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2-Carboxyethylgermanium sesquioxide, a synthetic organogermanium compound, as an inducer of contrasuppressor T cells

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Abstract. 2-Carboxyethylgermanium sesquioxide (Ge-132), a synthesized organogermanium compound with immunomodulaing activities, was shown to be an inducer of anti-suppressor T cells in normal mice. The suppressor cell activity of T6S cells, a clone of burn-induced $CD8^+$ IL-4-producing suppressor T cells, was clearly inhibited when a mixed lymphocyte-tumor cell reaction of the clone was conducted with splenic mononuclear cells from mice treated orally with a 100 mg/kg dose of Ge-132. The activity of anti-suppressor cells was demonstrated in spleens of mice 2 days after treatment with Ge-132 and reached its peak on day 3. The anti-suppressor cells induced by the compound were of a contrasuppressor T cell-linage, because they were characterized as $CD4^+$ $CD28^+$ $TCR\alpha/\beta^+$ *Vicia villosa* lectin-adherent T cells. These cells produced IFN-γ but did not produce IL-2, IL-4, IL-6 or IL-10 in their culture fluids. $CD4^+$ anti-suppressor T cells induced by Ge-132 may be different from other subsets of $CD4^+$ T cells because Th1 and Th2 cells generated in our laboratory did not adhere to *Vicia villosa* lectin-coated petri dishes, and each produced specific cytokines. Th1 cells produced IFN-γ and IL-2 while Th2 cells produce IL-4 and IL-10 in vitro. These results suggest that Ge-132 may be useful as an inducer of contrasuppressor T cells in immunocompromised individuals bearing suppressor T cells. To eliminate suppressor T cells from immunocompromised hosts may result in improved resistance from various opportunistic infections.

Key words. Organogermanium compound; contrasuppressor T cells; suppressor T cells.

Introduction

Suppressor T cells generated in patients or animals bearing malignancies, infections or thermal injury have been reported in many papers[1–4], and the decreased cell-mediated immunity in these individuals may be associated, in part, with the appearance of suppressor T cells[5]. Recent studies have revealed that type 1 T cells (Th1 cells or cytotoxic T lymphocytes) play a role in the host's protective response against various pathogens[6,7], while type 2 T cells (Th2 cells or $CD8^+$ suppressor T cells) produce increased susceptibility in individuals to various infections[6,7]. It has been described by Jayaraman and coworkers[8] that the adoptive transfer of $CD4^+$ type 2 T cells (Th2 cells) into mice resulted in the accelerated onset and the increased severity of stromal keratititis in recipient mice during herpesvirus infections. Kupper and coworkers have reported[9] that septic bacterial infections were established when normal mice were inoculated with burn-induced $CD8^+$ suppressor T cells after a cecal ligation and puncture. We have also demonstrated[10] that the generation of $CD8^+$ suppressor T cells and the increased susceptibility to the opportunistic herpesvirus infection in thermally injured mice are linked. The susceptibility of normal mice to the herpesvirus infection was increased to levels observed in thermally injured mice when burn-induced $CD8^+$ suppressor T cells were adoptively transferred into these mice. The suppressor T cells generated in spleens of mice 6 days after thermal injury have been identified as $CD8^+$ $CD11b^+$ $TCR\gamma/\delta^+$ IL-4-producing T cells[11]. All of these facts demonstrated by us and others suggest that both $CD4^+$ and $CD8^+$ type 2 T cells may play a role on the increased susceptibility of the immunocompromised host for opportunistic viral or bacterial infections. Some recent studies[12,13] have attempted to improve the resistance of immunocompromised hosts to opportunistic infections through the regulation of type 2 T cell activities. Administration of a blocker of histamine type 2 receptors or a low dose of cyclophosphamide has also been reported to increase the resistance of immunocompromised hosts to septic infections through the inhibition of suppressor T cells[12–14]. However, these blockers are not satisfactory against various opportunistic infections in immunocompromised hosts, because these agents only partially eliminate the suppressor cell activity. Therefore, a more effective way is needed to control opportunistic infections in immunocompromised hosts bearing suppressor T cells.

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2-Carboxyethylgermanium sesquioxide [$O_3(GeCH_2CH_2COOH)_2$] (Ge-132), synthesized from trichlorogermane and acrylonitrile[15], has been shown to exhibit antitumor activities in mice[16–18] and in rats[19], as revealed by the inhibition of tumor growth and increased mean survival time in these animals. The antitumor activity of the compound appeared through the induction of IFN$-\gamma$[16,17,21], augmentation of the NK cell activity[17,20] and generation of cytotoxic macrophages from resting macrophages[16,17,21]. Ge-132 has also been shown to express antiviral activities in mice infected with influenza virus[22]. The anti-influenza activity of Ge-132 was expressed through the NK cell activity stimulated by the agent-induced IFN$-\gamma$[22]. We have recently reported[23] that the generation of suppressor macrophages was inhibited in mice immunized with allogenic lymphocytes after treatment of these mice with Ge-132.

In the present study, we demonstrated the contrasuppressor T cell-inducing activity of Ge-132 in normal mice. The activity of a clone of burn-induced $CD8^+$ IL-4-producing suppressor T cells was markedly inhibited by anti-suppressor T cells induced by Ge-132. The anti-suppressor T cells induced by Ge-132 were characterized as $CD4^+$ $CD28^+$ TCRα/β^+ *Vicia villosa* lectin-adherent T cells, whcih were distinguished from standard Th1 and Th2 cells by their cytokine-producing profiles and adherence to *Vicia villosa* lectin.

Materials and methods

Mice, cells, media and reagents. Eight-week-old BALB/c mice purchased from Jackson Laboratories, Bar Harbor, (Maine, USA) were used in these experiments. EL-4 thymoma cells, maintained serially in vitro in our laboratory, were used as stimulators in a mixed lymphocyte-tumor cell reaction (MLTR)[24]. CTLL-2 cells (a cell line for IL-2 and IL-4 dependent murine cytotoxic T cells), 14.8 cells (anti-CD45RA mAb-producing cells), S4B6 cells (anti-IL-2mAb-producing cells), 11B11 cells (anti-IL-4 mAb-producing cells), 7TD1 cells (IL-6 dependent cell line) were purchased from the American Type Culture Collection (Rockville, Maryland, USA) and serially maintained in our laboratory. Murine L (L-Galveston) cells, maintained in our laboratory, were used for the assay of IFN-γ[25]. RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine and antibiotics (complete media) was the media for cultivation of EL-4, S4B6 and 11B11 cells. MEM supplemented with 10% FBS, 2 mM L-glutamine and antibiotics was the media for cultivation of 14.8 cells and L-Galveston cells. CTLL-2 cells were cultured with complete media supplemented with 100 U/ml of IL-2. Complete media, supplemented with 30 mM HEPES buffer and 5×10^{-5} M 2-ME, were used for the MLTR[24] and bioassay of IL-2[26], IL-4[26] and IL-6[27]. The Indiana strain of vesicular stomatitis virus grown in monolayer cultures of L-Galveston cells was used for the IFN titration. Other reagents used in these experiments were: anti-CD3ε mAb (Boehringer-Mannheim Biochemicals, Indianapolis, Indiana, USA); anti-L3T4 and anti-Lyt 2.2 mAbs (Accurate Chemical and Scientific Corp., Westbury, New York, USA); anti-CD11b, anti-CD28, anti-TCRα/β and anti-IL-10 mAbs (Pharmingen, San Diego, California, USA); anti-mouse-Ig antiserum (Cappel Laboratory, Cochranville, Pennsylvania, USA); low-tox-M rabbit complement (C, Cedarlane Laboratories, Hoenby, Ontario, Canada); *Vicia villosa* lectin (EY Laboratories, San Meteo, California, USA); *N*-acetyl-*D*-galactosamine, carbonyl iron and mitomycin C (Sigma Chemical Co., St. Louis, Missouri, USA); murine rIL-2, IL-4, IL-6 and IL-10 (Genzyme, Cambridge, Masachusetts, USA).

Ge-132. 2-Carboxyethylgermanium sesquioxide (Ge-132) was kindly supplied by the Asai Germanium Research Institute, Tokyo (Japan). Ge-132 was dissolved in sterile physiological saline and administered orally to mice at appropriate doses. In the majority of experiments a 100 mg/kg dose of Ge-132 was administered to mice because our previous results showed[16] that the activated macrophages were effectively induced in mice by this dose of the agent.

Preparation of mononuclear cells (MNC). MNC were prepared from spleens of mice treated orally with Ge-132 or saline, as described previously[28]. Briefly, spleens were obtained from various groups of mice and teased through a steel mesh to prepare a single cell suspension. Removal of phagocytic cells from MNC suspensions was performed by treatment with carbonyl iron, as described previously[28]. Cells which ingested carbonyl iron were depleted by Ficoll-Hypaque sedimentation (density 1.075 g/ml, 400 g for 30 min). More than 99% of phagocytic cells were removed by this procedure. The viability of MNC obtained was shown to be more than 99% by the trypan blue dye-exclusion test.

Preparation of standard Th1 and Th2 cells. Th1 cells were prepared from mice treated with an arabinomannan extracted from *Mycobacterium tuberculosis*, as described previously[29,30]. In the presence of antigen-presenting cells and antigen, Th2 cells were generated in vitro from splenic T cells of mice immunized with KLH[31].

A clone of suppressor T cells. A suppressor cell clone, designated as T6S cells, was isolated from burn-induced $CD8^+$ IL-4-producing suppressor T cells generated in spleens of mice 6 days after thermal injury, as described previously[11]. T6S cells were serially maintained in our laboratory in complete media supplemented with 10 U/ml of IL-2. T6S cells were used as a standard suppressor T cell in the MLTR when testing for the activity of anti-suppressor cells[11].

Assay of suppressor cell activity. As described previously[11,24], the suppressor cell activity of T6S cells was

assayed in a one-way MLTR. Responders (MNC from normal BALB/c mice, 5×10^4 cells/well) and stimulators (EL-4 thymoma cells, 5×10^4 cells/well) were co-cultured with or without T6S cells (5×10^3 cells/well) in a 96-well round-bottomed microtiter plate for 3 days at 37 °C in 5% CO_2. Before subjection to the MLTR, all cells except responders were treated with mitomycin C, as described previously[11,24]. [^{3}H]-thymidine (0.5 μCi/well) was added to the plate for the last 12 h of the incubation, and the [^{3}H]-thymidine uptake by responder cells was measured, as described previously[11,24]. As compared with the incorporation of [^{3}H]-thymidine into responder cells co-cultured with stimulator cells alone, the reduction of the isotope incorporation into the responder cells co-cultured with stimulator cells and T6S cells was considered to be suppressor cell activities of T6S cells. Each assay was performed three times, and the results were expressed as the mean of these three tests. The suppressor cell activity was calculated by the following formula: Suppression (%) = [1 − (cpm in the presence of T6S cells/cpm in the absence of T6S cells)] × 100.

Assay of the anti-suppressor cell activity. The anti-suppressor cell activity of various cells derived from Ge-132-treated mice was determined in the MLTR assay system for suppressor cells, as described previously[11]. T6S cells were employed as the standard suppressor cells in the MLTR assay[11]. Responders (MNC from BALB/c mice, 5×10^4 cells/well), stimulators (EL-4 thymoma) and T6S cells were co-cultured in the presence of putative anti-suppressor cells in the MLTR. Before subjection to the assay, all cells except responder cells were treated with mitomycin C, as described above. Responders, stimulators and T6S cells were co-cultured with various numbers of MNC from normal mice or anti-suppressor cells for 3 days at 37 °C in 5% CO_2. [^{3}H]-thymidine (0.5 μCi/well) was added to each well 12 h before being harvested. The incorporation of [^{3}H]-thymidine into responder cells was measured by a liquid scintillation counter. The reduction of the suppressor cell activity of T6S cells co-cultured with anti-suppressor cells in the MLTR was considered to be anti-suppressor cell activity. Each assay was performed three times, and the results were expressed as the mean of these three tests. The anti-suppressor cell activity was calculated using the following formula: Contrasuppression (%) = [1-(% suppression of MLTR containing T6S cells and test cells/% suppression of MLTR containing T6S cells)] × 100.

Characterization of anti-suppressor cells. To determine the type of cell responsible for anti-suppressor cell activity, various splenic cell populations were obtained from Ge-132-treated mice. These cells were purified and treated with various mAbs followed by C and then assayed for their inhibitory activities in the MLTR[11]. To obtain whole T cells, CD4$^+$ T cells or CD8$^+$ T cells, splenic MNC (1×10^7 cells/ml) were passed through the T Cell Enrichment Column, CD4 Subset Column or CD8 Subset Column (R&D Systems, Minneapolis, Minnesota, USA), respectively[10,11]. When the whole T cell fraction obtained was treated with anti-Ig antiserum and C, only a 3% reduction in viable cells was demonstrated,whereas treatment of these cells with anti-CD3ε mAb followed by C caused a 98% reduction in the number of viable cells. When the CD4$^+$ T cell or CD8$^+$ T cell fraction obtained was treated with anti-L3T4 mAb followed by C, 96% or 3% of viable cells were lysed. When they were treated with anti-Lyt 2.2 mAb followed by C, 2% or 97% of viable cells were lysed, respectively. These results suggest that purity of these three cell preparations (whole T cells, CD4$^+$ T cells and CD8$^+$ T cells) is more than 96%. CD4$^+$ T cells were further characterized phenotypically using various mAbs (4 °C, 30 min) plus C (1:30 dilution, 37 °C, 30 min), as described previously[32]. Monoclonal antibodies used in this experiment included; anti-CD11b (1:100 dilution), anti-CD28 (1:50 dilution) and anti-TCRα/β (1:100 dilution)[10,11]. For depletion of CD45RA$^+$ T cells, CD4$^+$ T cells (5×10^6 cells/5 ml) were placed into the petri dishes (15×100 mm) coated with anti-CD45RA mAb (purified conditioning media of 14.8 cells) and incubated for 1 h at 4 °C, then non-adherent cells were harvested by 3 to 4 gentle washes with cold media[33]. For preparation of *Vicia villosa* lectin-adherent cells, CD4$^+$ T cells were introduced into petri dishes coated with the lectin, as described previously[34,35]. Briefly, 5 ml of the lectin solution (0.05 mg/ml) was added to a petri dish (15×100 mm) and incubated at room temperature for 2 h. After the petri dish was rinsed with PBS, a 2% FBS solution in PBS was added to the dish and incubated for 15 min at room temperature. Five milliliters of the cell suspension containing 1×10^7 cells/ml of CD4$^+$ T cells from mice treated with Ge-132 was added to the petri dish and incubated for 45 min at 37 °C. The non-adherent cells were harvested from the petri dish with warm PBS. *Vicia villosa* lectin-adherent T cells were harvested by adding 5 ml of *N*-acetyl-*D*-galactosamine (1 mg/ml) dissolved in PBS to the petri dish for 15 min at 37 °C.

Measurement of cytokine activities. For induction of various cytokines, 2×10^6 cells/ml of CD4$^+$ CD28$^+$ TCR α/β^+ *Vicia villosa* lectin-adherent T cells purified from splenic mononuclear cells of Ge-132-treated mice were cultured without any stimulation for 24 to 72 h at 37 °C[26]. Culture fluids harvested from these cell cultures were assayed for their cytokine activities. The IFN activity was determined by means of a plaque reduction assay utilizing L-Galveston cells infected with vesicular stomatitis virus, as described previously[25]. The IFN titer in the fluids was determined by the reciprocal of the

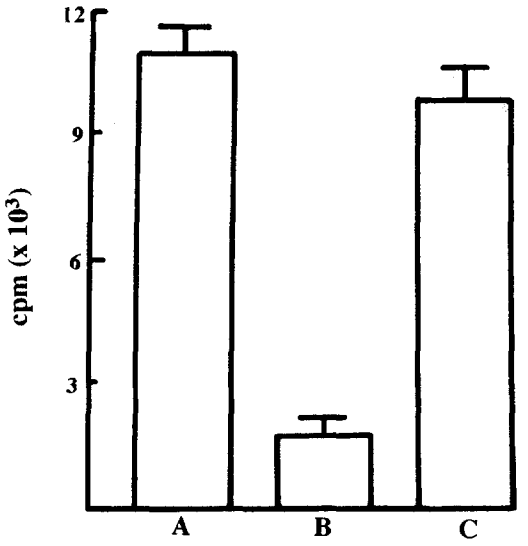

Figure 1. Inhibition of T6S suppressor cell activity by splenic lymphocytes from mice treated with Ge-132. Five × 10^3 cells/ml of splenic mononuclear cells (MNC), prepared from mice 3 days after treatment with a 100 mg/kg dose of Ge-132, were co-cultured with responder cells (5 × 10^4 cells/well, splenic MNC from BALB/c mice), stimulator cells (5 × 10^4 cells/well, EL-4 thymoma cells) and T6S cells (5 × 10^3 cells/well) for 3 days at 37 °C in a one-way MLTR (*C*), as described in the 'Materials and methods' section. As a control, responder cells were co-cultured with stimulator cells (*A*) in the presence of T6S cells (*B*) in the MLTR. The reduction of the suppressor cell activity of T6S cells was considered to be anti-suppressor cell activity and the results shown in the figure were expressed as the the [^{3}H]-thymidine incorporation into responder cells in each group.

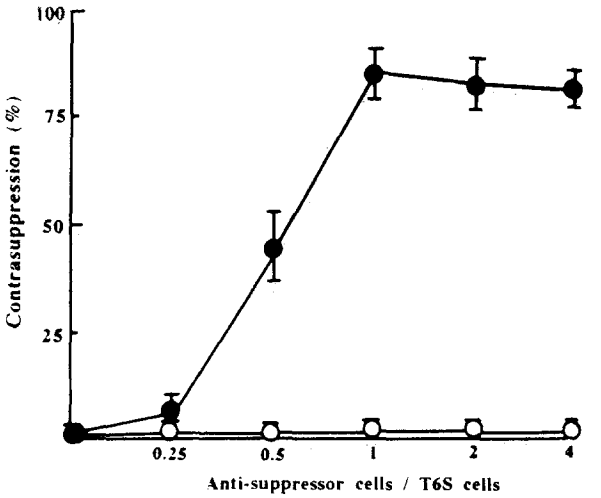

Figure 2. Effect of various effector to suppressor cell ratios on the anti-suppressor cell activity of splenic MNC from mice treated wth Ge-132. Five × 10^3 cells/well of T6S cells were co-cultured with graded numbers of anti-suppressor cells induced by Ge-132 (●) in the one-way MLTR. As a control (○), various numbers of splenic MNC from normal mice were co-cultured with responders and stimulators in the presence of T6S cells (5 × 10^3 cells). Contrasuppression (%) was calculated as the percent reduction of the T6S suppressor cell activity in the MLTR.

greatest dilution of the test sample that reduced virus plaques by 50% and compared to standard murine rIFN-γ[25]. The activity of IL-2 and IL-4 was determined by CTLL-2 cells which are IL-2 and IL-4-dependent[26]. The IL-6 activity was assayed using 7TD1 cells, an IL-6-dependent cell line[27], as described previously. The IL-10 activity was measured by ELISA using anti-IL-10 mAb[36]. Each assay was performed three times, and the results obtained were expressed as the mean of these three tests.

Statistical analysis. Results were analyzed statistically using Student's *t*-test. If a P value was lower than 0.05, the result obtained was considered significant.

Results

As shown in figure 1, the proliferation of responder cells stimulated with allogeneic EL-4 cells was inhibited by T6S cells when they were co-cultured in the MLTR at a ratio of 1:1:0.1. However, this activity of T6S cells was clearly inhibited when the MLTR was conducted with splenic MNC from normal mice 3 days after treatment with Ge-132. When T6S cells were co-cultured with responders, stimulators and splenic MNC from Ge-132-treated mice at a ratio of 1:1:0.1:0.1 in the MLTR, only 15% of the proliferative response of responder cells was inhibited, as compared to 80% suppression of [^{3}H]-thymidine incorporation into responder cells co-cultured with stimulator cells and T6S cells ($p < 0.001$). This means that splenic lymphocytes (macrophage-depleted splenic MNC) from Ge-132-treated mice were able to inhibit 81% of T6S suppressor cell activities. Similar anti-suppressor cell activity by splenic lymphocytes from Ge-132-treated mice was demonstrated when $CD8^+$ suppressor T cells naturally generated in mice 6 days after thermal injury[10,11] were used as suppressors in the MLTR (data not shown). In addition, these anti-suppressor cell activities were not detected when cell-lysate derived from splenic lymphocytes of Ge-132-treated mice or 1 to 500 µg/ml of Ge-132 was added to the MLTR for the assay of suppressor cells (data not shown). This suggests that Ge-132 has no direct-inhibitory effects on the suppressor cell activity of T6S cells in the MLTR, and viable cells were required for the inhibition of the T6S suppressor cell activity. A 50% inhibition of the T6S cell activity was observed when T6S cells and splenic MNC from Ge-132-treated mice were co-cultured at a ratio of 1:0.5 (fig. 2). However, various numbers of MNC from normal mice did not inhibit suppressor cell activities in the MLTR (fig. 2). These results suggested that the anti-suppressor cells were generated in spleens of normal mice after treatment with Ge-132. Therefore, kinetics of the anti-suppressor cell appearance in normal mice treated with Ge-132 was examined next. As shown in figure 3, the anti-suppressor cell activity was first detected in spleens of mice 2 days after treatment with

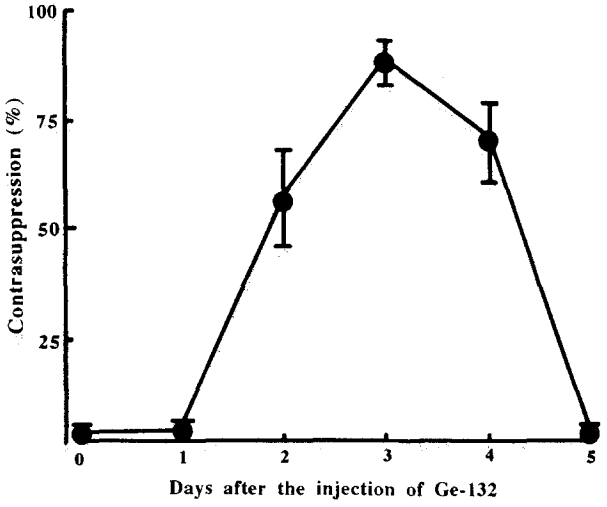

Figure 3. The time course on the appearance of anti-suppressor cells in spleens of Ge-132-treated mice. Splenic MNC, prepared from mice various days after Ge-132 treatment, were assayed for their anti-suppressor cell activities in the MLTR. Contrasuppression (%) was calculated as described in the legend for figure 2.

Ge-132, and reached its peak 3 days after the treatment, then decreased gradually.

In the first step, splenic MNC from mice treated with Ge-132 were fractionated by a column coated with various mAbs, and assay for their anti-suppressor cell activities in the MLTR. Results obtained were shown in table 1. The anti-suppressor cell activity was shown in whole T cells and $CD4^+$ T cells derived from splenic MNC of Ge-132-treated mice, whereas the fraction of $CD8^+$ T cells did not display any inhibitory activities on T6S suppressor cells. These results suggested that $CD4^+$ T cells in these splenic MNC worked as the anti-suppressor cells. To determine phenotypic properties of anti-suppressor cells, in the next series of experiments these $CD4^+$ T cells were treated with various mAbs followed by C, and the remaining cells were assayed for their anti-suppressor cell activities in the MLTR. When these cells were treated with anti-CD28 mAb or anti-TCRα/β mAb plus C, their anti-suppressor cell activities were eliminated (table 2). When these cells were treated with anti-CD11b mAb plus C or an anti-CD45RA mAb-coated dish, no change in the anti-suppressor cell activity of these cells was demonstrated (table 2). In addition, a *Vicia villosa* lectin-adherent population of $CD4^+$ T cells from Ge-132-treated mice counteracted the suppressor cell activity of T6S cells (table 3). These results suggested that $CD4^+$ $CD28^+$ TCRα/β^+ *Vicia villosa* lectin-adherent T cells were shown to be anti-suppressor cells induced by Ge-132, and these phenotypic properties of anti-suppressor cells induced by Ge-132 were shown to resemble contrasuppressor T cells described previously by Gershon and coworkers in 1981[37]. However, we could not determine the phenotypic similarities between the contrasuppressor T cells described previously and anti-suppressor T cells induced by Ge-132, because there is not enough information for phenotypic analysis of contrasuppressor T cells in their reports[37].

Next $CD4^+$ $CD28^+$ TCRα/β^+ *Vicia villosa* lectin-adherent T cells purified from splenic MNC of Ge-132-treated mice were examined for their ability to produce cytokines *in vitro*. After incubation at 37 °C for 24 to 72 h, no significant amounts of IL-2, IL-4, IL-6 or IL-10 were detected in the culture fluids of these cells. However, IFN-γ was detected in the culture fluids of $CD4^+$ $CD28^+$ TCRα/β^+ *Vicia villosa* lectin-adherent T cells, with the maximum levels of 170 U/ml being observed 48 h after incubation. As controls the Th1 and Th2 cells were also assayed. Both of these cells produced the maximum cytokine levels 48 h after incubation. Th1 cells produced: 190 U/ml of IFN-γ, 160 U/ml

Table 1. Anti-suppressor cell activities of various cell preparations from spleens of mice treated with Ge-132[a].

The MLTR was conducted with T6S cells and[b]:	cpm[c] (mean ± SE)	Suppression of the MLTR (%)	Contrasuppression (%)
MLTR control	10,304 ± 1,343	-	-
T6S control	2,062 ± 267[d]	80	-
Splenic MNC	8,490 ± 1,126[d]	18	78
Whole T cell preparations	8,737 ± 926[d]	15	81
$CD4^+$ T cell preparations	8,861 ± 1,050[d]	14	83
$CD8^+$ T cell preparations	2,164 ± 420	79	1

[a]Splenic MNC, whole T, $CD4^+$ T and $CD8^+$ T cells were prepared from spleens of mice 3 days after treatment with a 100 mg/kg dose of Ge-132.

[b]The MLTR was conducted with responders, stimulators and T6S cells in the presence of putative anti-suppressor cells at a ratio of 1:1:0.1:0.1. As a MLTR control, responder cells were co-cultured with only stimulator cells. As a control of the suppressor cells, responder cells and stimulator cells were co-cultured with T6S cells.

[c][^{3}H]-thymidine incorporation into responder cells.

[d]Student's *t*-test, $p < 0.001$: suppression, MLTR control vs T6S cell control; contrasuppression, T6S cell control vs tested cell preparations.

Table 2. Phenotypic properties of anti-suppressor cells induced in normal mice by Ge-132[a].

The activity of anti-suppressor cells treated with[b]:	cpm[c] (mean ± SE)	Suppression of the MLTR (%)	Contrasuppression (%)
MLTR control	12,985 ± 1,511	-	-
T6S control	2,467 ± 375[d]	81	-
C alone	11,037 ± 1,396[d]	15	80
Anti-CD11b mAb plus C	10,907 ± 1,320[d]	16	79
Anti-CD28 mAb plus C	2,337 ± 447	82	0
Anti-CD45RA mAb-coated petri dishes	11,037 ± 1,328[d]	14	82
Anti-TCRα/β mAb plus C	3,176 ± 509	76	6

[a]As anti-suppressor cells, CD4$^+$ T cells were prepared from spleens of mice 3 days after the treatment with a dose of 100 mg/kg of Ge-132.
[b]The MLTR was conducted with responders, stimulators and T6S cells in the presence of tested cells at a ratio of 1:1:0.1:0.1. As a MLTR control, responder cells were co-cultured with only stimulator cells. As a control of the suppressor cells, responder cells and stimulator cells were co-cultured with T6S cells.
[c][^{3}H]-thymidine incorporation into responder cells.
[d]Student's t-test, $p < 0.001$: suppression, MLTR control vs T6S cell control; contrasuppression, T6S cell control vs tested cell preparations.

Table 3. Effect of *Vicia villosa* lectin treatment on the activity of CD4$^+$ anti-suppressor cells induced by Ge-132[a].

The MLTR was conducted with T6S cells and CD4$^+$ cells[b]:	cpm[c] (mean ± SE)	Suppression of the MLTR (%)	Contrasuppression (%)
MLTR control	15,403 ± 1,735	-	-
T6S cell control	3,081 ± 271[d]	80	-
Treated with media	12,466 ± 1,430[d]	19	76
Not adhered to *Vicia villosa* lectin	2,320 ± 386	85	0
Adhered to *Vicia villosa* lectin	13,247 ± 1,241[d]	14	82

[a]As anti-suppressor cells, CD4$^+$ cells were prepared from mice 3 days after treatment with a 100 mg/kg dose of Ge-132.
[b]The MLTR was conducted with responders, stimulators and T6S cells in the presence of tested cells at a ratio of 1:1:0.1:0.1. As a MLTR control, responder cells were co-cultured with only stimulator cells. As a control of the suppressor cells, responder cells and stimulator cells were co-cultured with T6S cells.
[c][^{3}H]-thymidine incorporation into responder cells.
[d]Student's t-test, $p < 0.001$: suppression, MLTR control vs T6S cell control; contrasuppression, T6S cell control vs tested cell preparations.

of IL-2, less than 5 U/ml of IL-4, 80 U/ml of IL-6 and less than 2 pg/ml of IL-10 (fig. 4). The Th2 cells produced less than 4 U/ml of IFN-γ, IL-2 and IL-6, 160 U/ml of IL-4 and 70 pg/ml of IL-10. These results suggest that anti-suppressor cells induced in normal mice by Ge-132 may be distinguished from other CD4$^+$ T cells, such as Th1 and Th2 cells, by their cytokine-producing profiles.

Discussion

In 1981 contrasuppressor T cells were first reported as an essential cell in immunoregulation[37]. They were demonstrated following the generation of suppressor T cells[37–42]. Contrasuppressor T cells play a role in regulating the suppression of antibody production in vitro and in vivo[38,39], preventing the suppression of DTH[40] and depressing the oral tolerance induced by sheep erythrocytes[34]. Kupper and Green described[41] that the generation of contrasuppressor T cells was involved in recovery from the immunosuppression observed after burn injury. We recently reported[42] that the adoptive transfer of contrasuppressor T cells (which were generated naturally in thermally injured mice) to thermally injured mice just after the injury resulted in restoration of resistance to herpesvirus infections, to levels observed in normal mice.

In the present study, Ge-132 was shown to be an inducer of anti-suppressor cells. When normal mice were teated orally with a 100 mg/kg dose of Ge-132, cells which were able to counteract the activity of suppressor cells were detected in the spleens. This anti-suppressor cell activity, detected in the MLTR performed with a clone of burn-induced CD8$^+$ IL-4 producing suppressor T cells (T6S cells), was first demonstrated in spleens of mice 2 days after the treatment and reached its peak 3 days after the treatment with Ge-132. The anti-suppressor cells detected in spleens of Ge-132-treated mice were determined to be CD4$^+$ CD28$^+$ TCRα/β^+ *Vicia villosa* lectin-adherent T cells. IL-2,

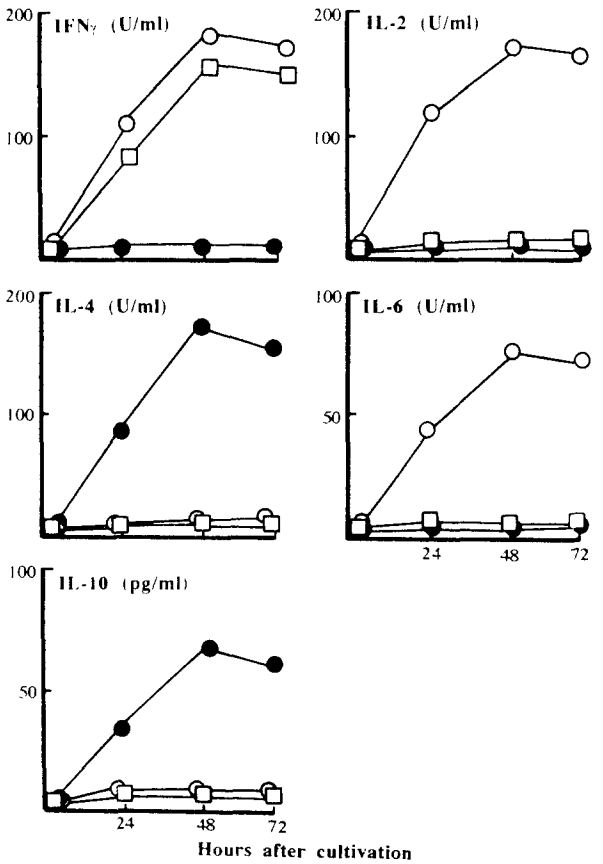

Figure 4. Cytokine-producing activities of Th1 cells, Th2 cells and CD4$^+$ CD28$^+$ TCRα/β^+ *Vicia villosa* lectin-adherent T cells from Ge-132-treated mice in vitro. Th1 cells and Th2 cells were prepared, as described in the 'Materials and methods' section. Anti-suppressor T cells induced in normal mice by Ge-132 (□), Th1 cells (○), Th2 cells (●) were cultured for 24 to 72 h at 37 °C in CO_2. Cytokine activities in culture fluids of these cells were determined as described in the 'Materials and methods' section.

IL-4, IL-6 and IL-10 were not produced by CD4$^+$ anti-suppressor T cells induced by Ge-132. The anti-suppressor T cells induced by Ge-132 may belong to contrasuppressor T cells previously described by Gershon and coworkers[37], because contrasuppressor T cells had been characterized as CD4$^+$ T cells with *Vicia villosa* lectin-adherence properties[37].

It has been described in many papers[4,6–8] that Th1 cells and Th2 cells are distinguished from each other by their cytokine-producing profiles. In this study, Th1 cells induced in spleens of normal mice, which were previously treated with arabinomannan extracted from *Mycobacterium tuberculosis*[29,30], produced IL-2 and IL-6 in their culture fluids. Th2 cells, generated in mice immunized with KLH[31] produced IL-4 and IL-10 in their culture fluids without any stimulation. However, as described above, CD4$^+$ anti-suppressor T cells induced in normal mice by Ge-132 produced no detectable cytokines in their culture fluids except IFN-γ. IFN-γ activities were detected in the culture fluids of Th1 and CD4$^+$ anti-suppressor T cells. These results suggest that CD4$^+$ anti-suppressor T cells induced by Ge-132 might be distinguished from other CD4$^+$ T cells (Th1 and Th2 cells) by their cytokine-producing profiles. In addition, CD4$^+$ *Vicia villosa* lectin-adherent anti-suppressor T cells induced by Ge-132 showed no helper cell activities when they were subjected to the hemolysin production assay and no killer cell activity when they were tested by ^{51}Cr-release assay (data not shown). The activity of Ge-132-induced anti-suppressor T cells did not change when assayed in the MLTR (responders, stimulators, T6S cells plus anti-suppressor T cells) in the presence of anti-TNF-α, anti-IL-2, anti-IL-3, anti-TGF-β and anti-GM-CSF mAbs. However, the culture fluids harvested 48 h after cultivation of Ge-132-induced anti-suppressor T cells significantly decreased the suppressor cell activity of T6S cells (data not shown). This suggests that there is a soluble factor(s), which has anti-suppressor T cell activity, released from Ge-132 induced anti-suppressor T cells. However, TNF-α, IL-2, IL-3, TGF-β and GM-CSF were not part of this soluble factor. We have also attempted to generate anti-supporessor T cells in vitro. Anti-suppressor T cells were generated when splenic MNC were stimulated in vitro with the anti-suppressor cell inducer for 24 to 72 h. The activity of anti-suppressor T cells was only detected when suppressor macrophages, which may appear during the cultivation, were depleted for effector cell preparations. However, anti-suppressor T cells were not generated in vitro when macrophage depleted MNC were stimulated with the inducer. Since IFN-γ was produced from anti-suppressor T cells induced by Ge-132, the role of IFN-γ on the activity of Ge-132-induced anti-suppressor T cells was examined in the MLTR supplemented with anti-IFN-γ mAb. When Ge-132-induced anti-suppressor T cells were co-cultured with T6S cells in the MLTR in the presence of 100 μg/ml of the mAb, the MLTR was cleraly inhibited (85% inhibition). This suggests that IFN-γ, may be released from the anti-suppressor T cells, is not involved on the inhibition of T6S, is not involved on the inhibition of T6S suppressor cell activities by Ge-132 induced anti-suppressor T cells.

Recent descriptions from several laboratories are showing[43,44] that skewing toward Th2 cells resulted in decreased cell-mediated immunity. Cytokine products (IL-4 and IL-10) from Th2 cells have been shown to inhibit: IFN-γ production[43,44], establishment of DTH[43,44] and activity of macrophage or NK cells[43,44]. Both murine and human studies demonstrated[6,7,45] that shifting of cellular functions to Th2 cells from Th1 cells may lead to decreased resistance against various infections including *Leishmania major*[46]. *Listeria monocytogenes*[47], *Candida albicans*[48], murine leukemia virus[49] and HIV[50]. The increased susceptibility associated with the Th1 to Th2 cell shift has also been reported in individuals bearing CD8$^+$ suppressor T cells[4,6,7]. We

have described[10] that the susceptibility of normal mice to opportunistic herpesvirus infections was increased to levels observed in thermally injured mice when burn-associated $CD8^+$ IL-4-producing type 2 T cells were adoptively transferred into these mice. In addition, adoptive transfer of contrasuppressor T cells, generated naturally in thermally injured mice, to herpesvirus-exposed thermally injured mice results in the improved resistane of these mice to HSV infection.

In this study Ge-132 was shown to be an inducer of contrasuppressor T cells which are able to effectively inhibit the suppressor cell activity of type 2 T cells. Ge-132 may be useful against various opportunistic infections which develop in immunocompromised hosts bearing type 2 T cells.

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