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Antiviral Activity of Carboxyethylgermanium Sesquioxide (Ge-132) in Mice Infected with Influenza Virus

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Summary: The protective effect of carboxyethylgermanium sesquioxide (Ge-132) in mice infected with a mouse-adapted strain of influenza virus (H_2N_2) was investigated. When mice were exposed to a 10 LD_{50} dose of influenza virus via aerosol and were treated orally with 20 or 100 mg/kg of Ge-132 daily for 6 consecutive days, a significant protective effect was demonstrated. The antiviral effect of Ge-132 was indicated by an increase of survivors, a prolongation of mean survival days, an inhibition of the development of lung consolidation, and a decrease of virus titer in lung tissues, as compared to infected control mice treated with phosphate-buffered saline. Natural killer (NK) cell activity in the spleens and lungs of the infected mice was also significantly augmented after the oral administration of Ge-132. In addition, NK cells stimulated with Ge-132 in vivo showed killing activity against NK-insensitive Meth-A cells infected with influenza virus. Because no virucidal or virustatic activities of Ge-132 on the virus were found in vitro, this protective effect in mice against influenza virus infection may be displayed through immunomodulating activities of this compound such as the augmentation of NK cell activity. **Key Words:** Ge-132—Germanium compound—Antiviral activity—Influenza virus infection—NK cell activity.

During the course of influenza virus infection in mice, several pathological changes occur in the lungs. Mononuclear cells infiltrate the infected organ, with the concomitant development of consolidation. When mice are treated with antilymphocyte serum before infection, the development of consolidation is postponed or even inhibited (1). Cyclophosphamide-treated mice (2,3), x-ray-irra-

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diated mice, and nude mice (4,5) react similarly to influenza virus infection. However, in immunodeficient models, the virus also persists in the lungs for a longer period of time than in the immunocompetent controls. The cell-mediated immune response may be beneficial in the recovery of the host from the infection; the responses may be detrimental by contributing to the immunopathological effects without helping in recovery (1–8).

Natural killer (NK) cells are now widely believed to be one of the most important effector cells in immunologic surveillance against tumor cells and virus-infected cells (9–12). Previous studies have established that there is a positive correlation between resistance to the lethal effects of virus infection and the level of augmentation of NK cells (13), and that the adoptive transfer of NK cells protects mice against virus infection (14). Furthermore, it has been reported that the infiltrating cells in the lungs express NK cell activity (4,12). Therefore, NK cells play an important role in protecting a host from disease during virus infection.

Ge-132 [carboxyethylgermanium sesquioxide, $O_3(GeCH_2CH_2COOH)_2$] (15), an organogermanium compound, has been shown to have antitumor activity in mice (16–18) and rats (19), as revealed by the inhibition of tumor growth and prolongation of mean survival time in these animals. It has also been shown to have, in both humans (20) and mice (21,22), immunopotentiating activities through both the induction of interferon-γ (IFN-γ) in serum and the augmentation of NK cell activity (21). Following these observations, we report the effect of Ge-132 on influenza virus infection in mice.

MATERIALS AND METHODS

Mice

Six-week-old inbred DDI mice were obtained from the Institute for Experimental Animals, Tohoku University School of Medicine, where care was taken to prevent infections with Sendai virus and *Mycoplasma*.

Medium

RPMI 1640 medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Microbiological Associates, Walkersville, MD, U.S.A.), 100 U/ml of penicillin, and 100 μg/ml of streptomycin was used as the culture medium throughout the experiments.

Ge-132

Ge-132 was supplied by the Asai Germanium Research Institute (Tokyo, Japan) (15). The drug was dissolved in sterile phosphate-buffered saline (PBS, pH 7.4) immediately before use.

Virus

The mouse-adapted Kumamoto strain of influenza A_2 (H_2N_2) was used (1). The virus strain was propagated in the allantoic cavity of embryonated eggs. The egg

infectious dose (EID_{50}) of the allantoic fluid was $10^{9.0}$/ml, which was equivalent to $10^{6.0}$ LD_{50} in DDI mice inoculated intranasally by our standard inhalation procedure (1). In all experiments, DDI mice were infected with 10 LD_{50} of the virus.

Virus Inoculation Procedures

Virus infection was induced by the inhalation of the influenza A_2 virus (23). A vaponephrine-type nebulizer was connected to a compressor to spray approximately 30 ml of diluted allantoic fluid at 500 mm Hg pressure in 30 min.

IFN Preparations

Pooled sera were obtained from DDI mice 24 h after the oral administration of Ge-132 (100 mg/kg) and were designated as Ge-132-induced serum IFN (Ge-IFN). The antiviral activity in this sera was 300 U/ml, which was expressed in international reference units based on the NIH reference mouse IFN (Catalog No. 022-904-511). The titration of IFN activity was carried out by the 50% plaque-reduction method on L929 cells with vesicular stomatitis virus (21). Interferon-α/β (specific activity: 1×10^7 U/mg protein), a mouse L-cell IFN preparation, was supplied from the Basic Research Laboratory, Toray Industries, Inc. (Kanagawa, Japan).

Consolidation Score and Virus Titer

The grades of lung consolidation were determined for each mouse according to a modification of the method of Horsfall (24) as follows: 5 = death or 100% consolidation; 4.5 = 90%; 4 = 80%; 3.5 = 70%; 3 = 60%; 2.5 = 50%; 2 = 40%; 1.5 = 30%; 1 = 20%; 0.5 = 10%; 0 = 0% consolidation. The lungs obtained from infected mice were disrupted with a homogenizer (Polytron, type PT 10.35, Kinematica, Switzerland) and a 10% suspension in PBS was prepared. This suspension was clarified by centrifugation at 2000 × *g* for 30 min. The virus titers of the supernatants were determined by the hemagglutinin (HA) test (23).

Assay for NK Activity

Target cells (YAC-1 and Meth-A) were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ (Daiichi Radioisotope Laboratory Co., Tokyo, Japan) in 200 μl of 0.85% NaCl solution at 37°C for 1 h. The cells were washed, resuspended in serum-free medium, and incubated at 37°C for 1 h. The cells were washed again three times with serum-free medium and resuspended in the culture medium at a cell density of 5×10^5 cells/ml. This procedure was effective in reducing the spontaneous release of ^{51}Cr. ^{51}Cr-labeled Meth-A cells incubated with $10^{9.0}$ EID_{50} of influenza A_2 virus at 37°C for 1 h were designated as Inf–Meth-A. Spleen cells and mononuclear cells prepared from mouse lungs were used as effector cells. Effector cells (5×10^5 cells/200 μl) were mixed with ^{51}Cr-labeled target cells (1×10^4 cells/20 μl) in triplicate and cultured in U-bottomed microtiter plates (Nunc, Roskilde, Denmark) at 37°C for 4 h. The plates were then centrifuged at 300 × *g* for 6 min, and the released ^{51}Cr in the culture supernatants was measured by a gamma counter

(model ARC-500, Aloka Co. Ltd., Mitaka, Japan). The spontaneous release was determined from the supernatants of the target cells incubated with medium alone. The maximum release was determined by incubating the target cells with 3.5*N* hydrochloric acid. The spontaneous release ranged from 8–12% of maximum release. Cytotoxicity was calculated as follows:

$$\% \text{ lysis} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximal cpm} - \text{spontaneous cpm}} \times 100$$

Statistical Analysis

The survival curve was statistically analyzed by the generalized Wilcoxon test (U test). The other results were statistically analyzed by Student's *t* test. The antiviral effect of Ge-132 was considered significant if the *p* value was less than 0.05.

RESULTS

Protection of Mice Infected with Influenza A_2 Virus by the Oral Administration of Ge-132

We first examined the effects of Ge-132 on influenza virus infection in mice. A 20, 100, or 500 mg/kg dose of Ge-132 or PBS (0.2 ml/mouse) was given orally once daily for 6 consecutive days starting on the day of the infection. As shown in Fig. 1, while all control mice died within 14 days after infection, mice treated with the compound survived at the rate of 40% (20 mg/kg), 60% (100 mg/kg), and 10% (500 mg/kg) more than 30 days after infection. While the mean survival days of control mice were determined to be 11.6 ± 1.5 days, mice receiving Ge-132 at doses of 20,

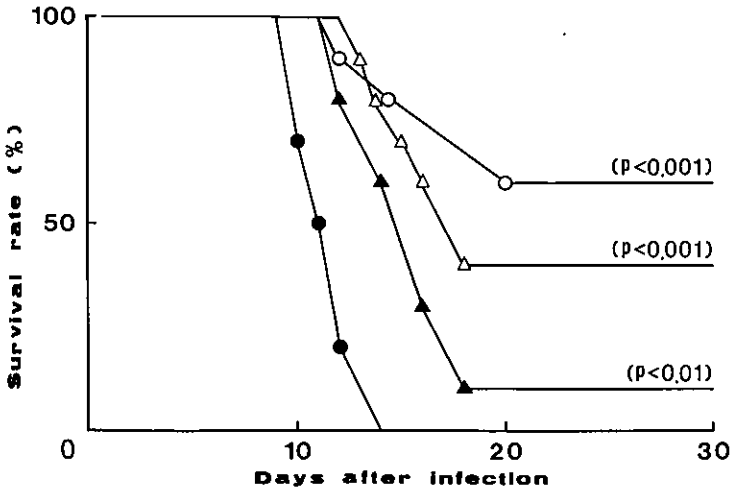

FIG. 1. Protective effect of Ge-132 in mice infected with a lethal dose of influenza virus. Mice infected with 10 LD_{50} of influenza virus were given various oral doses of Ge-132 once daily for 6 consecutive days after infection. The generalized Wilcoxon test (U test; $r = 10$) was used for statistical analysis. Control PBS, 0.2 ml/mouse (●); Ge-132, 20 mg/kg (△); Ge-132, 100 mg/kg (○); Ge-132, 500 mg/kg (▲).

100, and 500 mg/kg survived at 21.4 ± 7.6 ($p < 0.001$), 24.6 ± 7.4 ($p < 0.001$), and 16.6 ± 5.1 days ($p < 0.05$), respectively.

Effect of Ge-132 on Developmental Lung Consolidations

The lung consolidation score system was described in the Materials and Methods section. On day 5, consolidation scores of mice treated with various doses of Ge-132 were demonstrably higher than those obtained in the control group (Table 1). On day 11, a reversal of this trend was observed (PBS control, 4.5 ± 0.4; mice receiving Ge-132 at doses of 100 mg/kg, 2.5 ± 0.5; 20 mg/kg, 2.8 ± 0.3). The difference in consolidation scores between controls and Ge-132-treated mice was significant ($p < 0.001$).

Inhibition of Virus Growth

The virus growth in lungs of mice infected with influenza virus was determined by the HA test (Fig. 2). There was no difference in virus growth in the lungs between controls and groups treated with various doses of Ge-132 on day 3. On day 5, however, when the virus titer of the control group was shown to be 6400 HAU/lung, an inhibitory effect of Ge-132 on virus growth in the lungs was demonstrated. In groups treated with Ge-132, virus titers of 320 HAU/lung (20 mg/kg) and 200 HAU/lung (100 mg/kg) were detected. Therefore, the virus growth in lungs was inhibited by Ge-132 administration.

Enhancement of NK Cell Activity by Ge-132

As indicated in Table 2, splenic NK cell activity was augmented on days 1 and 3 in normal mice treated with multiple administrations of Ge-132, while no sig-

TABLE 1. *Effect of Ge-132 on the development of lung consolidation in influenza virus-infected mice*[a]

Treatment	Consolidation score,[b] days after infection			
	3	5	7	11
PBS	1.0 ± 0.0[c]	1.2 ± 0.3	2.8 ± 0.4	4.5 ± 0.4
Ge-132				
20 mg/kg	1.1 ± 0.2	2.2 ± 0.3[d]	2.7 ± 0.3	2.5 ± 0.3[d]
100 mg/kg	0.2 ± 0.3	2.0 ± 0.4[e]	2.5 ± 0.4	2.5 ± 0.5[d]
500 mg/kg	1.2 ± 0.3	2.3 ± 0.3[d]	2.7 ± 0.3	3.5 ± 0.6[f]

[a] Mice infected with a 10 LD_{50} dose of influenza virus received various oral doses of Ge-132 once daily for 6 days after virus infection.

[b] The grade of virus-induced lung consolidation was determined as described in the Materials and Methods section as follows: 5 = death or 100% consolidation; 4.5 = 90%; 4 = 80%; 3.5 = 70%; 3 = 60%; 2.5 = 50%; 2 = 40%; 1.5 = 30%; 1 = 20%; 0.5 = 10%; 0 = 0% consolidation.

[c] Mean ± SD ($n = 5$).

Compared to control treated with PBS (Student's t test: [d] $p < 0.001$, [e] $p < 0.01$, [f] $p < 0.05$).

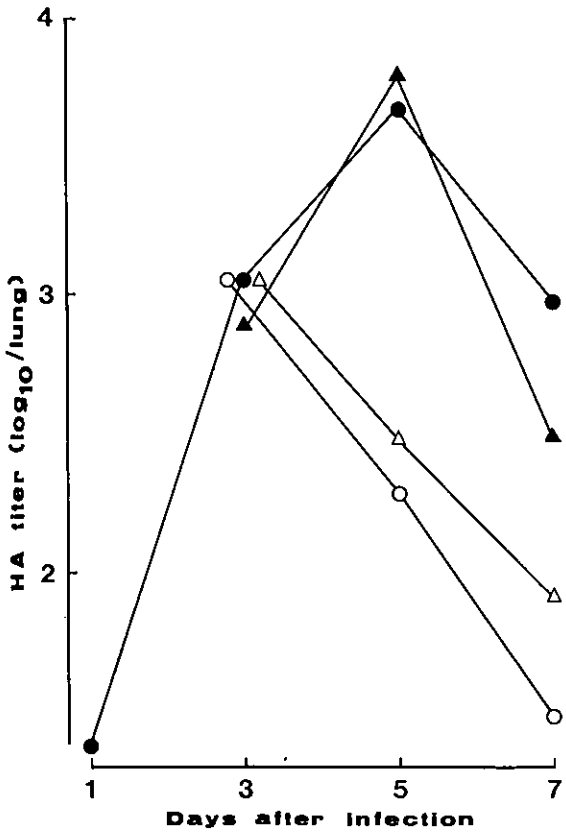

FIG. 2. Effect of Ge-132 on virus growth in the lungs of mice infected with influenza A_2 virus. Mice infected with a 10 LD_{50} dose of influenza virus received various oral doses of Ge-132 once daily for 6 days after virus infection. The HA titer of lung homogenates was calculated by the method of Reed and Meunch (see the Materials and Methods section). Control PBS, 0.2 ml/mouse (●); Ge-132, 20 mg/kg (△); Ge-132, 100 mg/kg (○); Ge-132, 500 mg/kg (▲).

nificant NK cell activity was detected in spleens of mice treated with PBS. In virus-infected mice, however, increased NK cell activity was detected in the spleens of mice treated with PBS (on day 1, 7.8% lysis of target cells; day 3, 28.8%; day 5, 43.6%; day 7, 15.1%). When virus-infected mice received multiple

TABLE 2. *Effect of Ge-132 on splenic NK cell activity in mice infected with influenza virus*[a]

Treatment	Infection	% Lysis,[b] days after infection			
		1	3	5	7
PBS	−	4.2 ± 1.7	3.0 ± 1.7	5.6 ± 1.2	6.4 ± 2.4
Ge-132	−	12.0 ± 1.1[c]	13.4 ± 0.5[d]	6.2 ± 1.4	5.6 ± 1.1
PBS	+	7.8 ± 2.1	28.8 ± 2.5	43.6 ± 2.2	15.1 ± 2.1
Ge-132	+	8.1 ± 2.0	40.0 ± 4.0[e]	63.4 ± 3.2[d]	26.1 ± 2.6[d]

[a] Mice were infected with 10 LD_{50} of influenza A_2 (H_2N_2) virus by inhalation. Ge-132 (100 mg/kg) was administered p.o. once daily for 6 consecutive days after infection. Control mice were treated with PBS (0.2 ml/mouse, p.o.). NK cell cytotoxicity was determined in a 4 h ^{51}Cr-release assay at an effector to target cell ratio of 50:1.

[b] Mean ± SD (n = 3).

Compared to each PBS control (Student's t test: [c]$p < 0.01$, [d]$p < 0.001$, [e]$p < 0.05$).

oral doses of Ge-132, a greater enhancement of NK cell activity was demonstrated (on day 1, 8.1% lysis; day 3, 40.0%; day 5, 63.4%; day 7, 26.1%). This increase of specific lysis on days 5 and 7 in infected mice was statistically significant when compared with that of the PBS-treated control.

NK cell activity in lymphocytes obtained from the lungs of infected mice treated with Ge-132 was also investigated. Although the augmentation of NK cell activity in the lungs of uninfected mice by Ge-132 was not detected, increased NK cell activity was demonstrated in lymphocytes prepared from lungs of virus-infected Ge-132-treated mice at 3 days (24.0% lysis), 5 days (42.4% lysis), and 7 days (60.3% lysis) (Table 3). While the augmentation of NK cell activity was also observed in infected mice treated with PBS, the administration of Ge-132 induced a more pronounced and rapid increase of lung NK cell activity.

In Vitro Augmentation of NK Cell Activity by Ge-IFN

The effect of Ge-IFN and Ge-132 itself on the augmentation of splenic NK cell activity in vitro was studied. As shown in Table 4, while the level of NK cell activity was not altered by 2 to 200 μg/ml of Ge-132, a significant increase in NK cell activity was detected in splenocytes treated with Ge-IFN as low as 0.3 U/ml. These results suggest that the augmentation of NK activity in mice after the oral administration of Ge-132 is mediated by the induction of IFN.

Enhancement of Virus-Specific NK Cell Activity Directed Against Target Cells Treated with Influenza A_2 Virus

After a single oral administration of Ge-132 (100 mg/kg), the NK activity of spleen cells against YAC-1 targets cells that were known to be sensitive to killing by NK cells was augmented, but not against Meth-A cells, which were known to be insensitive to NK cells (Table 5). However, NK cells stimulated by Ge-132 showed cytotoxicity against Meth-A cells that were infected with influenza virus. This phenomenon was also observed in splenic lymphocytes isolated from influenza virus-infected mice treated with Ge-132 (Table 5).

TABLE 3. *Effect of Ge-132 on NK cell activity in lungs of mice infected with influenza virus*[a]

Treatment	Infection	% Lysis,[b] days after infection			
		1	3	5	7
PBS	−	1.0 ± 0.3	3.3 ± 0.1	2.4 ± 1.7	3.7 ± 1.7
Ge-132	−	1.0 ± 0.5	2.2 ± 0.3	1.2 ± 0.7	2.4 ± 0.3
PBS	+	1.1 ± 1.3	13.3 ± 1.5	36.5 ± 3.7	36.1 ± 2.9
Ge-132	+	2.2 ± 2.5	24.0 ± 1.8[c]	42.4 ± 1.8	60.3 ± 3.4[d]

[a] Mice and treatment protocols were the same as in Table 2.
[b] Mean ± SD (n = 3).
Compared to control treated with PBS (Student's t test: [c]$p < 0.01$, [d]$p < 0.001$).

TABLE 4. *Effect of Ge-132 and Ge-IFN on splenic NK cell activity in vitro*[a]

Treatment		% Lysis[b]
None[c]		4.0 ± 0.8
Ge-132	2 μg/ml	3.3 ± 1.0
	20 μg/ml	4.4 ± 0.2
	200 μg/ml	4.1 ± 1.4
Ge-IFN	0.3 U/ml	8.0 ± 1.2[d]
	3 U/ml	18.5 ± 2.1[e]
	30 U/ml	20.0 ± 0.5[e]
IFN-α/β	100 U/ml	19.2 ± 0.7[e]

[a] Splenic lymphocytes (2.5×10^6 cells/ml) of normal mice were cultured with or without Ge-132, Ge-IFN, or IFN-α/β preparations. Twenty-four hours after cultivation, NK cell activity was determined in a 4 h ^{51}Cr-release assay at an effector to target cell ratio of 50:1.

[b] Mean ± SD (n = 3).

[c] Control.

Compared to the control (Student's t test): [d]$p < 0.01$, [e]$p < 0.001$).

DISCUSSION

The present study demonstrated that mice infected with a lethal dose of influenza virus were protected by multiple oral administrations of Ge-132. The protective activity of Ge-132 against influenza virus infection in mice was demonstrated by the increase of survival rate, the prolongation of mean survival days, an inhibition of lung consolidation development, and a suppression of virus growth in the lungs. Since it has been reported previously (25) that Ge-132 has no virucidal

TABLE 5. *NK cell activity against various target cells pretreated with or without influenza virus*[a]

Treatment	Infection	% Lysis,[b] target cells		
		YAC-1	Meth-A	Inf-Meth-A[c]
PBS	−	3.3 ± 0.5	0.0 ± 1.0	4.5 ± 1.3
Ge-132	−	17.2 ± 1.8[d]	2.1 ± 1.3	10.2 ± 0.8[d]
PBS	+	32.0 ± 2.8	3.5 ± 1.6	19.0 ± 2.1
Ge-132	+	48.0 ± 1.2[d]	5.0 ± 3.2	28.0 ± 1.1[e]

[a] Five days after mice were infected with a 10 LD_{50} dose of influenza virus, splenic lymphocytes from Ge-132- or PBS-treated mice were isolated and used as effector cells in a 4 h ^{51}Cr-release assay to determine NK cell activity. Splenocytes from uninfected mice were obtained 24 h after the administration of Ge-132 (100 mg/kg). As a control, 0.2 ml/mouse of PBS was administered to the mice.

[b] Mean ± SD (n = 3).

[c] ^{51}Cr-labeled Meth-A cells were incubated with $10^{9.0}$ EID_{50} of influenza A_2 virus at 37°C for 1 hr.

Compared to each control (Student's t test: [d]$p < 0.001$, [e]$p < 0.01$).

or virustatic activities against influenza virus in vitro, Ge-132 may express this antiviral activity through a potentiation of the host's defense functions.

As shown in a previous report (21), NK cell activity in spleens of normal mice was augmented by the administration of this compound. While we could not detect NK cell activity in lymphocytes prepared from normal mouse lungs, the same preparations obtained from lungs of virus-infected mice showed significantly elevated NK cell activity against target cells treated with or without influenza virus. Additionally, NK cell activity in lymphocytes isolated from influenza virus-infected mice treated with Ge-132 was detected against various tumor target cells, including NK cell-resistant Meth-A cells at a greater level than that observed in infected PBS-treated mice (Table 5). It has been previously reported that Ge-132 can induce circulating IFN-γ in mice (21,22). Since Ge-132 itself could not augment NK cells in vitro, Ge-132-induced IFN-γ may increase NK cell activity. Since influenza virus-treated target cells were killed by NK cells obtained from the spleens of Ge-132-treated and virus-infected mice, the protective effect of Ge-132 against influenza virus infection in vivo may depend upon the enhanced NK cell activity stimulated by Ge-132-induced IFN-γ.

Bancroft et al. (13) found that beige mice with low levels of NK activity during murine cytomegalovirus (MCMV) infection often have a greater susceptibility to MCMV infection than their normal littermates. In addition, Bukowski et al. (14) clearly described the role of NK cells in resistance to MCMV and lymphocytic choriomeningitic virus by the adoptive transfer of NK cells. The present experiments investigating the antiviral effect of Ge-132 have suggested that NK cells may be important in the protection of mice from influenza virus infection.

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