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Effect of a conjugate of daunomycin and antibodies to rat α-fetoprotein on the growth of α-fetoprotein-producing tumor cells

(hepatoma/anti-α-fetoprotein antibody/site-directed therapy/immunochemotherapy)

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ABSTRACT **Daunomycin was covalently attached via a dextran bridge to specific antibodies against rat α-fetoprotein produced in a horse. The effect of this conjugate on an α-fetoprotein-producing tumor was investigated in terms of cytotoxicity and inhibition or retardation of tumor development. Under the experimental conditions used, the covalent conjugate was by both criteria more efficient than either daunomycin alone or a mixture of daunomycin and specific antibodies or a conjugate of daunomycin with horse immunoglobulin. These results show that the conjugate may be useful as a specific cytotoxic agent against α-fetoprotein-producing tumors.**

The cytotoxic activity of a specific antiserum to α-fetoprotein (AFP) on AFP-producing tumors was determined both *in vitro* (1, 2, ‡) and *in vivo* (3, 4). The observation that tumor cells that produce very low levels of AFP are able to survive selectively on treatment with the antiserum *in vitro* (5) suggests that the cytotoxic activity of the antiserum may depend on the level of AFP production by the tumor cells. Recent studies (1, 6, 7) have shown that AFP is detectable on the cell surface of AFP-producing tumor cells and suggest that the binding of antibody to AFP may have a fundamental role in its cytotoxic effect on such cells.

Daunomycin (8, 9) is a well-known antitumor drug, widely used in cancer therapy. The major drawback, however, is that daunomycin, like many other drugs effective in killing tumor cells, also has detrimental effects on rapidly proliferating normal cells. This toxicity, which limits the effective use of chemotherapy in treatment of neoplastic diseases, may perhaps be overcome or reduced by binding the drug to a carrier that has specific affinity to the tumor cells (10, 11). In previous studies, we have tested the effects of daunomycin–antitumor immunoglobulin conjugates on various tumors (12). These conjugates retained most of their original drug and antibody activities (13, 14). Conjugates with specific antibodies were more effective than drug conjugates with normal immunoglobulin and, under certain conditions, were an improvement over the free drug (15).

The aim of this study was to test specific antibodies produced in a horse against rat AFP as carriers of daunomycin. Daunomycin–anti-AFP conjugates were tested for their effect on rat hepatoma and compared with free daunomycin, anti-AFP, and a mixture of anti-AFP and daunomycin linked to normal horse immunoglobulin. A preliminary report of these studies has been presented.§ A report describing the *in vitro* effect of daunomycin attached by a direct linkage to anti-mouse α-fetoprotein has also appeared (16).

MATERIALS AND METHODS

Specific Antibodies to AFP. Specific antiserum to rat AFP was produced in a horse by weekly subcutaneous injections of 1 mg of purified AFP (17) emulsified in Freund's complete adjuvant. Specific antibodies to rat AFP were purified by affinity chromatography on activated Sepharose 4B coupled to rat AFP (18). $(Fab')_2$ fragment from the specific antibody was prepared by pepsin digestion in 0.1 M citric acid/NaOH, pH 4, for 18 hr at 37°C. The resulting $(Fab')_2$ was separated from small peptides by gel filtration.¶

Tumor Cells. The rat ascites cell line AH66 was used throughout the experiments. It was maintained by intraperitoneal passage in syngeneic Donryu rats or cultivated *in vitro* (19, 20).

Chemicals and Reagents. Daunomycin hydrochloride was from Farmitalia (Milano, Italy). [^{3}H]Thymidine (specific activity, 25 Ci/nmol; 1 Ci = 3.7×10^{10} becquerels) was from the Radiochemical Centre (Amersham, England). Sodium periodate and sodium borohydride were from British Drug House (Poole, England). Sepharose 4B and Dextran T 10 (M_r 10,000) were from Pharmacia (Uppsala, Sweden), Bio-Gel P-60 was from Bio-Rad, and other chemicals and reagents were from Wako Pure Chemicals (Japan).

Binding of Antibody to Daunomycin. Normal horse IgG or specific antibodies to rat AFP were linked to daunomycin via a dextran bridge. The binding procedure was previously reported (13). In brief, oxidized dextran to which daunomycin had been linked was incubated overnight with specific antibodies at 4°C and the resulting complex was reduced by $NaBH_4$. Daunomycin–dextran-bound antibodies were separated from free daunomycin by gel filtration.

Activity of Specific Antibody, Daunomycin, and the Conjugates. The pharmacological activities of the specific antibodies, daunomycin, and the conjugates were measured by inhibition of DNA synthesis. The assay was carried out in microtest plates (Falcon No. 2024) in Eagle's minimal essential medium/kanamycin (Nisui, Japan). Cells were suspended in medium at 5×10^4/ml and dispensed into the wells of the plates in 50-μl aliquots. Drugs were diluted in 0.15 M NaCl/0.01 M sodium phosphate, pH 7.2, and then added to the cells in 50-μl portions. The plates were incubated for 2 hr at 37°C in humidified 5% CO_2/95% air. Then, 10 μCi of [^{3}H]thymidine was added to each well; after another 2 hr of incubation, 25 μl of 25% trichloroacetic acid was added, and the plates were kept at 4°C overnight. Trichloroacetic acid precipitates were washed, solubilized in tissue solubilizer (NCS, Amersham), and transferred

Abbreviation: AFP, α-fetoprotein.

‡ Tsukada, Y. & Hirai, H. (1973) *Abstracts of the 32nd General Assembly of Japan Cancer Association*, Tokyo, Japan, p. 63 (abstr.).

§ Tsukada, Y., Bischof, W. K.-D., Hurwitz, E., Sela, M. & Hirai, H. (1980) *Eighth Meeting of the International Society for Oncodevelopmental Biology and Medicine*, Tallin, U.S.S.R.

¶ Kobayashi, K., Hara, A. & Nishi, S. (1979) *Abstracts of the Third Conference for Anti-AFP Antiserum*, Tokyo, Japan, pp. 7–8 (abstr.).

to vials for assay in a liquid scintillation counter. Assays were performed in triplicate and generally showed < 10% variation. Another assay of drug activity used was the measurement of cytotoxicity as judged by trypan blue dye uptake.

Determination of the Presence of AFP-Producing Tumor Cells. Tumor cells (5×10^4/ml) were incubated with various amounts of specific antibodies to rat AFP or the appropriate controls and then washed three times with 0.15 M NaCl and incubated with 100 μl of ^{125}I-labeled protein A (1 μCi/ng) at 4°C overnight. Then, the tubes containing the tumor cells were washed thoroughly with 0.15 M NaCl, and the cells were assayed for radioactivity with a gamma counter (Aloka, Japan).

Determination of Antibody Activity of the Conjugate. The antibody activity of the conjugates was measured by solid-phase radioimmunoassay. Paper discs coated with rat AFP (2 μg per disc) were put into the wells of microtest plates (Falcon No. 2024), anti-rat AFP-specific antibodies (100 μl) or specific antibody–daunomycin conjugates (5–20 μg of antibody protein per ml) were added to each well, and the mixtures were incubated for 2 hr at room temperature. Then, the wells were washed, 50 μl of a solution of ^{125}I-labeled protein was added, and these mixtures were incubated with shaking at 4°C overnight. The plates were washed three times with 0.15 M NaCl, and the discs were transferred to new tubes for assay by a gamma counter.

***In Vitro* Studies.** The hepatoma cells were maintained in basal medium containing various amounts of conjugate or control substance for 48 hr. The growth and viability of the cells were judged by counting the cells with a hemocytometer using trypan blue dye uptake.

***In Vivo* Studies.** Hepatoma cells that had been treated with conjugate or various controls were tested for their ability to grow after transplantation, and the effect of the conjugate was evaluated by the prolongation of survival time and serum AFP levels in recipient rats.

Determination of AFP. The amount of AFP in recipient rats was determined by Mancini's radial immunodiffusion technique (21). To detect AFP at a concentration of < 5–10 μg/ml, which is the sensitivity limit of gel-precipitation techniques, we used a radioimmunoassay procedure modified by Nishie *et al.*‖ from a method described by Ceska and Lundkvist (22) for the determination of IgE.

RESULTS

Formation of AFP–Anti-AFP Antibody Complexes on Tumor Cells *in Vitro*. Rat hepatoma cells producing AFP were incubated with specific antibody to rat AFP or various controls. The cells incubated with the specific antibodies showed dose-dependent reactivity with ^{125}I-labeled protein A (Fig. 1). In contrast, the cells incubated with normal horse IgG or with $(Fab')_2$ purified from specific antibodies to rat AFP showed little reactivity with ^{125}I-labeled protein A. Some binding of ^{125}I-labeled protein A was also found when the cells were incubated with specific antibodies to human AFP.

Antibody Activity of the Conjugate. The antibody activity of anti-AFP and of daunomycin–dextran–anti-AFP conjugates was measured indirectly by the binding of radioactive protein A (Fig. 2). Only ≈10% of the original antibody activity was retained when protein A binding was used as the criterion for antibody activity. However, this is probably a minimum value; the actual antigen binding activity retained by the conjugates may be much higher, as some of the sites binding to protein A may be blocked by daunomycin–dextran.

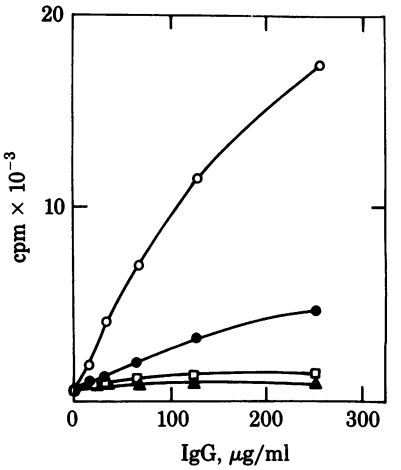

FIG. 1. Binding of anti-rat AFP-specific antibody on the surface of AFP-producing tumor cells. AH66 hepatoma cells (1×10^4 per ml) were incubated with various amounts of antibody to rat AFP or control substance for 2 hr at 37°C, washed three times with 0.15 M NaCl, incubated with ^{125}I-labeled protein A at 4°C overnight, rewashed, and assayed for radioactivity. ○, Anti-rat AFP; ▲, $(Fab')_2$ of anti-rat AFP; ●, anti-human AFP; □, normal horse immunoglobulin.

Pharmacological Activity of Daunomycin–Anti-Rat AFP. The inhibition of [^{3}H]thymidine incorporation by tumor cells, which was used as the criterion for drug activity, was measured by incubating the hepatoma cells with daunomycin–anti-AFP conjugate, free drug, or specific antibody (Fig. 3). The specific antibody by itself inhibited 30% of [^{3}H]thymidine incorporation at 400 μg/ml. The inhibition by the daunomycin–dextran–anti-AFP conjugate [antibody/drug, 50:1 (mol/mol)] was compared with that by equivalent concentrations of free daunomycin. As indicated by the similar inhibition curves, the conjugated drug maintained most of the original cytotoxic activity.

Specific Cytotoxicity of Antibody–Daunomycin Conjugate and Various Controls on Tumor *in Vitro*. Inhibition of tumor growth was tested by using an *in vitro* system (Fig. 4). The control substances included free daunomycin, normal horse IgG,

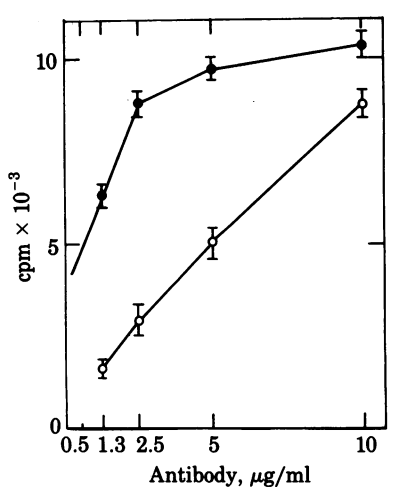

FIG. 2. Antigen binding activity of the antibodies. Discs coated with rat AFP were incubated with various amounts of horse anti-rat AFP (●) or a daunomycin–dextran–anti-AFP conjugate [50:1 (mol/mol)] (○) for 2 hr at room temperature. The discs were then washed, treated with ^{125}I-labeled protein A, incubated overnight at 4°C, rewashed, and assayed for radioactivity.

‖ Nishi, S., Kobayashi, K. & Hirai, H. (1974) *Proceedings of the 33rd Annual Meeting of the Japanese Cancer Association*, Tokyo, Japan, p. 161.

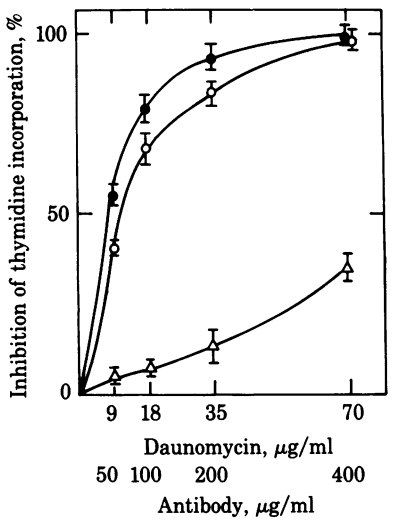

FIG. 3. Inhibition of [^{3}H]thymidine incorporation by daunomycin or an anti-AFP–daunomycin conjugate. AH66 hepatoma cells (50 μl; 5 × 10^4/ml) were incubated with 50μl-portions of solutions of various concentrations of daunomycin (●), anti-AFP (△), or daunomycin–anti-AFP conjugate [50:1 (mol/mol)] (○) for 2 hr at 37°C and then treated with 10 μl of [^{3}H]thymidine (1 μCi) for 2 hr at 37°C. The reaction was terminated by precipitation with trichloroacetic acid, and the precipitates were washed and assayed in a liquid scintillation counter.

specific antibodies to rat AFP, a conjugate or mixture of normal horse IgG and daunomycin, and a mixture of the specific antibodies and daunomycin. For every concentration of daunomycin and immunoglobulins or antibodies used, the highest cytotoxic effect was found with the specific antibody–daunomycin conjugate. The effect was 100-fold that of a mixture of specific antibodies and daunomycin, which showed a synergistic effect in comparison with the effects of either free daunomycin or specific antibodies alone. The conjugate or mixture of normal horse IgG and daunomycin showed almost the same effect as that of free daunomycin.

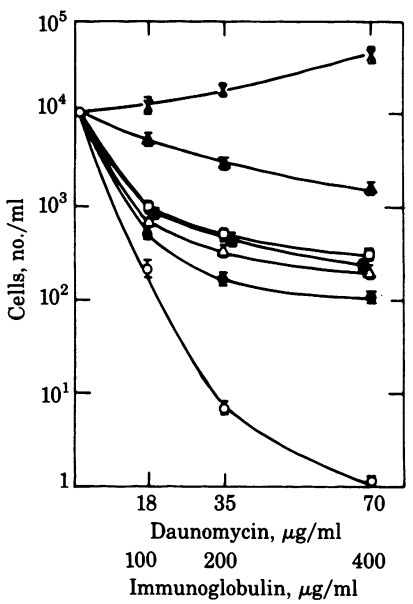

FIG. 4. Specific cytotoxicity of the conjugate *in vitro*. AH66 hepatoma cells were incubated with various amounts of conjugate or control substance for 48 hr at 37°C, and then the viable cells were counted by a hemocytometer. ×, Normal horse IgG; △, daunomycin; ▲, specific antibodies to AFP; ■, normal horse IgG/daunomycin; ●, specific antibodies to rat AFP/daunomycin; □, conjugate of daunomycin with normal horse IgG; ○, conjugate of daunomycin with specific antibodies to rat AFP.

***In Vivo* Studies.** *Treatment of tumor cells with specific antibody–daunomycin conjugate.* The conjugate was tested for effect on tumor cell growth. AH66 hepatoma cells were treated with the conjugate at 37°C for 15 min, and then the cells were transplanted intraperitoneally into syngeneic Donryu rats. Survival times of rats receiving 10^4 cells each are shown in Fig. 5. The mean survival times of rats receiving cells treated with 0.15 M NaCl or normal horse IgG were 17 and 21.4 days, respectively. Two-fold prolongation of survival time was observed in the group of rats receiving cells treated with free daunomycin or specific antibody. The conjugate or a mixture of normal horse IgG and daunomycin showed an effect similar to that of free daunomycin. A slightly synergistic effect was observed in the group of rats receiving cells treated with the mixture of specific antibodies and daunomycin. The group having the highest survival rate, however, was that receiving cells treated with the conjugate of specific antibody and daunomycin. Five out of 10 rats survived and showed resistance against repeated challenge with the tumor cells.

Therapeutic efficacy of daunomycin–anti-rat-AFP conjugate. Rats treated with 1 × 10^4 hepatoma cells received intraperitoneal injections of the conjugate and the various controls on the third, fifth, and seventh day after transplantation, and their survival was followed (Fig. 6). The mean survival times of rats injected with 0.15 M NaCl or normal horse IgG were 16.2 and 20.4 days, respectively. The survival time of rats injected with specific antibodies, daunomycin, the conjugate or a mixture of normal horse IgG and daunomycin, or a mixture of specific antibody and daunomycin were 33.3–45.2 days (i.e., those groups had survival times 1.5–2.3 times those of rats injected with normal horse IgG). The longest survival time (64 days) was that for the group of rats injected with the conjugate of specific antibodies and daunomycin.

The AFP levels in the ascites were quantitated in this experiment (Fig. 7). High AFP levels, which were accompanied by rapid growth of tumor cells, were observed in the groups of

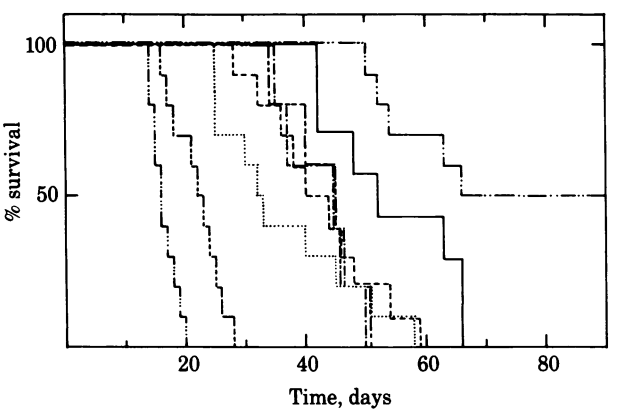

FIG. 5. The effect of previous treatment of AFP-producing tumor cells with daunomycin–anti-AFP conjugates on tumor growth *in vivo*. AH66 hepatoma cells were treated for 15 min *in vitro* with various specific and nonspecific conjugates and controls and injected into rats. Treatment: —···—, 0.15 M NaCl; — - - - —, normal horse IgG (400 μg/ml); ···, specific antibodies to rat AFP (400 μg/ml); - - -, daunomycin (70 μg/ml); —·—, normal horse IgG/daunomycin, 400:70 (wt/wt); — - - —, conjugate of daunomycin and normal horse IgG (70 μg of daunomycin bound via dextran to 400 μg of IgG); —, specific anti-AFP/daunomycin (400 μg of antibodies and 70 μg of daunomycin/ml); —···—, conjugate of daunomycin and specific antibodies to rat AFP (70 μg of daunomycin bound via dextran to 400 μg of antibodies). Cells were then washed and transplanted into syngeneic Donryu rats (10^4 cells per rat). Each group had 5–10 rats.

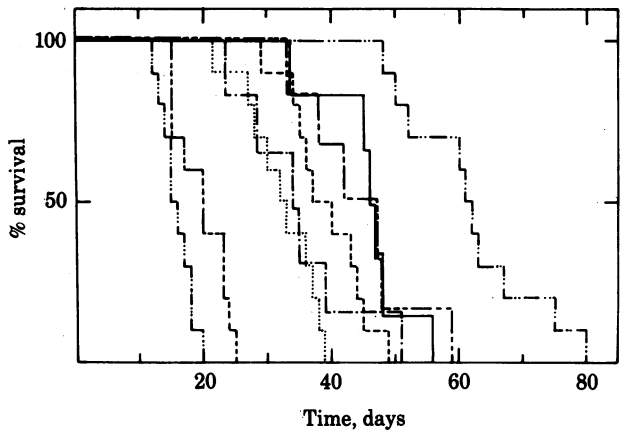

FIG. 6. Therapeutic effects of daunomycin–anti-AFP conjugates and controls on the development of AFP-producing tumor cells. AH66 hepatoma cells were injected into rats at 10^4/rat. Treatments were given 3, 5, and 7 days later. Symbols are the same as in Fig. 5. Each group had 5–10 rats.

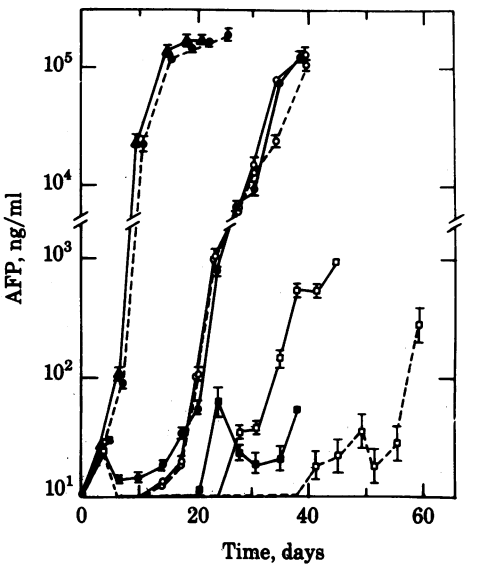

FIG. 7. AFP levels in rat ascites treated with daunomycin–antibody conjugate, daunomycin, and control substances. AFP levels in the ascites of rats that had been treated with the conjugate or a control substance (the experiment described in Fig. 6) were plotted vs. time after the injection. ▲, 0.15 M NaCl; ●---●, normal horse IgG; ●——●, daunomycin; ○——○, daunomycin/normal horse IgG; ○---○, conjugate of daunomycin and normal horse IgG; ■, specific antibodies to rat AFP; □——□, specific antibodies/daunomycin; □---□, conjugate of daunomycin and antibodies to rat AFP.

rats injected with 0.15 M NaCl or normal horse IgG. Transient decreases in the AFP levels, which were followed by abrupt increases, were observed in the groups of rats injected with free daunomycin or with the conjugate or with a mixture of daunomycin and normal horse IgG. Low AFP levels were maintained in the groups of rats injected with specific antibodies or with the conjugate and a mixture of specific antibodies and daunomycin, although gradual increases in the AFP levels were observed at terminal stages in each of these groups.

Therapeutic and Toxic Effects of the Specific Anti-AFP–Daunomycin Conjugate on Hepatoma-Inoculated Rats. The therapeutic and toxic effects of the specific anti-AFP–daunomycin conjugate at increasing doses were tested and compared with the following control treatments: free daunomycin, daunomycin–dextran, daunomycin–dextran–normal horse IgG, and daunomycin mixed with specific antibodies (Table 1). Rats were injected simultaneously with 1×10^4 tumor cells and the test substance(s), and the survival time was followed. The rats receiving 0.2 or 0.5 mg of daunomycin, or mixtures containing equivalent amounts, showed a dose-dependent increase in median survival time. The conjugate of specific antibodies and daunomycin was the most effective therapeutically.

At 2 mg per rat, free daunomycin was toxic, whereas the specific antibody–daunomycin conjugate became more effective than at lower doses, with 80% of the rats being completely cured by this treatment. The conjugate of normal horse IgG and daunomycin also showed a dose-dependent antitumor effect and was not toxic at the 2-mg dose, but it was much less effective than the specific conjugate.

DISCUSSION

Our results suggest that the anti-rat AFP-specific antibody–daunomycin conjugate has a preferential cytotoxicity against AFP-producing target tumor cells. Once the specific attachment of the conjugate to the target cells occurred, subsequent growth of the tumor cells was retarded or prevented. Specific binding of anti-rat AFP antibodies to AFP-producing cells suggests the presence of AFP on the cell surface (Fig. 1). The small amount of reactivity of anti-human AFP antibodies may be due to the immunological crossreactivity between rat and human AFP. The lack of reactivity of the $(Fab')_2$ of anti-rat AFP antibody was due to the absence of binding sites for protein A, which was used as the criterion for antibody activity. The antitumor effect of antibody–daunomycin conjugate indicates that the attachment of the conjugate to the cells must have persisted *in vivo* as well as *in vitro* (23). This persistent attachment must have been due to the antibody activity of the conjugate, because the conjugate of normal horse IgG and daunomycin was much less effective than that of the conjugate of specific antibodies and daunomycin (Figs. 4–6). In systems in which anti-IgG antibodies react with IgG-producing normal or tumor cells, there

Table 1. Toxicity of the daunomycin–anti-AFP conjugate vs. that of daunomycin and control substances

Treatment	0.2 mg of daunomycin/rat		0.5 mg of daunomycin/rat		2 mg of daunomycin/rat	
	Life span,* days	Survival,† %	Life span,* days	Survival,† %	Life span,* days	Survival,† %
Dau	21	0	30	0	12	0
Dau–dex	20	0	44	0	24	0
Dau–dex–NIgG	24	0	48	0	47	20
Dau/sp ab	28	0	32	0	28	0
Dau–dex–sp ab	38	0	54	20	60	80

Donyru rats (200 g) were injected intraperitoneally with 10^4 tumor cells and simultaneously with daunomycin–anti-AFP conjugate or a control substance. Dau, daunomycin; dex, dextran; NIgG, normal goat IgG; sp ab, specific anti-rat AFP antibodies.

* Median value.

† Calculated by dividing the number of rats surviving >2 months by the number(s) of rats in the group.

is ample evidence for the internalization of the surface-bound antibodies or their conjugates (24, 25); thus, daunomycin–antibody conjugates could also gain access by this route.

One of the important problems studied here is retaining both the activity of the drug—i.e., its cytotoxicity—and the activity of the antibody—i.e., the capacity to bind AFP. The drug activity of the conjugates was quantitated by the inhibition of [^{3}H]thymidine incorporation into target cells. Binding of daunomycin via a dextran bridge caused reduction in its activity but this could be compensated for by increasing the concentration (Fig. 2). The cell-killing effects, as estimated by trypan blue uptake, confirmed the results of the [^{3}H]thymidine incorporation assay.

The antibody activities of the conjugate were diminished as a result of the extent of drug substitution. However, because the antibody binding was measured indirectly by the binding of ^{125}I-labeled protein A, it is possible that at least some of this decrease was due to blocking of the protein A sites, whereas the antigen binding sites may have been much less affected. Experiments using the drug–antibody conjugates *in vivo* indeed suggest that a higher extent of antibody activity was maintained.

The specific cytotoxicity of the conjugate toward AFP-producing tumor cells was shown by incubating these cells in medium containing the conjugate and various controls. The cytotoxicity of the conjugate was assessed by the inhibition of cell growth for 48 hr. Marked cytotoxicity was observed in the cells to which the conjugate was added (Fig. 4). The results indicate that it is possible to obtain conjugates of daunomycin with specific antibody to AFP with sufficient retention of both drug and antibody activity to be potentially useful.

The cell-killing effect of the conjugate was also assessed by interference with the transplant capability of tumor cells and their capacity to develop into ascitic tumors in recipient rats. The growth of the tumor cells subsequent to *in vitro* treatment with the conjugate was prevented entirely in 50% of the recipient rats (Fig. 5). The therapeutic effect of the conjugate was evaluated by testing its ability to prevent *in vivo* development of tumors. The effect was assessed by the suppression of tumor growth as indicated by prolongation of the life span of the recipient rats (Fig. 6). In this experiment, the conjugate of daunomycin and specific antibodies to AFP showed a higher inhibitory effect on tumor growth than any of the controls. The persistant low levels of AFP in the group of rats receiving the conjugate clearly show that it may selectively affect AFP-producing tumor cells (Fig. 7). It is still too early to decide whether this approach will develop into a therapeutic procedure for the treatment of cancer. The results described in Table 1 are encouraging.

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1. Tsukada, Y., Mikuni, M., Watabe, H., Nishi, S. & Hirai, H. (1974) *Int. J. Cancer* **13**, 187–195.
2. Mizejewski, G. J., Young, S. R. & Allen, R. P. (1975) *J. Natl. Cancer Inst.* **54**, 1361–1365.
3. Mizejewski, G. J. & Allen, R. P. (1971) *Nature (London)* **250**, 50–52.
4. Hirai, H. (1979) in *Carcinoembryonic Proteins*, ed. Lehmann, F.-G. (Elsevier/North-Holland, Amsterdam), Vol. 1, pp. 527–538.
5. Tsukada, Y., Mikuni, M. & Hirai, H. (1974) *Int. J. Cancer* **13**, 196–202.
6. Tsukada, Y. & Hirai, H. (1975) *Ann. N.Y. Acad. Sci.* **259**, 37–46.
7. Mizejewski, G. J. & Allen, R. P. (1978) *Clin. Immunol. Immunopathol.* **11**, 307–317.
8. Calendi, E., DiMarco, A., Reggiani, M., Scarpinato, B. & Valentini, L. (1965) *Biochim. Biophys. Acta* **103**, 25–49.
9. Rusconi, A. (1966) *Biochim. Biophys. Acta* **123**, 627–630.
10. O'Neill, G. J. (1979) in *Drug Carriers in Biology and Medicine*, ed. Gregoriadis, G. (Academic, New York), pp. 23–43.
11. Blythman, H. E., Casellas, P., Gros, O., Gros, P., Jansen, F. K., Paolucci, F., Pau, B. & Vidal, H. (1981) *Nature (London)* **290**, 145–146.
12. Sela, M., Hurwitz, E. & Maron, R. (1979) in *The Role of Non-Specific Immunity in the Prevention and Treatment of Cancer*, ed. Sela, M. (Elsevier/North-Holland, Amsterdam), pp. 481–507.
13. Hurwitz, E., Levy, R., Maron, R., Wilchek, M., Arnon, R. & Sela, M. (1975) *Cancer Res.* **35**, 1175–1181.
14. Levy, R., Hurwitz, E., Maron, R., Arnon, R. & Sela, M. (1975) *Cancer Res.* **35**, 1182–1186.
15. Hurwitz, E., Maron, R., Arnon, R., Wilchek, M. & Sela, M. (1978) *Eur. J. Cancer* **14**, 1213–1220.
16. Belles-Isles, M. & Page, M. (1980) *Br. J. Cancer* **41**, 841–842.
17. Nishi, S. (1970) *Cancer Res.* **30**, 2507–2513.
18. Nishi, S. & Hirai, H. (1972) *Biochim. Biophys. Acta* **278**, 293–298.
19. Isaka, H., Umehara, S., Yoshii, H., Tsukada, Y. & Hirai, H. (1976) *GANN* **67**, 131–135.
20. Tsukada, Y. & Hirai, H. (1976) in *Oncodevelopmental Gene Expression*, eds. Fishman, W. H. & Sell, S. (Academic, New York), pp. 639–646.
21. Mancini, G., Carbonara, A. O. & Heremans, J. F. (1965) *Immunochemistry* **2**, 235–254.
22. Ceska, M. & Lundkvist, U. (1972) *Immunochemistry* **9**, 1021–1030.
23. Hurwitz, E., Maron, R., Arnon, R., Wilchek, M. & Sela, M. (1978) *Eur. J. Cancer* **14**, 1213–1220.
24. DePetris, S. & Raff, M. C. (1973) *Nature (London) New Biol.* **241**, 257–262.
25. Antoine, J.-C., Avrameas, S., Gonatas, N. K., Stieber, A. & Gonatas, J. O. (1974) *J. Cell Biol.* **63**, 12–20.