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Evaluation of a conjugate of purified antibodies against human AFP-dextran-daunorubicin to human AFP-producing yolk sac tumor cell lines

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Summary. The anticancer drug, DNR, was conjugated to an affinity-purified horse antibody to human AFP (aAFP) via a dextran bridge. The conjugate (immunoglobulin : DNR molar ratio, 1 : 50) was twice as potent as free DNR in an in vitro cytotoxicity assay against an AFP-producing human yolk sac tumor. The in vivo effect of aAFP, DNR, and the conjugate was tested against the human yolk sac tumor growing in nude mice. The conjugate, at a concentration of DNR containing the equivalent amount of 20 μg or 70 μg/mouse significantly retarded tumor growth whereas free aAFP showed only a slight inhibition of tumor growth compared to the PBS-treated control. Mice which received 20 μg/mouse of free DNR showed a moderate retardation of tumor growth whereas those which received 70 μg/mouse of DNR or a mixture of DNR and aAFP showed emaciation and early death due to acute toxicity of the drug. These results suggest that the anti-body-drug conjugate accumulated preferentially on the AFP-producing tumor cells and that cytotoxicity occurred.

Introduction

In recent years, the use of either polyclonal or monoclonal antibodies against tumor associated antigens for targeting therapeutic agents to tumors has become a major area of interest in tumor immunology [1, 2, 13, 19, 20, 21, 22]. The cytotoxic activity and the mechanism by which specific antiserum to AFP can kill AFP-producing tumor cells both in vitro [5, 10, 18] and in vivo [4, 23] has been studied. Because AFP is detectable on the surface of AFP-producing tumor cells [18, 19] it has been suggested that AFP may be considered to be a membrane associated antigen. We have previously reported that the conjugate of DNR and antibody to rat AFP, via the dextran bridge, can significantly decrease AFP-producing rat hepatoma cells [19, 20].

It is now possible to evaluate many experimental chemotherapies to human tumors using xenotransplantation where human tumors are grown in nude mice. There appears to be a good correlation between results seen with this system and those seen in patients [12, 14, 15, 16].

In this manuscript we report that a conjugate of aAFP-DNR, linked via a periodate-oxidized dextran bridge had in vitro and in vivo antitumor activity against AFP-producing human yolk sac tumor cell lines.

Materials and methods

Tumor. The yolk sac tumor (JTG-1) used in this study was established by inoculation of fresh tumor tissue into nude mice in our laboratory as described previously [9]. Another tumor cell line (JOG-9) was established from a tumor obtained from a 14-year-old female, who was diagnosed as having a pure yolk sac tumor of the ovary. Tumors were passaged by the s.c. inoculation of tumor pieces of 2–3 mm size into the lateral abdominal wall of nude mice. The tumor volume doubling time for JTG-1 was 15 days and for JOG-9 it was 20 days. The histologic appearance of the serially transplanted tumors was identical to those of the original tumors. For these studies tumors were taken between the 17th and 35th passage in nude mice. The tumor-take rate of the tumor lines was 90%–100%. Both cell lines have the ability to produce AFP and several kinds of other human serum proteins [8].

An in vitro tumor cell line was established from the JTG-1 nude mouse tumor line using the method of Okabe et al. [11] for elimination of mouse fibroblasts. The in vitro tumor cell line was designated TG-1. The tumor cells were spherical in shape and produced AFP. MKN45 (human gastric cancer cell line) [6], was kindly provided by Dr. T. Suzuki, Niigata Univ. Sch. Med. All in vitro tumor cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (Gibco, Grand Island, NY, USA) under 5% CO_2, 95% humidified air at 37 °C. Cells were passaged routinely after detachment with PBS solution containing 0.02% EDTA.

Animals. Female BALB/c athymic nude mice 4 to 8 weeks old were obtained from CLEA Japan Inc. They were kept under specific pathogen-free conditions.

Specific antibody against human AFP (aAFP). Specific antiserum to human AFP was produced in a horse by weekly s. c. injections of 1 mg of purified AFP emulsified in Freund's complete adjuvant. The aAFP was purified by af-

Abbreviations used: AFP = alpha-fetoprotein; aAFP = purified antibodies to human AFP; dex = dextran; DNR = daunorubicin; PBS = Ca^{2+}- Mg^{2+}-free Dulbecco's phosphate-buffered saline; RV = relative tumor volume; T/C = percent ratio of RV for treated mice to RV for control

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finity chromatography on activated Sepharose 4B (Pharmacia, Uppsala, Sweden) and coupled to human AFP as previously described [7].

Coupling method of aAFP to DNR. aAFP was linked to DNR via a dextran bridge. The binding procedure has previously been described [3, 19]. Briefly, oxidized dextran to which DNR had been linked was incubated overnight with specific antibodies at 4 °C. The resulting aAFP-dex-DNR conjugates (immunoglobulin : DNR molar ratio, 1 : 50) were separated from free DNR by Sephadex G200 (Pharmacia) gel filtration.

Determination of binding activity of conjugate. ^{125}I-labeled human AFP (sp. act. 100 mCi/mg protein) diluted with 10% normal horse serum in PBS (50 µl) was mixed with either aAFP or aAFP-dex-DNR conjugates (0.1–100 µg specific antibody/ml, 100 µl) in microtiter wells (Corning, No. 25860, NY, USA), and the wells were incubated for 2 h at room temperature on a horizontal shaker. Then paper discs coated with aAFP (10 µg/disc) were put into the wells and the mixture was reincubated for 18 h at room temperature. The discs were washed three times with 200 µl of PBS and were then transferred to new tubes for counting (Dinabot gamma counter, Japan). The results were expressed as % ^{125}I-AFP bound as follows: % binding = 100 × (cpm on disc of test material − cpm on disc of PBS containing 10% horse serum)/ total cpm added.

Cytotoxicity test. TG-1 cells were harvested with EDTA in PBS, washed with PBS, and resuspended in 10% fetal calf serum-containing RPMI 1640. Two kinds of cytotoxicity tests were used to assess the cytotoxic activity of the antibody, drug, or antibody-drug conjugates.

(1) The 48-h incubation test. 1×10^5 cells in 0.5 ml growth media per well were plated in 24-well microplates (Corning, No. 25820) and incubated for 2 h to allow the cells to become adherent. The drug or conjugate was filtrated through a 0.22 µm Millex filter (Millipore) to ensure sterility. Various dilutions in 0.5 ml growth media were then added to each well after which the plates were incubated for 48 h at 37 °C.

(2) The 2-h pretreatment test. Aliquots of 1×10^6 cells in growth media were spun down in plastic centrifuge tubes (Corning, No. 25330). The pellets were resuspended in 0.5 ml growth media or in 0.5 ml of various dilutions of antibody, drug, or conjugate in growth media. The cells were incubated for 2 h at 37 °C and then centrifuged. The supernatants were removed and the cells were washed once in 15 ml of growth media followed by final resuspension in growth media. Aliquots of growth media containing 10^5 cells were plated in 24-well microplates and the plates were cultured at 37 °C for 48 h.

After 48-h incubation, 10 µCi ^{3}H-thymidine (sp. act. 20 Ci/mmol; New England Nuclear, England) was added to each well and incubated for further 2 h. Then 1 ml of 10% trichloroacetic acid (TCA) was added to each well and the plates were kept at 4 °C overnight. The TCA precipitates were washed, solubilized in 0.5 ml of 1 *N* NaOH, transferred to vials, mixed with Triton-X 100 scintillator for counting in a liquid scintillation counter (Aloka LSC900 liquid scintillation counter, Japan). Viable cell number was also determined by trypan blue dye exclusion method as previously described [10].

In vivo therapy. Nude mice with progressively growing tumors were divided into groups of 5 animals. Two experiments were performed. In one study treatment was initiated on day 10 when tumors had reached a volume of 20 mm^3, whereas in a second study treatment was initiated at 8 weeks when the tumor volume was 12,000 mm^3. The drugs were dissolved in 0.1 ml PBS and injected i. p. into the tumor-bearing nude mice every 5 days. Control mice were given the same volume of PBS.

Evaluation of therapeutic effects. The size of the growing transplanted tumors was measured with sliding calipers every 5 days, and the volume was calculated by the formula as follows; $V\ (mm^3) = W^2 \times L \times 1/2$, where W and L are the width and length in millimeters. For comparison of different groups, the RV for each group was calculated using the formula $RV = Vi/V_0$, where Vi is the mean tumor volume at any given time and V_0 is the mean initial tumor volume when the treatment started. This was done to minimize the inherent variability seen with individual tumors. The ratio of treated/control × 100 (T/C) was calculated each time the tumors were measured. At the end of the studies, all treated and control mice were sacrified. The tumors were resected and weighed.

Histologic examination. Autopsies were performed on all animals at the end of the treatment. The tumors were dissected free, and the volume and weight determined. Histopathologic sections were made from all tumors and from other organs such as lung, heart, spleen, liver, and kidney. Tissues were fixed in buffered formalin and stained with hematoxylin and eosin.

Serum AFP determination. Serum AFP levels of tumor-bearing nude mice were measured by sandwich radioimmunoassay [7] at regular intervals. Serum was obtained from the tail vein of each mouse.

Drug toxicity. To monitor the side effects of the test materials, the body weight of the mice was measured once a week during the experimental period.

Results

Antibody activity of the conjugate

The antibody activity of aAFP and of the aAFP-dex-DNR conjugate was measured using the competitive binding radioimmunoassay for AFP. The binding activity of antibody in the conjugate was roughly equivalent to that of the original antibody used for conjugation (Fig. 1).

Specific cytotoxicity of aAFP-dex-DNR conjugate and various controls against tumor cells in vitro. Cytotoxicity tests were performed in which target cells were continuously incubated with the conjugate or various controls for 48 h. The control substances included free DNR, aAFP, and a mixture of aAFP and DNR. Figure 2 shows the antitumor activity of the conjugate to AFP-producing TG-1 and AFP-nonproducing MKN45 cells, respectively. The concentration of the conjugate which showed a 50% inhibitory effect of DNA synthesis in TG-1 corresponded to 186 ng of antibody and to 35 ng of DNR/ml. This DNR concentration in the conjugate was found to be less than 40% of the DNR concentration which was required to cause a 50%

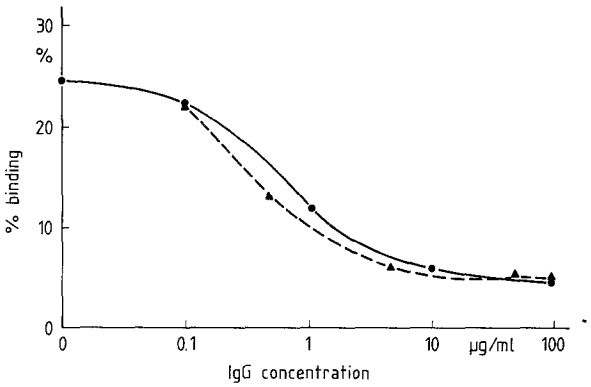


Fig. 1. Antibody activity of aAFP and conjugate. The antibody activity of aAFP (●) and of conjugate (▲) was measured with competitive binding radio-immunoassay. ^{125}I-labeled human AFP was mixed with either aAFP or conjugate in microtiter wells and incubated for 2 h at room temperature. Then aAFP-coated discs were put into the wells and further incubated for 18 h at room temperature. The discs were washed with PBS and counted in a gamma counter. Each point represents the mean ± SD of triplicate assays. The points where bars are absent indicate that the SD is less than the plotted point size

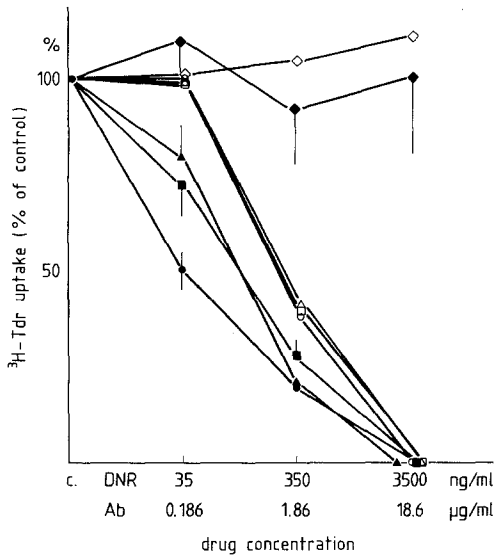


Fig. 2. Retention of drug cytotoxic activity in aAFP and conjugate. The cytotoxic effect of free DNR (squares), aAFP (diamonds), a mixture of DNR and aAFP (triangles), or conjugate (circles) on AFP-producing TG-1 (closed markers) and AFP non-producing MKN45 (open markers) cells in a 48-h cytotoxicity test. Percent ^{3}H-thymidine uptake was compared to that of controls incubated with medium. Each point represents the mean ± SE of triplicate assays and where bars are absent indicate that the SE was less than the plotted point size

inhibitory effect on DNA synthesis when only DNR alone or a mixture of aAFP and DNR was studied. The inhibitory effect of 10 or 100 times greater concentration of conjugate in the medium showed the same inhibitory effect as that of DNR alone or a mixture of aAFP and DNR (Fig. 2). By contrast, no inhibitory effect on the AFP-non-producing tumor cell line MKN45 was observed at a DNR concentration of 35 ng/ml in the conjugate. The inhibitory

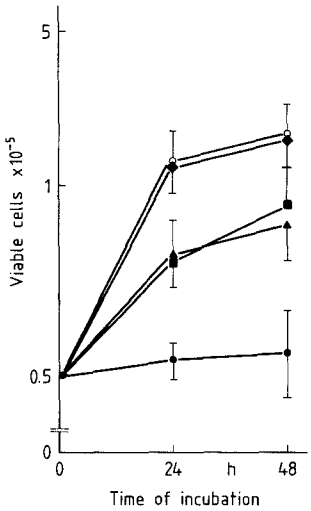
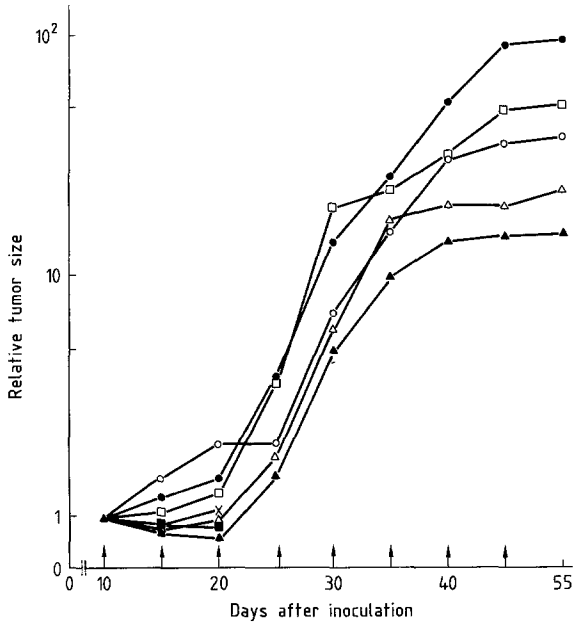


Fig. 3. Specific cytotoxicity of aAFP and conjugate. The cytotoxic effect of PBS (○) free DNR (■), aAFP (◆), a mixture of DNR and aAFP (▲), or conjugate (●) on TG-1 cells in a 2-h pretreatment test as measured by numbers of viable cells. Error bars demonstrate the SE of each point and where absent indicate that SE was less than the plotted size

effect of the conjugate was essentially the same as that of free DNR or a mixture of aAFP and DNR, implying that all activity was because of the drug (Fig. 2). Extreme reduction of the viable cell counts was also observed in the group which was treated with the aAFP-dex-DNR conjugate (data not shown). A study was undertaken to see if there was some selective cytotoxicity of the conjugate, as compared to antibody, drug, or the mixture of drug-antibody. Since the binding of the antibody to TG-1 cells had been shown to reach a plateau within 2 h at 37 °C (data not shown), tumor cells were exposed to these agents for 2 h and then incubated in fresh growth media for an additional 48 h. The concentration of each agent was selected to be equivalent to that in the conjugate which showed a 50% inhibitory effect in the 48-h test (aAFP 186 ng and DNR 35 ng/ml). As shown in Fig. 3 practically complete inhibition by the conjugate, moderate inhibition by free DNR or mixture of aAFP and DNR, and no inhibition by aAFP were observed. The inhibitory effect of DNA synthesis on the conjugate in this experiment was also observed (data not shown).

Therapeutic effect of aAFP-dex-DNR conjugate

The in vivo antitumor activity of the aAFP-dex-DNR conjugate against the AFP-producing human yolk sac tumor (JOG-9) transplanted in nude mice was studied. The in vitro cytotoxicity test of tumor cells of JOG-9 could not be performed because of the impossibility of in vitro culturing of JOG-9 cells during this period. Groups of mice which were inoculated s. c. with small pieces of tumors (day 0) were treated with i. p. injections of the drug conjugate or the appropriate control treatment (aAFP 370 µg/mouse, DNR 20 or 70 µg/mouse, DNR dose of 70 µg/mouse is the LD_{50}). In the first experiment (experiment A) treatment was started on day 10 and in the second experiment (experiment B) treatment was started on day 56. Treatment was given every 5 days for a total of 8 doses, and tumor volumes were measured. Experimental data obtained is shown in Figs. 4a, b and Table 1. The results are



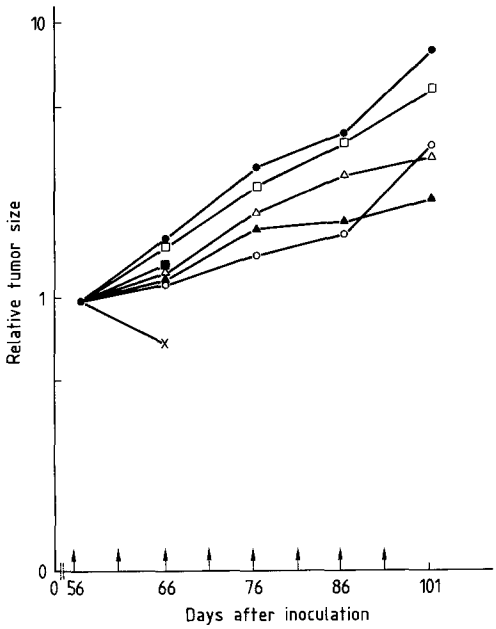


Fig. 4a, b. Effect of test materials on growth of human yolk sac tumor xenografts. Groups of 5 athymic nude mice were inoculated with tumors on day 0 and treated with i. p. (arrows) injections of test material every 5 days starting from day 10 (experiment A, Fig. 4a) or day 56 (experiment B, Fig. 4b) for a total of 8 doses. One dose includes as follows: PBS, ●; aAFP (370 μg), ○; DNR (20 μg), □: DNR (70 μg), X; mixture of aAFP (370 μg) and DNR (70 μg), ■; conjugate of aAFP (106 μg) and DNR (20 μg), △; and conjugate of aAFP (370 μg) and DNR (70 μg) ▲. Results are expressed as mean RV for each group

Table 1. Summary of the effect of the conjugate on human yolk sac tumor (JOG-9) transplanted in nude mice

Materials	One dose[a] (μg/mouse)	No. of mice	T/C[b]	
			Exp. A	Exp. B
aAFP	106	–	NT[c]	NT
	370	5	38±12[e]	47±10[e]
DNR	20	5	55±18[e]	81±25
	70	5	dead[d]	dead
aAFP/DNR	106/20	–	NT	NT
mixture	370/70	5	Dead	Dead
aAFP/DNR	106/20	5	24±5[e]	40±15[e]
conjugate	370/70	5	14±8[e]	32±2[e]

[a] Groups of 5 mice were inoculated with tumor on day 0 and treated with i.p. injections of test material every 5 days starting from day 10 (experiment A, Exp. A) or day 56 (experiment B, Exp. B) for a total of 8 doses

[b] The ratio of treated/control × 100 (T/C) was calculated from the final set of tumor volume (RV) measurements at 10 days after the final treatment. Values are mean ± SE

[c] NT: not tested

[d] Dead: death due to drug toxicity

[e] Statistics by Student's *t*-test are differences significant to $P<0.05$

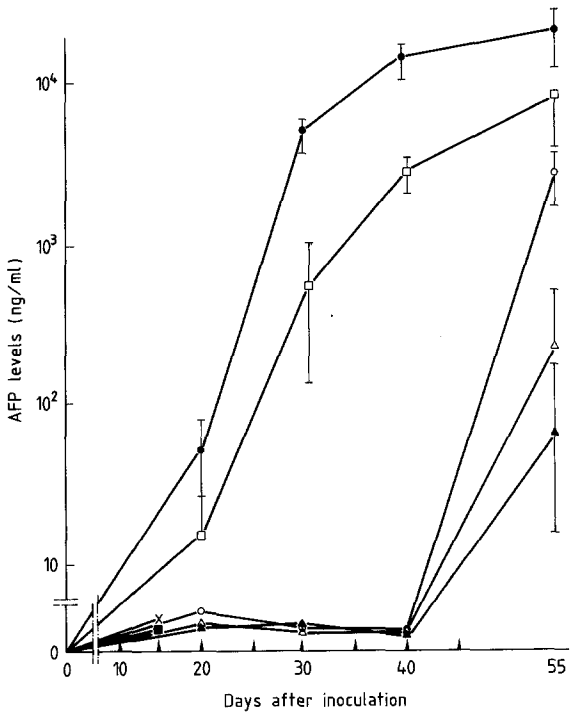


Fig. 5. Serum AFP levels in nude mice sera treated with test materials in experiment A of Fig. 4. Symbols are same as Fig. 4. Results are expressed as mean ± SE

presented as mean relative tumor volumes both in treated and control groups. The aAFP slightly inhibited the tumor growth (T/C) values, 38±12 in experiment A and 47±10 in experiment B). The conjugate was most efficacious and significantly reduced the tumor volume. The estimated T/C value at 10 days after the final treatment was 24±5 at a DNR dose of 20 μg/mouse or 14±8 at a DNR dose of 70 μg/mouse in experiment A, and 40±15 at a DNR dose of 20 μg/mouse or 32±2 at a DNR dose of 70 μg/mouse in experiment B, respectively. Free DNR was toxic at the 70 μg/mouse dose, and mice injected with DNR or a mixture of aAFP and DNR died within 15 days after initiation of treatment, whereas the DNR dose of 20 μg/mouse showed moderate inhibition of the tumor growth. The serum AFP levels correlated with the tumor volumes observed with each group (Fig. 5). Low AFP levels due to the

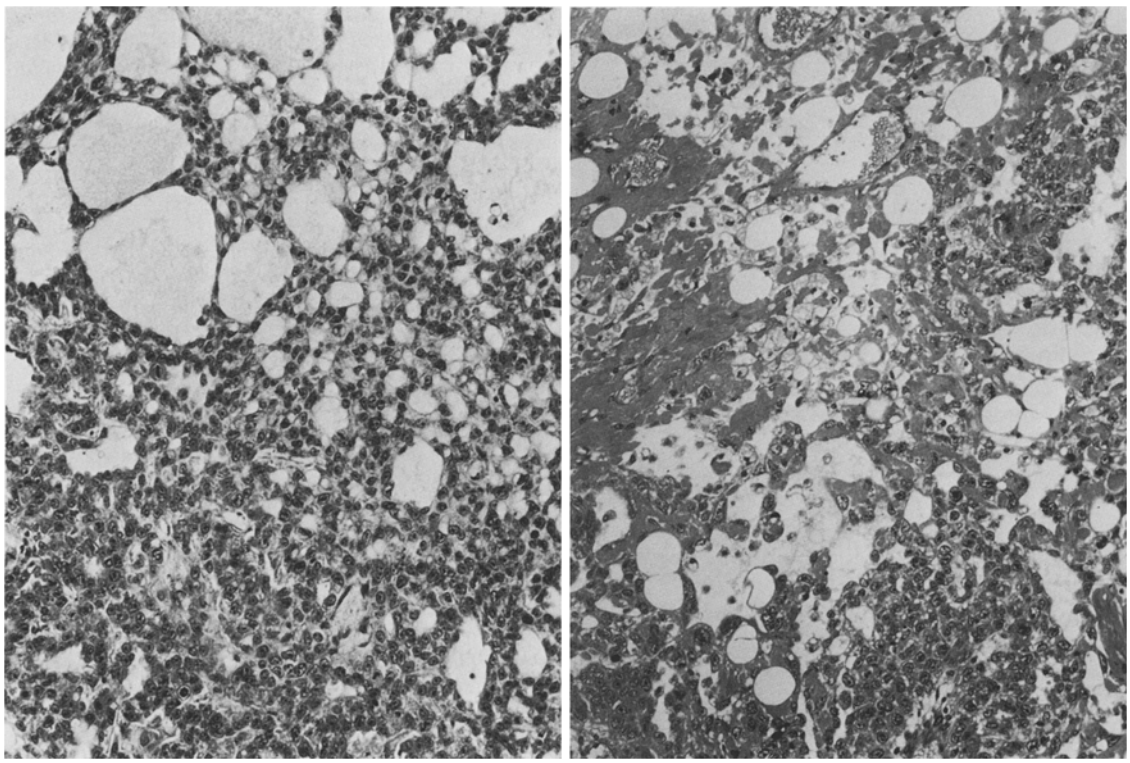


Fig. 6. Histology of tumor treated with conjugate. Tumors treated with PBS as a control consisted of the typical yolk sac elements characterized by three major patterns, reticular, endodermal sinus, and solid pattern. Tumor cells were polygonal in shape and had bizarre nuclei with prominent nucleoli. Flocculent eosinophilic substances were often seen in the stroma (*left*). The degenerative tumor cells treated with conjugate were surrounded with amorphous eosinophilic fibrin-like materials and were markedly swollen with frequent cytoplasmic vacuolization with pyknotic nuclei. A few viable cells were observed in perivascular areas *(right)*

neutralization of AFP by aAFP as previously described [19, 20, 23] were maintained in the group of mice injected with aAFP or with the conjugate, although gradual increase in AFP levels were found at the terminal stages in each of these groups.

Histologic examination of tumors which displayed tumor regression showed degenerative tumor cells embedded in amorphous eosinophilic fibrin-like material. The tumor cells showed marked swelling with frequent cytoplasmic vacuolization and pyknotic nuclei indicating cell death (Fig. 6).

Drug toxicity

A significant weight loss of 30% was noted in the mice treated with DNR alone or a mixture of aAFP and DNR in experiment A as well as in experiment B at a DNR dose of 70 μg/mouse. Moderate but significant weight loss of 10% was also observed in the mice treated with DNR at 20 μg/mouse. A slight but insignificant weight loss was noted in the mice treated with the conjugate when compared to the control mice (data not shown).

Discussion

The complete success of anticancer chemotherapy depends on a sufficient quantity of the cytotoxic agent being specifically directed to the target tissue and/or cell with minimal effect on normal tissues. In pursuit of this aim we have previously reported the antitumor effect of not only aAFP-dex-DNR conjugates but also of aAFP-DNR conjugate with poly L-glutamic acid as the intermediate drug carrier using both rat and human hepatoma cells [19, 20, 21, 22]. This manuscript describes for the first time the in vitro and in vivo antitumor effect of the aAFP-dex-DNR conjugate on the AFP-producing human yolk sac tumor. The results presented show that the conjugate retains both antigen-binding activity and anticancer drug activity against the target yolk sac tumor cell lines. Human yolk sac tumor-derived AFP contains a high proportion of AFP which does not bind to lectin, whereas up to 90% of hepatoma-derived AFP does bind to various types of lectin [17]. The biological significance of this heterogeneity of AFP, if any, remains to be determined. The aAFP used in this study was purified polyclonal antibody to AFP and the conjugate containing the aAFP was found to be as effective to yolk sac tumor as it was to hepatomas. These facts suggest that this aAFP equally recognizes the AFP antigenic sites on both hepatomas and yolk sac tumors. The use of monoclonal antibodies to AFP for the analysis of membrane bound AFP may provide further information which will be valuable to additonal immunochemotherapy studies. The antigen-binding activity of the conjugate was practically unchanged as assayed by the competitive binding radioimmunoassay of aAFP and the conjugate to ^{125}I-labeled AFP.

The specific cytotoxicity of the conjugate for AFP-producing tumor cells was shown by incubating these cells in medium containing the conjugate and comparing this to control groups. Marked and no reduction of drug cytotoxicity was observed in the tumor cells to which the conjugate was exposed for only 2 h as well as when the cells were continuously exposed for 48 h. This short-term cyto-

toxicity test in which the conjugate is briefly exposed to the target tumor cells we believe is an effective method for evaluation and speculation of the in vivo therapeutic activity of antibody-drug conjugations [2].

The therapeutic effect at the two doses of the conjugate was also evaluated by the growth inhibitory test using the human yolk sac tumor growing in nude mice. The effect was assessed by the suppression of tumor growth as indicated by estimation of T/C values, by histologic examination, and by a correlation with serum AFP levels. In this experiment, the aAFP-dex-DNR conjugate at both dosages showed a more specific antitumor effect than the free drug, that is a greater tumor inhibition was possible with less systemic toxicity, for an overall enhancement in apparent therapeutic index. Although tumor growth inhibition of the aAFP-dex-DNR conjugate at a DNR concentration of 70 μg/mouse was found, the same dose of free DNR and the mixture of aAFP and DNR showed early death due to acute drug toxicity. As a single injection of 70 μg of DNR to a mouse is equivalent to the LD_{50} dose level, mice would not survive due to systemic drug toxicity if the specific accumulation of the drug-conjugate to the tumor tissues does not occur. These later observations suggest that it may be possible to increase the dosage of drug without producing any irreversible adverse effect by antibody-mediated selective localization and targeting to the target tumor tissues.

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