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EFFECT OF E-64, A THIOL PROTEASE INHIBITOR, ON ANTIBODY FORMATION IN MICE

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Summary: E-64, L-trans-epoxysuccinyl-leucylamido (4-guanidino) butane, a specific inhibitor of thiol proteases originally isolated from a culture broth of fungi, and its synthetic analogues, were examined for immune responses to the splenocytes of BDF_1 mice. In the cultures of 2-day-primed splenocytes of the mice, E-64 and its close analogues, increased the number of direct splenic hemolytic plaque forming cells (PFC). In addition, it was demonstrated that E-64 enhanced the PFC responses in the mice. These results suggested that some thiol proteases might be involved in the immune response process in mice.

There are several reports showing that lymphocytes could be activated by serine proteases(1-5). It appeared that endogenous serine proteases might be located on the surface of lymphocytes which could be activated by mitogens and antigens, and that this proteolytic activity was essential as a "trigger" for a subsequent blast transformation (3, 4). In this connection, it was also demonstrated that specific inhibitors of serine proteases, such as diisopropylfluorophosphate, α-aminocapric acid, N-α-tosyl-L-lysyl-chloromethyl-ketone, Trasylol, leupeptin and soybean trypsin inhibitors could suppress the triggering of a lymphocyte blast transformation (6-9). The majority of these reports dealt with the relationship between the triggering effects of serine proteases and the suppressive effects of the serine protease inhibitors regarding lymphocytes, mainly using the method of counting tritiated thymidine incorporated into the

Abbreviation : E-64, L-trans-epoxysuccinyl-leucylamido (4-guanidino) butane.

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DNA of activated lymphocytes. On the other hand, there are a few reports demonstrating that papain, a thiol protease, enhanced antibody forming cell *in vitro* (10), and that chymopapain C sharply inhibited the primary immune response and secondary response to antibody formation *in vivo* (11, 12).

E-64, isolated from *Aspergillus japonicus* (13, 14), has a specific and potent inhibitory effect on the thiol proteases. Some close analogues of E-64 have been synthesized (15, 16). They inhibit not only plant thiol proteases (14), but also mammal thiol proteases (17-20). In this paper, we have described the enhancing effect of thiol proteases regarding the immune responses in mice.

MATERIALS AND METHOD

Chemicals: E-64 and its analogues, were synthesized in our laboratory. Their structures are shown in Table 1.

Antigen: Normal sheep red blood cells (SRBC) was obtained in Alsever's solution and washed three times with saline and Eagle's minimal essential medium, for use as antigens in mice and immunogens in the spleen cultures of mice, respectively.

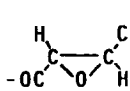
Mice: Both male and female BDF_1 mice were used at the age of 8-10 weeks.

Priming: Mice were intravenously injected with 4×10^6 or 7.8×10^8 SRBC.

Preparation of cell suspension: Single cell suspensions were made from the spleens of mice by using a stainless steel sieve.

Table 1. The Structures of Thiol Protease Inhibitors

Chemicals	Structures	Inhibition to thiol protease
E-64	HO-ES-Leu-Agm	+
Ep-0	HO-ES-OH	-
EP-459	HO-ES-Leu-DBA	+
Ep-475	HO-ES-Leu-IAA	+

Abbreviations : ES,  ; Leu, $(CH_3)_2CHCH_2\overset{-NH}{\underset{|}{C}}HCO-$;

Agm, $-NH(CH_2)_4NH\overset{NH}{\overset{\|}{C}}NH_2$; DBA, $-NH(CH_2)_4NH_2$; IAA, $(CH_3)_2CHCH_2CH_2NH-$.

The resulting cells were gently aspirated with a Pastuer pipette and followed by filtration through siliconized cotton wool. The single cells obtained were washed twice, resuspended, and counted with a hemocytometer. The viability of nucleated cells was estimated by the trypan blue exclusion test.

Culture: Mishell-Dutton's method (21) was used with a slight modification. In brief, spleen cells were cultured in a siliconized Widal's test tube at 37 °C in a humidified atmosphere of 5% CO_2 in air. Each tube contained 2 x 10^6 of splenocytes with 1 x 10 of SRBC as immunogens in 0.4 ml of the culture medium. The medium was RPMI-1640 supplemented with 1 mM L-glutamine, 5% FCS, 5 x 10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, and kanamycin 100 ug/ml.

Assay for plaque-forming cells: Single-cell suspensions were prepared by collecting them from individual cultures or by dissecting spleens of the mice. The number of direct anti-SRBC PFC was determined by Cunningham's technique (22).

RESULTS

Two days after priming, the splenocytes were obtained from the mice and cultured in a medium containing E-64 and its analogues. After culturing for 2 days, the number of PFC was counted. E-64 and Ep-459 had an enhancing effect on the anti-SRBC response, while Ep-475 produced two contrasting effects on the anti-SRBC response, namely, enhancement at 10^{-7} M and suppression at 10^{-5} and 10^{-4} M. On the other hand, Ep-0 provided no influence on PFC (Table 2). Viability of the recovered splenocytes from individual cultures treated with E-64, Ep-459 and Ep-0, was more than 95% of that of the control, but only Ep-475 gave 85%.

The experiment was designed to ascertain whether E-64 and its analogues were active in the anti-SRBC response in mice. Two hours after immunization with 7.8 x 10^8 SRBC, the mice were given E-64 or its analogues intraperitoneally at doses of 1, 10 and 30 mg/kg. On the 4th day, the PFC was determined. The results indicated that E-64 and Ep-475 had significant effects on PFC responses in mice, but Ep-459 did not (Fig. 1).

Two hours after priming with 4 x 10^6 SRBC, the mice were given E-64 intraperitoneally at doses of 10, 30 and 100 mg/kg. On the 2th, 4th, 8th and 10th day, the direct PFC was individual-

Table 2. Effect of E-64 and its Analogues on Anti-SRBC Response to the Cultures of 2-Day-Primed Spleen Cells *in vivo*

	Dose M	PFC/10^6 Spleen cells a)	Percent of control
E-64	0	244 ± 47	100
	10^{-6}	387 ± 28* b)	159
	10^{-5}	361 ± 61*	148
	10^{-4}	258 ± 96	106
Ep-0	0	246 ± 59	100
	10^{-6}	243 ± 16	99
	10^{-5}	244 ± 40	99
	10^{-4}	214 ± 53	86
Ep-459	0	277 ± 37	100
	10^{-6}	323 ± 26	116
	10^{-5}	365 ± 74*	132
	10^{-4}	227 ± 14	82
Ep-475	0	246 ± 59	100
	10^{-7}	333 ± 96*	135
	10^{-6}	215 ± 58	87
	10^{-5}	165 ± 75*	68
	10^{-4}	118 ± 61**	48

Spleen cells obtained from 2-day-primed mice were cultured. Varying concentrations of E-64 and its analogues were added to the medium at the culture initiation. Direct PFC was determined on the 4th day. a) Each value represents the mean of 6 cultures and standard deviation. b) Statistical significance symbols, (*) and (**) represent $p < 0.05$, $p < 0.01$, respectively.

ly determined. E-64 produced a significant increase in the number of PFC on days 4, 6 and 8 at doses of 30 and 100 mg/kg (Table 3).

DISCUSSION

E-64 and its analogues, have been found to possess an enhancing activity as regards the anti-SRBC PFC response.

In an earlier study, the inhibitors showed mitogenic activity on the normal spleen cells of mice. Moreover, E-64 and Ep-459 provided a significant stimulator for the splenocytes induced by the sub-optimal concentration of concanavalin A (23).

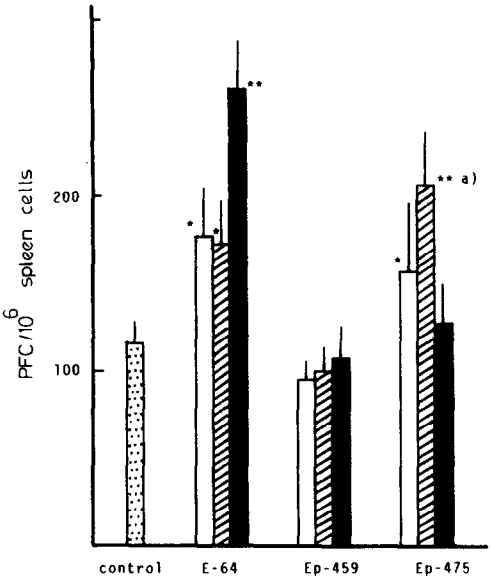


Fig. 1. Effect of E-64 and its analogues on the anti-SRBC response in vivo. Two hours after immunization, the mice were intraperitoneally injected with 1, 10 and 30 mg/kg of E-64 and its analogues. On the 4th day, PFC was determined. Open, hatched and solid columns indicate 1, 10 and 30 mg/kg, respectively. Each column and each vertical bar represent the mean of 8 mice and the standard deviation, respectively. a) Statistical significance symbols, (*) and (**) represent $p < 0.05$, $p < 0.01$, respectively.

In this study, E-64, Ep-459 and Ep-475 enhanced PFC, but Ep-0, having no inhibitory activity on the thiol protease, showed no augmenting effect (Table 2). This enhancing effect was not dependent on the increasing dose of E-64 and its analogues, but only on their optimum doses. Ep-475 has a proliferative effect on the PFC response at the low concentration, while it was suppressive at the high concentrations. Thus, Ep-475 had dual contrasting effects on the PFC responses in the 2-day-primed splenocytes. In the in vivo PFC test, E-64 and Ep-475 enhanced the PFC from 1.4 to 2.5 times, but Ep-459 had no apparent effect. The results obtained from this experiment did not agree with the results of the splenocytes cultures in vitro. This might be due to the metabolic behavior of these inhibitors. E-64, the originally isolated inhibitor, was revealed to have the strongest effect among the inhibitors (Fig. 1).

Table 3. Time Course of Effect of E-64 on the Anti-SRBC Responses in vivo

PFC Assay	Dose mg/kg	PFC/10^6 Spleen cells [a]	Percent of control
On day 2	0	1.2 ± 0.3	100
	100	0.9 ± 0.2	75
On day 4	0	50.0 ± 17	100
	100	68.7 ± 2* [b]	137
On day 6	0	76.0 ± 8	100
	10	85.0 ± 2	112
	30	102.0 ± 12**	135
	100	180.0 ± 38**	237
On day 8	0	10.0 ± 4	100
	10	11.5 ± 1	115
	30	19.2 ± 2**	192
	100	24.1 ± 12*	241
On day 10	0	5.3 ± 2	100
	100	5.8 ± 1	109

Two hours after immunization, the mice were given 10, 30 and 100 mg/kg of E-64 by intraperitoneal injection. On the 2th,4th, 6th, 8th and 10th day, the direct PFC was determined. a) Each value represents the mean for 5 mice and the standard deviation. b) Statistical significance symbols, (*) and (**) represent $p < 0.05$, $p < 0.01$, respectively.

Stein-Streilein et al. reported that exogenous thiol protease papain was able to enhance the PFC response in the cultures of the lymphocytes of hamsters, and suggested that proteolytic events could be effective in enhancing the quantity of specific effector cells (10). Claget et al. demonstrated that chymopapain C, a thiol protease, depressed the primary immune response to SRBC and caused a change in the secondary response in C57BL/6 mice when it preceded the "priming" SRBC injection (13, 14). These data seemed to support the theory that the exogenous thiol protease was "trigger", since it short-circuited the endogenous thiol protease step supposed to be common to antibody formation.

The results of the above examination of the effects of E-64 on the anti-SRBC responses in the mice and the results reported from other laboratories, indicate that a thiol protease might be involved in the process of immune response in mice.

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