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Bioconjugation of Tumor Necrosis Factor-α with the Copolymer of Divinyl Ether and Maleic Anhydride Increasing Its Antitumor Potency

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To enhance the therapeutic usefulness of antitumor cytokines *in vivo*, we synthesized bioconjugated tumor necrosis factor-α (TNF-α) with divinyl ether and maleic anhydride copolymer (DIVEMA), which has intrinsic antitumor activity as a synthetic biological response modifier. The degree of modification could be controlled by the addition of 2,3-dimethylmaleic anhydride (DMMAn), which binds to amino groups of TNF-α by changing the pH. In addition, the specific activity of DIVEMA-TNF-α was hardly decreased *in vitro*. DIVEMA-TNF-α showed a marked antitumor effect compared to native TNF-α without any side effects such as sudden death, body-weight reduction, and decrease in platelet count on mice bearing solid tumors. These results suggest that DIVEMA is a useful polymeric modifier for bioconjugation of TNF-α in order to increase its antitumor activity, and multifunctionally bioconjugated TNF-α may be a potentiated antitumor agent for therapeutic use.

Key Words: **tumor necrosis factor-α; copolymer of divinyl ether and maleic anhydride; bioconjugation; antitumor effect.**

Tumor necrosis factor-α (TNF-α) is a macrophage-derived bioactive protein produced in the serum of *Bacillus Calmette-Guerin* (BCG)-infected mice after administration of lipopolysaccharide (1). TNF-α has numerous biological activities, such as direct cytotoxicity against tumor cells *in vitro* (2) and *in vivo* (3), activation of the immune response (4) and selective impairment of tumor blood vessels without any cytotoxic effect on normal blood vessels (5). The antitumor effect of TNF-α has been reported on murine tumors transplanted into syngeneic mice (6) and human tumors transplanted into nude mice (7). Recently, clinical applications of TNF-α have been attempted as specific antineoplastic agents to tumors instead of traditional antitumor drugs (8). However, since the administration of TNF-α induces various side effects such as chills, tachycardia, hypertension, headache and pyrexia, its clinical applications are limited (9). These unexpected side effects are due to a breakdown in the balance of the cytokine network by frequent high-dose administration.

In recent years, in drug development that considers the drug delivery system, bioconjugation of water-soluble polymers to the surface of bioactive proteins has been devised (10, 11, 12). Many approaches based on bioconjugation have aimed at increasing the biological activities of proteins such as interleukin-2 (13) and asparaginase (10). In fact, polyethylene glycol-modified adenosine deaminase (PEG-ADA) and poly(styrene-co-maleic acid)-conjugated neocarzinostatin (SMANCS) shows marked therapeutic effects than native drugs and clinical applications have already been realized (14, 15). These results suggested that the bioconjugation is a pragmatic approach for successful therapies with various proteodrugs such as enzymes and antitumor agents. Bioconjugated proteins may be more extensively applied to clinical use from this now on.

We reported that chemical modification of TNF-α with PEG enhanced its antitumor potency when compared to native TNF-α (16). In addition, we found that an optimal modification with polymer (targeting molec-

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Abbreviations used are: TNF-α, tumor necrosis factor-α; DIVEMA, divinyl ether and maleic anhydride copolymer; DMMAn, 2,3-dimethylmaleic anhydride; JRU, Japan Reference Units; IL-2, interleukin-2; PEG, polyethylene glycol; PEG-ADA, polyethylene glycol-modified adenosine deaminase; SMANCS, poly(styrene-co-maleic acid)-conjugated neocarzinostatin; S-180, Sarcoma-180; FBS, fetal bovine serum; MEM, minimal essential medium.

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ular size, remaining activity *in vitro,* and degree of modification) could optimize drug therapy based on bioconjugation *in vivo* (17). Polymeric modifiers such as PEG and dextran have been most widely used because of their low toxicity, low antigenicity, and low immunogenicity. However, in order to achieve the optimum delivery of bioactive proteins utilizing bioconjugation with polymeric modifiers for clinical application, the selection of optimal modifiers specific to the purpose of bioconjugation and the properties of each protein becomes important.

The copolymer of divinyl ether and maleic anhydride (DIVEMA), a water-soluble polymeric modifier, is known as a synthetic biological response modifier that shows an enhancement of resistance to infections and various tumors in mice (18, 19). These activities of DIVEMA have been attributed to its ability to activate macrophages (18) and natural killer cells (20, 21), to induce interferon, and to increase antibody-dependent cellular cytotoxicity. These various biological activities of DIVEMA seem to be useful in increasing the antitumor potency of TNF-α *in vivo.*

In the present study, we synthesized DIVEMA-TNF-α in order to increase the antitumor activity. However, the conjugation between maleic anhydrides of DIVEMA and Lys amino groups of TNF-α seems to be complicated. Thus, we attempted to establish a novel method of bioconjugation by use of 2,3-dimethylmaleic anhydride (DMMAn) and we controlled the molecular size by protecting several amino groups of TNF-α. DIVEMA-TNF-α was separated into fractions by gel filtration chromatography, after which, the remaining activity and molecular size were determined. In addition, we evaluated the usefulness of DIVEMA on bioconjugation. These approaches enable us to design an intelligent TNF-α product suitable for clinical application.

METHODS

Materials. Natural human TNF-α was generously provided by Hayashibara Biological Laboratories, Okayama, Japan. DIVEMA (Mw: 3×10^4, Mw/Mn: 1.7) was synthesized as we previously reported (22). 2,3-dimethylmaleic anhydride (DMMAn) was purchased from ACROS, New Jersey, USA. Fluorescamine was purchased from Fluka, Tokyo, Japan. Cellpack was purchased from Sysmex, Kobe, Japan. All other chemicals were from commercial sources.

Animals and cells. Female Balb/c mice (5 weeks old) and male ddY mice (5 weeks old) were obtained from SLC, Hamamatsu, Japan. Meth-A fibrosarcoma cells and Sarcoma-180 (S-180) cells were maintained intraperitoneally on Balb/c mice and ddY mice respectively. L-M cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 1% fetal bovine serum (FBS).

Bioconjugation of TNF-α with DIVEMA and monitoring the reaction by the fluorescamine method. Natural human TNF-α was conjugated with DIVEMA by allowing formation of amido bonds between Lys amino groups of TNF-α and maleic anhydride. TNF-α with a 10-fold molar excess of Lys amino groups in pH 8.5 buffer solution was reacted with DMMAn at room temperature. After reacting for 45 min, DIVEMA (which was present in twice the quantity of TNF-α in dimethyl sulfoxide) was dripped into the reaction mixture for 60 min. The reaction mixture was adjusted to pH 6.0 by 0.1N NaOH in order to detach DMMAn from TNF-α. The reaction was monitored by the fluorescamine method in which fluorescamine reacts with the primary amino groups (23). Five μl of reaction mixture were collected at indicated times, and added to 595 μl of pH 8.5 borate buffer. The sample solution was admixed with 500 μl of fluorescamine solution, and the fluorescent intensity (390-nm excitation, 475-nm emission) was measured. DIVEMA-TNF-α was separated into fractions by gel filtration chromatography (GFC) (Superose 12 HR, Pharmacia Biotech, Uppsala, Sweden) in pH 7.2 phosphate buffer solution. The protein concentration of DIVEMA-TNF-α was determined by absorbance at 280 nm. The number-average molecular weight of native TNF-α and of DIVEMA-TNF-α was estimated by GFC analysis by comparison with protein standards. The specific activity of TNF-α and DIVEMA-TNF-α was estimated by the L-M cell cytotoxicity assay according to the method described by Yamazaki *et al.* (24).

Antitumor effect of native TNF-α or DIVEMA-TNF-α. The antitumor effect of DIVEMA-TNF-α *in vivo* was estimated on mice bearing S-180 solid tumors or Meth-A solid tumors. S-180 cells were implanted (5×10^5/200 μl/site) intradermally into male ddY mice. On day 11, native TNF-α or DIVEMA-TNF-α was administered i.v. as a single injection. Mice were used in groups of five. Meth-A cells were also implanted (5×10^5/200 μl/site) intradermally into female Balb/c mice. In this tumor model, native TNF-α or DIVEMA-TNF-α was administered i.v. twice a week for 2 weeks starting from 11 days after tumor inoculation. Mice were also used in groups of five. The antitumor potency was evaluated by tumor volume, scores of tumor hemorrhagic necrosis and animal lifespan. The tumor volume was calculated from the formula described by Haranaka *et al* (7). Tumor hemorrhagic necrosis was scored according to the method described by Carswell *et al* (1).

Body-weight change and platelet count in mice bearing Meth-A tumors with DIVEMA-TNF-α. The side effects of DIVEMA-TNF-α *in vivo* were assessed by body-weight change and platelet count. Groups of five female Balb/c mice were inoculated i.v. with native TNF-α or DIVEMA-TNF-α in various doses. The body weight after i.v. treatment was recorded over a 25-day period for comparison with the weight before administration. The platelet count was measured by using peripheral blood. Blood was collected from the tail vein into Cellpack® containing 5% EDTA and 0.1% bovine serum albumin. The platelet level on the indicated day was determined by a blood-cell counter (F-820, Sysmex, Kobe, Japan).

Statistical analysis. The hemorrhagic necrotic scores and tumor volume were statistically evaluated by the Student *t*-test.

RESULTS

Bioconjugation of TNF-α with DIVEMA. As DIVEMA has many anhydride residues, the complicated reactions between DIVEMA and proteins were expected. Therefore, the conjugation of TNF-α and DIVEMA is carried out as shown Figure 1a. The % modification of TNF-α was determined by fluorescamine, which reacts with substances containing primary amino groups to yield fluorescent products. About 50% of the Lys amino groups were first protected by addition of DMMAn, which bound to amino groups with a pH change (Fig.1b), thus resulting in a 10-fold molar excess of Lys amino groups (Fig. 2). The % protection could be controlled by the amount of DMMAn (data not shown). After conjugation with DMMAn, DIVEMA was reacted with TNF-α. DIVEMA was applied to a 10-fold molar excess of total Lys amino groups with TNF-α,

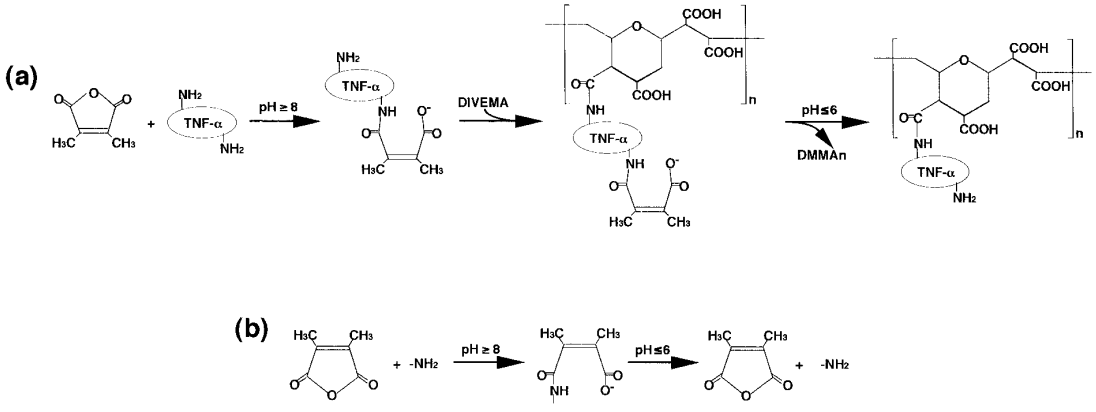

FIG. 1. Bioconjugation of TNF-α with DIVEMA. TNF-α was conjugated with DIVEMA by permitting amido bonds to form between the Lys amino groups of TNF-α and maleic anhydride (a). In order to block the complicated reactions between TNF-α and DIVEMA, the pH-reversible agent 2,3-dimethylmaleic anhydride (DMMAn), which binds amino groups at basic pH was reacted with Lys amino groups of TNF-α in pH 8.5 buffer (b).

and 15% of the amino groups remaining free after binding of DMMAn were modified by the anhydride residues of DIVEMA. After adjustment to pH 6.0, the DMMAn was removed. The reaction mixture was purified and separated by gel filtration chromatography to study the relationship between the degree of modification and the specific activity. Molecular sizes was determined with the aid of protein standards. Table 1 shows the characterization of DIVEMA-TNF-α. Fraction 2 of DIVEMA-TNF-α had one TNF-α moiety per unit of DIVEMA-TNF-α. The specific activity of DIVEMA-TNF-α fraction 1, which contained the complex conjugates, was markedly decreased when compared with native TNF-α, but fraction 2 retained the normal specific activity.

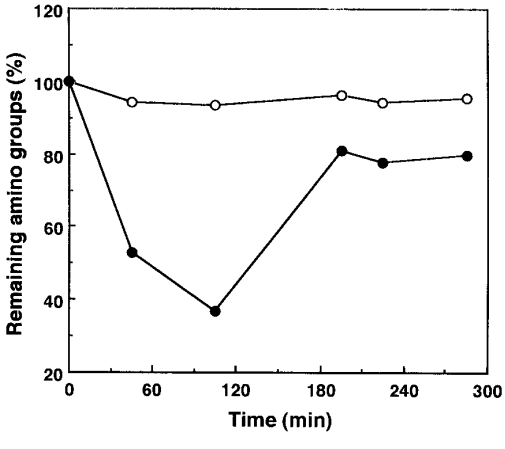

FIG. 2. Monitoring the bioconjugation of TNF-α with DIVEMA by the fluorescamine method. The synthesis of DIVEMA-TNF-α was monitored by measuring the remaining free amino groups of TNF-α. The degree of modification was estimated by comparing the free amino groups of native TNF-α (○) with those of DIVEMA-TNF-α (●).

Antitumor effect of native TNF-α and DIVEMA-TNF-α. The antitumor effects of a single i.v. injection of DIVEMA-TNF-α on mice bearing Sarcoma-180 solid tumors was studied. Native TNF-α at doses of 5,000 JRU and 10,000 JRU dramatically showed hemorrhagic necrotic effects 24 h after i.v. injection (Fig. 3). However, three of the five mice administered native TNF-α at a dose of 10,000 JRU died within 24 h of injection, and the remaining mice showed toxic side effects such as tremors and piloerection. By contrast, DIVEMA-TNF-α fraction 2 showed an antitumor effect with administration of only 1,000 JRU per mouse without toxic side effects (Fig. 3). Three μg of DIVEMA, which was the maximal dose in fraction 2 had no antitumor potency in itself by i.v. injection. Furthermore, a mixture of DIVEMA and 1,000 JRU of TNF-α did not show the hemorrhagic necrotic effects. Complete regression was not obtained by administration of 10,000 JRU of native TNF-α, which was the maximal achievable therapeutic dose. However, only 1,000 JRU

TABLE 1

Characterization of DIVEMA-TNF-α

Run	Molecular size (kD)[a]	Specific activity ($\times 10^4$ JRU/mg)[b]	Remaining activity (%)
Native TNF-α	32	223.2	100.0
DIVEMA-TNF-α			
Fr.1	$\geqslant$92	37.5	16.8
Fr.2	63	213.2	95.5

[a] The molecular size was determined by comparison with protein standards.

[b] The specific activities of native TNF-a and DIVEMA-TNF-a were measured by cytotoxic activity against L-M cells and are expressed in terms of JRU (Japan Reference Units).

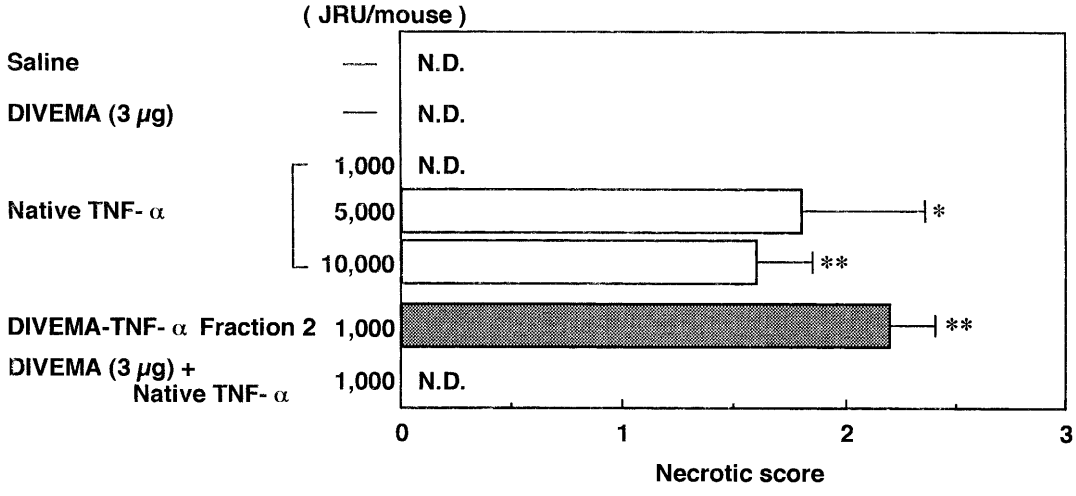

FIG. 3. Tumor necrotic effects of native TNF-α and DIVEMA-TNF-α on Sarcoma-180 solid tumors. Mice were used in groups of five. Each value represents the mean ± S.E.M. The maximal necrotic response (score 3) indicates that 50% or more of tumor mass is necrotic; the moderate response (score 2), 25-50% necrotic; the minimal response (score 1), less than 25% necrotic; and no response (score 0), no visible necrosis. Statistical significance compared with saline control: *P $<$ 0.01, **P $<$ 0.001.

of DIVEMA-TNF-α induced complete regression of one of five mice (data not shown). In addition, in order to more thoroughly investigate the usefulness of DIVEMA-TNF-α, the antitumor effects by scheduled i.v. administration on Meth-A solid tumors was examined. Native TNF-α at a dose of 5,000 JRU or 10,000 JRU completely inhibited tumor growth up to day 24 after tumor inoculation, but tumor growth was observed after that (Fig. 4a). DIVEMA alone at a dose of 3 μg or a mixture of TNF-α and DIVEMA also did not inhibit

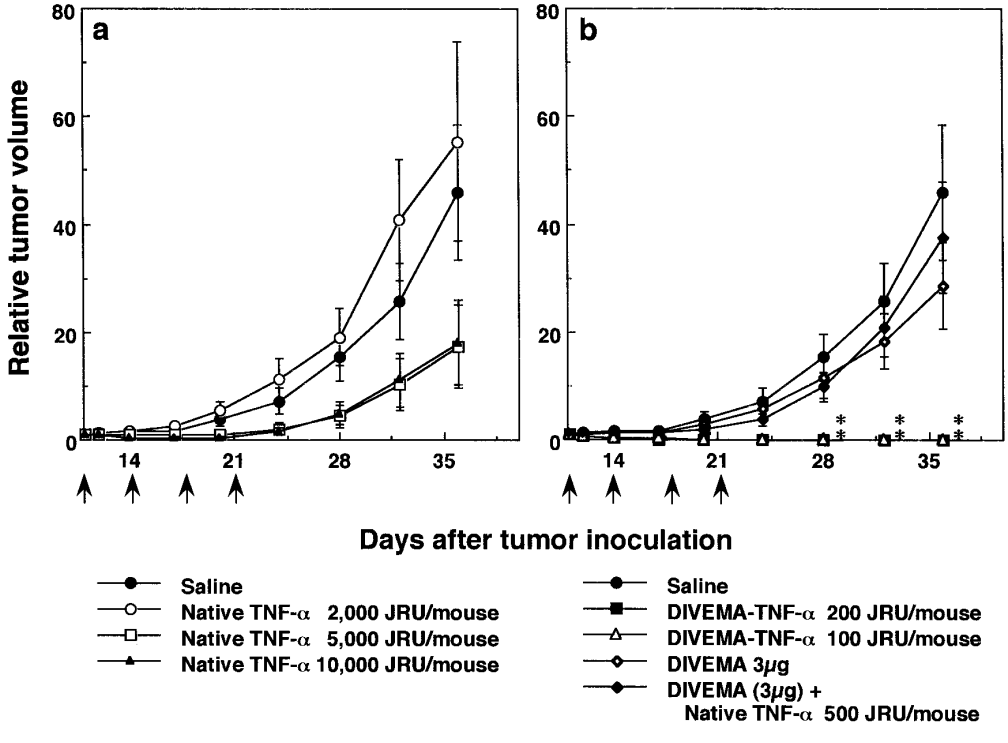

FIG. 4. Antitumor effect of native TNF-α and DIVEMA-TNF-α on Meth-A solid tumors in mice. Eleven days after tumor inoculation, native TNF-α (a) or DIVEMA-TNF-α (b) was given as i.v. injections twice a week for 2 weeks (arrows). Mice were used in groups of five. Data are expressed as relative tumor volume by using the equation: relative tumor volume = mean tumor volume at a given time / mean tumor volume on day 11. Each value represents the mean ± S.E.M. Statistical significance compared with saline control: **P $<$ 0.005.

tumor growth (Fig. 4b). On the other hand, DIVEMA-TNF-α at a dose of only 100 JRU per mouse showed a dramatic antitumor effect: tumor growth was completely inhibited for the observation period (Fig. 4b). Complete regression was obtained in only one of five mice at a dose of 10,000 JRU of native TNF-α, but DIVEMA-TNF-α showed complete regression in all mice at a dose of only 100 JRU (data not shown). The antitumor effect of DIVEMA-TNF-α at a dose of 100 JRU was superior to that of native TNF-α at a dose of 10,000 JRU.

Body-weight and platelet level in mice bearing Meth-A tumors treated with native TNF-α or DIVEMA-TNF-α. The toxic side effects of scheduled i.v. injection of DIVEMA-TNF-α on mice with Meth-A tumors were evaluated by body-weight and platelet level. A body-weight reduction was not observed with administration of DIVEMA-TNF-α at all doses (Fig. 5a). However, native TNF-α at a dose of 10,000 JRU caused a transient body-weight reduction during the administration period: the body-weight was minimum on day 17 after tumor inoculation. A platelet reduction was also observed with administration of native TNF-α, and the platelet count was reduced in a dose-dependent manner on day 15 (Fig. 5b). Native TNF-α at a dose of 10,000 JRU reduced the platelets about 50% on day 4 after the first administration. However, DIVEMA-TNF-α at all doses did not reduce the number of platelets.

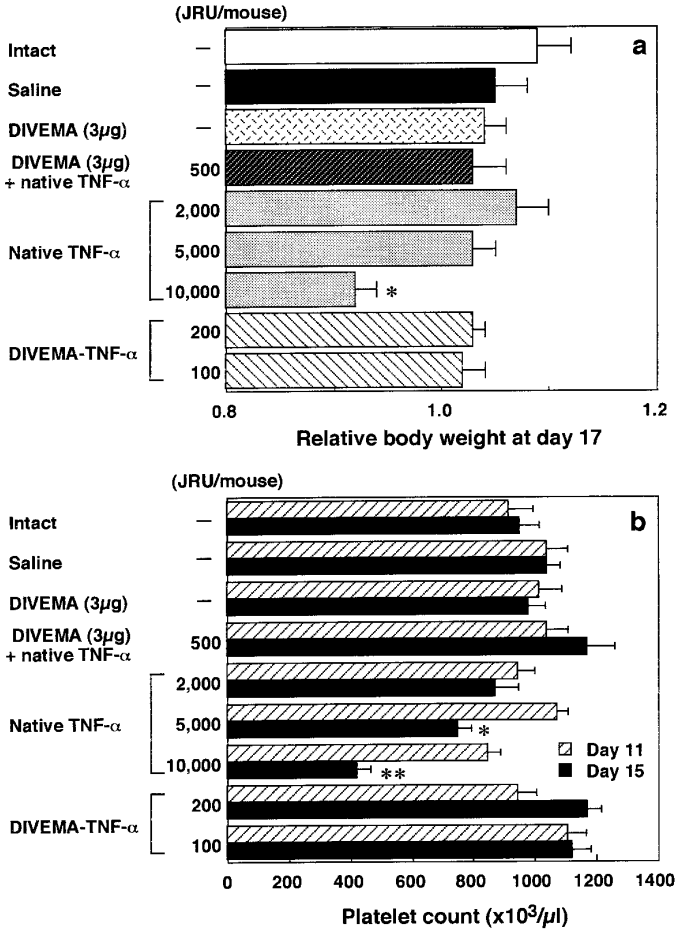

FIG. 5. Body-weight changes and platelet counts in mice with Meth-A tumors treated with native TNF-α or DIVEMA-TNF-α. a, relative body weight at 17 days after tumor inoculation. Data are expressed as relative body weight (day 17/day 11). Mice were used in groups of five. Each value represents the mean ± S.E.M. *P < 0.01 compared to that for the intact animal. b, platelet count 15 days after tumor inoculation. The platelet count was measured by using peripheral blood at the indicated time. Mice were also used in groups of five. Each value represents the mean S.E.M. *P < 0.005, **P < 0.001 compared to that for the intact animal.

DISCUSSION

In this study, we estimated the function of the biological response modifier DIVEMA by its bioconjugation with TNF-α and established a methodology for cytokine bioconjugation using polymeric modifiers binding at multiple points of the side chain.

The inhibition of binding between the proteins and control of the molecular size could be accomplished by first protecting the amino groups with DMMAn (Figs.1, 2). These results suggested that bioconjugation of cytokines with polymeric modifiers that bind at multiple points of the side chain was able to control the degree of modification by DMMAn addition. This novel methodology for bioconjugation with the use of DMMAn might also be applied to the PEG conjugation of various bioactive proteins. In addition, since DMMAn binds to reactive amino groups, conjugation with partial protection of amino groups leads to the synthesis of highly active forms. In fact, DIVEMA-TNF-α retained high activity *in vitro* (Table 1), and showed a dramatic effect on both S-180 and Meth-A solid tumors without any side effect (Figs. 3, 4, 5). In addition, the therapeutic effect of DIVEMA-TNF-α was superior to that of PEG-modified TNF-α in which 56% of the lysine residues were coupled to PEG (MPEG-TNF-α) on Meth-A tumors in mice. Until now, cancer therapy with TNF-α has been limited to specified tumors because of unexpected side effects. However, the intravenous administration of DIVEMA-TNF-α alone markedly inhibited the tumor growth and induced complete regression without any side effects. These findings strongly suggest that TNF-α bioconjugated with polymeric modifiers of specific design may be useful as novel antitumor therapeutic agents for clinical use.

However, it is not clear why the bioconjugation by DIVEMA increases antitumor potency *in vivo*. We previously reported that MPEG-TNF-α was well balanced relative to plasma clearance, tissue transport, and specific activity (17). On the contrary, LPEG-TNF-α, which is 84 kD, was cleared more rapidly and accumulated

to a lesser extent in the tumor than MPEG-TNF-α (17). The molecular size of DIVEMA-TNF-α is 63 kD, so that prolongation of plasma half-life may not affect the increase of antitumor activity *in vivo*. Two other possible mechanisms for the synergistic effect due to DIVEMA modification are: i) The tissue distribution of DIVEMA-TNF-α is improved, and ii) the immune system is modulated. The mechanism needs further study for optimal clinical application of bioconjugated TNF-α. Our findings in this report led to the conclusion that DIVEMA is one of the optimal polymeric modifier for antitumor cytokine therapy. In addition, we established a novel method of bioconjugation for cytokine by use of DMMAn. These designed approaches may enable us to establish a definitive bioconjugated cytokine therapy for clinical use.

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