The *Limulus* Coagulation Test for Endotoxin. A Comparison with Other Assay Methods[1]

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The present paper reports recent studies of the *Limulus polyphemus* horseshoe crab) coagulation test for endotoxin of Levin and Bang (1). The test utilizes a lysate of the blood cells (amebocyte) from *Limulus,* which forms a gel when exposed to trace amounts of bacterial endotoxin. The mechanism of this reaction is unknown, but an analogy between this reaction and the altered coagulability of blood in animals with gram-negative sepsis has been suggested (1). Under ideal circumstances this test can detect as little as 10^{-6} mg of endotoxin.

Method. The lysate is prepared as follows. A 15-gauge needle is inserted into the junction of the thoracic and abdominal segments of the *Limulus* after the area has been cleansed and flamed. The blood is allowed to flow into a sterile pyrogen-free flask containing pyrogen-free solution of NEM, *i.e.,* 3% saline to which *N*-ethylmaleimide (NEM) is added to a concentration of 0.125%. After sedimentation, which requires 1–4 hr, the plasma is discarded and the cells washed three times with NEM solution, and finally with pyrogen-free artificial sea water. The sedimented cells are then ruptured by adding 7.5 ml of pyrogen-free distilled water per 100 ml of the total volume of blood from which the cells were harvested. The cell debris is removed by centrifugation at 2500 rpm for 15 min. The lysate, which is stored at 4°, retains clotting activity for at least 4 weeks.

Before use the lysate is diluted with an equal volume of phosphate buffered (0.05 *M*) saline at pH 7.2. One-tenth ml or more of test material is added to 0.9 ml of the diluted lysate and incubated at 37°. The formation of a gel, *i.e.,* no fluid displacement on slanting the tube to 45°, within 1 hour is taken as evidence of the presence of endotoxin.

Normal plasma and whole blood, with rare exceptions, do not cause gel formation. Table I shows the results of testing for specificity by comparing endotoxin with all the known vasoactive substances, except angiotensin, present in normal plasma. The data show that none of these vasoactive components cause gel formation.

Table II presents the data on the relative sensitivity of three different tests for endotoxin: (i) the *Limulus* test; (ii) the intracutaneous epinephrine sensitivity test of Thomas which, as modified by Kass (2), requires keeping the test animal (rabbit) at an ambient temperature of 37°; and (iii) the immunoassay of Skarnes (3), which identifies the precipitation arcs of two major antigenic components of an endotoxin. Various concentrations of endotoxin were tested after incubation in saline and after detoxification by three different inactivating media: (i) fresh splenic extract (4); (ii) ACD plasma (3); and (iii) 0.025% NaOH (3). The substances were incubated for 3 hr at 37° in all but the last, which was run at 60°. Since samples of all the incubation mixtures taken during the earlier phases of the inactivation period coagulated the gel, one can exclude inhibition of the coagulation properties of the gel by the inactivating media.

Results. For quantities of endotoxin which

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TABLE I. Specificity Tests for Clot Formation of Amebocyte Lysate of *Limulus*.

	Titer in normal plasma (mg)	Test substance (mg/ml of lysate)						
		1×10^{-8}	1×10^{-7}	4×10^{-6}	1×10^{-5}	1×10^{-4}	1×10^{-3}	1×10^{-2}
Serotonin	$1–3.3 \times 10^{-4}$				0	0	0	
Histamine	$2–8 \times 10^{-5}$			0	0	0	0	
Epinephrine	1×10^{-7}	0	0	0				
Norepinephrine	5×10^{-7}	0	0	0				
Bradykinin	?					0	0	0
Endotoxin			0	+	+	+	+	+

[a] The least amount of endotoxin detectable by most preparations of the lysate by the technique described is 4×10^{-6} mg.

all three tests can detect, *i.e.*, 1.4×10^{-3} mg or more, the results were identical. However, the *Limulus* test was clearly the most sensitive of the three, as it was consistently positive in all endotoxin dilutions tested. The epinephrine skin sensitivity test was not consistently positive at endotoxin levels below 1 μg, and the immunoassay test was not of sufficient sensitivity below 1.4 μg. The immunoassay test detects detoxification by the loss of precipitation of one of the major antigenic components. Since there is good agreement between the *Limulus* test and the immunoassay test for the measurement of detoxification, it may be expected that the use of the *Limulus* test for monitoring endotoxemia produced by injected endotoxin will not give positive reactions with endotoxin which has been detoxified.

Table III gives the results obtained by the *Limulus* test for monitoring endotoxemia produced by injecting iv an MLD/80 dose of endotoxin (1 mg/kg of *E. coli* $011b_4$) into each rabbit in three groups of rabbits: (a) normal rabbits, (b) rabbits made resistant to endotoxin by increasing doses of endotoxin ranging from 0.05 to 2 mg every 48 hr for 10 days, and (c) rabbits with denervated spleens produced by injecting 2 ml of Cyclane (Merck Sharp Dohme; 5 mg/ml) into the splenic pedicle exposed by a laparotomy incision. This incision was performed on all animals in each group. Table III shows that endotoxin injected intravenously continues to circulate in a toxic form in normal rabbits for at least 3–4 hr after injection. This is in accord with the observations of others (5). On the other hand, most rabbits which are made resistant to endotoxin clear the circulation of the toxin within 15–30 min. Table III shows further that denervating the spleen, which by preventing vasoconstriction keeps the reticuloendothelial system in this organ fully available for clearance (6), achieves a

TABLE II. Sensitivity Tests for Endotoxin.[a]

Endotoxin (mg/test)	*Limulus* clot (Levin and Bang)				Immunoassay[b] (Skarnes)				Epinephrine skin sensitivity (Thomas and Kass)			
	Pl	SE	Alk	Saline[c]	Pl	SE	Alk	Saline[c]	Pl	SE	Alk	Saline[c]
4×10^{-6}	0	0	0	+ (5/5)	—	—	—	—	—	—	—	—
4×10^{-5}	0	0	0	+ (5/5)	—	—	—	—	0	0	0	0 (8/8)
4×10^{-4}	0	0	0	+ (5/5)	—	—	—	—	0	0	0	+ (3/6)
1×10^{-3}	0	0	0	+ (5/5)	—	—	—	—	0	0	0	+ (5/5)
1.4×10^{-3}	0	0	0	+ (5/5)	0	0	0	+ (5/5)	—	—	—	—

[a] All tests performed at pH 7.3.

[b] Immunoassay test insensitive for amounts below 1.4×10^{-3}.

[c] Pl = ACD plasma; SE = splenic extract; Alk = 0.025 NaOH. Figures in parentheses: denominator gives number of tests by each technique.

TABLE III. *Limulus* Assay for Clearance of Endotoxin from Plasma.[a]

	After intravenous injection of endotoxin						
(min):	0	15	30	60	120	180	240
Normal rabbits, control	0/10[b]	10/10	10/10	10/10	10/10	9/10	8/10
Rabbits with denervated spleen	0/10	9/10	8/10	2/10	0/10	0/10	0/10
Endotoxin resistant rabbits	0/10	4/10	2/10	0/10	0/10	0/10	0/10

[a] Positive test for endotoxin is formation of a firm gel in 60 min at 37°.

[b] Numerator = number of tests positive for endotoxin; denominator = number of rabbits tested.

reduction in the titer of circulating endotoxin that is nearly equal to that of the resistant group.

Summary. The foregoing data, together with previous data on such other tests as the chick embryo and pertussis-treated mouse (7), indicate that the *Limulus* gel test is the most sensitive test available for the detection of endotoxin in its toxic or undegraded state. The test appears to be specific for endotoxin, since the amebocyte lysate is not clotted by normal blood or plasma or the individual vasoactive constituents known to be present in plasma of rabbits, dogs, or man. Because of its advantages in cost, simplicity, and sensitivity over all other available tests, it is to be preferred for monitoring a known endotoxemia, and for the detection of spontaneous endotoxemia.

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