Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 3778–3781, May 1989
Immunology

Inhibition of human tumor growth in nude mice by a conjugate of doxorubicin with monoclonal antibodies to epidermal growth factor receptor

(growth factor receptors/cytotoxicity)

ESTHER ABOUD-PIRAK*, ESTHER HURWITZ*, FRANCOISE BELLOT†, JOSEPH SCHLESSINGER*†, AND MICHAEL SELA*

*Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel; and †Rorer Biotechnology, Inc., 680 Allendale Road, King of Prussia, PA 19406

Contributed by Michael Sela, February 13, 1989

ABSTRACT **Monoclonal antibodies that recognize the extracellular domain of the epidermal growth factor receptor (mAb108) were conjugated with doxorubicin through a dextran bridge. Several antibody–drug conjugates, containing different amounts of doxorubicin, retained binding capacity to human epidermoid carcinoma (KB) cells overexpressing epidermal growth factor receptors. Slight decrease in drug cytotoxicity was seen in *in vitro* tests, as determined either by inhibition of thymidine incorporation into cells or by reduction in number and size of KB-cell colonies. Yet, when tested *in vivo* against KB tumor xenografted into nude mice, the anti-epidermal growth factor–receptor drug conjugates with high drug-substitution levels were significantly more effective than free doxorubicin, antibody alone, mixture of dextran–doxorubicin and antibody, or drug conjugated with irrelevant antibody. When the labile covalent bonds linking antibody to dextran bridge were stabilized by reduction, the therapeutic efficacy of the conjugate was markedly decreased. These results show that antibodies against the extracellular domain of the epidermal growth factor can deliver doxorubicin specifically and efficiently to tumor sites that express high receptor levels exerting a specific antitumor effect.**

The high toxicity of currently used anticancer drugs dictates a careful balance in treatment schedules between killing the tumor cells and excessive toxicity to the patient. Such considerations often impose administration of doses that are limited in efficiency. The purpose of drug targeting is to deliver the toxic agents selectively to the tumor cells while sparing normal cells. Overexpression of epidermal growth factor (EGF) receptor was found in various types of human cancers, including gliomas and both epidermoid and squamous carcinoma cells (1–7), thus offering a potential recognition site for specific delivery of cytotoxic agents to these tumors. Moreover, the EGF receptor plays an important role in regulating cell growth and oncogenesis and, therefore, blocking this receptor by antibodies may interfere with signal-transduction pathways in addition to cytotoxicity *per se*. Indeed, we have recently demonstrated that monoclonal antibodies (mAb) against the extracellular domain of the EGF receptor (mAb108) can concentrate on subcutaneous xenografts of KB cells in nude mice and retard cellular growth (8). The antiproliferative effect of mAb108 was potentiated when various antineoplastic drugs were added together with the anti-EGF-receptor antibodies (8).

In the present study we examine the possibility of enhancing the antitumor effect of mAb108 by conjugating it to an antineoplastic drug, doxorubicin. The drug was conjugated by means of periodate-oxidized dextran to the antibodies. We had previously shown that daunorubicin conjugated to antibodies against rat α-fetoprotein was very effective in prolonging the survival time of intraperitoneally hepatoma-challenged rats (9). This work was recently successfully extended to subcutaneous hepatoma tumors that metastasize to the lungs with or without surgical resection of the subcutaneous tumor (10). We report here results showing high efficacy of doxorubicin–anti-EGF-receptor conjugates in the treatment of subcutaneous xenografts of human KB cells in nude mice. The specific conjugate was significantly more effective than free drug, drug linked to an irrelevant antibody, and various other pertinent controls.

MATERIALS AND METHODS

Animals. CD1 nude mice (aged 4–8 weeks) obtained from Charles River Breeding Laboratories were maintained at the Experimental Animals Center of the Weizmann Institute of Science.

Cells. The KB human tumor cell line derived from oral epidermoid carcinoma was obtained from The American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (depleted of complement activity), glutamine, penicillin, streptomycin, and sodium pyruvate, at 37°C in 5% CO_2/95% air.

Antibodies. The mAb108 IgG2a hybridoma line was generated by immunizing mice with Chinese hamster ovary cells expressing the extracellular domain of the human EGF receptor (11). The mAb were purified as described (8).

Binding of Antibody to Doxorubicin. mAb108 or anti-dinitrophenyl (DNP) mAb were linked to doxorubicin (lot 50432619, Farmitalia) through a dextran bridge, as reported (12) with some modifications. Briefly, dextran T10 (Pharmacia), 0.5 g, was oxidized by adding 30 ml of 0.03 M $NaIO_4$ (Merck) in 0.1 M sodium acetate buffer, pH 5.5, for 48 hr at 4°C. Oxidized dextran was mixed with doxorubicin overnight at 4°C and finally with the mAbs for 24 hr at the same temperature. Doxorubicin–dextran-bound mAbs were separated from free doxorubicin and dextran–doxorubicin by gel filtration on Biogel P-60 (Bio-Rad) or by precipitation of the conjugates in 55% saturated $(NH_4)_2SO_4$ and dialysis against phosphate buffered saline (0.01 M sodium phosphate/0.15 M sodium chloride, pH 7.4) (PBS). For reduction, $NaCNBH_3$ (Sigma) was added in equivalent amounts of conjugated drug after fractionation. The molecular mass of the conjugates was estimated to be 180–250 kDa by sucrose-gradient centrifugation.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: mAb, monoclonal antibodies; EGF, epidermal growth factor; DNP, dinitrophenyl; PBS, phosphate-buffered saline.

Table 1. Antibody activity of the conjugates

	Degree of substitution, mol of Dox/mol of mAb	Concentration to replace 50% of ^{125}I-labeled mAb108 bound, μg of mAb/ml	Activity, %
	Experiment 1		
mAb108–Dex–Dox	14	0.30	100
mAb108		0.30	
	Experiment 2		
mAb108–Dex–Dox	10	2.15	47
mAb108		1.00	
	Experiment 3		
mAb108–Dex–Dox	26	0.92	52
mAb108		0.48	
	Experiment 4		
mAb108–Dex–Dox	82	0.76	65
Reduced mAb108–Dex–Dox	97	0.90	55
mAb108		0.50	

Dex, dextran; Dox, doxorubicin.

Antibody Activity of the Conjugate. Antibody activity of the conjugates was assayed by measuring their capacity to compete with the binding of native ^{125}I-labeled mAb to KB cells. Labeling of the mAb was performed by the chloramine-T method (13). KB cells (10^5 cells per well in 24-well plates, Nunc) were grown for 24 hr, washed with PBS, and incubated with different concentrations of either conjugated or native mAb for 1 hr at room temperature in the presence of ^{125}I-labeled mAb ($\approx 1.10^6$ cpm/ml; 3.10^6 cpm/μg). The cells were then washed and solubilized in 0.5 NaOH, and the associated radioactivity was determined in a γ counter (Kontron, Münchenstein, Switzerland). Concentrations of conjugated mAbs necessary to compete with binding of free labeled mAb were determined, and values at 50% competition were compared with those obtained by unconjugated mAb.

Drug Activity of the Conjugates. Drug activity was measured by the capacity of the conjugates to inhibit KB colony formation. KB cells were seeded in tissue culture dishes (50 × 15 mm^2; Nunc) at 100 cells per ml and 2.5 ml per dish. After 16–24 hr the medium was replaced, and different concentrations of either free or mAb-conjugated doxorubicin were added for 15 min at 4°C. The cells were washed once with PBS and incubated for 14 days. The cultures were then washed with PBS, fixed with 4% (vol/vol) formaldehyde for 15 min, and stained with hematoxylin. The number of colonies formed (>25 cells) was then determined. In addition, we assayed the conjugates for the inhibition of [^{3}H]thymidine incorporation as described (14).

The Antitumor Activity of mAb108–Dextran–Doxorubicin in Nude Mice. The antitumor activity of mAb108–dextran–doxorubicin conjugates was studied on s.c. xenografts of the human epidermoid carcinoma in nude mice as described (8). Briefly, KB cells ($0.5–1.10^6$) were injected s.c. into nude mice; this injection produced tumors in 100% of the mice 10 days after cell implantation. Tumor weight was 0.6–1.3 g after 27–37 days, depending on the initial cell inoculum. Treatment was started 1 day after tumor transplantation by three i.v. injections at 3- to 4-day intervals. Each treatment group consisted of at least six animals. Tumor parameters were measured twice a week with a caliper, and the volume of a tumor was calculated according to the standard formula: tumor volume in mm^3 = length × width × height. To validate volume measurements, correlation between tumor volume and tumor weight at death was assessed.

RESULTS

We have first examined the capacity of anti EGF-receptor antibodies (mAb108) conjugated with dextran–doxorubicin to bind to the EGF receptor of cultured KB cells. A displacement assay in which the conjugated antibodies were used to block the binding of ^{125}I-labeled mAb108 to cultured KB cells was used to determine binding specificity. Table 1 summarizes the binding properties of various mAb preparations containing different amounts of drug ranging from 10 to 92 doxorubicin molecules per mAb molecule. The drug was bound to the mAb through a bridge, and, therefore, no direct correlation between extent of substitution and preservation of antibody activity was found. The most-substituted mAb conjugate (drug-to-mAb molar ratio of 82:1) retained 65% of the original mAb binding activity. Highly substituted reduced conjugate (97:1 molar ratio) retained 55% of mAb–receptor binding activity.

The Effect of Conjugated mAb108 on the Growth of Cultured KB Cells. The inhibition of KB-cell colony formation was used as a measure for drug activity *in vitro* and was determined by exposing cultured KB cells (250 in 2.5 ml) for 15 min at 4°C (Fig. 1) with either mAb108–dextran–doxorubicin (experiment 3, 26:1 molar ratio of drug to mAb), anti-DNP–dextran–doxorubicin (same drug substitution), or free doxorubicin. The drug concentrations required for a 50% reduction in the number of KB cell colonies were 0.28, 0.47, and 0.15 μg/ml, respectively. These results indicated that conjugated mAb were less potent than the drug alone, but more potent than doxorubicin attached to control anti-DNP antibodies.

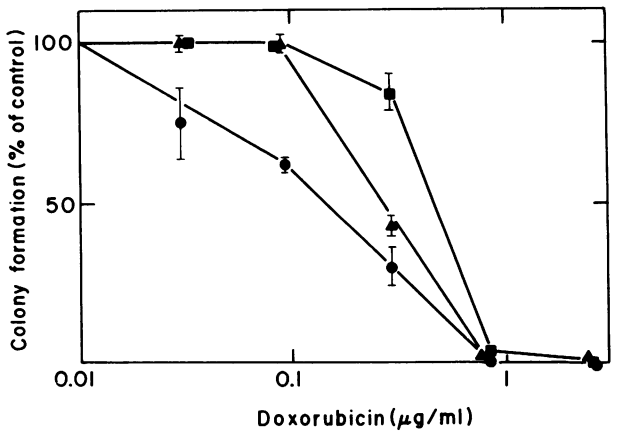

FIG. 1. Drug activity of mAb108–dextran–doxorubicin conjugate. The assay was performed as described. ●, Doxorubicin; ▲, mAb108–dextran–doxorubicin; and ■, anti-DNP mAb–dextran–doxorubicin (average of three independent measurements).

Inhibition of [^{3}H]thymidine incorporation into KB cells by the conjugated antibodies was tested. In these experiments cells were treated for 4 hr with either intact or reduced mAb108–dextran–doxorubicin conjugates and also with the controls, anti-DNP–dextran–doxorubicin or free doxorubicin. These various treatments resulted in 50% of total [^{3}H]-thymidine incorporation at doxorubicin concentrations of 0.85, 0.93, 0.83, and 0.5 μg/ml, respectively. mAb108 alone in corresponding concentrations had no effect either on colony formation or on [^{3}H]thymidine incorporation under similar experimental conditions.

Effects of Conjugated Antibodies on Xenografts of KB Cells in Nude Mice. The various conjugates were tested for their effect on the growth of KB cells in nude mice. Specificity of the antitumor effect was established by comparing the effect of conjugated mAb108 with the effect of conjugated anti-DNP antibodies. The antitumor effect was also compared with that of mixtures of dextran–doxorubicin or free doxorubicin with the specific antibody. Table 2 summarizes the influence of various conjugates under different dosages on growth of s.c. tumor xenografts as measured by their volume. From experiments 1–3 the differences obtained between the mAb108 conjugate-treated group and those treated with mixtures of antibody and dextran–doxorubicin or antibody with free doxorubicin appeared to be statistically insignificant. The degrees of drug substitution in these experiments were 14, 10, and 26 drug molecules per mAb molecule, respectively. However, the conjugate used in experiment 4 contained 82 molecules of drug per mAb molecule, thus enabling the delivery of 100 μg of doxorubicin per 325 μg of mAb108. This antibody conjugate was more effective than the various control treatments, including antibody alone, free drug, conjugates with control antibody with similar drug substitution, or a mixture of dextran–doxorubicin and mAb108 (Mann–Whitney U test; $P < 0.04$; $P < 0.03$; $P < 0.03$; and $P < 0.008$, respectively, for the above-mentioned groups). The high drug content of the conjugate enabled use of a generally low mAb dose in the treatment (325 μg per mouse)—namely, a dose lower than that needed to produce a therapeutic effect with mAb alone (8). In the group treated with the specific mAb conjugate in which the drug bonds to the dextran were chemically reduced, dramatic reduction in the antitumor effect was seen (Table 2). The group treated with this reduced conjugate showed no statistically significant difference from the placebo group and other controls. Fig. 2 describes the mean tumor volumes for the different groups of experiment 4.

DISCUSSION

The close similarity between the phenotype of tumor cells and their corresponding normal cells creates a major obstacle in cancer chemotherapy, in general, and in antibody-directed targeting, in particular. The EGF receptor is overexpressed on the cell surface of a large number of tumors—mainly in brain and in squamous epidermoid tumors. Hence, anti-EGF-receptor antibodies could be applied as carriers of drugs for specific target therapy. mAbs against the EGF receptor (mAb108) were found to retard growth of human epidermoid carcinoma (KB) cells as xenografts in nude mice (8); the mAbs concentrated and localized in the tumor mass. Thus, the mAb-mediated antitumor effect could conceivably be enhanced by conjugating the mAbs to doxorubicin, a drug widely used in solid-tumor chemotherapy despite its high toxicity.

Conjugates with doxorubicin were prepared successfully in our laboratory (9, 12, 14), as well as by others (15, 16). We report here the preparation of drug–mAb conjugates of high drug-to-mAb molar ratio, which specifically bind to the EGF receptor and exert cytotoxic effects on KB tumors in nude mice. The conjugate has an average molecular mass of ≈200 kDa, as revealed by sucrose-gradient centrifugation, indicating that the preparation mainly contains a monomeric form composed of three to five molecules of dextran per mAb molecule. Another group reported the use of dextran as a spacer for the construction of conjugates, reaching a drug-to-antibody ratio of only 3.5–4, and they obtained a molecular mass of 1.5×10^6 for the antibody conjugates (15).

The antitumor effect of the antibodies to the external domain of the EGF receptor conjugated to doxorubicin was assayed on s.c. xenografts of KB cells. Conjugates were prepared with different extents of substitution. In experiments 1–3, in which drug substitution values were low (10–26 mol of drug per mol of antibody), even though the specific conjugate was most effective, no significant difference was seen compared with a mixture of antibody and dextran–doxorubicin. Yet, the anti-EGF receptor-containing conjugates were more potent than conjugates attached to irrelevant antibodies, suggesting that targeting to the tumor site had

Table 2. Effect of mAb108 dextran–doxorubicin conjugates substituted to different extents on human epidermoid carcinoma (KB) s.c. xenografts in nude mice

Group	Treatment	Tumor volume, % PBS control			
		Exp. 1*	Exp. 2†	Exp. 3‡	Exp. 4§
1	mAb108	67 (<0.08)	49	54	112
2	Doxorubicin	81	60	52	80
3	Dextran–doxorubicin	88	87		94
4	mAb108 + dextran–doxorubicin	57	39 (<0.01)	22 (<0.01)	95
5	mAb108 + doxorubicin			26 (<0.04)	
6	αDNP mAb–dextran–doxorubicin		95	50	116
7	mAb108–dextran–doxorubicin	56 (<0.05)	39 (<0.02)	21 (<0.003)	19 (<0.001) (<0.008)

Exp., experiment. Numbers in parentheses are probabilities as determined by the Mann–Whitney U test. Mean tumor volumes in treated groups were compared with those of PBS-treated group. In Exp. 4 values for the specific conjugate-treated group were compared, as well, with those of the group treated with a mixture of drug and antibody (lower number in parentheses).

*Animals received a single injection of 50 μg of doxorubicin and 1 mg of mAb in the indicated forms; tumor volume was determined at day 27.

†Animals received three injections at days 1, 3, and 7 of a total dose of 103 μg of doxorubicin and 3 mg of mAb in the indicated form; tumor volume was determined at day 32.

‡Animals received three injections at days 1, 3, and 7 of a total dose of 150 μg of doxorubicin and 1.5 mg of mAb in the indicated form; tumor volume was determined at day 37.

§Animals received three injections at days 1, 3, and 7 of a total dose of 100 μg of doxorubicin and 325 μg of mAb in the indicated form; tumor volume was determined at day 33. For the nonreduced conjugate a total dose of 100 μg of doxorubicin and 275 μg of mAb108 was given.

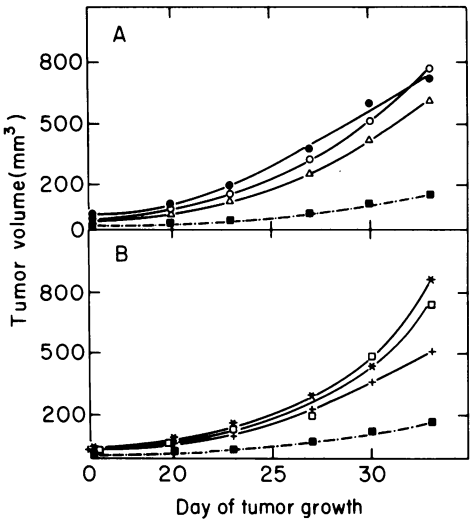

FIG. 2. Antitumor activity of mAb108–dextran–doxorubicin conjugate on s.c. xenografts of KB cells in nude mice. The experiment was done as described in text and in Table 2, experiment 4. (*A* and *B*) Results of the same experiment. The specific conjugate mAb108–dextran–doxorubicin (■) is shown in both parts of this figure. Animals were treated with mAb108 (○), doxorubicin (△), dextran–doxorubicin (●), reduced mAb108–dextran–doxorubicin (+), anti-DNP mAb–dextran–doxorubicin (*), and a mixture of mAb108 and dextran–doxorubicin (□).

been achieved. Dissociation between drug and antibody was unlikely because we have shown that 70% of the conjugate in the serum of nude mice remained intact during 48 hr (data not shown). We postulated, therefore, that the conjugated mAbs were concentrated at the tumor mass, but drug substitution probably had adverse effects on either antigen binding or effector mechanisms related to the Fc portion of the antibody.

When a conjugate with a higher degree of drug substitution was used (experiment 4), superior efficacy was seen over all control groups, including a mixture of mAb and dextran–doxorubicin. Evidently a high degree of drug substitution can compensate for the loss of mAb activity. Moreover, when a similar conjugate, containing reduced-Schiff base linkages, was used for bridging antibody to dextran, the conjugate was less effective, indicating that drug release at the tumor site did not occur. This last observation is consistent with the notion that internalization of the drug is necessary for efficient drug action. A recent report by Yang and Reisfeld (16) further supports this idea; they used antibodies to human melanoma-associated proteoglycan linked by means of an acid-sensitive linker, *cis*-aconitic anhydride, to doxorubicin. The potency of specific conjugates was explained by their stability at the intravascular pH, followed by endocytosis and cleavage in the more acidic environment of endosomes and lysosomes.

The accessibility of antibodies or drug–antibody conjugates to their target cells is a relevant variable affecting the efficacy of drug targeting. To assess this function we have checked the effect of the conjugates on KB tumor nodules in the lungs of nude mice. Preliminary results indicate that lung metastasis was also affected by the specific antibody conjugate.

From these results, it is evident that targeting of drugs to tumor-specific sites is feasible and that the use of an active antibody may contribute to the desirable anti-tumor effect of that conjugate.

1. Lyall, M. L., Hwang, J., Cordarelli, C., FitzGerald, D., Akiyama, S. I. & Gottesmann, M. M. (1987) *Cancer Res.* **47**, 2961–2966.
2. Libermann, T. A., Razon, N., Bartal, A. D., Yarden, Y., Schlessinger, J. & Souq, H. (1984) *Cancer Res.* **44**, 753–760.
3. Ozawa, S., Vuda, M., Audo, N., Abe, O. & Nobuyoshi, S. (1987) *Int. J. Cancer* **39**, 333–337.
4. Guillick, W. J., Marsden, J. J., Whittle, N., Ward, B., Bobrow, L. & Waterfield, M. D. (1986) *Cancer Res.* **46**, 285–292.
5. Yamamota, T., Kamato, N., Kawano, H., Shimizu, S., Kuraki, T., Toyoshima, K., Rikimoru, K., Nomura, N., Ishizaki, R., Pastan, I., Gainou, S. & Shimizo, N. (1986) *Cancer Res.* **46**, 414–416.
6. Ozanne, B., Shum, A., Richards, C. S., Cassells, D., Grossman, D., Trent, J., Gusterson, B. & Hendler, F. (1985) in *Cancer Cells*, eds. Faramisco, B., Ozanne, B. & Stiler, C. (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp. 41–49.
7. Jungsil, R., North, S. M., Gallick, G. E., Hortobagyi, G. N., Gutterman, J. U. & Blick, M. (1988) *Cancer Res.* **48**, 161–164.
8. Aboud-Pirak, E., Hurwitz, E., Pirak, M. E., Bellot, F., Schlessinger, J. & Sela, M. (1988) *J. Natl. Cancer Inst.* **80**, 1605–1610.
9. Tsukada, Y., Bishof, W. K. D., Hibi, N., Hirai, H., Hurwitz, E. & Sela, M. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 621–625.
10. Tsukada, Y., Ohkawa, K. & Hibi, N. (1987) *Cancer Res.* **47**, 4293–4295.
11. Schlessinger, J. (1986) *J. Cell Biol.* **103**, 2067–2072.
12. Hurwitz, E., Maron, R., Bernstein, A., Wilchek, M., Sela, M. & Arnon, R. (1978) *Int. J. Cancer* **21**, 747–755.
13. Hunter, M. W. & Greenwood, F. (1962) *Nature (London)* **194**, 495–496.
14. Hurwitz, E., Levy, R., Maron, R., Wilchek, M., Arnon, R. & Sela, M. (1975) *Cancer Res.* **35**, 1175–1181.
15. Dillman, R. O., Shawler, D. L., Johnson, D. E., Meyer, D. L., Koziol, J. A. & Frincke, J. M. (1986) *Cancer Res.* **46**, 4886–4891.
16. Yang, H. M. & Reisfeld, R. A. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1189–1193.