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Tohoku Journal of Agricultural Research
Vol. 19, No. 4, Dec. 1968
Printed in Japan

Effects of Mouse Tissue Extracts on Bacterial Endotoxin*

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(Received, October 7, 1968)

Summary

Extracts of mouse liver, kidney and spleen inactivated the antigenicity and the toxicity of bacterial endotoxin *in vitro*. Concerning the inactivation of the antigenicity, the liver was the most effective organ and the spleen was the least, however, regarding the inactivation of the toxicity the liver, kidney and spleen were equally effective.

Fractionation of liver extract with ammonium sulfate distinguished the factor which inactivated the antigenicity of the endotoxin, from that which inactivated the toxicity.

Though these factors had characteristics of protein, they were not antibody against *Salmonella abortus equi* from which the endotoxin was extracted.

From these results, it seems likely that mice possess both the substance which inactivates the antigenicity and that which inactivates the toxicity.

During the past decade, it has been reported that sera or plasmas of various animal species are capable of altering bacterial endotoxins *in vitro* so that they no longer elicit the characteristic toxicity (1, 2, 3, 4) and antigenicity (5, 6, 7, 8).

Homogenates of various animal tissues also are capable of enzymatically detoxifying bacterial endotoxins *in vitro*. It was reported by Keen (9) that *in vitro* incubation of extracts of rabbit liver, kidney, spleen and heart with bacterial endotoxin resulted in inactivation of its pyrogenic and tumor-necrotizing properties. Trapani et al. (10) observed that soluble extracts of rabbit liver, adrenal, kidney and lymph node inactivated the pyrogenicity and the tumor-necrotizing activity in mice, however, those of brain, heart and spleen did not. Smith et al. (11) reported that a protein with the properties of an esterase type of enzyme, which inactivated the lethal activity of bacterial endotoxin on the ten-day-old chick embryos, was more active in dog spleen than liver, whereas, kidney, heart and skeletal

* The oral presentation of the abstract was made at the 61st Meeting of Japanese Society of Veterinary Science on April 1, 1966.

muscle were inactive. Corwin and Farrer (12) investigated that the lethal activity of endotoxin on the ten-day-old chick embryos was inactivated by guinea pig liver mitochondria and concluded that the inactivation was completed by a process of activation and subsequent oxidation of the fatty acid portion of the endotoxin molecule.

It is the purpose of this report to find whether tissue extracts of mice display the activities of the inactivation of the endotoxin, in regard to both its antigenicity and its toxicity on chick embryos.

Materials and Methods

Tissue Extracts

Adult DD strain mice used were obtained from the Mouse Center, Faculty of Medicine, Tohoku University. Mice were paralyzed by a blow on the head, exsanginated by cutting off the head with a pair of scissors. The organs to be tested were quickly removed, weighed and placed in two volumes of cold 0.02 M phosphate buffer solution as aseptically as possible. They were homogenated for one minute with a Waring Blendor, cooling with ice and water. The homogenates were centrifuged at 15,000 rpm at 4°C for 30 minutes. Then, the supernatants were tested for their ability to inactivate toxic property and antigenicity of bacterial endotoxin.

Fractionation of Liver Extract by Ammonium Sulfate

The liver extract with cold deionized water was fractionated into three parts with cold saturated ammonium sulfate solution and solid salt. After standing for three hours at 4°C, the solution was centrifuged at 10,000 rpm for 30 minutes. The precipitate was collected, washed three times and dissolved in cold deionized water. After dialysis against saline to remove excess ammonium sulfate, the solution was centrifuged at 10,000 rpm at 4°C for 30 minutes to obtain the supernatant. Each supernatant, adjusted with saline to one-half the original volume, was assayed for inactivation of antigencity and toxicity of endotoxin.

Bacterial Endotoxin

The endotoxin used throughout this study was derived from frozen *Salmonella abortus equi* with trichloroacetic acid, according to the method of Boivin et al (13). It contained 2.5 per cent nitrogen and 38.5 per cent polysaccharide, calculated from the value of rhamnose analyzed (14, 15). The fifty per cent lethal dose on ten-day-old chick embryos was 0.51μg.

Antiserum to Salmonella abortus equi

Antiserum was prepared by immunizing Japan Albino rabbits with heat-killed bacteria.

Incubation Test System for Antigenicity

It was carried out by incubating endotoxin with the tissue extract at 37°C for one hour. The reaction mixture contained 1.5 ml of tissue extract, 0.4 ml of 0.02M phosphate buffer solution (pH 7.7) and 0.1ml endotoxin solution which contained 200μg in 0.02 M phosphate buffer solution. Following incubation, the reaction mixture was heated in a boiling water bath for five minutes and centrifuged at 4,000 rpm for ten minutes. The supernatant was assayed for the residual antigenicity.

Assay of Antigenicity

The supernatant of the reaction mixture was serially diluted with saline, using a twofold falling dilution. The titer of the residual antigenicity was determined by the ring test with the above mentioned anti-*Salmonella abortus equi* serum. The final determination of the titer was made one hour after layering.

Incubation Test System for Toxicity

The incubation system was the same as that for antigenicity except for the amount of endotoxin. The amount was sufficient to give a final concentration of 80 per cent chick embryo mortality per 0.1 ml of the reaction mixture. In addition, the supernatant obtained from the incubated reaction mixture was sterilized in a boiling water bath for five minutes and assayed for the residual toxicity with ten-day-old chick embryos.

Assay of Toxicity

According to the method of Smith and Thomas (16), ten-day-old chick embryos of White Leg Horn were used throughout this study. The eggs were incubated in a self-turning incubator regulated at 38.5±0.5°C. The mortality was determined after 20 to 24 hour inoculation.

Results and Discussion

Effects of Tissue Extracts on Endotoxin.

Table 1 shows the reduction of the antigenicity of the endotoxin by liver, spleen and kidney. The residual antigenic titer of endotoxin solution incubated with liver extract was one-eighth of that of the control and the titer of that with spleen was one-half. This result shows that the tissue extract inactivated the antigenic activity of the endotoxin. Liver was the most active organ in the inactivation of the antigencity of the endotoxin, spleen was the least and kidney showed intermediate activity.

Table 2 shows the reduction of the lethal activity of endotoxin by liver, spleen and kidney. The mortality of the endotoxin incubated with liver extract was

TABLE 1. *Reduction of Antigenic Activity of Endotoxin by Tissue Extracts*

Organ	Residual antigenicity				
	1	2	4	8	16[1]
Liver	±	−	−	−	−
Spleen	+	+	±	−	−
Kidney	+	±	−	−	−
Control[2]	++	+	+	±	−

Reaction mixture (2 ml) contained 1.5 ml of tissue extract, 200 μg endotoxin and 1/50 M phosphate buffer, pH 7.7.
Condition of reaction: 37°C, 1 hr.
1) Dilution of the reaction mixture in saline.
2) Endotoxin and phosphate buffer.

TABLE 2. *Reduction of Toxic Property of Endotoxin by Tissue Extracts*

Organ	Mortality
Liver	3/12[1] (25.0%)[2]
Spleen	1/10 (10.0%)
Kidney	2/ 9 (22.2%)
Control[3]	8/10 (80.0%)

Reaction mixture (2 ml) contained 1.5 ml of tissue extract, 1/50M phosphate buffer, pH 7.7 and 80 per cent lethal dose of endotoxin per 0.1 ml reaction mixture.
Condition of reaction: 37°C, 1hr.
1) Deaths/total eggs.
2) Death percentage.
3) Endotoxin and phosphate buffer.

25.0 per cent, that with spleen extract was 10.0 per cent and that with kidney extract was 22.2 per cent. This means, also, that the tissue extracts inactivated the lethal activity of the endotoxin. Liver, spleen and kidney were equally effective in the inactivation of the lethal activity of the endotoxin within the limits of accuracy of the assay system used. In addition, the liver extract had no agglutination of heat-killed bacteria from which the endotoxin was extracted and the inactivating properties were not decreased by being absrobed with heat-killed bacteria. So it is thought that the properties were independent of the antibody.

Trapani et al. (10) reported that rabbit liver displayed high effectiveness in the inactivation of pyrogenicity, while kidney showed significant and spleen no effectivness. Keene (9), on the other hand, detected the effectivness of the inactivation of tumor-necrotizing property in rabbit spleen, liver and kidney. On the inactivation of the toxicity on chick embryos by tissue extracts, Smith et al. (11) found dog spleen to be more effective than liver, whereas, kidney was ineffective. Farrer (17) observed guinea pig liver, kidney and spleen were equally effective.

It seems that the results that tissue extracts of mice inactivated endotoxin were similar to those of Keene and of Farrer on the inactivation of toxicity. There, however, has been almost no report on the inactivation of the antigenicity of the endotoxin by tissue extracts.

Comparing the results shown in Tables 1 and 2, the effectiveness in inactivating the antigenicity was not in parallel with that of the inactivation of toxicity on ten-day-old chick embryos. This suggests that there are two factors inactivating endotoxin in different ways.

Effective Fractions of Liver Extract.

This experiment was performed to separate those factors which inactivated the antigenicity and the toxicity of endotoxin, respectively. At first it was fractionated into 0–33 per cent, 33–50 per cent and 50–100 per cent saturated fractions.

TABLE 3. *Reduction of Antigenic Activity by Fractions of Liver Extract with* $(NH_4)_2SO_4$

$(NH_4)_2SO_4$ fractions (%saturation)	Residual antigenicity				
	1	2	4	8	16[1]
0— 33	++	+	+	−	−
33— 50	−	−	−	−	−
50—100	+	±	−	−	−
Control[2]	++	+	+	±	−

Reaction mixture (2 ml) contained 1.5 ml of the fraction, 200 μg endotoxin and 1/50M phosphate buffer, pH 7.7.
Condition of reaction: 37°C, 1 hr.
1) Dilution of the reaction mixture in saline.
2) Endotoxin and phosphate buffer.

TABLE 4. *Reduction of Toxic Property by Fractions of Liver Extract with* $(NH_4)_2SO_4$

$(NH_4)_2SO_4$ fractions (%saturation)	Mortality
0— 33	4/24[1] (16.7%)[2]
33— 50	5/24 (20.8%)
50—100	11/16 (68.8%)
Control[3]	11/18 (61.1%)

Reaction mixture (2 ml) contained 1.5 ml of the fraction, 1/50 M phosphate buffer, pH 7.7 and 80 per cent lethal dose of endotoxin per 0.1 ml reaction mixture.
Condition of reaction: 37°C, 1 hr.
1) Deaths/total eggs.
2) Death percentage.
3) Endotoxin and phosphate buffer.

As shown in Table 3, the inactivating effectiveness of the antigenicity was observed in the highest degree in the 33–50 per cent saturated fraction, while the 50–100 per cent one exhibited only little effectiveness and the 0–33 per cent one possessed almost no effectiveness.

Table 4 shows that the effectiveness in inactivating the toxicity was observed both in the 0–33 per cent and in the 33–50 per cent saturated fractions, while the 50–100 per cent one possessed none.

From the results in Tables 3 and 4, it was found that a factor which inactivated only the toxicity of the endotoxin was contained in the 0–33 per cent saturated fraction. The 33–50 per cent one possessed inactivating potential both for antigenicity and toxicity. The 50–100 per cent one displayed this potential for only antigenicity.

TABLE 5. *Reduction of Antigenic Activity by Fractions of Liver Extract with* $(NH_4)_2SO_4$

$(NH_4)_2SO_4$ fractions (%saturation)	Residual antigenicity				
	1	2	4	8	16
0— 35	−	−	−	−	−
35— 50	−	−	−	−	−
50—100	+	±	−	−	−
Control	++	+	+	±	−

See the legend of the Table 3.

TABLE 6. *Reduction of Toxic Property by Fractions of Liver Extract with* $(HN_4)_2SO_4$

$(NH_4)_2SO_4$ fractions (%saturation)	Mortality
0— 35	0/ 6(0 %)
35— 50	4/ 6(66.6%)
50—100	11/16(68.8%)
Control	11/18(61.1%)

See the legened of Table 4.

Subsequently, an attempt was made to separate these factors more distinctively and to obtain a more effective fraction for inactivating the antigenicity. The liver extract was fractionated into 0–35 per cent, 35–50 per cent and 50–100 per cent saturated fractions with ammonium sulfate.

As shown in Table 5, the inactivating potential for the antigenicity was found potent both in the 0–35 per cent and in the 35–50 per cent saturated fractions. Table 6 shows that the inactivating potential toward toxicity was contained only in the 0–35 per cent one.

From the results in Tables 5 and 6, the 0–35 per cent saturated fraction

displayed the inactivating potential in antigenicity and toxicity. The 35–50 per cent one possessed only the factor inactivating the toxicity.

Thus, the factor inactivating the toxicity was found in the 0–33 per cent saturated fraction and that inactivating the antigenicity in the 35–100 per cent one. It was likely that mouse liver possessed two kind of substances which inactivated the antigenicity and the toxicity of the endotoxin respectively and were distinguished by ammonium sulfate. They were inactivated by heating at 100°C for five minutes. These data suggest that the substances were proteinlike.

So far, it has been reported that the reduction of the antigenicity with serum or plasma accompanied the abolishment of the toxicity (5, 6, 8, 18). On the other hand, chemical modification of endotoxin resulted in reduction of the toxicity without affecting immunogenic potency and serological activity of the original (19). In another report, an epimeraseless mutant of *Salmonella*, which has an entirely different polysaccharide structure, has endotoxin of the same potency as the parent strain (20).

These observations that the endotoxin molecule has respective sites for the antigenicity and the toxicity support the results reported here.

Thus, it is said that mouse liver is capable of inactivating the antigenicity and the toxicity of the endotoxin respectively in different ways.

Acknowledgement

The authors are indebted for many valuable suggestions to Professor Akihiko Toba, the Faculty of Agriculture, Tokyo University of Agriculture and Technology, Dr. Yoshio Oki, the National Institute of Animal Health and to Mr. Shigeyoshi Hoshi and to Mrs. Tokiko Omori of this laboratory for their kind assistance. Thanks are also expressed to Professor Shusaku Nishida and other members, the Animal Breeding Laboratory, Faculty of Agriculture, Tohoku University, for their kind advice on usage of chick embryos, and Mr. Ryuichiro Iwaya and Mr. Kiyoshi Watanabe for giving them fertilized eggs.

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