Immunopharmacologic Profiles of a Thiol Protease Inhibitor, 
L-Trans-Dicyclohexyl Epoxysuccinate

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Abstract—Ep-1, L-trans-Dicyclohexyl epoxysuccinate, is a synthetic and specific 
inhibitor of thiol proteases. The effects of this inhibitor on some immunological 
parameters were examined in normal and immunity-impaired mice and rats. In 
the cultures of splenocytes obtained from the mice treated with Ep-1, it enhanced 
the lymphocyte blast transformation induced by both suboptimal and optimal 
concentrations of concanavalin A (Con A) and Lens culinaris (LC). The in vivo 
administration of Ep-1 caused a depression of the plaque forming cells (PFC) for 
sheep red blood cell (SRBC) and enhanced the delayed-type hypersensitivity 
(DTH) for bovine serum albumin (BSA) as well as mixed lymphocytes cultures 
(MLC). Furthermore, Ep-1 demonstrated a preventive effect on adjuvant arthritic 
rats. The relevance of immunological regulation and the mode of action of Ep-1 
as a thiol protease inhibitor are discussed in these findings.

Recently, the physiological role of thiol 
proteases has been recognized as one of the 
important factors in the regulation of 
biological functions (1, 2), and their 
pathogenic role has also become apparent 
in Arthus- and heat-inflammation (3, 4) and 
in muscular dystrophy (5). Especially in 
immunology, which is a subject of absorbing 
interest to us, there are several reports which 
show that chymopapain C completely inhibits 
the primary immune response and alters the 
secondary response to antibody formation in 
C57BL/6 mice (6, 7) and that papain is 
able to enhance the quantity of the in vitro 
plaque forming cell (PFC) (8).

However, as far as the immune response is 
concerned, little is known about thiol 
protease inhibitors except for those reported 
by Järvinen et al. (9) and by Udaka and 
Hayashi (3), independently, appearing in the 
superficial layers of newborn rat epidermis 
and in the healing phase of the Arthus 
reaction. These workers felt that some 
inhibitors might act as a defense mechanism 
against foreign substances.

Ep-1 is one of the synthetic analogues 
that was derived for practical use from E-64 
which is a specific and potent inhibitor of 
thiol proteases originally isolated from 
Aspergillus Japonicus (10, 11) and inhibits 
not only plant thiol proteases (12, 13) but 
also mammalian ones such as calcium-
activated neutral protease (14) and lysosomal 
cathepsins B, H and L of rat liver in vitro as 
well as in vivo (15–17).

In this paper, we have described the in vivo 
effects of Ep-1 on lymphocyte transformation 
and mixed lymphocyte culture (MLC), using 
the splenocytes of mice. Moreover, the in 
vivo effect was studied in mice by determining 
delayed-type hypersensitivity and PFC 
response and in rats by observation of 
adjuvant arthritus.

Materials and Methods

Animals: Both male and female C3H/He, 
C57BL/6 and CDF1 mice at the age of 8–12 
weeks and female Wistar rats ranging in age 
from 8–12 weeks were obtained from Japan 
Charles River Co. for all the experiment. Mice
were maintained under laminar air-flow isolation.

**Drugs:** Ep-1, L-trans-Dicyclohexyl epoxy-succinate, the thiol protease inhibitor, was synthesized in our laboratory and suspended in a 5% gum arabic solution for intraperitoneal injections or in a saline solution containing 0.5% CMC and 0.1% Tween 80 for oral administration individually at the indicated doses and times.

**Preparation of cell suspension:** Mice were sacrificed by exsanguination from the carotid arteries. Their spleens were aseptically removed, shredded with scissors, and strained through a 60-gauge stainless-steel sieve in Eagle’s minimal essential medium (MEM, Nissui Seiyaku Co., Ltd., Tokyo) adjusted pH to 7.2 with sodium bicarbonate. Further disruption was achieved by gentle aspiration with a Pasteur pipette, followed by filtration through siliconized cotton wool (Fuji Systems Co., Ltd., Tokyo). The single cells thus obtained were washed twice with MEM by low-speed centrifugation for 7 min at 180 x g, resuspended, and counted with a hemocytometer (Kayagaki Irika Kogyo Co., Ltd., Tokyo). The viability of nucleated cells was estimated by the trypan-blue exclusion test.

**Lymphocyte blast transformation test:** 2 x 10^5 spleen cells of C3H/He mice were cultured with three mitogens, concanavalin A (Con A), *Lensi culinaris* (LC) and lipopolysaccharide (LPS) at suboptimal and optimal concentrations in RPMI-1640 medium (GIBCO) with an additional 5% heat-inactivated fetal calf serum (FCS from GIBCO) and 100 μg/ml kanamycin in a microtest plate II (Falcon Plastics) at 37°C in a humidified atmosphere of 5% CO₂-air atmosphere. Fifteen hours before the termination of the culture, 0.5 μCi of methyl-³H-thymidine (³H-TdR, specific activity of 5 mCi/mM) was added to each well. Stimulation index (SI) was calculated as follows: Ratio (mean counts/min in MLC—mean counts/min in USC) in treated culture to (mean counts/min in MLC—mean counts/min in USC) in non-treated culture. The ³H-TdR uptakes were determined by the method described above in the lymphocyte blast transformation test.

**Delayed-type hypersensitivity (DTH):** The effect of Ep-1 on DTH for bovine serum albumin (BSA: NBCo) was tested by the methods described by Katura et al. (20, 21). Freund’s complete-type adjuvant containing a high concentration of mycobacteria was prepared by the addition of 10 mg of the heat-killed Aoyama B strain to 1 ml of Freund’s incomplete adjuvant (Difco Laboratories). CDF₁ mice were injected subcu-
taneously in the back with 0.2 ml of the emulsion which contained 1 mg of mycobacteria and 1 mg of BSA. Fourteen days after immunization, DTH response was elicited by an injection of 0.02 ml of alum-precipitated BSA (AP-BSA) into the left hind foot pad.

AP-BSA was prepared by an addition of 1 ml of 10% potassium alum into a 3.5 ml of BSA solution (containing 20 mg BSA), followed by neutralization pH 6.3 with 10% sodium carbonate. The dorsoventral thickness of the hind footpads was measured with dial-gauged calipers (Ozaki Co., Ltd.) immediately before and at different time intervals after the antigen injection (at 3. 6, 9.5, 24, 28.5 and 28 hr), and the increment (swelling) in thickness during the time before and after the injection of the antigen was calculated. Both hind footpads in one of the animals were subjected to the test, and the mean of their swelling was regarded as the value of the animal.

Plaque forming cell (PFC) response: Mishell-Dutton’s method (22) was used with a slight modification. The spleen cells obtained from the mice treated by Ep-1 were cultured in siliconized Widal’s test tubes at 37°C in a humidified air atmosphere of 5% CO₂. Each tube contained 2×10⁶ of spleen cells and 1×10⁶ of sheep red blood cells (SRBC) in 0.4 ml of the culture medium. SRBC was commercially obtained in Alsever's solution and washed three times with MEM for use as immunogens in spleen cell cultures. The medium for the culture was RPMI-1640 supplemented with 1 mM L-glutamine, 5% FCS, 5×10⁻⁶ M 2-mercaptoethanol, 1 mM sodium pyruvate, and kanamycin. After a four-day culture, the number of direct PFC (IgM) was determined by Cunningham’s technique (23).

Adjuvant arthritis (AA): Female Wistar rats, 8–14 weeks old, were inoculated intracutaneously in the tail with 0.6 mg of heat-killed Mycobacterium tuberculosis Aoyama B strain suspended in 0.1 ml of liquid paraffin. Ep-1 was suspended in a saline solution containing both 0.5% of CMC and 0.1% Tween 80, and it was administered orally. The rats were examined daily for 24 days to record the severity of the arthritis. Arthritic signs were evaluated as described by Otomo et al. (24). Briefly, the lesions of the four paws and ears were each graded from 0 to 4 according to the increasing extent of the erythema and swelling of the periarticular tissues, as well as the enlargement, distortion or ankylosis of the joints. The maximal possible score per rat was 24.

Statistical analysis: The significance of differences between the means was determined by Student’s t-test. Willcoxon’s rank-sum test was employed to assess the severity of AA.

Results

Effect of mitogens on the cultured splenocytes obtained from the mice treated with Ep-1: In the initial series of experiments, C3H/He mice were intraperitoneally injected with Ep-1 at a dose of 100 mg/kg successively for 3 days. Individually, after an interval of 1, 3 and 5 days, spleen cells (2×10⁵) were cultured at suboptimal and optimal concentrations of Con A, LC and LPS in the medium. As shown in Table 1, Ep-1 seemed to have little or no mitogenic effect since the increase of the ³H-TdR uptake into the cultured splenocytes of the mice treated with Ep-1 was observed in experiment I, but not in II and III. Moreover, the significant enhancement of the ³H-TdR uptake was induced by suboptimal and optimal concentrations of T-cell mitogens, Con A and LC, in all experiments. On the contrary, a slight but significant inhibition of the ³H-TdR uptake was caused by the B-cell mitogen LPS in experiments I and II.

MLC and PFC response of the splenocytes of the mice treated with Ep-1: C3H/He mice were intraperitoneally injected with Ep-1 at a dose of 100 mg/kg successively for 3 days. Individually, after an interval of 3 and 5 days, the spleen cells obtained from the mice treated with Ep-1 were applied to MLC and PFC to test their effect on the cellular and humoral immune response. As shown in Table 2, in experiment I, the ³H-TdR uptake was slightly but significantly enhanced in the USC response as well as in the MLC response, and there was no difference in the SI value between the mice treated by Ep-1 and the ones not treated. The number of PFC
Table 1. Effect of mitogens on the cultured splenocytes of mice administered with Ep-1

| Experiment | Interval after treatment (days) | Treatment mg/kg 3 days | \(^3\)H-TdR (cpm/culture \(\times 10^{-3}\)) |
|------------|---------------------------------|------------------------|------------------------------------------|
|            |                                 |                        | Con A  | LC    | LPS    |
|            |                                 |                        | \(\mu g/culture\) | \(\mu g/culture\) | \(\mu g/culture\) |
| I          | 1                               | –                      | 1.3±0.1 | 5.0±0.1 | 25.1±0.9 | 8.1±0.1 | 12.1±0.6 | 12.5±0.9 | 17.2±1.3 |
|            |                                 | +                      | 1.6±0.1** | 6.1±0.1** | 27.1±0.4** | 8.6±0.4* | 15.4±0.5** | 10.5±0.7** | 13.7±1.1** |
| II         | 3                               | –                      | 1.1±0.1 | 3.8±0.2 | 20.9±0.9 | 7.4±0.5 | 24.9±0.7 | 10.8±0.4 | 14.1±0.1 |
|            |                                 | +                      | 1.0±0.1 | 5.6±0.1** | 26.2±0.2** | 12.2±0.3** | 33.7±0.6** | 8.8±0.2** | 11.5±0.2** |
| III        | 10                              | –                      | 1.0±0.2 | 4.3±0.1 | 23.6±0.9 | 6.9±0.9 | 22.0±1.8 | N.D.     | N.D.     |
|            |                                 | +                      | 1.2±0.1 | 10.3±0.9** | 53.4±2.5** | 15.5±0.3** | 44.8±3.1** | N.D.     | N.D.     |

C3H/He mice were i.p. injected with Ep-1 at a dose of 100 mg/kg successively for 3 days. After an interval of 1, 3 and 10 days, spleen cells \(2 \times 10^8\) were cultured with each optimal and suboptimal concentration of Con A, LC and LPS for 48 hr. Each value represents the mean±standard deviation of 5 mice. Statistical significance symbols, * and **, represent P<0.05, P<0.01, respectively. N.D.: not done.
was significantly decreased in the mice that were treated. In contrast, in experiment II, there were no difference in the MLC, USC and PFC test between the mice treated by Ep-1 and the ones not treated.

**DTH response of mice treated with Ep-1:** CDF1 mice were immunized, and at the same time, Ep-1 was administered orally successively at a dose of 100 mg/kg for 10 days, and the DTH response was elicited as described in Materials and Methods. As shown in Fig. 1, in the control mice, a fairly strong hypersensitive reaction which subsided within 9.5 hr was observed, and the DTH reaction appeared at after 24 hr after the challenge. Ep-1 augmented the establishment of the Arthus-type and DTH response.

**Effect of Ep-1 on the induction or on the suppression of the establishment of adjuvant arthritis:** Two experiments were carried out to examine the dose response and a suitable treatment period before or after the initiation of arthritis. The first experiment: after the rats were inoculated with the heat-killed *Mycobacterium tuberculosis* to induce AA, they were orally administered with various doses of Ep-1 for 23 days from the day of inoculation to the end of observations. As shown in Fig. 2, the rats given a low dose of Ep-1 developed less severe arthritis than those of the other groups given large doses of it. Treatment with Ep-1 at a dose of 25 mg/kg showed a suppressive effect, and at doses of 50 and 100 mg/kg, this effect was diminished.

The second experiment: Ep-1 was administered in two ways, one method was to administer it from day 0 to day 9, and the other method was to administer it from day 10 to day 19. The preventive and therapeutic effects of Ep-1 at a dose of 100 mg/kg on the development of AA were examined. As shown in Fig. 3, a significant suppressive effect was observed in the preventive experiment.

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**Table 2.** MLC and PFC responses of the splenocytes of the mice administered Ep-1

| Experiment | An interval after treatment (days) | Ep-1 treatment | MLC (cpm/culture × 10⁻³) | SI |
|------------|----------------------------------|----------------|--------------------------|----|
| I          | 3                                | −              | 4.6±0.5                  | 2.6| 70.8±18 |
|            |                                  | +              | 5.5±0.7*                 | 2.5| 34.8± 8** |
| II         | 10                               | −              | 5.0±0.5                  | 2.4| 66.3±10 |
|            |                                  | +              | 4.3±0.6                  | 2.8| 86.0±20 |

C3H/He mice i.p. injected with Ep-1 at a dose of 100 mg/kg successively for 3 days. After an interval of 3 and 10 days, one way mixed lymphocytes culture (MLC), unstimulated culture (USC) and the culture for PFC responses were performed. Each value represents the mean±standard deviation of 5 mice. Statistical symbols, * and **, represent P<0.05, P<0.01, respectively.
Fig. 2. Effect of Ep-1 on the course of adjuvant arthritis in rats. Ep-1 was administered orally at doses of 25 (△), 50 (△) and 100 (△) mg/kg for 23 days. Each point refers to the mean of 10 rats. Control: (●). * and ** represent P<0.05 and P<0.01, respectively.

Fig. 3. Effect of Ep-1 on the course of adjuvant arthritis in rats. Ep-1 was administered orally at a dose of 100 mg/kg, (●) from day 0 to day 9 and (●) from day 10 to day 19. Each point refers to the mean of 10 rats. Control: (●). * represents P<0.05.

Discussion
The above data indicate that Ep-1, a specific inhibitor of thiol proteases, has an effect on humoral and cellular immune responses in mice or rats.

In the lymphocyte blast transformation test using the splenocytes obtained from the mice treated with Ep-1, it showed the enhancement of the ³H-TdR uptake into the cells induced at the widespread concentrations of T- and B-cell mitogens such as Con A and LPS. Ep-1 is likely to be long-acting since it prolonged the stimulation of splenocytes until 10 days after the end of the administration. In contrast, it was short-acting with regards to mitogenicity since it had little or no mitogenic activity when determined at an interval of 3 or 10 days after the end of the treatment (Table 1). In this connection, we tried to examine the in vitro MLC and PFC responses of splenocytes of the mice treated with Ep-1 at a dose of 100 mg/kg successively for 3 days. A significant increase of MLC and USC and a decrease in PFC were respectively observed when both MLC and PFC responses were determined at an interval of 3 days after the end of the administration (Table 2). Immunological actions on the lymphocytes were shown in two ways: augmentation of cellular immunity and suppression of humoral immunity. As one possible explanation for these results, the effects of Ep-1 may be caused by the induction of regulatory cells such as helper and suppressor cells (25).
Clagett et al. (6, 7) demonstrated that chymopapain C, a thiol protease, suppressed the PFC response for SRBC in mice, and such an effect might be caused by the contribution of chymopapain C to membrane alterations of the lymphoid cell. Stein-Streilein et al. (8) found that papain, a thiol protease, was able to enhance the quantity of PFC which developed and indicated that protease-protease inhibitor systems might be involved in the proliferation stages of the immune response. However, no report has suggested that an enhanced or a suppressed immune response can develop as a consequence of thiol protease stimulation of the lymphocytes by a thiol protease inhibitor. In order to ascertain whether Ep-1, a thiol protease inhibitor, was effective or not in the establishment of DTH in mice, it was administered to them at a dose of 100 mg/kg for 10 successive days. Ep-1 strongly augmented the DTH response as well as the Arthus-type reaction in the early stage (Fig. 1). These data are consistent with those of the MLC responses.

AA is one of animal models of rheumatoid arthritis and can be produced in rats by a single injection of heat-killed Mycobacterium tuberculosis. Kayashima et al. (26, 27) reported that the subpopulation of T-cells, which were thymus-derived, short-lived and radiation-sensitive ones, might regulate the process of the AA disease. When Ep-1 was administered at a dose of 100 mg/kg for 10 successive days before or after the initiation of arthritis, it was observed that Ep-1 could prevent the induction of AA, but showed no improvement in established AA (Fig. 3). From these data, it seems reasonable to assume that the suppression of AA by the treatment of Ep-1 was brought about by the induction of the regulatory cells competing against the suppressor cells which might regulate AA disease (26).

It is possible that the practical use of Ep-1 may be carried out in some immunological process of the disease. Of course, the question still remains to be answered.

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