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On a Role of the Endotoxin-Inactivating Agent in the Inactivation of Bacterial Endotoxin*

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Summary

These experiments were undertaken to investigate the mechanisms of the inactivation of endotoxin by the endotoxin-inactivating agent (EIA) partially purified from pig liver. The results obtained are as follows.

1. The agar gel diffusion and the immunoelectrophoretic analysis revealed a decrease in the density of the precipitate band, when the endotoxin was inactivated. Moreover, it was found that the greater part of the polysaccharide of the inactivated endotoxin was retained near the origin by the cellulose-acetate electrophoresis.

2. It was found from the agar gel diffusion and the starch gel electrophoresis that there were two components in a preparation of the EIA, one of which seemed to react with endotoxin.

3. The gelfiltration study of the incubation mixture exhibited the formation of a complex between the endotoxin and the endotoxin-inactivating agent, but not any release of mono- or oligo-saccharide from the endotoxin nor depolymerization into subunits.

4. Thus, the serological inactivation of endotoxin by the EIA from pig liver was due to the formation of the complex between the two substances rather than to the degradation of the endotoxin.

It has been well known that human serum (1, 2, 3), horse serum (4) and others (1, 5) were able to alter the serological and immunological properties of endotoxin.

On the mechanisms of this alteration with these sera, Rudbach and Johnson (6) indicated that the inactivation of endotoxin with Cohn fraction IV-1 derived from normal human serum by cold ethanol fractionation was due to formation of a haptenic polysaccharide, but not to the release of a major determinant sugar. Subsequently, it was shown that the activity of endotoxin, which was altered by the incubation with human plasma or Cohn fraction IV-1 of human plasma, was restored by treating the incubation mixture with a proteolytic enzyme and by ethyl alcohol precipitation (7, 8). Thus, the inactivation of endotoxin by plasma

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resulted from a reversible complexing with plasma proteins rather than from enzymatic degradation. Moreover, Ribi et al. (9) found that a bile salt, sodium deoxycholate, depolymerized endotoxin and that human plasma would stabilize the dissociated endotoxin. Yoshioka (4) observed, however, that α_2-macroglobulin from horse serum depolymerized endotoxin reversibly without a bile salt.

The present investigation was undertaken to inquire into the mechanisms of the inactivation of endotoxin by the endotoxin-inactivating agent (EIA) prepared from pig liver which was characterized by manganese ion for the inactivation of endotoxin (10) and inactivated the immunological and pyrogenic properties of the endotoxin but not the hemorrhagically necrotic and lethal properties (11).

Materials and Methods

Bacterial Endotoxin and Antiserum. These were made according to the method reported in our previous paper (12).

Endotoxin-Inactivating Agent (EIA). Extraction and purification of this agent from pig liver were performed according to the method described in our previous paper (10).

Antiserum to Liver Extract. Pig liver was homogenated in two volumes of cold deionized water with a Waring Blendor and centrifuged at 13,000 rpm for 45 minutes at 4°C. The supernatant was used as the liver extract. The antiserum was made by immunizing albino rabbits with this extract.

Incubation Test System for Inactivation of Endotoxin. The reaction mixture, the volume of which was 1.0 ml, consisted of 1.2 mg of the inactivating agent, 1 mg of endotoxin and 10^{-3} M $MnCl_2$ in 1/50 M phosphate buffer (pH 6.0), excepting case of gelfitration. As the manganese ion was necessary for the inactivation of endotoxin (10), an incubation mixture without Mn^{2+} was used as one of the controls for all experiments. The conditions of the incubation were the same as in our previous paper (10).

Gelfiltration by Sephadex G-200. Sephadex (Pharmacia) gel was packed into a column the dimensions of which were 2.0 cm × 40 cm. Three ml of each sample were applied for gelfiltration and eluted with 0.15 M NaCl solution containing 10^{-3} M Mn^{2+} except for the control. The effluent was collected by 5 ml fractions. Each fraction was determined an amount of protein according to the Cu-Folin method (13) and of saccharide by the method of Dische and Shettles (14).

Agar Gel Diffusion. The micro-diffusion method was employed (15). The agar solution was made with 1.0% Special Agar-Noble (Difco) in 0.15 M NaCl solution containing 1:10,000 thimerosal as a preservative and poured 2 mm thick onto microscopic slide glasses (Matsunami, No. 1). Then, wells for antigen and a trough for antibody were made. The antigens were pipetted into wells and

antibody into the trough. The slide glasses were set in a Petri dishes containing moistened gauze, kept at room temperature for 24 hours and then observed.

Immunoelectrophoresis. Micro-method was employed (15). Agar gel was prepared, solving 1.0% Special Agar-Noble (Difco) in Veronal buffer (pH 8.2, ionicity 0.02) containing 1:10,000 thimerosal. The electrophoresis was performed under a potential of 6 V/cm for 120 minutes. The other preparations were the same as those described above in the agar gel diffusion.

Cellulose-Acetate Electrophoresis. This was done according to the method of Ogawa (16). The cellulose-acetate membrane (Separax) and the apparatus used were manufactured by Jōkō Sangyo Co. The volume of samples was 0.8 μl. Electrophoresis was carried out under the condition of 0.6 mA/cm for 50 to 60 minutes, using 0.07 M barbital buffer, pH 8.6. After the electrephoresis, the membrane strips were stained with Schiff reagent (17).

Starch Gel Electrophoresis. Vertical starch gel electrophoresis was used according to the method described by Smithies (18), using a discontinuous buffer system (19). The gel, however, was made with a buffer consisting of 0.0375 M tris-aminomethane (Sigma) and 0.0025 M citric acid. Electrophoresis was performed for 16 to 17 hours with a potentiality of 140 V. Then, the starch gel was stained with amido-black 10B for protein detection (18).

Results

On the State of the Endotoxin after the Incubation with the Endotoxin-Inactivating Agent (EIA). These experiments using the methods of agar gel diffusion, immunoelectrophoresis and cellulose-acetate electrophoresis were performed to clarify how the endotoxin was altered during incubation with the endotoxin-inactivating agent (EIA). As shown in Fig. 1, the agar gel diffusion study of endotoxin showed no difference in precipitate bands between the endotoxin alone and that treated with the EIA without Mn^{2+}, though in the case of endotoxin treated with the EIA and Mn^{2+}, the precipitate band was found to be weaker in density than in that of endotoxin alone.

Moreover, the results of Fig. 2 obtained from the immunoelectrophoresis were similar to those from the agar gel diffusion. Namely, the band of the precipitate of the endotoxin treated with the EIA and Mn^{2+} was weaker in density than those of both the endotoxin alone and of that treated with only the EIA. From these results, it was confirmed that the serological activity of endotoxin was decreased by the incubation with the EIA and Mn^{2+}, and that there was a necessity for the manganese ion for the inactivation of endotoxin with the EIA. At the same time it was found that the endotoxin treated with both the agent and ion moved a shorter distance toward the cathode from the origin than the unaffected one in a diffused pattern.

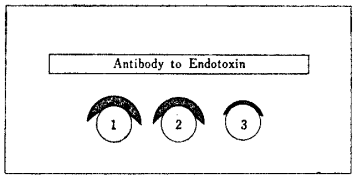

FIG. 1.

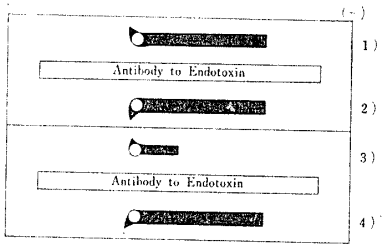

FIG. 2.

FIG. 1. Agar gel diffusion of endotoxin.
Incubation mixture consisting of only the endotoxin was in well 1, that of EIA and endotoxin in well 2, and that of EIA, endotoxin and Mn^{2+} in the well 3.

FIG. 2. Immunoelectrophoresis of endotoxin.
1) and 4) represent the incubation mixture containing only endotoxin, 2) that containing endotoxin and EIA, and 3) that containing endotoxin, EIA and Mn^{2+}.

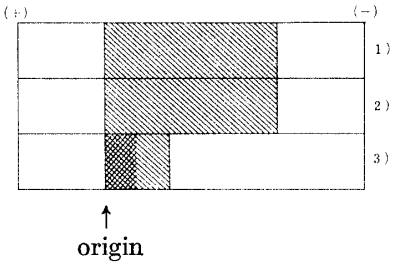

FIG. 3.

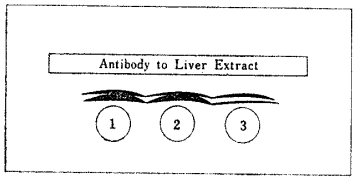

FIG. 4.

FIG. 3. Cellulose-acetate electrophoresis.
See the legend of Fig. 2.

FIG. 4. Agar gel diffusion of the endotoxin-inactivating agent.
See the legend of Fig. 1.

Similar results were obtained from the cellulose-acetate electrophoretic study as shown in Fig. 3. The electrophoretic patterns of the untreated endotoxin and of that treated with only the EIA were diffused a long way from the origin toward the cathode. On the contrary, that of the endotoxin treated with the EIA and Mn^{2+} diffused a shorter distance and was stained denser near to the origin.

Thus, from these results it was confirmed that the EIA affected an electric property of the endotoxin, when a loss of the serological activity of the endotoxin was observed.

On the State of the Endotoxin-Inactivating Agent (EIA) after Incubation. This experiment was carried out to investigate by means of the agar gel diffusion, whether or not the EIA was altered, when it was incubated with endotoxin. In Fig. 4, it was observed that there were two precipitate bands, one of which was situated near to the antibody trough and the other near to the antigen well. When the EIA was incubated with endotoxin and Mn^{2+}, the density of the precipitate band near to the antigen well became weaker than that of the unincubated EIA or of that incubated with only endotoxin. However, no alteration in the precipitate band

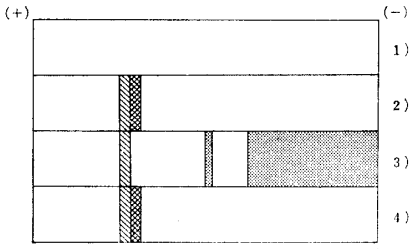

FIG. 5.

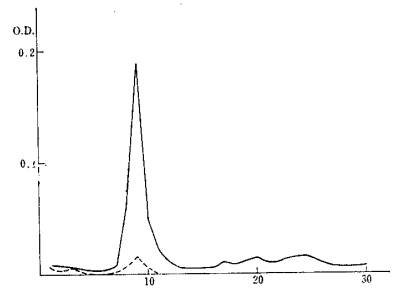

FIG. 6.

FIG. 5. Starch gel electrophoresis of the endotoxin-inactivating agent.
1) represents the incubation mixture containing only endotoxin, 2) that containing EIA and endotoxin, 3) that containing EIA, endotoxin and Mn^{2+}, and 4) that containing only EIA.

FIG. 6. Gelfiltration of endotoxin by Sephadex G-200.
Incubation mixture (3 ml) contained 3 mg endotoxin. The mixture without endotoxin was incubated for 30 minutes at 37°C, added endotoxin, and then incubated for more 30 minutes. After the incubation, the mixture (3 ml) was gelfiltrated. 0.15 M NaCl solution was used as the eluant. A fourfold dilution of effluent was used for protein determination and twofold one for saccharide determination.

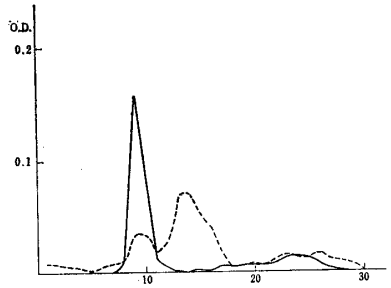

FIG. 7.

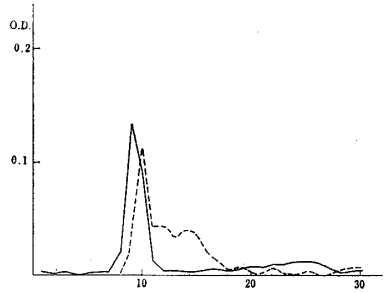

FIG. 8.

FIG. 7. Gelfiltration of incubation product without Mn^{2+} by Sephadex G-200.
Incubation mixture (3 ml) contained 3 mg endotoxin, 3.9 mg EIA in 1/50 M phosphate buffer, pH 6.0. Other procedures were described in the legend of Fig. 6.

FIG. 8. Gelfiltration of incubation product with Mn^{2+} by Sephadex G-200.
Incubation mixture (3 ml) contained 3 mg endotoxin, 3.9 mg EIA and 10^{-3}M Mn^{2+} in 1/50 M phosphate buffer, pH 6.0. In this case, the eluant was 0.15 M NaCl solution containing 10^{-3} M Mn^{2+}. Other procedures were described in the legend of Fig. 6.

near to the antibody trough was observed. These facts show that the EIA prepared by our method contains two components, one of which is lost serologically by the incubation with endotoxin.

With the same purpose, the study of the starch gel electrophoresis was performed. As shown in Fig. 5, the electrophoretic pattern showed that the EIA consisted of two components. The pattern of the EIA incubated with endotoxin

alone was similar to that of the EIA alone. When the inactivation of endotoxin occurred in the presence of Mn^{2+}, one of the two components which was found at the side close to the origin almost disappeared and a diffusely stained area was found near the origin. This altered component of the EIA should be involved in the endotoxin-inactivation. Though the new band which had a slower mobility appeared, this seemed to be independent from the endotoxin-inactivation, for the other preparation was occasionally accompanied with this band.

Role of the Endotoxin-Inactivating Agent (EIA). As described above, when the serological activity of the endotoxin was lost, the altered endotoxin and a part of the EIA decreased their electrophoretic mobility. This fact suggests that there may be a complex between both substances. To clarify this question, the gelfiltration studies were carried out. Fig. 6 shows the gelfiltration pattern of the endotoxin, Fig. 7 that of the incubation mixture without Mn^{2+} and Fig. 8 that of one with Mn^{2+}, respectively. In the case of the incubation mixture without Mn^{2+}, the elution patterns of the protein and saccharide were not different from those of the EIA and endotoxin, respectively. However, when the inactivation of endotoxin occurred, the peaks of the protein and saccharide were found at almost the same position. Also, a degradation of endotoxin into small molecules was not found. This means that the inactivation of endotoxin was due to a formation of some kind of complex between the EIA and endotoxin, though there is a problem whether an intact or depolymerized endotoxin will combine with EIA.

From these results it seems that the inactivation of endotoxin by the EIA is due to the formation of some kind of complex between the endotoxin and the EIA, but not to the depolymerization of endotoxin into subunits nor to the degradation of endotoxin into small molecules such as mono- and/or oligo-saccharide which are acknowledged to play a role as an immunological determinant in O-antigen.

Discussion

These experiments were carried out to investigate the mechanisms of the endotoxin-inactivation by EIA which was partially purified from pig liver according to the method described in our previous paper (10).

The results obtained from the agar gel diffusion and the immunoelectrophoresis showed that the precipitate band of endotoxin became weaker in density when the endotoxin was inactivated. The same thing happened to the EIA. Furthermore, the starch gel electrophoresis revealed a decrease of protein. It seems that these results on the EIA support those obtained from the gefiltration study that revealed the formation of a complex between the EIA and the endotoxin.

The gelfiltration study of the incubation mixture indicated, when the inactivation of endotoxin occurred, the formation of a complex between the endotoxin and the EIA, but not release of small molecules such as mono and/or

oligo-saccharide. Rudbach and Johnson (6) observed that the loss in serological activity of endotoxin by Cohn fraction IV-1 from human serum was not due to a release of a major determinant sugar. Our results were similar to these.

Cluff (20), Rudbach and Johnson (6) stated that, using the method of agar gel diffusion, endotoxin treated with serum component displayed a dispersion of a major antigen, associating a formation of a more rapidly migrating component. Moreover, Yoshioka (4) obtained a similar result from an electron microscopic study. These results indicated that endotoxin was depolymerized into subunits by the serum component. Though our results obtained here were not similar to others, it was not obvious what made this difference. It is thought, however, that there is a possibility that this is due to the origin of the EIA, that is, a serum origin or an organ origin.

So far, there have been interpretations of the role of the EIA in the inactivation of endotoxin. Ribi et al (9) suggested that a bile salt, sodium deoxycholate, dissociated endotoxin into its subunits and human plasma protein which bound endotoxin prevented reaggregation of the subunits. On the contrary, Yoshioka (4) observed that no bile salt was found in his preparation, so he suggested that α_2-macroglobulin from horse serum split endotoxin into the subunits and maintained them. It was not observed whether or not the bile salt was necessary for the inactivation of endotoxin in our experiment. In the case of our preparation, however, this inactivation of endotoxin seems to be independent from the bile salt because the inactivation occurred only in the presence of the manganese ion. It is unknown if our preparation contains the bile salt.

On the nature of the complex formed between the endotoxin and the serum component, it was reported that the altered activity of the endotoxin was able to be restored from this complex by treating with a proteolytic enzyme such as pronase (4, 7, 8). This meant that the inactivation of the endotoxin was due to a formation of a reversible complex. Concerning this reversibility, we would like to examine the nature of the complex in the case of our preparation from a liver origin.

Finally, comparing the results of other investigators, it was found that there was some difference between our results and others, in the fashion of the endotoxin-inactivation, which was thought to be mainly due to the difference of the origin of the EIA.

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