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Augmentation of Host Defense by a Unicellular Green Alga, *Chlorella vulgaris*, to *Escherichia coli* Infection

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Protection against *Escherichia coli* inoculated intraperitoneally into mice was enhanced by intraperitoneal, intravenous, or subcutaneous administration of a water-soluble, high-molecular-weight fraction extracted from a dialyzed hot-water extract from a strain of *Chlorella vulgaris* (CVE-A). The enhancing effect was detected with doses over 2.0 mg/kg and when doses were administered 1, 4, or 7 days before the infection. The elimination of bacteria from the spleen of CVE-A-treated mice was increased, and this enhanced elimination may have been related to the acceleration of superoxide generation and chemokinesis in polymorphonuclear leucocytes by CVE-A treatment. A cyclophosphamide-induced decrease in protection against *E. coli* could be prevented by subcutaneous administration of CVE-A.

Chlorella vulgaris, an unicellular green algae, possesses a potent activity to augment host-defense mechanisms in a murine model. The growth of transplantable Meth A tumor was significantly suppressed in mice administered a water-soluble extract from *C. vulgaris* (CVE), and this effect was attributed to an augmented host defense, not to a direct action of CVE on tumor cells (14). When the Winn-type assay was used to monitor the antitumor effects of CVE on cells from CVE-treated mice, polymorphonuclear leucocytes (PMNs) showed an enhanced antitumor activity (7). The precise mechanisms for this enhanced activity of PMNs was unclear, but the contribution of active oxygen derivatives should be taken into consideration. Nathan and Cohn (9, 10) demonstrated that hydrogen peroxide, an active oxygen derivative, contributes in part to the antineoplastic activity of macrophages. Active oxygen derivatives are generated not only in macrophages but also in PMNs. It is also well-known that oxygen derivatives are generated in higher amounts by PMNs than by macrophages. Therefore, it is plausible that the generation of active oxygen derivatives is enhanced in PMNs obtained from CVE-treated mice.

PMNs play an important role in protecting the host against infection by a variety of bacteria or fungi. The bactericidal activity of PMNs is important especially in the defense against infection by *Escherichia coli* or *Pseudomonas aeruginosa* (15, 17). It has been reported that the killing of *E. coli* by human PMNs is mainly mediated by active oxygen derivatives (4, 11). These gram-negative rods have been widely isolated as causative agents for opportunistic infections in so-called immunocompromised states, including malignancy, chronic disease, and organ transplantation. In bone marrow transplantation, prophylactic granulocyte transfusions have been reported to be effective in preventing septicemia, which develops with a high incidence in marrow recipients (18). In renal transplant patients, ascorbic acid has been found to improve impaired PMN functions (16). These findings suggest that modulation of PMN functions may be beneficial for the treatment or prevention of bacterial infections.

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In the present study, the effect of high-molecular-weight extract (CVE-A) from *C. vulgaris* on the infection of mice by *E. coli* was studied. The effect on PMN functions, including superoxide generation, was also examined, following systemic administration.

MATERIALS AND METHODS

Animals. Female strain CDF1 (BALB/c × DBA/2) mice were obtained from the Shizuoka Laboratory Animal Center, Hamamatsu, Japan, and were used for experiments at 8 to 10 weeks of age. Each experimental group consisted of 5 to 12 mice. Animals were housed in sterile, plastic, filter-covered cages in groups of four or five and fed autoclaved laboratory food pellets and sterile tap water.

Microorganisms. *Escherichia coli* (E77156:06:H1) was prepared as previously described (17) and used for the experiments. Bacteria were stored at −75°C and were used after culturing in tryptic soy broth for 16 h.

Preparation of CVE-A. The CVE-A of a strain of unicellular green algae, *Chlorella vulgaris* (CVE), obtained from Chlorella Industry Co., Ltd., was dialyzed and lyophilized; then the lyophilisate (CVE-A) was dissolved in physiological saline. Chemical analysis revealed that CVE-A contained 44.3 g of proteins, 39.5 g of carbohydrates, and 15.4 g of nucleic acids in 100 g (dry weight) of whole material. Lipids could not be detected. Concentrations were adjusted to give an appropriate dry weight per milliliter of saline.

Treatment of mice with CVE-A. Mice were administered CVE-A intraperitoneally (i.p.), intravenously (i.v.), or subcutaneously (s.c.). The standard dose of CVE-A was 50 mg/kg, but doses in the range of 0.05 to 50 mg/kg were evaluated. Mice injected with saline were used as the control group.

Challenge infection and determination of protection. Mice were challenged i.p. with varying doses of viable *E. coli* at 1, 4, or 7 days after treatment with CVE-A. The protection afforded by treatment with CVE-A was determined by comparing the survival rate and the survival time in treated mice to those in control mice. Survival was observed for 5 days after infection. To observe the bacterial growth in spleen, the mice were decapitated, and the spleens were removed aseptically. Each individual organ was homoge-

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TABLE 1. Effect of route of CVE-A administration on survival of *E. coli*-infected mice[a]

Expt (no. of *E. coli* cells injected)	No. of mice surviving/total mice[b] after CVE-A injection:			
	None	i.p.	i.v.	s.c.
Expt 1				
6.8×10^6	2/6	6/6	5/6	6/6
2.7×10^7	0/6	6/6	6/6	5/6
1.1×10^8	0/6	0/6	0/6	0/6
Expt 2				
6.8×10^6	4/10	10/10	10/10	10/10
2.7×10^7	1/10	10/10	9/10	9/10
1.1×10^8	0/10	1/10	0/10	1/10

[a] CVE-A (50 mg/kg) was injected s.c. at 24 h before i.p. infection with *E. coli*.
[b] Values were recorded after 5 days of observation.

nized in phosphate-buffered saline, and dilutions of the homogenates were plated on nutrient agar for colony counts. Results were expressed as the $\log_{10}$ viable number of *E. coli* per spleen.

Assay of superoxide generation. CVE-A was injected s.c. into mice 24 h before casein administration. Normal or CVE-A-treated mice were injected i.p. with 2 ml of 2% casein, and casein-induced peritoneal-exudate cells (PEC) were collected 3.5 h later. Cells were centrifuged at 110 × *g* for 10 min and counted, and smear specimens were stained with Giemsa solution to determine the differential number of PMNs, macrophages, and lymphocytes. Usually, casein-induced PEC contained 55 to 70% PMNs, so they were used as PMN-rich PEC.

To determine superoxide generation, PEC were adjusted to 5.0×10^6 or 3.0×10^6 cells per ml with phosphate-buffered saline. In a plastic cuvette for spectrophotometry, 0.1 ml of PEC suspension, 0.02 ml of 5 mM cytochrome *c*, and 0.85 ml of reaction buffer (pH 7.4; 17 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid], 120 mM NaCl, 5 mM KCl, 2 mM $MgSO_4$, 0.5 mM $CaCl_2$, 5 mM glucose) were mixed, and the mixture was preincubated at 37°C for 7 to 10 min. After preincubation, 10 μl of 1 M NaN_3 was added to the mixture, and the cuvette was set into the double-beam spectrophotometer (Hitachi 556) with a temperature regulation at 37°C. After the addition of 2 μl of phorbol myristate acetate (1 mg/ml) to the cuvette, the mixture was mixed rapidly, and superoxide generation induced by phorbol myristate acetate was determined continuously by recording the absorption increase at 550 to 540 nm.

Assay of in vitro chemokinesis. Casein-induced PEC were collected and adjusted to 5×10^5 cells per ml with RPMI 1640 medium supplemented with 0.5% bovine serum albumin. The effect of CVE-A on the chemokinesis of PMN-rich PEC was examined by a Checkerboard assay (19) with a chemokinesis chamber (Blind Chamber; Nucleopore Corp., Pleasanton, Calif.). Various concentrations of CVE-A were added to upper or lower chambers or to both, and PEC was added to the upper chamber. The chambers were incubated at 37°C in 5% CO_2 for 90 min, the filter was removed and stained, and the number of cells migrating through a filter (pore size, 5 μm) was counted under ×400 magnification. The mean number of cells from triplicate chambers was determined.

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 considered significant.

RESULTS

Effects of route, dose, and timing of CVE-A administration on the survival after infection with *E. coli*. The effect of pretreatment of mice with single i.p., s.c. or i.v. administrations of CVE-A (50 mg/kg) was examined with regard to survival after infection with *E. coli* (Table 1). CVE-A was injected 24 h before i.p. infection with *E. coli*. The survival rate of mice at 5 days after *E. coli* infection was remarkably increased not only by pretreatment via the i.p. route, but also via the i.v. or s.c. route. There was no significant difference among these routes of administration with regard to the survival rate.

Mice injected s.c. with various doses of CVE-A were infected i.p. with lethal doses of *E. coli* 24 h later. Significant increases in survival rates were observed after a single s.c. administration of over 2.0 mg of CVE-A per kg (Table 2).

The relationship between the timing and effect of CVE-A administration was examined in mice given various doses of *E. coli*. CVE-A (50 mg/kg) was administered s.c. into mice at 1, 4, or 7 days before the challenge. When mice were challenged with low doses of *E. coli*, a significant protective effect was observed with a single administration of CVE-A irrespective of the timing of administration (Table 3). However, against challenge with a high dose of *E. coli*, mice were protected only when CVE-A was administered 24 h previously. From these results, we concluded that s.c. administration of CVE-A in a dose of 50 mg/kg given 24 h before challenge was the most effective treatment.

Effect of pretreatment of mice with CVE-A on the growth of *E. coli* in spleen. When mice were infected with 2×10^7 *E. coli* cells, all control mice died between 4.9 and 24 h after the infection, while all CVE-A (50 mg/kg)-infected mice were alive even at 48 h after infection (Table 4). After the injection of 5×10^7 *E. coli* cells, all mice in both groups died within 1

TABLE 2. Dose-dependent effect of CVE-A on survival of *E. coli*-infected mice

No. of *E. coli* cells injected	No. of mice surviving/total mice[b] in indicated expt after CVE-A injection at dose (mg/kg)[a]:							
	Expt 1				Expt 2			
	None	2.0	10.0	50.0	None	0.05	0.50	5.0
2.6×10^6	10/10	10/10	10/10	10/10				
1.1×10^7	3/10	9/10	10/10	10/10				
4.2×10^7	1/10	10/10	8/10	8/10				
5.3×10^6					10/10	9/10	10/10	10/10
2.1×10^7					3/10	3/10	4/10	9/10
8.5×10^7					0/10	0/10	10/10	1/10

[a] Indicated doses of CVE-A were injected s.c. at 24 h before i.p. infection with *E. coli*. Values were recorded after 5 days of observation.

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TABLE 3. Effect of timing of administration of CVE-A on survival of *E. coli*-infected mice[a]

No. of *E. coli* cells injected	No. of mice surviving/total mice after CVE-A injection at day before infection:			
	None	−7	−4	−1
8.4×10^5	8/10	10/10	10/10	10/10
3.3×10^6	2/10	9/10	10/10	10/10
1.3×10^7	0/10	1/10	0/10	8/10

[a] CVE-A was injected s.c. at 1, 4, or 7 day(s) before i.p. infection with *E. coli*. Values were recorded after 5 days of observation.

TABLE 5. Effect of CVE-A on the number of *E. coli* cells in spleen[a]

Times after *E. coli* infection	Mean $\log_{10}$ CFU ± SD in spleen after treatment:	
	None	+CVE-A
10 min	6.15 ± 0.10	6.35 ± 0.12
1 h	6.02 ± 0.08	6.32 ± 0.03
3 h	6.21 ± 0.08	6.59 ± 0.12
5 h	7.25 ± 0.35	6.05 ± 0.22

[a] CVE-A (50 mg/kg) was injected s.c. 24 h before infection and the spleen was harvested at 10 min or at 1, 3, or 5 h after i.p. infection with 2×10^7 *E. coli* cells.

day. All control mice died within 4.7 h after the *E. coli* infection, but the CVE-A-injected mice survived for 6 h.

Mice treated s.c. with CVE-A 24 h previously were infected i.p. with 2×10^7 *E. coli* cells, and the numbers of bacteria in the spleen were determined at various intervals. Bacterial numbers in the spleen of both groups were much the same or slightly increased for 3 h after infection (Table 5). At 5 h after infection, the number in the spleen of the CVE-A-treated group decreased to about one-third of that at 3 h. On the other hand, the number in the spleen of the control group showed a 14-fold increase.

Superoxide generation by PMN-rich PEC from CVE-A-treated mice. To determine whether CVE-A administration would have any effect on PMN activity, superoxide generation by the PMN-rich PEC was examined. Mice treated s.c. with 50 mg of CVE-A per kg 24 h previously were injected i.p. with 2 ml of 2% casein. PEC were obtained 3.5 h after casein treatment, and the superoxide generated by the PMN-rich PEC was measured. In experiment 1, PEC from one group were pooled, and measurement was repeated 5 times. In experiment 2, PEC from two mice were pooled as one specimen, and five specimens in each group were examined for superoxide generation. The total numbers and % PMNs in PEC from the CVE-A-treated group were much the same as those from the saline-injected control group (Table 6). The superoxide generated by the PEC from the CVE-A-treated group was approximately 1.6-fold higher than that from the control group. When the intracellular abilities to kill *E. coli* were compared between casein-induced PMNs from saline-treated and CVE-A-treated mice, the percent killing in 30 min was 45.0 ± 17.3% and 67.2 ± 17.2%, respectively, suggesting that CVE-A treatment enhanced the killing ability of PMNs.

Effect of CVE-A on in vitro chemokinesis. The effect of CVE-A on chemokinesis of casein-induced PEC in vitro was examined by the Checkerboard assay. Casein-induced PEC were obtained 3 h after i.p. administration of 2% casein (2 ml). When CVE-A was placed in only the lower part of the chemotaxis chamber, the migration of PMN-rich PEC was enhanced (Table 7). The Checkerboard assay revealed that the enhanced migration of PMN-rich PEC was attributable to the enhanced chemokinesis but not to chemotaxis by CVE-A.

Effect of CVE-A on the protection of mice pretreated with cyclophosphamide. When 150 mg of cyclophosphamide (CY) was injected i.p. into mice on various days before *E. coli* infection, the mice treated with CY 4 days before infection became highly susceptible to infection with *E. coli* (data not shown). Using these mice, we examined whether CVE-A could restore the CY-induced impairment of protection. Mice were treated with CY on day −4, CVE-A was administered on day −1, and *E. coli* cells were injected i.p. on day 0. Significant protection was afforded by CVE-A in the CY-treated group against infection with 4.0×10^6 or 1.6×10^7 *E. coli* cells (Table 8).

DISCUSSION

The present study demonstrated that the protection of mice against *E. coli* infection was enhanced by systemic administration of CVE-A. When CVE-A was administered i.p., i.v., or s.c. to mice at 1, 4, or 7 day(s) before the i.p. infection with *E. coli*, the survival rate was significantly increased. The proliferation of *E. coli* in the spleen was significantly suppressed by CVE-A pretreatment. The activities of PMN-rich PEC, as examined by superoxide generation and chemokinesis, were also enhanced by in vivo or in vitro treatment with CVE-A. These results suggest that, in CVE-A-treated mice, PMNs may migrate more effectively to the infected site and may engulf infecting bacteria and kill them more rapidly by enhanced superoxide generation, thus resulting in enhanced protection in vivo.

TABLE 4. Effect of CVE-A on the survival duration after i.p. infection of *E. coli*[a]

No. of *E. coli* cells injected	No. of mice surviving/total mice (survival duration) in treatment group:	
	CVE-A-treated mice	Control mice
8×10^6	5/5	3/5
2×10^7	5/5 (>48)[b]	0/5 (4.9–24.0 [5.0 ± 7.4][c])[b]
5×10^7	0/5 (6–24)[b]	0/5 (3.3–4.7 [4.0 ± 0.5][c])[b]

[a] CVE-A (50 mg/kg) was injected s.c. at 24 h before infection. Values were recorded after 48 h of observation.

[b] Hours of survival, after bacterial infection, of shortest-lived mouse and longest-lived mouse.

[c] Values in brackets are mean hours of survival plus or minus standard deviations.

TABLE 6. Effect of CVE-A on activity of superoxide generation of 2% casein-induced peritoneal-exudate cells[a]

Expt and CVE-A treatment	Mean % PMNs ± SD in PEC	No. (10^6) of PEC/ml in the assay	Mean superoxide generation ± SD (nmol of O_2^- per min per 10^5 phagocytes)
Expt 1			
−	70	5.0	0.406 ± 0.024
+	70	5.0	0.642 ± 0.030
Expt 2			
−	55.3 ± 0.6	3.0	0.548 ± 0.217
+	59.7 ± 4.7	3.0	0.927 ± 0.131

[a] CVE-A (50 mg/kg) was injected s.c. at 24 h before i.p. injection of 2 ml of 2% casein. PEC was harvested 3.5 h after casein injection.

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TABLE 7. Effect of CVE-A on the in vitro chemokinesis of casein-induced PEC[a]

CVE-A concn in lower chamber	No. of PMNs[b] migrating at upper-chamber CVE-A concn (μg/ml):		
	0	10	100
0	1.7	14.5	20.0
10	8.7	18.7	9.3
100	22.0	18.0	26.0

[a] CVE-A was added into the upper or lower chamber or into both as indicated.

[b] Values are expressed as the number of PMNs migrating during 90 min of incubation per one chamber.

The enhanced protection was evident shortly after and also 4 or 7 days after a single administration of CVE-A (Table 3, 4). Such long-lasting protection cannot be explained by the activation of PMNs, in the conventional sense, since PMNs have a very short life-span. However, several mechanisms have to be considered: (i) CVE-A and its metabolites may exist at the infected site or systemically and exert effects on PMN activation at the infected site or on proliferation in the bone marrow; (ii) PMNs may live longer after being activated by CVE-A. In a recent study, we found that CVE-induced PMNs were viable for over 3 days in the peritoneal cavity (7). Morikawa et al. divided various inducers for peritoneal-exudate cells into five groups with respect to PMN-inducing ability (8). They showed that PMNs induced with TAK (β-1-3-glucan from *Alcaligenes faecalis*) in large numbers existed for over 2 days in the peritoneal cavity. CVE-A may exert the same effect on PMNs.

Death occurred at 4 or 5 h when normal mice were inoculated with over 2×10^7 bacteria (Table 4). Endotoxin released from *E. coli* may explain the early death. If this is the case, controlling the susceptibility to endotoxin would be beneficial, in addition to controlling the bacterial number. CVE-A treatment was also effective against this type of early death. Prolonged survival or an increase in the survival rate were observed in CVE-A-injected mice (Table 4). The mechanism of CVE-A action in this type of early death cannot be explained only by the activation of PMNs, because the number of PMNs in the peritoneal cavity started to increase 3 to 5 h after infection (data not shown).

In the spleen of normal mice, a 14-fold increase was observed in the number of viable *E. coli* cells at 5 h after infection. On the other hand, the numbers of bacteria in the spleens of CVE-A-treated mice decreased to about one-third of that at 3 h. When we examined the numbers of peritoneal leucocytes of mice in control and CVE-A-treated groups, a

TABLE 8. Effect of CVE-A on the survival of *E. coli*-infected mice treated with CY[a]

No. of *E. coli* cells injected and	CY treatment	No. of mice surviving/total mice after CVE-A injection (mg/kg):			
		None	0.5	5.0	50.0
4.0×10^6	−	9/10		10/10	
	+	4/10	8/10	9/10	8/10
1.6×10^7	−	4/10		10/10	
	+	0/10	0/10	2/10	7/10
6.4×10^7	−	0/10		0/10	
	+	0/10	0/10	0/10	0/10

[a] CY (150 mg/kg) was injected i.p. on day 4, CVE-A was injected s.c. on day −1, and *E. coli* was infected i.p. on day 0. Values were recorded after 5 days of observation.

twofold increase was observed between 3 h and 5 h after *E. coli* infection in both groups, without a significant difference (data not shown). This finding also suggests that CVE-A qualitatively activates the PMNs.

CY is often used as a therapeutic agent for malignancy but is known to cause severe granulocytopenia, one of most serious side effects. Harvath et al. (5) showed that combined therapy with immunization and granulocyte transfusions was effective in preventing bacteremia during periods of leukopenia, determined in transiently neutropenic dogs given CY. The host defense to *E. coli* infection was abrogated by CY treatment, but this type of impaired host defense could be restored to some extent by systemic administration of CVE-A (Table 8). This indicates that CVE-A is effective even in granulocytopenic states.

Opportunistic infection caused by gram-negative rods, including *E. coli*, is the most serious complication in immunosuppressed patients; thus, protection in the early stages of infection is most important. Renal-transplant recipients are put on immunosuppressants after surgery, and this in turn gives rise to their susceptibility to infection. Neutrophils are decreased in these cases. In renal-transplant patients, leucocytes show reduced superoxide production and chemiluminescence response (13). Lowered PMN functions were observed in many types of patients. Therapeutic or experimental trials to activate PMNs have been attempted (2, 3, 6, 12, 16). Vitamin E has been used in premature newborn babies to accelerate the normalization of phagocytic functions in the neonatal period (2). Cationic polyamino acids promote the phagocytosis and killing of some bacterial strains (12). Most of these trials were not so successful, and a long-term administration was required.

By activating PMNs, CVE-A seems to be of significant value for the prevention of opportunistic infections resulting from chronic diseases or organ transplantation. It is also a useful tool for analyzing the precise mechanisms of protection against infection with bacteria.

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