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Effects of the Endotoxin-Inactivating Agent on the Biological Properties of Bacterial Endotoxin*

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The present investigation deals with alterations of the immunological and pharmacological properties of *Salmonella* endotoxin by the endotoxin-inactivating agent (EIA), considerably purified, from pig liver. The results obtained are as follows.

1) The specific antibody production in mice and rabbits which were immunized with the endotoxin treated with the EIA was reduced more than those immunized with the untreated endotoxin.

2) The treatment of endotoxin with the EIA reduced its capacity to elicit the pyrogenic response in rabbits.

3) There was no difference of the lethal rate of ten-day old chick embyros between the group injected with the untreated endotoxin and that injected with the endotoxin treated with the EIA. Also, on tumor hemorrhagic property of the endotoxin in mice, there was no difference between the untreated and the EIA-treated endotoxin.

4) No alteration of the capacity of the endotoxin by the treatment with the EIA to prepare for and provoke the dermal Shwartzman reaction of rabbits was observed.

5) These results clearly show that the EIA alters the endotoxin's immunogenic and pyrogenic properties, but not its hemorrhagically necrotic and hemorrhagically lethal properties.

Many investigators have studied the inactivation of endotoxin with the plasmas or the sera of human and other mammals (1, 2, 3, 4, 5, 6, 7), their protein fractions (1, 8, 9, 10, 11), homogenates of organs such as liver (12, 13, 14, 15) or spleen (16), proteolytic enzyme such as a papain (17), or surfactant such as a bile salt (18, 19, 20). In these related reports, the pharmacological and immunological properties of the altered endotoxin, including pyrogenicity (1, 2, 3, 6, 9, 10), tumor damage (4, 7, 21) and a lethal property on chick embryos (14, 15, 16, 22), mice (7, 23) and rabbits (24), also a Shwartzman reaction (20, 24), a leucopenic response (2, 3), an antigencity (1, 5, 6, 8, 9, 10, 15, 25, 26) and others (20), were observed.

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In most cases, however, only one or two kinds were observed. So, there are difficulties in making a comparison of characteristics among these endotoxin-inactivating agents and in determining their functions on the alteration of the various properties of endotoxin. Also, the extent of inactivation of the properties of the endotoxin by each agent is not clear.

In this paper, the effects of an endotoxin-inactivating agent (EIA), which has high specific-activity on the alteration of the antigenicity, prepared from pig liver, on several biological properties of a *Salmonella* endotoxin were studied and the detailed nature of the agent will be clarified, especially the immunological property i.e., antigenicity and immunogenicity, and the toxic properties, i.e., pyrogenicity and hemorrhagic action of the endotoxin.

Materials and Methods

Bacterial Endotoxin and Endotoxin-Inactivating Agent (EIA).

These were prepared according to the method described in our previous paper (15, 25). This time, however, the method of preparation of the EIA was slightly modified, that is, the acid treatment was performed at pH 5.1 instead of pH 5.0. The endotoxin solution was sterilized by heating for 10 mimutes in a boiling water bath and the EIA solution by filtering with a Millipore HA filter.

Animals. The dd strain mice were purchased from the Mouse-Center of Tohoku University and Funabashi Nojo (Animal Farm). Randomly bred rabbits were supplied from animal dealers. Embryonated eggs of White Leghorn were used. In each experiment, except for the Schwartzman reaction, these animals were divided into three groups; one was treated with an inactivated endotoxin in the incubation mixture which had been diluted suitably for each experiment, the second, as the control, were treated with intact endotoxin and the third with EIA dissolved in buffer solution at the same concentrations.

Incubation Test System for Endotoxin-Inactivation. The incubation mixture, the volume of which was 1 ml, consisted of 2.3 mg of the EIA, 0.5 mg of endotoxin and 10^{-3} M Mn^{2+} in 1/50 M phosphate buffer, pH 6.0. The conditions of incubation were the same as reported in our other paper.* Thus, the EIA-treated, inactivated, endotoxin was obtained.

Test for Immunogenicity. To adult mice, 0.1 ml of incubation mixture which contained 50 μg endotoxin was injected intraperitoneally twice. The second injection was one week after the first. Blood samples were gained by cardiopuncture one week after the last injection. The sera obtained through the ordinary method were inactivated at 56°C for 30 minutes. The titer of the specific antibody was determined by the agglutination test against *Sal. abortus equi* from which the endotoxin was extracted. The rabbits were intravenously injected

* Inoue, T., and Katsuno, M.. *Tohoku J. Agr. Res.*, **21**, (1970), (in press).

with a mixture containing 1 μg endotoxin on the first day and 2 μg on the next. Other preparations and the determination of the antibody titer were the same as those used in the case of mice.

Shwartzman Reaction. Approximately 2.0~2.5 kg male rabbits were used for this test and the hair of the back was removed with a paste consisting of BaS and flour on the day before the preparatory injection. An intradermal injection of 0.1 ml of serial dilutions (0.032 μg–0.50 μg) of the treated and untreated endotoxin as a preparatory injection was performed and then the animals were devided into two groups. After 24 hours, the one group was intravenously administered with 100 μg of the untreated endotoxin, and the other with the same dose of the treated endotoxin, as a provocative injection. In addition, as a control, the EIA was injected preparatively in each animal. Four hours later, the result was observed grossly from the inner surface of the skin stripped off from the subcutaneous tissue and the diameters of the hemorrhagic lesions were measured.

Results

On the Immunogenicity. As shown in Table 1, all the mice immunized with untreated endotoxin produced specific antibody, the mean agglutinating titer of which was $10\times2^{6.3}$. On the contrary, in the group of mice immunized with the endotoxin treated with the EIA, it was $10\times2^{4.2}$. No detectable antibody against the endotoxin was present in the mice injected only with EIA. Moreover, concerning the geometrical mean titer of the antibody produced, there was a significant difference at the 1% level between the group immunized with the EIA-treated endotoxin and that with the untreated endotoxin.

Also, in the case of rabbits, the results were similar to those of mice as shown in Table 2. The mean titer of the antibody produced was $10\times2^{6.8}$ in the group immunized with the untreated endotoxin, though in the one immunized with the EIA-treated endotoxin the mean titer was $10\times2^{5.3}$. The rabbits injected only with EIA produced almost no detectable antibody.

TABLE 1. *Effect of EIA on the Immunogenicity of Endotoxin for Mouse.*

Inoculum	Total No. of animal	Mean titer[1)]
Untreated endotoxin	10	$10\times2^{6.3\pm0.8}$
EIA-treated endotoxin	10	$10\times2^{4.2\pm1.4}$**
Only EIA	10	$<10\times2^{1}$

1) Mean ±S.D. **, $P<0.01$, group injected with untreated endotoxin compared with that injected with EIA-treated endotoxin.

TABLE 2. *Effect of EIA on the Immunogenicity of Endotoxin for Rabbit.*

Inoculum	Total No. of animal	Mean titer
Untreated endotoxin	8	$10\times2^{6.8\pm1.0}$
EIA-treated endotoxin	6	$10\times2^{5.3\pm1.0}$
Only EIA	5	$<10\times2^{1}$

See the legened of Table 1.

From these results obtained in mice and rabbits, it is known that the immunogenicity of endotoxin was inactivated by the EIA considerably purified from pig liver.

On the Pyrogenicity. Fig. 1 shows the febrile response of rabbits to the untreated endotoxin, the EIA-treated endotoxin and the EIA, respectively. Each cruve indicates the mean temperature of each group elevated from the normal one measured before an administration of the materials above mentioned. The group injected with untreated endotoxin exhibited a biphasic temperature curve, the maximal height of which was 1.3°C, while the group injected with EIA-treated endotoxin exhibited a suppressed temperature curve and the highest elevation was 0.7°C. The fevering period was also shortened, compared with that of the former group. The pyrogenic activity of EIA could almost be neglected. These results mean also that the pyrogenic property of the endotoxin was altered by this EIA.

On the Lethal Property on Ten-Day Old Chick Embryos. Results are shown in Table 3. It is found that there was no difference in the lethality of chick embryos between the group which was given the untreated endotoxin and the group given

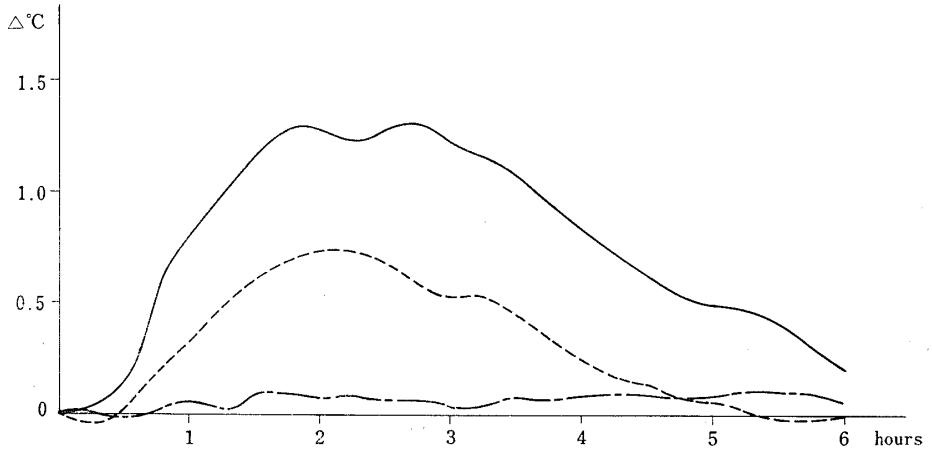

FIG. 1. Effect of EIA on the Pyrogenicity of Endotoxin on Rabbits.
Each curve is an average elevation of rectal temperature of 3 to 4 rabbits.
——— untreated endotoxin, - - - - - - - - EIA treated endotoxin,
—·—·— EIA
Rabbits were injected 1 μg endotoxin.

TABLE 3. *Effect of EIA on the Lethal Property of Endotoxin on Ten-Day Old Chick Embryos.*

Inoculum	Mortality
Untreated endotoxin	8/11[1] (72.7%)
EIA-treated endotoxin	8/11 (72.7%)
Only EIA	1/10 (10.0%)

The amount of endotoxin administered was 50 μg in 0.1 ml of the incubation mixture. On the method, see the reference (15).
1) Deaths/total eggs.

TABLE 4. *Effect of EIA on the Tumor-Hemorrhagic Property of Endotoxin.*

Inoculum	Tumor-hemorrhage
Untreated endotoxin	7/11[1] (63.6%)
EIA-treated endotoxin	7/11 (63.6%)
Only EIA	2/11 (18.2%)

Ehrlich ascite tumor was used. The amount of endotoxin was 50 μg per mouse.
1) Mouse bearing tumor-hemorrhage/total mice

the EIA-treated one. Also, it was made sure that the EIA had almost no lethal property. This means that the EIA did not change the lethal property of the endotoxin.

On the Tumor Hemorrhaging Property. As shown in Table 4, there was no difference between the rates of tumor hemorrhage induced by the untreated endotoxin and by the EIA-treated endotoxin. Though there were two mice which bore hemorrhagic tumor in the group injected with EIA only, it is supposed that this was due to a spontaneous hemorrhage. These results mean that the tumor hemorrhaging property of the endotoxin is not affected by the EIA.

On the Shwartzman Reaction. The results are shown in Table 5. The preparatory activity of the EIA-treated endotoxin was not different from that of the untreated endotoxin in both groups i.e., the intravenously injected untreated endotoxin (No. 1$\sim$3) and the treated endotoxin (No. 4$\sim$6), respectively. The EIA had almost no preparatory activity at this time. Next, no difference was observed in diameters and strength of hemorrhagic lesions between the two groups. These facts indicate that preparatory and provocative activities of the endotoxin were not affected by the EIA.

Putting these results together, it is obvious that the EIA prepared from pig liver affected *Salmonella* endotoxin to alter its immunogenic and pyrogenic properties but not its hemorrhagic properties.

TABLE 5. *Effect of EIA on the Shwartzman Reactive Property of Endotoxin.*

Provocative[1] injection	Untreated endotoxin		EIA-treated endotoxin			
Preparatory injection	Untreated endotoxin		EIA-treated endotoxin		Untreated endotoxin		EIA-treated endotoxin	
	No. 1[4]				No. 4			
0.125 μg[2]	12×11[5]	+[6]	12×12	+	6×6	+′	7×7	+
0.063	10×9	+	11×10	+	5×5	+″	6×5	+
0.032	10×8	∔	8×7	+	−	±	−	±
EIA[3]	4×3	±	5×5	±	−	−	−	−
	No. 2				No. 5			
0.125 μg	5×5	+″	5×5	+	10×9	+″	8×6	+′
0.063	3×3	+″	5×5	+	10×8	+″	9×7	+
0.032	3×3	±	4×3	∔″	9×7	+‴	5×5	+′
EIA	−	−	−	−	−	−	−	−
	No. 3				No. 6			
0.125 μg	6×6	+‴	6×6	+‴	9×7	+‴	9×11	+′
0.063	4×4	+‴	4×4	+‴	6×4	+″	7×8	+‴
0.032	3×3	+‴	3×3	±	5×5	+‴	5×4	±
EIA	−	−	−	−	−	−	−	−

1) The amount of each endotoxin injected was 100 μg.
2) Numbers indicated the amount of endotoxin contained in 0.1 ml of the diluted incubation mixture.
3) The volume injected was 0.1 ml of the 50-times-diluted incubation mixture composed only of the EIA.
4) Number of rabbit.
5) Diameters of hemorrhagic lesions expressed in mm.
6) Degree of hemorrahge: ∔>∔′>∔″>∔‴>+>+′>+″>+‴>±>−

Discussion

We (25) obtained the fraction purified from pig liver by means of salt-out and gelfiltration, which exhibited fairly high specific-activity in the inactivation of the antigenicity of endotoxin. Here, the effects of the same fraction (EIA) on biological properties of the *Salmonella abortus equi* endotoxin were studied.

From the results shown in this paper, it was found that the EIA inactivated the immunogenicity as well as the antigenicity, but not the hemorrhagic properties including the tumor-hemorrhage, the lethal property on chick embyros and the Shwartzman reaction. Moreover, we (15) already reported that by a fractionation of mouse liver extract with ammonium sulfate, there were two kind of endotoxin-inactivating agents which inactivated the antigenicity of endotoxin and the lethal property on chick embryos, respectively. Similarly the results obtained here, using pig liver, indicate that the agent which inactivated the immunogenicity is different from the one which inactivated the hemorrhagic property. These results also should mean indirectly that the site responsible for the immunological property of the endotoxin and that for the hemorrhagic property are different. At

the same time, these show that the mechanism for the inactivation of the immunological property by the EIA is not similar to that reported by Ribi et al (26) who investigated the loss of biological properties of endotoxin by hydrolysis. Though there have been many publications on the inactivation of endotoxin by sera and tissue extracts of mammals, as above mentioned, there were not so many reports on the inactivation of endotoxin by a purified preparation which has a high specific-activity. Our observations, using the purified fraction from pig liver, are similar to those of Yoshioka et al (10) indicating that Cohn fractions IV-1 and III-O of human serum inactivated the antigenicity and the pyrogenicity of endotoxin. However, as they did not study the inactivation of the hemorrhagic property, it is not obvious whether or not their inactivating agent responsible for the inactivation of the antigenicity and the pyrogenicity was able to inactivate the hemorrhagic property.

From the results reported by Ribi et al (18) and Tarmina et al (19, 20), it was clarified that sodium deoxycholate inhibited the antigenicity, the immunogenicity, the pyrogenicity, the hemorrhagic property on chick embryos and the Shwartzman reaction. However, as our EIA did not inactivate the hemorrhagic property, the mechanism of the inactivation of endotoxin by EIA did not seem to be similar to that by sodium deoxycholate. Moreover, it was reported that papain (17) inactivated the pyrogenicity and the tumor-hemorrhagic property. However, from the stand point that the EIA inactivated only the former, the function of the EIA is not an enzymatic one as of a papain.

On the other hand, the results that the EIA inactivated both the pyrogenicity and the immunogenicity, though the degree of inactivation was not studied quantitatively, suggest that the EIA may consists of two kinds of inactivating agents which inactivated both the properties respectively, or that the active sites of endotoxin carrying both the properties may be closely located or similar in their functions.

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