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

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Summary Sera from C57B1/6 mice treated orally with Ge-132 exhibited antitumour activity against Ehrlich (allogeneic) and RL\textsubscript{51} (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\textgamma 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\textgamma.

Certain immunopotentiators, derived from a variety of sources, have not only an antitumour effect (Mastrangelo et al., 1981), but also induce interferon (IFN) production \textit{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\textphi) to become tumoricidal, generate cytotoxic T lymphocytes (CTL), and enhance various nonspecific immune responses both \textit{in vivo} and \textit{in vitro} (Mastrangelo et al., 1981; Vilecek et al., 1980). Carboxyethylgermanium sesquioxide \((\text{O}_2\text{GeCH}_2\text{CH}_2\text{COOH})_2\), an organogermain compound originally synthesized at Asai Germanium Research Institute, Tokyo, Japan (Tsutsui et al., 1976), is an immunopotentiating agent (Arimori et al., 1981\textbf{b}; 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., 1981\textbf{a}; 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\textsuperscript{-1}) or oral (30–3,000 mg kg\textsuperscript{-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 \textit{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\textsuperscript{+} cells from mice (Nakayama & Unaka, 1985; Opitz et al., 1982), or macrophage (M\phi) blockers, such as trypsin blue and carrageenan (Chaout & Howard, 1976; Hibbs, 1975). However, the antitumour effect of Ge-132 was not blocked by \textit{in vivo} treatment with anti-asialo GMI 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\phi (Suzuki, 1985\textbf{a},\textbf{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) (Muhradt & Opitz, 1982) and IFN gamma (IFN\gamma) 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\gamma (Kasahara et al., 1983; Torres et al., 1982; Weigert et al., 1983). The oral administration of Ge-132 was reported to stimulate the production of IFN\gamma 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...
paper, we investigated the role of IFNγ, 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 C57Bl/6 mice were used (Suzuki, 1985a,b). Ehrlich (allogeneic), and RL-31 (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 IFNγ and anti-mouse IFNγ antiserum

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

Preparation of sera

The C57Bl/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 IFNγ (400 U ml⁻¹) were mixed with an equal volume of anti-IFNγ (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 IFNγ by trypsin or acid pH treatment was performed as previously described (Aso et al., 1985). Briefly, both IFNγ preparations were treated with 200 μg 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-31 tumour cell inoculation (1 × 10⁴ 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 U kg⁻¹ body wt i.p. dose of murine IFNγ, 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 IFNγ. 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 χ² 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
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 (RL31) in vivo, tumour-bearing mice were treated in the same fashion as in the Ehrlich experiments. As shown in Table IB, sera from Ge-mice also inhibited the growth of RL31 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 RL31 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.

**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
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 antisera 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
(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 U kg\(^{-1}\) body wt, 20 mice, 40% survival, P<0.001), or IFN-containing serum from Ge-mice (4,500 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 (RL31) 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 Mφ, since the administration of Mφ blockers prevented the expression of the antitumour activity of Ge-132 (Suzuki, 1985a), the passive transfer of Mφ from Ge-mice conferred antitumour resistance to untreated tumour-bearing mice (Suzuki, 1985b), and Mφ 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 Mφ from a resting state requires IFN γ which induced the priming step (Pace et al., 1982) in Mφ activation, or acts as a cofactor associated with Mφ-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

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**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 IFN neutralizing U ml\(^{-1}\)), and 0.5 ml of these preparations was injected to mice bearing \(1 \times 10^5\) Ehrlich cells (neutralized serum IFN, △; neutralized murine IFN γ, []). As positive or negative controls, tumour-bearing mice were given i.p. 0.5 ml of serum IFN (4,500 IFN U kg\(^{-1}\) body wt, △), murine IFN γ (5,000 IFN U kg\(^{-1}\) body wt, □), and saline (●).
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 Mφ blockers, (ii) Mφ 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 Mφ to become tumouricidal. This suggests that lymphokine(s) such as IFN γ induced by the compound, may activate Mφ to become tumouricidal and mediate the antitumour activity observed in mice treated with Ge-132.

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