).
37) Carlsson, J., Drevin, H. and Axen, R. Protein thiolation and reversible protein-protein conjugation. *Biochem. J.,* **173,** 723–737 (1978).
38) Trowbridge, I. S. and Omary, M. B. Human cell surface glycoprotein related to cell proliferation is the receptor for transferrin. *Proc. Natl. Acad. Sci. USA,* **78,** 3030–3043 (1981).
39) Trowbridge, I. S. and Lopez, F. Monoclonal antibody to transferrin receptor blocks transferrin binding and inhibits human tumor cell growth *in vitro. Proc. Natl. Acad. Sci. USA,* **79,** 1175–1179 (1982).
40) Mendelsohn, J., Trowbridge, I. and Castagnola, J. Inhibition of human lymphocyte proliferation by monoclonal antibody to transferrin receptor. *Blood,* **62,** 821–826 (1983).
41) Haynes, B. F., Hemler, M., Cotner, T., Mann, D. L., Eisenbarth, G. S., Strominger, J. L. and Fauci, A. S. Characterization of a monoclonal antibody (5E9) that defines a human cell surface antigen of cell activation. *J. Immunol.,* **127,** 347–351 (1981).
42) Shindelman, J. E., Ortmeyer, A. E. and Sussman, H. H. Demonstration of the transferrin receptor in human breast cancer tissue. Potential marker for identifying dividing cells. *Int. J. Cancer,* **27,** 329–334 (1981).
43) Vodinelich, L., Sutherland, R., Schneider, C., Newman, R. and Greaves, M. Receptor for transferrin may be a "target" structure for natural killer cells. *Proc. Natl. Acad. Sci. USA,* **80,** 835–839 (1983).
44) Trowbridge, I. S. and Domingo, D. L. Anti-transferrin receptor monoclonal antibody and toxin-antibody conjugates affect growth of human tumor cells. *Nature,* **294,** 171–173 (1981).
45) Betra, J. K., Jinno, Y., Chaudhary, V. K., Kondo, T., Willingham, M. C., FitzGerald, D. J. and Pastan, I. 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 (1989).