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THE LIMULUS AMOEBOCYTE LYSATE TEST: DETECTION OF ENDOTOXIN IN PLASMA OF SWINE AND CATTLE

II. IN VIVO INVESTIGATIONS

By

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BINDER, M. and K. MORTENSEN: *The Limulus Amoebocyte Lysate test: Detection of endotoxin in plasma of swine and cattle. II. In vivo investigations.* Acta vet. scand. 1985, *26*, 246—261. — Endotoxin was detected by the LAL test in the plasma of swine and cattle following in vivo injection of endotoxin in order to evaluate the applicability of the test in veterinary medicine for detection of endotoxemia.

Clinical symptoms of endotoxemia occurred after the injection into 3 swine of 0.10—0.25 mg endotoxin/100 kg bwt and after the infusion during 1—1½ h of 2.0—2.2 mg endotoxin/100 kg bwt into 3 calves. The concentration of endotoxin detected by the LAL test in the experimental animals ranged from 0.15 ng to 6.0 ng endotoxin per ml crude plasma. As positive LAL reactions were obtained only in close connection to the administration of endotoxin, clearance of endotoxin to levels below the sensitivity of the test was fast. In spite of the fast clearance, light symptoms of endotoxemia could be seen as long as 24 h after the last sample showing a positive test result. The applied technique for LAL analysis on blood, therefore, was not adequate for detection of endotoxin at sufficiently low concentrations and some possibilities of improving the technique are discussed.

Though leukocytosis were found not to influence the outcome of the LAL test on blood, leukocytic mediators released by endotoxin or endotoxin-derived injuries may still have caused the persistence of the symptoms of endotoxemia, and this question is disputed in relation to the benefit of improving the technique.

Especielly in the veterinary clinic, great precautions are necessary to obviate false positive test samples resulting from, e.g., external contamination or transient stress caused by excitement, and it is concluded that the possible application of the LAL test for detection of endotoxemia in veterinary medicine is restricted to surveillance of hospitalized animals and to research purposes.

clearance; diagnostic sensitivity; diagnostic specificity; veterinary applications.

For clinical use in human medicine the Limulus Amoebocyte Lysate (LAL) test is now generally accepted as an adjunct to isolation and identification of bacteria from body fluids (*Levin* 1979), and a similar application of this test seems appropriate in veterinary medicine, where sepsis and pyemia are not at all seldom events. If the sensitivity of the lysate is adequate, a 100 % correlation may be obtained between positive LAL tests and culture-proven gram-negative bacteremia (*Corrigan & Kiernat* 1979, *Fink et al.* 1981). Even if the blood culture is negative, as for instance because of administration of antibiotics or in individuals with circulatory shock, endotoxin originating from dead gram-negative bacteria in the blood or escaping from the gastro-intestinal tract, respectively (*Ravin et al.* 1960, *Tamakuma et al.* 1971, *Caridis et al.* 1973, *Walker & Porvaznik* 1978) may be detected by the LAL test. Repeated frequently, this test may, therefore, be valuable for surveillance of patients at high risk of developing endotoxin shock and for evaluating the response of such patients to treatment (*Fossard et al.* 1974). Furthermore, detection of endotoxins by the LAL test in body fluids not quite as complex as blood, e.g., synovial fluid (*Elin et al.* 1978) and peritoneal fluid (*Beger et al.* 1981, *McClure et al.* 1982) may also prove valuable in the veterinary clinic, but is outside the scope of this paper.

The purpose of the present study is to detect endotoxin in plasma of swine and cattle following in vivo injection of the endotoxin and to evaluate the utility of the LAL test for detection of endotoxemia in the veterinary medicine, when the test is performed by the method previously described (*Mortensen & Binder* 1985).

MATERIALS AND METHODS

Laboratory procedures and collection of blood samples were performed as previously described, except that before blood was drawn for the LAL analyses, some blood was drawn and stabilized for analysis of such blood constituents, that could minimize the risk of false positive LAL tests by making possible a discrimination between endotoxemia and external contamination of the blood sample with endotoxin. The assay was conducted after heat-extraction of the blood and additional serial 2-fold dilutions in water. The detected concentration of endotoxin in the crude plasma sample was calculated by multiplying the sensitivity of the lysate in water by the end-point dilution factor.

Our routine prescribes a rather low concentration of heparin, since the first lysate[1] we tested, in the absence of added salts, was

[1] Lysate prepared by T. Mikkelsen, Denmark; lot. No. 801103.

highly susceptible to heparin (*Sullivan et al.* 1976). The problem of samples clotting spontaneously has been solved by incubating tubes with heat-extracted plasma without lysate along with tubes containing plasma and lysate. Spontaneous clotting appeared as a firm gel or as increased viscosity in tubes containing plasma only, but there were never any changes in turbidity, and distinction from gelation pertinent to the LAL test was easy.

Experiment 1: Detection of induced endotoxemia in swine

Endotoxin, 0.25 mg/100 kg bwt, was injected into sow No. 3 and sow No. 25 on day 1 post partum through a vein catheter[2] inserted approximately 30 cm into an earvein, taking surgical precautions. Before collection of blood, the catheter was rinsed by injection of 20 ml saline and then filled with 1.5 ml of heparin.

Endotoxin, 0.10 mg/100 kg bwt, was injected into pig No. 24 through an earvein and blood samples were collected from the jugular vein.

Time schedule for the samples assayed for endotoxin is shown in Table 1. Plasma samples were stored at —18°C until analysed. The incubation was 1 h at 37°C, negative controls included water and heat-extracted plasma.

Experiment 2: Detection of induced endotoxemia in cattle

Endotoxin, 2.0 mg/100 kg bwt, was infused through 1 h into 2 calves (Nos. 21 & 26, 110 kg bwt). The endotoxin stock solution containing 10^{-2} g/ml was diluted to the appropriate concentration in pyrogen-free saline and administered at a rate of 2 ml/min into a digital artery via a catheter[3]. Blood samples were collected by the stated method through a Branüle in the left jugular vein. Time schedule for the samples assayed for endotoxin is shown in Fig. 1.

On 3 successive days, endotoxin, 2.2 mg/100 kg bwt, was infused through 1½ h into 1 calf (No. 14, 170 kg bwt) via a Branüle in the right jugular vein. Preparation of the endotoxin, infusion rate, and blood sampling were performed as above. Time schedule for the samples assayed for endotoxin is shown in Table 2.

Generally, plasma samples were stored at —18°C until analysed. The assays were performed as in Experiment 1, except that each sample was incubated in duplicate for 1 h at 37°C as well as for 1 h at 37°C followed by 22 h at 20—23°C.

RESULTS

Experiment 1: Detection of induced endotoxemia in swine

Following intravenous injection of 0.25 mg endotoxin/100 kg bwt the 2 sows developed symptoms of endotoxemia, lasting for about 6 h. The symptoms included pyrexia, depression, signs of pain, circulatory disturbances, ataxia, hypogalactia, and leukopenia, followed by leukocytosis (*Binder* 1984). The apparent presence of endotoxin in the peripheral blood of sow No. 25 was confirmed by the LAL test, the detected concentration being 3.00 ng per ml crude plasma at 20 min after the injection, see Table 1.

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[2] Intracath®, Cat. No. 3184, Deseret Pharmaceutical Co., Inc. Sandy, Utah, U.S.A.

[3] E-ZCATH®, Cat. No. 2252, Deseret Pharmaceutical Co., Inc., Sandy, Utah, U.S.A.

Table 1. Induced endotoxemia in swine. Detection by the LAL test following heat-extraction of the plasma and serial 2-fold dilutions in water.

Swine No.	kg bwt	mg endotoxin/100 kg bwt	Day post partum	Sensitivity ng per ml water	Test result[1]/detected concentration, ng per ml crude plasma								
					min after the injection								
					0	5	10	15	20	30	45	60	120
25	260	0.25	1	0.50	—	ND[2]	ND	ND	+/3.00	ND	ND	—	—
3	190	0.25	1	0.25 0.10	—	ND	ND	ND	—	—	—	(+)/<0.75 (+)/<0.30	—
24	16	0.10		0.50 0.05	—	+/1.50 +/0.30[3]	—	(+)/1.50	ND	(+)/<1.50	ND	—	ND

[1] + = Positive reaction; cf. *Mortensen & Binder* (1985)
(+) = Intermediate reaction; cf. „ „ „ „
— = Negative reaction; cf. „ „ „ „

[2] ND = Not done

[3] Sample analysed after storage for 1 year at —18°C.

For sow No. 3 only a weak positive result was obtained. Though the clinical symptoms in the 2 sows did not differ essentially, the possibility of an individual variation in susceptibility to endotoxin was supported by the white blood cell count, in that the leukopenia persisted for 24 h in sow No. 25 as compared to 8 h in sow No. 3. Pig No. 24 developed light symptoms of endotoxemia after intravenous injection of 0.10 mg endotoxin/100 kg bwt; the symptoms, consisting in a slight rise in temperature, depression, and circulatory disturbances, lasted for up to 3 h. The maximum concentration of endotoxin detected per ml crude plasma was 1.50 ng at 5 min after the injection (Table 1).

Experiment 2: Detection of induced endotoxemia in cattle

Symptoms of endotoxin shock occurred in all 3 calves during and after the 1 or 1½ h when the infusion of 2.0—2.2 mg endotoxin/100 kg bwt took place. All calves survived the predetermined period (i.e., for calf No. 26: 0 h, for calf No. 21: 24 h, and for calf No. 14: 8 days) after which they were killed for postmortem examination. There were great differencies between the 3 calves in the severity of the symptoms and calf No. 14, which was inoculated on 3 successive days, showed symptoms of varying severity on those 3 days (*Mortensen et al.* 1985). Clinical symptoms included changes in temperature, depression, increase in pulse and respiration rate, dyspnoea, rumen stasis and tympany, diarrhoea, signs of pain, synovitis, and ataxia. Light symptoms persisted even after 24 h.

Table 2. Induced endotoxemia in cattle[1]. Detection by the LAL test following heat-extraction of the plasma. The effect of prolonged incubation.

Incubation	Sensitivity ng per ml water	Test result[2] Day 1 10 min after infusion	Day 2 1 min after infusion	Day 3 10 min after infusion	Detected concentration ng per ml crude plasma
1 h at 37°C	0.10	(—)	(—)	—	<0.30
1 h at 37°C & 22 h at 20—23°C	0.05	+	+	+	0.15

[1] Calf No. 14, rate of infusion: 2.2 mg endotoxin/100 kg bwt through 1½ h.
[2] + = Positive reaction; cf. *Mortensen & Binder* (1985)
(—) = Intermediate reaction; cf. „ „ „ „
— = Negative reaction; cf. „ „ „ „

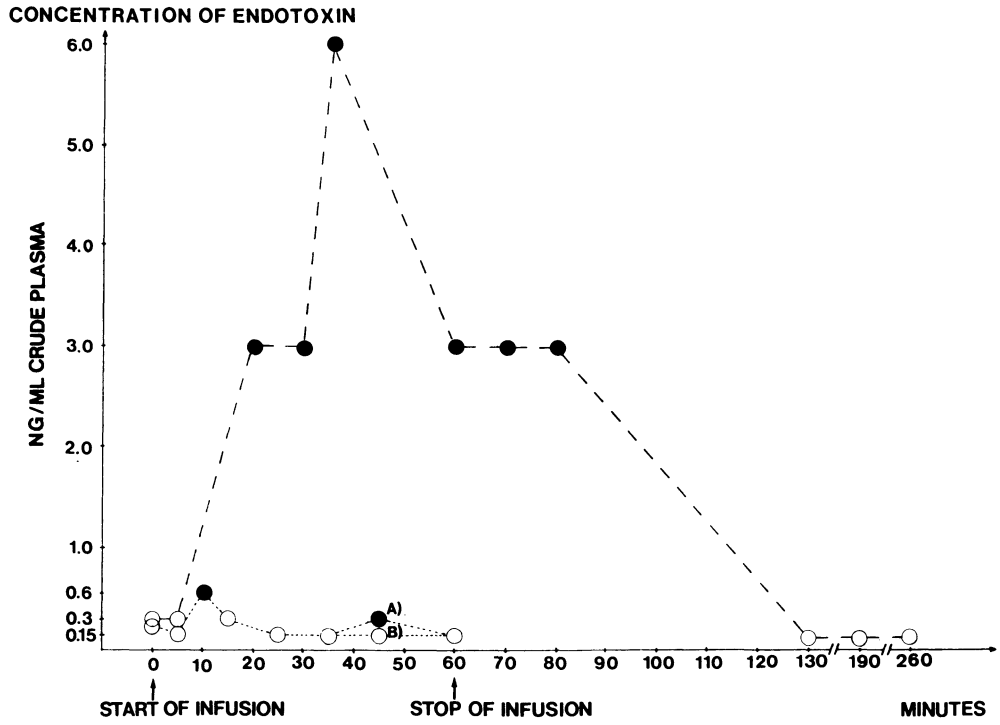

Figure 1. Induced endotoxemia in cattle. Rate of infusion: 2.0 mg endotoxin/100 kg bwt/h. Detection by the LAL test following heat-extraction of the plasma and serial 2-fold dilutions in water.

——— = calf No. 21 (killed 24 h after the infusion)
------- = calf No. 26 (killed immediately after the infusion)
● = positive test result: +
○ = negative test result: —, the concentration of endotoxin per ml crude plasma being less than the sensitivity of the lysate multiplied by 3 (the dilution factor of the heat-extraction)
A) = sample analysed immediately after collection
B) = sample analysed after storage for 1 year at —18°C.

The apparent presence of endotoxin in the peripheral blood was confirmed by the LAL test, the detected concentration of endotoxin ranging from 0.15 to 6.00 ng per ml crude plasma, cf. Fig. 1 and Table 2. For each calf there was a good correlation between the severity of the clinical symptoms and a positive LAL test. Changes concerning blood constituents, e.g., leukopenia followed by leukocytosis, thrombocytopenia, increased plasma lactate, hypocalcemia, and hypozincemia, verified the endotoxemia.

As demonstrated in Table 2, the prolonged incubation made the detection of endotoxin possible at low plasma concentrations.

DISCUSSION AND CONCLUSION

In the present clinical experiments, the plasma of the swine and the cattle positive in the LAL test only during and/or shortly after the administration of endotoxin. The clinical symptoms and changes in blood constituents were consistent with the findings of *van Miert & Frens* (1968) concerning the reaction of swine and cattle to endotoxin.

The 0.25 mg endotoxin/100 kg bwt injected into the sows was far from the lethal dose found by *Berczi et al.* (1966) to be 500 mg/100 kg bwt on intraperitoneal administration, but in spite of this symptoms of endotoxemia occurred and leukopenia lasting for 8—24 h developed. In the calves, the infused dosage was sublethal, as all 3 calves survived the predetermined period though symptoms of endotoxin shock occurred and light symptoms persisted 24 h after the infusion. The observed individual difference in susceptibility to endotoxin is consistent with the finding of *Wray & Thomlinson* (1972). The total dose injected during 1—1½ h approached the lethal dose found by *Berczi et al.* (1966) to be 2.5 mg/100 kg bwt on intraperitoneal administration, but did not exceed the lethal dose found by *Raškova et al.* (1980) to be 0.04 mg/min/100 kg bwt.

The prognostic significance of a positive LAL test in the blood of a patient is difficult to estimate and the most profitable information is obtained by repeated blood analyses combined with coagulation studies (*Meyers* 1982) and isolation of bacteria, as a positive LAL test associated with symptoms of endotoxemia is not tantamount to a poor prognosis. This was demonstrated in the experiment on swine, where no permanent injury was observed. In human patients the state of the liver has been shown to have a pronounced influence on the prognosis of patients with symptoms of endotoxemia and positive LAL reaction in the blood; when hepatic damage is absent, the prognosis may be good (cf., e.g., *Caridis et al.* 1972, *Stumacher et al.* 1973, *Fossard et al.* 1974), whereas a poor prognosis is presented by patients, where the hepatic clearance of endotoxin is impaired (cf., e.g., *Wilkinson et al.* 1974, *Jacob et al.* 1977, *Tarao et al.* 1977).

Adding to the above difficulties of interpreting the outcome of the LAL test on blood are problems concerning the sensitivity

and specificity of the test. The diagnostic sensitivity of the LAL test for endotoxemia is probably rather low, as the number of individuals with verified false negative blood reactions may be rather high, proved in human patients with consumption coagulopathy by the use of 2 different techniques (*Das et al.* 1973), and observed in human patients with circulating immune complexes and typical symptoms of endotoxemia (*Young* 1975, *Koster et al.* 1978).

Judged by the discrepancy between the duration of the symptoms of endotoxemia and the occurrence of positive LAL tests only in close connection to the administration of endotoxin, the mentioned sensitivity may well have been rather low in the present experiments, too. Especially in the calves, injected with sublethal doses of endotoxin, one could reasonably have expected the LAL test to be positive during all of the infusion period and for more than 20 min after the infusion was stopped. Consistent with the finding of *Maxie et al.* (1974) clearance of endotoxin from the circulatory system to levels below the test sensitivity consequently must have been fast, and the applied technique not adequate for detection of endotoxin in plasma at sufficiently low concentrations. This is clearly demonstrated in Table 2, where a 2-fold increase of the lysate sensitivity as a result of prolonged incubation admits of the detection of endotoxin in the plasma. The use of platelet-rich plasma probably could have lowered the detection limit further (*Das et al.* 1973). Moreover, the in vitro investigations (*Mortensen & Binder* 1985) showed, that some inhibition of the LAL test persisted at low endotoxin concentrations even in heat-extracted plasma, thus implying that if the calculated concentration of endotoxin was, e.g., 0.30 ng per ml crude plasma then the actual concentration in bovine plasma was approximately 1.7 ng per ml crude plasma. Hence, it is to be expected that a considerable gain in the diagnostic sensitivity of the LAL test may be achieved by development of improved techniques for the LAL analysis on blood.

The diagnostic specificity of the LAL test is influenced by the number of individuals with verified false positive blood reactions, and especially the specificity of the lysate for gram-negative bacterial endotoxin is a source of many conflicting reports (for references see *Mortensen & Binder* 1985). Because of the uncertain results provided by radioisotopic labeling of endotoxin (*Skarnes & Rosen* 1971) no attempts were made at this early

stage to ascertain whether the endotoxin detected by the LAL test was actually the injected endotoxin and not endotoxin of gastro-intestinal origin, or if it was not endotoxin at all but merely endotoxin-like activity. However, the short period where LAL positive blood samples could be obtained made it less plausible that endotoxin from the gastro-intestinal tract had bypassed the liver and entered the circulatory system. By comparison, *Ziv et al.* (1976) were able to detect endotoxin in the blood of 3 cows 6—24 h after intramammary administration of 10 mg endotoxin, and *Elmore et al.* (1978) detected endotoxin in the peripheral blood of postparturient sows up to 94 h after intramammary or intrauterine administration of 33—132 mg endotoxin/100 kg bwt, thus indicating a protracted absorption of endotoxin and/or mobilization of endotoxin from the gastro-intestinal tract of the shocked animals.

Endotoxin-like activity is believed by *Elin et al.* (1978) and *Fink et al.* (1981) to be caused by an elevated white blood cell count and the last-mentioned investigators proved, that heat-labile factors, presumably leukocytic endogenous mediators from phagocytic leukocytes (*Kampschmidt* 1980), could induce a positive LAL test. However, the heat-extraction performed on plasma by the investigators and also in this study should eliminate heat-labile factors from the plasma sample, and leukocytosis, therefore, should not affect the specificity of the test. Leukocytosis was found in human patients with LAL positive blood reactions by *Prytz et al.* (1976), while *Koster et al.* (1978) found LAL positive blood reactions in patients with leukopenia as well as leukocytosis, and in both reports the association is attributed to the feature of the white blood cell count in endotoxemia, i.e., an initial leukopenia followed by leukocytosis. In the present experiments, positive LAL reactions in the blood of both the swine and the cattle was only detected when the white blood cell count was decreasing or even when the animals were leukopenic, which is consistent with the findings of *Corrigan & Kiernat* (1979) in rabbits, *Elmore et al.* (1981) and *Vogelweid et al.* (1981) in swine, and *McClure et al.* (1982) in ponies. Hence, there is sufficient evidence to presume, that leukocytosis, generally, does not decrease the specificity of the LAL test but is rather a confounding factor of the test because of the correlation to endotoxemia.

Endotoxin tolerance may limit the clinical applications of the LAL test, as it has been observed in human patients with hepa-

tic cirrhosis (*Tarao et al.* 1977); the LAL test on the blood was positive, but the patients had no symptoms of endotoxemia as tolerance was induced by the sustained endotoxemia. Though limited in extent the experiment on the calf, infused on 3 successive days, presented a similar trend, as the LAL test on the blood was positive shortly after each administration, but clinically the last infusion was best tolerated.

Further limitations to the clinical utility of the test are brought about by 2 factors especially important in the veterinary clinic, i.e., transient stress caused by excitement and external contamination. In a clinical experiment *Morkoc* (1982) thus found positive LAL reactions in the blood of 8 cows (32 %) with agalactia and in 1 clinically healthy sow (13 %). Likewise, *Ross et al.* (1981) found 20—140 ng endotoxin per ml blood in all sows tested, the controls included, and *Dougherty et al.* (1976) found the plasma samples from 3 sheep and 1 steer positive before the start of an experiment. As described, great efforts were made in these experiments to successfully obviate false positive test results, but the extreme precautions necessary limit the applicability of the test considerably.

In order to augment the clinical utility of the LAL test for analysis on blood improvements described previously are needed (*Mortensen & Binder* 1985), and further improvements may be obtained by a more appropriate storage of plasma samples containing endotoxin. Only small amounts of endotoxin were lost during short-time storage at —18°C and this loss, probably owing to adhesion to the glass tubes (*Rastogi et al.* 1979), could be minimized by a thorough mixing of all thawed samples for 3 min prior to the heat-extraction. However, substantial amounts of endotoxin were lost during prolonged storage of untreated plasma samples containing endotoxin, probably owing to irreversible detoxification by plasma esterases. For instance, when plasma drawn from pig No. 24 five min after endotoxin injection was first thawed and assayed, the concentration was 1.50 ng/ml. One year later, the concentration had dropped to 0.30 ng/ml (Table 1). A similar trend was observed with the plasma of calf No. 26, sampled 45 min after the start of the endotoxin infusion; the concentration of endotoxin in the unfrozen sample was 0.30 ng/ml (Fig. 1), whereas 1 year later it had been reduced to less than 0.15 ng/ml. As the endotoxin detoxifying plasma esterases are heat-labile (*Skarnes et al.* 1958), the loss of endotoxin could

probably have been prevented if plasma samples that were to be stored for a prolonged period, were heated before they were frozen at the lowest possible temperature.

As mentioned, some investigators use platelet-rich plasma instead of platelet-poor plasma (*Hollander & Harding* 1976, *Corrigan & Kiernat* 1979, *Fink et al.* 1981, *Thomas et al.* 1981), as *Das et al.* (1973) demonstrated that much of the endotoxin added to human and rabbit platelet-rich plasma could be detected by the LAL test in the platelet fraction. *Springer & Adye* (1976) further proved, that endotoxin was bound to human platelets, and *Meyers et al.* (1982) demonstrated that approximately 25 % of the endotoxin added to equine and bovine platelet-rich plasma was bound by non-specific binding to the platelets, and this binding could be markedly reduced by gel-filtration. A disadvantage in the use of platelet-rich plasma could be occasioned by the presence of a certain amount of leukocytes, as they may reduce the lysate sensitivity (*Young* 1975), but in spite of this, the good results provided by the use of platelet-rich plasma with the LAL test are in favour of its use.

Though the discrepancy between the duration of the symptoms of endotoxemia and the occurrence of positive LAL tests may be diminished by development of improved techniques, including improved elimination of plasma inhibitors, full agreement might not be expected as mediators released during the course of the endotoxemia by endotoxin (cf. *Proctor et al.* 1980, *Schade & Rietschel* 1980) or released by endotoxin-derived injuries (*Verheijden et al.* 1981) may be responsible for the persistence of the symptoms. Consistent with this, *Corrigan & Kiernat* (1977) found that when endotoxin had once exerted its effects in the rabbits, treatment with an endotoxin-binding antibiotic was of no effect. Therefore, it must be concluded from the literature and the present in vivo investigations, that further knowledge about the interactions of blood, endotoxin, LAL, and host defense mechanisms are necessary in order to establish the clinical and prognostic significance of LAL analysis on blood. As the LAL test at the present is the most sensitive test available for bacterial endotoxin (*Weary & Baker* 1977) it may still prove to be valuable in the veterinary medicine, but the elaborate technique required for blood analyses will restrict the possible future applications of the LAL test to surveillance of hospitalized animals and to research purposes.

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SAMMENDRAG

Limulus Amøbocyt Lysat testen: Påvisning af endotoksin i svine- og kvægplasma. II. In vivo undersøgelser.

Ved hjælp af LAL testen blev endotoksin påvist i svine- og kvægplasma efter in vivo injektion med henblik på at vurdere anvendeligheden af testen i veterinærmedicinen til påvisning af endotoksinæmi.

Kliniske symptomer på endotoksinæmi opstod efter injektion af 0,10—0,25 mg endotoxin/100 kg lgv. i 3 svin og efter 1—1½ times infusion af 2,0—2,2 mg endotoksin/100 kg lgv. i 3 kalve. Koncentrationen of endotoksin, der blev påvist ved hjælp af LAL testen i forsøgsdyrene, varierede fra 0,15 ng til 6,0 ng endotoksin pr. ml ubehandlet plasma. På grund af en hurtig clearance blev koncentration af endotoksin hurtigt lavere end testens følsomhed, hvorfor positive LAL reaktioner kun forekom i nær tilknytning til administrationen af endo-

toksin. På trods af den hurtige clearance kunne lette symptomer på endotoksinæmi ses så længe som 24 timer efter den sidste positive prøve. Derfor var den anvendte teknik til LAL analyse af blod ikke god nok til påvisning af endotoksin i tilstrækkelig lave koncentrationer, og nogle muligheder for at forbedre teknikken diskuteres.

Selv om det blev fundet, at leukocytose ikke influerede på udfaldet af LAL testen på blod, kan de vedvarende symptomer på endotoksinæmi stadig være forårsaget enten af mediatorer frigjort fra leukocyter under endotoksinæmien eller af endotoksin-betingede skader, og dette spørgsmål drøftes i relation til den fordel, der kan opnås ved at forbedre teknikken.

Især i den veterinære klinik er det nødvendigt med mange foranstaltninger for at undgå falske positive prøver, der f. eks. kan skyldes kontamination med endotoksin eller forbigående stress i forbindelse med ophidselse, og det konkluderes, at den mulige anvendelse af LAL testen til påvisning af endotoksinæmi i veterinærmedicinen er begrænset til overvågning af hospitalsindlagte dyr og til forskningsrelaterede formål.

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