JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1978, p. 139-141
0095-1137/78/0007-0139$02.00/0

Vol. 7, No. 2
Printed in U.S.A.

Survey for Positive *Limulus* Amoebocyte Lysate Test in Plasma from Humans and Common Research Animals

D. DUBOSE,* M. LEMAIRE, J. BROWN, D. WOLFE, AND M. HAMLET

Department of the Army, U.S. Army Research Institute of Environmental Medicine, Natick, Massachusetts 01760

Received for publication 27 October 1977

A survey for positive *Limulus* amoebocyte lysate tests was conducted on apparently healthy humans, mongrel dogs, rats, mice, rabbits, and squirrel monkeys. Only mongrel dog (45.8%) and human (32.8%) plasma samples gave positive tests. In dogs, a significant correlation between positive *Limulus* amoebocyte lysate tests and the presence of intestinal parasites was found. Positives found in human plasma samples were thought to be due to the presence of background levels of endotoxin or some possible mimicker substance found in the plasma after chloroform extraction. It was concluded that there was a need to distinguish between these positive *Limulus* tests and those which represent significant endotoxemia.

The *Limulus* amoebocyte lysate (LAL) test has been used to indicate the presence of endotoxin in a variety of test samples and to detect gram-negative sepsis in urine, spinal fluids, joint fluids, and blood (1, 2, 5, 8, 11, 13, 16-18). Investigators have stated that normal plasma is negative (11, 14). To determine whether background levels of endotoxin might contribute to the ambiguities found in previous studies employing the LAL test in blood plasma (3, 16), a survey for positive LAL tests in apparently healthy humans, mongrel dogs, rats, mice, rabbits, and squirrel monkeys was conducted.

MATERIALS AND METHODS

Animals from research laboratory colonies were selected and given an examination by a veterinarian to determine state of health. In the dogs, in addition to a complete physical examination, blood chemistries were run. Only dogs showing a normal blood chemistry were selected for LAL testing. From laboratory personnel, subjects were selected for LAL testing after screening by a physician to ensure that no subject had elevated body temperatures or obvious infections. The site for bleeding was scrubbed three times with betadine soap and alcohol rinse. Blood samples were collected by percutaneous puncture using pyrogen-free syringes and needles. In four dogs (see Table 2), in addition to percutaneous puncture, a vein was exposed for bleeding by using aseptic surgical technique, to determine whether percutaneous puncture led to contamination of sample. Five-milliliter samples of blood were placed in endotoxin-free vials containing 300 U of endotoxin-free heparin. These heparinized vials were obtained from Sigma Chemical Co. (St. Louis, Mo.). LAL testing used Sigma Chemical Co. lysate (Etoxate) following the procedures found in Sigma bulletin no. 210 for chloroform extraction and testing of plasma samples. *Escherichia coli* endotoxin 0113, PP-E-434 (Associates of Cape Cod, Woods Hole, Mass.), was used to determine lysate sensitivity. Only those lots of lysate which gave a solid gel with at least 1 ng of this endotoxin per ml in 1 h of incubation at 37°C were used. To ensure proper chloroform extraction of inhibitors found in plasma, each sample tested had a positive plasma control, which consisted of the addition of a final concentration of 1 ng of endotoxin per ml to the extracted test plasma. Negative controls were set up to ensure that glassware and lysate were not contaminated with endotoxin. In addition to these controls, each test had a negative plasma control consisting of 0.2 ml of extracted test plasma without lysate. This was set up to detect any false positives due to spontaneous gelling of extracted plasma. All readings were made at 1 h except where otherwise noted. The degree of positive reaction was rated between +4, a firm gel, and +1, representing a change in viscosity in the reaction. In one group of dogs (see Table 3), in addition to plasma LAL testing, stool samples were taken and examined microscopically for parasites to determine the relationship between positive LAL test and presence of intestinal parasites. In this group, an 18-h reading (first hour at 37°C, next 17 h at room temperature) was included, since it was assumed that endotoxin level would be low in these dogs due to the prophylactic treatment to control dog parasites in our colony. These dogs were also obtained from a source different from those dogs found in Table 1, and fewer dogs were found to have parasites.

RESULTS

Table 1 shows that there were no positives found in rats, mice, rabbits, or monkeys. In dogs, 44.8% were found to be positive, and, in humans, 31.8%.

Table 2 shows a comparison of percutaneous

puncture with vein exposure as a method for collecting dog blood samples without contamination. Results were the same with both methods.

To assess parasitic infestation as a factor involved in positive LAL tests, 13 parasite-positive dogs and 13 parasite-negative dogs were tested. Table 3 shows that a total of 8 out of the 13 (61.5%) parasite-positive dogs had a positive LAL test by the 18-h reading, whereas only one of the dogs negative for parasites had a positive LAL test. A chi-square test of the frequencies of positive LAL tests indicated a significant difference between the groups ($\chi^2 = 8.32$, df = 1, $P = 0.01$). The one parasite-negative dog which yielded a positive LAL test was monitored for the presence of parasites and positive LAL test for a 13-day period. In all tests, this dog was found to be negative for parasites. Three out of four LAL tests were found to be positive. Table 3 also shows that of the 13 dogs positive for parasites 7 had hookworms (*Ancylostoma caninum*) and 6 had roundworms (*Toxocara canis*). Of hookworm-infested dogs, 71.4% (5 of 7) had positive LAL tests by the 18-h reading; in roundworm-infested dogs, 50.0% (3 of 6) were positive. A chi-square test of the frequencies of positive LAL tests (18-h reading) showed no significant difference between the two types of parasitic infestations ($\chi^2 = 0.627$, df = 1, $P = 0.05$).

TABLE 1. *LAL test on normal populations of humans, mongrel dogs, rats, mice, rabbits, and squirrel monkeys*

Species tested	LAL (+)/total tested (1-h reading)	% Positive
Dog	13/29	44.8
Human	7/22	31.8
Rat	0/14	00.0
Mouse	0/16	00.0
Rabbit	0/06	00.0
Monkey	0/10	00.0

TABLE 2. *Comparison of percutaneous puncture with vein exposure as a means for collection of dog blood used in LAL testing*

Dog no.	*Limulus* reaction at 1 h[a]	
	Percutaneous puncture	Vein exposure
1	−	−
2	+4	+4
3	−	−
4	+4	+4

[a] +4, Solid gel; −, no gel.

TABLE 3. *Relationship of positive LAL test and presence of parasites in dogs*

Type or state of infestation	LAL (+)/no. tested at reaction time:	
	1 h	18 h (%)
Hookworm	0/7	5/7 (71.4)
Roundworm	2/6	3/6 (50.0)
All infested dogs	2/13	8/13 (61.5)
Noninfested dogs	0/13	1/13 (7.7)

DISCUSSION

It appears that apparently healthy members of certain species can have positive plasma LAL tests. Since percutaneous blood drawing did not show an increase in positive tests over vein exposure for blood collecting, these positive tests do not represent contamination of the sample during collection. Positive test results due to contaminated glassware or poor technique were improbable, as indicated by the negative controls as well as by the negative tests found in rat, mouse, rabbit, and monkey samples.

Since not all parasite-infested dogs had positive LAL tests (8 of 13), it can be speculated that positive LAL tests in these dogs are a function of the degree of infestation and perhaps the type of infestation or stage of infestation. The hookworm is a bloodsucker and can cause blood loss and hemorrhage from the small intestine (4). These parasitic feeding sites might serve as sites for endotoxin leakage from the gut. Heavy roundworm infestations are known to cause abdominal distention and diarrhea in juvenile dogs (4). In adult dogs, the presence of roundworms may be associated with injury of the intestinal wall, allowing for endotoxin leakage. Our observations on dogs were in accord with the findings of Klein et al. in malnourished children with ascariasis (9). The finding of one dog that had positive LAL tests but was negative for parasites may be an indication of some other means of leakage of endotoxin into the circulation, such as via the oral cavity (15), or presence of some blood factor that can mimic endotoxin in the LAL test.

Previous investigations have found positive LAL tests in human patients without detectable infections (10, 12). Caridis et al. (1) attributed this to endotoxin released from the gastrointestinal tract which, due to a poorly functioning reticuloendothelial system, could not be detoxified. As seen in the present results, positive LAL tests were found in apparently healthy human subjects. These positives could be due to some intestinal or oral cavity disorder leading to the circulation of background endotoxin, or perhaps these positives again point to the lack of specificity of the LAL test in blood. At present there is some question as to the specificity of the LAL

test due to possible mimickers found in blood (3, 19). Since blood factors have been considered possible mimickers of the LAL test, some procedure is needed to remove not only inhibitors but mimickers. Goldstein et al. (6) have suggested heating diluted plasma to 95°C to inactivate all plasma proteins and other substances that may inhibit or cause gelation of the LAL test. The gel filtration technique as described by Hollander and Harding (7) may also be a means by which plasma endotoxin could be separated from inhibitors and mimickers. With these techniques, however, there remains the question whether a negative test represents the absence of endotoxin or the dilution of endotoxin beyond the range of sensitivity of the lysate. Chloroform extraction for removal of inhibitors of the test has also been speculated to be a possible reason for confusing results (18). It has been our experience when reading tests of human plasma samples at 18 h that the plasma control becomes quite viscous, making it difficult to interpret readings at this time. This is thought to be due to the chloroform extractions, since it had been noted that the longer the extraction the more viscous the test sample became. If these positives are due to mimickers, then our results indicate that this may be specific to human and possibly dog plasma samples, since positives were not found in the other species tested.

In conclusion, the LAL test is an extremely sensitive technique for indicating the presence of endotoxin in a variety of samples, but previous investigators have had ambiguous results when testing blood plasma samples. In the six animal species surveyed, only human and dog plasma samples had positive LAL tests. These positives were not considered to be due to sample contamination. In dogs, evidence was found to indicate the positive test could be attributed to intestinal parasites. A number of possible reasons for positive human plasma samples were presented. In any case, the finding of positive LAL tests in apparently normal human subjects points out the need to distinguish between these positives and those which might indicate or lead to significant endotoxemia.

ACKNOWLEDGMENT

We thank G. Bynum for screening human subjects and collecting human plasma samples.

LITERATURE CITED

1. **Caridis, D. T., R. B. Reinhold, P. W. H. Woodruff, and J. Fine.** 1972. Endotoxin in man. Lancet i:1381-1386.
2. **Cuevas, P., and J. Fine.** 1971. Production of fatal endotoxic shock by vasoactive substances. Gastroenterology **64**:285-291.
3. **Elin, R. J., and S. M. Wolff.** 1973. Nonspecificity of the limulus amebocyte lysate test: positive reactions with polynucleotides and proteins. J. Infect. Dis. **128**:349-352.
4. **Flynn, R. J.** 1973. Parasites of laboratory animals, p. 207-209 and 229-232. The Iowa State University Press, Ames.
5. **Fumarola, D., and E. Jirillo.** 1976. Limulus test, parenteral drugs and biological products: an approach. Estratto dal Boll. 1st Sieroter. Milanese **55**:460-462.
6. **Goldstein, J. A., R. L. Barth, and W. L. Wang.** 1976. Limulus amebocyte lysate test in neonates. Am. J. Clin. Pathol. **66**:1012-1015.
7. **Hollander, V. P., and W. C. Harding.** 1976. A sensitive spectrophotometric method for measurement of plasma endotoxin. Biochem. Med. **15**:28-33.
8. **Jorgensen, J. H., H. F. Carvajal, B. E. Chipps, and R. F. Smith.** 1973. Rapid detection of gram-negative bacteriuria by use of the *Limulus* endotoxin assay. Appl. Microbiol. **26**:38-42.
9. **Klein, K., R. M. Suskind, P. Kulapongs, G. Mertz, and R. E. Olson.** 1977. Endotoxemia, a possible cause of decreased complement activity in malnourished Thai children, p. 321-328. *In* R. M. Suskind (ed.), Malnutrition and the immune response. Raven Press, New York.
10. **Levin, J.** 1973. Endotoxin and endotoxemia. N. Engl. J. Med. **288**:1297-1298.
11. **Levin, J., T. E. Poore, N. P. Zauber, and R. S. Oser.** 1970. Detection of endotoxin in the blood of patients with sepsis due to gram-negative bacteria. N. Engl. J. Med. **283**:1313-1316.
12. **Liehr, H., M. Grun, D. Brunswig, and T. Sautter.** 1975. Endotoxemia in liver cirrhosis: treatment with polymyxin B. Lancet i:810-811.
13. **Nachum, R., A. Lipsey, and S. E. Siegel.** 1973. Rapid detection of gram-negative bacterial meningitis by the limulus lysate test. N. Engl. J. Med. **289**:931-934.
14. **Rojas-Corona, R. R., R. Skarnes, S. Tamakuma, and J. Fine.** 1969. The *Limulus* coagulation test for endotoxin. A comparison with other assay methods. Proc. Soc. Exp. Biol. Med. **132**:599-601.
15. **Schwartz, J., F. L. Stinson, and R. B. Parker.** 1972. The passage of tritiated bacterial endotoxin across intact gingival crevicular epithelium. J. Peridontol. **43**:270-276.
16. **Stumacher, R. J., M. J. Kovnat, and W. R. McCabe.** 1973. Limitations of the usefulness of the Limulus assay for endotoxin. N. Engl. J. Med. **288**:1261-1264.
17. **Trippodo, N. C., J. H. Jorgersen, L. L. Priano, and D. L. Traber.** 1973. Cerebrospinal fluid levels of endotoxin during endotoxemia. Proc. Soc. Exp. Biol. Med. **143**:932-937.
18. **Tuazon, C. U., A. A. Perez, R. J. Elin, and J. N. Sheagren.** 1977. Detection of endotoxin in cerebrospinal and joint fluids by Limulus assay. Arch. Intern. Med. **137**:55-56.
19. **Wildfeuer, A., B. Heymer, K. H. Schleifer, and O. Haferkamp.** 1974. Investigations on the specificity of the Limulus test for the detection of endotoxin. Appl. Microbiol. **28**:867-871.