Ability of sera from mice treated with Ge-132, an organic germanium compound, to inhibit experimental murine ascites tumours

F. Suzuki^{1,2}, R.R. Bratkiewicz² & R.B. Pollard²

¹Department of Microbiology, Kumamoto University Medical School, Kumamoto 860, Japan; and ²Division of Infectious Diseases, Department of Internal Medicine and Department of Microbiology, University of Texas Medical Branch, and Virology Division, Shriners Burns Institute, Galveston, Texas 77550, USA.

Summary Sera from C57B1/6 mice treated orally with Ge-132 exhibited antitumour activity against Ehrlich (allogeneic) and RL β 1 (syngeneic) ascites tumours in BALB/c mice. Sera obtained from mice 24 h after Ge-132 administration displayed the greatest antitumour effect and this was dose dependent. Sera prepared from mice 12, 36, or 48 h after Ge-132 treatment had no protective effect. Circulating interferon (IFN) was induced at 24 h after administration of Ge-132 but was not detected in the sera at 12, 36, or 48 h after administration. The antiviral activity of sera from Ge-132-treated mice was inactivated by treatments with trypsin, low pH, and anti-IFN γ antiserum. The inactivated preparations of serum IFN induced by Ge-132 did not exhibit antitumour activity when administered to tumour-bearing mice. These results suggest that antitumour activity in the sera of Ge-132-treated mice may be expressed through activities of Ge-132-induced lymphokine(s), such as IFN γ .

Certain immunopotentiators, derived from a variety of sources, have not only an antitumour effect (Mastrangelo *et al.*, 1981), but also induce interferon (IFN) production *in vivo* (Matsubara *et al.*, 1980). Some immunopotentiators, as well as IFN, have been reported to augment natural killer (NK) cell activity, stimulate macrophages (M ϕ) to become tumoricidal, generate cytotoxic T lymphocytes (CTL), and enhance various nonspecific immune responses both *in vivo* and *in vitro* (Mastrangelo *et al.*, 1981; Vilcek *et al.*, 1980). Carboxyethyl-germanium sesquioxide [O₃(GeCH₂CH₂COOH)₂], an organogermanium compound originally synthesized at Asai Germanium Research Institute, Tokyo, Japan (Tsutsui *et al.*, 1976), is an immunopotentiating agent (Arimori *et al.*, 1981b; Mizushima *et al.*, 1980; Suzuki *et al.*, 1984) with IFN-inducing (Aso *et al.*, 1985; Miyao *et al.*, 1980) and antitumour activities (Kumano *et al.*, 1980; Arimori *et al.*, 1981a; Satoh & Iwaguchi, 1979). Acute and chronic toxicities of the compound were determined in Wistar rats (acute and chronic) or beagle dogs (chronic) by i.v. (125-500 mg kg⁻¹) or oral (30-3,000 mg kg⁻¹) administration. At all doses examined, no significant toxicity was detected (Miyao *et al.*, 1980; Nagata *et al.*, 1978). The physicochemical characteristics and pharmacokinetics of the compound have been described previously (Miyao *et al.*, 1980; Tomisawa *et al.*, 1978). As an immunotherapeutic agent for cancer,

this compound has undergone some clinical testing in Japan.

The antitumour activity of Ge-132 in ascites tumour-bearing mice was eliminated by the *in vivo* administration of monoclonal anti-Thy 1.2 antibody (anti-Thy1.2 mAb), which has been reported to cause a disappearance of Thy-1.2⁺ cells from mice (Nakayama & Uenaka, 1985; Opitz *et al.*, 1982), or macrophage (M ϕ) blockers, such as trypan blue and carrageenan (Chaout & Howard, 1976; Hibbs, 1975). However, the antitumour effect of Ge-132 was not blocked by *in vivo* treatment with anti-asialo GM1 antiserum which has been reported to eliminate NK cell activity (Habu *et al.*, 1981). This suggested that the protective effect displayed by Ge-132 was expressed through the function of T lymphocytes and/or M ϕ (Suzuki, 1985a, b).

The elimination of T-cells by anti-Thy 1.2 antibody and complement has been shown to result in the suppression of interleukin 2 (IL-2) (Muhlradt & Opitz, 1982) and IFN gamma (IFN γ) production (Sonnenfeld *et al.*, 1979), as well as the generation of CTL (Wong *et al.*, 1977). It has also been reported that IL-2 production, CTL generation, and the augmentation of NK cell activity may be influenced by IFN γ (Kasahara *et al.*, 1983; Torres *et al.*, 1982; Weigent *et al.*, 1983). The oral administration of Ge-132 was reported to stimulate the production of IFN γ in the sera of mice (Aso *et al.*, 1985), and this type of IFN appears to be produced by T lymphocytes (Chang *et al.*, 1982; Sonnenfeld *et al.*, 1979). Therefore, in the present

Correspondence: F. Suzuki.

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paper, we investigated the role of IFN γ , one of the lymphokines (Epstein, 1981) induced by Ge-132 in the sera of mice, on the antitumour properties of the compound.

Materials and methods

Mice and tumours

Eight-week-old male and female inbred BALB/c and C57B1/6 mice were used (Suzuki, 1985a,b). Ehrlich (allogeneic), and RL δ 1 (syngeneic) ascites tumours were serially passaged in BALB/c mice by i.p. injection of 1×10^6 cells per mouse (Suzuki, 1985a,b).

IFN assay and IFN standard

Mouse IFN activity was assayed by a microplaque reduction method (Suzuki & Pollard, 1982). The reference standard of murine IFN (G-002-904-511) was obtained from the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda, MD, USA and used to standardize the IFN titration.

Murine IFN γ and anti-mouse IFN γ antiserum

Murine IFN γ (Osborne *et al.*, 1979) was kindly provided by Dr H.M. Johnson, University of Texas Medical Branch, Galveston, TX. Anti-mouse IFN γ antiserum (anti-IFN γ) was also supplied by Dr H.M. Johnson. The anti-IFN γ has been shown to preferentially inactivate the antiviral activity of IFN γ *in vitro* while not reacting with IFN α or IFN β (Osborne *et al.*, 1980a,b).

Preparation of sera

The C57B1/6 mice received a 100 mg kg^{-1} body wt oral dose of Ge-132 or a 0.5 ml per mouse oral dose of saline as a control. Twelve to 48 h later the mice were sacrificed and blood samples were maintained at 4°C overnight, centrifuged at 1,250 g for 30 min, and the supernatants (serum specimens) were stored at -70°C.

Inactivation of IFN activity

Twenty ml of serum IFN (360 U ml^{-1}) obtained from mice 24 h after Ge-132 administration or murine IFN γ (400 U ml^{-1}) were mixed with an equal volume of anti-IFN γ (400 IFN neutralizing U ml^{-1}) or normal rabbit serum (control) and kept at room temperature for 1 h (Osborne *et al.*, 1980a,b). Then, 0.5 ml was injected i.p. to tumour-

bearing mice. The inactivation of the serum IFN induced by Ge-132 and murine IFN γ by trypsin or acid pH treatment was performed as previously described (Aso *et al.*, 1985). Briefly, both IFN γ preparations were treated with $200 \text{ } \mu\text{g ml}^{-1}$ of trypsin at 37°C for 2 h or dialyzed against 0.1M glycine-HCl buffer (pH 2.0) at 4°C for 12 h, and then redialyzed against 0.1M PBS (pH 7.0). After inactivation, these preparations were stored at -70°C.

Antitumour assay of serum specimens in vivo

One day before Ehrlich or RL δ 1 tumour cell inoculation (1×10^5 cells per mouse, i.p.), BALB/c mice received an i.p. injection of 0.5 ml of either a 1:5 dilution of serum obtained from mice at various intervals after oral Ge-132 (100 mg kg^{-1} body wt) administration, the same dilution of control serum obtained from mice 24 h after oral saline (0.5 ml per mouse) injection, a $5,000 \text{ U kg}^{-1}$ body wt i.p. dose of murine IFN γ , or a 100 mg kg^{-1} body wt oral dose of Ge-132. In some experiments, tumour-bearing mice received an i.p. injection of serum IFN inactivated by trypsin or acid pH treatments, or with antiserum-inactivated IFN γ . These treatments of tumour-bearing mice were performed once every other day for a total of 10 injections. Mice were observed daily in order to determine the mean survival days (MSD). Each experiment was terminated 50 days after tumour inoculation, and the percent survival was calculated from the number of mice surviving more than 50 days. The influence of these preparations on tumour growth was evaluated by MSD and survival percent as compared with controls (Suzuki, 1985a).

Statistical analysis

As described previously (Suzuki, 1985a,b), statistical significance was determined by Student's *t*-test or χ^2 analysis. The results were considered significant if $P < 0.05$.

Results

Antitumour activity of sera

To determine the antitumour activity of sera obtained 24 h after Ge-132 administration, 20 BALB/c mice bearing Ehrlich tumours were treated i.p. with 0.5 ml of a 1:5 dilution of the serum from Ge-132-treated mice (Ge-mice) or normal control mice. As a positive control, one group of 30 mice was treated with a 100 mg kg^{-1} body wt oral dose of Ge-132, and a group of 40 mice was injected orally with 0.5 ml per mouse of

Table I Antitumour effect of sera from mice treated with or without Ge-132 on Ehrlich or RL δ 1 ascites tumours in mice

Tumour-bearing mice treated with; ^a	No. of mice	Mean survival days (Range)	P	50 day survivors(%)	P
A. Ehrlich carcinoma					
Sera from mice treated with Ge-132	20	27.6(14->50)	<0.001	8(40)	<0.001
Sera from normal mice	20	19.5(15-23)	NS	0	
Ge-132	30	28.8(14->50)	<0.001	11(37)	<0.001
Saline (tumour control)	40	18.6(14-24)	—	0	—
B RLδ1 leukaemia					
Sera from mice treated with Ge-132	10	38.4(25->50)	<0.001	4(40)	0.025
Sera from normal mice	10	24.6(23-34)	NS	0	
Ge-132	20	35.6(22->50)	<0.001	9(45)	<0.001
Saline (tumour control)	20	20.3(22-23)	—	0	—

^aMice bearing 1×10^5 Ehrlich or RL δ 1 tumour cells were treated with sera (0.1 ml per mouse, i.p.), Ge-132 (100 mg kg⁻¹ body wt, orally) or saline (0.5 ml per mouse, orally) 24 h before tumour inoculation and every other day thereafter for a total of 10 treatments. Serum specimens were obtained from mice 24 h after Ge-132 or saline injection.

saline as a tumour control. As shown in Table IA, Ge-132 and serum from Ge-mice significantly increased the MSD of tumour-bearing mice compared to those of tumour controls conferring 37 and 40% survival, respectively. In order to determine if sera from Ge-mice were also effective against a syngeneic ascites tumour (RL δ 1) *in vivo*, tumour-bearing mice were treated in the same fashion as in the Ehrlich experiments. As shown Table IB, sera from Ge-mice also inhibited the growth of RL δ 1 ascites tumours in BALB/c mice (40% survival, $P=0.025$) as did Ge-132 administration (45% survival, $P<0.001$), while all control mice treated with saline or normal mouse serum died within 34 days after tumour inoculation. Thus, sera from Ge-mice were as active *in vivo* against Ehrlich and RL δ 1 ascites tumours as Ge-132 administration.

Dose response effect of sera

Various dilutions of sera obtained 24 h after administration of Ge-132 were injected into mice bearing Ehrlich tumours. Eight groups of tumour-bearing mice (20 mice each) received i.p. injections of 0.5 ml of a 1:1 (0.5 ml), 1:4 (0.12 ml), 1:16 (0.03 ml), or 1:64 (0.007 ml) dilution of serum obtained from Ge-mice or normal mice. As illustrated in Figure 1, significant antitumour activity of sera from Ge-mice was noted at dosages of 0.5 ml per mouse (70% survival, $P<0.001$) and 0.12 ml per mouse (40% survival, $P=0.002$), while <0.03 ml per mouse and all concentrations of normal mouse serum had no effect. These results indicate that the antitumour properties of sera from Ge-mice was dose dependent.

Figure 1. Dose response protective effect of sera from mice treated with Ge-132 (Ge-mice) against Ehrlich ascites tumours *in vivo*. Mice (20 mice each) bearing 1×10^5 Ehrlich ascites tumour cells were treated i.p. with 0.5 ml of various dilutions of sera obtained from normal (●) or Ge-mice (○), one day before and one day after tumour inoculation, and every 2 days subsequently for a total of 10 treatments.

Correlation between antitumour activity of sera and appearance of IFN

To determine if there was a correlation between the appearance of IFN and the antitumour activity induced by Ge-132, sera were harvested after an oral dose of Ge-132 (100 mg kg⁻¹ body wt) every 6 h for 48 h. As shown in Figure 2, IFN appeared 18 h after Ge-132 administration with maximum levels (360 U ml⁻¹) detected at 24 h and gradually decreased thereafter until 42 h when no IFN was observed. Sera obtained from mice 12, 24, 36 and 48 h after Ge-132 administration, and labelled A, B, C, and D, respectively, were tested for their antitumour activity in Ehrlich-bearing mice. Five groups (20 mice each) were treated i.p. with 0.5 ml of a 1:5 dilution of specimens A to D or 0.5 ml per mouse of saline, and observed for survival. As

Figure 2. Antitumour activity of sera obtained from mice various times after Ge-132 administration. Tumour-bearing mice received i.p. injections of sera (every other day, 10 total treatments, beginning one day before tumour inoculation, 0.1 ml per mouse) obtained from mice 12 h (A, $\blacktriangle$), 24 h (B, $\circ$), 36 h (C, $\triangle$) or 48 h (D, $\square$) after oral Ge-132 (100 mg kg^{-1} body wt) or saline (0.5 ml, $\bullet$) administration.

shown in Figure 2, specimens A, C, and D had no antitumour activity, while samples B and C produced 40% ($P < 0.001$) and 10% survival, respectively. Therefore, the maximum antitumour activity correlated with the highest IFN titre which was noted 24 h after Ge-132 administration.

Inactivation of IFN activity

To determine if the serum IFN induced by Ge-132 was responsible for the observed antitumour effect, IFN activity was inactivated by treatment with trypsin, acid pH or anti-IFN γ . The IFN activity was completely inactivated by dialyzing against

acidic buffer and trypsin treatments. In addition, the IFN activity was neutralized by treatment with anti-IFN γ , as well as the murine IFN γ . Since it has been reported (Osborne *et al.*, 1980a,b) that this antiserum did not neutralize the antiviral activity of IFN α or IFN β , the IFN in sera from Ge-mice was confirmed as an IFN γ . The antitumour activities of inactivated sera were next evaluated in mice bearing Ehrlich tumours. Groups of mice (10 mice each) were treated with inactivated or unactivated sera and a group of 30 mice treated with saline was served as a tumour control. As shown in Figure 3, while sera kept at 37°C for 2 h (control for trypsin digestion) and 4°C for 12 h

Figure 3. Antitumour activity of inactivated sera from mice treated with Ge-132 (Ge-mice) against Ehrlich ascites tumours *in vivo*. Tumour-bearing mice received i.p. injections of 0.5 ml saline per mouse ($\bullet$), or sera from Ge-mice which were subjected to pH 2.0 dialysis (4°C, 12 h, $\blacktriangle$), pH 7.0 dialysis (4°C, 12 h, $\triangle$), trypsin treatment ($200 \mu\text{g ml}^{-1}$, 37°C, 2 h, $\blacksquare$), or incubation without trypsin at 37°C (2 h, $\square$), every 2 days for a total of 10 treatments.

Figure 4. Failure of protection of tumour-bearing mice by two IFN preparations treated with anti-IFN γ antiserum. 20 ml of serum IFN (360 U ml^{-1} of IFN activity) obtained from Ge-mice and murine IFN γ (400 U ml^{-1} of IFN activity) were treated with the same amount of anti-mouse IFN γ antiserum ($400 \text{ IFN neutralizing U ml}^{-1}$), and 0.5 ml of these preparations was injected to mice bearing 1×10^5 Ehrlich cells (neutralized serum IFN, $\blacktriangle$; neutralized murine IFN γ , $\blacksquare$). As positive or negative controls, tumour-bearing mice were given i.p. 0.5 ml of serum IFN ($4,500 \text{ IFN U kg}^{-1}$ body wt, $\triangle$), murine IFN γ ($5,000 \text{ IFN U kg}^{-1}$ body wt, $\square$), and saline ($\bullet$).

(control for dialysis) protected 50% ($P < 0.001$) and 40% ($P < 0.001$) of the mice, respectively, serum exposed to pH 2.0 or trypsin treatment did not influence survival. In addition, when IFN-containing samples were treated with anti-IFN γ , no antitumour effect was noted in a group of 10 tumour-bearing mice (Figure 4). Increased survival was observed in mice treated with murine IFN γ ($5,000 \text{ U kg}^{-1}$ body wt, 20 mice, 40% survival, $P < 0.001$), or IFN-containing serum from Ge-mice ($4,500 \text{ U kg}^{-1}$ body wt, 40% survival, 20 mice, $P < 0.001$). The tumour controls (30 mice) died within 23 days of inoculation.

Discussion

The antitumour activity of sera obtained from Ge-mice against syngeneic (RL δ 1) or allogeneic (Ehrlich) ascites tumours in mice appeared similar to that observed with the compound itself. In addition, the serum-mediated antitumour effect correlated with IFN levels present and this association was confirmed by kinetic and inactivation studies. The maximum antitumour activity in the serum was observed at the time of the highest serum IFN titer (24 h after Ge-132 administration). The inactivation of the IFN activity in sera from Ge-mice was accomplished by treatments with acid pH, trypsin or anti-IFN γ and abolished antitumour activity. The serum IFN induced by Ge-132 in mice was identified as IFN γ since its activity was eliminated by treatment with

low pH and anti-IFN γ . These results indicate that the antitumour activity of the compound in mice bearing experimental ascites tumours is reconstituted by administration of the IFN γ containing sera from Ge-mice.

As compared to IFN α and β , IFN γ has been demonstrated to be a more potent mediator of antitumour activities (Crane *et al.*, 1978). Therefore, repeated administration of mouse IFN γ preparations resulted in the marked inhibition of the growth of transplantable murine tumours and increased the survival of tumour-inoculated mice (Crane *et al.*, 1978; Salvin *et al.*, 1975; Gresser, 1983). On the other hand, the antitumour activity of Ge-132 appears to involve $M\phi$, since the administration of $M\phi$ blockers prevented the expression of the antitumour activity of Ge-132 (Suzuki, 1985a), the passive transfer of $M\phi$ from Ge-mice conferred antitumour resistance to untreated tumour-bearing mice (Suzuki, 1985b), and $M\phi$ from Ge-Mice also had cytotoxic properties *in vitro* against certain tumours which were sensitive to the antitumour activity of Ge-132 *in vivo* (Suzuki, 1985b). Some reports (Kleinschmidt & Schultz, 1982; Robert & Vasil, 1982) suggest that the induction of tumouricidal $M\phi$ from a resting state requires IFN γ which induced the priming step (Pace *et al.*, 1982) in $M\phi$ activation, or acts as a cofactor associated with $M\phi$ -activating factor (Mannel & Falk, 1983). In addition, it was also demonstrated that the *in vivo* administration of anti-Thy1.2 mAb, effectively prevented the expression of antitumour activity of Ge-132

(Suzuki, 1985a). As presented here, the antitumour activity of Ge-132 appeared to be expressed through the induction of lymphokine(s), such as IFN γ , and since lymphokines have been shown to be produced by T lymphocytes (Epstein, 1981), it is possible that the activity of Ge-132 against tumours may be due to the following properties: (i) the compound exhibits antitumour activity which can be blocked by the exogenous administration of T-cell or M ϕ blockers, (ii) M ϕ obtained from Ge-mice display antitumour activity *in vivo* and *in vitro*, (iii) sera from Ge-mice contain IFN γ , one of the lymphokines which is produced by T-cells, (iv)

lymphokine(s), or IFN γ , can activate M ϕ to become tumouricidal. This suggests that lymphokine(s) such as IFN γ induced by the compound, may activate M ϕ to become tumouricidal and mediate the antitumour activity observed in mice treated with Ge-132.

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