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[54] **QUANTITATIVE DETECTION OF ENDOTOXIN IN BIOLOGICAL FLUIDS**

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Related U.S. Application Data

[63] Continuation of Ser. No. 40,348, May 25, 1970, abandoned.

[52] **U.S. Cl.** ..... **195/103.5 R;** 23/230 B; 424/2; 424/95; 424/101

[51] **Int. Cl.²** **G01N 31/00;** C12K 1/00

[58] **Field of Search** ..... 195/103.5; 23/230 B; 424/95, 101

[56] **References Cited**

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[57] **ABSTRACT**

An in vitro method as well as an article is provided for quantitative detection of endotoxin in biologicals, biological fluids, such as blood, and any protein containing material by an indicator comprising amebocyte lysate. This method or technique consists in first centrifuging a blood sample to obtain plasma, and then admixing chloroform with the plasma to precipitate certain protein fractions of the plasma. The mixture of chloroform and plasma is then centrifuged to sediment the precipitated protein and results in the formation of an aqueous layer, an intermediate layer, and a chloroform layer. The intermediate layer is then removed, and a clottable substrate, namely amebocyte lysate is then admixed with the removed intermediate layer. The rate of reaction which is proportional to the concentration of endotoxin in the sample is measured as manifested by the increase in turbidity thereof. The clottable substrate, namely amebocyte lysate, is obtained from amebocytes of Limulus and is provided as an article of manufacture for use in the detection of the endotoxin in the biological fluid sample.

7 Claims, No Drawings

QUANTITATIVE DETECTION OF ENDOTOXIN IN BIOLOGICAL FLUIDS

This is a continuation of application Ser. No. 40,348, filed May 25, 1970, now abandoned.

This invention relates generally to hematology and infectious diseases, and more particularly it pertains to an indicator and method for detecting bacterial endotoxin in protein containing materials, such as blood.

In connection with this invention, biologicals are defined as medicinal perparations made from living organisms and their products, including serums, vaccines, antigens, and antitoxins, all of which contain protein, while biological fluids are defined as various substances obtained from living organisms, such as blood, cerebrospinal fluid, exudates, and transudates. It is to be noted that while the present invention is illustrated in connection with determining endotoxin in blood, it is not limited thereto as any biologicals, biological fluids, or protein containing substance can be used in this process and the article pertaining thereto is equally applicable.

Bacterial endotoxin is defined as some part of the bacterial cell wall of organisms classified as gram-negative, that is, they do not take gram stain. Generally, such organisms contain endotoxin and many of their biological effects and the clinical illnesses they cause are believed due to the endotoxin which is an intrinsic part of their structures.

The chemical nature of endotoxin while broadly described as a lipopolysaccharide, has not been clearly defined. Which part of the endotoxin molecule is responsible for its biological effects in certain clinical illnesses is still not clear.

The significance of a positive test would be that in the case of a patient, the physician would know that he had detected endotoxin in the blood (i.e. endotoxemia). He then would be able to initiate appropriate therapy for the effects of the endotoxin or for an underlying disease which might account for its presence.

Another use of the test would be by pharmaceutical companies or other facilities that require pyrogen testing; that is, detection of the presence of endotoxin in biologicals, biological fluids, and other protein containing materials for human use.

Conventional methods for the detection of endotoxin have in the past involved biological assays using test animals, such as rabbits. The response of the animal was taken as a measure of the presence of endotoxin in the fluid administered.

It is an object of this invention, therefore, to provide a simple and rapid method for the detection of endotoxin, which is clinically practical and which can be done in vitro (i.e., does not require test animals).

Another object of this invention is to provide a method of preparing a specimen of biological fluid (i.e., blood) for endotoxin testing in a manner which yields a high sensitivity.

Clottable protein in the amebocytes of the horseshoe crab (Limulus) is a sensitive indicator of bacterial endotoxin. Table I below, for example, shows the effect of endotoxin concentration upon the rate of increase in light scattering, where the reaction mixture consisted of 0.9 ml of lysate (clottable substrate) and 0.1 ml of endotoxin. The final endotoxin concentration (μg/ml) is shown. It is to be noted that the Δ light scattering was the maximum percent increase in light scattering per minute.

TABLE I

Effect of Endotoxin Concentration Upon the Rate of Increase in Light Scattering

Endotoxin (μg/ml)	Latent Period (min)	Δ Light Scattering (Maximum/min)
100	5	8.7
25	7	9.5
10	9	7.0
2	15.5	4.4
1	23.0	3.9
0.5	22.5	3.2
0.05	40.0	1.3
0.02	56.0	0.38
0.01	77.0	0.20
0.0075	110.0	—

The clottable protein in the horseshoe crab can detect as little as five ten-thousandths (0.0005 μg/ml) microgram of endotoxin per milliliter.

This sensitivity, however, has not been achieved in the past with in vitro or in vivo testing because of an inhibitory effect of blood which is not well understood, as indicated by Table II below. Table II shows the effect of blood and blood coagulation on the detection of endotoxin, in vitro.

TABLE II

Effect of Blood and Blood Coagulation on the Detection of Endotoxin, in vitro*

Initial Endotoxin Concentration (μg/ml)	Plasma	Serum	NaCl
0.001	Neg.	Neg.	Pos.
0.02	Pos.	Neg.	Pos.
0.1	Pos.	Neg.	Pos.
0.2	Pos.	Neg.	Pos.
0.4	Neg.	Neg.	Pos.
2.0	Pos.	Pos.	Pos.
5.0	Pos.	Neg.	Pos.

*It is to be noted that the plasma and serum (each obtained from the same donor) were separated from blood to which endotoxin had been added, and then tested for endotoxin activity after their removal from the blood cells or fibrin clot. NaCl indicates the controls in which identical concentrations of endotoxin were present in saline. In every instance in which endotoxin was detected in plasma and saline, the rate of gelation was faster in the NaCl control.

The decrease in the ability to detect endotoxin after exposure to blood is presumably the result of inactivation of the biologically active ("endotoxic") polysaccharides by serum or plasma.

According to the present invention, a technique has been devised for the preparation of blood samples so that endotoxin may be detected by the aforesaid clottable protein. See Table III below which gives data relating to the recovery of endotoxin activity from plasma following extraction with chloroform.

The blood sample in which endotoxin is suspected to be present is centrifuged so that the plasma is separated from the blood cells. The plasma which is obtained from the heparinized blood is then mixed with chloroform, one part chloroform to four parts plasma.

The mixture is then vigorously agitated for one hour and the material is then re-centrifuged so that the aqueous layer is on top and the chloroform layer containing precipitated protein is at the bottom. This results in an interface between the aqueous and chloroform layers which has a cloudy appearance. This intermediate (middle) layer contains a concentration of detectable endotoxin almost equal to that present in the original sample of whole blood. However, endotoxin can also be detected in the top layer, and there are instances when this layer may be used.

The cloudy middle layer is removed by pipette or needle and syringe and mixed with an equal volume of amebocyte lysate. The incubation mixture is incubated at 37°C. in a water bath and observed visually. Other temperatures can be selected for the incubation. The reaction occurs at a rate proportional to the concentration of endotoxin in the blood or other solution tested. It is manifested by an increase in cloudiness of the mixture, which can be recognized with the eye or measured more accurately with any type of light scattering apparatus. Thus, increased turbidity of the mixture indicates the presence of endotoxin. It is also to be understood that increased viscosity of the mixture indicates the presence of endotoxin.

During the observation, it is desirable to incubate the assay mixture in a 37°C. water bath and visually observe the reaction, first at short intervals, and then, at longer intervals. Optionally, the increase in light scattering in the incubation mixture may be monitored on a turbidity meter (nephelometrically).

TABLE III

Recovery of Endotoxin Activity from Plasma Following Extraction with Chloroform*

Endotoxin Concentration (μg/ml)	Duration of Exposure of Plasma to Chloroform 15 min.**	30 min.**	60 min.**	60 min.***
0.1	5/7	3/3		1/1
0.01	5/14	4/4		1/1
0.005	1/4	3/3	2/2	1/1
0.001	0/3	0/4	3/4	7/7
0.0005		0/1	3/4	3/3
0.0001		0/1	0/3	0/3
Control	0/9	0/8	0/4	0/7

*The incubation mixture consisted of 1 part middle layer and 1 part amebocyte lysate. The fractions indicate the number of positive tests/total number of experiments at each endotoxin concentration. Thirty-eight different blood donors were used in these experiments.

**Endotoxin was added to plasma.

***Endotoxin was initially added to whole heparinized blood before the plasma was prepared.

The clottable substrate referred to herein essentially comprises plasma-free Limulus amebocytes which are separated from plasma in a sample of Limulus blood, the Limulus used being the horseshoe crab. The blood is drawn directly into sterile, pyrogen free, siliconized syringes or flasks preheated to 40°C and containing tris-buffered N-ethyl maleimide (NEM) at pH 7.28 and 40°C. The ratio of whole blood to NEM is 1:1 producing a final concentration of NEM of $5 \times 10^{-}$M. The NEM is used to prevent [3]aggregation.

After centrifuging the blood samples at 600 rpm for one minute, or allowing the cells to sediment for one hour, the supernatent material is poured off and the sedimented cells resuspended in 10 ml. of buffered NEM, 1×10^{-2} at 40°C. This procedure is repeated twice.

The amebocytes are then twice washed and resuspended in artificial buffered sea water. These washed cells are then lysed by freezing and thawing four times in dry ice in acetone, or by exposure to distilled water. The cell lysate is then cleared by centrifugation at 2000 rpm for five minutes, yielding a clear colorless liquid composed of cellular lysate in artificial sea water or distilled water, which then may be used to detect endotoxin. This form of the material is stable and will keep for many months at 4°C. without losing potency.

Obviously many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims the invention may be practiced otherwise than as specifically described.

What is claimed is:

1. An in vitro method for quantitative detection of endotoxin in a sample of protein-containing material, comprising:

admixing chloroform with the sample to precipitate certain proteins therefrom;

separating the precipitated proteins from the sample;

admixing lysate of plasma-free Limulus amebocytes with said sample from which the precipitated proteins have been separated; and,

measuring the rate of reaction between endotoxin in the sample and the lysate, the rate of reaction being proportional to the concentration of endotoxin in the sample as manifested by the increased turbidity or viscosity thereof.

2. The method of claim 1 wherein the second-mentioned step comprises centrifuging the sample from which certain proteins have been precipitated and removing said precipitated proteins from the sample.

3. The method of claim 1 and further comprising:

sedimenting the sample to which chloroform has been added to form an aqueous layer, an intermediate layer, and a chloroform layer in the sample;

removing at least a portion of said intermediate layer; and,

admixing said removed portion of said intermediate layer with lysate of plasma-free Limulus amebocytes.

4. An in vitro method for quantitative detection of endotoxin in a protein containing material, comprising, admixing chloroform with said protein containing material to precipitate certain proteins from said protein containing material, centrifuging said precipitated proteins to obtain a sample contaminated with endotoxin, admixing Limulus amebocyte lysate with said sample, and then measuring the rate of reaction which is proportional to the concentration of endotoxin in said sample as manifested by the increased turbidity or viscosity thereof.

5. An in vitro method for quantitative detection of endotoxin in biological fluids, comprising, admixing chloroform with a biological fluid to precipitate certain proteins of said biological fluid, centrifuging said mixture of chloroform and biological fluid to sediment the precipitated proteins resulting in the formation of an aqueous layer, an intermediate layer, and a chloroform layer, removing a sample from said intermediate layer, admixing Limulus amebocyte lysate with said removed sample, and then measuring the rate of reaction which is proportional to the concentration of endotoxin in said sample as manifested by the increased turbidity or viscosity thereof.

6. An in vitro method for quantitive detection of endotoxin in blood, comprising, admixing chloroform with a blood plasma to precipitate certain proteins of said plasma, centrifuging said mixture of chloroform and plasma to sediment the precipitated proteins resulting in the formation of an aqueous layer, an intermediate layer, and a chloroform layer, removing a sample from said intermediate layer, admixing Limulus amebocyte lysate with said removed sample, and then measuring the rate of reaction which is proportional to the concentration of endotoxin in the sample as manifested by the increased turbidity or viscosity thereof.

7. An in vitro method for quantitative detection of endotoxin in blood, comprising, centrifuging a sample of blood to obtain plasma, admixing chloroform with said plasma to precipitate certain proteins of said plasma, centrifuging said mixture of chloroform and biologial fluid to sediment the precipitated proteins resulting in the formation of an aqueous layer, an intermediate layer, and a chloroform layer, removing a sample from said intermediate layer, admixing Limulus amebocyte lysate with said removed sample layer, and then measuring the rate of reaction which is proportional to the concentration of endotoxin in the sample as manifested by the increased turbidity or viscosity thereof.

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