Effect of Cysteine Ethylester Hydrochloride (Cystanin®) on Host Defense Mechanisms (IV): Potentiating Effects on the Function of Peritoneal or Spleen Macrophages

Masao HISADOME, Yuka KIMURA, Kiyoteru IKEGAMI and Michio TERASAWA
Research Laboratories, Yoshitomi Pharmaceutical Industries, Ltd., Yoshitomi-cho, Chikujo-gun, Fukuoka 871, Japan
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Abstract—L-Cysteine ethylester hydrochloride (ethylcysteine; 30 mg/kg, p.o.) increased the number of la-positive cells (antigen presenting cells) in spleen adherent cells (SAC) and that of Lyt 1.2-positive cells (helper T cells), but not that of Lyt 2.2-positive cells (suppressor T cells) of C57BL/6 mice immunized with sheep red blood cells. The production of hemolytic plaque forming cells (HPFC) in spleens of syngeneic recipient mice was enhanced by the transfer of SAC or spleen lymphocytes of the donor mice pretreated with ethylcysteine. This drug augmented phagocytosis of yeast particles by peritoneal macrophages of ICR mice at concentrations of 1–100 µM. In ex vivo experiments, this drug (30 mg/kg, p.o.) augmented the phagocytosis of yeast particles by mouse macrophages and showed a tendency to increase the macrophage number in the peritoneal cavity. Ethylcysteine (30 mg/kg, p.o.) significantly accelerated the decrease of viable E. coli number in the liver of normal mice 2 and 48 hr after challenge. Furthermore, this drug at the same dose restored the suppression of the decrease of E. coli number in the blood and liver of mice treated with cyclophosphamide (200 mg/kg, i.p.). These results suggest that ethylcysteine augments the functions of macrophages in vitro and ex vivo, and these enhancing effects may lead to the enhancement of host resistance to infections in compromised hosts.

It has been shown that L-cysteine ethylester hydrochloride (Cystanin®, ethylcysteine) potentiated the phagocytic activity and nitroblue tetrazolium (NBT) reducing activity of rat peritoneal leukocytes and augmented the antibody production in mice (1, 2). Recently, SA 96, D-penicillamine and levamisole which have sulphydryl groups or a sulphur-containing ring have been reported to activate the function of normal peritoneal cells that contain resident macrophages in vivo (3). These immunomodulators have been developed for the treatment of autoimmune diseases through the activation and suppression of the immune functions of T cells, B cells and macrophages (4–9). The effects of ethylcysteine on macrophage function and the mode of action of this drug on the immunopotentiation in mice remain unclear. In addition, several reports suggest that many patients with autoimmune diseases have complications due to infectious diseases (10, 11). In this study, we investigated the effect of ethylcysteine on the phagocytic activity of mouse peritoneal macrophages, on subpopulations of macrophages and T cells of spleens of mice immunized with sheep red blood cells (SRBC) and on the clearance of Escherichia coli (E. coli) in the blood and liver of mice.

Materials and Methods

Animals: Female C57BL/6 mice and male ICR mice, 4 weeks of age, were purchased from Charles River Laboratory (Japan). Mice were housed at 24±2°C and 50–60% hu-
midity. Mice of 5–6 weeks of age were used for all experiments.

**Drugs:** L-Cysteine ethylester hydrochloride (Cystamin®, ethylcysteine; Yoshitomi Pharmaceutical Industries, Japan) and cyclophosphamide (Endoxan®; Shionogi Pharmaceutical Industries, Japan) were used as test drugs.

In the in vivo and ex vivo experiments, ethylcysteine and cyclophosphamide were dissolved in 0.5% (w/v) methylcellulose solution and 0.9% (w/v) NaCl solution, respectively. In in vitro experiments, ethylcysteine was dissolved in Hanks' balanced salt solution without phenol red (HBSS) at pH 7.4.

**Antigen and immunization:** SRBC were obtained from Nippon Bio-Test Laboratories Inc. (Tokyo, Japan). SRBC were centrifuged at 700×g for 5 min and washed three times with phosphate-buffered saline at pH 7.2. These cells were resuspended in phosphate-buffered saline at a concentration of 5×10⁹/ml.

C57BL/6 mice were immunized i.p. with 5×10⁸ SRBC (0.1 ml/mouse), and spleen cells were removed from the mice 4 days after immunization.

**Preparation of spleen cells or spleen adherent cells (SAC):** Spleen cells and SAC obtained from immunized mice with SRBC were prepared in Eagle’s MEM as previously described (1). SAC were prepared in Eagle’s MEM by the method described by Nomoto (12). Viable cells were counted by the trypan blue exclusion test and suspended at appropriate concentrations.

**Treatment of spleen cells or SAC with antibody and complement:** The treatment of spleen cells with anti-Lyt 1.2 antibody (Cedarlane) or anti Lyt 2.2 antibody (Cedarlane) plus rabbit complement (Cedarlane) were performed as previously described (13, 14). The effect of treatment of SAC with anti-la antibody (Cedarlane) plus complement was assessed as previously described (15).

Cell viability of spleen cells or SAC treated with or without antibody plus complement was assessed according to the 0.1% trypan blue exclusion test.

**Cell transfer:** Donor C57BL/6 mice were primed with 5×10⁸ SRBC on day 0 and treated with ethylcysteine on days 0 and 1. The spleen cells (5×10⁸ cells) or SAC (5×10⁶ cells) obtained on day 4 were transferred i.v. into normal recipient mice, and the transferred mice were immediately immunized with 5×10⁸ SRBC.

Spleen cells were removed from the recipient mice 4 days after immunization, and the direct hemolytic plaque forming cells (HPFC) assay was carried out.

**Direct HPFC assay:** The splenic direct HPFC assay in mice was performed according to the method of Hashimoto and Ohshima (16).

**Phagocytosis of yeast particles by macrophages:** ICR mice were injected intraperitoneally with 2 ml of casein suspension. Peritoneal exudates were collected 3 days after casein injection and were centrifuged at 150×g for 10 min. The peritoneal exudate cells obtained were washed three times with HBSS.

About 70% of the exudate cells was found to be macrophages. In in vitro experiments, 100 μl of the cell suspension (5×10⁶ cells/ml) was preincubated with 20 μl of ethylcysteine at 37°C for 10 min, and thereafter, these mixtures were incubated with 50 μl of mouse serum and 50 μl of yeast suspension (Sigma, Type II; 2.5×10⁸/ml) at 37°C for 20 min. About 200 cells of the macrophages were counted in a hemocytometer according to the method of 1% basic fuchsin-dyeing. The results are expressed as a ratio (%) of a number of the macrophages which ingested more than one yeast particle to total cell number.

In ex vivo experiments, ethylcysteine was administered orally on days 0, 1 and 2 after casein injection (on day 0). Peritoneal exudates collected on day 3 were prepared according to the above-described methods.

**Viable E. coli number in blood and liver of infected mice:** ICR mice were treated with ethylcysteine (30 mg/kg, p.o.) immediately after i.v. challenge with E. coli 0–111 12×10⁷ colony forming units (CFU).

In another experiment, cyclophosphamide was intraperitoneally administered to mice, and ethylcysteine (30 mg/kg, p.o.) was administered on days –3, –2, –1; on days –1, 0, 1; or on days 1, 2, 3. Then the mice were
challenged with E. coli 0-111 on day 4.

Blood and liver of normal mice or cyclophosphamide-treated mice were obtained at appropriate times after the challenge. Each sample was serially diluted 10-fold with saline. A suspension of 0.2 ml of each sample (triplicate) was plated onto a MacConkey agar (Nissui Seiyaku, Co., Japan) plate and spread with a Conradi rod. The number of CFU was counted 18 hr after incubation at 37°C.

Statistics: Results were expressed as a mean value or mean±S.D. The statistical analysis was performed using a one way layout.

Results

1. Effect on HPFC production to SRBC: The HPFC number of spleens obtained from the immunized C57BL/6 mice treated without or with ethylcysteine (30 mg/kg, p.o.) was 9.7±1.2 or 16.2±1.7 (×10⁴/spleen, N=6), respectively. The cell viability of SAC of control mice was decreased by the treatment with anti-Ia antibody and complement. Ethylcysteine (30 mg/kg, p.o.) potentiated the decrease of the cell viability by the same treatment. The cell viability of spleen lymphocytes of mice treated with this drug was markedly decreased by the treatment with anti-Lyt 1.2 antibody, but not by the treatment with anti-Lyt 2.2 antibody (Fig. 1). SAC or spleen lymphocytes of donor mice treated with ethylcysteine were transferred to syngeneic recipient mice, and the immunization was performed. The HPFC number of spleens of recipient mice was further increased by the transfer of SAC or spleen lymphocytes of mice pretreated with ethylcysteine (30 mg/kg, p.o.) (Table 1). The intensity of enhancing effect of ethylcysteine on the HPFC production of recipient mice was almost to the same degree as that of donor mice.

2. Effect on phagocytosis of yeast particles by mouse peritoneal macrophages: Ethylcysteine potentiated the phagocytosis of yeast particles by mouse peritoneal macrophages at concentrations of 1–100 µM (Fig. 2). About 70% of the peritoneal exudate cells 3 days after casein injection was found to be macrophages in the mice treated with or without this drug, and most of the other contaminating cells were lymphocytes. In ex vivo experiments, this drug (3–30 mg/kg, p.o.) augmented phagocytosis of yeast particles in a dose-dependent manner. Ethyl-

| Drug       | Dose (mg/kg) | Treatment with SAC |
|------------|--------------|--------------------|
| Control    | 0            | No treatment       |
| Ethylcysteine | 30           | Anti-Ia + C        |

| Drug       | Dose (mg/kg) | Treatment with lymphocytes |
|------------|--------------|---------------------------|
| Control    | 0            | No treatment              |
| Ethylcysteine | 30           | Anti-Lyt 1.2 + C          |

Fig. 1. Subpopulations of spleen cells in C57BL/6 mice pretreated with ethylcysteine. Spleen adherent cells (SAC) or spleen lymphocytes were obtained from mice immunized with SRBC. These cells were treated with anti-Ia antibody, anti-Lyt 1.2 antibody or anti-Lyt 2.2 antibody and complement. Cell viability was assessed according to the trypan blue exclusion method. Results are given as the mean values (N=2).
cysteine (30 mg/kg, p.o.) showed a tendency to increase the number of macrophages in the peritoneal cavity (Table 2).

Table 1. Effect of ethylcysteine on immune response to SRBC in C57BL/6 mice

| Drug       | Dose (mg/kg, p.o.) | Spleen cells transferred | HPFC |
|------------|--------------------|--------------------------|------|
| Control    | 0                  | None                     | 20.4±4.0 | 25.9±6.8 |
| Control    | 0                  | Macrophages              | 30.3±4.1** | 35.2±5.3** |
| Ethylcysteine | 30              | Macrophages              | 53.3±10.3†† | 49.4±8.5†† |
| Control    | 0                  | Lymphocytes              | 36.4±4.5** | 45.9±3.0** |
| Ethylcysteine | 30              | Lymphocytes              | 44.7±7.6†† | 59.6±15.1†† |

Donor mice were intraperitoneally immunized with 5×10⁶ SRBC on day 0. Ethylcysteine was administered orally on days 0 and 1. Spleen adherent cells (5×10⁶ cells) or spleen lymphocytes (5×10⁶ cells) obtained on day 4 were transferred to recipient mice before immunization with 5×10⁶ SRBC. HPFC were assessed on day 4. Results are given as the mean±S.D. of 6 animals. **P<0.01: Significantly different from the control group. †P<0.05. ††P<0.01: Significantly different from the control group treated with cell-transfer.

Fig. 2. Effect of ethylcysteine on phagocytosis of yeast by mouse peritoneal macrophages in vitro. ICR mice were injected intraperitoneally with 2 ml of 5% casein suspension. Exudates were collected 3 days after casein injection. After the cell suspension (5×10⁶ cells/ml; 100 μl) was preincubated with ethylcysteine (20 μl) at 37°C for 10 min, these mixtures were incubated with mouse serum (50 μl) and yeast suspension (2.5×10⁶/ml, 50 μl) at 37°C for 20 min. Results are given as the mean±S.D. (N=3). **P<0.01: Significantly different from the control.

cysteine (30 mg/kg, p.o.) showed a tendency to increase the number of macrophages in the peritoneal cavity (Table 2).

3. Effect on viable E. coli number in blood and liver of infected mice: The viable E. coli number in blood and liver of normal mice infected with E. coli was decreased up to 48 hr in a time-dependent manner after challenge. Ethylcysteine (30 mg/kg, p.o.) significantly accelerated the decrease of viable E. coli number in the liver of normal mice 2 and 48 hr after E. coli challenge, but not that in the blood of infected mice (Table 3). The white blood cell number in the blood of mice treated with cyclophosphamide (200 mg/kg, i.p.) and that of normal mice was 29±3 and 89±10 (×10⁹/mm³, N=10), respectively. The viable E. coli number in the blood and liver of cyclophosphamide-treated mice infected with E. coli was decreased more slowly than that of normal mice when assayed 2 hr after challenge. Ethylcysteine (30 mg/kg, p.o.) did not restore the decrease of white blood cell number in cyclophosphamide-treated mice (data not shown), but accelerated the decrease of viable E. coli number in the blood and liver of mice treated with cyclophosphamide (200 mg/kg, i.p.; on day 0) during the administration-schedule on days -3, -2, -1 and on days 1, 2, 3. In addition, the de-
Table 2. Effect of ethylcysteine on phagocytosis of yeast by mouse peritoneal macrophages ex vivo

| Drug      | Dose (mg/kg) p.o. | Macrophage number (×10⁶) | Phagocytosis of yeast (%) |
|-----------|-------------------|--------------------------|--------------------------|
| Control   | 0                 | 1.4±0.2                  | 5.8±2.1 (100%)           |
| Ethylcysteine | 3             | 1.3±0.3                  | 16.3±3.1 (281)**         |
| Ethylcysteine | 30            | 3.1±1.7                  | 21.7±0.4 (374)**         |

ICR mice were intraperitoneally injected with 2 ml of 5% casein suspension. Ethylcysteine was administered orally on days 0, 1 and 2 after casein injection. Exudates were collected 3 days after casein injection. The cell suspension (5×10⁶ cells/ml, 100 μl) was incubated with mouse serum (50 μl) and yeast suspension (2.5×10⁹/ml, 50 μl) at 37°C for 20 min. Results are given as the mean±S.D. (N=4). Figures in parentheses show percent of the control. **P<0.01: Significantly different from the control group.

Table 3. Effect of ethylcysteine on viable E. coli number in blood and liver of normal mice infected with E. coli

| Time (hr) after E. coli challenge | Ethylcysteine (mg/kg) p.o. | Number of viable E. coli in blood (×10⁴ CFU/ml) | Number of viable E. coli in liver (×10⁴ CFU) |
|----------------------------------|-----------------------------|--------------------------------------------------|---------------------------------------------|
| 0.5                             | 0                           | 1208±888                                         | 781±238                                     |
| 2                               | 0                           | 98±28                                            | 315±136                                     |
| 2                               | 30                          | 80±42                                            | 133±132*                                    |
| 48                              | 0                           | 0                                                | 1.7±0.7                                     |
| 48                              | 30                          | 0                                                | 0.4±0.2*                                    |

Mice were treated p.o. with ethylcysteine immediately after the i.v. challenge with E. coli 0–111 (2×10⁹ CFU). The viable counts of E. coli in blood and liver were determined by plating on a MacConkey agar plate. Results are given as the mean±S.D. of 5 animals. *P<0.05: Significantly different from the control group.

Table 4. Effect of ethylcysteine on viable E. coli number in blood and liver of cyclophosphamide-treated mice infected with E. coli

| Drug               | Time schedule (on day) | No. of mice | Number of viable E. coli in blood (×10⁴ CFU/ml) | Number of viable E. coli in liver (×10⁴ CFU) |
|--------------------|-------------------------|-------------|--------------------------------------------------|---------------------------------------------|
| Cyclophosphamide   | 0                       | 10          | 360±184 (100%)                                   | 386±96 (100%)                               |
| +Ethylcysteine     | –3, –2, –1              | 5           | 102±131 (28)**                                   | 179±31 (46)**                               |
| +Ethylcysteine     | –1, 0, 1                | 5           | 216±119 (50)**                                   | 185±39 (48)**                               |
| +Ethylcysteine     | 1, 2, 3                 | 5           | 114±129 (32)**                                   | 146±41 (38)**                               |
| Control            | 0                       | 5           | 128±162 (36)**                                   | 148±28 (38)**                               |
| Control (0.5 hr)   | 0                       | 5           | 1166±510 (324)**                                 | 1086±182 (281)**                            |

Mice were pretreated i.p. with cyclophosphamide (200 mg/kg) on day 0 and treated p.o. with ethylcysteine (30 mg/kg). They were challenged i.v. with E. coli 0–111 (2×10⁹ CFU) on day 4. Blood and liver of infected mice were obtained 2 hr after the challenge. The viable counts of E. coli in blood and liver were determined by plating on a MacConkey agar plate. Results are given as the mean±S.D. **P<0.01: Significantly different from cyclophosphamide-treatment.
crease of viable *E. coli* number in liver of mice was accelerated by the administration of this drug on days -1, 0 and 1 (Table 4).

**Discussion**

It has been reported that levamisole, SA 96 and D-penicillamine enhance the immune response to SRBC through macrophages, helper T cells and/or B cell functions, and they suppress the immune response through the induction and activation of suppressor T cells of mice (4–9). A preceding paper (13) showed that ethylcysteine restored the suppression of the HPFC production to SRBC in immunosuppressed mice treated with cyclophosphamide or carrageenan, and it seems likely that this drug may restore the antibody production by a similar mechanism to levamisole and D-penicillamine. In addition, the HPFC production in response to dinitrophenylated-Ficoll or trinitrophenylated (TNP)-Ficoll (T cell-independent antigen) was augmented by SA 96 and levamisole, suggesting that these agents may act directly on B cells. The fact that ethylcysteine did not influence the immune response to a T cell-independent antigen such as TNP-polyvinylpyrrolidone (1) suggests that the potentiating effects of this drug on the immune response are not directly attributed to the activation of B cells. Thus, thiol compounds may be augmenting the antibody production by a different mechanism.

In this study, the present data indicated that ethylcysteine could potentiate the HPFC production in lower responder mice (C57BL/6 mice) (1), and that these potentiating effects were attributed to the induction of LA-positive macrophages (antigen presenting cells) and Lyt 1,2-positive cells (helper T cells). Moreover, the results of cell-transfer suggest that the effects of this drug were attributed to the activation of macrophages and T cells. These findings suggest that ethylcysteine regulates the immune response through the induction and activation of macrophages and helper T cells in lower responder mice as well as in immunosuppressed mice.

Macrophages also play an important role not only in the immune response but also in the non-immune response which includes the enhancement of host resistance to infections. An earlier paper (2) showed that ethylcysteine could enhance the phagocytic and NBT reducing activities of peritoneal leukocytes in mice and guinea pigs. Since macrophages have the same activities as leukocytes, ethylcysteine may be expected to potentiate these activities of macrophages. In in vitro and ex vivo studies, this drug potentiated the phagocytic activities of peritoneal macrophages in mice. These findings indicate that ethylcysteine potentiates the functions of macrophages. The mode of action of this drug still remains unclear, but the activation of the oxidase system generating the superoxide radicals and the stimulation of macrophage secretory processes by this drug could be expected. It may be necessary to examine the effect of this drug on the above reaction system.

Furthermore, the findings that this drug stimulates the decrease of viable *E. coli* number in the blood and liver of normal mice and immunosuppressed mice pretreated with cyclophosphamide suggest that this drug may enhance the host resistance to infections in immunocompromised hosts. The decrease of bacteria in blood and liver was suggested to be due to the activation of the functions of blood cells, which contain leukocytes and monocytes, Kupffer cells and pulmonary macrophages. It may be also necessary to examine the effects of this drug on the functions of the above cells and the protective effects in mice infected with other bacteria such as *Pseudomonas aeruginosa* or viruses.

It has been reported that levamisole activates the phagocytic cells (17, 18) and reduces the severity of chronic infection (19–21). In spite of the unknown mode of its action, ethylcysteine is a very interesting drug exhibiting prophylactic and therapeutic effects on the clearance of micro-organisms. This drug may be suitable for the treatment of opportunistic infections in immunocompromised hosts.

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