Quantitative Evaluation for the Endotoxin Adsorption-Ability of Medicinal Carbon

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The therapy of mass alimentary intoxication caused by Salmonella and Enteropathogenic Escherichia coli is discussed. Endotoxin causes fervescence, disseminated intravascular coagulation or shock. When antibiotics are administered for the treatment of alimentary intoxication, toxicity of the endotoxin from the defluvium of bacillus mort body can cause serious symptoms.

We noted the strong adsorption force of medicinal carbon and evaluated the endotoxin adsorption ability of medicinal carbon quantitatively using a chromogenic endotoxin specific assay quantitatively. Methods for evaluating damage due to the oral invasion of endotoxin have hardly been developed. The presence of alimentary intoxication due to a large quantity of endotoxin in intestine was assumed, and an in vivo test was performed to evaluate the endotoxin adsorption ability of medicinal carbon based on the influence of particle size. An in vitro test showed the adsorption coefficient to increase with the reaction times of medicinal carbon and endotoxin. The quantity of adsorption of endotoxin decreased as the particle size of medicinal carbon increased. As in vivo test showed that a change in the body temperature of mouse, which is an index of the physiological activity of endotoxin, was reduced by the oral administration of medicinal carbon. Consisting of small sized particles is thus considered to have a useful synergistic effect on the antibiotic treatment of food poisoning.

Keywords — medicinal carbon, food poisoning, limulus test, chromogenic endotoxin specific assay

Introduction

In late years, large outbreaks of food poisoning caused by Salmonella or enteropathogenic Escherichia coli have been occurred in various countries in the world, and the therapies for them are argued†0. The pathogenesis of bacterial food poisoning is mainly based on the bacterial invasion into a tissue and/or endotoxin excreted by the organisms. On the other hand, the causative gram negative bacteria have an endotoxin in the outer membrane of their cell envelopes. The endotoxin consists of a complex of a lipopolysaccharide (LPS) and protein(s). The lipopolysaccharide consists of a
complex of lipid, called lipid A, to which is attached a polysaccharide of a core and terminal series of repeat units.

Lipid A consists of a chain of glucosamine disaccharide units connected by pyrophosphate bridges, to which a number of long chain fatty acids are attached. LPS that has a moiety of toxic lipid A is causative in vivo of the various symptoms such as the fever, disseminated intravascular coagulation (DIC), and a shock. In the therapy for food poisonings, easy antibiotic administration leads to the leak of endotoxin from the killed organisms and provokes further serious symptoms in the patient.

Endotoxin examination has been evaluated up to now qualitatively or semi-quantitatively by the pyrogen test or the limulus test which used rabbit or the limulus amoebocyte lysate. Recently, the limulus test was remarkably improved and the alternative chromogenic substrate method was developed, that allowed to detect endotoxins quantitatively.

We noted the property of medicinal carbon to adsorb various kinds of substances on the surface of structure, and the evaluation of medicinal carbon as an endotoxin adsorbent was carried out quantitatively using the chromogenic method.

Materials and methods

1. Chemical reagents

The medicinal carbon described on the Japanese pharmacopoeia was obtained from Oriental Co., Ltd., Yamagata, Japan, and it was fractionated according to the particle size to four classes of sizes through the sieves of (A) 60 to 100 meshes, (B) 100 to 150 meshes, (C) 150 to 200 meshes, and (D) 200 mesh passed. Ordinary Kremezin (Kure emergence studies, Sankyo, Tokyo) which had been administered to the patient with renal failure as a control pharmaceutical preparation was used. In vivo test, the standard solution of endotoxin (57.4EU/mL) was purchased from Sigma Chemical Co., St. Louis, Mo. USA and prepared with asepsis. All glassware and buffer solutions were sterilized by heating at 200°C for 2.0h and autoclaved at 120°C for 1.5h, respectively. Both reagents, an Endospacy (ES-20S kit) and an Endospeck (ES test TE diazo-coupling reagent) were purchased from Seikagaku Kogyo, Co., Ltd., Japan, and were used for experiments using the chromogenic method.

2. Quantification of endotoxin

The ability of medicinal carbon to adsorb endotoxin was evaluated by measuring the quantities of medicinal carbon and endotoxin were mixed of non-adsorbed endotoxin upon the known together.

As shown in Fig. 1, 0.01g of medicinal carbon and 0.5 mL of standard endotoxin solution (0.12EU/mL, Seikagaku Kogyo, Co., Ltd., Japan) were added into an endotoxin-free test tube and the mixture was shaked at room temperature. After the shaking for 30min, the mixture was filtered through a membrane filter of 0.45μm pore size (Miles HV, Nihon Milipore Ltd., Japan). A 100μL aliquots of the filtrate was used as a sample. Endotoxin in the sample was quantitated by the chromogenic substrate method using an Endospacy (ES-20S kit) and an Endospeck (ES test TE diazo-coupling reagent set).

Endotoxin-free water for the oral administration was used for a blank solution. Net amounts of endotoxin were calculated from the calibration curve that was preliminally prepared.

3. Studies on the factors affecting endotoxin adsorption

3-1. Quantity added with medicinal carbon

In order to determine the amount of medicinal carbon that should adsorb the known quantities of endotoxin, various amounts of medicinal carbon (0.005g, 0.010g, 0.020g and 0.050g) were added to the 0.5mL of standard endotoxin solution, respectively. The endotoxin that was not adsorbed with the medicinal carbon was determined according to the procedure described above. Measurement was repeated 5 times for each preparation, and a mean value was obtained.

3-2. The particle size of the medicinal carbon

Each fraction of charcoal (A to D) was used for the adsorption of endotoxin, and the endotoxin remained in the filtrate was determined as described above, and their abilities of adsorption were compared each other. Measurement was...
repeated 3 times and a mean value was calculated.

3-3. Reaction time for the medicinal carbon and endotoxin
Charcoal was added to the solution of endotoxin and the interaction between them was carried out in the test tube for 1, 5 and 30 min, respectively. After filtration, each quantity of endotoxins in the filtrate was determined according to the procedure described above. Measurement was repeated 3 times, and a mean value was calculated.

3-4. Reaction temperature
Interactions between medicinal carbon and endotoxin were carried out at 25°C and 37°C. The quantity of endotoxin which had not been adsorbed on the medicinal carbon at each reaction temperature was compared with each other. The reaction was carried out in the incubator established in a constant temperature. Measurement was repeated 3 times and a mean value was calculated.

3-5. In vivo examination
One milliliter of the standard endotoxin solution was given through an oral sonde to each of 4 weeks old male ICR mice which were fasted from the day before. Thirty minutes after the oral administration, 0.12 mL of medicinal carbon that contained 0.04 g of medicinal carbon fraction (B) or (C) was administered by the same manner as described above to the mice. Before the endotoxin dosage, we measured rectal temperature of the mice using a digital thermometer PC 94000, SATO Co. Ltd., Tokyo, and the temperature was compared with that of the same mice in 3 h after oral administration of endotoxin. As a control, we established two groups of mice. One group of mice was administered with 1 mL of endotoxin-free water and another group of mice was not administered with the medicinal carbon. At the same time of the measurement of the rectal temperature, we observed the animal behavior. For the experiment, each group of 5 mice was used. A mean value was calculated after 3 repeated measurements.

Results

1. Effectiveness of a calibration curve
In order to confirm the reliability of the measurement kit used in the present study, we prepared calibration curve. In the chromogenic substrate method, in which the chromogenic substrate is used in place of limulus amoebocyte lysate for the horseshoe crab clotting enzyme, it is possible to calculate quantity of endotoxin by single point method using a reference standard. In the curve, X axis is for the value of absorbance at 545 nm wave length, and Y axis is for the endotoxin-density. The curve showed a linear expression between the endotoxin-density at 0.12 EU/mL and that at 0.001875 EU/mL. This curve gave the following first order equation:

\[ Y = 0.16483X - 0.00228 \quad (r^2 = 0.998) \]

From first order equation, reliability of this assay was confirmed.

2. Decision of medicinal carbon amount added into solution
As the amount of medicinal carbon added into the solution increased, a quantity of the endotoxin remained in the filtrate decreased. When the 0.020 g of medicinal carbon was added to the endotoxin solution, almost 100% of the endotoxin was adsorbed (Fig. 2). According to the Figure 2, we considered that 0.010 g of medicinal carbon should be enough as the amounts to be added in the following experiments.

3. Effect of particle sizes of medicinal carbon on endotoxin adsorption ability
Each fraction (A to D) of the medicinal carbon was evaluated at the point of view of the endotoxin adsorption. After addition of each fraction, the quantity of endotoxin remained in the filtrate was determined (Fig. 3). Among the all fractions, even the fraction A that corresponds to the largest size of particles (60-100 meshes), adsorbed endotoxin dramatically. Approximately 90% of the endotoxin was adsorbed by the fraction A. Endotoxin remained in the filtrate was determined as 0.01275 ± 0.00451 EU/mL.

When the fraction B, C, or D was added, more amounts of endotoxin were adsorbed. Quantity of endotoxin remained in the filtrate was 0.002034 ± 0.00087 EU/mL, 0.00161 ± 0.00121 EU/mL, and 0.0000249 ± 0.00000 EU/mL, respectively, indicating surface coverage of them was approximately 98%, 99% and 100%, respectively. In this study,
the larger sizes of particles gave the large amount of the remained endotoxin, indicating the tendency that smaller sizes of particles adsorbed more endotoxin because of the larger surface area.

On the other hand, the same amount of a pharmaceutical preparation (Kremezin®), which had been marketed as a capsules, was used for the endotoxin adsorption test. Almost all amount of endotoxin was not adsorbed by the Kremezin® particles and approximately 100% of endotoxin was found in the filtrate. It seems that this is the result due to the spherical medicinal carbon-particles in a capsule which was designed for adsorption of the substances having molecular weight less than 1000.

4. Effects of the reaction time on the endotoxin-adsorption ability of medicinal carbon

We show the relationship between the reaction time and the quantity of non-adsorbed endotoxin in Fig. 4. The quantity of endotoxin remained in the filtrate was 0.0305 EU/mL in a 1 min-reaction time, and 0.0078EU/mL in a 5 min-reaction time, indicating the surface coverage was approximately 75% and 93.5%, respectively.

On the other hand, the quantity remained in the filtrate after a 30 min reaction was 0.0016EU/mL and the detection was impossible in 37°C Thus, assuming the reaction in a body, the adsorption ability should be increased by the temperature higher than the room temperature (Fig. 5).

6. Oral administration of endotoxin and medicinal carbon to mice

A change of body temperature after oral administration of endotoxin and medicinal carbon to mice was shown in
Table 1. The rectal temperature in each group of mice, which was given with water, endotoxin standard solution, medicinal carbon fraction A plus endotoxin standard solution, and medicinal carbon fraction C plus endotoxin standard solution, were 37.7±0.3°C, 37.9±0.7°C, 38.0±0.4°C and 38.1±0.4°C, respectively. The rectal temperature start after 3h descended inconsiderably, and each differences became -1.10±0.44°C, -0.19±0.60°C, -0.45±0.78°C and -1.23±0.79°C, respectively. The changes of the rectal temperatures in mice given water and carbon fraction A with endotoxin standard solution were significantly low (p<0.05 in t-test) compared with those given endotoxin standard solution. The results showed the administration of medicinal carbon to the mice brought about the restraint of rectal temperature change. The behavior of mice given with only endotoxin standard solution showed the tendency to become slow activity compared with the mice those were administered with medicinal carbon. These findings suggest that the rectal temperature of the mice raised by the oral administration of endotoxin, and that the rise of temperature was restrained by the additional administration of medicinal carbon. It was also found that the effect of medicinal carbon was dependent on its particle size and the smaller size of particle was more effective in the restraint.

Discussion

Endotoxin is a strong pyrogen and has various biological activities. It is a component of gram-negative bacteria, some of which causes a large outbreak of food poisoning, and provokes further serious symptoms in the patient\(^{12,13}\). There are several methods to detect endotoxin including the pyrogen test using rabbits\(^{14}\) and the limulus test\(^{15,16}\). Among them, the limulus test had been begun to develop with the gelation method that utilized the principle that limulus amoebocyte extract was solidified by endotoxin. Using the chromogenic substrate method, we made possible to evaluate the effect of medicinal carbon quantitatively, which should be used for the treatment of food poisonings.

On the other hand, it seems that parenterally discharged endotoxin and endotoxins derived from live and/or killed enteric bacteria will give various influences to a body. However, the evaluation method for that is not known at all so long as we know. Antibiotic abuse leads to the killing of a large number of bacteria existing in the intestine, and by it a large quantity of endotoxins should be released to the intestinal tract\(^{17}\). We administered a large quantity of endotoxin into a murine intestine experimentally, and established the method to evaluate the influence of particle size and endotoxin-adsorption ability of medicinal carbon.

As the results, medicinal carbon with the small particle size showed the higher ability to adsorb the endotoxin. Clinically, it is hoped that oral administration of the medicinal carbon with small sizes would be useful for a supporting agent upon the antibiotics therapy.

Food poisonings by the microorganisms have been occurred world-wide\(^{18}\). Among the developing countries where the knowledge and techniques for the medicine have not spread and not easy to use expensive antibiotics. It has been known that antibiotic abuse allows the production and increase of resistant microbes. As a circle of an alternative medicine, we noted the utilization of medicinal carbon for the medicine, especially for food poisonings. Medicinal carbon is amorphous carbon from the incomplete combustion of animal or vegetable matter such as a wood, and it will be possible to get it in plenty and easily in many developing countries in the world. From the ancient times in the Orient, it has been said that carbon powder has the actions of intestinal detoxification and purifications in a body. In the present study, we demonstrated experimentally a part of usefulness of medicinal carbon for food poisonings.

Because the medicinal carbon has been experimentally suggested to be effective for the prevention and treatment of food poisonings, it will be perspective to develop the new carbon preparation for them.

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References


