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Enhanced resistance against *Escherichia coli* infection by subcutaneous administration of the hot-water extract of *Chlorella vulgaris* in cyclophosphamide-treated mice

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Summary. The effects of *Chlorella vulgaris* extract (CVE-A) on the recovery of leukocyte number and the augmentation of resistance to bacterial infection were examined in CDF1 mice made neutropenic by cyclophosphamide (CY). They were treated intraperitoneally with CY (150 mg/kg) on day 0, and were given CVE-A (50 mg/kg) subcutaneously (s.c.) every other day from day 1 to day 13 after CY treatment. CVE-A accelerated the recovery of polymorphonuclear leukocytes (PMN) in the peripheral blood in CY-treated mice. The number of granulocyte/monocyte-progenitor cells (CFU-GM) in the spleen increased rapidly and highly after the administration of CVE-A in CY-treated mice, in contrast to the absence of change due to CVE-A in the number of bone marrow cells in CY-treated mice. Administration of CVE-A in CY-treated mice enhanced the accumulation of PMN in the inflammatory site and the activity of the accumulated leukocyte cells in luminol-dependent chemiluminescence. The mice became highly susceptible to an intraperitoneal infection with *E.coli* on day 4 after CY treatment, whereas the mice given CVE-A showed an enhanced resistance against *E.coli* infection, irrespective of the timing of challenge. The bacterial number in CY-treated mice increased explosively after inoculation, resulting in death within 24 h. A progressive elimination of bacteria was observed from 6 h in the peritoneal cavity, spleen and liver of CY-treated mice given CVE-A s.c. These results indicate that CVE-A can be used as a potent stimulant of nonspecific resistance to infection in neutropenic mice.

Introduction

Treatment with chemotherapeutic or immunosuppressive agents or radiotherapy for malignancy, autoimmune disease, or organ transplantation frequently results in neutropenia. As neutrophil suppression becomes more profound and has a longer duration, patients are at a progressively greater risk of developing either severe or life-threatening infections. In these patients, the improvement of polymorphonuclear leukocyte (PMN) generation or function by some means would be beneficial for both the treatment and prevention of bacterial infection.

Previous studies from our laboratory have demonstrated the efficacy of CVE-A, glycoproteins [13] that have been partially purified from the unicellular green algae, *Chlorella vulgaris*, in a host defense against tumor and bacteria. CVE-A showed antitumor activity after intratumor injection in a syngeneic system of a methylcholanthrene-induced fibrosarcoma, MethA, and BALB/c mice [20]. The antitumor activity was mediated by CVE-A-induced PMN but not by CVE-A-induced macrophages as assessed by the Winn assay [11]. CVE-A also augmented resistance against an i.p. infection with *E.coli* by its intraperitoneal (i.p.), intravenous (i.v.) or subcutaneous (s.c.) administration in mice [21] and by its oral administration in rats [8]. Moreover, CVE-A enhanced superoxide generation and chemokinesis of PMN of mice in vitro and in vivo [21].

In this paper, we evaluate the effects of s.c. administration of CVE-A on the accumulation and activation of PMN and on resistance to *E.coli* infection in mice made neutropenic by cyclophosphamide (CY). The data show that CVE-A is a potent stimulator of both the regeneration of PMN and antibacterial resistance in neutropenic mice. We propose the possibility that CVE-A may be of clinical value as an adjunctive therapy in neutropenic patients.

Materials and methods

Animals. Specific-pathogen-free female CDF1 (BALB/c × DBA/2) mice were obtained from Japan SLC Inc. (Shizuoka) and were used for experiments at 8–10 weeks of age.

Preparation of CVE-A. The CVE-A of a strain of unicellular green algae, *Chlorella vulgaris*, obtained from Chlorella Industry Co. Ltd. (Tokyo),

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was dialyzed and lyophilized; the lyophilisate (CVE-A) was then dissolved in physiological saline. Chemical analysis revealed that CVE-A contained 44.3 g protein, 39.5 g carbohydrates, and 15.4 g nucleic acids in 100 g (dry weight) whole material. No lipids could be detected. The concentration was adjusted to give an appropriate dry weight per milliliter of saline.

Treatment of mice with CVE-A and CY. Mice were given 50 mg/kg CVE-A s.c. every other day from 1 day after intraperitoneal (i.p.) injection with 150 mg/kg CY.

Bacterial infection and determination of protection. Escherichia coli (E77156:0.6:H1) were prepared as previously described [23] and used for the experiments. The bacteria were stored at –75° C and used after cultivation in a tryptic soy broth for 16 h at 37° C. The mice were challenged by i.p. inoculation with $5 \times 10^6 - 2.5 \times 10^7$ *E.coli* on various days after injection of CY and CVE-A. The survival of the mice was observed for 5 days. The bacterial number in the peritoneal cavity was determined at various intervals after the challenge. The peritoneal cavity was lavaged with 3 ml phosphate-buffered saline (PBS), and 0.1 ml lavaged fluid was diluted tenfold with distilled water and vortexed to lyse cells. After a serial tenfold dilution of the specimens, 0.1 ml each sample was spread on nutrient agar plates containing 0.4% glucose. Colonies were counted after overnight incubation and the number was expressed as $\log_{10}$ (colony-forming units). Each experimental group consisted of four mice.

Counting of leukocytes. Bone marrow cells were obtained by excising the femur and flushing the marrow with 5 ml RPMI-1640 medium. Single-cell suspensions from the spleen were prepared by using two glass slides in a petri dish containing 5 ml RPMI-1640 supplemented with 5% fetal bovine serum. Peritoneal cells were washed out with 3 ml PBS. Each cell suspension was diluted with Turk solution and counted in a hemocytometer. Smear specimens for differential counts were prepared with May-Giemsa staining and examined under oil immersion.

Assay of granulocyte/macrophage-colony-forming units (CFU-GM). To determine the number of CFU-GM, a modification of a previously described in vitro soft agar culture technique was used [3]. Briefly, unfractionated 5×10^5 bone marrow cells or 2.5×10^7 spleen cells were suspended in 5 ml minimal essential alpha medium containing 0.3% agar, 10% fetal bovine serum and 50 units of mouse granulocyte/macrophage-colony-stimulating factor (GM-CSF; Genzyme, Boston) as a CSF source, and 1 ml of the cell suspension was plated into a 35-mm petri dish and cultured. On the 5th day, colonies (>50 cells) were counted and classified as CFU-GM.

Opsonization of E.coli. Cells of 10^9 *E.coli* were opsonized by incubation in 4 ml 75% fresh guinea-pig serum in PBS at 37° C for 15 min. Opsonized bacteria were washed and resuspended in 1 ml buffer II (5 mM KCl, 145 mM NaCl, 5.5 mM glucose, 10 mM HEPES and 0.1 M NaOH to adjust to ph 7.4), and kept on ice until use.

Preparation of phorbol myristate acetate (PMA) and formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe). PMA (Sigma Chemical Co. St. Louis., Mo) was prepared in 0.1 mM solution with dimethylsulfoxide and stored at –25° C. fMet-Leu-Phe (Sigma Chemical Co. St. Louis., Mo) was diluted with PBS to 0.1 mM and stored at –25° C. The reagents were thawed just before use and diluted to appropriate concentrations using buffer II.

Chemiluminescence assay procedure. For detecting the respiratory burst of peritoneal leukocytes, the chemiluminescence response was measured using a Packard Luminometer analyzer (Packard Instrument Co., Downer Grove Ill). A vial containing 100 µl peritoneal cells (1×10^7/ml buffer II), 50 µl buffer I (buffer II+1 mM $CaCl_2$) and 100 µl luminol solution (diluted fivefold with buffer II, Labo Science, Tokyo) were preincubated at 37° C for 10 min before the addition of 50 µl stimuli: fMet-Leu-Phe, opsonized *E.coli* or PMA. Chemiluminescence was measured every 10 s after the addition of stimuli. The data were presented as the maximum counts per minute.

Statistics. The statistical significance of the data was determined by Student's *t*-test and the Mann-Whitney *U* test. All *P* values less than 0.05 were taken as significant.

Results

Effect of CVE-A treatment on the recovery of leukocyte number in the peripheral blood of CY-treated mice

The effect of CVE-A on the number of leukocytes was examined in CY-treated mice. A marked decrease was observed in the number of leukocytes in the peripheral blood by day 4 after CY treatment alone; the leukocyte

Table 1. Effect of *Chlorella vulgaris* extract (CVE-A) treatment on restoration of leukocytes in the peripheral blood of cyclophosphamide (CY)-treated mice

Treatment[a]		Total leukocytes ($\times 10^6$/ml)	$10^{-3}\times$ Differential leukocyte counts[b] (ml^{-1})				
CY	CVE-A		Stab	Seg	Lymp	Mon	Eosin
–	–	6.02±1.08[c]	138± 90	1306±409	436±571	120±60	18±36
2d	–	1.76±0.65	83± 28	533± 28	1114±227	30±26	0± 0
2d	×1	1.59±0.28	41± 21	396± 72	1142± 51	11±14	0± 0
4d	–	0.81±0.16	10± 4	14± 4	774± 5	12± 5	0± 0
4d	×2	0.83±0.50	8± 0	50± 22	722± 25	50±17[d]	0± 0
6d	–	2.00±0.55	26± 12	326± 64	1580±112	66±40	0± 0
6d	×3	6.72±1.70[d]	316± 81[d]	5127±208[d]	1,163±282	114±81	0± 0
8d	–	4.36±0.46	161± 52	2354± 74	1626± 92	218±44	0± 0
8d	×4	7.92±0.87[d]	499±119[d]	5330±562[d]	1766±372	317±95	0± 0
14d	–	3.74±0.40	112± 37	1683±355	1821±348	100±45	26±22
14d	×7	5.21±0.64[d]	141± 62	2813±359[d]	2152±437	68±78	36±31

[a] Mice were treated i.p. with 150 mg/kg CY on day 0 and s.c. with CVE-A every other day (d) from day 1. Leukocyte counts were determined 24 h after the last injection of CVE-A

[b] Stab, Stab-form leukocytes; Seg, segmented leukocytes; Lymp, lymphocytes; Mono, monocytes; Eosin, eosinophils were differentiated with May-Giemsa staining

[c] Each value represents the mean ± SD for three mice

[d] Significantly different from CY-treated control group ($P < 0.05$)

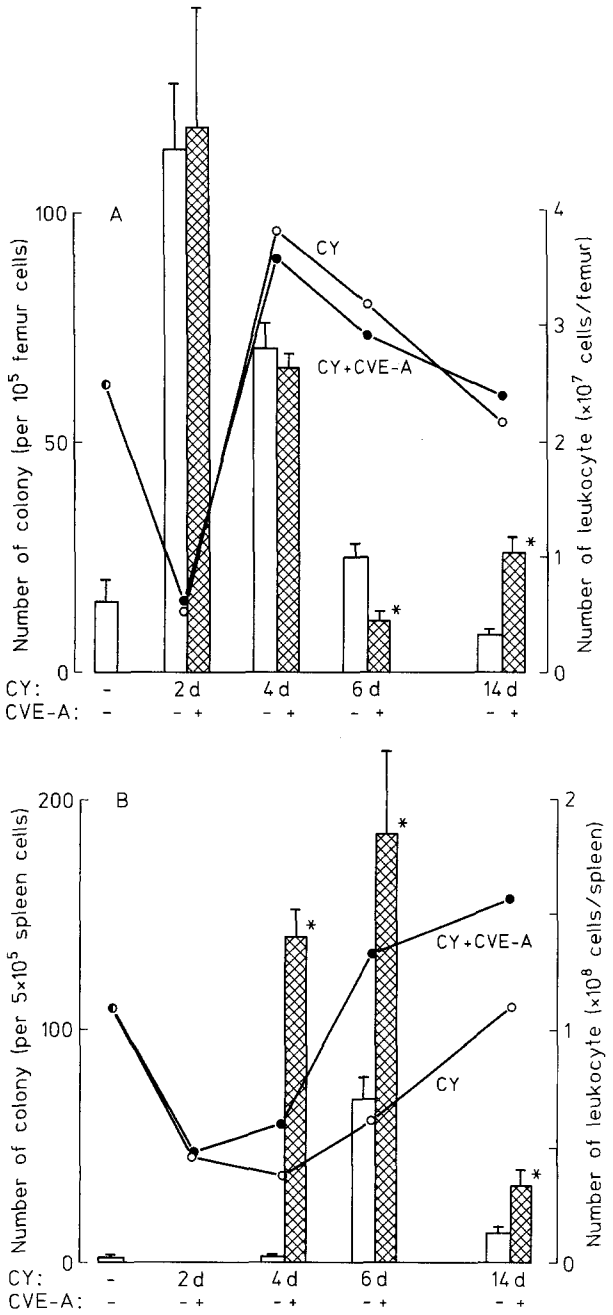

Fig. 1. The counts of granulocyte/macrophage-colony-forming units (CFU-GM) and leukocytes in the femur (**A**) and spleen (**B**) after treatment with cyclophosphamide (*CY*) were examined. Mice were injected i.p. with 150 mg/kg CY on day 0 and s.c. with 50 mg/kg CVE-A every other day from day 1. ▩, ○, CFU-GM and leukocytes in CY-treated mice, respectively; ▩, ●, CFU-GM and leukocytes in CY-and CVE-A-treated mice, respectively; *, significantly different from CY-treated group ($P < 0.05$)

number then recovered slowly to reach a normal level by day 8 or 10 (Table 1). In mice given CVE-A s.c. every other day after CY treatment, an initial decrease in the leukocyte count was also observed on day 4 after CY treatment. The number of leukocytes in the peripheral blood of CVE-A-administered mice increased more rapidly, as compared to that in CY-treated mice, reaching 1.5-fold to 2-fold higher levels than that in normal mice. Among leukocyte cells, PMN showed a quick recovery when the mice were given CVE-A s.c. There was no marked effect of CVE-A on lymphocytes. CVE-A appeared mainly to accelerate the recovery of PMN number after CY-induced suppression.

Effect of CVE-A treatment on the numbers of CFU-GM in the bone marrow and spleen of CY-treated mice

To determine the effect of CVE-A on the hematopoiesis in CY-treated mice, the numbers of CFU-GM in the bone marrow and spleen were examined in mice given CVE-A s.c. after CY treatment. There was no difference in the number of total leukocytes and the frequency of CFU-GM in the bone marrow between both groups of CY-treated mice whether or not they had been given CVE-A (Fig. 1 A) at any stage after CY treatment. On the other hand, the frequency of CFU-GM in the spleen from mice treated with CY only had not increased by day 4, whereas those from CY-treated and CVE-A-administered mice had by that time already increased to 70 times as many as those from normal mice (Fig. 1 B). It was apparent that CVE-A promoted the regeneration of granulocyte/monocyte-progenitor cells under neutropenic conditions.

Effect of CVE-A treatment on the accumulation of PMN into the inflammatory site in CY-treated mice

To test whether CVE-A administration would enhance the delivery of cells to the inflammatory site in CY-treated mice, the PMN count in the peritoneal cavity after an i.p. challenge with casein was measured. PMN increased in number 18 h after the i.p. challenge with casein in normal mice, whereas they could scarcely be detected 18 h after the challenge on day 3 in CY-treated mice given CVE-A or not (Table 2, expt. 1). The PMN count increased in the inflammatory site of CY-treated mice given CVE-A s.c. after the challenge on day 4 after CY treatment (Table 2, expt. 2), whereas that in CY-treated mice without CVE-A remained at a lower level. Such an accelerating accumulation of PMN into the inoculated peritoneal cavity of CY-treated mice given CVE-A s.c. was also detected after the inoculation of viable or heat-killed *E.coli* (Table 2, expt. 2). Thus, recovery of PMN in CVE-A-administered mice was remarkable and significantly higher than that in CY-treated control mice.

Effect of CVE-A treatment on the luminol-dependent chemiluminescent activity of the cells accumulating in the peritoneal cavity

To determine whether the restorative effect of CVE-A in CY-treated mice is exerted on the function of phagocytic cells, chemiluminescent activity was assessed. On day 4, killed *E.coli* or casein was injected into the peritoneal cavity, then peritoneal cells were harvested and pooled 6 h after injection. The cell suspension prepared from each group of mice was examined for chemiluminescent activity using fMet-Leu-Phe, opsonized *E.coli* and PMA as triggers

Table 2. Effect of CVE-A treatment on accumulation of polymorphonuclear leukocytes (PMN) in the peritoneal cavity after i.p. challenge with casein or *E.coli* in CY-treated mice[a]

Before challenge		Challenge	10^{-3} × number of leukocytes	
CY	CVE-A		PMN	Others
Expt. 1				
–	–	–	185 ± 135	5386 ± 110
–	–	Casein	5704 ± 400	2635 ± 684
1d	–	Casein	4384 ± 539	1116 ± 567
1d	1d	Casein	3680 ± 92	920 ± 133
3d	–	Casein	63 ± 29	857 ± 32
3d	1, 3d	Casein	63 ± 22	837 ± 71
5d	–	Casein	2008 ± 271	3762 ± 271
5d	1, 3, 5d	Casein	9295 ± 1193[c]	4415 ± 1343
Expt. 2				
4d	–	–	41 ± 15	2099 ± 15
4d	1, 3d	–	31 ± 15	2169 ± 15
4d	–	Casein	454 ± 38	2986 ± 65
4d	1, 3d	Casein	9229 ± 2404[c]	3648 ± 2427
4d	–	v-*E.coli*	144 ± 81	2386 ± 192
4d	1, 3d	v-*E.coli*	1281 ± 664[c]	3429 ± 702[c]
4d	–	hk-*E.coli*	87 ± 59	1173 ± 98
4d	1, 3d	hk-*E.coli*	1205 ± 406[c]	2305 ± 445[c]

[a] Mice were treated i.p. with 150 mg/kg CY on day 1 and s.c. with 50 mg/kg CVE-A ever other day from day 1. Peritoneal exudate cells were obtained after 18 h in expt. 1 or 16 h in expt. 2 following i.p. injection of 2% casein (2 ml), 5×10^5 viable (v) *E.coli* or 6×10^6 heat-killed (hk) *E.coli*

[b] Each value represents the mean ± SD for four mice

[c] Significantly different from CY-treated control group ($P < 0.05$)

of response. Resident peritoneal cells obtained from all mice without stimulation did not show chemiluminescent activity in response to any of these triggers (data not shown). Cells taken from normal mice 6 h after injection with killed bacteria responded very well to each trigger, whereas those from CY-treated mice did not (Table 3). Chemiluminescent activity from cells of mice treated with CY followed by CVE-A administration was significantly higher than that from cells of CY-treated mice. Such activity was also observed in cells obtained from all mice after injection with casein. When triggered with PMA, cells from CY- and CVE-A-treated mice showed significantly higher chemiluminescent activity than those from normal mice ($P < 0.05$). The percentage of PMN in cell suspensions of CY- and CVE-A-treated mice was about five times of that in CY-treated mice and about one-third that of the group of normal mice. It was obvious that the level of chemiluminescence in each group of cells is not a simple reflection of PMN number.

Effect of CVE-A treatment on the resistance to E.coli infection in CY-treated mice

The elimination of bacteria after *E.coli* infection is known to depend mainly on the function of PMN [6, 23]. Our data indicate that s.c. treatment with CVE-A accelerated the generation and enhanced the activity of PMN in CY-treated mice. Therefore, we examined the ability of CVE-A to enhance survival after *E.coli* infection in CY-treated mice. The mice were treated with CY on day 0, and half of the mice were administered CVE-A s.c. every other day from day 1. On various days, the mice were challenged with 1×10^7 or 2×10^7 *E.coli* and the survival was recorded. Without CVE-A administration, the mice became highly susceptible to *E.coli* infection 4 days after CY treatment. Thereafter, a spontaneous recovery of the protection against *E.coli* was observed in CY-treated mice (Table 4). In CY-treated mice given CVE-A, survival after the challenge with 1×10^7 *E.coli* was enhanced markedly, irrespective of the timing of bacterial challenge. Even against challenge with a lethal dose (2×10^7) of *E.coli* in normal mice, they showed a good protection. When the mice were challenged with *E.coli* on day 4 after CY treatment, there was a prominent difference in the survival between groups of mice given CVE-A or not. CVE-A thus has a restorative effect on the impaired resistance to *E.coli* in CY-treated mice.

To discover whether the restoration of an impaired resistance in CY-treated mice by CVE-A administration is due to an improved bacterial elimination, the growth of *E.coli* and the appearance of PMN were monitored in the

Table 3. Effect of CVE-A treatment on luminol-dependent chemiluminescence activity of inflammatory peritoneal cells in CY-treated mice[a]

Treatment			PMN in PEC (%)	10^{-3} × Max. chemiluminescence (cpm/10^6 cells)			
CY	CVE-A	Inducer		–	fMet-Leu-Phe	O-*E.coli*	PMA
–	–	*E.coli*	14.2	4.7 ± 0.2[b]	123.1 ± 22.2	54.1 ± 14.8	303.3 ± 121.2
4d	–	*E.coli*	1.9	1.5 ± 0.1	2.4 ± 0.2	1.6 ± 0.3	20.6 ± 1.4
4d	1, 3d	*E.coli*	9.4[d]	4.8 ± 0.3[d]	25.4 ± 7.5[d]	71.8 ± 14.8[d]	669.0 ± 88.3[c, d]
–	–	Casein	67.5	22.0	1,047.5	85.2	ND
4d	–	Casein	28.3	3.0	17.3	5.6	ND
4d	1, 3d	Casein	53.3	14.5	120.5	47.0	ND

[a] Mice were treated i.p. with 150 mg/kg CY on day 1, s.c. with 50 mg/kg CVE-A on days 1 and 3 and i.p. with hk-*E.coli* or casein on day 4. Peritoneal exudate cells (PEC), obtained from three or nine mice 6 h after challenge with hk-*E.coli* or casein, were pooled as one specimen, and one or three specimens in each group were examined. PMA, phorbol myristate acetate; O-*E.coli*, opsonized *E.coli*

[b] Each value represents the mean ± SD

[c] Significantly different from nontreated group ($P < 0.05$)

[d] Significantly different from CY-treated control group ($P < 0.05$)

Table 4. Effect of CVE-A treatment on survival after i.p. infection *E.coli* in CY-treated mice[a]

Treatment before challenge		Survival (%) of mice challenged with *E.coli*	
CY	CVE-A	1×10^7	2×10^7
–	–	82[b]	12
–	1, 3d	100	100[c]
2d	–	30	0
2d	1d	80[d]	0
4d	–	0	0
4d	1, 3d	100[d]	83[c, d]
6d	–	55	13
6d	1, 3, 5d	100[d]	100[c, d]

[a] Mice were administered i.p. 150 mg/kg CY on day 0 and s.c. 50 mg/kg CVE-A every other day from day 1. Challenge was done on various days after CY treatment

[b] Data show percentage of mice surviving out of 15–20 mice used after 5 days of observation

[c] Significantly different from nontreated group

[d] Significantly different from CY-treated control group

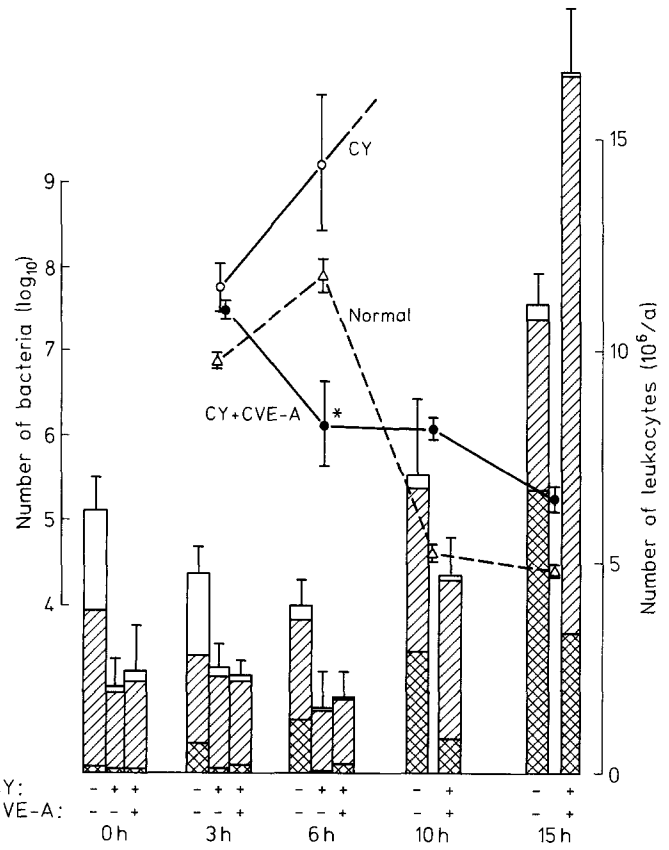

Fig. 2. The number of bacteria and differential leukocyte counts in the peritoneal cavity were determined 3, 6, 10 and 15 h after an i.p. challenge with 6.7×10^6 bacteria on day 4 after CY treatment. △, Untreated mice; ○, CY-treated mice; ●, CY- and CVE-A-treated mice; ▩, PMN; ▨, macrophages; □, lymphocytes; *, significantly different from CY-treated control group ($P < 0.05$). Each point represents the mean plus SD of data obtained from four mice

peritoneal cavity after i.p. challenge on day 4. In CY-treated mice without CVE-A, the increase of PMN was not detected and the bacterial number increased explosively just after the inoculation, resulting in the death of the mice within 24 h (Fig. 2). CVE-A administration to CY-treated mice effectively restored the ability to eliminate bacteria and this correlated with the increased number of PMN. In contrast to the progressive growth of bacteria in CY-treated mice without CVE-A, a progressive elimination of bacteria and an increase of PMN were observed from 6 h in the peritoneal cavity of CY- and CVE-A-treated mice. The growth of bacteria in the spleen and liver of each group was almost the same as that in the peritoneal cavity (data not shown).

Discussion

CY, used as a therapeutic agent for malignancy or immunosuppression, induces a decrease of hematopoietic cells and impaired resistance to gram-negative bacterial infection [2, 16]. In the present study, we have obtained evidence that CVE-A augments the resistance to *E.coli* infection in CY-induced neutropenic mice when CVE-A is administered s.c. after CY treatment. CVE-A treatment seems to enhance not only the quantity but also the quality of PMN in these mice. PMN have been found to be the principal effector cells in the resistance to *Pseudomonas* and *E.coli* infections [6, 22, 23]. A plausible mechanism for such a restoration of resistance by CVE-A would be an increased accumulation of activated PMN to infected sites on the basis of enhanced restoration in PMN generation in hematopoietic organs, and an enhanced bacteriocidal function of such phagocyte cells.

A large number of PMN are stored in the peripheral blood. However, since PMN are short-lived ($t_{1/2}$ is 2.3 h in murine blood [12]) daily generation by hematopoiesis in the bone marrow and spleen is the most important source of PMN. The remarkable increase of PMN in the peripheral blood after CVE-A injection in neutropenic mice appeared later following the increase in CFU-GM in the spleen. Furthermore, the number of PMN accumulated in the inflammatory site in neutropenic mice given CVE-A s.c. was correlated with the recovery of PMN in the peripheral blood. The PMN number in the peripheral blood transiently increased after the challenge with *E.coli* in normal mice, whereas that in neutropenic mice given CVE-A s.c. increased independently without the challenge with casein or *E.coli* (data not shown). These results suggest that the promotion of granulocyte/monocyte-progenitor cells by CVE-A contributes to the restoration of PMN number in neutropenic mice. Alternatively, it is possible that CVE-A enhances the responsiveness of newly generated PMN progenitors to colony-stimulating factor (CSF) to expand their pool. In aged mice, the responsiveness of the bone marrow cells to CSF was found to be depressed [15]. Recently restoration of the bone marrow function and the augmentation of resistance to bacterial infection have been reported using cytokines such as rG-CSF [19] or rGM-CSF [18], recombinant interleukin-1 [17, 24] and recombinant interferon [5, 9, 26]. Our preliminary studies indicate that

CVE-A stimulates the production of some CSF (unpublished data). CVE-A may contribute to the promotion of the progenitor cells through augmenting the production of a cytokine/cell growth factor, such as GM-CSF, and/or the responsiveness of the progenitor to CSF.

Luminol-dependent chemiluminescence is generated by many species of activated oxygen produced by phagocytes and is, therefore, useful to determine phagocytic activity directly related with antibacterial defense [14, 25]. In CVE-A- and CY-treated mice 6 h after challenge, the chemiluminescent activity of peritoneal exudate cells was stronger than that in normal mice, although the PMN number in these mice was lower than that in normal mice. CVE-A has been previously reported to enhance the superoxide generation of PMN in normal mice [21]. Kojima et al. reported that polysaccharide from *Chlorella ellipsoideus* enhanced the phagocytic activity of the reticuloendothelial system [10]. These results suggest that CVE-A administration enhances the function of phagocytic cells such as PMN and macrophages in neutropenic mice. Peritoneal cells from CVE-A-administered mice responded well to triggering by opsonized *E.coli* and PMA but poorly to that by fMet-Leu-Phe. Such a difference may be ascribed to the difference in the mode of action of each agent. There are distinctive differences in the mode of action among those agents; that is, fMet-Leu-Phe stimulates the chemiluminescent response via specific receptors on the cell surface [1], whereas *E.coli* opsonized by normal serum stimulate the response via binding to C3 receptors and undergoing phagocytosis [7] and PMA stimulates protein C kinase directly [4]. There is an alternative possibility that cells capable of responding to each agent differ in cell type or in maturational stage within the same lineage. Whatever are the exact mechanisms for the activation by CVE-A, it is clear that CVE-A is capable of enhancing the function of phagocytic cells.

The s.c. administration with CVE-A restored the impairment of resistance to *E.coli* infection in neutropenic mice. PMN have been known as the main effector cells in the early protection of gram-negative bacterial infection [6, 22]. In CY-treated mice, the enhanced resistance to *E.coli* infection by CVE-A injection may be attributed to the rapid restoration of PMN count, since the progressive elimination of bacteria was accompanied by the accumulation of PMN in the infection sites. However, this enhanced resistance by CVE-A cannot be explained by the quantitative increase of PMN alone. In CY- and CVE-A-treated mice, the resistance to *E.coli* infection was greater than in normal mice albeit fewer PMN accumulated in the infection site. The ability to eliminate bacteria in the peritoneal cavity 6 h after challenge was comparable to the chemiluminescent activity of peritoneal cells in each group of normal mice and CY- and CVE-A-treated mice. The activation of phagocytic cells by CVE-A may also contribute to the enhanced resistance to infection in neutropenic mice.

In other studies, in which we examined the influence of CVE-A on the therapeutic activity of CY, we found that CVE-A had no effect on the therapeutic activity of CY, whereas it relieved the side-effects of CY. This suggests that the s.c. administration of CVE-A in combination with CY may allow for the increased dosage of alkylating agent to heighten the therapeutic effect.

In conclusion, CVE-A enhanced not only the restoration of PMN generation in hematopoietic organs, but also the bacteriocidal activity of PMN in CY-treated mice. There seems to be a good possibility that CVE-A may be a useful substance for protecting patients undergoing extensive chemotherapy against opportunistic infections. Now, preclinical experiments with CVE-A need to be done on the application to humans.

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