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THE CHROMOGENIC LIMULUS AMOEBOCYTE LYSATE (LAL) ASSAY FOR ENDOTOXIN: DEVELOPMENTS AND APPLICATIONS

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Presented for the degree of Doctor of Philosophy in the Faculty of Medicine, University of Glasgow.

Department of Bacteriology and Immunology, July 1986.

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I dedicate this work to Mr. and Mrs. Patrick and Helen Buchanan of Touch, Stirling, without whose care, love and support it would not have been completed.

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SUMMARY

Endotoxin (lipopolysaccharide, LPS) is a part of the envelope of Gram-negative bacteria. Among the different methods for the detection and measurement of endotoxin, the Limulus amoebocyte lysate (LAL) assay is the most widely used. The reagent used in this assay is the lysate of blood cells (amoebocytes) of one of the species of horseshoe crab, usually Limulus polyphemus.

The most recent development among the objective and quantitative LAL assay methods is a chromogenic method, which uses a synthetic chromogenic peptide substrate. This method is based on the principle that endotoxin-activated lysate releases from the colourless substrate yellow p-nitroaniline (pNA), which can be measured spectrophotometrically.

In this study, the original two-stage assay, as recommended by the LAL manufacturer, was optimized. This resulted in a ten-fold increase in sensitivity of the assay and a two-fold decrease in the volumes of expensive reagents. The processing time of large numbers of samples was also significantly decreased by using microplates for spectrophotometric readings in a MICROELISA MiniReader.

Subsequently, the two-stage assay was further modified and a one-stage method was developed. This resulted in a further five-fold increase in sensitivity of the assay, to 0.2 pg endotoxin/ml of pyrogen-free water, which is the equivalent of 0.002 Endotoxin Units (EU)/ml of pyrogen-free water, with the linearity of the standard curve in the range of 0-2 pg/ml or 0-0.023 EU/ml. In plasma, the threshold of sensitivity was 1 pg/ml (0.012 EU/ml) and the standard curve was linear in the range 0-30 pg/ml (0-0.345 EU/ml). Other advantages of the one-stage assay were: a further decrease in reagent volumes and reduction of the time required for processing large numbers of samples.

In this study, the chromogenic LAL assay was used for detection of endotoxin in clinical and non-clinical studies. Endotoxin is thought to be an important factor in the pathogenesis of Gram-negative infections, although its exact role remains unknown. However, difficulties arise when the LAL assay is used for the detection of endotoxin in human blood. This is because of the presence in blood of inhibitors and non-specific activators of the assay.

The clinical studies involved three trials: septic shock, cyclic neutropenia and acute pancreatitis.

The septic shock trial consisted of two groups of patients:

Group 1 - ten critically ill, but not necessarily septic shock patients with daily LAL assays and simultaneous blood cultures;

Group 2 - eight acute septic shock patients, who had four-hourly LAL assays for the first 48 hours, and thereafter daily. Blood for blood cultures and fibronectin was taken simultaneously with blood for LAL assays. Cardiovascular and respiratory functions were also measured at the same time.

The septic shock trial demonstrated the need for frequent monitoring of endotoxin levels in acute conditions. In Group 2, an inverse correlation between endotoxin levels and plasma fibronectin was shown. In most cases, endotoxin levels correlated with cardiovascular and respiratory indices. However, there seemed to be no correlation between the severity of septic shock and absolute levels of endotoxin. Several hypotheses are discussed for future verification.

The cyclic neutropenia trial involved one patient. In the acute pancreatitis trial 16 patients were examined . The results of both studies were unsatisfactory. This was most likely because of inadequate sampling. Therefore no conclusions could be made about the role of endotoxin in these conditions. A different approach is suggested for future studies.

In the non-clinical studies, the LAL assay was used to examine two practical problems. One was the removal of endotoxin from human blood and the other was investigation of the role of endotoxin as an occupational hazard. Thus the performance of an extra-corporeal filter (DEP-1) for removal of endotoxin from human blood was tested. The filter appeared to be incapable of extracting clinically significant amounts of endotoxin from human plasma. Endotoxin levels were measured in baffle plate material from the humidification system of a semi-conductor factory and in a dust sample from books in an archive library. Endotoxin, which was found in both, could have been at least partially responsible for the clinical symptoms reported by the factory workers and library staff.

I. INTRODUCTION

1.1. Endotoxin

1.1.1. History

The history of fever goes back 2 500 years and is as old as the history of medicine itself (644). Since then, throughout the centuries, fever has been seen not only as a threat, symptom or expression of disease, but also as an indication of activated defence, which should be promoted by the physician (644, 383). The Greek physician Hippocrates in the 4th century B.C. described many diseases accompanied by fever. In c. 500 B.C., another Greek physician, Parmenides, was praising the beneficial effects of fever as a cure for all illnesses. His fellow countryman, Galen, in the 2nd century A.D., reported fever as a cure, or as having at least a favourable influence on a variety of conditions.

In the modern era, fever therapy has been applied to treatment of psychiatric conditions (459, 579), paralysis (625, 626), cancer (78, 79, 80, 111, 127), bacterial endocarditis (538), herpes zoster (34), encephalitis, encephalomyelitis, rheumatoid arthritis, chorea, various eye and skin diseases (579), neurosyphilis (126, 217, 365), abdominal typhus (467), gonorrhoea and many other conditions (14).

There were also several commercial bacterial preparations developed for fever induction, which were used for treatment of different clinical conditions (385). The first of these, "Pyrifer", was a preparation obtained by growing a non-pathogenic 'colon' bacterium in milk. It was marketed in seven strengths, ranging from 50 to 500 bacteria per ml. "Pyromen" was an endotoxin preparation obtained after isolation and partial purification of endotoxin from Pseudomonas aeruginosa. It was prepared and marketed by Baxter Laboratories. "Pyrexal"

("Lipexal") was a product developed by Vander GmbH, Bern, Switzerland, which contained a purified and highly potent endotoxin, obtained according to the method of Westphal (660).

The term "pyrogenic substance" was first used in 1862 by T.Billroth (38). In 1875, J. Burdon-Sanderson named the fever-producing bacterial substances "pyrogen" (55). In 1892, Pfeiffer observed that cholera vibrios, in addition to the toxin which they excreted into the culture medium - the exotoxin - produced another, quite different toxin, which was tightly anchored to the bacterial cell (420). Pfeiffer coined the term "endotoxin" for it (658).

The latter name referred both to pathophysiological properties of the bacterial substance, which exerted a specific toxic action, and to its structural property of being tightly anchored to the bacterial cell.

In 1884 the Danish physician Hans C.J.Gram made an accidental discovery of a new staining technique (182), which led to classifying bacteria into two major groups: Gram-negative and Gram-positive.

Endotoxins were first isolated in 1933 by Boivin and Mesrobeanu (47, 48), who also first determined their biochemical nature. Their technique involved extraction with cold trichloroacetic acid and provided LPS preparations which also contained significant quantities of protein and lipid. Endotoxin-associated protein later found with LPS prepared by this method is mitogenic for B lymphocytes (221, 334, 383, 562, 563), irrespective of any mitogenic effects of LPS, which is reflected in high immunologic activity of Boivin-type LPS preparations.

Boivin made also a remarkable discovery, viz, that the bacteria which produced endotoxins were all of the Gram-negative type.

The term "lipopolysaccharide" (LPS) is most frequently used to describe the endotoxins obtained by the extraction procedure of Westphal and Lüderitz (383). They utilized hot aqueous phenol for extraction (643). This technique provides LPS practically free of protein and is probably nowadays the most extensively used method for production of LPS preparations (306). However, the term "lipopolysaccharide" merely describes endotoxins biochemically, and the terms lipopolysaccharide (LPS), glycolipid (for endotoxins from rough mutants), O-antigen (O-somatic or O-specific antigen), bacterial pyrogen and endotoxin have been used interchangeably (306).

1.1.2. Biochemistry

The envelope of most Gram-negative bacteria is built up of the following layers outwards from the cytoplasm of the cell (456):

1. The plasma or cytoplasmic membrane appearing as a unit membrane of three layers;
2. A thin peptidoglycan layer (called also murein or mucopeptide (53)).
3. A usually somewhat structureless lipoprotein zone (periplasmic space);
4. An outer phospholipid-protein membrane appearing as a second unit membrane of three layers;
5. Lipopolysaccharide that can usually only be made visible by application of suitably labelled antibodies, such as those conjugated with ferritin.

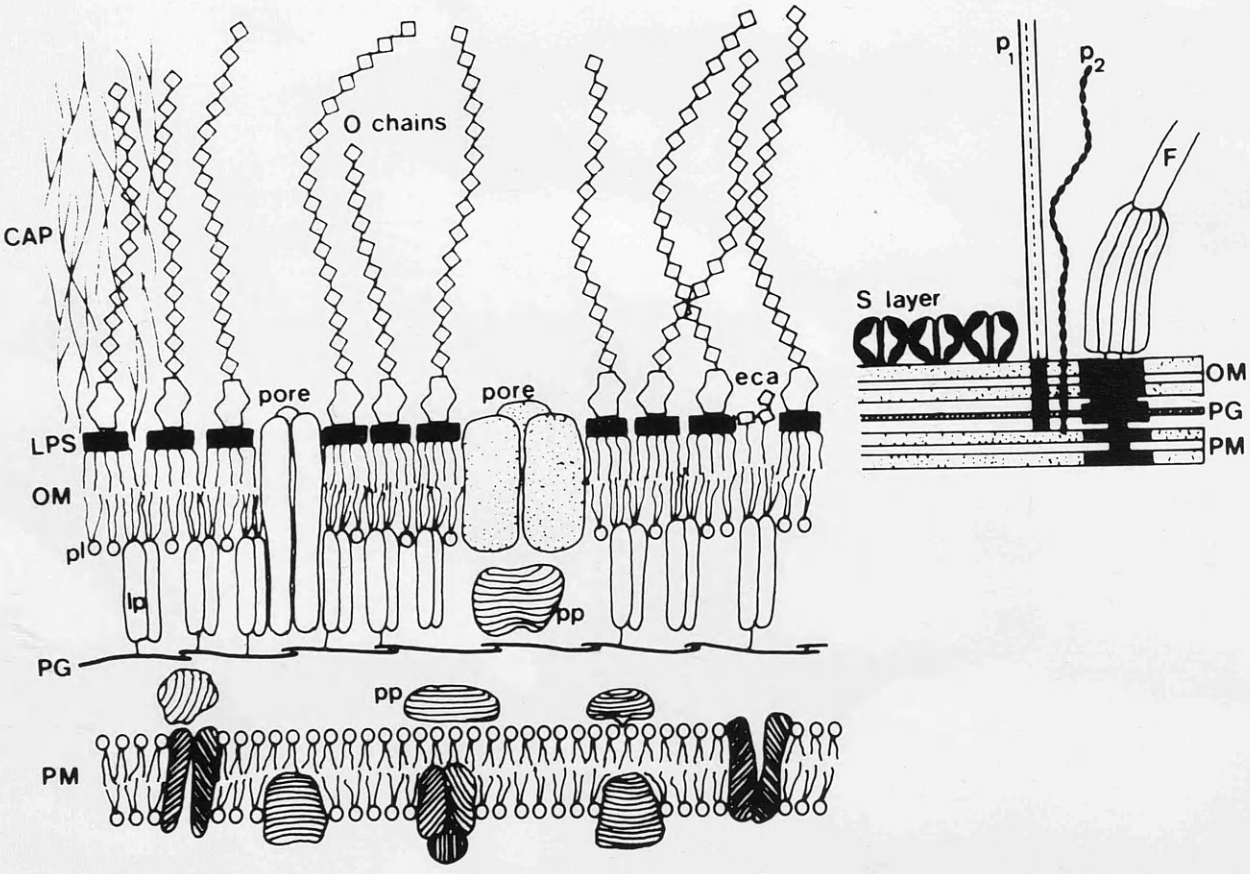
CAP
O chains
pore
pore
eca
LPS
OM
pl
lp
pp
PG
pp
PM
S layer
p_1
p_2
F
OM
PG
PM

Fig. 1

Fig.1 Cell wall of Gram-negative bacteria.

PM - plasma membrane

PG - rigid or R layer (peptidoglycan)

OM - outer membrane

LPS - lipopolysaccharide

CAP - polysaccharide capsules

pp - proteins

lp - lipoproteins

pl - phospholipids

eca - enterobacterial common antigen

F - flagellum

p_1 - fimbria

p_2 - pilus

From: Freer, J.H.: Illustrated guide to the anatomy of the bacterial cell envelope. In: D.E.S. Stewart-Tull, M. Davies (eds.): Immunology of the Bacterial Cell Envelope. John Wiley & Sons, Chichester 1985, pp.355-383.

Differences in distances of separation between the layers, if not in the number of layers, exist in various Gram-negative species. Also, many Gram-negative bacteria have an outermost surrounding envelope, variously termed capsules with Klebsiella spp. and Meningococcus spp., K antigen with ~~(E coli)~~ Escherichia coli, and Vi antigen with Salmonella typhi. Some bacteria also have two forms of filiform extensions - flagella and pili or fimbriae. Capsules, Vi antigen and most K antigens are polysaccharides but a few K antigens are proteins (306).

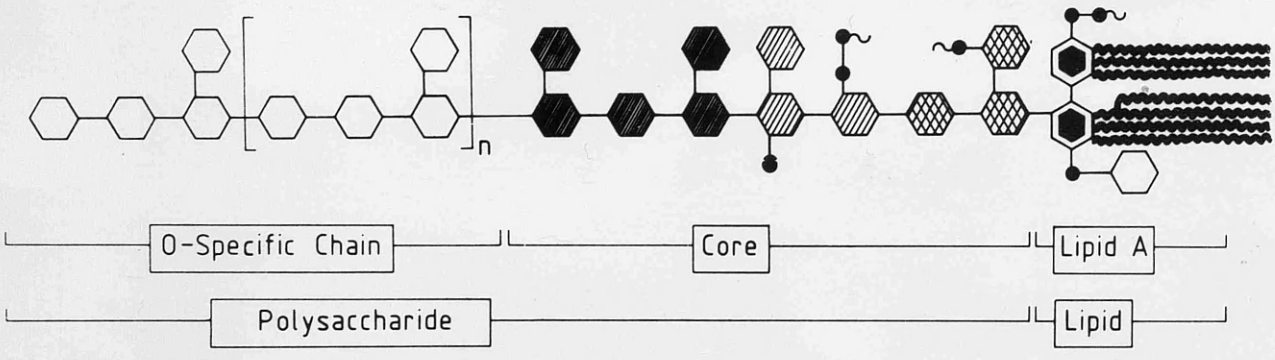
n
O-Specific Chain
Core
Lipid A
Polysaccharide
Lipid
:Monosaccharide, ● :Phosphate, ∼ :Ethanolamine
:Long Chain(Hydroxy) Fatty Acid

Fig. 2

Lipopolysaccharides are characteristically found in the Gram-negative bacteria and they are known to represent the endotoxic principle of Gram-negative organisms (299). However, an endotoxin-like material, chemically very similar to Gram-negative endotoxins, has been isolated from the Gram-positive organism Listeria monocytogenes. In contrast, the Gram-negative spirochaetes Borrelia recurrentis, Borrelia hispanica and Treponema pallidum lack LPS (57, 164, 199). Also, apart from the "structural endotoxin", which builds walls of Gram-negative bacteria, there is also "free endotoxin" which is released from cells during normal healthy growth (91, 101, 384, 473). From the point of view of physico-chemical properties, endotoxins are high molecular weight (~0.2 x 10^6 to 1.0 x 10^6) (309, 436), heat stable (100° C) substances. Endotoxins from different groups of Gram-negative bacteria conform to a common structural principle: they consist of a polysaccharide and a covalently bound lipid component, termed lipid A (642). The polysaccharide component consists of two subcomponents: the O-specific chain and the core. As an example, Fig. 2 shows schematically the architecture of a Salmonella lipopolysaccharide.

Fig 2 Schematic structure of a Salmonella lipopolysaccharide.
From: Rietschel, E. T., Schade, U., Jensen, M., Wollenweber, H.-W., Lüderitz, O., Greisman, S.G.: Bacterial endotoxins: chemical structure, biological activity and role in septicaemia. Scand. J. Infect. Dis. 1982, Suppl. 31: 8-21.

The heteropolysaccharide (O-specific chain and core) carries those structures which are recognized by lectins, specific antibodies and bacteriophages (300). It is also involved in bacterial virulence (293), the induction of certain host derived mediators (382, 610) and the tumour necrotic activity of lipopolysaccharides (450).

On the other hand, lipid A is responsible for the endotoxic properties of lipopolysaccharides (163).

The O-specific chains are polymers of oligosaccharide molecules, and they consist of a sequence of identical units, the so-called "repeating units" (301). In rare cases, O-specific chains may represent a homopolysaccharide, such as a mannan or a galactan. Each serotype of Gram-negative bacterium is characterized by a unique structure in the O-specific chain, thus creating a great variety of lipopolysaccharide structure and composition.

Studies on O-specific chains have revealed many new sugar classes as well as unusual sugar derivatives. The average length of O-specific chains varies from one species to another, but a high degree of heterogeneity is seen within species (174,400).

Core polysaccharide consists of an outer core and an inner core (52). The outer core in the Salmonella species is a lipid A-distal branched oligosaccharide containing the common hexoses: N-acetyl-D-glucosamine, D-glucose and D-galactose. The inner core is a lipid A-proximal branched oligosaccharide containing unique, core-specific sugars, L-glycero-D-mannoheptose (L, D-Hep, Hep) and 2-keto-3-deoxy-D-mannooctonate (KDO) (301). The O chain is linked to the subterminal glucose residue; the reducing KDO group forms the link to lipid A. Heptose and KDO residues are substituted by phosphate, phosphoryl - and pyrophosphoryl - ethanolamine groups (280). Together with the carboxyl groups of KDO, these confer negative charge to the molecule, which is neutralized by a mixture of cations, including metal ions and polyamines. These cations are always present in original lipopolysaccharide preparations (162).

The highly charged inner core region may be potentially important for the physiological functions of translocation and integration of lipopolysaccharides into the outer membrane, and for conferring stability on the outer membrane. This stability may occur through cation-mediated ionic linkages between lipopolysaccharide molecules themselves and between lipopolysaccharides and proteins (485).

All Salmonella species synthesize LPS with a similarly structured core. However, in other Enterobacteriaceae, such as E coli, Shigella spp, Arizona spp and in other bacterial families, structurally distinct core types have been identified (639,649).

The above complete LPS structure is synthesized by wild S (smooth) form of bacteria, many of which are pathogenic in man and in experimental animals. From many Gram-negative organisms, R (rough) mutants have also been isolated. As a result of genetic defects they synthesize incomplete LPS, from which the O-polysaccharide is absent and the core is present in different degrees of completion. Although R form bacteria are usually non-pathogenic, the incomplete LPS which they possess exhibits all toxic and other biological activities expressed by complete LPS. Their non-pathogenic character is mainly due to their inability to invade and proliferate in the macroorganism (160). In Salmonella species, five main classes (and a number of sub-classes) of R mutants have been recognized, and designated R_a to R_e in the order of increasing defect.

R mutants made structural, biosynthetic and genetic investigations of the core region of lipopolysaccharides feasible. It was also shown that incomplete LPS from all mutants had endotoxic activity similar to that of the complete LPS from

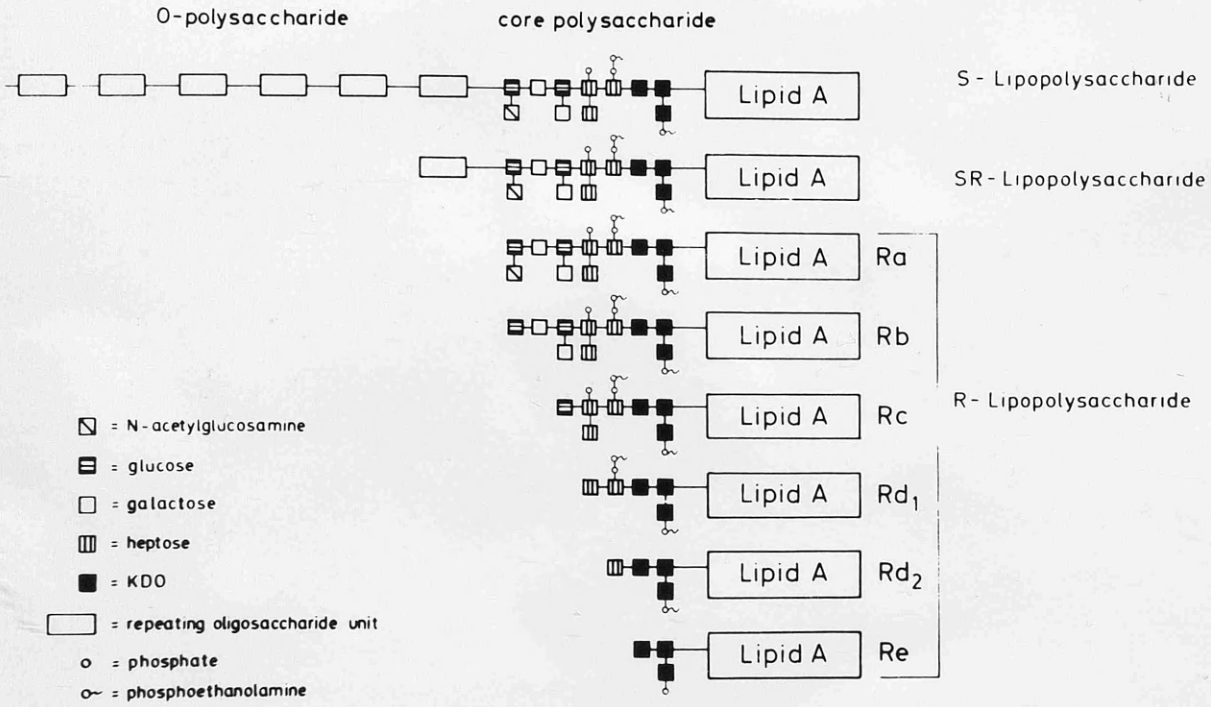
O-polysaccharide
core polysaccharide
Lipid A
S - Lipopolysaccharide
SR - Lipopolysaccharide
Ra
Rb
Rc
Rd1
Rd2
Re
R - Lipopolysaccharide
= N-acetylglucosamine
= glucose
= galactose
= heptose
= KDO
= repeating oligosaccharide unit
= phosphate
= phosphoethanolamine

Fig. 3

the parent strain. This indicated that the polysaccharide part was not essential for endotoxicity. Further work demonstrated that free lipid A exhibited the characteristic biological activities of LPS and was the endotoxic principle of lipopolysaccharides (for the review of literature see 301,309).

Fig.3. Schematically shown various R classes in genus Salmonella.
From: Galanos, C., Freudenberg, M. A., Lüderitz, O., Rietschel, E T., Westphal, O.: Chemical, physicochemical and biological properties of bacterial lipopolysaccharides. In:, E Cohen (ed.), Prog. Clin. Biol. Res. vol.29: Biomedical Applications of the Horseshoe Crab (Limulidae). Alan R. Liss, Inc., New York 1979, pp 321-332.

Lipid A, which is covalently bound to the core polysaccharide, consists of a phosphorylated hexosamine disaccharide backbone (central phosphorylated β - 1 6-linked D-glucosamine disaccharide), to which long chain D-3-hydroxy fatty acids are ester or amide linked. The composition of the fatty acids in lipid A can be influenced even by medium composition (173, 255). However, as only minor differences exist in the lipid A portion of LPS moiety from taxonomically remote bacteria, and it is constant for larger groups of bacteria, lipid A represents therefore the most conservative and least variable structural element of LPS (451). However, photosynthetic bacteria , such as Rhodopseudomonas viridis, Rh. palustris, Rh. sulfoviridis, Rh. sphaeroides, Chromatium vinosum, Thiocapsa roseopersicina and Rhodomicrobium vanielli, as well as cyanobacteria, two Nitrobacter species and two saprophytic representatives of genus Pseudomonas: Ps. diminuta and Ps. vesicularis lack the usual backbone structure of lipid A (106, 227,322, 323,324,554,580, 639,650). As a result, both the LPS and the free lipid A of

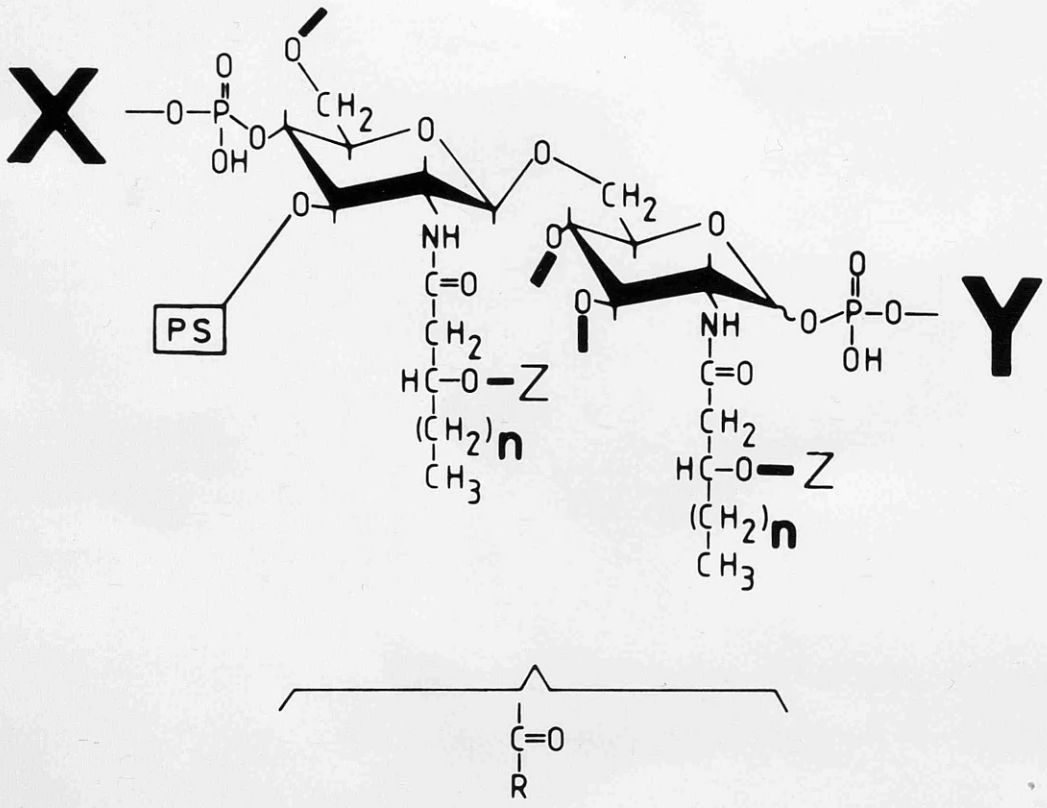

Fig. 4

most of these species are nontoxic (324).

Fig.4. Generalized structure of lipid A.

X	Y	Z	n	Bacterium
H	P	COR	10	Escherichia coli
H	PEtN	COR	10	Vibrio cholerae
4-AraN	H	COR	10	Proteus mirabilis
4-AraN	PEtN	COR	10	Salmonella minnesota
4-AraN	GlcN	H	8	Chromobacterium violaceum

P = phosphate

4-AraN = 4 - amino - 4 - deoxy - L - arabinose

GlcN = 2 - amino - 2 - deoxy - D - glucose

PEtN = phosphorylethanolamine

PS = polysaccharide

From: Rietschel, E. T., Schade, U., Jensen, M., Wollenweber, H.-W., Lüderitz, O., Greisman, S. G.: Bacterial endotoxins: chemical structure, biological activity and role in septicaemia. Scand. J. Infect. Dis. 1982, Suppl. 31: 8-21.

The chemical structure of lipid A makes it an amphipathic molecule (with hydrophobic and hydrophilic regions) as well as an amphoteric molecule (with both acidic and basic residues). These unique characteristics endow lipid A with distinct properties, which make it a vital constituent of the cell wall of Gram-negative bacteria, where it has both structural and functional roles.

Because of their compact and ordered arrangement, the lipid A fatty acids form a comparatively rigid structure. The dense packing of the hydrophobic moiety of lipid A, which is favoured by the absence of unsaturated fatty acids, causes a high viscosity of the outer membrane. This inhibits hydrophobic molecules from diffusing into the outer membrane and therefore is responsible for the marked impermeability of the membrane to bile acids, detergents and lipophilic antibiotics. In this way, lipid A plays an essential role in the function of the outer membrane as a permeation barrier (133, 369). In addition to its important structural role, lipid A is essential for the maintenance of the integrity of the outer membrane and cell shape.

1.1.3. Biological activity

Endotoxins exert numerous physiopathological effects upon living organisms and their organs. Table 1 summarizes various biological activities demonstrated so far with endotoxin.

Table 1. Biological activities of endotoxin.

Stimulation of the immune system	Others
Replacement of helper T lymphocytes in induction of antibody response	Pyrogenicity
B-cell mitogenicity	Hypothermia
Polyclonal antibody induction	Abortion
Adjuvanticity	Foetopathy
Macrophage activation	Haemorrhagic tumour necrosis
Immunogenicity	Lethal toxicity
Complement activation	Endotoxic shock
Stem cell mobilisation (CFU-S)	Enhanced resistance to irradiation lethality
IgG synthesis in newborn mice	Leucopenia
Induction of non-specific resistance to infection	Leucocytosis
Induction of tolerance to endotoxin	Local Shwartzman reaction
	Generalized Shwartzman reaction
Stimulation of mediator production	Bone marrow necrosis
Interleukin - 1	Embryonic bone resorption
Colony stimulating factor	Hypotension
Interferon	Hypertension
Glucocorticoid-antagonizing factor	Platelet aggregation
Tumour-necrotizing factor	Thrombocytopenia
Thymidine	*Limulus* lysate gelation
Arginase	Toxicity enhanced by BCG
Endogenous pyrogen	Toxicity enhanced by adrenalectomy
Plasminogen activating factor	Enhanced dermal reactivity to epinephrine
Hageman (XII) factor	Induction of early refractory state to temperature change (endotoxin pyrogenicity)
Neutrophil immobilizing factor	Type C RNA virus release from mouse spleen cells
Leucocyte endogenous mediator	Helper activity for Friend Spleen Focus Forming Virus in mice
Lymphocyte activating factor	Inhibition of phosphoenolpyruvate carboxykinase, glycogen synthase,
Lysosomal hydrolases	
Collagenase	
Elastase	
Prostaglandins	
Kinin	

Table 1. Biological activities of endotoxin – continuation

Stimulation of mediator production (continuation)	Others (continuation)
Superoxide anion	fructose – 1,6 – diphosphatase and glucose – 6 phosphatase
Histamine and serotonin	Enhanced activity of pyruvate kinase
	Disseminated Intravascular Coagulation
	Hyperglycaemia
	Hypoglycaemia
	Hypoferraemia
	Decrease in iron absorption from the gut
	Decreased serum zinc
	Decreased serum transferrin
	Increased serum copper
	Decrease in water and food intake
	Arthritis and synovial inflammatory reactions
	Oliguria
	Polyuria
	Spermatozoa agglutination *in vitro*
	Hyperlipidaemia
	Fibrinogen decrease

Modified from: Gallahon, K.A., Michalek, S.M., Wannemuehler, M.J., McGhee, J.R.: Lipopolysaccharide regulation of the immune response. In: A. Nowotny (ed.): *Beneficial Effects of Endotoxins*. Plenum Press, New York and London 1983, pp. 327–353.

Recently it has been shown that free lipid A also exhibits these activities.

Endotoxin acts as a mitogen for B-lymphocytes and a polyclonal activator of these cells, thus replacing helper T-lymphocytes in induction of antibody responses

(366). The apparent specificity of the B-lymphocyte proliferative response to endotoxin would suggest that such responses might prove to be useful as a general means of defining B-cell populations. However, results obtained by a number of investigations have indicated significant species variability in the capacity of B-lymphocytes to respond to endotoxin. For example, endotoxin was unable to elicit proliferative responses in cultures of human lymphocytes (397, 415), unless defined culture systems were used (273, 328). Mice are the most commonly used model for proliferation of B-lympohocyte population in response to endotoxin. Rat lymphocytes have characteristic responses to endotoxin very much like those of a mouse (618). Results obtained for rabbit (49, 508) and guinea pig (27, 113) B-cells are contradictory. B-cell proliferative responses are also observed in chicken (637), axolotl and newt (81), hamster (207) and the rainbow trout (123).

Although both lipopolysaccharide and free lipid A activate complement, the mechanism of this process is different; lipopolysaccharide activates complement through the alternative pathway (i.e. in the absence of antibody), while free lipid A activates complement through the classical pathway in the absence of antibody (335). In the presence of specific antibody, LPS may also activate the classical pathway (347).

Pyrogenicity is one of the most sensitive indicators of endotoxin activity, but this assay is used primarily in rabbits, since LPS normally induces a hypothermic response in other common laboratory animals - mice and rats - and no reaction in guinea pigs (616). However, it is possible to obtain hyperthermic response in mice after preconditioning them at 36° C (429).

Humans appear to be the species most sensitive to endotoxin. The threshold dose for pyrogenicity of LPS is similar to that in rabbits, thus making results of the Rabbit Pyrogen Test for Endotoxin valid with regard to humans; but the dose-response curve is steeper (185). In contrast, sub-human primates (baboons, vervets) are relatively resistant to the pyrogenic effects of LPS. The susceptibility of humans and baboons to endotoxin pyrogenicity differs by a factor of more than 1:100 000 (301).

The degree of susceptibility to endotoxin between different species varies depending on the parameter being assessed, but van Miert and Frens (616) made the following list of diminishing sensitivity: rabbit, horse, goat, dog, cow, sheep, cat, swine and chicken. As the mouse, rat and guinea pig exhibit neither a fever reaction nor a leucocyte response to endotoxin, they could not be compared with the others. There are several experimental models, however, in which the natural sensitivity of animals towards LPS may be increased. Enhanced susceptibility to endotoxin lethality develops when the phagocytic activity of the RES (rediculoendothelial system) is stimulated with zymosan (28), triolein (glycergl trioleate) (555), glucan (84), BCG, (565) group A streptococcal pyrogenic exotoxin (SPE C) (487), Corynebacterium parvum (128), muramyl dipeptide (MDP) (447), graft-versus-host reactions (230), brucellosis (603), typhoid fever and tularemia (187), as well as a number of other infections (564), and antigen-antibody complexes (159). The fact that reticuloendothelial stimulation by this group of agents significantly enhances endotoxin sensitivity is a cause for concern. This concern is based upon the application of many of these agents in experimental clinical immunotherapy, as well as the potential wide-spread therapeutic employment of these agents (103).

Agents which depress RES phagocytic function may also increase susceptibility to endotoxin. To this group belong the following: carbon tetrachloride (125, 141), thorotrast (colloidal thorium dioxide) (24, 25, 474, 656), lead acetate (129, 274, 503), cadmium (83), and carrageenan (23). The protein synthesis inhibitors, actinomycin D (37, 421) and α-amanitin (506) are other sensitizing materials. Other pharmacological conditioning, which greatly increases sensitivity to LPS, involves adrenalectomy (67, 504) and treatment with galactosamine (161).

The age of the individual is also a factor influencing reaction to pyrogens. In general, susceptibility to endotoxin increases with age. Adult rabbits are approximately 50 times more sensitive than infants (532). Young mice are also less susceptible than more mature animals to the lethal and sublethal action of pyrogens (439). Similarly, the pyrogenic response to endotoxin increases with age in man (305). The hypothesis which attempts to explain this suggests that the biological activities of endotoxin may depend on the degree of sensitization of the host to the endotoxin from the alimentary tract. This spontaneous sensitization is a result of a cross-allergic reaction with intestinal bacterial flora (276, 425, 531, 552, 633). With regard to chickens, on the other hand, chick embryos are extremely sensitive and fully grown chickens are almost completely resistant to the lethal effects of LPS (533). Similarly, new-born guinea pigs (604) and young dogs (441) are more sensitive than adult animals. Also, individuals of the same age within the same species may vary considerably in their susceptibility to LPS. This explains the poor precision of the Rabbit Pyrogen Test for Endotoxin (22, 262).

LPS has a profound effect on polymorphonuclear leucocytes (PMNL) <u>in vivo</u>; there is an initial marked leucopenia, followed by a leucocytosis (15). This may in some

instances be of diagnostic value (*e.g.* in the diagnosis of neonatal sepsis) (552). Endotoxin is also responsible for hypotension (158), which is one of the important diagnostic criteria of septic shock (279). Hypotension occurs as a result of activation of one of mediators - the kinin system. However, Wolff and colleagues noted an increase in pulse pressure, after administration of relatively small doses of endotoxin to human volunteers (660). At these doses, they did not observe a fall in systolic blood pressure.

LPS may also affect the carbohydrate metabolism. Shortly after injection of LPS, blood sugar levels increase and reach a maximum (several times normal levels) in approximately two hours. This transient hyperglycaemia is followed by hypoglycaemia, which becomes profound (657). A further noteworthy fact is that endotoxin can account for either oliguria or polyguria (181, 629). In infusion studies, a polyguric phase commonly precedes the development of oliguria (82, 181, 629).

1.1.4. Clinical aspects

The role of endotoxin in pathogenesis of bacterial infections is increasing. This is because of the rising proportion of Gram-negative sepsis in the total number of septic cases (3, 118, 260, 270, 271, 438) which at present amounts to 60%-70% (279, 303). An increase in Gram-negative sepsis appears to coincide with the "antibiotic era". Despite antibiotics and recent technological advances in medicine and surgery, mortality due to Gram-negative septicaemia is increasing (7, 142, 270, 313, 363, 550). In some forms of Gram-negative septicaemia, mortality may be even as high as 80%-90% (260, 279, 303, 363), although on average mortality due to this cause is claimed to range between 30% and 50% (438).

The precise aetiology of shock in association with sepsis is uncertain and clearly also involves factors other than bacteraemia (279). Nevertheless, the presence of bacteria in the bloodstream of shocked patients cannot be ignored. As many as half of the patients with septicaemia due to aerobic Gram-negative bacilli, and one third of those with Bacteroides species as the infecting organism develop shock. In contrast, shock occurs in only one patient out of ten suffering from septicaemia due to Staphylococcus aureus (505). The majority of patients who develop septic shock become infected while in hospital. This frequently follows operations on the gastrointestinal or genito-urinary systems, particularly when surgery has been performed under emergency or semi-elective conditions (279). Major trauma and burns also carry an increased risk of Gram-negative sepsis and septic shock (212, 329). In addition, the transplant patient who is rendered immunocompromised has an increased risk of developing these complications. The non-surgical causes of septic shock include pneumonia, endocarditis, meningitis and dermatological infections. Advanced age, diabetes, malnutrition, uraemia, malignancy and immunological defects, and treatment with chemotherapeutic and corticosteroid drugs can also be predisposing factors (279).

Intensive care patients are a group especially at risk of developing septicaemia (672). The combination of their illness (trauma, burn, surgery, metabolic coma, etc.) and iatrogenic factors (foreign bodies, ventilation, drugs, etc.) render them more susceptible to severe infections. One of the frustrations in modern intensive care medicine is that, because of improvement in techniques of resuscitation, the patient survives the period of shock itself, only to die as a consequence of the delayed effects with multiple organ failure (611). This is where prediction, diagnosis, monitoring and treatment of septic shock come as an acute problem. In early phases of septic shock the features of infection predominate, e.g. fever,

tachycardia, hyperventilation and warm extremities, together with a degree of hypotension (the hyperdynamic phase). Cyanosis, vasoconstriction, oliguria and confusion, together with more marked hypotension, are later features (the hypodynamic phase) (279). Clinical indices of shock are fever, urine flow of less than 20 ml per hour and a blood pressure usually below 90 mm Hg (512).

Nevertheless, the precise role of endotoxin during Gram-negative bacterial sepsis remains unresolved (451). In part, this may be due to the vast array of biological effects produced by LPS (see Table 1). Many of these are nonspecific and can be induced by other agents. Consequently, it is not surprising that some manifestations similar to those produced by endotoxin will be found in other conditions, unrelated to the presence of endotoxin. On the other hand, the presence of endotoxin in the circulation does not necessarily result in any clinical manifestations. Transient endotoxaemia has been observed following urethral instrumentation (524), colonoscopy (263) and other endoscopic and roentgenological manipulations but was not accompanied by a rise in temperature or any other sign of malaise. In contrast, in septic shock, where endotoxin is implicated as a major pathogenic factor, levels of endotoxin sometimes may be relatively low (308). This may be explained, at least partially, by the finding that man becomes remarkably hyperreactive to circulating endotoxin during those Gram-negative bacterial infections in which reactivity was tested, i.e. typhoid fever, tularemia and brucellosis (451). Also, it is likely that factors other than endotoxin are more critical in determining the lethal outcome of sepsis (259). Although the exact pathogenesis of the septic response remains undetermined, the response appears to be host-determined (548). It has been suggested that mediators may have a role in this response (36).

1.1.5. Assays for endotoxin

A large number of different techniques has been introduced for the identification and quantitation of endotoxin. This fact reflects the limitations of many of the procedures and shows that the existing assays are unsatisfactory. The usefulness of the individual method is largely dependent upon both the sensitivity (Table 2) and the specificity of the technique itself. Other factors which determine the value of the assay are: ease or difficulty of performance, cost, time requirements, and the circumstances for which the procedure is utilised.

Table 2 Sensitivity of assays for detection of endotoxin

Assay method	Smallest detectable level (approximate) in µg of endotoxin
Lethality in adult rats (LD_{50})†	750
Lethality in adult rabbits (LD_{50})	500
Lethality in adult mice (LD_{50})	150
Inhibition of haemagglutination	10
Induction of local Shwartzman reaction	10
Damage to mouse sarcoma 37	10
Fever in man	3·5–0·07
Hypothermia in mice	2
Lethality in lead acetate treated rats (LD_{50})	1·2
Abortion in mice	1·0
Lethality in thorotrast-treated rabbits (LD_{100})	1·0–0·1
Epinephrine-induced rabbit skin necrosis, at 23°C	1·0–0·1
Complement activation	0·2
Dermal inflammation (SLD_{50})*	0·15
Stimulation of haemagglutinating antibody (rabbit)	0·1
Lethality in histamine-sensitized mice (LD_{50})	0·05–0·007
Lethality in adrenalectomized mice (LD_{50})	0·03
Leucocytosis in mice	0·025
Epinephrine-induced skin necrosis, at 37°C	0·02
Chick embryo lethality (LD_{50})	0·01
Fever in rabbits	0·004–0·000 5
Actinomycin D-enhanced mouse lethality (LD_{50})	0·002
B-cell mitogenicity	0·001
Limulus gelation assay	0·001–0·000 000 1
Hypoferraemia in rats	0·000 1
Fever in rabbits (intrathecal injection)	< 0·000 01

† LD_{50} : Lethal dose 50

*SLD_{50} : Skin lesion dose 50

Fig. 6 L. polyphemus: ●, T. tridentatus: △, T. gigas: ×, C. rotundicauda: ○.

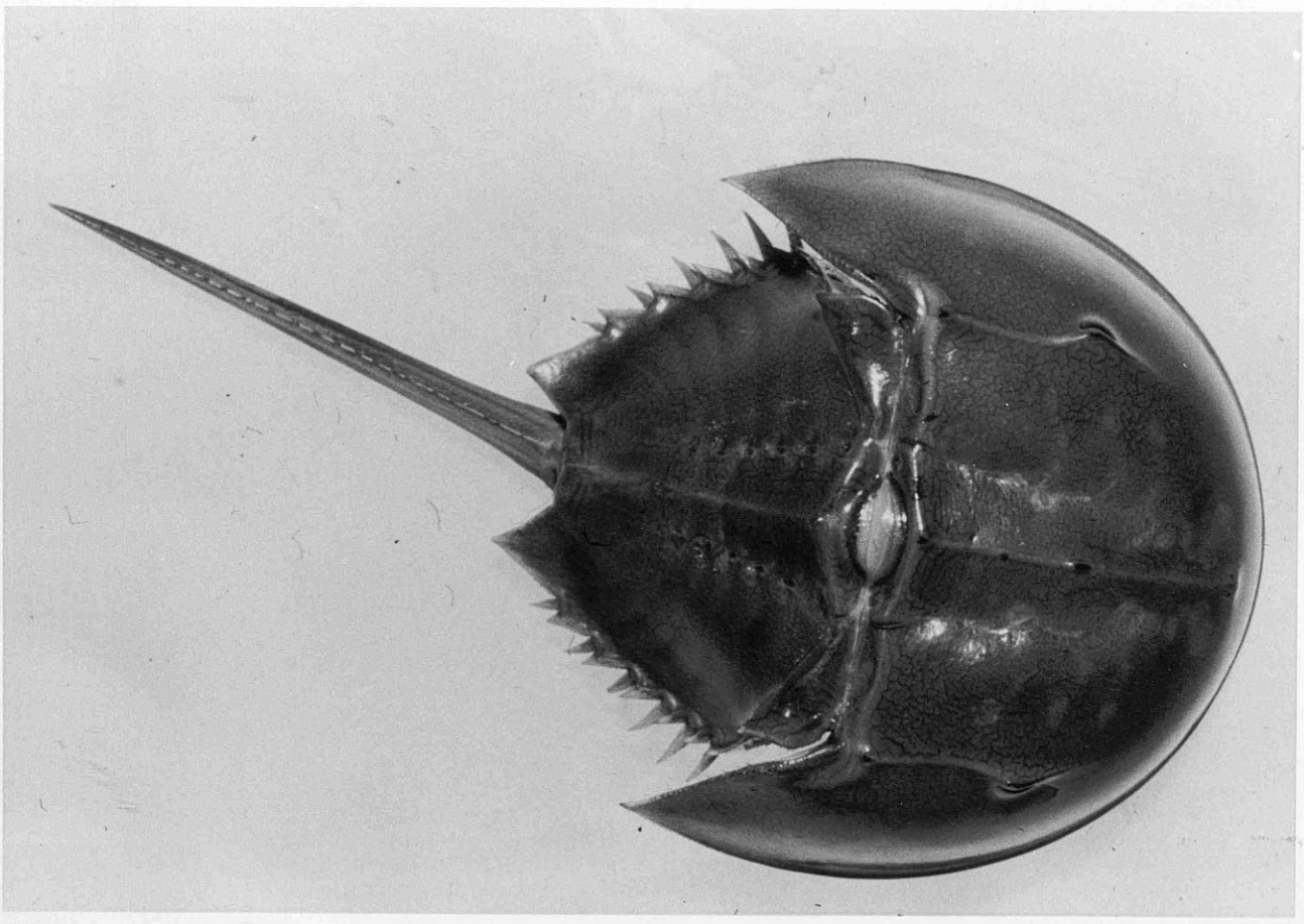

Fig. 5a

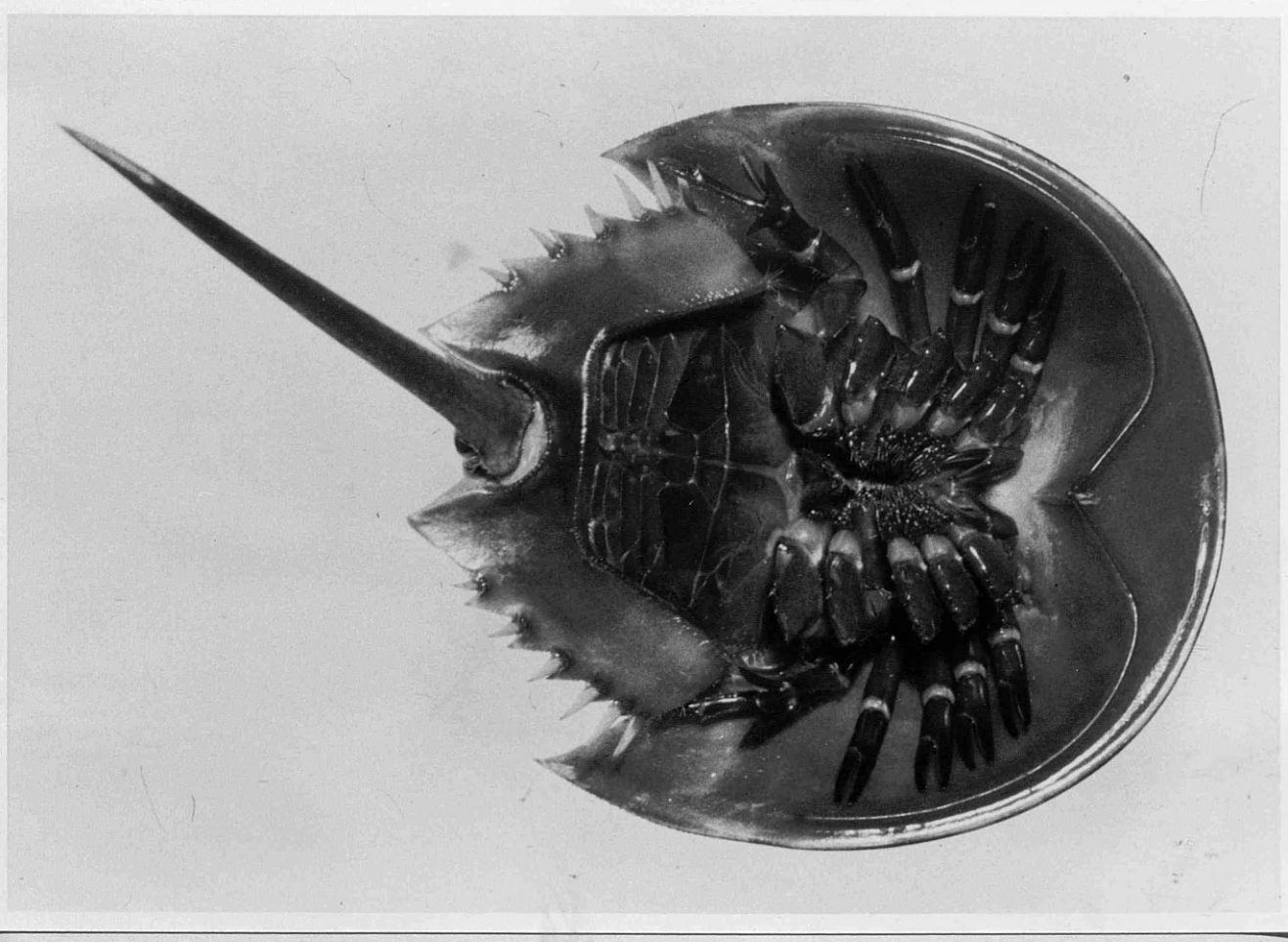

Fig. 5b

Modified from McCabe, W.: Endotoxin: microbiological, chemical, pathophysiologic and clinical correlations. In: L. Weinstein, B N Fields (eds.): Seminars in Infectious Disease, vol III, Thieme-Stratton Inc., New York 1980, pp 38-88.

and

Braude, A. J: Absorption, distribution and elimination of endotoxins and their derivatives. In: M. Landy, W. Braun (eds.): Bacterial Endotoxins, Rutgers University Press, New Brunswick, N J. 1964, pp 98-109.

1 2. Limulus amoebocyte lysate assay

1 2.1. Natural history

Limulus amoebocyte lysate is obtained by lysis of circulating blood cells (amoebocytes) of the horseshoe crab. Nowadays, there are four extant species of horseshoe crabs, in three genera: Limulus (= Xiphosura, the sword tail) polyphemus (Fig 5), Tachypleus tridentatus, Tachypleus gigas and Carcinoscorpius rotundicauda. (466, 519) Serologically, three of these species, i.e. L. polyphemus, T gigas and C rotundicauda appear to be con-generic (517). Figure 6 shows the distribution of each species.

Fig 5. Limulus polyphemus: a) dorsal view; b) ventral view.

Fig 6. Distribution of extant species of horseshoe crabs

From: Sekiguchi, K., Nakamura, K.: Ecology of the extant horseshoe crabs. In: E. Cohen (ed.): Prog. Clin. Biol. Res. . vol 29: Biomedical Applications of the Horseshoe Crab (Limulidae). Alan R Liss, Inc., New York 1979, p p. 37-45.

L. polyphemus occurs on the Atlantic coast of North America, and is widely distributed from Maine to Yucatan (518). The other three species occur in the Pacific Ocean. T. tridentatus occurs in western and southern Japan, Taiwan, the Philippines, North Borneo and Sumatra (502). T. gigas and C. rotundicauda live in almost the same area (502). They have been found along the shores of the Bay of Bengal, Thailand, Malaysia, the Philippines and Borneo. T. gigas has also been recorded in the Torres Straits. L. polyphemus and T. tridentatus are distributed from north to south, while T. gigas and C rotundicauda are distributed from east to west.

All four species are typical benthic animals, digging in the mud and sand of estuaries and shallow marine waters near the shore (553). C. rotundicauda is also able, however, to migrate up rivers into fresh water (11).

In the taxonomic sense, though, the horseshoe crab is a misnomer, which wrongly classifies the family Limulidae as being in the class Crustacea, while in fact they belong to the class Merostomata (520)and are thus much more closely related to scorpions, spiders, mites, ticks and other representatives of the class Arachnida, than to crabs, lobsters or shrimps.

Table 3. Taxonomy of horseshoe crabs.

Phylum	: Arthropoda
Subphylum	: Chelicerata
Class	: Merostomata
Subclass	: Xiphosura
Order	: Xiphosurida
Suborder	: Limulina
Superfamily	: Limulacea
Family	: Limulidae
Genera	: Limulus, Tachypleus, Carcinoscorpius

Modified from: Stermer, L.: Phylogeny and taxonomy of fossil horseshoe crabs. J. Paleontol. 1952; 26(4), 630-639.

and

Shuster, C. N. Jr.: Xiphosurida. Encyclopedia of Science and Technology, McGraw-Hill Book Comp. New York 1982, pp. 766-770.

Limulidae are called "living fossils", as their earliest ancestors (order Aglaspida) from the Cambrian period, dating back 500 million years, were clearly recognizable as horseshoe crabs (379), which are thus also called "Paleozoic relics" (466). Forms nearly identical to Limulus date from the Triassic some 230 million years ago, and by the late Jurassic (135 million years ago) Mesolimulus, the nearest ancestor of today's horseshoe crab to be found in the fossil record, had appeared (553). Fossils of this creature exhibit morphology virtually identical with that of the extant Limulus.

The horseshoe crab owes its name to its characteristic shape. Its other common names include: horsefoot crab, horsefeet, pan crab, piggyback crab and king crab (520). The name "king crab" (11, 218, 401, 530) is due to the crab's blue blood. The

blood circulates in the open cardiovascular system (442), pouring into haemocoel, and consists of one type of circulating blood cell, the amoebocyte (haemocyte), and haemolymph. The blue colour of haemolymph is due to the respiratory pigment - haemocyanin, its predominant protein, in which a molecule of oxygen is carried between two copper atoms. Another important haemolymph protein - haemagglutinin - comprises only a small percentage (approximately 2%) (316) of the total protein in serum. This invertebrate lectin from haemolymph of Limulus polyphemus was first described by Noguchi in 1903 (373). It is capable of agglutinating bacterial cells (422). It was also found to have affinity for sialic acid (N-acetyl neuraminic acid, NANA) residues (74). Sialic acid-containing glycoconjugates are present in every mammalian cell membrane thus far examined(138). These sialic acid residues occupy a terminal position in oligosaccharide chains and thus tend to have an external position in the membrane (598). Therefore the Limulus haemagglutinin should be very useful in the study and purification of membrane glycoproteins, although as yet it has not been widely exploited for this purpose (138). The Limulus haemagglutinin was first isolated and characterized in 1968 by Marchalonis and Edelman (315).

Now there is evidence for two separate agglutinins in Limulus serum. One, directed towards Gram-negative bacteria, has probable specificity for the 2-keto-3-deoxy-octonate (KDO) moiety of the LPS molecule (as reported by Rostam-Abadi and Pistole) (462). Preliminary evidence suggests that this agglutinin is identical to the serum haemagglutinin with reported specificity for NANA (462), which was termed "limulin" by Roche and Monsigny (455). The other agglutinin, isolated by Gilbride and Pistole, is reactive with Gram-positive microorganisms and appears to have a molecular specificity for galactose-like

residues (75, 172, 613, 619). These agglutinins may serve as "opsonins" for the phagocytic removal of foreign cells from the haemolymph of the horseshoe crab (172, 422, 424). They represent also an enormous potential as tools for selective isolation of different types of cells, in particular lymphocyte subpopulations, and also as "cell probe" reagents for investigating the participation of specific cell surface glycoproteins in the initiation of blastogenesis and differentiation (75, 316). The amoebocyte is the only type of circulating cell in the blood of Limulus polyphemus (110, 297). It is a nucleated cell, the cytoplasm of which is packed with granules (286). However, Jorgensen and Smith have shown that haemolymph contains two different cell types, one with large granules and another with no granules, but many mitochondria (250).

The Limulus amoebocyte contains the entire coagulation system of this marine invertebrate (290, 671). Cell-free plasma does not clot and is not required for coagulation, since extracts of washed amoebocytes (amoebocyte lysate) gel following incubation with bacterial endotoxins (289, 290).

Exposure of Limulus blood to bacterial endotoxin results in aggregation of the amoebocytes. Aggregation is asociated with marked changes in cell shape, flattening with an apparent increase in cell diameter, development of long processes that extend from cell to cell, striking degranulation and disruption of amoebocytes, and subsequent extracellular clot formation (110, 282, 288). This endotoxin-induced clotting phenomenon has been characterized as a possible defence mechanism, serving to immobilize invading Gram-negative bacteria (21, 284) and it has been postulated that the Limulus amoebocyte may play an important role in host defence against bacterial invasion and dissemination (21, 282, 290, 513). Additional evidence for the role of the Limulus amoebocyte in host

defence was reported by Nachum and colleagues, who showed that the amoebocyte contains an endotoxin inactivating capability (353). The bactericidal activity remains when the amoebocyte lysate is treated with mild heat (60°C for 10 minutes) (351) in order to inactivate the clotting system (561). This indicates a system at least partly distinct from the LPS activation of the clotting system (423).

Endotoxin cannot enter the intact cell. Consequently, in vivo, endotoxin must mediate the degranulation and lysis of amoebocytes before the activation of the clotting system. Liu and colleagues proposed the existence of endotoxin receptor site(s) on the outer membrane of Limulus amoebocyte. They isolated and purified a protein with a molecular weight of about 80 000 from amoebocyte membranes (296). This protein binds with endotoxin and enhances the Limulus lysate coagulation process as effectively as about a 100-fold excess of endotoxin. It is possible that the "endotoxin-receptor protein" plays a role in the Limulus coagulating system.

Secretion in Limulus amoebocytes is by exocytosis (398). Amoebocyte secretory granules move towards the plasmalemma with outer granules fusing with the plasmalemma, followed by sequential exocytosis of more internal granules.

Amoebocytes appear unable to phagocytize E. coli (13). In the presence of the bacteria, the cells degranulated, flattened, became immobile, and massive amounts of extracellular gel were formed. However, amoebocytes were able to phagocytize carbonyl iron particles, especially when these had previously been rendered endotoxin-free by heating (13). It was suggested that Limulus amoebocytes could handle foreign particles in two ways. The extracellular

clotting system deals with Gram-negative organisms and phagocytosis deals with other types of foreign particles.

The major biomedical applications of Limulidae are: the detection of bacterial endotoxin, using a lysate from the amoebocytes, and the probing of cellular membrane constituents, using agglutinins from the haemolymph (520). The credit for the Limulus amoebocyte lysate (LAL) test for detection of endotoxin goes to Dr. Frederik Bang. In 1956 he observed that when horseshoe crabs were injected with live or dead Gram-negative bacteria, their blood clotted, resulting in fatal intravascular coagulation (21). Subsequently, in 1964, Levin and Bang discovered that the agent responsible for the clotting reaction in the horseshoe crab was localized in the amoebocytes and that the lipopolysaccharide fraction of the cell wall of the Gram-negative organisms triggered the clotting reaction (288, 289). In 1968, Levin first reported the use of Limulus lysate bioassay to detect the presence of endotoxin (283).

Amoebocyte lysate is prepared from washed amoebocytes (286), which in the original method have been disrupted by osmotic lysis by suspension in distilled water, or by freezing and thawing (290). Further modifications of cell rupture methods involved the use of:

- Shaking during lysis with distilled water
- vortex mixing
- tissue grinding (blender or homogenizer)
- sonification
- use of Tris (tris [hydroxymethyl] aminomethane) - buffered 3% saline pH 7.0 as a medium to suspend cells for lysis (223, 600, 667).

The method of producing LAL with the maximum sensitivity to LPS involved:

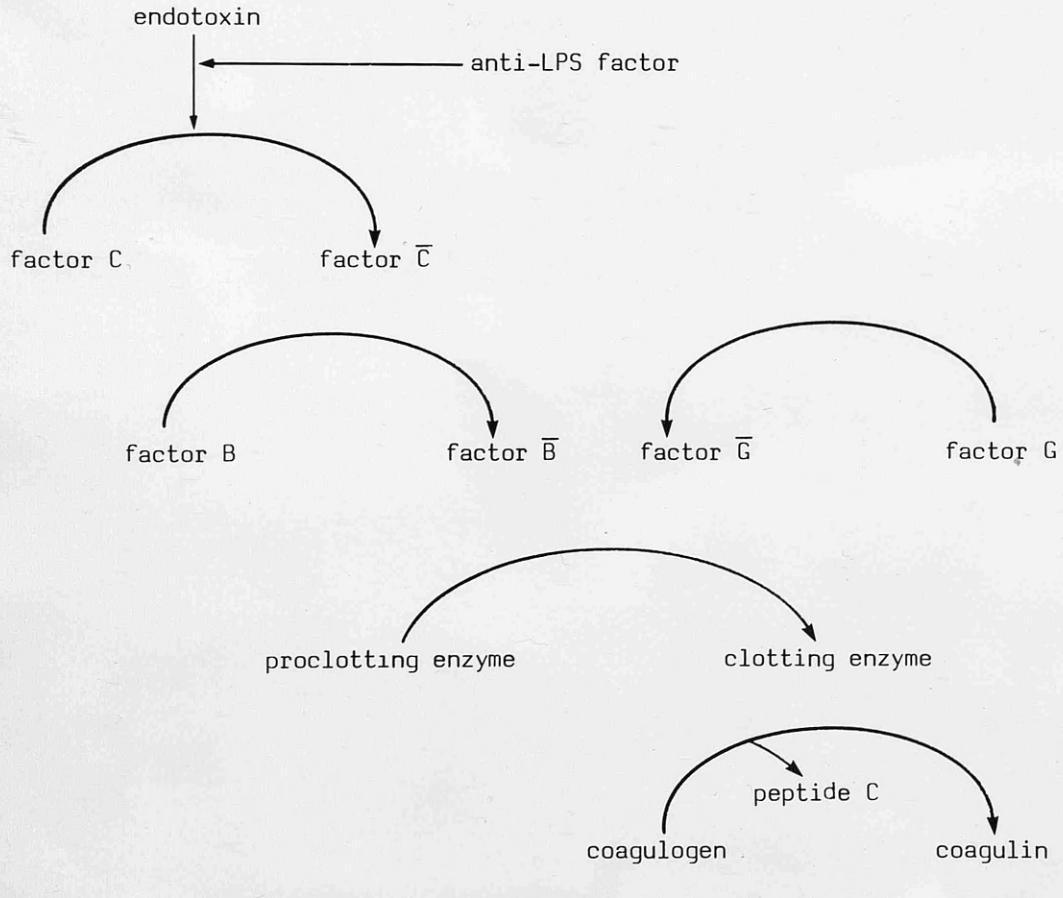
endotoxin
anti-LPS factor
factor C
factor $\overline{C}$
factor B
factor $\overline{B}$
factor $\overline{G}$
factor G
proclotting enzyme
clotting enzyme
peptide C
coagulogen
coagulin

Fig. 7

avoidance of the anticoagulant N-ethylmaleimide (NEM) in the procedure of bleeding crabs, effective mechanical homogenization of cells and optimal pyrogen-free conditions (600). Also, Tris-buffered saline seems to have an advantage over distilled water (223).

1.2.2. Biochemistry

Reaction between Limulus amoebocyte lysate and endotoxin

Endotoxin activates the clotting cascade of Limulus amoebocyte lysate (Fig.7). Endotoxin reacts with LAL in conjunction with an appropriate concentration of divalent cations, usually Ca^{++} and/or Mg^{++} (268,372,559,594).

Fig.7. Clotting cascade of endotoxin - activated Limulus amoebocyte lysate.

C,B,G - proenzymes

$\bar{C},\bar{B},\bar{G}$ - activated enzymes

Modified from the presentation of T. Morita during the "International Conference on Endotoxin Assays", May 25-26, 1984, Academic Medical Centre, Amsterdam, the Netherlands, in the paper: Biochemical characterization of Limulus clotting factors and inhibitors which interact with bacterial endotoxins.

Components of the Limulus amoebocyte lysate taking part in the reaction with endotoxin

Factor C was discovered by Nakamura and Levin, who called it Protease II (355). Tsuji in his model proposes that Protease II consists of two separate proenzyme-enzyme systems (594). Factor B, discovered by Ohki and colleagues (389), is also

called proactivator (355). It is a precursor of the proclotting enzyme activating factor, designated activator. Both the activator and the clotting enzyme are trypsin-type serine proteases. Thus, limited proteolysis by the activator of an Arg-X or Lys-X bond(s) in the proclotting enzyme molecule probably causes activation of the proenzyme (356). This is similar to blood coagulation mechanisms of higher animals, in which a cascade-like activation by limited proteolysis of several protease precursors is involved (98).

The intact molecule of the activator (M.W. 50 000) presumably is composed of two identical subunit chains (M.W. 22 000) associated by noncovalent bond(s). Probably each of the subunits consists of multiple polypeptide chains, including a heavy chain (M.W. 17 000), linked by disulphide bond(s) (355). Active factor B converts proclotting enzyme to the clotting enzyme (203).

The clotting enzyme, also termed coagulase (486, 594), was isolated by Sullivan and Watson (561) and the proclotting enzyme was purified by Tai and Liu (568). The proclotting enzyme, also known as the procoagulant enzyme (286, 430), or procoagulase (486), appears to be a protein consisting of a single peptide chain (M.W. 150 000). It contains γ-carboxyglutamic acid (569). The clotting enzyme in the study of Sullivan and Watson had a molecular weight of 84 000 and consisted of two identical subunits (M.W. 43 000). These findings were later confirmed by Seid and Liu (499). They demonstrated that, in the Limulus amoebocyte lysate, two active forms of the clotting enzyme existed, with the molecular weight of 79 000 and 40 000. The amino acid compositions of the two proteins were very similar, indicating a "monomer" - "dimer" relationship, but atempts to interconvert the 79 000 protein into the 40 000 protein have not been successful.

Clotting enzyme displays substrate specificity similar to that of mammalian blood coagulation factor, factor Xa (357). The clotting enzyme from Tachypleus tridentatus, which has been purified and characterized (357), shows very similar properties.

Limulus amoebocytes contain at least two independent coagulation pathways, endotoxin-mediated and (1 -> 3) - β - D - glucan - mediated (Fig.7), both of which result in the transformation of coagulogen to coagulin. (1 -> 3) - β - D - glucan sensitive factor is named factor G and acts on the proclotting enzyme (332).

Coagulogen, also called the clottable protein (286, 430, 539), constitutes approximately 50% of amoebocyte lysate (354, 539, 540). Reported estimates of the size of the Limulus polyphemus amoebocyte lysate coagulogen indicate that its molecular weight lies between 19 000 and 27 000 (154, 341, 569, 671) and that each molecule consists of a single chain (540, 569). However, Mosesson and colleagues (341) concluded that coagulogen consists of two chains of equal mass, connected by a disulphide bridge. The first coagulogen to have the amino acid sequence determined was that of Tachypleus tridentatus (360, 571). Its chain consisted of 175 residues (571); the molecular weight was between 16 000 and 23 000 (354, 514). Furthermore, under the same conditions the molecular weight of coagulogens from all four species of horseshoe crab was found to be identical (515) and equal to 20 000. However, immunological studies have shown that although coagulogens of all three Asian species were closely related to one another, they were very different from the American species (515). Physicochemical properties, the gelation mechanism and amino acid sequences of all four coagulogens obtained from the extant species of horseshoe crabs have been elucidated in the work of

Nakamura and colleagues (359).

The clotting enzyme causes limited proteolysis of the Limulus coagulogen (Fig.7) by release of C-peptide containing 45 amino acids. The remaining fragment, designated coagulin or the N-peptide and consisting of A and B chains, polymerizes via disulphide cross-links to form a clot (309,569). C-peptide, which is released during coagulation from the coagulogen of Tachypleus contains 28 amino acids and appears homologous to primate fibrinopeptide B.

Specificity of the reaction between Limulus amoebocyte lysate and endotoxin.

The LAL assay has been considered very specific for endotoxin, despite reports by a number of investigators about the activation of LAL by other substances (411). Many of these are high-molecular-weight carbohydrates, like peptidoglycan (647), synthetic dextrans (566), carrageenan (408), (1->3) - β - D - glucan (254,332), mannans and dextrans (327), and lipoteichoic acid (645).

These reports about non-specificity of the LAL test have been dismissed on the grounds that polysaccharide concentrations required for a positive reaction were far in excess of the endotoxin concentrations required to produce a positive Limulus test. For example, Levin calculated that as little as 0.1% - 0.000 25% contamination with endotoxin could have been responsible for the positive results of peptidoglycan in the LAL assay reported by Wildfeuer and colleagues, although they did attempt to exclude contamination (287). Similar claims of reactivity in the LAL assay have been refuted, also on the basis of endotoxin contamination (497, 521, 666), for dithiols (426), thrombin, thromboplastin, polyribonucleotides and ribonuclease (117).

Recent studies, however, have shown that a cellulose-derived material can mediate a LAL-coagulation pathway, which is independent from that mediated by endotoxin (215, 408). This LAL-reactive material (LAL-RM) was shown to be nonpyrogenic, as, at tested doses between 380 and 2 000 ng per kg, it failed to elicit a pyrogenic response in rabbits. It was also unreactive in a number of in vitro biological assays - human leucocytic pyrogen (LP), lymphocyte-activating factor (LAF), peritoneal macrophage tumouricidal activity and arginase release (412). Also, the reactivity of LAL-RM with LAL varies depending upon the lysate used. Although all four LAL preparations used were standardized to the US national reference endotoxin prepared from E. coli, the Mallinckrodt LAL consistently yielded negative results, regardless of concentration. Furthermore, cellulase reduced the activity of LAL-RM but had no effect on endotoxin. Gas chromatographic and mass spectrophotometric analyses also indicate that a cellulose-derived material is the source of LAL-RM. This is indicated by the LAL activity of the 24 000 - molecular weight peak, and by the fact that both this fraction and 95% of the Limulus activity are reduced by treatment with cellulase. Also, although LAL-RM can be virtually eliminated by constant rinsing, the level of LAL-RM returns overnight, typically to 25% of the starting value (408). It was shown additionally that endotoxin exposed to conditions occurring in the process of industrial manufacturing of this cellulose-derived material was degraded and washed out, while LAL-RM, after exposure to the conditions of the production process, retains its endotoxin-like activity (215). As LAL-RM gives positive results in the chromogenic LAL assay, it suggests that the activation of LAL occurs at or before the proclotting enzyme stage (see Figs. 7 and 8) (408).

1.2.3. Limulus amoebocyte lysate assays for endotoxin

Introductory remarks

The LAL test may be applied in various areas of research into endotoxin, but its single most important use is as an alternative test to the official Rabbit Pyrogen Test for Endotoxin

The LAL test has numerous and great advantages compared with the rabbit test. Of these, the most important are speed, simplicity, quantification and reduced sample volume. The LAL assay is much more sensitive than the Rabbit Pyrogen Test. It is also much more specific, since not all pyrogens are endotoxins. The LAL assay does not suffer from the disadvantages of a bioassay, such as great variability of results. Age, weight, sex, route of administration, previous history of the test animal, its health and genetic make up all influence an animal's response. Even if all of these factors could be controlled, there would still be certain biological variations in the response of identical members of the same species to drugs and toxins. One individual will not respond identically to the same dose of a substance when it is administered on two separate occasions either. Also, some substances have the capacity not only to elicit many biological effects directly, but also to activate various body systems to produce other physiological and pathological changes in the organism indirectly.

Many species have systems to potentiate or inhibit the action of drugs or toxins. Furthermore, these modifying systems may not be equally manifested in all individuals of the same species. The signs and symptoms shown by test animals after drug or toxin administration are, then, the sum of the effects of the substance itself plus the effects of the activated potentiating or inhibiting system which existed in the animal prior to drug administration. All of these factors

may be active when endotoxin is administered to test animals (589). There are also cyclic and chronobiological factors, both circadian (26, 209) and circannual (26, 525) which may affect the Rabbit Pyrogen Test. In addition, the Rabbit Pyrogen Test is unsuitable for testing of some drugs because of their pharmacological effect upon rabbits. For example, epinephrine, methylene blue, and amphotericin B are inherently pyrogenic (64). Pyrogen-free phosphate ion may induce a febrile response when injected in sufficient quantities (418). Conversely, calcium gluconate, some sedatives and anaesthetics, corticosteroids, phenothiazine derivatives and antipyretic drugs may lower rabbit temperatures and mask the pyrogenic potential of test solutions (29, 32, 418).

Certain drug classes are inappropriately pyrogen tested because of various properties. Examples include cancer chemotherapeutic agents, such as L-asparaginase (88), and antibiotics like lincomycin (206). Short-lived radioactive drugs also are not appropriately tested by the Rabbit Pyrogen Test because of time, sample size, problems of personnel exposure to radiation and control of radioactive waste (85, 87). The rabbit bioassay is unsuitable for screening intrathecal products on acount of its insensitivity (86). The intrathecal route of administration is exquisitely sensitive to the presence of pyrogen (30). The sensitivity of the Limulus test is especially advantageous for screening drugs designed for intrathecal administration, such as: contrast media, cancer chemotherapeutic agents, antibiotics, radiopharmaceuticals, and chemical dyes (85).

Finally, the susceptibility of rabbits to nonspecific febrile responses, the development of endotoxic tolerance and the restrictions for testing biological agents which contain antigenic materials are additional problems that may be

encountered with the rabbit assay (185,188,418,578).

The Limulus amoebocyte lysate assay correlates well with the accepted, but less specific and less sensitive assays for endotoxin (Table 2) and it has been approved by the United States Pharmacopeia as the official Bacterial Endotoxins Test (606). This, after meeting certain requirements, allows the use of LAL for end-product testing. However, the European Pharmacopoeia has not yet recognized the LAL assay. However, although the existing national legislation excludes its application for end-product testing, the LAL test has become useful to European manufacturers of drugs and medical devices as an in-process control of raw materials, components and of various stages during the manufacture of drugs and devices (350, 620, 621). The high sensitivity of the LAL test in comparison with the rabbit test ensures a wide safety margin.

The rapid nature, simplicity and low cost of the LAL test allow the extensive testing necessary to evaluate variations in manufacturing procedures that will ultimately improve the purity of the final product (224).

Limulus amoebocyte lysate assays

The original LAL assay was a simple gelation test (gel-clot test) (283). Subsequent modifications have involved a micro-slide method (145, 180), a micro-method with dye and phase contrast (390), a micro-slide method in wells with capillaries (139), a micro-slide method in wells with dye (325), a micro-slide dry-up method (178), a micro-capillary method with dye (168), a micro-capillary method based on hydrostatic pressure (364), a micro-plate method with capillaries (269), a micro-plate microdilution method with dye (431) and a LAL-bead assay (203). The gel-clot test is cheap and very simple but has a major disadvantage, namely that

it is not quantitative. Also, its sensitivity, at 0.02 - 1.0 EU per ml (375) is low. In the turbidimetric (nephelometric) test, the optical density of a turbid mixture of LAL and endotoxin is measured in the spectrophotometer or in a nephelometer. The most commonly used wavelength is 360 nm (optimum) (375, 577). This is a quantitative test, as turbidity is proportional to the amount of active clotting enzyme, and hence to the amount of endotoxin present in the test solution. It can be performed in two ways: as an end-point method or as a kinetic assay. The latter was first described by Hollander and Harding in 1976 (226). Kinetic assays offer sensitivity as high as 0.0005 EU/ml (104, 249, 375, 381), but are expensive and require highly specialised equipment. End-point methods are usually cheaper to perform, but are much less sensitive. Their threshold of sensitivity in most cases is only 0.01 EU/ml (5, 107, 577). However, Fink and colleagues (131), and Valois (614) reported obtaining much higher sensitivities which were comparable with those of the kinetic assays.

The colorimetric test is also quantitative, but unlike the turbidimetric method, the reading of the endpoint is indirect. The precipitated clot is assayed by the Lowry procedure for protein measurement, which requires a spectrophotometer with visible light wavelength capability (usually 500 or 700 nm) (375). Since the protein in the precipitate is directly proportional to the amount of coagulogen cleaved by active clotting enzyme, a standard curve can be constructed for determination of endotoxin concentration in the tested samples. This method was first reported by Iiiva and colleagues in 1974 (371). Later it was improved by Nandan and Brown (362), allowing detection of 0.05 EU/ml.

The radioisotopic method, which is based either on measurement of ^{125}I-labelled coagulogen (348) or ^{125}I-labelled antibodies to O-polysaccharides of Escherichia

coli (281), is quantitative and has a sensitivity of up to 0.05 EU/ml (375), but it is labour-intensive, and requires special facilities and expensive equipment.

The rocket immunoelectrophoresis assay, which involves crossed immunoelectrophoresis of LAL against rabbit antiserum (20) is semiquantitative, with a sensitivity very similar to that of the radioisotope method (375). It is also expensive and requires specialized equipment.

The fluorogenic assay, which uses amoebocyte lysate labelled with a fluorescent probe, fluorescamine, is based on measurement of changes of fluorescent polarization. These changes are proportional to the amount of endotoxin present in the test solution (668, 669).

Finally, there are enzymatic methods, which measure cleavage of a synthetic chromogenic peptide substrate by the clotting enzyme (196, 233, 357, 389). The chromogenic LAL assay will be discussed in more detail below.

Chromogenic LAL assay

The chromogenic LAL assay was first described in 1977 by Nakamura and colleagues (358). In this method the coagulogen is completely or partially replaced by a chromogenic substrate. The chromogenic substrate is a small synthetic peptide covalently bound to a chromophore. The synthetic peptide contains the same, or a similar, sequence of amino acids as coagulogen. Thus the clotting enzyme acts on the chromogenic substrate in the same way as on coagulogen. The chromogenic substrates currently available for LAL all contain para-nitroaniline (pNA) as the chromophore (233). When linked to the synthetic

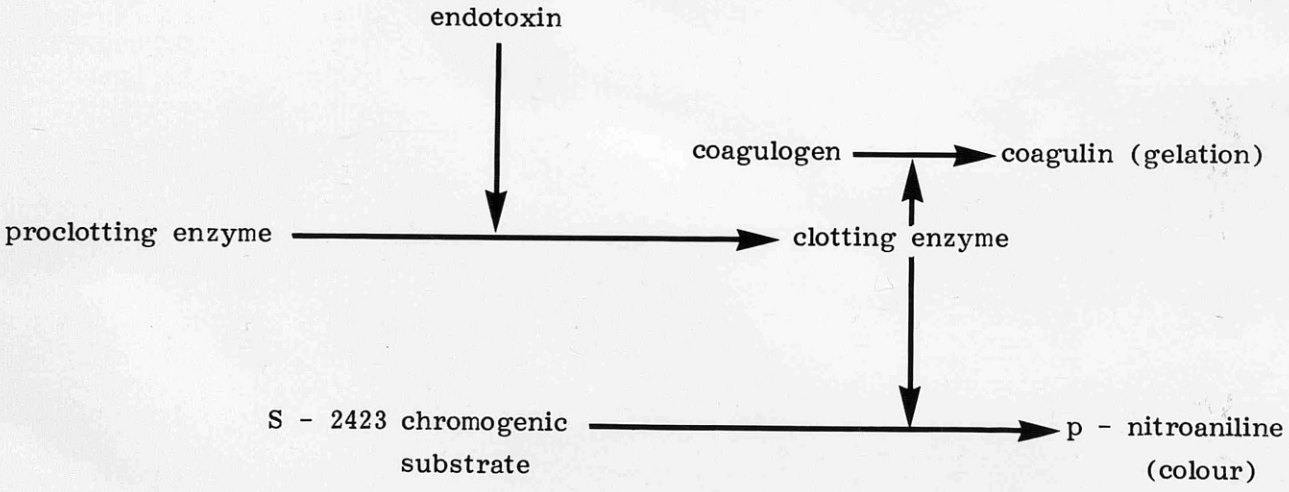
endotoxin
coagulogen
coagulin (gelation)
proclotting enzyme
clotting enzyme
S - 2423 chromogenic substrate
p - nitroaniline (colour)

Fig. 8

peptide, para-nitroaniline is colourless and when free, it is yellow. It is released in proportion to the amount of endotoxin present in a sample (Fig 8). Optical measurement of the colour makes this method quantitative.

Fig.8 The principle of the Limulus amoebocyte lysate assay with the chromogenic substrate S-2423.

The Limulus clotting enzyme exhibits amidase activity. This Factor Xa-like serine proteinase appeared to have a substrate specificity similar to blood clotting factors and their related enzymes. Thus it acted upon a number of chromogenic substrates, which were originally developed for determination of components of the coagulation and fibrinolytic systems (2,233,655; see also monographs : 1,43,147,495,534).

The clotting enzyme displays a high specificity towards para-nitroanilide (peptide-pNA), with the COOH-terminal Gly-Arg sequence (196). Furthermore, the clotting enzyme has preference for hydrophobic residues (Leu or Val) in the P_3 position. Increasing the length of the peptide chain has little effect on the reactivity of the substrate. Therefore, Harada-Suzuki and colleagues recommended the tripeptides, Boc-Leu-Gly-Arg-pNA and Bz-Val-Gly-Arg-pNA (where Boc = tert-butoxycarbonyl and Bz = benzyl) as substrates for the clotting enzyme (197). And, indeed, commercial peptide substrates, which have been used in the chromogenic LAL assay, i.e. S-2222, S-2422, S-2433, S-2423 are all tetrapeptides (2).

The chromogenic LAL assay is usually performed in two stages, since optimal conditions for the activation of the proclotting enzyme by endotoxin are: pH 6.0-7.5 and relatively high ionic strength, while optimum pH for most available substrates is usually greater than 8.0 with low ionic strength (375).

Furthermore, coagulogen present in the lysate competitively inhibits the amidase activity of the clotting enzyme on a chromogenic substrate. This renders many LAL preparations incompatible with chromogenic substrates, which is seen as a loss of sensitivity of the method.

Generally, however, the method appears to be much more sensitive than the Limulus gelation test. Harada-Suzuki and colleagues in their work reported that, under certain conditions, the chromogenic substrate assay method was 50 times more sensitive than the Limulus gelation test (197).

Applications of the LAL assay

There are now many applications of the LAL test. The general areas of application are given in Table 4. Further details are given below.

Table 4. Applications of the LAL assay.

Non-clinical	Clinical
mains water	blood
industrial water	cerebrospinal fluid
wastewater	urine
food products	synovial fluid
airborne dusts	gingival specimens
biological reagents	peritoneal fluid
pharmaceutical products	urethral and cervical exudates
medical devices	ocular aspirates and scrapings
	ear specimens

Non-clinical applications

Endotoxin is ubiquitous and varied levels are found throughout the environment. Müller-Calgan (350), in a study in the West German town of Darmstadt, found 0.2 ng of endotoxin per ml in central pump water, 0.4 ng per ml in central town water, 0.8 ng per ml in domestic drinking water, and 1.6 ng per ml in drinking water in an office supply. Water in a natural swimming pool in Darmstadt contained 25.6 µg of endoxin per ml.

Müller-Calgan also reported that the endotoxin level in grape sugar from emergency rations was 256 ng/g. Furthermore, Limulus amoebocyte lysate assay has been used for testing other food products to determine their quality. Among

these products were ground beef (236,237,238) and other kinds of meat (500), lean fish (557) and milk (195,208,331).

The assay has been shown to be of value in determination of bacteriological quality of water (124,248) and in assessment of the effectiveness of wastewater renovation (247). It has also confirmed the large endotoxin load of the marine environment (634), which is the habitat of horseshoe crabs.

The Limulus amoebocyte lysate assay has been used to screen various occupational environments for the presence of airborne endotoxin. Large quantities of viable bacteria and related endotoxins can be found in dusts in animal confinement units (72, 581), compost plants (71, 302), cereal grain elevators, mills and other processing plants (99, 393, 395), poultry processing plants (394,582), plants processing vegetable fibres such as cotton (33,70,134,391, 392,484), flax and grass (478,484) and also in air humidification systems (135,136, 475,477). Airborne endotoxin in occupational environments is thought to be the cause of various hypersensitivity reactions which are mainly respiratory (63,65, 229,476,479,535,615). These reports may be of value in the application of the LAL assay in routine environmental screen tests. This is because endotoxin levels can be used potentially as measures of "toxicity" or "cleanliness" of airborne dusts (391,393).

Limulus amoebocyte lysate assay has also been used for testing biological reagents for endotoxin contamination. Since endotoxin is very potent in a wide range of biological activities (See section 1.3), it can alter many biological assays and processes. The LAL assay has been employed for detection of endotoxin in some mitogens, such as concanavalin A (Con A) (150, 448), phytohaemagglutinin

(PHA) and pokeweed mitogen (PWM) (152); in some batches of horse serum, used in tissue culture media (152); in some therapeutic agents, used in experimental research, such as antilymphocyte serum (ALS) and transfer factor (TF) (152), interferon (IFN) and lymphokine (LK) preparations (41), erythropoietin (152, 437, 545); and in type C RNA virus reagents, used to study cell mediated immune (CMI) responses (105).

The LAL assay has been employed routinely in the pharmaceutical industry for both in-process and final pyrogen testing of injectable pharmaceutical products, and for testing of medical devices before release.

Endotoxic reactions are usually systemic, and are manifested by fever, chills, rigors, tachycardia, headaches, hypotension, diarrhoea, myalgia, nausea, vomiting and other adverse host responses (330). In the most severe cases the patient may develop endotoxic shock, which may be fatal. For this reason, manufacturers are legally obliged to test injectables and certain medical devices for endotoxin contamination. Where the legislation of the given country permits, the LAL assay for endotoxin is the only test employed; otherwise, it is used as an auxiliary test (320, 635). Local reactions to endotoxin are also known. The LAL ssay established that contact dermatitis in a worker using irradiated latex gloves was caused by endotoxin (516). This case highlights problems associated with endotoxin contamination of "plastic software" for the pharmaceutical industry and difficulties connected with re-use of medical devices. Indeed, the LAL assay has been developed into a method for determining pyrogen burden (pyroburden) (378) on packaging components used in sterile manufacturing (214).

The LAL assay has been used by the Centers for Disease Control (CDC), USA, in investigating outbreaks of pyrogenic reactions associated with parenteral fluids and medical devices, as part of their Hospital Infections Program (219, 220). The LAL assay has been used for detection of endotoxin in reusable cardiac catheters (272, 446), hepatic catheters (591) and Foley catheters (170) after outbreaks of pyrogenic reactions in patients. Inadequate sterilization, which did not render the catheters pyrogen-free, was blamed in these cases, which occurred in the USA. Interestingly, plasticware is pyrogen-free immediately following moulding (318, 378) but rapidly becomes contaminated if the manufacturing environment is not controlled. Also, the risk of endotoxin contamination increases if the final product is a result of a complex process, as is the case with catheters (170). Also, in the USA, there is a legal requirement for catheters to be sterile and to pass lead content, acute toxicity and animal implantation tests according to the Federal Hazardous Substances Act, but they are not required to be endotoxin or pyrogen-free, or to be routinely tested for pyrogenicity. This legal loophole may be a reason for pyrogenicity of some brand new medical devices (170).

The Limulus amoebocyte lysate assay has also been applied to routine pyrogen testing of parenteral products . The US Pharmacopeia designates the LAL test as the "official" pyrogen test for water for injection and 29 different radiopharmaceuticals (376). The LAL assay, among others, has been employed for LVPs (Large Volume Parenterals) (151, 318, 319, 410, 590, 601), SVPs (Small Volume Parenterals) (601), vaccines (bacterial as well as live and inactivated viral) (88, 222, 269, 275, 386, 440), allergenic extracts (54, 222), viscous, opaque solutions of iron dextran (155), a variety of antitumour agents (151, 152, 523), intravenous fat emulsions (193, 403), antibiotics (206, 311, 367, 572, 595),

radiographic contrast media (RCM) and coagulation factor preparations (152, 222), plasma fractions (albumin, immunoglobulin, cryopoor plasma) (167, 222, 251, 317, 319, 407, 549), peritoneal dialysis solutions (319) and radiopharmaceuticals (151, 222, 349, 445, 601).

Clinical applications

The Limulus amoebocyte lysate assay has been used for detection of endotoxin in gingival inflammation (507), synovial fluid (114, 115, 596), ascites fluid (73, 576), urine (242, 243, 244, 245, 307), cerebrospinal fluid (112, 246, 352, 460, 522, 586), urethral (253, 431, 432, 434, 541) and cervical exudates (433, 542), ocular aspirates and scrapings (304, 661) and ear specimens (35).

The LAL test for detection of endotoxin in blood has also been described (104, 131, 202, 291, 584) but it is complicated by the presence in blood of natural inhibitors of the test (292), and the usefulness of the LAL assay in plasma/serum has been disputed (114, 116, 285, 438, 463, 556).

Theoretically, three types of factors may affect the chromogenic LAL assay:

those which interfere with LAL;

those which interfere with endotoxin; and

those which interfere with the chromogenic substrate.

Apparently, all three are present in blood and in the LAL itself.

As mentioned above in subsection 1.2.2., the clotting enzyme of the LAL cascade displays substrate specificity similar to that of factor Xa, which is a blood coagulation factor. In this way, factor Xa can replace the clotting enzyme in the

LAL coagulation pathway, cleaving coagulogen, which results in the formation of coagulin and subsequent non-specific gel formation. Factor Xa can also compete with the clotting enzyme for the chromogenic substrate which in fact was initially synthetized for factor Xa. Apart from factor Xa, blood also contains several other enzymes with amidase activity, e.g. plasmin, thrombin and urokinase, which cleave some substrates having the -Gly-Arg-pNA terminal sequence (149,638). Further, coagulogen, if present in significant quantities in LAL designated for use in the chromogenic assay, will inhibit the chromogenic reaction, as the clotting enzyme will preferentially cleave it to the exclusion of the chromogenic substrate (competitive inhibition).

In order to obtain Limulus amoebocyte lysate, which would be highly specific to endotoxin, it is also necessary to remove factor G. Factor G is β-D-glucan sensitive and acts upon the proclotting enzyme (see Fig.7). On the other hand, antithrombin III and α_2-plasmin inhibitor (α_2-antiplasmin), which are proteinase inhibitors naturally occuring in blood plasma, very strongly inhibit the amidase activity of the clotting enzyme (357, 457). Purified antithrombin II was also found to have a strong inhibitory effect on the LAL assay (638). Since the active consituents of the LAL are proteins, common protein denaturants, such as sodium dodecyl sulphate (SDS) and urea also prevent gelation of the lysate by LPS (558). Moreover, anticoagulants can have a profound, dose related, inhibitory effect on LAL. For example, it has been shown that the LAL reagent can be inhibited by chelating agents which remove the divalent cations essential for the complete activation of the LAL proclotting enzyme (289,380,558). Citrate and EDTA (ethylenediamine tetraacetic acid) are such agents.

EDTA has also been shown to cause structural changes in endotoxin due to the removal of divalent cations. Such modified LPS would not clot the LAL (558). Anticoagulants which depress plasma calcium levels should also be avoided since depression of plasma calcium levels results in the activation of globulins with esterase activity, which disaggregate and detoxify endotoxin (458).

With heparin, early reports stated that doses of up to 100 units per ml of plasma (292), or even of 1 000 units/ml (288, 289) had no effect on the gelation of LAL, and values up to 200 units/ml were recommended for anticoagulation (449). However, Sullivan and Watson reported that as little as one unit of heparin per ml was inhibitory to the LAL gelation test, although this inhibition was overcome by the addition of calcium chloride and sodium chloride to LAL (560). McConnell and Cohen also found that, with concentrations of heparin of approximately 30 units/ml, there was a 90% reduction in endotoxin detectable by the chromogenic LAL assay (310). However, the inhibitory effect of up to 100 units/ml could be neutralized by the addition of protamine sulphate. In contrast, Ronneberger found inhibition of gel formation only with concentrations of heparin exceeding 50 units/ml (457). This difference in concentrations of heparin affecting the LAL assay may be due to a difference in sensitivity between the chromogenic and gel tests.

Endotoxin inhibitors present in human blood can also interfere with the LAL assay. Webster showed that at 37°C, endotoxin in serum was inactivated rapidly at a rate of up to 600 ng/ml/hour. At 5°C inactivation was slower, but still accounted for the disappearance of a substantial fraction of the added endotoxin after 24 hours (638).

Levin and colleagues suggested that the inhibitory nature of blood was primarily the result of reversible binding between endotoxin and serum protein(s), rather than destruction of endotoxin (292). Das and colleagues found that endotoxin in plasma was bound to the surface of platelets (97). This was thought to be an intermediate step in detoxification by the reticuloendothelial system.

Skarnes and Chedid demonstrated that a biological inactivation of endotoxin was a two-step reaction (528); the first step being degradation of endotoxin by the α-globulin fraction of serum, followed by a second step of detoxification. Later Skarnes postulated that α-globulin, which caused disaggregation of LPS, was a heat-stable α_1 lipoprotein esterase. Detoxification was effected by the second α_1-globulin esterase, which was organophosphate-resistant and heat-labile and which reacted enzymatically with the disaggregated LPS (526, 527, 529). Similar two-step, albeit non-enzymatic detoxification of LPS was postulated by Ulevitch and Johnston (605). Johnson and colleagues also demonstrated that α-globulin was an endotoxin inactivator (240). But their studies sugested that it was a single protein which inactivated endotoxin by disaggregation, and that it was neither a lipoprotein nor a serine esterase. In contrast, earlier studies by Schultz and Becker, and later studies by the Galanos group, suggested inactivation of endotoxin by lipoprotein (146, 489). It has also been suggested that the complement system may participate in the detoxification of LPS in plasma (239, 321). Finally, antibodies were also indicated as factors reversibly inactivating endotoxin (292, 465, 670).

Despite all of the difficulties inherent in testing blood for endotoxin in the LAL assay, and despite the value of this application of the assay being often

questioned, it is still potentially an invaluable tool in cases of endotoxaemia, bacteraemia, septicaemia and septic shock.

All shock, including septic shock, is essentially an inadequate capillary perfusion (198), a state of systemic imbalance between supply and demand for oxygenated blood (512). Gram-negative organisms are responsible for two-thirds of the cases of septic shock, and Gram-positive organisms for the remainder, and they are in decline (512). However, it is still unclear whether endotoxin is a primary cause of Gram-negative septic shock. Several other questions also remain to be answered, namely the role of endotoxin in the pathogenesis of shock, whether there is dose-related correlation between endotoxin levels and the severity of the septic shock, and whether endotoxin assay may be of value in prediction, diagnosis and prognosis of the shock (306, 438, 451).

The pathogenesis of acute pancreatitis, although still unclear, seems to be quite different. The favoured theory is that of pancreatic autodigestion, which was introduced by Klebs in 1876 (266). This theory proposes that pancreatic zymogens (proenzymes) are in some way activated within the pancreas itself. The active enzymes (particularly trypsin) then activate other proenzymes (chymotrypsin, elastase and phospholipase A), which leads to digestion of pancreatic and peripancreatic tissues (548, 587, 641). However, endotoxaemia has been reported to accompany acute pancreatitis (61, 142). In this clinical condition, endotoxin seems to be of intestinal origin, escaping from portal blood into the systemic circulation, due to impaired hepatic reticuloendothelial system function at the onset of pancreatitis (130, 143, 264). The Liehr and Foulis groups found that the presence of endotoxaemia seemed to correlate with the outcome and the severity of the disease (144, 295). However, it still remains unclear whether there is any

correlation between the presence of endotoxin and the course and outcome of the illness. It has not yet been demonstrated whether endotoxin may be an indication of poor prognosis in patients with fulminant (haemorrhagic) pancreatitis (264).

In neutropenic patients, systemic endotoxaemia without bacteraemia has been reported as a cause of fever (204). Endotoxin in this clinical condition was thought to permeate from the intestinal tract into the circulation as a consequence of intestinal mucosal damage by cytotoxic agents, or because of numerical and functional defects of neutrophils, or because of impaired hepatic reticuloendothelial cell clearance of endotoxin (204). Gram-negative bacteraemia originating from the digestive tract in neutropenic patients has also been described (375).

1.3. Fibronectin

1.3.1. Biochemistry

Fibronectin was isolated and partially purified from human plasma by Morrison and colleagues in 1948 (337). It was termed "cold insoluble globulin" or CIg. In 1970, it was purified to homogeneity and investigated by Mosesson and Umfleet (340). Other synonyms used in the literature are: large, external transformation-sensitive (LETS) protein (663), cell surface protein (CSP) (231, 665), fibroblast surface antigen (FSA) (471, 472), anti-gelatin factor (659), opsonic α_2-surface binding (SB) glycoprotein (45, 46), galactoprotein a (62, 157), cell attachment or cell adhesion factor or protein (189, 265, 404), zeta protein (454), cell spreading factor (189), and humoral recognition factor (258, 480).

Fibronectin exists in two forms: as soluble plasma fibronectin and insoluble cell surface (cellular) fibronectin (340). Soluble fibronectin, apart from in plasma, exists in cerebrospinal fluid, amniotic fluid, joint fluid, seminal plasma, lymph, tissue fluid and other body fluids (4,468,536), while cellular fibronectin has been found in most, if not all, body tissues (4). Fibronectin is produced by fibroblasts (471, 662), platelets (673), neutrophils (225), vascular endothelial cells (235), epithelial cells (69, 140), astroglial cells (612), smooth muscle cells (56), macrophages (6), hepatocytes (8,622), chondrocytes (100), and other selected cell types.

Plasma fibronectin is antigenically related to cell surface fibronectin (471, 663), but apart from the difference in solubility of the two forms, there may be slight differences in their amino acid sequences, since monoclonal antibodies can be generated, which recognize only one form of the molecule (16, 261). There is also a possibility that a strand of the plasma form is simply shorter, as it was found that the molecular weight of plasma fibronectin was 10 000 daltons less than the cell surface form of the molecule (662). Plasma fibronectin is a strand-like molecule with a diameter of 2 nm and a length of 120–160 nm (119,120).

Fibronectin from both plasma and tissue appears to have a molecular weight of 440 000 – 450 000 daltons, and consists of two very similar disulphide-bonded subunits of 220 000 – 230 000 daltons (340,342,405).

Fibronectin is a large glycoprotein, with carbohydrate content in both plasma and cell surface fibronectins of 4.4 – 5.8 % (62,339,623,664), while the carbohydrate content of amniotic fluid fibronectin is 9.5–9.6% (469). The main carbohydrate components of fibronectin are: mannose, galactose, N-acetylglucose and sialic acids (588). Structural studies of fibronectin indicate that there are at least ten

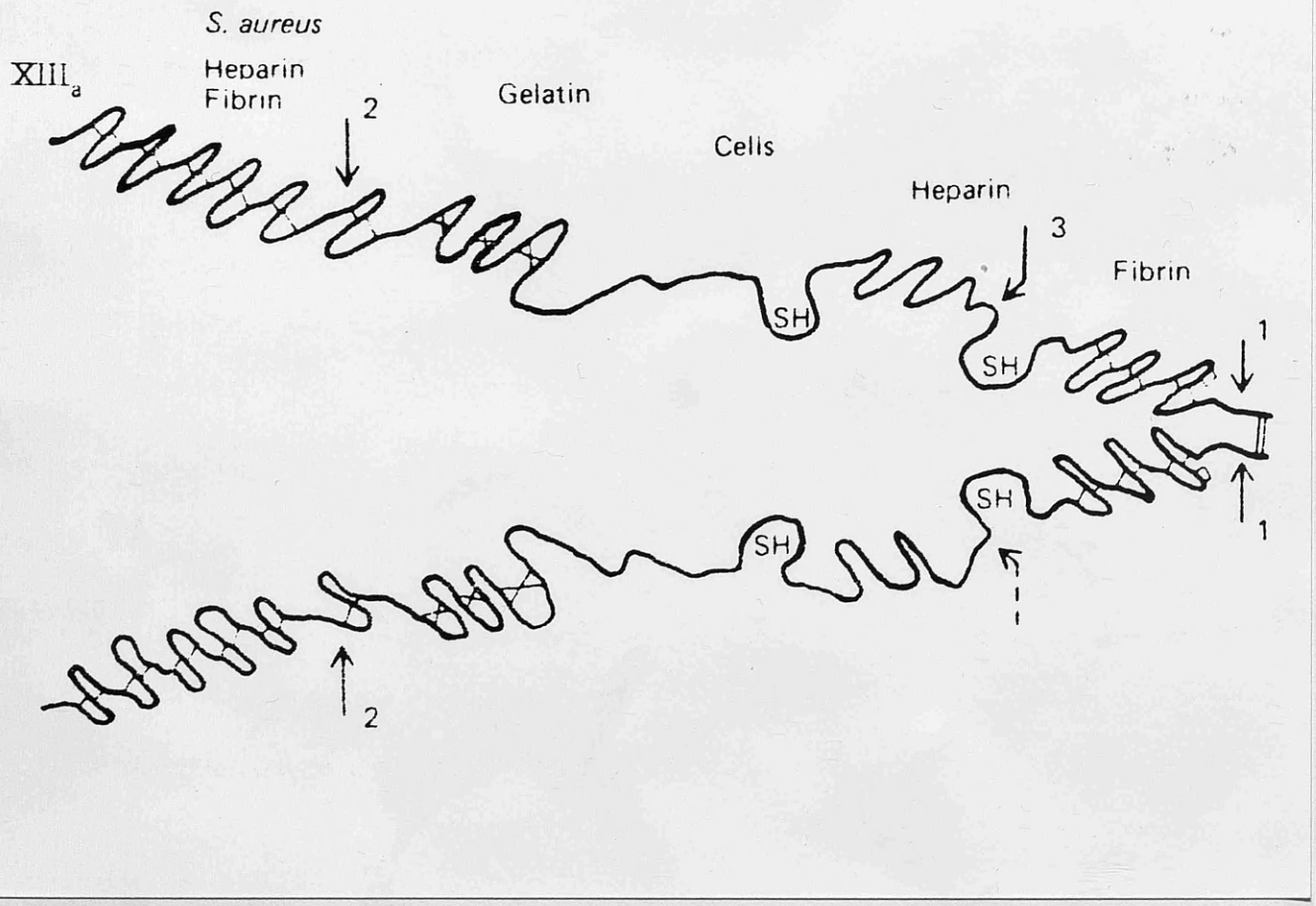
S. aureus
XIII$_a$
Heparin
Fibrin
2
Gelatin
Cells
Heparin
3
Fibrin
SH
SH
1
SH
SH
1
2

Fig. 9

repeating units of homologous "finger" sequences, each stabilized by an invariant pair of intrachain disulphide bonds. In addition, there are repeating units of two other types of homologous sequences (419).

Fibronectin binds to collagen, gelatin, fibrin, hyaluronic acid, heparin or heparan sulphate, Staphylococcus aureus and other bacteria, actin, sulphated proteoglycans, sphingolipids (gangliosides), DNA, transglutaminase (factor XIII a) and the C1q complement component (39, 153, 211, 232, 267, 326, 345, 417, 501).

Fig 9 Diagram of human fibronectin dimer.

The NH_2 termini are to the left; the COOH-termini to the right. Disulphide bonds are shown as thin lines connecting two portions of the polypeptide strand. Loops containing similar sets of disulphide bonds are homologous. Numbered arrows indicate the order of sequential proteolytic cleavage by plasmin or trypsin. The dotted arrow depicts a tryptic cleavage site which is evidently missing in one-half of the subunits. The approximate sites of transamidation by factor XIIIa; of binding to Staphylococcus aureus, heparin, fibrin, gelatin and cells; and of the cryptic free sulphydryl (SH) groups are indicated.

From: Mosher, D. F., Mosesson, M. W.: Plasma fibronectin: roles in hemostasis, macrophage function and related processes. In: E J. Bowie, A A. Sharp (eds.): Hemostasis and Thrombosis. Butterworths International Medical Reviews: Hematology, vol 2. Butterworths, Woburn, MA. 1985, pp 173-196.

1.3.2. Clinical significance

Low fibronectin levels have been reported in the clinical conditions of sepsis (277, 388, 428, 464, 492, 547), disseminated intravascular coagulation (DIC) (122, 346, 388, 547), adult respiratory distress syndrome (ARDS) (90, 388), hepatic failure with or without gastrointestinal bleeding (177, 388), burn injury (121, 192, 278), accidental and surgical trauma (122, 256, 257, 452, 483, 493) and after transfusion of stored blood (because of microaggregate debris) (537).

The functions and pathophysiological role of plasma fibronectin are uncertain, and all the evidence is indirect. However, it is thought that fibronectin may be of clinical importance as a non-invasive measurement of RES function (257, 278), since it is postulated that fibronectin may be a non-specific opsonin responsible for uptake and clearance of predominantly non-bacterial particulates, such as collagenous tissue debris, cellular debris, immune complexes, denaturated proteins and products of intravascular coagulation (343, 480, 483).

To date, there have been several clinical studies on fibronectin replacement therapy using cryoprecipitate in critically ill septic patients with multisystem failure. Cryoprecipitate is prepared from plasma (427) and is enriched in plasma fibronectin and several other plasma proteins (9, 68, 338, 340). The results of cryoprecipitate administration to septic trauma, burn or complicated surgical patients with multiple organ failure and low fibronectin levels, show general improvement in the patients' condition, a reversal of the febrile and septic state, increased alertness, and improvement in cardiovascular, pulmonary and renal functions (12, 453, 481, 483, 491, 492).

Unfortunately, the studies in which these interesting results were obtained were uncontrolled, non-randomized and non-double blind. Furthermore, the cryoprecipitate used as the source of fibronectin is also enriched in fibrinogen, factor VIII coagulant activity and von Willebrand factor (344). This may be important in reevaluation of the significance of fibronectin in cryoprecipitate for critically ill patients, as it has been found that the fibronectin molecules recovered from plasma cryoprecipitates lack the polypeptide domain required for binding to Staphylococcus aureus (547). Other data suggest that opsonic activity of cryoprecipitate declines with storage in excess of approximately four months (480). This would indicate that the alleged improvement in patients' condition obtained in some studies after administration of cryoprecipitate was due to factors other than fibronectin. In fact, the decrease in the fibronectin level of many critically ill patients may be merely a reflection of their acute or chronic nutritional deficiencies; indeed, fibronectin has been postulated as a new nutritional parameter (314), in view of the fact that plasma fibronectin has a half-life of approximately 15-24 hours (183, 482) and its levels have been shown to drop rapidly in response to starvation in healthy volunteers, and return to normal soon after refeeding (102, 490).

Further, none of animal models to date supports the hypothesized relationship between sepsis or endotoxaemia and fibronectin depletion. Rats given intravenous endotoxin showed either no change or increase in bioassayable fibronectin levels (298). Fibronectin concentrations in rats remained unchanged, or increased as a result of septic shock or Escherichia coli bacteraemia (18, 258). Generalized Shwartzman reaction in rabbits, and intraabdominal abscesses in rats, generated a significant increase in fibronectin levels, at a time when RES function was depressed (190).

In view of the above, clinical trials with intravenous administration of plasma fibronectin should be conducted with extreme caution, as they may actually cause organ failure. It has been postulated that fibronectin infusion in the presence of already elevated plasma fibronectin may increase the deposition of exogenous and/or endogenous particles in pulmonary and other capillary beds (18,480).

AIMS OF THE STUDY

The aims of this study were twofold:

1. to develop a sensitive, quantitative, simple and inexpensive Limulus amoebocyte lysate (LAL) assay for the detection of endotoxin;

2. to apply this assay in clinical studies (septic shock, cyclic neutropenia and acute pancreatitis trials) and in non-clinical studies (performance of extra-corporeal filter for removal of endotoxin from human blood, measurement of endotoxin content in baffle plate material from the humidifier system and in a dust sample from archive books).

II MATERIALS, METHODS AND PATIENTS

2.1. Equipment

2.1.1. Disposable plasticware

For dilutions of endotoxin and storage of plasma samples, sterile, polystyrene test-tubes with caps by "Falcon" (12x75 mm, cat.no. 2003, Division of Becton, Dickinson and Co., Cockeysville, MD 21030, USA) were employed.

LAL tests were performed in "Falcon" sterile polypropylene test-tubes with caps (12x75 mm, cat.no.2063).

Plastic tips for volumes 0-200 µl were from L.I.P. Equipment and Services (Shipley, West Yorkshire, BD17 7AS) or from Gilson Co. (for handling blood only) (Gilson France SA, 95400 Villiers le Bel, France).

"Volac" tips for volumes 0-1000 µl were manufactured by John Poulten Ltd. (Barking, Essex, IG11 8QD).

All plasticware was shown not to bind endotoxin.

Plastic tips were rendered pyrogen-free by autoclaving in standard conditions, i.e. at 121°C at 15 lb/inch^2 for 20 minutes.

Microplates for spectrophotometric readings were non-cobalt treated EIA microtitration plates (Dynatech Laboratories Ltd., Billingshurst, Sussex).

2.1.2. Glassware

Pyrex disposable screw cap culture tubes made from borosilicate glass (13x100 mm, cat. no 99449.13, Corning Glass Works, Corning, NY 14830, USA) were employed. In order to obtain pyrogen-free heparinized test-tubes, caps and tubes were soaked in 1% Decon 90 overnight, then rinsed, washed six times in tap water and subsequently six times in distilled water, dried and after closing tubes with similarly pretreated caps, they were heated at 180°C for at least 12 hours. Then 0.1 ml of heparin was added to all tubes, which subsequently were dried in a drying oven to obtain a shell of heparin.

2.1.3. Spectrophotometer

Absorbance of samples was determined at 410 nm in a MICROELISA MiniReader MR 590 (Dynatech Laboratories Ltd., Sussex).

2.2. Reagents

2.2.1. Heparin

For heparinizing test-tubes, pyrogen-free heparin without preservatives, at a concentration of 1000 units per ml, was used.

2.2.2. Boiling buffer

This buffer, used for extraction of endotoxin from plasma, was prepared as follows:

6.06g TRIS

2.03g $MgCl_2$

0.88g NaCl

distilled water to 1 litre.

The pH was adjusted to 7.4 with NaOH or HCl, accordingly. To render the buffer pyrogen-free, 2.5g of asbestos was added to every 2 l volume of the buffer. Then the suspension was mixed thoroughly and left overnight. The supernatant was subsequently filtered through a 0.22 µm millipore filter. The filtrate was divided into approximately 10 ml aliquots, which were autoclaved at 121°C at 15 lb/inch^2 for 20 minutes.

2.2.3. Limulus amoebocyte lysate and chromogenic substrate

In the early part of this work, a commercially available Limulus Amebocyte Lysate Chromogenic Reagent Set (M.A. Bioproducts, Walkersville, MD 21793, USA) was used. This was complemented by S-2423 chromogenic substrate (Kabi Diagnostica, Stockholm, Sweden).

Later, the Quantitative Chromogenic LAL (QCL-1000) kits (M.A. Bioproducts) which included the chromogenic substrate, were used.

Coatest Endotoxin kits (Kabi Diagnostica) and the Limulus Amebocyte Lysate Color Chemistry kits (Mallinckrodt, St. Louis, MO 63134, USA) were also tested.

2.3. Blood collection and plasma preparation

Human venous blood was drawn with stringent precautions. The venepuncture site was treated with 2% (w/v) iodine in alcohol and allowed to dry. Blood was drawn into a sterile disposable plastic syringe. After removing the needle, blood was transferred into the screw-capped pyrogen-free heparinized glass test-tube. Blood in a tightly capped tube was then mixed by slow, gentle 180° inversion of the tube several times. Tubes were immediately placed on ice.

Subsequently, blood was centrifuged at +4°C at 100 x g for 10 minutes to obtain platelet rich plasma (PRP), and if not used immediately, the plasma was stored at -20°C.

Just before the assay, plasma was put on ice to thaw and kept on ice throughout the extraction procedure.

The extraction procedure consisted of two steps:

1) 50 µl of PRP plus 150 µl of boiling buffer, plus 50 µl of pyrogen-free water (PFW) or appropriate dilution of endotoxin in pyrogen-free water. PRP for the standard curve was pooled from 5-8 healthy volunteers, whose plasma had been shown to be endotoxin-free. To the 0 pg/ml sample for the standard curve and to the patient's plasma, PFW was added instead of endotoxin dilution.

2) heating of diluted plasma at 100°C for 1 minute in a boiling water bath.

The precipitate formed was sedimented by centrifugation at 1500 x g at room temperature for 10 minutes and the supernatant containing the plasma extract was removed. All samples were prepared in duplicate.

2.4. Preparation of baffle plate material and dust for endotoxin assay

Baffle plate material (from the semiconductor factory) was soaked in PFW (proportion 1:4 w/v) for 1 hour at room temperature (27°C). The container was agitated a few times during the procedure and some soaking solution was removed. Subsequently, serial ten-fold dilutions of the soaking solution were tested in the one-stage LAL assay.

A dust sample was taken from the surfaces of a collection of old, water-damaged books, which are held in the Ayrshire branch of Strathclyde Regional Council Archives.

0.1g of dust was diluted with 1ml of PFW and soaked for 15 minutes at 37°C. Then the container was agitated and centrifuged for 5 minutes at 1500 x g at room temperature. The supernatant was used as a starting point for serial, ten-fold dilutions, which were subsequently tested in the one-stage LAL assay.

2.5. LAL assay

2.5.1. Two-stage assay

In this assay, 50 µl of tested sample were incubated at 37°C in a water bath with 50 µl of LAL. Then 100 µl of S-2423 were added and the mixture was further incubated at 37°C. The reaction was terminated by the addition of 50% (v/v) acetic acid. Each sample mixture was then centrifuged at 1500 x g for 10 minutes. Two hundred and fifty microlitres of supernatant from each sample were then transferred to an individual well of a micro-titre plate and absorbance of each sample was measured in a MICROELISA MiniReader.

With each set of patients' samples, the following controls were set up:

1. Reagent control

   For this, 50 µl of PFW replaced 50 µl of plasma extract, and was incubated with LAL and chromogenic substrate in the same way as plasma extract;

2. Standard curve background control

   Plasma extract was prepared, as for the 0 pg/ml value of the standard curve,

and incubated with LAL as usual, but 100 µl of acetic acid was added prior to 100 µl of chromogenic substrate in order to prevent any chromogenic reaction;

3. Patient plasma background control

This was set up as the standard curve background control, but using patient plasma.

2.5.2. One-stage assay

In this modification, 100 µl of test sample were incubated with 100 µl of a mixture of LAL and chromogenic substrate. The reaction was terminated with 100 µl of 50% (v/v) acetic acid. The absorbance of 250 µl of each sample was subsequently measured in the MICROELISA MiniReader, in the same manner as in the two-stage method. The same controls were also set up.

2.6. Comparison of three commercial kits for the chromogenic LAL assay

In this comparison, the assay used to test the Coatest Endotoxin kits (Kabi Diagnostica) and the Quantitative Chromogenic LAL (QCL-1000) kits was a one-stage modification, as described in part 3.2.2.

The Mallinckrodt kits were tested in accordance with the manufacturer's instructions, i.e. a 1:4 mixture of LAL and the chromogenic substrate was incubated according to the label incubation time claim, which is different for every batch.

2.7. Calculations

2.7.1. Calculation of endotoxin concentration in test samples

Endotoxin potencies in assayed samples were either determined graphically from the plotted standard curve or (particularly in cases of multi-sample experiments) were determined with the aid of the Hewlett-Packard (model 10) calculator, using the linear regression programme.

2.7.2 The intra-assay coefficient of variation (C.V.) was calculated according to the formula:

$$C.V. = \frac{S.D.}{\bar{x}} \times 100$$

1) where S.D. = Standard Deviation of group of values

2) $\bar{x}$ = mean value;

$$S.D. = \sqrt{\frac{\Sigma d^2}{n-1}}$$

3) where d (deviation) $= |x_n - \bar{x}|$

4) x_n = value of sample

5) $\bar{x}$ (mean value) $= \frac{\Sigma x}{n}$

6) n = number of values

2.8. Study on the extra-corporeal filter to remove endotoxin from human blood.

For this study, the two-stage LAL assay was employed, but the incubation time with S-2423 was 3 minutes, and all volumes doubled in comparison with the method described above.

Experiments assessing the ability of this small particle-size sorbent filter (1-DEP) to remove endotoxin from human plasma were carried out at room temperature, using human plasma obtained by plasmaphoresis and from patients requiring venesection for haemochromatosis and polycythaemia.

The endotoxin used in this study was from E. coli Endotoxin Sets (5 x 2.5 mg per vial, code F 185, Mallinckrodt Inc., St. Louis, Mo. 63134, USA). Each vial was reconstituted with 10 ml of pyrogen-free water.

Concentrations of endotoxin used in different experiments were 25 ng/ml of plasma and 250 pg/ml of plasma. The samples were drawn before and after the filter at different time intervals.

2.9. Patients

2.9.1. Septic shock trial patients

These patients were evaluated in two groups:

Group 1 - ten critically ill, but not necessarily septic shock, patients, with daily LAL assays and simultaneous blood cultures;

Group 2 eight acute septic shock patients, who had four-hourly LAL assays for the first 48 hours, and thereafter daily. Blood for blood cultures and fibronectin was taken simultaneously with blood for LAL assays. Cardiovascular and respiratory parameters were also measured at the same time.

All blood samples from the above groups were drawn under the supervision of, and subsequently processed by, the author.

Blood cultures in this trial were processed routinely at the Department of Bacteriology and Immunology of the Western Infirmary, Glasgow. Dr Steven Edlin, of the Intensive Therapy Unit (ITU) at the Western Infirmary, measured and calculated patients' cardiovascular and respiratory parameters (Table 5).

Table 5. Cardiovascular and Respiratory Measurements

Cardiac Index (CI)	$= \frac{CO}{\text{Body Surface Area}}$ l/min/m^2
Systemic Vascular Resistance (SVR)	$= \frac{(MAP - RAP)}{CO} \times 79.9$ dyne sec/cm5
Pulmonary Vascular Resistance (PVR)	$= \frac{(MPAP - PAOP)}{CO} \times 79.9$ dyne sec/cm^5
Alveolar arterial oxygen difference (A-a)DO_2	$= [FIO_2 \times (Pb-47) - PaCO_2] - PaO_2$ mm Hg
Pulmonary shunt $\frac{Qs}{Qt}$	$= \frac{C\acute{c}O_2 - CaO_2}{C\acute{c}O_2 - CmvO_2}$ %

Abbreviations

CO	Cardiac Output
MAP	Mean Arterial Pressure
RAP	Right Atrial Pressure
MPAP	Mean Pulmonary Artery Pressure
PAOP	Pulmonary Artery Occlusion Pressure
FIO_2	Fractional Inspired Oxygen
Pb	Atmospheric Pressure

$PaCO_2$ Arterial Pressure of Carbon Dioxide

PaO_2 Arterial Pressure of Oxygen

$C\acute{c}O_2$ End Capillary Content of Oxygen

CaO_2 Arterial Content of Oxygen

$C\bar{m}vO_2$ Mixed Venous Content of Oxygen

Mr Paul Newman of the Department of Surgery at the Western Infirmary assayed plasma fibronectin in the patients' samples. The method of plasma preparation and the description of the assay are given below.

Two-ml blood samples for plasma fibronectin assay were drawn, either together with the blood for the LAL assay (i.e. from the venepuncture site) or separately, through the indwelling arterial line via a three-way tap, following the removal of a large flush sample. The blood was placed either in a Sterilin KE/5 vial with potassium EDTA for routine laboratory tests (Sterilin Ltd, Feltham, Middlesex, England) or in an Eppendorf 1.5 ml micro test-tube (Eppendorf Gerätebau, Netheler & Hinz GmbH, Hamburg 65, West Germany) containing 2mg of EDTA. The sample was immediately placed on ice. Subsequently, blood was centrifuged at 13 000 x g for 2 minutes in a microcentrifuge and, if not tested immediately, the plasma was stored at -20°C. If samples were stored over a period of time, aprotinin (Trasylol, Bayer AG, Leverkusen, West Germany) at a concentration of 40 IU/ml plasma was added. The assay method was a modification of a procedure recommended by Boehringer Mannheim Biochemica (Fibronectin Opsonic Protein, in vitro turbidimetric immunoassay for the quantitative determination of human opsonic protein). The Biochemica Test-Combination kits (Boehringer Mannheim)

were used for the assay. In this assay, 5 µl of patient's plasma was added to a reaction volume of 500 µl containing a 1 in 21 dilution of the fibronectin antiserum and the antiserum buffer. The sample was then mixed throughout and the optical density was read at 365 nm, using a Pye Unicam SP 450 spectrophotometer (Pye Unicam Ltd., Cambridge, England) at 1 minute and at 11 minutes. Two control samples were assayed with every series of measurements. They were prepared in the same way as the patients' samples, i.e. 5 µl of standard fibronectin from the kit (0 µg/ml or one of the following concentrations: 125 µg/ml, 250 µg/ml, 500 µg/ml, 750 µg/ml, 1000 µg/ml) was added to 500 µl of the reaction volume containing a 1 in 21 dilution of the fibronectin antiserum and the antiserum buffer. Plasma fibronectin concentrations in patients' samples were calculated from the standard curve plotted from the respective control samples.

2.9.2. Acute pancreatitis patients - preliminary trial

Blood from these patients was taken and plasma samples were prepared by Dr. Colin Wilson of the Department of Surgery at the Royal Infirmary, Glasgow. This heterogenous group consisted of 16 patients. Initial samples for the endotoxin assay were taken with delays of up to four days (two patients) from the onset of clinical illness. Seven patients in this group had mild pancreatitis diagnosed later, while five others, although primarily suspected of having some form of pancreatitis, had some other illness diagnosed later. Six patients had blood taken for endotoxin assay on one or two occasions only. On average, the patients were sampled once or twice daily. The clinical assessment of these patients was made by Dr. C. Wilson.

2.9.3. Cyclic neutropenia patient

Blood from this patient was taken and plasma samples were prepared by Dr. Moira Robertson of the Department of Haematology at the Western Infirmary, Glasgow. The clinical assessment of this patient was also made by Dr. Robertson.

This patient had cyclic neutropenia with neutrophils cycling on a 21 day basis, and reciprocal T_8 lymphocytosis. During periods of neutropenia (defined as $< 0.5 \times 10^9$ neutrophils/l), the patient usually had one or two mouth ulcers (occasionally Herpes simplex was grown), general malaise and occasional perianal problems. For this study, a two-stage LAL assay was employed, but the incubation time with S-2423 was 3 minutes, and all volumes were doubled in comparison with the method described above.

2.9.4. Cases of syndromes, due to the environmental endotoxin.

Semi-conductor factory workers.

A group of 19 normally healthy subjects (14 females: age range 28-52 years, mean 41 years; 5 males: age range 30-44 years, mean 39 years) working in this factory reported work-related respiratory and systemic symptoms, which in some were suggestive of humidifier fever (HF). All of them were found to have normal serology against a variety of infectious agents, including Legionella spp., but serum precipitating antibody against antigens in the factory humidifier water was present in 12 subjects, and was most pronounced in those with the symptoms most in keeping with HF. Extensive serological identity between these antigens and sera, and others from confined outbreaks of HF, was found. Following modification of the humidifier system in the factory, the symptoms resolved in 14

of the 19 subjects, and the serum IgG levels, which previously had been significantly raised, became significantly reduced. The clinical and laboratory assessment of these workers was conducted by Dr. Charles McSharry and his group. The individual symptom profiles which each individual attributed to their working environment are summarized in Table 6.

Table 6. The extent and nature of symptoms in 19 factory workers.

	Group 1										Group 2								
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9
Improvement June 84	y	y	y	y	y	y	y	y	y	y	n	n	n	n	n	n	y	y	y
Work specific	y	y	y	y	y	y	y	y	y	y	y	y	n	y	y	y	n	n	n
Time of symptoms	w	w	w	1	w	w	1	1	1	1	i	1	i	w	w	w	i	w	w
General tiredness	*	*	*	*	*	*	*	*	*	*	*	*		*	*	*	*	*	
Flu-like illness		*	*	*	*	*	*	*	*	*				*	*	*	*		*
Shortness of breath			*	*	*	*	*			*	*	*	*	*		*		*	*
Cough	*	*	*		*	*	*	*					*		*				*
Chest tightness	*		*		*			*		*			*	*	*	*	*		
Headache	*		*		*			*	*	*						*	*	*	
Fever		*		*	*	*	*	*	*	*						*			
Shivering and chills				*	*	*	*	*	*	*							*		
Wheeze						*	*				*		*	*	*	*			
Anorexia		*			*	*		*		*									
Weight loss						*													
Miscellaneous		a	a								b	a		a	f	e	b	a	a
			g										c	d					

Abbreviations

The extent of symptoms in 19 factory workers: y = yes; n = no; w = all week; 1 = first day back at work; i = intermittent.

The nature of the symptoms: a = dry sore throat; b = nausea; c = conjunctivitis; d – rhinitis; e = lightheadedness; f = rash; g = muscle pain.

Group 1	Considered to have humidifier fever (work-related symptoms)
Group 2	Humidifier fever unlikely (symptoms probably due to an abnormal, non-physiological work and sleep pattern, as all workers in this group were working night-shift).
June 1984	Time of clinical reappraisal (after modification of the humidification system).

Table from Dr. Charles McSharry, Department of Bacteriology and Immunology, Western Infirmary, Glasgow.

A member of staff in the Ayrshire branch of the Strathclyde Regional Council Archives

This woman complained of a "flu-like" illness, following several attempts to clean a collection of old, water-damaged books. Subsequent microbial analysis of the dust taken from the surfaces of the books demonstrated the presence of fungi and Gram-negative bacteria in quantity.

III RESULTS

3.1. LPS binding

Before starting this study, plastic tips and plastic test tubes were tested for binding endotoxin. Concentrations of 0, 5, 10 and 20 pg of endotoxin per ml of plasma were used in the test. For this study, the two-stage LAL assay was used, but the incubation time with S-2423 was 3 minutes and all reagent volumes were doubled.

Results of the test for glass pipettes and glass test tubes (i.e. disposable serological glass pipettes, volume 1ml, code D 80 4' PS, by John Poulten Ltd., Barking, Essex IG11 8QD, and disposable glass culture tubes, 13 x 100mm, cat. no. 99449.13 by Corning Ltd. - see "Materials, methods and patients") were compared with results for glass pipettes and plastic test tubes, and with results for glass test tubes and plastic tips.

There was no decrease in endotoxin levels when sets with plastic test tubes and sets with plastic tips were compared with sets composed of glassware alone. This indicated that there was no endotoxin binding by plasticware.

3.2. Development of the LAL assay

3.2.1. Two-stage assay

Endotoxin activity throughout is expressed in both picograms per millilitre (pg/ml) and Endotoxin Units per millilitre (EU/ml).

Early experiments with the chromogenic LAL assay method (as recommended by the manufacturer of the kits) were not satisfactory and prompted evaluation of the optimal conditions for the assay.

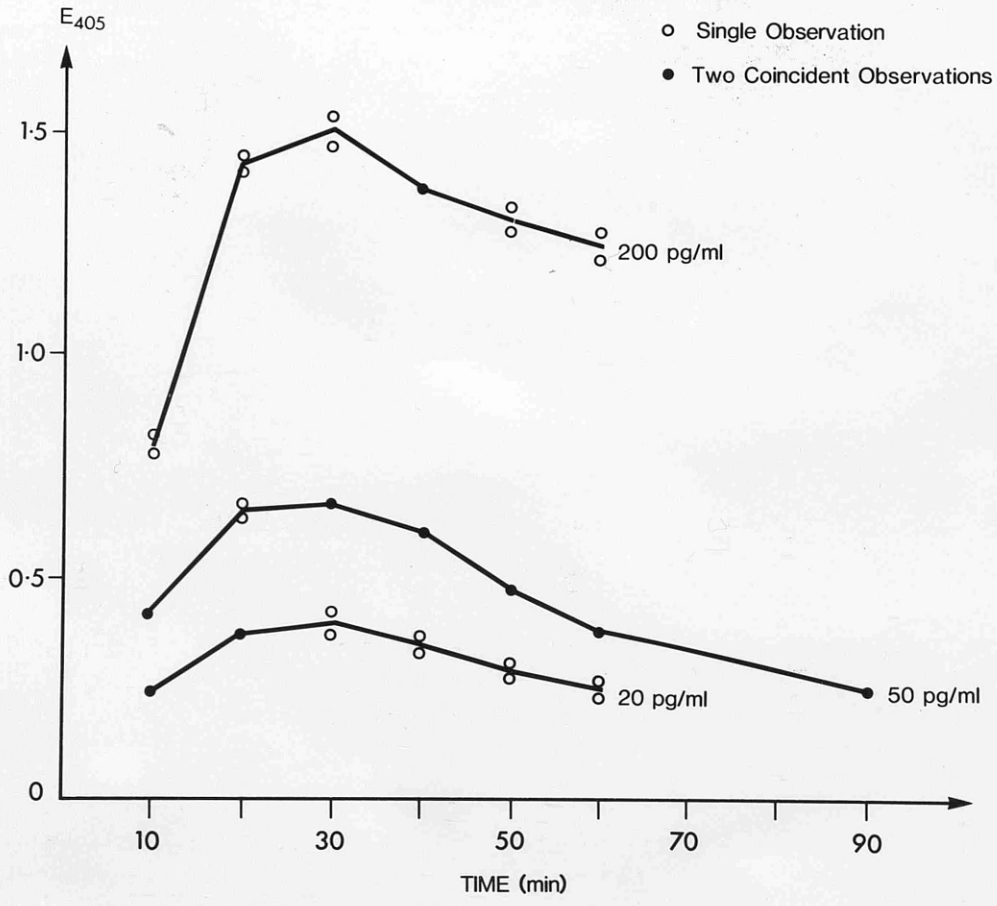
E405
Single Observation
Two Coincident Observations
1·5
1·0
0·5
0
200 pg/ml
20 pg/ml
50 pg/ml
10
30
50
70
90
TIME (min)

Fig. 10

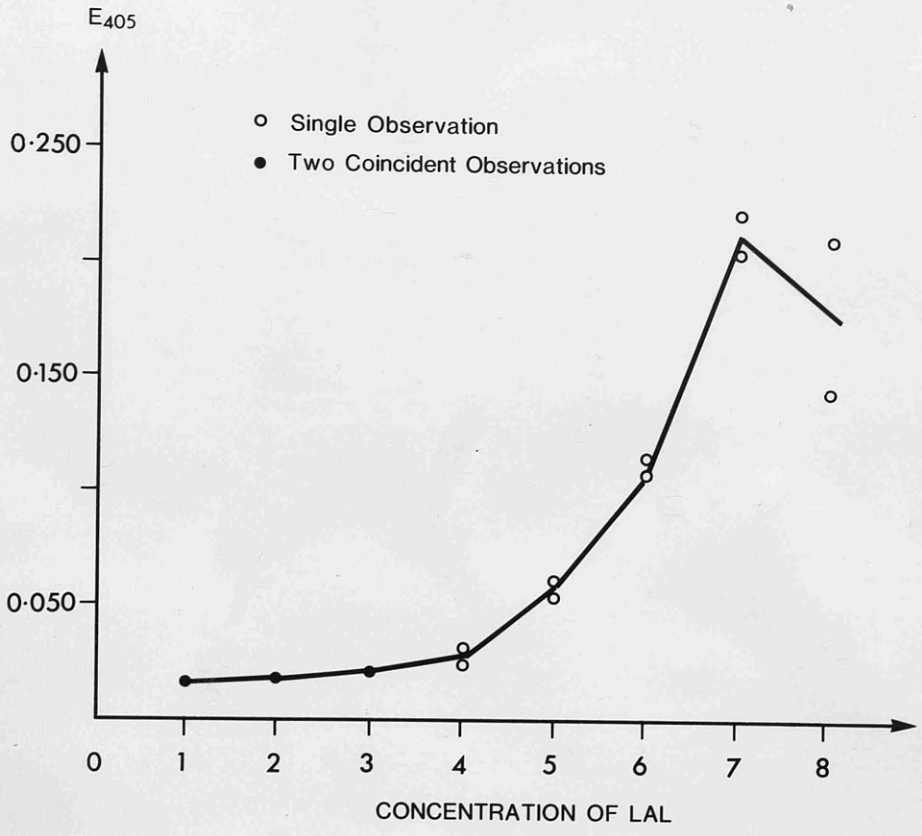
E405
Single Observation
Two Coincident Observations
0·250
0·150
0·050
0
1
2
3
4
5
6
7
8
CONCENTRATION OF LAL

Fig. 11

It was found that the optimal time of incubation of plasma extracts with LAL was 30 minutes (Fig.10).

Fig.10. The relationship between incubation time with LAL and extinction in plasma extracts with 20 pg/ml, 50 pg/ml and 200 pg/ml of endotoxin. The subsequent incubation time with S-2423 for all samples was 3 minutes.

The highest absorbance readings were obtained using a LAL vial reconstituted with 1.4ml PFW, as instructed by the manufacturer (M.A.Bioproducts) (Fig.11).

Fig.11 The relationship between LAL concentration, and the extinction after incubation for 30 minutes. The subsequent incubation time with S-2423 was 3 minutes.

Concentration of LAL:

1. 1 part PFW + 1 part PFW
2. 1 part PFW + 1 part LAL 1:32
3. 1 part PFW + 1 part LAL 1:16
4. 1 part PFW + 1 part LAL 1:8
5. 1 part PFW + 1 part LAL 1:4
6. 1 part PFW + 1 part LAL 1:2
7. 1 part PFW + 1 part LAL neat - as per reagent control
8. 1 part LAL neat + 1 part LAL neat.

The optimum time of incubation of samples with the chromogenic substrate S-2423 was chosen to be 8 minutes (Fig.12). Although when the incubation was extended beyond this time, the extinction was still slightly higher, the

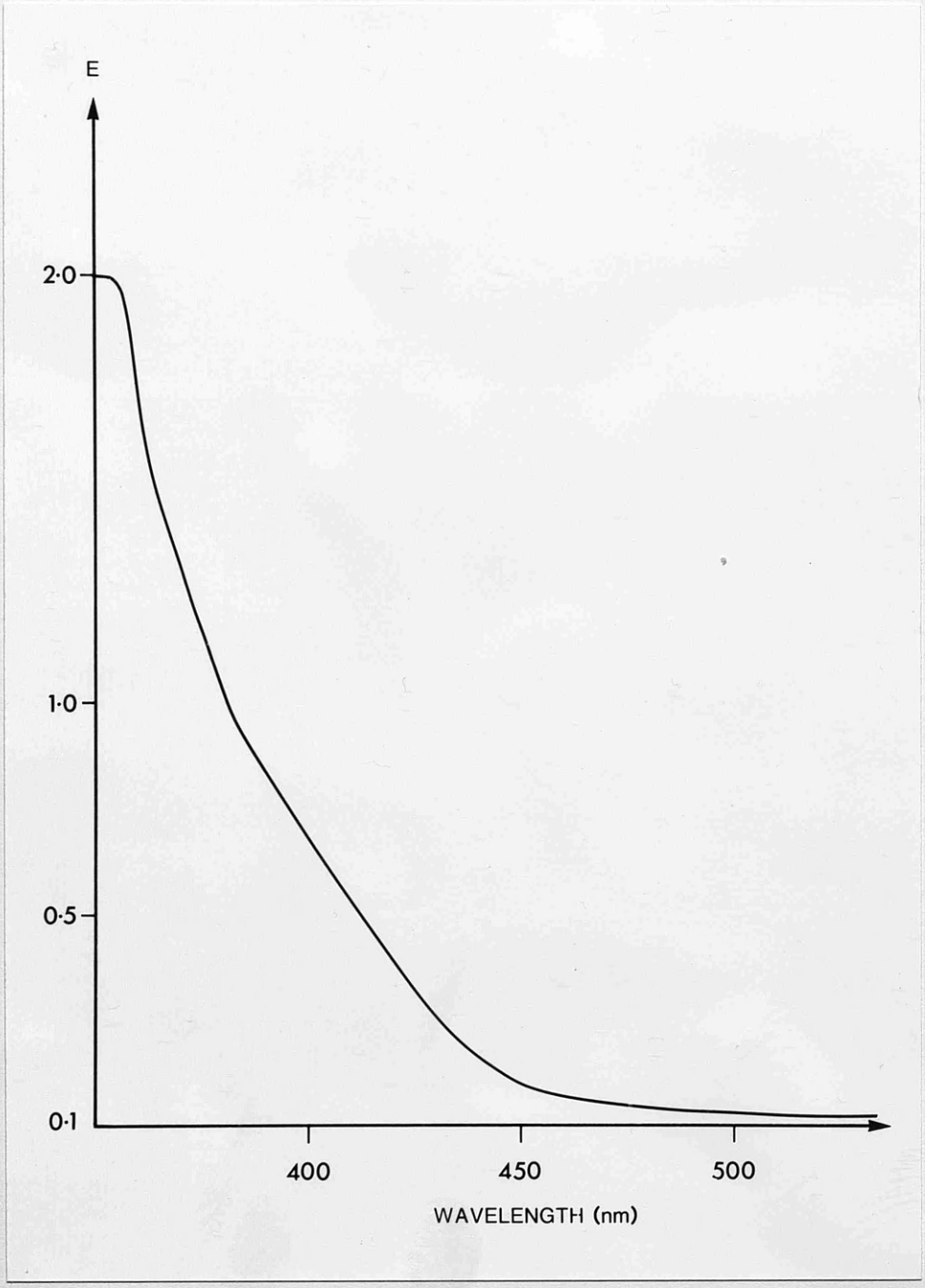

Fig. 13

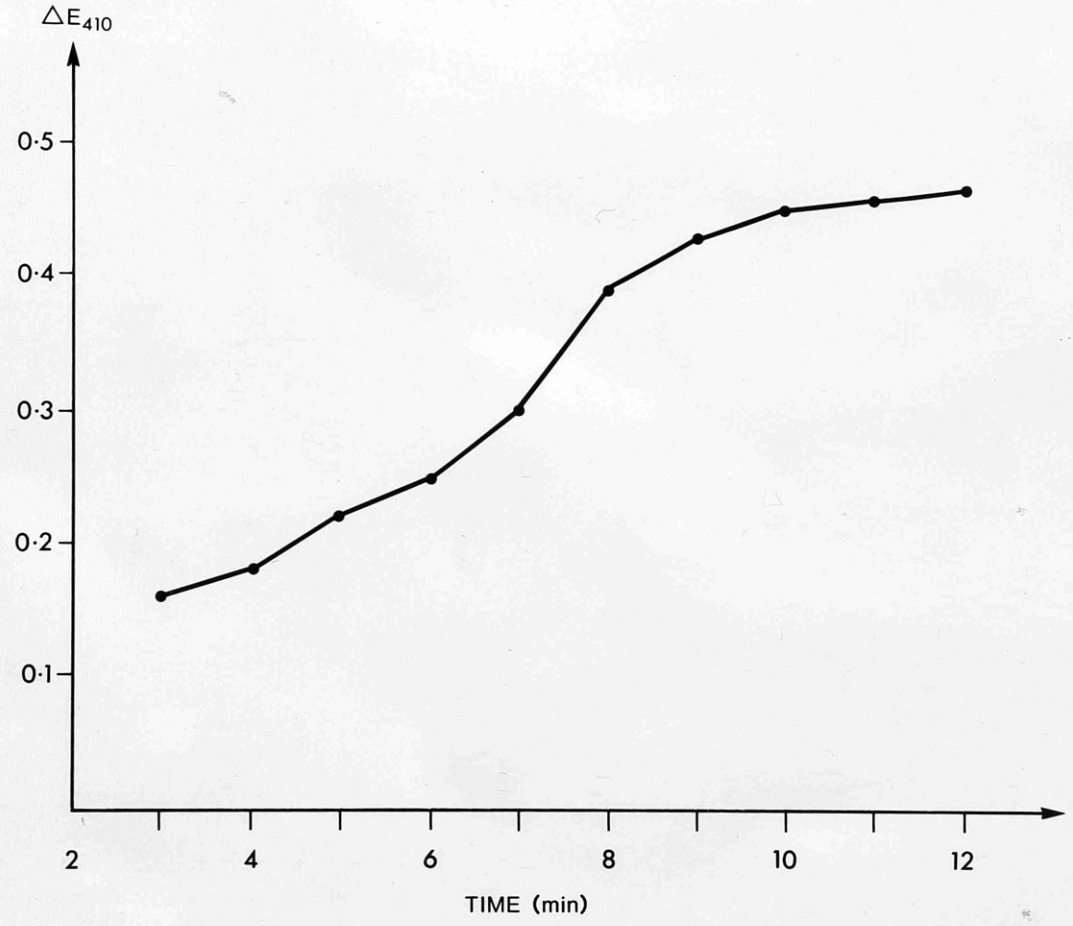

Fig. 12

improvement of results thus obtained did not compensate for the increased assay procedure time.

Fig.12. The relationship between different incubation times with S-2423 substrate and extinction. All plasma extracts were preincubated with LAL for 30 minutes. $\Delta E_{410} = E_{410}$ 100 pg endotoxin/ml plasma $- E_{410}$ 0 pg endotoxin/ml plasma.

The manufacturers of chromogenic substrates, including S-2423, recommend measuring absorbance at 405 nm. However, this wavelength appeared to be arbitrary (according to a personal communication with Kabi Diagnostica) and not optimal for the substrate, as shown in Fig.13. Thus the wavelength of 410 nm was chosen arbitrarily because of the convenience offered by using the MICROELISA MiniReader. This allowed significantly faster processing of large numbers of samples.

Fig.13 The absorbance spectrum of p-nitroaniline released from S-2423, between 350 nm and 550 nm.

Volumes of reagents used were reduced to half of those recommended by the manufacturer (M.A. Bioproducts). With the assay conditions described above, i.e. incubating plasma extracts with LAL for 30 minutes, and subsequently with S-2423 for 8 minutes, the standard curve was linear within the range 0-50 pg/ml of endotoxin (0-0.58 EU/ml) (Fig.14). The threshold of sensitivity of the assay was below 5 pg/ml (0.06 EU/ml). The intra-assay coefficient of variation was below 5%.

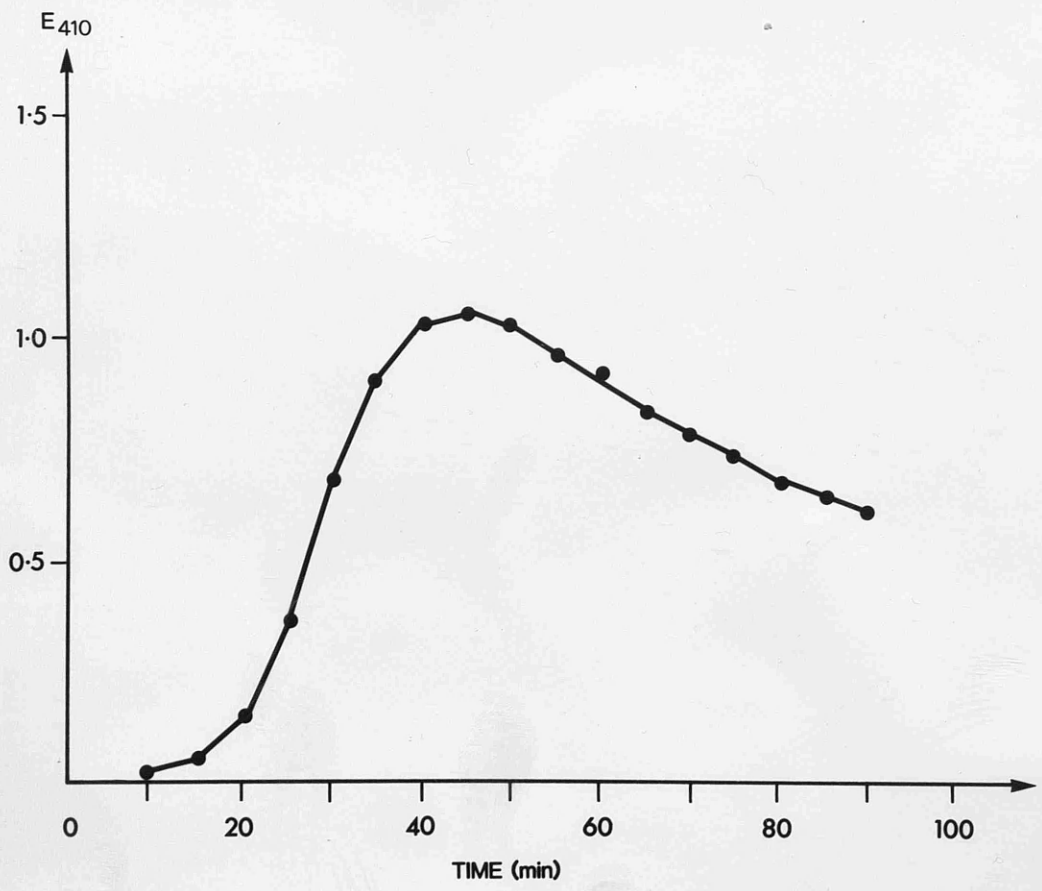

Fig. 16

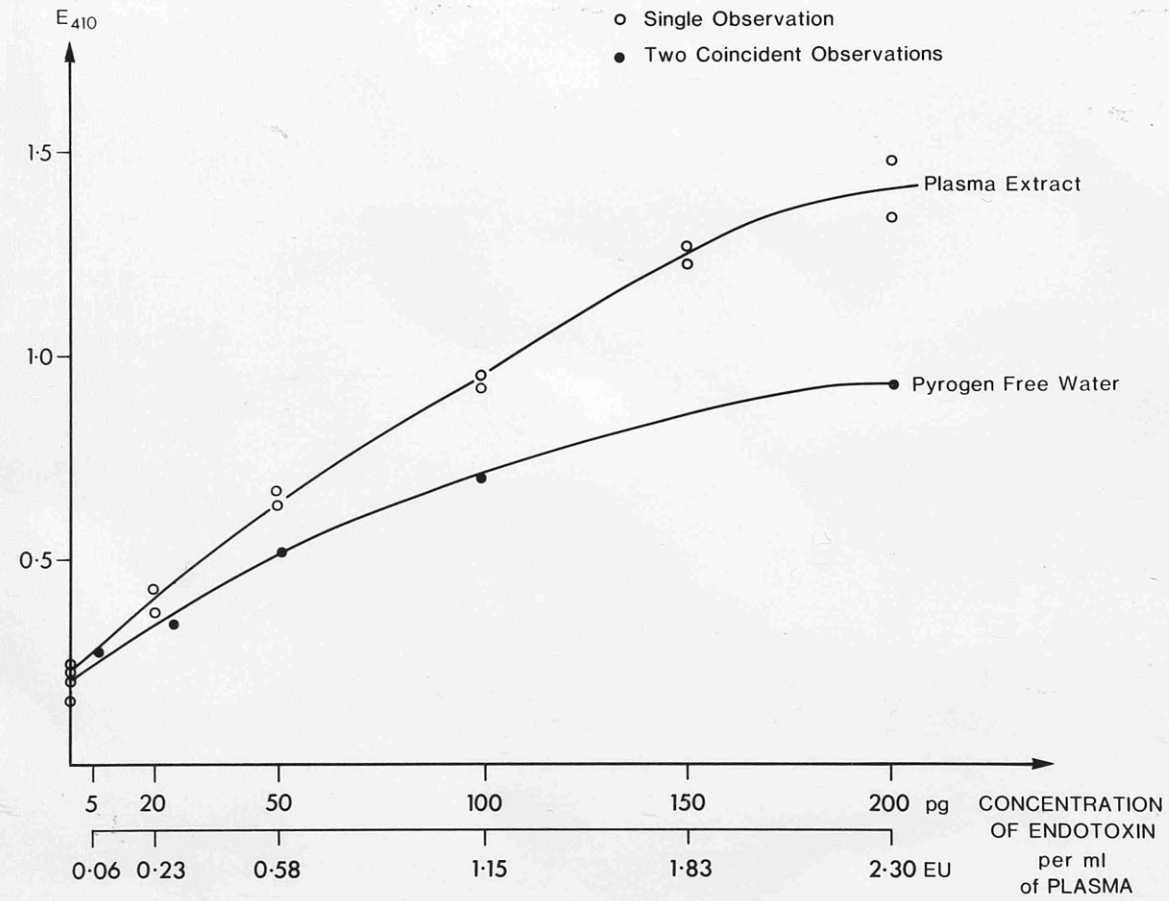
E410
Single Observation
Two Coincident Observations
1·5
1·0
0·5
Plasma Extract
Pyrogen Free Water
5
20
50
100
150
200 pg
CONCENTRATION OF ENDOTOXIN per ml of PLASMA
0·06
0·23
0·58
1·15
1·83
2·30 EU

Fig. 14

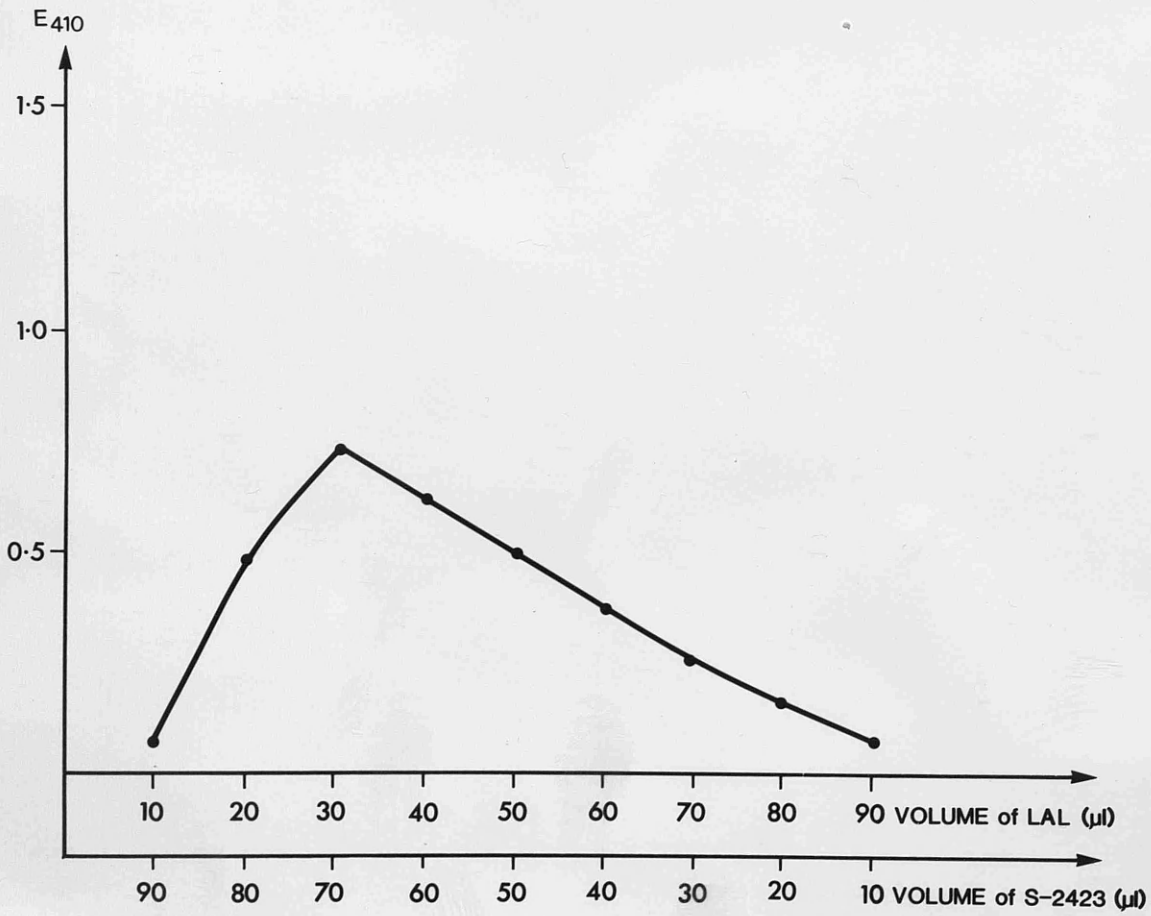
E410
1·5
1·0
0·5
10
20
30
40
50
60
70
80
90 VOLUME of LAL (µl)
90
80
70
60
50
40
30
20
10 VOLUME of S-2423 (µl)

Fig. 15

The standard curve in pyrogen-free water is also shown in Fig.14. It was linear within the range 0-10 pg endotoxin/ml (0-0.115 EU/ml), and with a sensitivity of 1 pg endotoxin/ml (0.012 EU/ml). The absorbance values for the standard curve of endotoxin in PFW were consistently lower than those of endotoxin in plasma.

Fig.14 Comparison of standard curves of endotoxin in plasma and in PFW.

3.2.2. One-stage assay

The modified two-stage assay described above, despite the advantage of sensitivity, was time-consuming. Furthermore, the two stages involved were associated with a high risk of contamination. Thus an attempt was made to modify the LAL assay even further, to a one-stage method, by adding the LAL and chromogenic substrate as a mixture.

It was found that the optimal proportion in the mixture of LAL and chromogenic substrate S-2423 was 30 µl LAL to 70 µl S-2423 (Fig.15). The optimal time for incubation of samples with the mixture of LAL and S-2423 was 45 minutes (Fig.16).

Fig.15 Optimal proportions of LAL and chromogenic substrate S-2423 for the endotoxin assay. $E_{410} = E_{410}$ 10 pg endotoxin/ml PFW (0.115 EU/ml PFW) - E_{410} 0pg endotoxin/ml PFW (0.000 EU/ml PFW).

Fig.16 Optimal incubation time of the mixture of LAL and chromogenic substrate S-2423 with endotoxin. $E_{410} = E_{410}$ 10 pg endotoxin/ml PFW (0.115 EU/ml PFW) - E_{410} 0pg endotoxin/ml PFW (0.000 EU/ml PFW).

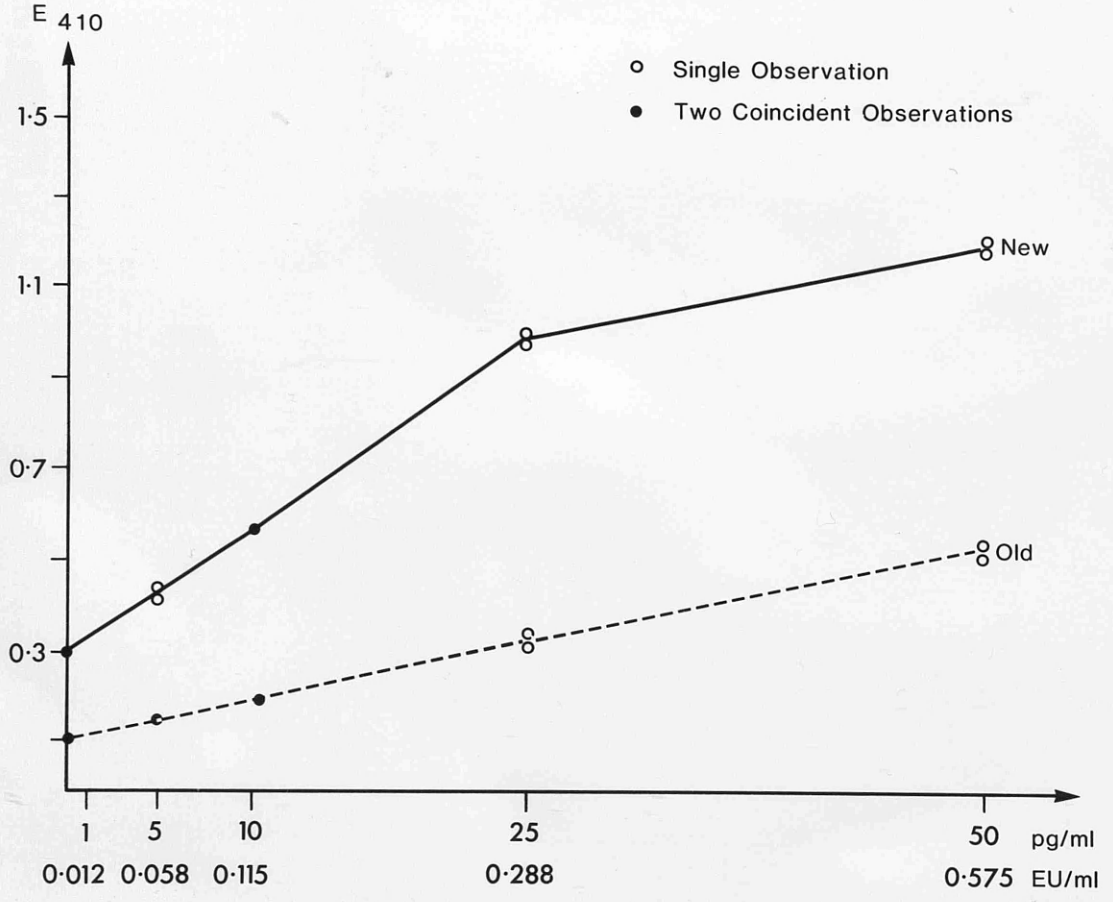
E 410
Single Observation
Two Coincident Observations
1·5
1·1
0·7
0·3
New
Old
1
5
10
25
50
pg/ml
0·012
0·058
0·115
0·288
0·575
EU/ml

Fig. 17

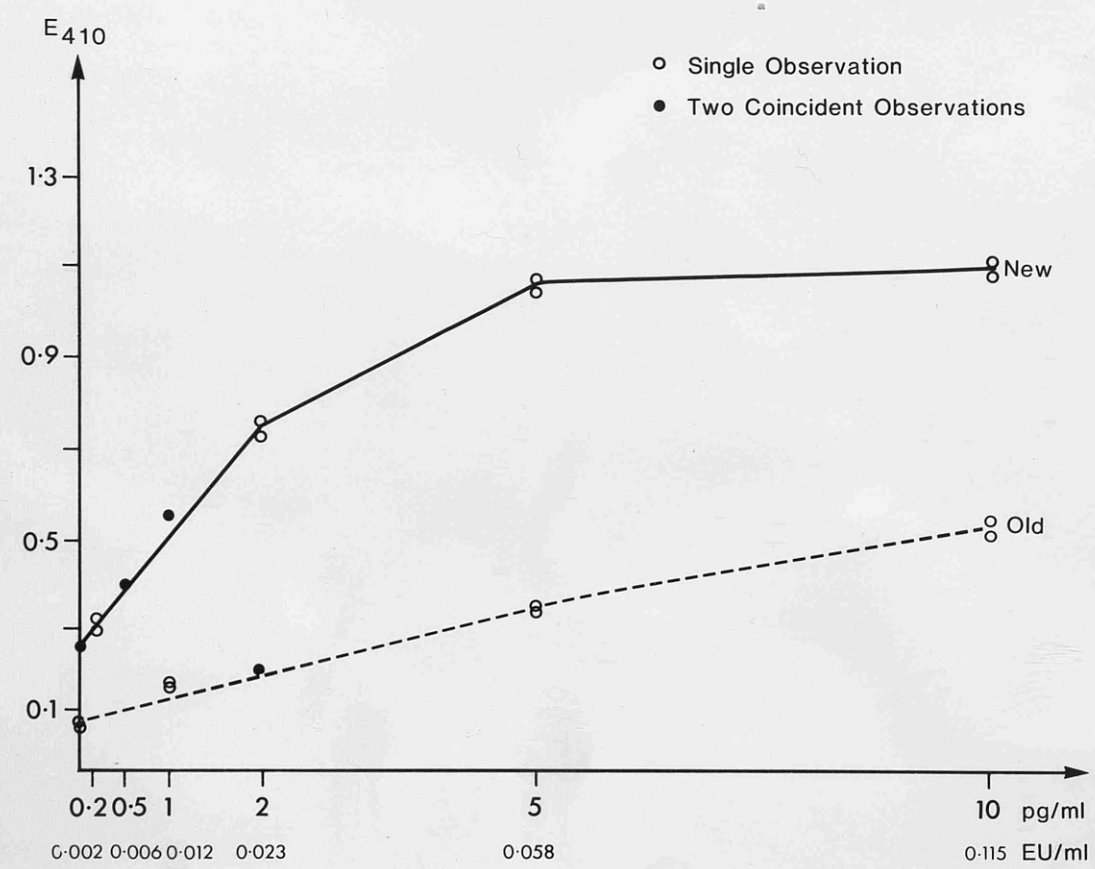
E410
Single Observation
Two Coincident Observations
1·3
0·9
0·5
0·1
New
Old
0·2
0·5
1
2
5
10
pg/ml
0·002
0·006
0·012
0·023
0·058
0·115
EU/ml

Fig. 18

The standard curve of endotoxin in plasma under these conditions was linear in the range 0–30 pg/ml (0–0.345 EU/ml) with a threshold of sensitivity at 1 pg/ml (0.012 EU/ml).

These results were greatly improved in comparison with those obtained in the earlier, two-stage method (Fig.17).

Fig.17 Comparison of standard curves in plasma obtained in the two-stage and one-stage methods.

The standard curve in pyrogen-free water in the one-stage method was linear in the range 0–2 pg endotoxin/ml PFW (0–0.023 EU/ml), against linearity of 0–10 pg endotoxin/ml (0–0.115 EU/ml) in the two-stage method, and with the sensitivity of 0.2 pg/ml (0.002 EU/ml), against sensitivity of 1 pg endotoxin/ml (0.012 EU/ml) in the two-stage method (Fig.18). The intra-assay coefficient of variation was less than 5%.

Fig.18 Comparison of standard curves in pyrogen-free water, obtained in the two-stage and one-stage methods.

Two-stage and one-stage modifications of the LAL assay in pyrogen-free water are compared with the original method recommended by MA. Bioproducts, the manufacturer of the Quantitative Chromogenic LAL (QCL-1000) kits, in Table 7.

Table 7. Comparison of the original LAL assay in pyrogen-free water, as recommended by M.A.Bioproducts , with two-stage and one-stage modifications.

	Sensitivity, pg/ml (EU/ml)	Linearity, pg/ml (EU/ml)	Total incubation time, minutes	Volume of reagents, µl	
				LAL	S-2423
QCL-1000 kits	10 (0.115)	0-100 (0-1.150)	13	100	200
Two stage modification	1 (0.012)	0-10 (0-0.115)	38	50	100
One-stage modification	0.2 (0.002)	0-2 (0-0.023)	45	30	70

Table 8 demonstrates the comparison of the two-stage and one-stage modifications of the LAL assay in plasma. MA Bioproducts does not recommend its QCL-1000 kits for determination of endotoxaemia in man. This results in a lack of comparative data for plasma samples.

Table 8 Comparison of two-stage and one-stage modifications of the LAL assay in plasma

	Sensitivity, pg/ml (EU/ml)	Linearity, pg/ml (EU/ml)
Two-stage modification	5 (0.060)	0-50 (0-0.580)
One-stage modification	1 (0.012)	0-30 (0-0.345)

However, this sensitive assay procedure was not suitable for those samples where high concentrations of endotoxin were expected. The assay procedure for such samples was modified by reducing the incubation period with the mixture of LAL and substrate. This eliminates the need for sample dilution.

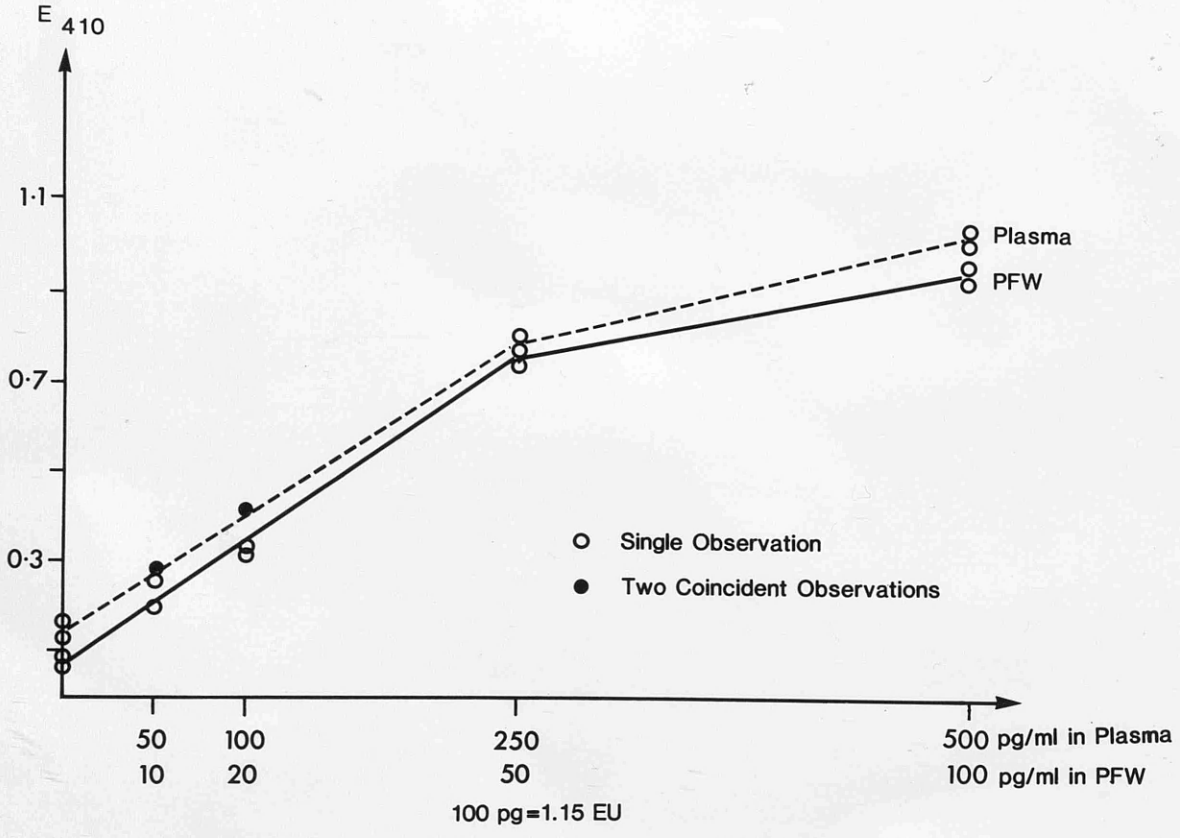
E 410
1·1
0·7
0·3
Plasma
PFW
Single Observation
Two Coincident Observations
50
10
100
20
250
50
500 pg/ml in Plasma
100 pg/ml in PFW
100 pg=1.15 EU

Fig. 19

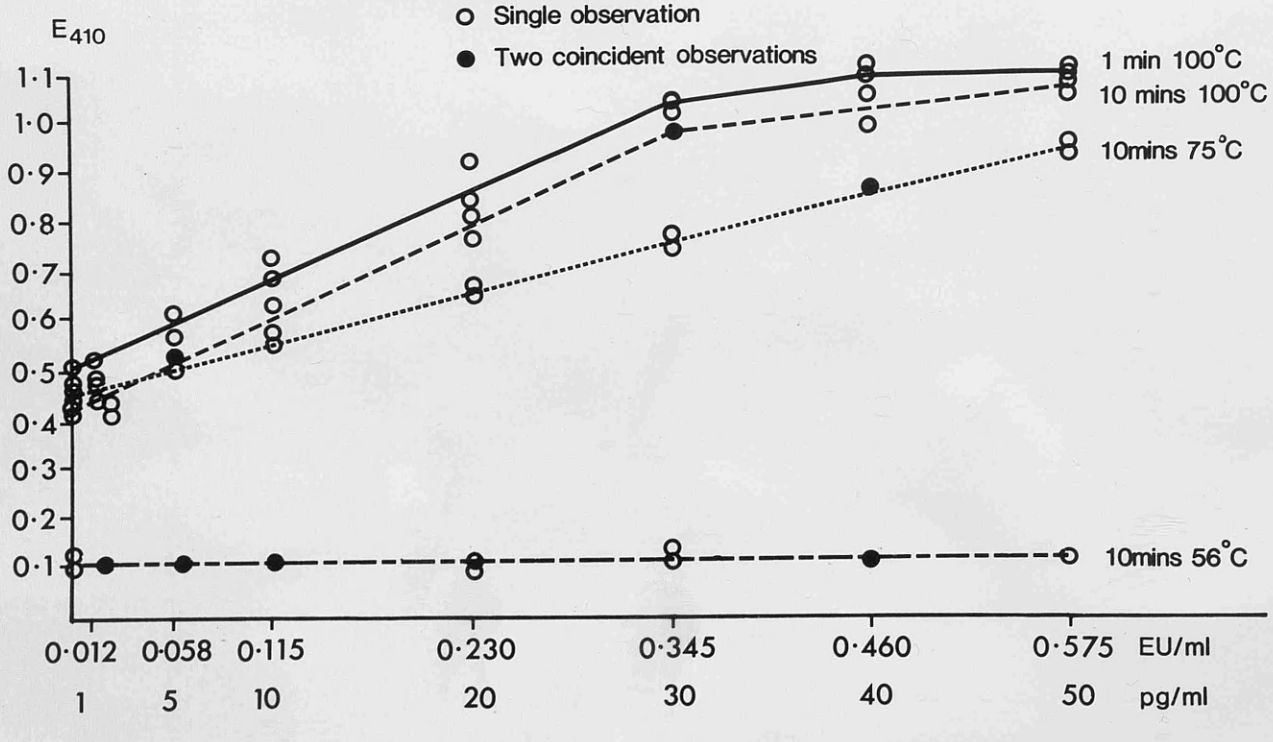
E410
Single observation
Two coincident observations
1 min 100°C
10 mins 100°C
10mins 75°C
10mins 56°C
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·012
1
0·058
5
0·115
10
0·230
20
0·345
30
0·460
40
0·575
50
EU/ml
pg/ml

Fig. 20

This modification of assay procedure was particularly important for plasma samples. Significant differences had been observed between plasma samples diluted before and after the boiling stage. As a result of these observations, in order to use reliably the standard curve for determination of endotoxin levels in unknown samples, the same dilution procedure would have to be adopted for both the unknown samples and the standard curve.

Reduction of the incubation time from 45 minutes to 20 minutes resulted in the extension of the linearity of the standard curves in plasma to 250 pg endotoxin/ml (2.875 EU/ml) and in water to 50 pg endotoxin/ml (0.575 EU/ml) (Fig.19).

Fig.19 Standard curves for high concentrations of endotoxin in plasma and pyrogen-free water.

3.3. Effect of heat on endotoxin in plasma and in pyrogen-free water

For this study, the one-stage method (see sub-section 3.2.2) was employed. Plasma used in this study was pre-diluted in the usual manner (i.e. 50 µl of PRP + 150 µl of boiling buffer + 50 µl of PFW or appropriate dilution of endotoxin in PFW). No significant differences were found between heating of plasma at 100°C for 1 minute and for 10 minutes. Also, the temperature of 100°C appeared to be superior to the temperature of 75°C. Surprisingly, the standard curve, after heating plasma for 10 minutes at 56°C was completely flat (Fig.20).

Fig.20 Comparison of four modes of heating of plasma.

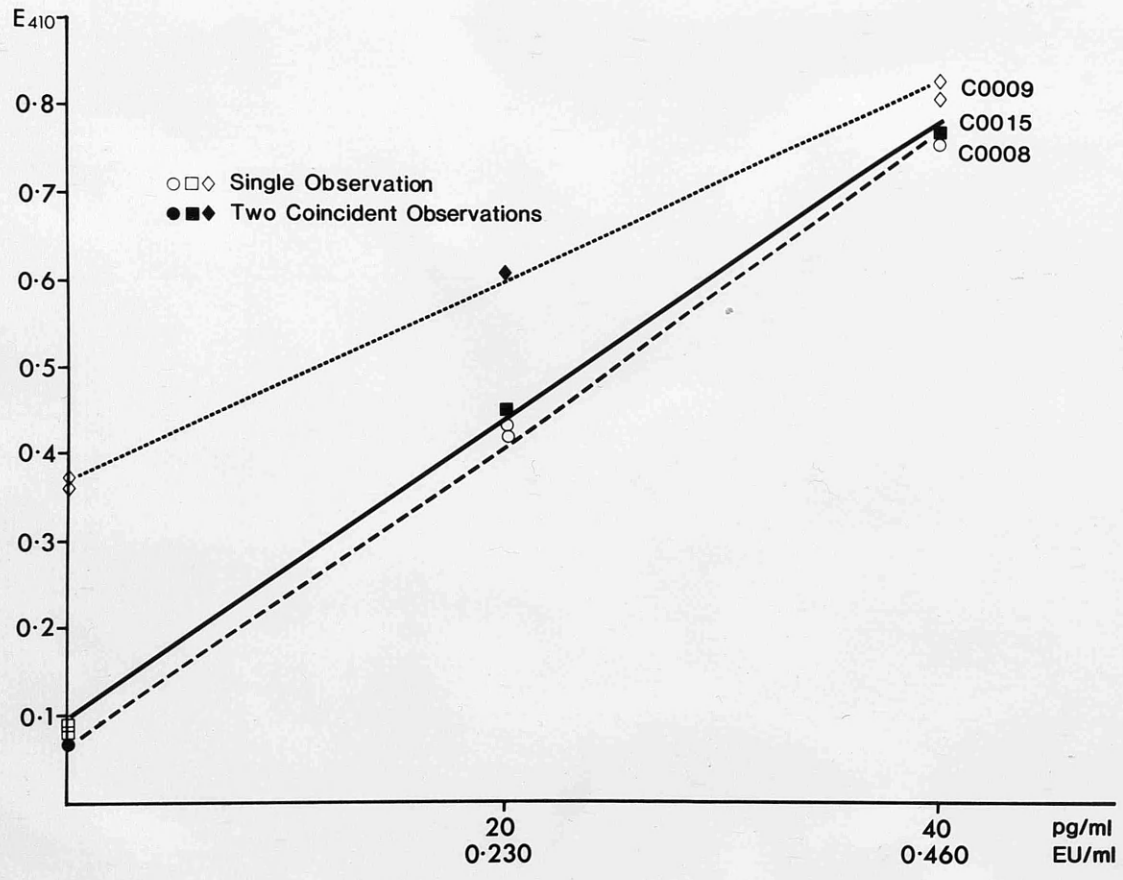
E410
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
○□◇ Single Observation
●■◆ Two Coincident Observations
◇ C0009
C0015
C0008
20
0·230
40
0·460
pg/ml
EU/ml

Fig. 23

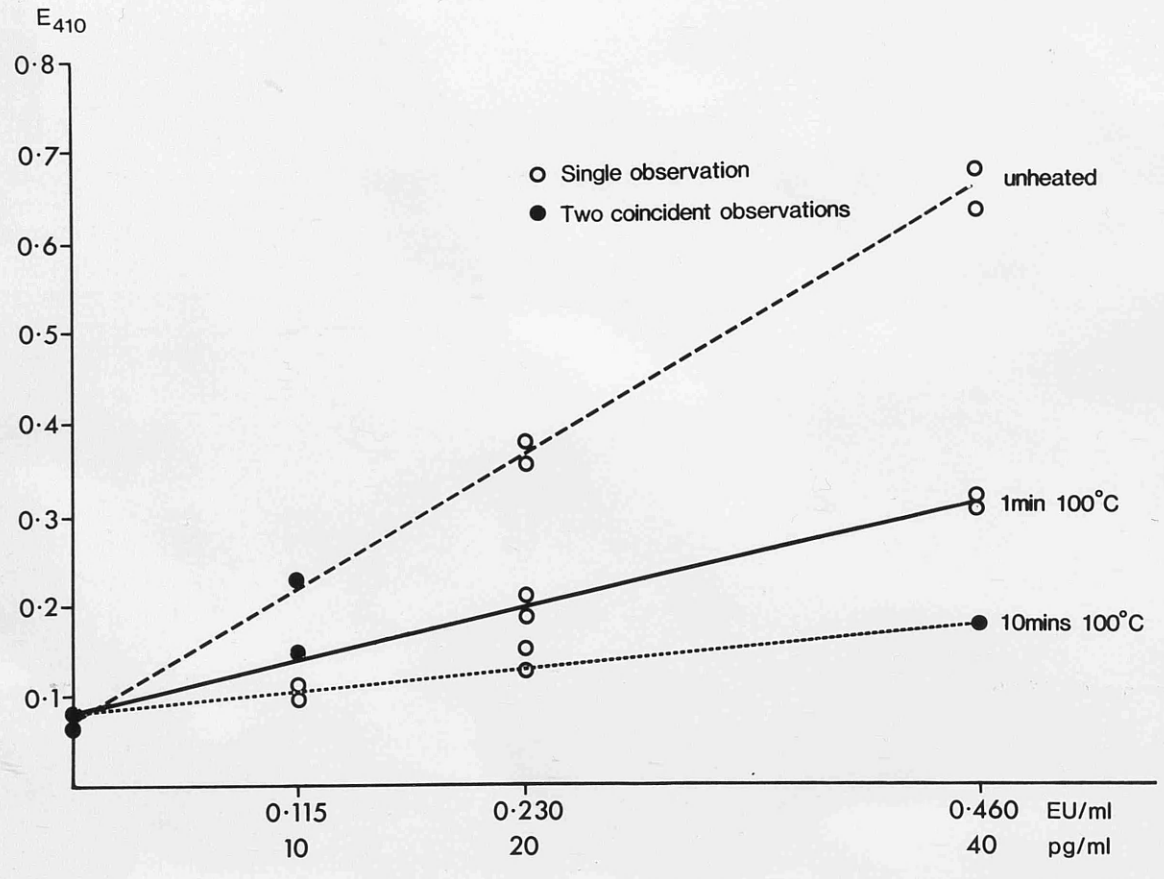
E_{410}
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
Single observation
Two coincident observations
unheated
1min 100°C
10mins 100°C
0·115
10
0·230
20
0·460
40
EU/ml
pg/ml

Fig. 21

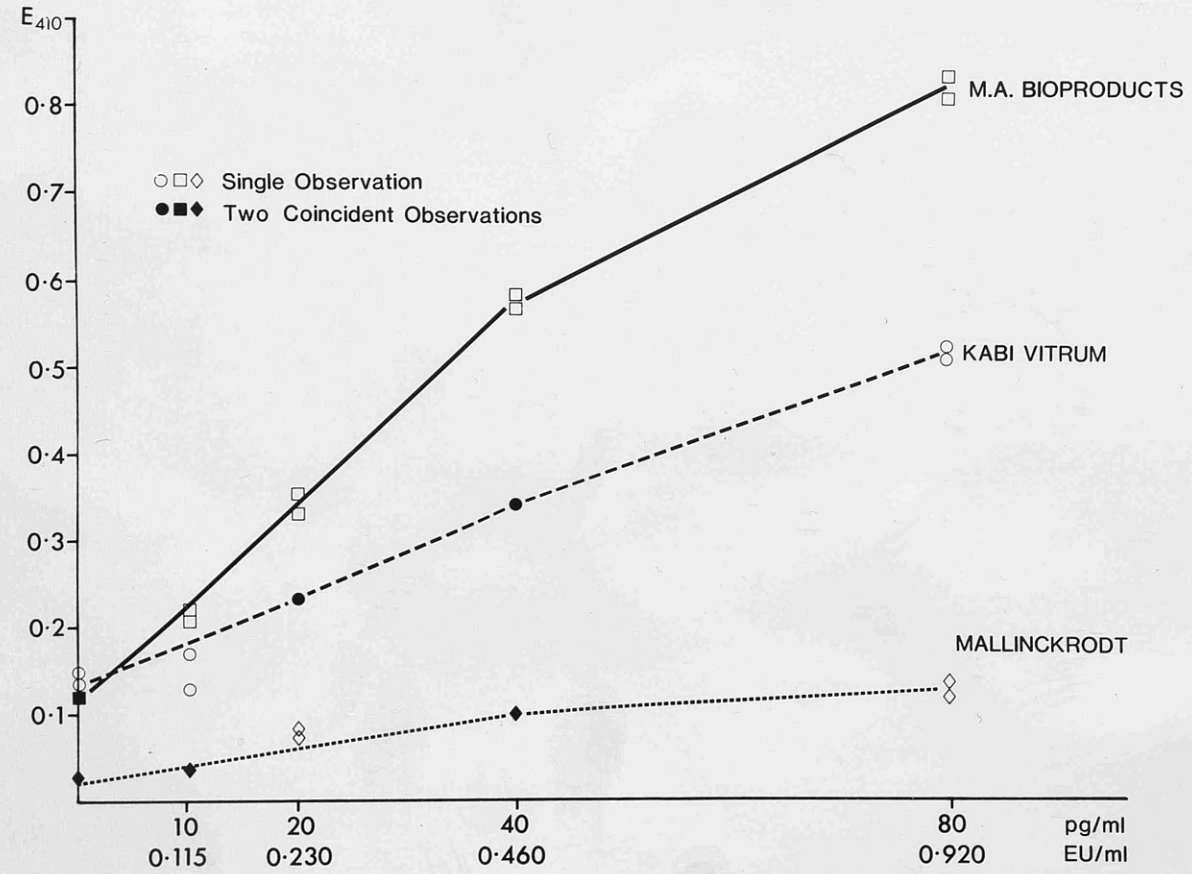
E_{410}
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
Single Observation
Two Coincident Observations
M.A. BIOPRODUCTS
KABI VITRUM
MALLINCKRODT
10
0·115
20
0·230
40
0·460
80
0·920
pg/ml
EU/ml

Fig. 22

It was also found that the standard curve of endotoxin heated in PFW was consistently lower than the standard curve of unheated endotoxin in PFW (Fig 21).

Fig.21 Influence of heating on endotoxin in pyrogen-free water.

3.4. Comparison of three commercial kits for the chromogenic LAL assay.

During the period of this study, two other chromogenic LAL assay kits (Kabi Diagnostica and Mallinckrodt) were marketed. The three commercial kits were compared (Fig.22). The poor performance of the Mallinckrodt kit was confirmed by using another batch of LAL and the substrate from the same manufacturer.

Fig. 22 Comparison of performance of chromogenic LAL assay kits from three different manufacturers.

Three different lots of LAL from M.A.Bioproducts' kits were also tested (Fig.23).

Fig.23. Comparison of performance of three chromogenic LAL kits from M. A. Bioproducts.

3.5. Clinical applications of the LAL assay

3.5.1 Cyclic neutropenia

The patient (M.B.) was a 35 year old woman with cyclic neutropenia (duration of cycle - 21 days).

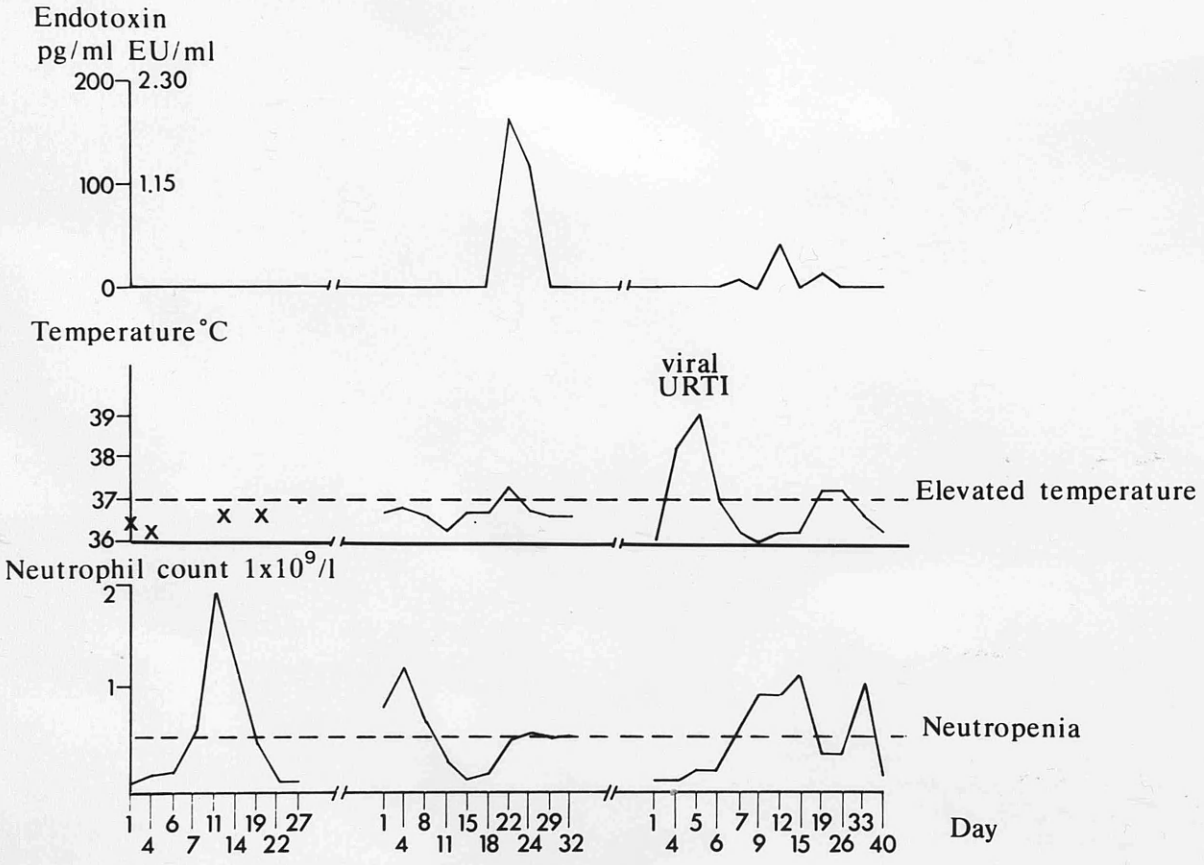
Endotoxin
pg/ml EU/ml
200
2.30
100
1.15
0
Temperature °C
viral
URTI
39
38
37
36
Elevated temperature
Neutrophil count 1x10⁹/l
2
1
Neutropenia
1 6 11 19 27
4 7 14 22
1 8 15 22 29
4 11 18 24 32
1 5 7 12 19 33
4 6 9 15 26 40
Day

Fig. 24

Endotoxin assays were carried out over three cycles of neutropenia in this patient to determine whether:

1. endotoxin was present in the patient's bloodstream during periods of neutropenia and malaise;
2. there was a correlation between the results of quantitative LAL assays and a rise in the body temperature of this patient.

Fig. 24 Endotoxin levels, body temperature and neutrophil count during three cycles of neutropenia in M B., 35 year old female patient.

In this patient, no temporal relationship between fever, endotoxin levels and neutrophil counts was found during three studied periods of neutropenia.

3.5.2. Septic shock trial

In an earlier study at the Western Infirmary, the semi-quantitative gel-clot LAL assay was one of a series of laboratory and clinical investigations on a group of patients with septic shock (308). This prospective study was undertaken to establish whether endotoxaemia was a constant feature of Gram-negative septic shock and to correlate the presence (or absence) of endotoxin in peripheral blood with the clinical course and prognosis. From this study, it was shown that endotoxin in the bloodstream was a feature of septic shock and that the continued presence of endotoxin in the peripheral blood was associated with a poor prognosis.

With the development of a quantitative chromogenic LAL assay (described in this thesis), it was decided to determine whether there was any correlation between

the quantitative levels of endotoxin in blood and the severity of sepsis. Ten critically ill but not necessarily septic shock patients were examined in the first group (Group 1). In this group (7 males and 3 females: age range 35–74 years, mean 56 years), there were five survivors and five non-survivors (Table 9). Septic status is a subjective clinical score.

Table 9. Clinical and laboratory findings in ten critically ill patients.

				Survivors		
Patient	Age	Pathology	LAL (no.)	Blood cultures (no.)	Septic status
1 J.M.	63	Small bowel infarct	Negative (4)	Negative (4)	±
2 E.M.	65	Perforated duodenal ulcer	Negative (4)	Negative (5)	++
3 H.H.	74	Perforated large bowel	Negative (2) Negative (2)	Negative (2) Positive (2 : 1 Gram +, 1 Gram -)	++
4 A.M.	50	Multiple injuries	Negative (14) Positive (5)	Negative (9) Negative (4)	++
5 R.O.	35	Multiple injuries	Negative (26) Positive (2) Negative (2) Positive (2)	Negative (37) Positive (2:Gram -) Positive (2 1:Gram +, 1:Gram -) Negative (5)	+++

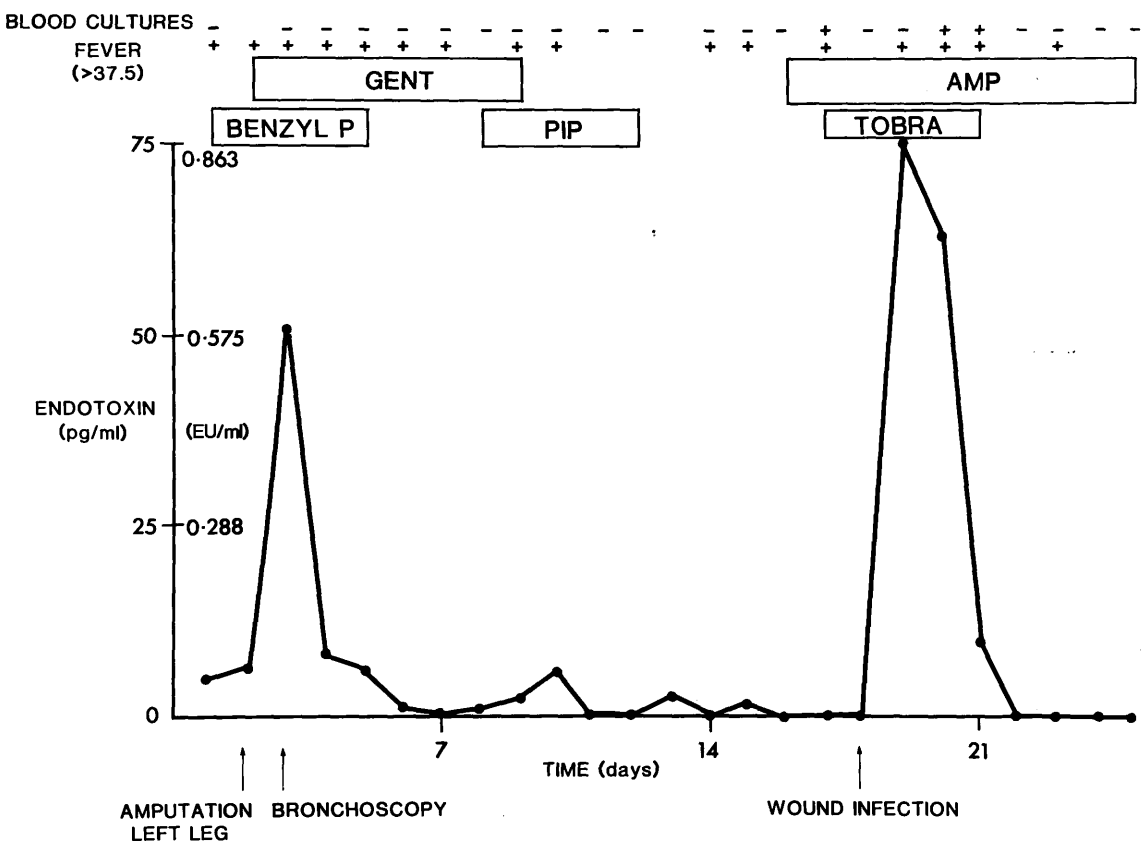
BLOOD CULTURES
FEVER
(>37.5)
GENT
AMP
BENZYL P
PIP
TOBRA
75
0·863
50
0·575
ENDOTOXIN
(pg/ml)
(EU/ml)
25
0·288
0
7
14
21
TIME (days)
AMPUTATION
LEFT LEG
BRONCHOSCOPY
WOUND INFECTION

Fig. 25

Non-Survivors

Patient	Age	Pathology	LAL(no.)	Blood cultures (no.)	Cause of death
1 K.R.	63	Large bowel obstruction	Negative (1)	Negative (1)	Sudden cardiovascular collapse
2 J.B.	52	Genito-urinary surgery	Negative (7)	Positive (2:Gram +) Negative (11)	Complication of sepsis
3 R.S.	55	Metabolic (neuroleptic-malignant syndrome)	Negative (3)	Negative (7)	Progressive coma
4 W.F.	43	Small bowel infarction (polyarteritis nodosa)	Negative (3)	Negative (3)	Renal failure (treatment discontinued)
5 A.M.	61	Perforated gastric ulcer (histology later malignant)	Negative (7)	Negative (9) Positive (4:Gram -)	Sepsis (treatment discontinued)

In this group of patients there was no close correlation between the results of blood cultures and the daily quantitative LAL assays. However, in one patient (R.O.), there was an apparent association between significant levels of endotoxin, positive blood cultures and acute septic episodes (Fig. 25).

Fig 25 R.O., 35 year old male patient with septic shock, who was admitted to the Intensive Therapy Unit with multiple injuries following a traffic accident: blood cultures, endotoxin assays and fever correlated with antibiotic therapy, clinical events and surgical care.

GENT = gentamicin; BENZYLP = benzylpenicillin; PIP = piperacillin; AMP = ampicillin; TOBRA = tobramycin.

Blood cultures: day 17 - Enterobacter cloacae, days 20 and 21 - Acinetobacter calcoaceticus, variant anitratus; wound infection - Pseudomonas aeruginosa and

Acinetobacter calcoaceticus, variant anitratus.

Early in this patient's course, transient endotoxaemia occurred after amputation and bronchoscopy in the absence of positive blood cultures. The wound infection on day 18 was associated with a high level of endotoxin in the bloodstream. Blood cultures subsequently became positive.

When the results from this group were reviewed, it was decided that for critical evaluation of the LAL assay in septic shock, frequent monitoring during acute septic episodes was essential. Thus septic shock patients in the second group (Group 2) were monitored at 4-hourly intervals for the first 48 hours at ITU and thereafter daily for one week.

This closely monitored group (Group 2) consisted of 8 patients (6 males and 2 females; age range 22-77 years, mean 57 years). Table 10 summarizes the primary pathology of these patients. Septic status is a subjective clinical score. Of the 8 patients studied, 4 did not survive.

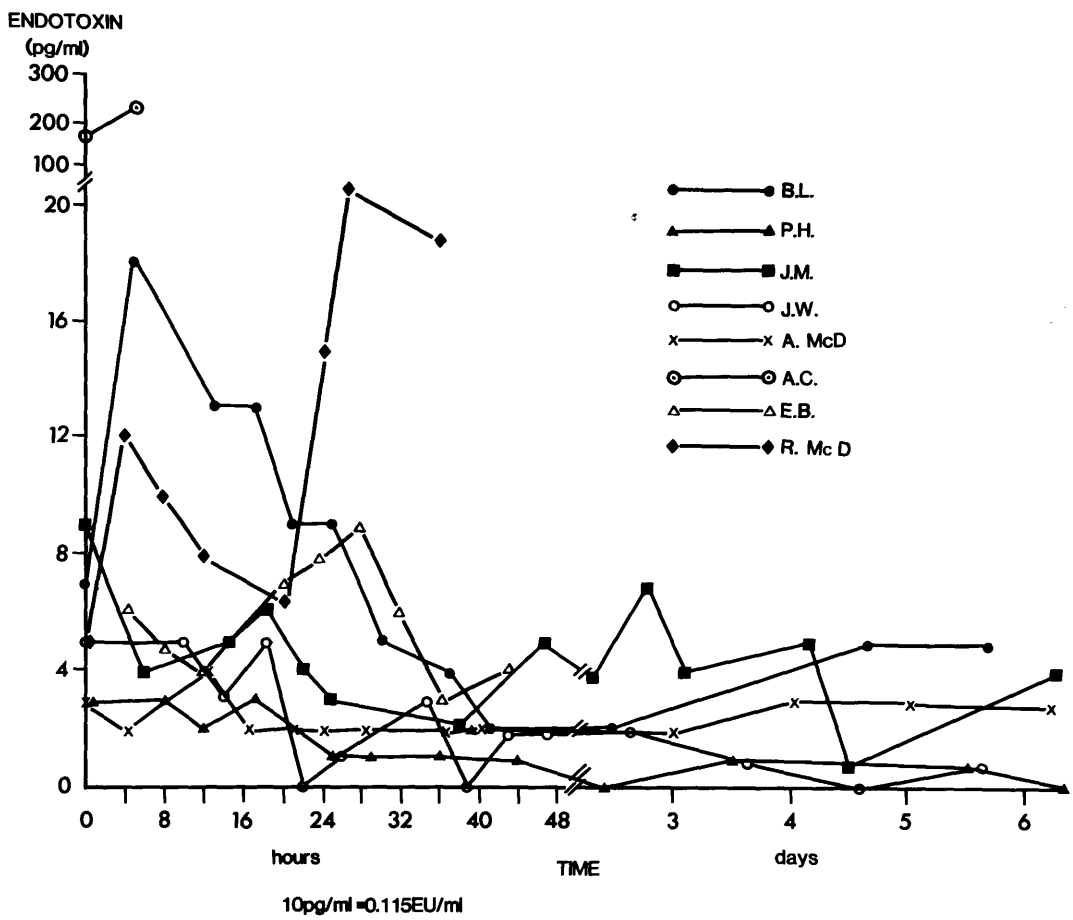
ENDOTOXIN
(pg/ml)
300
200
100
20
16
12
8
4
0
0
8
16
24
32
40
48
3
4
5
6
hours
TIME
days
B.L.
P.H.
J.M.
J.W.
A. McD
A.C.
E.B.
R. Mc D
10pg/ml =0.115EU/ml

Fig. 26

Table 10 Clinical information on 8 septic shock patients.

Patient	Age	Primary pathology	Septic status	Outcome
B L.	49	Post-operative genitourinary surgery	+++	Survived
J M.	65	Thoracic spinal abscess	++	Survived
P H.	22	Streptococcus viridans septicaemia (drug addiction)	++	Survived
J.W.	77	Post-operative perforated gastric ulcer	++	Survived
A McD.	72	Post-operative genitourinary surgery	+++	Died
A C.	60	Peritonitis	++++	Died
E B.	67	Infected varicose ulcers	+++	Died
R McD.	47	Multiple trauma	++++	Died

The time course of changes in endotoxin levels of all eight patients is shown in Fig.26.

Fig 26. The time course of the LAL assays

The case histories of the eight individual patients are given in detail below.

Patient B L.

This patient was a 49 year old woman who developed septic shock 24 hours after ileal conduit surgery. She had had no antibiotics. On admission to ITU she had a temperature of 39.2°C, a systolic blood pressure of 60 mm Hg and a pulse of 153 beats/minute. Treatment was commenced with plasma, mechanical ventilation

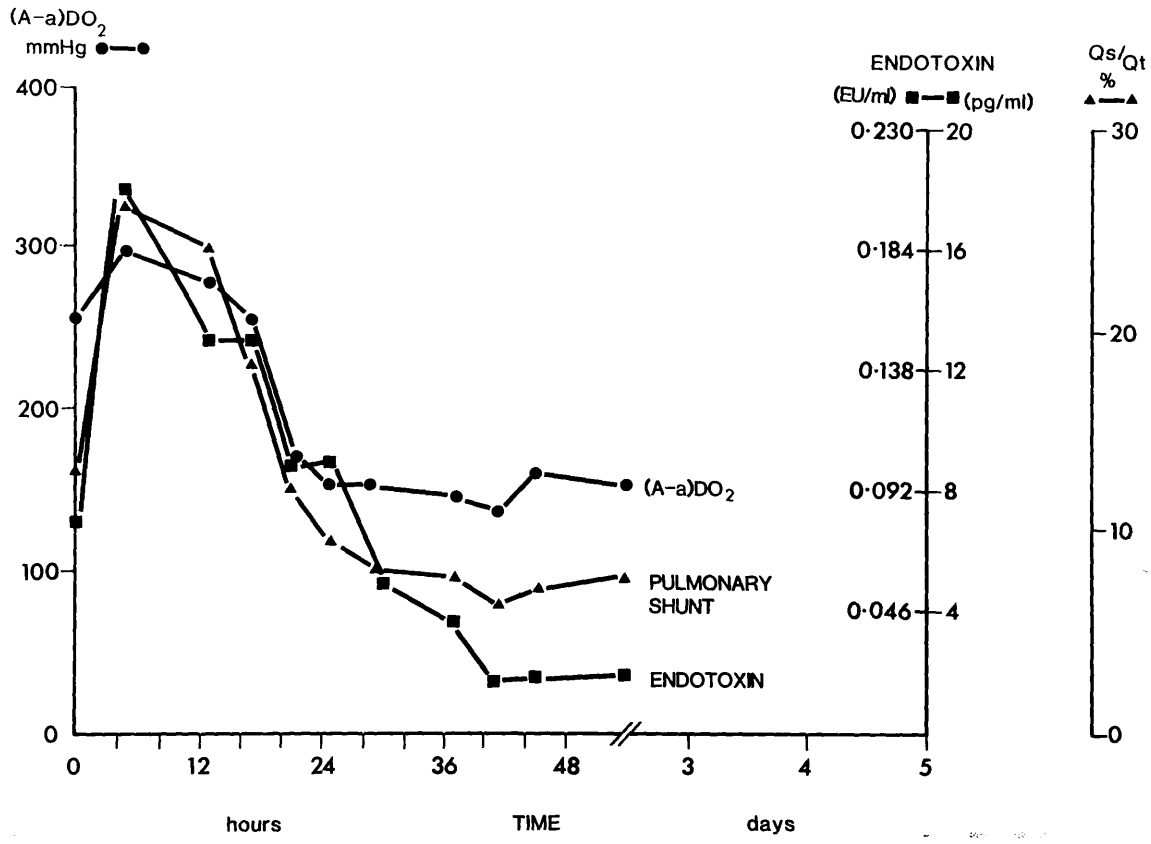
(A-a)DO2
mmHg
400
300
200
100
0
ENDOTOXIN
(EU/ml) (pg/ml)
0·230
0·184
0·138
0·092
0·046
20
16
12
8
4
Qs/Qt
%
30
20
10
0
(A-a)DO2
PULMONARY SHUNT
ENDOTOXIN
0
12
24
36
48
3
4
5
hours
TIME
days

Fig. 27

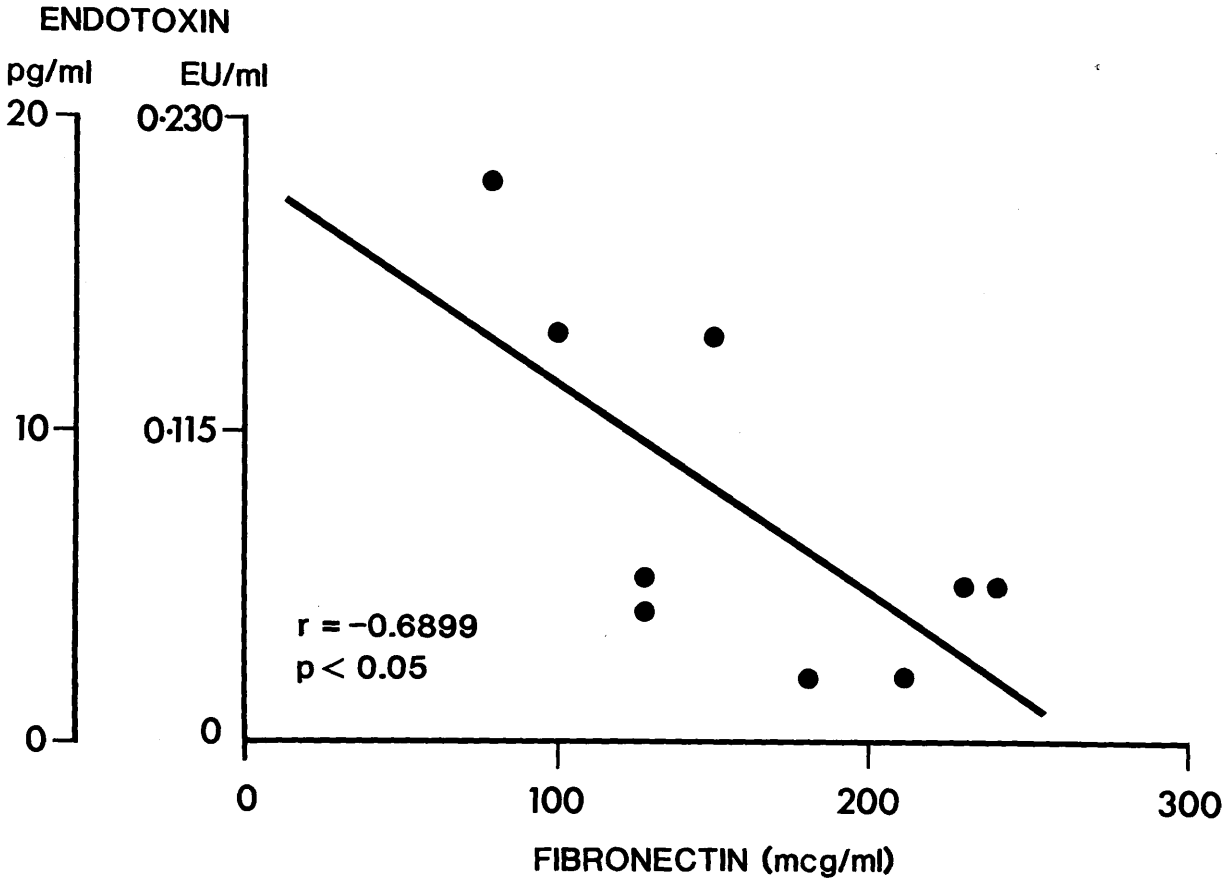
ENDOTOXIN
pg/ml
EU/ml
20
10
0
0·230
0·115
0
r = −0.6899
p < 0.05
0
100
200
300
FIBRONECTIN (mcg/ml)

Fig. 28

and an inotropic infusion of dopamine. A pulmonary artery flotation catheter was inserted and fluid infusion continued until the blood pressure was above 100 mm Hg systolic and the pulmonary artery occlusion pressure was greater than 8 mm Hg. Six litres of fluid were required. She was commenced on ampicillin, gentamicin and metronidazole. Fig.27 shows endotoxin levels, alveolar arterial oxygen difference and pulmonary shunt. Plasma endotoxin on admission was 6 pg/ml (0.072 EU/ml) and within four hours rose to 19 pg/ml (0.228 EU/ml). Thereafter it fell gradually over the following 36 hours.

Fig 27. Endotoxin levels, alveolar-arterial oxygen difference and pulmonary shunt in B.L., 49 year old female patient, admitted to ITU with septic shock 24 hours after ileal conduit surgery.

There was a close temporal relationship between endotoxin and pulmonary shunt ($r = 0.9211$) and endotoxin and alveolar-arterial oxygen differences ($r = 0.8852$).

In this patient there was also an inverse relationship between changes in plasma fibronectin and endotoxin levels, with $r = -0.6899$ and $p < 0.05$ (Fig 28). Plasma fibronectin recovered to within the normal range (275-300 µg/ml) as the endotoxin was eliminated from the bloodstream.

Fig 28. Inverse correlation between fibronectin and endotoxin levels in patient B.L.

In patient B.L. there was also good correlation between endotoxin and septic score, and fibronectin and septic score (Fig 29).

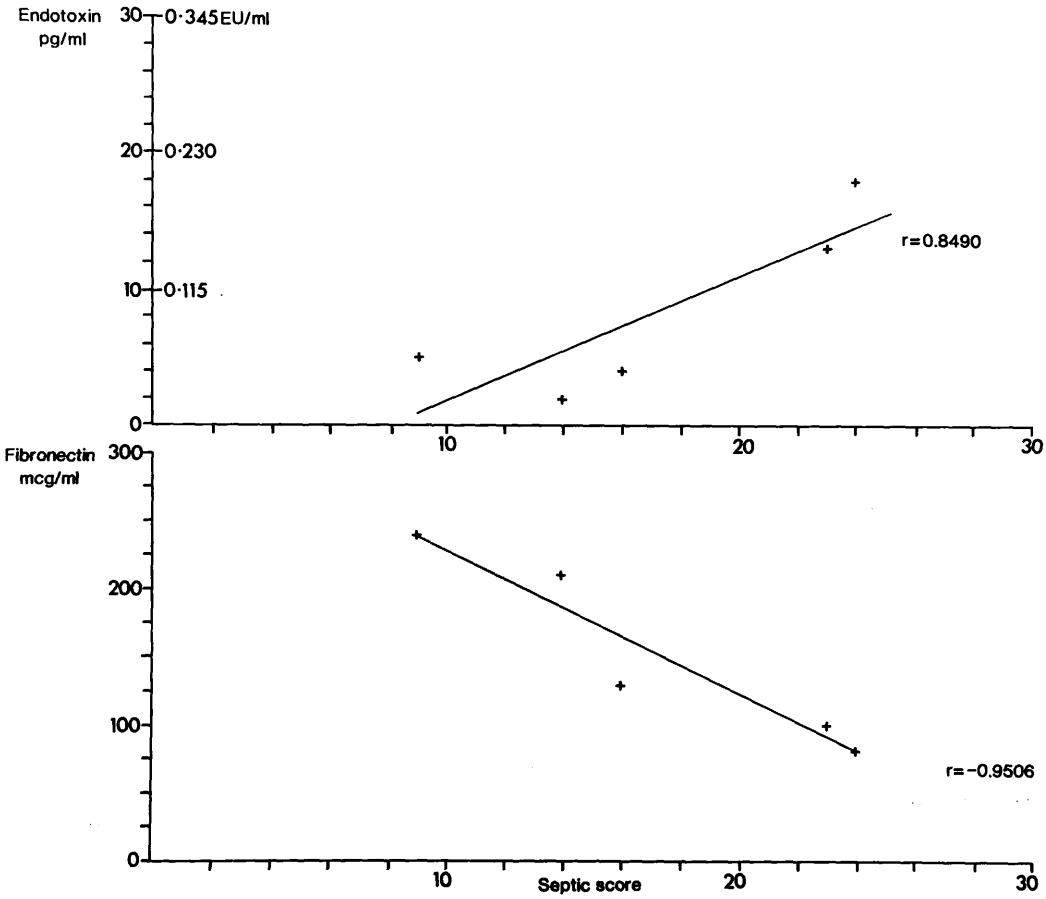
Endotoxin
pg/ml
30
0·345EU/ml
20
0·230
10
0·115
0
r=0.8490
10
20
30
Fibronectin
mcg/ml
300
200
100
0
r=-0.9506
10
Septic score
20
30

Fig. 29

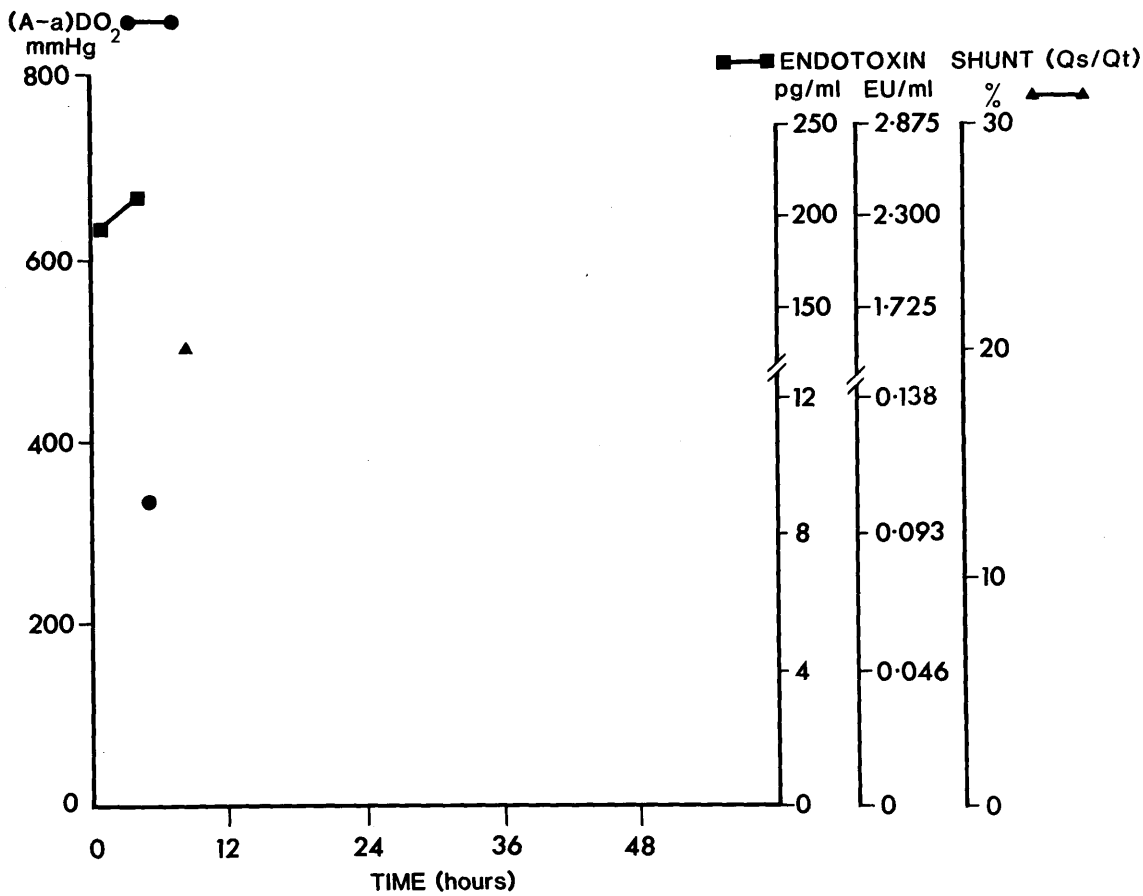
(A-a)DO2
mmHg
800
600
400
200
0
ENDOTOXIN
pg/ml
EU/ml
SHUNT (Qs/Qt)
%
250
2·875
200
2·300
150
1·725
12
0·138
8
0·093
4
0·046
0
30
20
10
0
0
12
24
36
48
TIME (hours)

Fig. 30

Fig 29. Relationship between endotoxin, fibronectin and septic score* in patient B L.

In this patient, Staphylococcus aureus was isolated from six of 10 blood cultures taken within the first 48 hours. Blood cultures taken on days 5 and 6 yielded Morganella morganii.

Patient A C.

This patient was a 60 year old man admitted to hospital with an 11 hour history of vomiting, rigors and persistent lower abdominal pain. He was pyrexial with a temperature of 39°C, a pulse of 120 beats per minute and a systolic blood pressure of 70 mm Hg. Shortly after admission, he was taken for investigative laparotomy to the operating theatre, where an enlarged cirrhotic liver was noted and 200 ml of yellow turbid peritoneal fluid was drained. Escherichia coli was cultured from this fluid. There was no evidence of perforation of any viscus. However, despite intensive therapy post-operatively, the patient died nine hours after admission. He had the highest endotoxin levels recorded in our septic shock patients (180 pg/ml = 2.16 EU/ml and 220 pg/ml = 2.64 EU/ml), despite only moderately elevated pulmonary shunt and alveolar-arterial oxygen difference (Fig30), and negative blood cultures.

Fig 30. Endotoxin levels, alveolar-arterial oxygen difference and pulmonary shunt in A C., 60 year old male patient with peritonitis.

The plasma fibronectin levels of this patient were almost undetectable at around 40 µg/ml.

*Septic score - see Elebute, E.A., Stoner, H.B.: The grading of sepsis. Br. J. Surg. 1983; 70: 29-31.

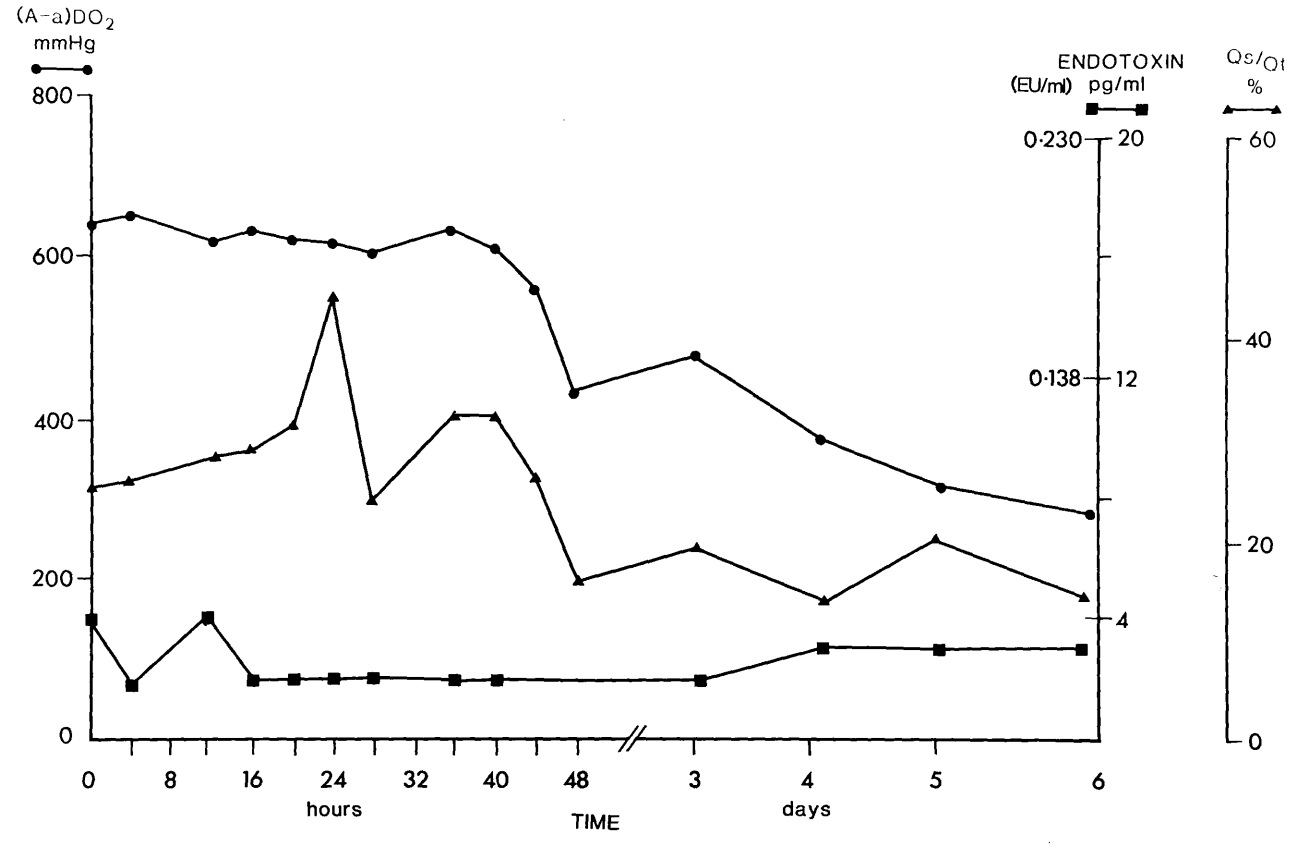
(A-a)DO2
mmHg
800
600
400
200
0
ENDOTOXIN
(EU/ml) pg/ml
0·230
0·138
20
12
4
Qs/Qt
%
60
40
20
0
0
8
16
24
32
40
48
3
4
5
6
hours
TIME
days

Fig. 31

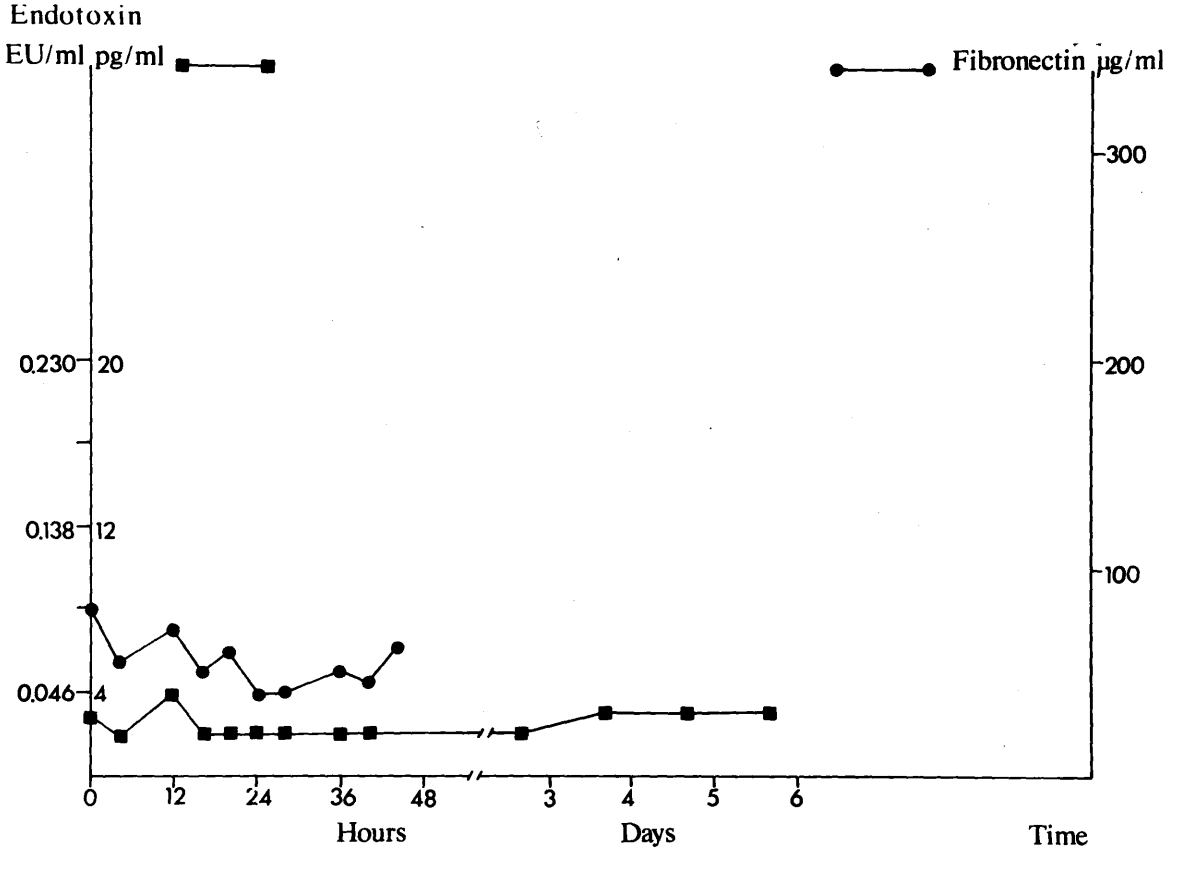
Endotoxin
EU/ml pg/ml
Fibronectin µg/ml
0.230
20
0.138
12
0.046
4
300
200
100
0
12
24
36
48
3
4
5
6
Hours
Days
Time

Fig. 32

Patient A.McD.

This patient was a 72 year old man admitted to a peripheral hospital six hours after having a transrectal prostate biopsy performed as an outpatient. He was in septic shock with a systolic blood pressure of 80 mm Hg, a pulse of 120 beats per minute and a temperature of 40°C. Escherichia coli was grown from blood cultures taken on admission. Despite appropriate antibiotics, he continued to deteriorate and 48 hours later he was transferred to ITU at the Western Infirmary. On admission, he was in severe septic shock with hypotension, tachycardia and pyrexia.

Patient A.McD. illustrates the opposite situation to patient A.C., as his endotoxin levels were negligible despite clinical evidence of overwhelming sepsis, grossly elevated pulmonary shunt and alveolar-arterial oxygen difference consistent with early Adult Respiratory Distress Syndrome (ARDS) (Fig 31).

Fig 31. Endotoxin levels, alveolar arterial oxygen difference and pulmonary shunt in A.McD., 72 year old male patient, admitted to ITU with severe septic shock after a transrectal prostate biopsy.

The corresponding plasma fibronectin levels of this patient are shown in Fig 32.

Fig 32. Fibronectin levels versus endotoxin levels in patient A.McD.

The initial level of plasma fibronectin in patient A. McD. was 80 µg/ml and remained very low during this patient's course prior to his death in ITU.

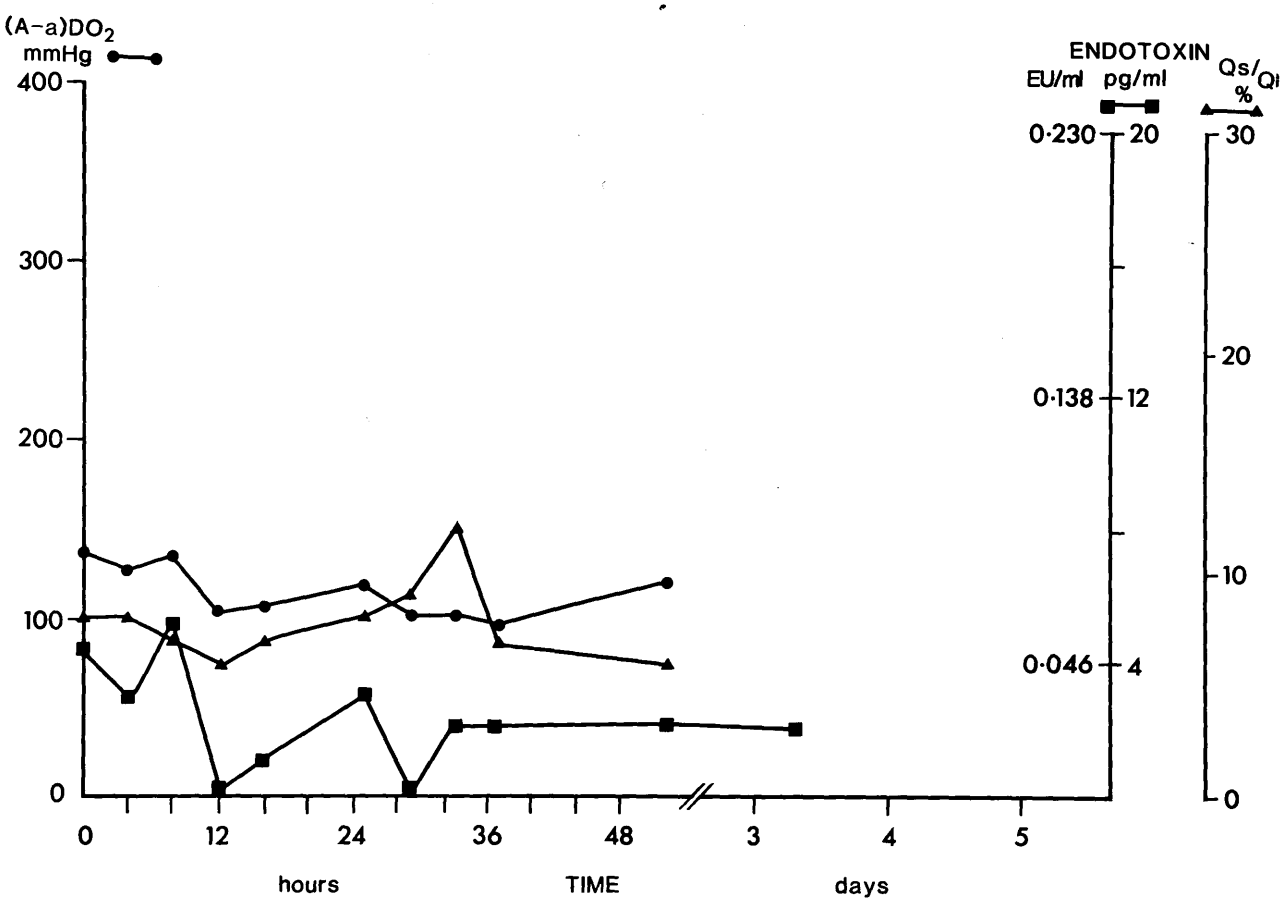
(A-a)DO2
mmHg
400
300
200
100
0
ENDOTOXIN
EU/ml
pg/ml
Qs/Qt
%
0·230
20
30
0·138
12
20
0·046
4
10
0
0
12
24
36
48
3
4
5
hours
TIME
days

Fig. 35

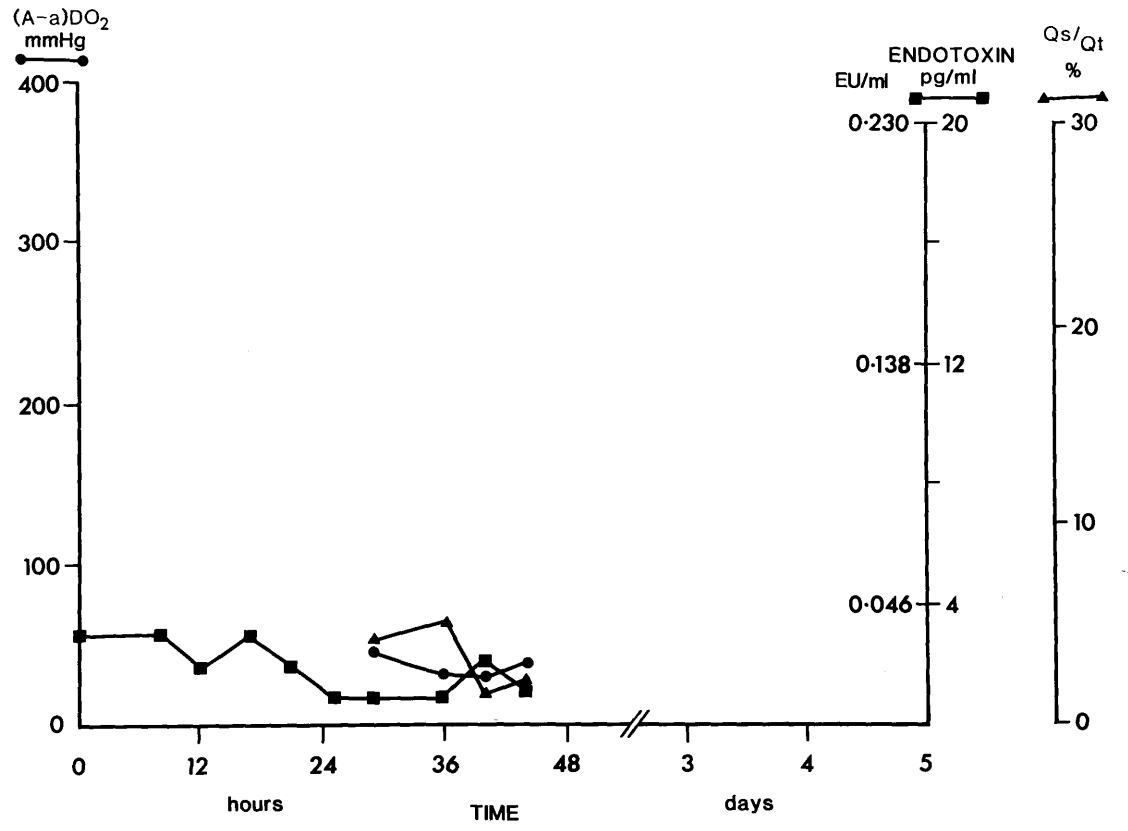
(A-a)DO2
mmHg
400
300
200
100
0
ENDOTOXIN
EU/ml pg/ml
0·230 20
0·138 12
0·046 4
Qs/Qt
%
30
20
10
0
0 12 24 36 48 3 4 5
hours
TIME
days

Fig. 33

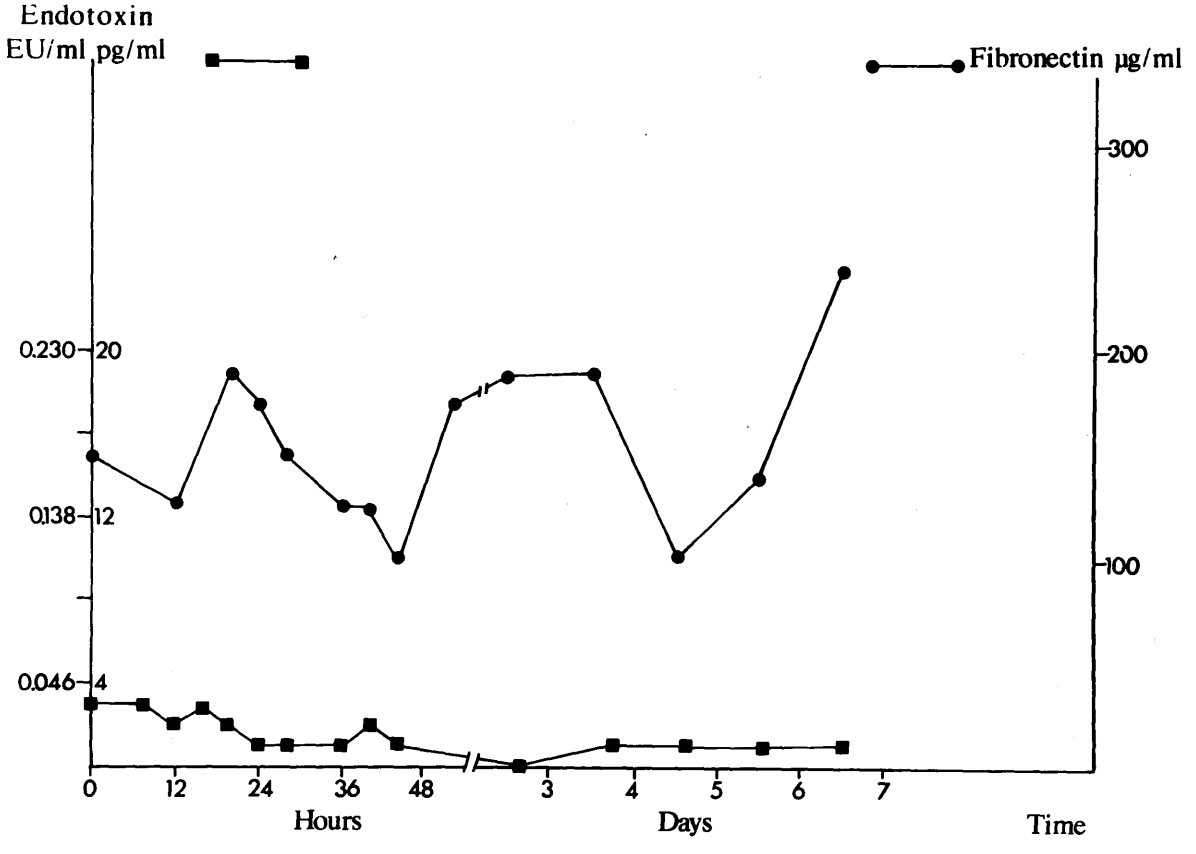
Endotoxin
EU/ml pg/ml
Fibronectin µg/ml
0.230 20
0.138 12
0.046 4
300
200
100
0 12 24 36 48 3 4 5 6 7
Hours
Days
Time

Fig. 34

Patient P H.

This patient was a 22 year old man who presented clinically with septic shock, but his endotoxin levels were negligible. Pulmonary shunt and alveolar-arterial oxygen differences were also low (Fig 33). The patient was a drug addict. Streptococcus viridans was isolated from blood cultures prior to the development of septic shock.

Fig 33. Endotoxin levels, alveolar-arterial oxygen difference and pulmonary shunt in P H., 22 year old male patient, drug addict, with Streptococcus viridans isolated from blood cultures.

Corresponding plasma fibronectin levels of this patient were not dramatically depressed (Fig 34).

Fig 34. Fibronectin levels versus endotoxin levels in patient P H.

Patients J.W. and J M.

Patient J.W. was a 77 year old man with a perforated gastric ulcer. Patient J M. was a 65 year old man with a thoracic spine abscess.

These patients, who presented clinically with septic shock, elevated pulmonary shunt and alveolar-arterial oxygen differences, nevertheless had negligible endotoxin levels (Figs. 35 and 36) and all their blood cultures were negative.

Fig 35. Endotoxin levels, alveolar-arterial oxygen difference and pulmonary shunt in J.W., 77 year old male patient with perforated gastric ulcer.

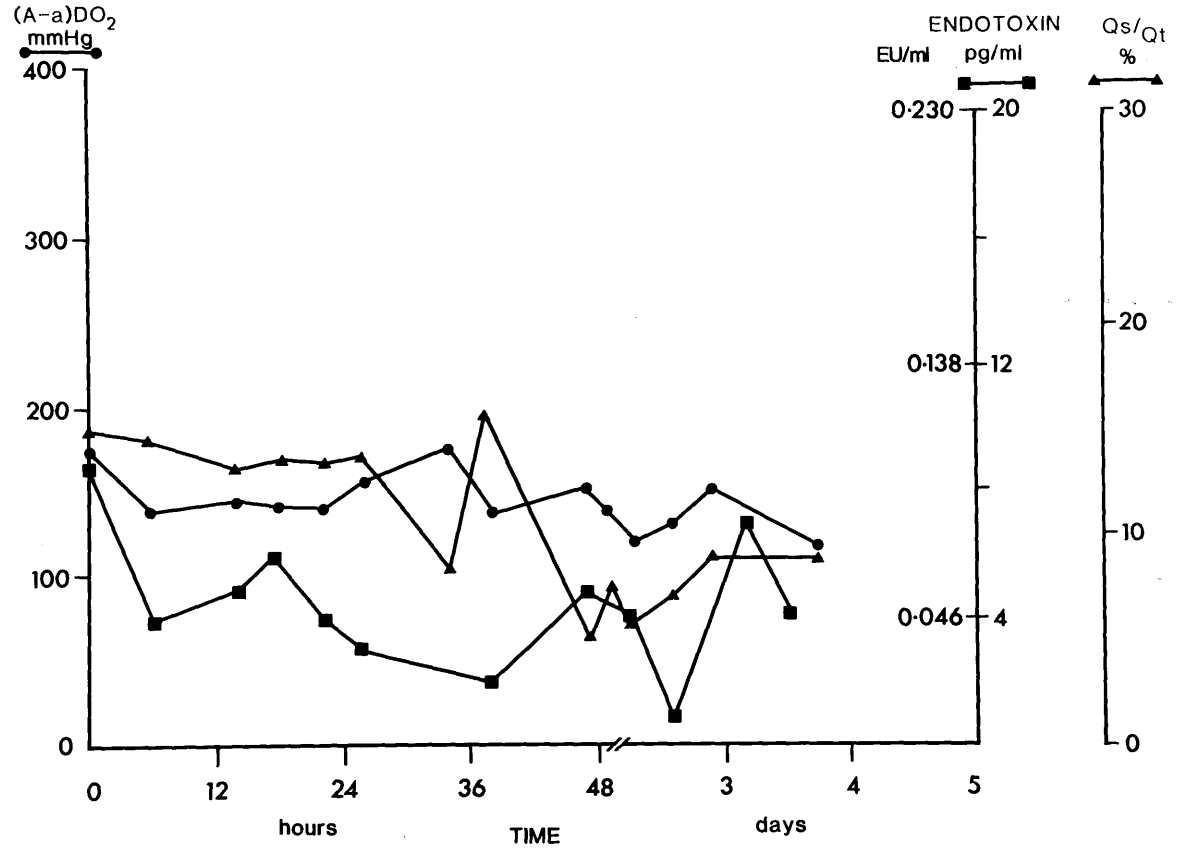
(A-a)DO2
mmHg
400
300
200
100
0
ENDOTOXIN
EU/ml pg/ml
0·230 20
0·138 12
0·046 4
Qs/Qt
%
30
20
10
0
0 12 24 36 48 3 4 5
hours
TIME
days

Fig. 36

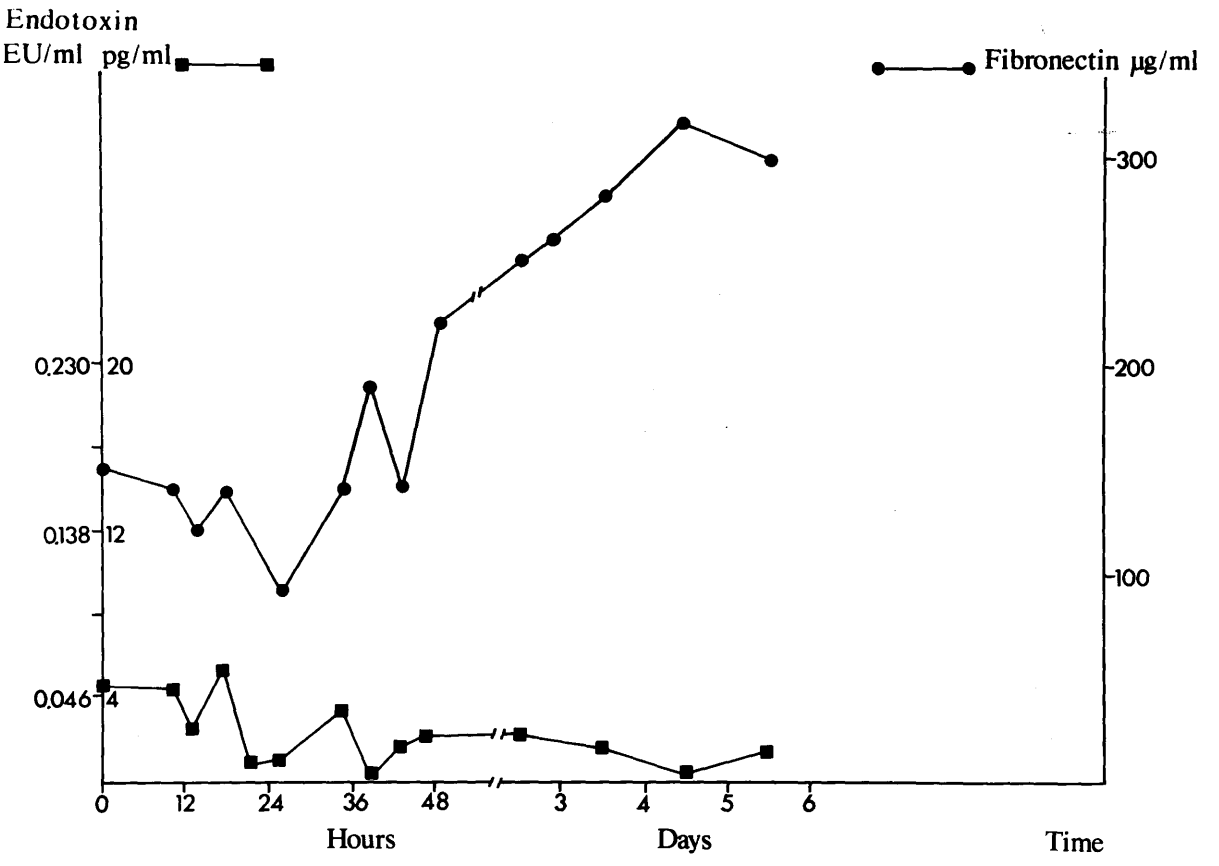
Endotoxin
EU/ml pg/ml
Fibronectin µg/ml
0.230 20
0.138 12
0.046 4
300
200
100
0 12 24 36 48 3 4 5 6
Hours
Days
Time

Fig. 37

Fig 36. Endotoxin levels, alveolar-arterial oxygen difference and pulmonary shunt in J.M., 65 year old male patient with thoracic spine abscess.

The corresponding plasma fibronectin levels of these two patients were not dramatically depressed (Figs. 37 and 38). It is interesting to note that both patients survived.

Fig 37. Fibronectin levels versus endotoxin levels in patient J.W.

Fig. 38. Fibronectin levels versus endotoxin levels in patient J.M.

Patient R. McD.

This patient was a 47 year old man, who sustained multiple trauma in a road traffic accident. He remained stable for five days but then developed septic shock. After 20 hours of monitoring, he was taken to the operating theatre in an attempt to locate a focus of sepsis. A four-fold rise in endotoxin levels was observed in association with the surgery (Fig 39).

Endotoxin levels in this patient were measured in optical density (O.D.) units, and not in pg/ml (EU/ml) At the time of assaying these samples, the standard endotoxin from the LAL kits did not react with the LAL reagent, and consequently no standard curve could be constructed. The endotoxin levels presented in Figs. 26 and 39, which are expressed in pg/ml, are only an estimation, which was made to allow comparison of the time course of endotoxin assays of this patient with other patients in this group.

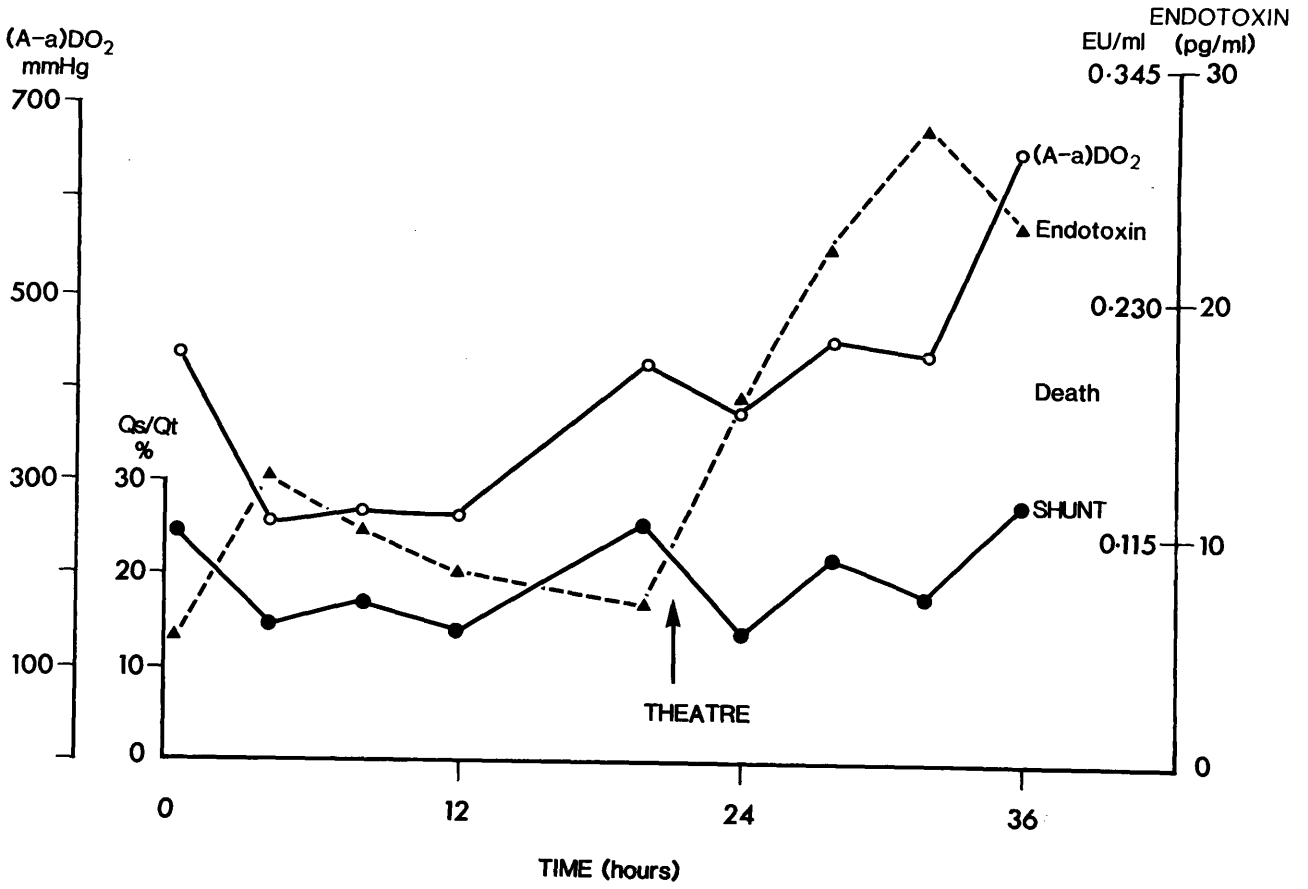
(A-a)DO2
mmHg
700
500
300
100
Qs/Qt
%
30
20
10
0
ENDOTOXIN
EU/ml (pg/ml)
0·345 30
0·230 20
0·115 10
0
(A-a)DO2
Endotoxin
Death
SHUNT
THEATRE
0
12
24
36
TIME (hours)

Fig. 39

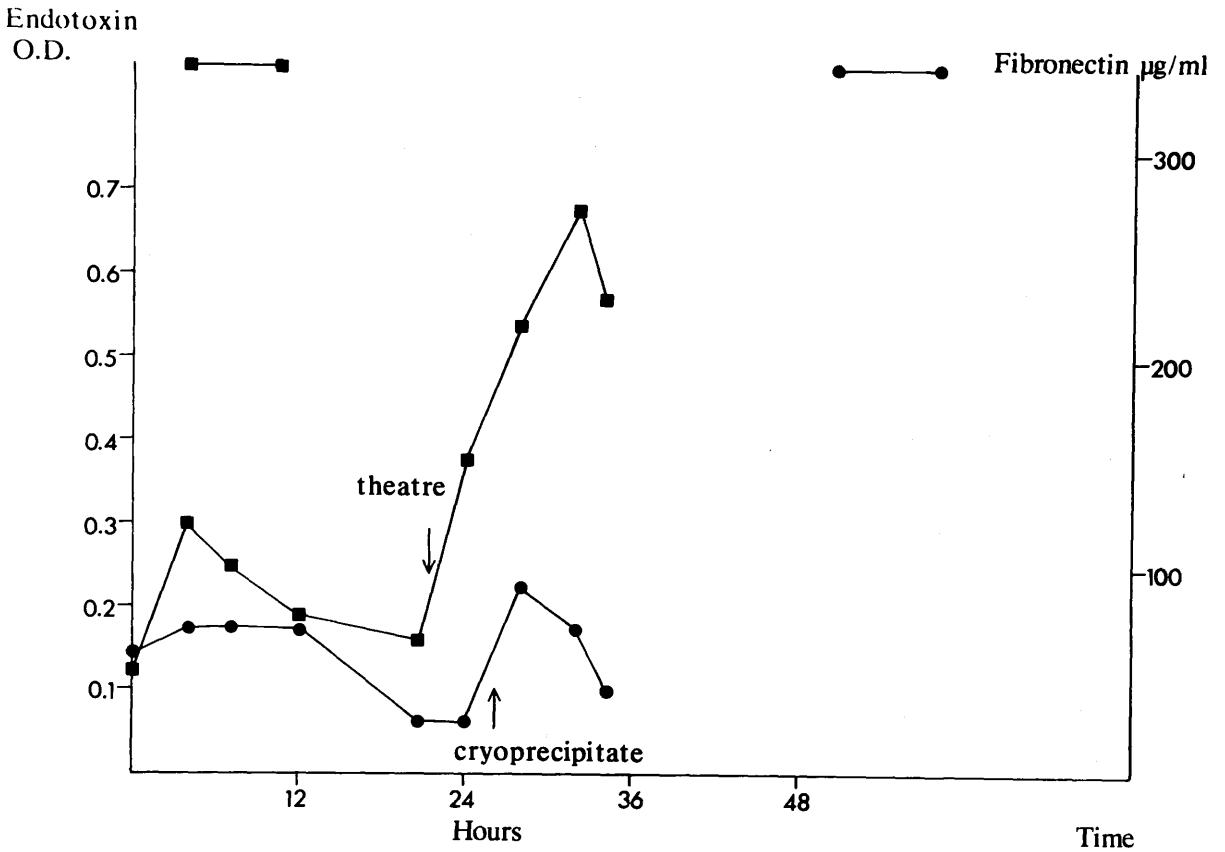
Endotoxin
O.D.
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Fibronectin µg/ml
300
200
100
theatre
cryoprecipitate
12
24
36
48
Hours
Time

Fig. 40

In this patient, there was a direct relationship between alveolar-arterial oxygen difference and endotoxin (r = 0.623) (Fig 39).

Fig 39. Endotoxin levels, alveolar-arterial oxygen difference and pulmonary shunt in R. McD., 47 year old male patient with multiple trauma.

The corresponding plasma fibronectin levels of this patient are shown in Fig 40.

Fig 40. Fibronectin levels versus endotoxin levels in patient R. McD.

After the initial period of 12 hours when plasma fibronectin remained stable at a relatively low level of 70 µg/ml, the fibronectin level then fell to 25 µg /ml. Despite a transient increase after administration of cryoprecipitate, the fibronectin level fell again. The patient died.

Patient E.B.

This patient was a 59 year old woman, with a history of rheumatoid arthritis, who developed septic shock from infected varicose ulcers. All blood cultures were negative, but Proteus spp., Enterobacter cloacae and Streptococcus faecalis were cultured from the varicose ulcers. Thirty six hours after admission, she developed ARDS (Adult Respiratory Distress Syndrome) and died 24 hours later. Her endotoxin levels were slightly elevated.

During this patient's course in ITU, cryoprecipitate was administered on several occasions. Since the fibronectin contained in the preparation may have influenced the clinical course, endotoxin levels and cardiovascular-respiratory measurements, this patient's results could not be analysed in detail.

3.5.3. Acute pancreatitis - preliminary trial

In an earlier study at the Western Infirmary, the semi-quantitative gel-clot LAL assay was one of a series of laboratory and clinical investigations on a group of patients with acute pancreatitis (144). This prospective study was designed to assess the incidence of endotoxaemia and complement activation in pancreatitis, in order to see whether they were related, and to correlate the presence of each with the development of complications. In this study, it was shown that half of the patients studied had endotoxaemia. Moreover, endotoxaemia was present in six out of seven patients with systemic complications of the disease. It was suggested that endotoxaemia could be at least partly responsible for the development of shock and other complications of acute pancreatitis.

With the development of a quantitative chromogenic LAL assay (described in this thesis), it was decided to determine whether there was any correlation between the quantitative levels of endotoxin in blood and the severity of pancreatitis. In this trial, a total of 16 patients was examined.

After clinical and laboratory assessment (including endotoxin assays), the group of 16 patients was divided into the following categories:

- severe pancreatitis with related endotoxaemia (one patient)
- severe pancreatitis with no significant endotoxaemia (one patient)
- endotoxaemia unrelated to pancreatitis (two patients)
- mild pancreatitis with no significant endotoxaemia (six patients)
- mild pancreatitis with unrelated endotoxaemia (one patient)
- a group of five patients with various conditions, rejected on the grounds of inadequate sampling (too few samples to allow drawing of any conclusions).

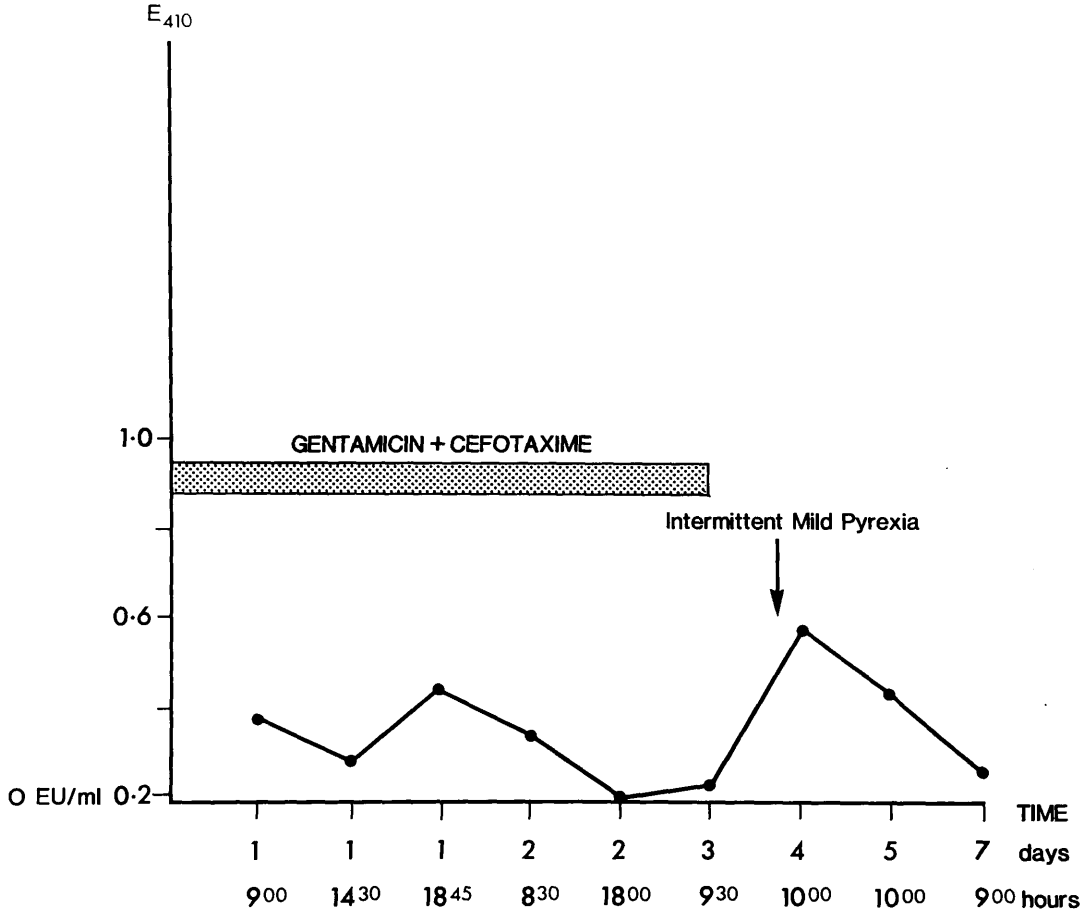
E410
1·0
GENTAMICIN + CEFOTAXIME
Intermittent Mild Pyrexia
0·6
O EU/ml 0·2
TIME
1 1 1 2 2 3 4 5 7 days
900 1430 1845 830 1800 930 1000 1000 900 hours

Fig. 41

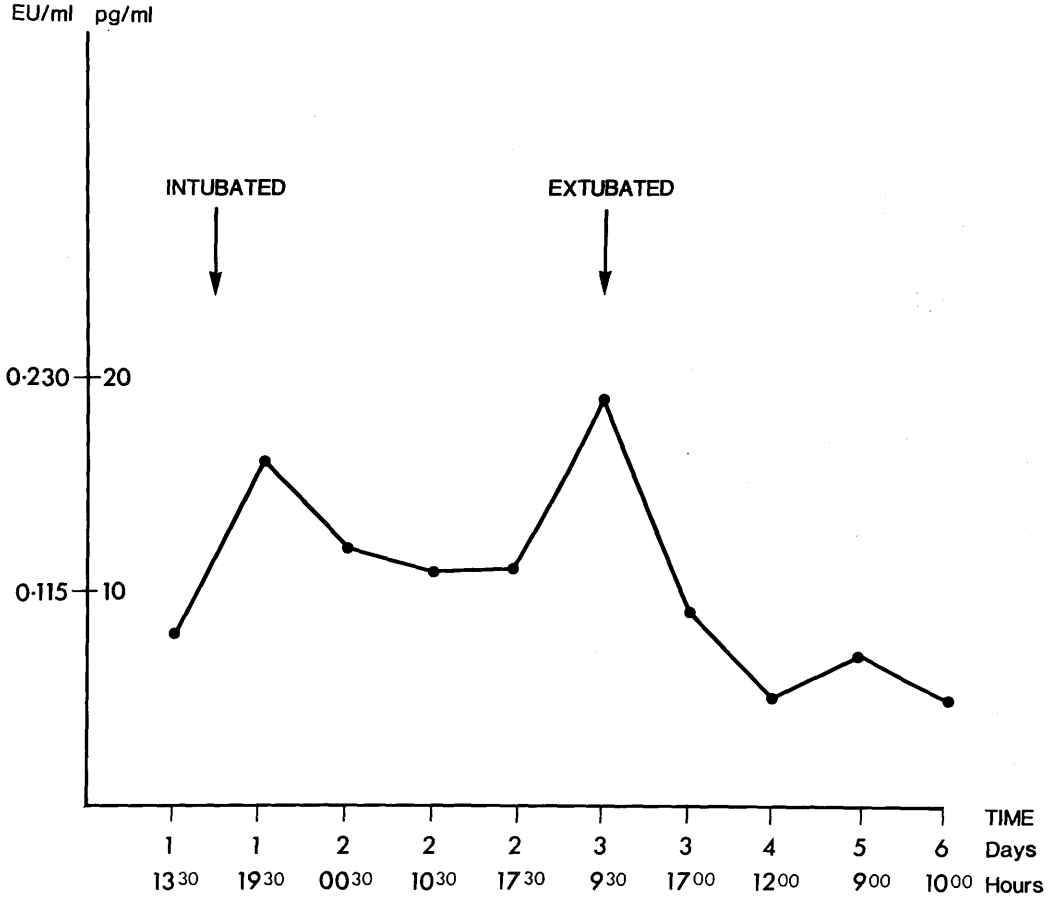
EU/ml pg/ml
INTUBATED
EXTUBATED
0·230 20
0·115 10
TIME
1 1 2 2 2 3 3 4 5 6 Days
1330 1930 0030 1030 1730 930 1700 1200 900 1000 Hours

Fig. 42

Endotoxin levels in all patients in this group, except for those of C.B., were measured in optical density (O.D.) units, due to the failure of standard endotoxin from LAL kits at the time of performing the assays.

Patient J.D., who had severe pancreatitis with related endotoxaemia, presented with abdominal pain and vomiting. Next day, he became pyrexial (to 40°C), developed rigors, hypotension and jaundice, and clinical diagnosis of cholangitis was made. Escherichia coli was grown from blood cultures. Severe acute pancreatitis was diagnosed 60 hours after the onset of pain, with serum amylase of 4400 IU/l (normal range 70-300 IU/l) and from this point endotoxin levels were monitored (Fig 41).

Fig 41. Time course of endotoxin levels in J.D., 70 year old male patient with severe acute pancreatitis.

In another patient with diagnosed severe acute pancreatitis, no significant levels of endotoxin were found. This may have been attributed to the delay of 96 hours after onset prior to the collection of samples for endotoxin assay.

Two other patients had endotoxaemia probably unrelated to pancreatitis (Figs. 42 and 43). Peaks in their endotoxin levels could be attributed to surgical manipulations (intubation and extubation).

Fig 42. Time course of endotoxin levels in C.B., 47 year old female patient. Severe acute pancreatitis (serum amylase 1360 IU/l) with rapid development of renal and respiratory failure. First endotoxin assay - 36 hours from the onset.

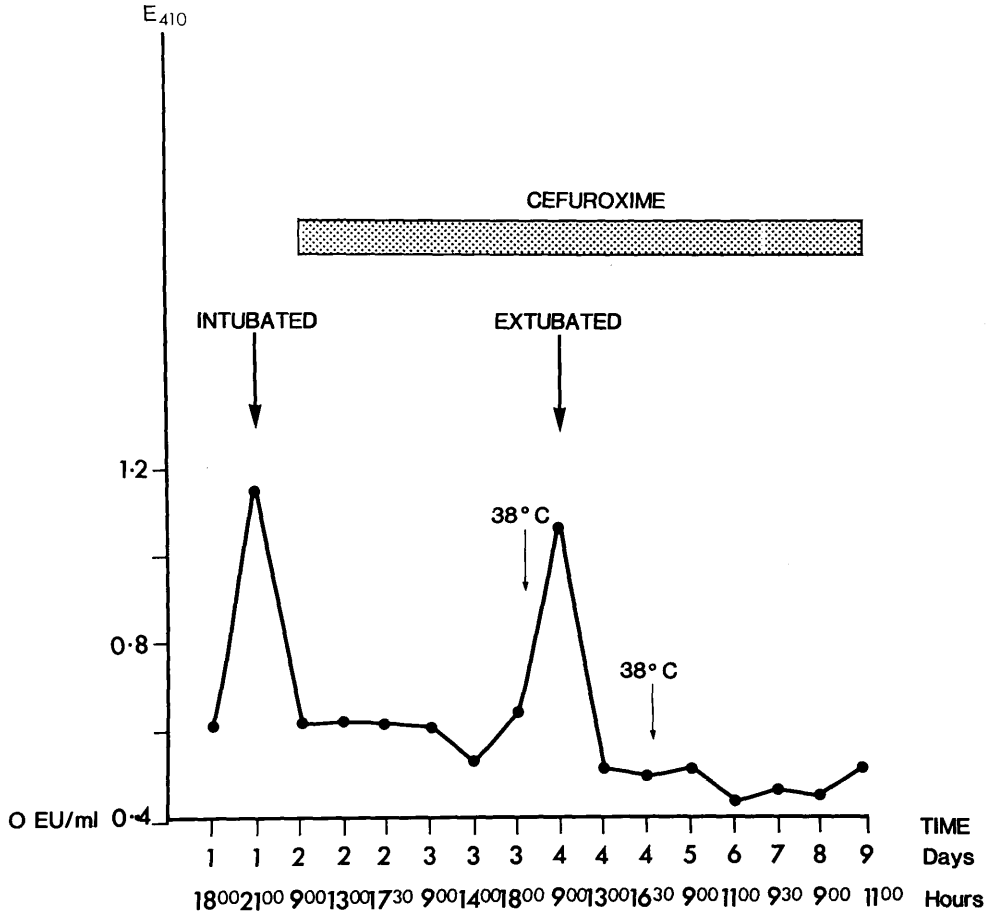
E410
CEFUROXIME
INTUBATED
EXTUBATED
1·2
38° C
0·8
38° C
O EU/ml 0·4
TIME
1 1 2 2 2 3 3 3 4 4 4 5 6 7 8 9 Days
1800 2100 900 1300 1730 900 1400 1800 900 1300 1630 900 1100 930 900 1100 Hours

Fig. 43

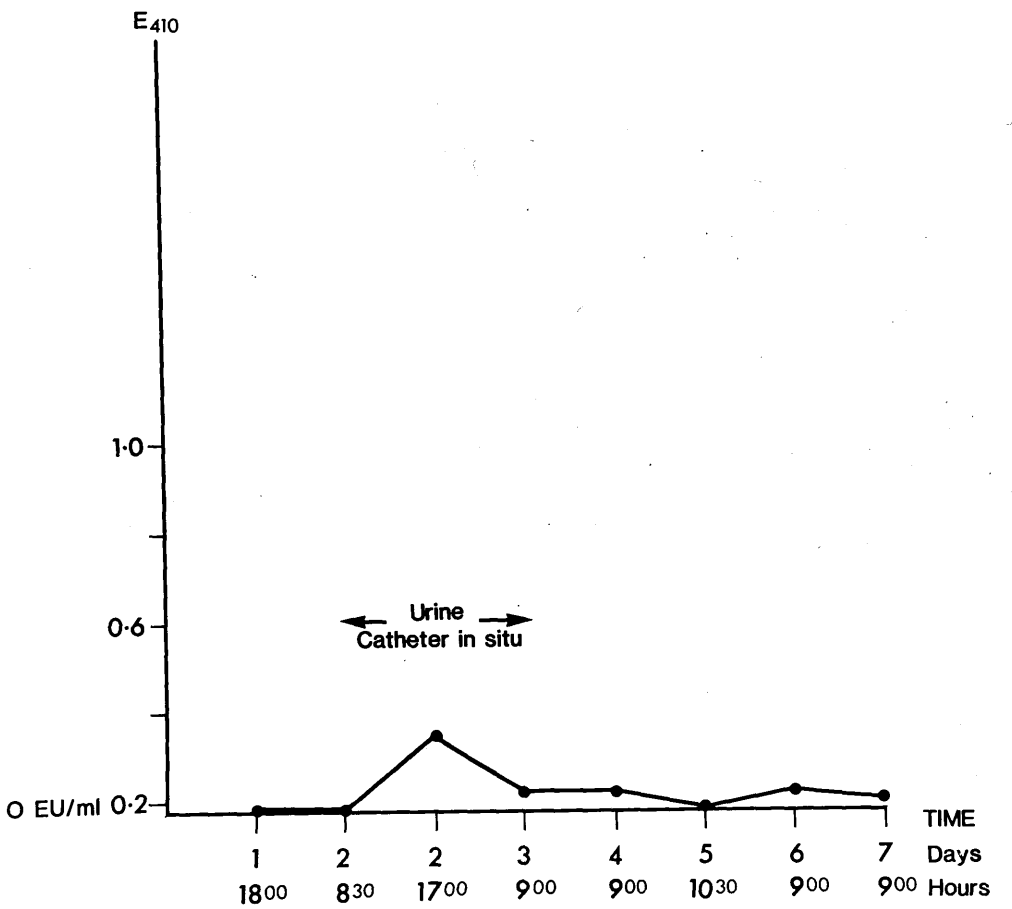
E410
1·0
0·6
Urine
Catheter in situ
O EU/ml 0·2
TIME
1 2 2 3 4 5 6 7 Days
1800 830 1700 900 900 1030 900 900 Hours

Fig. 44

Fig 43. Time course of endotoxin levels in N.C., 55 year old male patient; post mortem examination showed a perforated duodenal ulcer and broncho-pneumonia, with no evidence of acute pancreatitis. Monitoring of endotoxin commenced more than 96 hours after the onset.

Patient M.M. represents results obtained in a group of six patients with mild pancreatitis and no significant endotoxaemia (Fig 44).

Fig 44. Time course of endotoxin levels in M.M., 48 year old male patient with mild acute pancreatitis (serum amylase 1790 IU/l) of alcohol aetiology. Endotoxin monitoring started 22 hours from the onset.

3.5. Non-clinical applications of the LAL assay

3.5.1. Extra corporeal filter to remove endotoxin from human blood

The maximum rate of endotoxin extraction from human plasma, using the I-DEP filter, was 16% of a physiological endotoxin load (i.e. 250 pg/ml) over a period of 10 minutes. Thereafter, no significant endotoxin extraction was observed throughout the period of the runs (30 to 45 minutes). Both heparin and ACD (Acid Citrate Dextrose) were tested as anticoagulants in the system, and no difference in filter function was observed.

However, a significant spontaneous decrease with time in the amount of measurable endotoxin in human plasma at room temperature was noted. This phenomenon was observed in consecutive samples proximal to the filter. The fall in endotoxin levels was 36% after 15 minutes and 78% after 30 minutes respectively, and thus the mean rate of endotoxin decay was calculated to be 1.3 ng/ml/minute.

3.5.2. Baffle plate material

It was found that the concentration of extractable endotoxin in baffle plate material was 145 ng per ml of soaking solution (1667.5 EU/ml) (baffle plate material: PFW ratio = 1:4, w/w) or 580 ng/g of baffle plate material (6670 EU/g).

3.5.3. Dust sample

The sample contained extractable endotoxin activity in the LAL assay equivalent to 370 ng per gram of dust (4255 EU/g).

IV DISCUSSION

4.1 Comments on "Materials, methods and patients"

Studies by Harris and Feinstein demonstrated that certain hydrophobic, aliphatic polymers have a specific affinity for binding endotoxin (200, 201, 202). Generally, it is thought that polypropylene (as well as new glass) exhibits a high degree of adsorption of endotoxin, while polystyrene (and used glass) does not (377). Although it was shown that plastic test-tubes and plastic tips used in this study were pyrogen-free and did not bind endotoxin, for crucial stages in quantification of endotoxin (such as dilutions of standard endotoxin and storage of plasma samples), polystyrene test-tubes were used, despite their much higher cost in comparison with polypropylene ones (see "Materials, methods and patients").

Reagents for the LAL assay prepared in the laboratory (i.e. buffers), were depyrogenated with asbestos, following the experience of the Thomas group (585). An alternative, used by Tsuji and colleagues, was activated carbon (595).

Because of the dangers of handling asbestos in the laboratory, it may be useful to compare the endotoxin adsorbing properties of the two substances.

Autoclaving of plasticware used for the LAL assay, in conditions described in Chapter II ("Materials, methods and patients"), appeared to be a satisfactory means of rendering it pyrogen-free, despite the findings of Tsuji and Harrison (593). However, Tsuji and Harrison tested lipopolysaccharide inactivation only with ethylene oxide, ^{60}Co irradiation and dry-heat treatments. The present study showed that standard curves of endotoxin heated in pyrogen-free water were much lower than standard curves of unheated endotoxin in PFW (Fig 21). This decrease was time-dependent. This phenomenon may be responsible for the

satisfactory efficiency of wet-heat in depyrogenation of endotoxin-contaminated plasticware used for LAL assays, as found empirically in our laboratory.

Blood for the LAL assays was always obtained by venepuncture, despite the inconvenience and technical difficulties associated with repeated venepuncture in critically ill patients, who often present with vasoconstriction. Väisänen and colleagues reported that arterial catheters were not reliable for blood cultures and could not replace the venepuncture method (617), since they were often found to be colonized with bacteria. This makes catheters an unacceptable means of obtaining blood for LAL assays too.

Heparin, which was the anticoagulant of choice in this study, often posed a problem when it was used for blood of septic patients. It is generally postulated that the concentration of heparin of 20 units per ml of blood should not be exceeded, especially in light of evidence that heparin at concentrations as low as 1 unit per ml could be inhibitory to the LAL test (560). The results of McConnell and Cohen emphasize the importance of not exceeding this value if significant inhibition is to be avoided (310). However, in septic shock patients, many of whom present themselves with disseminated intravascular coagulation (DIC), this concentration of heparin frequently appeared not to be sufficient, and in order to prevent blood from clotting, concentrations of heparin between 30 and 40 units per ml were used. These values greatly exceed clinically relevant heparin concentrations. Clinical anticoagulation is usually achieved at concentrations not exceeding 2.5 heparin units per ml of blood (583).

In this study, platelet rich plasma was used in the LAL assay for endotoxin in blood. Although some authors did not find significant difference between platelet

poor plasma (PPP) and platelet rich plasma (PRP), in results of LAL tests (77, 104, 148, 205), it is necessary to note that these comparisons were carried out using relatively high concentrations of endotoxin. On the other hand, Das and colleagues reported that PRP improves recovery of endotoxin (97). This may be explained by the ability of endotoxin to bind selectively to platelets (97, 210, 216, 546, 631, 632). In view of this, the use of PPP (or serum) in the endotoxin assay may result in false-negative results, especially in cases where low endotoxin levels are present.

Endotoxin inhibitors present in plasma may be another reason for false-negative results of the LAL test in plasma. Strict adherence to proper blood collection and plasma preparation without any undue delay may be an important factor in obtaining reliable results in clinical studies. Delays in handling plasma samples may lead to rapid inactivation of endotoxin in plasma which has been shown to be as high as 600 ng per ml of plasma per hour at 37°C (600 ng/ml greatly exceeds the highest concentrations of endotoxin ever found in humans) (638).

The method of inactivation of endotoxin inhibitors in plasma used in this study was a combination of dilution and heating of the plasma. This procedure proved to be more effective than dilution alone, and pH shift with acetic acid reversed with dibasic potassium phosphate buffer. It was comparable only with chloroform extraction and gel filtration (108). Both of them, however, being very tedious and prone to accidental contamination with exogenous endotoxin, are unsuitable in the clinical situation. Also, Takagi and colleagues found amidase activity of serum samples against the chromogenic substrate remaining after chloroform treatment of human blood (570). However, alkali treatment with NaOH-KCl, as developed with this group, could remove the amidase activity

almost completely (370).

Later, the same group reported that a perchloric acid (PCA) treatment eliminated both nonspecific amidase activities and inhibitors from serum and plasma, with good recovery of added endotoxin (387,374).

Hakogi and Shimada proved that PCA pre-treatment of blood plasma from a range of domestic animals (cows, horses, pigs and chickens) accomplished complete removal of non-specific amidolytic activity and inhibitors, and ensured nearly 100% endotoxin recovery, irrespective of the species of animal. Thus they demonstrated the usefulness of this plasma pre-treatment method for the LAL test in the field of veterinary medicine (194).

Among the various methods for removal of plasma inhibitors and non-specific activators of the LAL test, extraction with chloroform, first developed by Levin and colleagues (292), is historically the oldest, along with the dilution method (292). Subsequently, other methods were devised: ether extraction by Niwa (370), alkali treatment by Takagi and colleagues (370), acid extraction by Reinhold and Fine (444), gel filtration by Hollander and Harding (226), and ammonium sulphate precipitation by Goto and Nakamura (178). However, dilution and heating, first reported by Cooperstock and colleagues (89), is considered by many groups as the method of choice (108, 131, 175, 385), particularly as it has been reported that heating not only inactivates inhibitors of the test, but also inactivates possible protein mimickers (175).

In this study, for the standard curve in plasma, blood was taken from normal, healthy volunteers. Samples of plasma were checked against stored "reference

plasma" which had been recognized as endotoxin-negative, and if the same level of absorbance was obtained, such blood was regarded as endotoxin-free and it was used as a reference. There is a lack of agreement among workers in this field as to whether normal humans are endotoxaemic or not and whether low-grade endotoxaemia is a physiological or pathological feature.

In early studies there was a consensus of opinion about the absence of a positive LAL reaction in the blood of healthy adults (203, 285, 291). However, the methods used at that time allowed the detection of high concentrations of endotoxin only. In 1980, DuBose and colleagues (108), upon comparison of Limulus amoebocyte lysate test results from healthy human plasma samples extracted by dilution and heating, or chloroform, concluded that lysate type and not extraction procedure was associated with previously reported questionable positive tests (109). However, more recent findings, which were obtained with much more sensitive methods, have been very confusing. Fink and colleagues reported that the LAL assay in plasma of healthy adults was negative (131), whereas the Pearson group found that a mean endotoxin level in blood of normal, healthy blood donors was between 0.02 and 1.57 pg/ml (409). A preliminary trial organized by Dr. P. Friberger from Kabi AB, Sweden, showed the level of endotoxin in normal humans to be 6 pg/ml ± 8 (396, 494).

Endotoxin (without bacteraemia) may be present in the circulation as a result of intestinal leakage of endotoxin or hepatic failure (608, 653). Low-grade endotoxaemia has been detected in portal venous blood of normal individuals, suggesting that penetration of endotoxins across the intestinal mucosa may be a physiological event (234, 435, 592). The normal, healthy liver presumably removes and detoxifies such molecules before they reach the systemic circulation (374).

Chronic systemic endotoxaemia has been documented in patients with cirrhosis and portosystemic venous shunts (294, 435, 576, 651). Fine and colleagues produced experimental evidence for the hypothesis that endotoxin may also be released from abnormally permeable bowel and absorbed into the circulation via the peritoneal cavity (92,93,573). The lymphatic route of entry was also indicated in pathological conditions (19, 96, 166, 396).

Nevertheless, endotoxin would normally be absent from the systemic circulation and it would appear there only as a pathological phenomenon. However, it is also suggested that low levels of endotoxin in the systemic circulation may be physiological, and that they take part in the normal functioning of the organism, being responsible for bone marrow stimulation and granulopoiesis (66, 333, 627). A major shortcoming of this hypothesis is the fact that a fundamental property of endotoxin is its ability to induce endotoxin tolerance in a host organism (Table 1). This biological activity of endotoxin is demonstrated as a result of repeated administration of non-lethal doses of LPS. Periodical release of endotoxin into the systemic circulation, which would be required if endotoxin were to play a role in normal granulopoiesis, would render a live organism tolerant and therefore unresponsive to all aspects of biological activities of endotoxin, including granulopoiesis. The tolerance thus induced would of course be temporary, but if endotoxin were to play such an important physiological role in normal organisms, it would have to penetrate into the peripheral blood throughout the ontogeny. In new-born babies, which have immature immunological systems, permanent tolerance could be induced in this way. Furthermore, the influence of maternal endotoxin cannot be discounted. It could penetrate through the placenta and thus be present in the foetal blood, thus

affecting their immunological systems at a still earlier stage. However, in fact, the whole range of diverse host responses of adult humans and individual human systems to endotoxin is unaffected. Indeed, they seem to be more pronounced with age; for example, the pyrogenic response to endotoxin increases with age in man (305). These are only rational premises. Experimental data is still lacking.

In order to obtain a definite answer to this question, an independent assay, as specific and sensitive as the LAL test, is required. Only such an assay would provide independent empirical data and would allow definite determination of whether low levels of endotoxin are continuously present in normal, healthy individuals. Results obtained to date, supporting the hypothesis of continuous presence of endotoxin in normal humans, may be simply a result of technical shortcomings of the methods applied. This view seems to be supported by the findings of Cohen and McConnell (77). They showed that there was a substantial variation (by more than 100%) in recovery of endotoxin added to plasma samples from six normal individuals.

In order to overcome variations in individual plasma samples ("sample-related interferences"), the Urbaschek group developed a method of sample internal standardization (104,608), which is compatible with their automated, kinetic LAL microtitre test.

In the present study, differences between samples from the same individual, taken at different times, and differences between samples from different individuals, were taken into account by:

1) setting up plasma background controls;

2) testing samples from the same individual serially, in the same experiment,

and

3) using uniform plasma for standard curves throughout blocks of experiments.

Methods of solubilization of endotoxin from baffle plate material and dust were devised according to Helme (214), and Ross and Bruch (461). These authors suggest that soaking tested material in pyrogen-free water for one hour at room temperature above 18°C can be used interchangeably with soaking it for 15 minutes at 37°C (461). However, the recommended 1:1 (w/v) proportion of tested material and PFW could not be applied, as the volumes of PFW indicated by this proportion would not cover completely the tested material. Altered volumes of PFW were taken into consideration while making calculations for concentrations of endotoxin present in tested materials.

Blood for LAL assays must be handled very carefully because of the high risk of contamination. Furthermore, it has to be processed promptly, because of the presence of fast-acting endotoxin inhibitors in plasma. Consequently, in clinical projects using the LAL assay, it is esential to have close participation of the research worker doing the LAL assays, and also to have good cooperation of all persons involved. The need for such involvement is clearly illustrated when the two major clinical projects (the septic shock trial and the pancreatitis trial) described in this thesis are compared.

In the septic shock trial, patients were chosen in a selective manner and, once included in the trial, they were monitored for purposes of this trial every four hours. In the acute pancreatitis trial, on the other hand, there were delays of up to four days from the onset of the illness before inclusion of a patient in the trial,

and blood samples were taken twice daily at the most. This frequency very often may be insufficient and may result in missing periods of endotoxaemia or, at the very best, missing endotoxin peaks. This may be illustrated by the example of patient B.L. from the septic shock trial (Fig 27). This patient's endotoxin levels changed dramatically within eight hours and became unremarkable within the first 32 hours. Yet another result of delay is that patients who are included in the trial are receiving treatment which may affect endotoxin levels, thus making interpretation of results very difficult or even impossible.

Three assay controls (see: "Materials, methods and patients", section 2.5. LAL assay) were always set up with each assay, for the following two reasons. Firstly, it was found that the performance of individual lots of LAL was significantly different. This difference was established by the reagent control. Secondly, considerable differences were often found in the optical properties of human plasma from different individuals (especially patients undergoing intensive treatment), or from the same individual when samples were taken at different times. In cases where patient plasma background control differed from the standard curve background control, the optical density value for a patient plasma background control was subtracted from the value obtained for the patient's plasma in the LAL test.

The finding that the reaction between LAL and endotoxin is in some way amplified in plasma extract, when compared with results obtained in pyrogen-free water (Figs 14 and 19), was confirmed by the results of some workers (104, 205), but contradicted by others (77, 148). Urbaschek and colleagues found that differences in amplification for each sample in plasma occurred to such an extent, that a single comparison to a common plasma standard curve was impossible (104).

This observation may be inherent to their method of assaying endotoxin, which was a kinetic turbidimetric test. In the present study, which used an end-point chromogenic assay and test controls, as described in "Materials, methods and patients", such a wide variability in the degree of enhancement of LAL assay results in normal plasma was never observed.

4.2. Discussion of results

4.2.1. Two-stage assay

The sensitivity of this method was below 1pg of endotoxin/ml of pyrogen-free water (0.012 EU/ml) and the standard curve was linear within the range of 0-10 pg of endotoxin/ml PFW (0-0.115 EU/ml) (Fig.18). For comparison, the manufacturers of QCL-1000 kits claimed a sensitivity of 10 pg of endotoxin/ml PFW (0.115 EU/ml), with the standard curve linear in the concentration range 0-100 pg/ml (0-1.150 EU/ml) (M.A. Bioproduct's brochure "QCL-1000"). In addition to increased sensitivity in this two-stage assay method, the reagent volumes were reduced by half, thus making the assay more economical. A disadvantage of this assay method over the manufacturer's method was a three-fold increase in incubation time. However, this was compensated for by using a microtitre plate for measuring absorbance of samples.

4.2.2. One-stage assay

In this modification, the mixture of LAL and chromogenic substrate was added to a tested sample. This increased the sensitivity of the LAL assay to 0.2 pg of endotoxin/ml PFW (0.002 EU/ml), in comparison with a threshold of sensitivity of 1 pg/ml (0.012 EU/ml) obtained in a two-stage method. This improvement was achieved despite the fact that the reaction was performed in conditions which

were not optimal for each of the reagents. The optimal conditions are: for the activation of proclotting enzyme pH 6.0–7.5 and relatively high ionic strength, and for a chromogenic substrate pH > 8.0 with low ionic strength (see: "Introduction", sub-section 1.2.3. Limulus amoebocyte lysate assay for endotoxin). However, the enhancement of activity of both reagents, when mixed together, clearly more than compensates for the loss of optimal conditions for both of them. This modification also allowed a reduction of two thirds in the volumes of expensive reagents (Table 7). As compared with the two-stage assay method, the incubation time was slightly longer (Table 7). However, the one-stage procedure minimized the risk of contamination and reduced the time required for processing large numbers of samples.

There is scope for further miniaturization of the assay. However, volumes would be too small for the present method, which essentially is a test-tube one, and the entire assay would have to be performed in microtitre plates. This development would also reduce considerably the time of processing samples. The use of multi-channel pipettes in the assay would be a further technical improvement. However, such developments would necessitate the use of expensive specialized equipment, such as a laminar air-flow cabinet (to counteract the increased risk of contamination), a heating block and a cold tray for microtitre plates. The advantage of the present method is that it requires no such equipment and could be performed, if necessary, at the patient's bedside, or in camp conditions, which has already drawn the attention of a WHO specialist, Dr. Podoprigora (personal communication).

The endotoxin levels in this study were converted from picograms into endotoxin units and expressed in both. It is thought that the conversion from weight

amounts of endotoxins to endotoxin units may help to overcome the great variability of activities of different endotoxin preparations and batches on a weight basis (607). The reason for this variability is that endotoxins from different Gram-negative bacteria, and even the same endotoxins, extracted by different methods from the parent bacteria, differ in their activity in the LAL assay (132, 137, 228, 414, 496, 636). Furthermore, the activity of endotoxins in the assay depends on their degree of purification. Thus, the potency of "environmental" or "natural" endotoxins, although varied, is very low in comparison with purified endotoxins (406, 413).

It is also possible that the activity of "free endotoxin", which is released from cells during normal, healthy growth, may differ in the LAL test, when compared to the activity of endotoxin released by cell lysis, although there is some evidence to contradict this (252). Therefore, to allow comparison of results of this study with those obtained by others and to comply with the FDA recommendations and appeals by workers in the field (132, 287), Endotoxin Units (EU) have been adopted to express endotoxin activity in the LAL assay. However, due to problems with LAL kits (see "Results", 3.5.2. Septic shock trial, and 3.5.3. Acute pancreatitis - preliminary trial), in some cases endotoxin activity in samples could only be asessed by measuring extinction of these samples, and was expressed in Optical Density (O.D.) units, which represent relative levels of endotoxin.

4.2.3. Effect of heat on endotoxin in plasma and in pyrogen-free water

For this study, dilution and heating were employed as the method of choice for removal of inhibitors and non-specific activators present in plasma. When different times and temperatures of heating were compared, it appeared that there was no difference between heating of plasma at 100°C for 10 minutes and for

1 minute (Fig 20). Therefore, the latter mode of heating plasma samples was chosen for the LAL assay for time-saving reasons. It was also shown that the standard curve, after heating plasma for 10 minutes at 56°C, was completely flat (Fig 20). This observation is supported by the findings of Cundell and colleagues, that pasteurization of plasma proteins reduces detectable endotoxin concentrations (95). As it is believed that heating to 60°C does not destroy endotoxin, one possible explanation is that, at temperatures up to 60°C, endotoxin inhibitors in plasma are not inactivated and they may be responsible for the disappearance of endotoxin from tested samples. A decrease in levels of endotoxin heated in pyrogen-free water was shown to be time-dependent (Fig 21). This finding is very surprising because of the known resistance of endotoxin to physical factors (593). Aggregation of lipopolysaccharide molecules in heated PFW may be a possible explanation. In water, LPS exists as large aggregates (368) and heat may encourage further aggregation. This endotoxin aggregation may be prevented in platelet-rich plasma, where endotoxin is bound to various blood components, such as platelets (632), specific antibodies and, possibly, fibronectin (44). This may be responsible also for amplified reaction between LAL and plasma endotoxin in comparison with endotoxin in PFW, as, even after the heating stage of the extraction procedure, plasma endotoxin, being bound to soluble plasma components, may be prevented from aggregation and thus may exhibit much higher activity in the reaction with LAL than aggregated endotoxin in water.

4.2.4. Comparison of three commercial kits for the chromogenic LAL assay

In this study, the method used to test the Coatest Endotoxin kits and QCL-1000 kits was our modification of the manufacturer's recommended method. This modification optimized the assay conditions. The one-stage assay procedure for high concentrations of endotoxin was applied to both kits to make the

modification comparable with the method recommended for the Mallinckrodt kits.

Comparison of all three kits showed poor performance of the Mallinckrodt kit (Fig. 22). The difference in performance of different lots of LAL from M A. Bioproducts (Fig. 23) indicates that there is a high batch to batch variability of the LAL reagent. However, the standard curve which is performed with every series of samples tested, renders the effects of this variability negligible. The observed differences in the LAL batches from M. A. Bioproducts suggest that the differences in performance noted between kits manufactured by M. A. Bioproducts and Kabi Diagnostica may not be significant.

The findings of this study, which showed considerable variability among the currently available chromogenic LAL kits, are supported by the results of Twohy et al. (602), who demonstrated similar variations in performance of LAL from different manufacturers in the gel-clot test.

4.2.5. Clinical applications of the LAL assay

Cyclic neutropenia

Cyclic neutropenia is a rare haematological disorder, characterized by the disappearance of neutrophils from the blood and bone marrow at regular intervals. In most cases, these neutropenic cycles occur every 21 days, although cases of cycles occurring every 15 to 35 days have been reported (399, 654). Between cycles, the neutrophil count rises, but seldom exceeds 50% of the differential count. In some patients, a compensatory monocytosis and/or eosinophilia permits the total white blood cell count to remain at a low normal level during the neutropenic cycles. This compensatory monocytosis during

periods of neutropenia is thought to be responsible for the fact that cyclic neutropenia, when compared with congenital neutropenia, leads less frequently to life-threatening diseases (213).

However, in other patients, the white blood cell count may fall to low levels during the neutropenic cycle and rise to near normal levels between cycles. The neutropenic phase of each cycle is characteristically associated with clinical symptoms, such as fever, malaise, chills, anorexia and ulcers of the oral mucous membrane. Additional symptoms in some patients include: intermittent arthralgia, abdominal pain, sore throat, lymphadenitis, ischiorectal infections, mental depression, conjunctivitis and cutaneous ulcers.

The clinical manifestations usually appear one to three days prior to any changes in the differential blood count and may persist from three or four days up to 10 days. The patient is generally healthy between the periods of neutropenia (543). It is thought that endotoxin may be responsible for fever and malaise in the neutropenic phase of the cycle. Thus Greenberg et al. demonstrated that the presence of systemic endotoxaemia or staphylococcal abscesses were associated with blood neutrophil nadirs, monocyte elevations, and peak urinary Colony-Stimulating Factor (C S F.) levels. In their study, they also observed the absence of endotoxaemia during four neutrophil nadirs. They attributed it to the patient having received oral antibiotics during these periods (184).

Such treatment decreases endotoxaemia in experimental animals by diminishing the content of Gram-negative organisms in the bowel (60). In a human study, selective decontamination with polymyxin to remove Enterobacteriaceae from the digestive tract, did cause a significant decrease in the faecal endotoxin

concentration in immunocompromised patients (624).

In our study, we failed to demonstrate any correlation between endotoxin levels, body temperature and neutrophil counts (Fig 24). This is probably a result of shortcomings in the design of this study. The patient was monitored with insufficient frequency, i.e. at intervals of a few days.

It is suggested that in future studies, relevant parameters should be measured daily, and even more frequently around the time of expected neutrophil nadir, i.e. at four-hourly intervals.

A rise in patient's temperature is an unsatisfactory indication for more frequent monitoring of endotoxin levels. This is because there is a latency period of a few hours between the appearance of endotoxin in the bloodstream and the release of the endogenous pyrogen (Leukocytic Endogenous Mediator, LEM), which mediates fever by direct action on the thermoregulatory centres in the hypothalamus.

With the "ephemeral" character of endotoxin in blood, there may not be any endotoxin detectable in the systemic circulation at the time when the body temperature rises.

If gut-derived endotoxin appears to play a significant role in clinical manifestations associated with the neutrophil nadir, it may prove beneficial for patients with cyclic neutropenia to undergo treatment with an oral endotoxin adsorbent prior to and during the neutrophil nadir.

Septic shock trial

The results of LAL assays of blood in a group of 10 critically ill, but not necessarily septic shock, patients were disappointing. However, there was a good correlation between significant levels of endotoxin, positive blood cultures and the clinical course in one patient, R.O. (Fig 25). Episodes of endotoxaemia after surgery, such as seen in this patient, have also been reported by Grundmann and Papavasiliou (191).

However, the results from this group of patients did suggest that more frequent monitoring of patients in septic shock could provide information on the possible relationship of endotoxin levels and severity of septic shock. This proved to be the right course, as best confirmed by the results obtained from patient B.L. (Fig.27). If this patient had been monitored daily, her septic episode would probably have been missed . However, there is a striking feature in this case of septic shock. A Gram-positive organism, Staphylococcus aureus, was isolated from six out of 10 blood cultures from this patient during the period corresponding with endotoxaemia. Gram-negative organisms were never isolated from this patient's blood cultures. Similarly, Harris and colleagues, in two of their patients with Gram-positive bacteraemia, also found systemic endotoxaemia (204). This may be explained in two ways. Firstly, this patient's Gram-positive septicaemia could have caused abnormal permeability of the gut. Endotoxin detected in the LAL assay would in this case be of intestinal origin. Secondly, this patient could have had an undetected septic focus outside the bloodstream. Endotoxin released by bacteria from this source could have been a reason for the endotoxaemia in this patient. According to Levin (285, 287), the presence of endotoxin in blood is not usually a direct consequence of the bacteria which are in the circulation at the time the sample is obtained, but more commonly is due to bacteria outside the

bloodstream, which have generated endotoxin which subsequently gains access to the circulation.

In this patient, there was a close temporal relationship between levels of endotoxin, and cardiovascular and respiratory indices (Fig.27). There was also an inverse correlation between plasma fibronectin and endotoxin levels (Fig.28). When the endotoxin was eliminated from the blood, recovery of plasma fibronectin to within the normal range was observed.

Patient A.C. had the highest endotoxin levels recorded in our septic shock patients (Fig.30), approximately 10 times higher than in other patients who presented with septic shock. His plasma fibronectin levels, although very low, correlated well (inversely) with high endotoxin levels. However, the corresponding pulmonary shunt and alveolar arterial oxygen difference were only moderately elevated. Despite the fact that the cardiovascular and respiratory indices of this patient were not high, and despite intensive therapy at ITU, he died nine hours after admission.

The hypothesis may be postulated that this patient might have died as a result of direct toxic action of endotoxin, while the condition of patient B.L. was caused by the indirect action of endotoxin. The septic shock in patient B.L. could have been caused by host mediators of shock, i.e. various enzymatic systems which were activated by endotoxin present in the circulation (94,336,567).

It also seems that patients at the highest risk of developing septic shock may be hypersensitive to endotoxin. Their hypersensitivity may be a result of their clinical condition or the treatment which they receive (see "Introduction", section

1.1.3. Biological activity). Fever may be yet another important factor which increases patients' sensitivity to endotoxin. It has been shown to decrease the clearance of certain agents (42) and may also affect the clearance of endotoxin (116). Also, the presence of endotoxin in the gastrointestinal tract (as a part of the normal intestinal flora) apparently seems to increase sensitivity to endotoxin in the bloodstream. Experimental data show that circulating endotoxin may mobilize additional endotoxin of gut origin. Thus endotoxaemia could become a self-sustaining process (60, 92).

This hypersensitivity to endotoxin of some individuals in certain conditions could be responsible for catheter fever in cases where endotoxin was found to contaminate catheters (170, 272, 446, 591). However, Garibaldi and colleagues reported that in patients with indwelling urinary catheters and no severe underlying disease, positive LAL tests were not accompanied by any clinical signs of endotoxaemia. They demonstrated, however, an excellent correlation between the presence of Gram-negative urinary tract infection in these patients and positive LAL tests (169). Similarly, Sim and McCartney, and Kiss et al, found that transient endotoxaemia in "normal" individuals, following urethral instrumentation (524) and colonoscopy (263), was not accompanied by any clinical manifestations of endotoxaemia, including fever.

This may be explained by rapid clearance of bolus-dose endotoxin from the bloodstream of normal, healthy individuals. The speed of this clearance may prevent endotoxin from triggering various systems of host mediators, including endogenous pyrogen, which is directly responsible for febrile reactions. Also, probably relatively low doses of endotoxin are involved in cases of post-instrumentation endotoxaemia without fever, and this contributes to the

lack of patient response to endotoxin present in their circulation.

For understandable reasons, there is little experimental data on the speed of endotoxin clearance in humans. However, Greisman and colleagues found that the half-time of clearance of small quantities of Pseudomonas sp. endotoxin in human volunteers was 8.2 ± 0.63 minutes when labelled with $^{51}CrCl_3$ and 8.2 ± 1.3 minutes when labelled with $Na_2{}^{51}CrO_4$ (186).

Experiments on animals provide much more data on the speed at which endotoxin is removed from the circulation. Herring and colleagues and the Carey group found that, after intravenous injection into rabbits of sublethal doses (0.006 - 0.100 mg) of E. coli endotoxin, 90% of circulating radioactivity is removed within five minutes and 60% appears in the liver within 15 minutes (51, 216). These observations were supported by Beeson and Atkins, who reported that endotoxin rapidly disappears from the bloodstream, so that little pyrogenic activity can be detected in rabbit serum 8-10 minutes after intravenous injection of endotoxin (17, 25). After intravenous injection into rabbits of large lethal doses (5.0 mg) of radiolabelled E. coli endotoxin, only 30-50% of radioactivity is removed from the blood during the first 15 minutes, 30-50% may be found there two hours later, and 20% still circulates after five hours (51).

Hepatic uptake of radioactivity in rabbits injected with 5.0 mg of E coli endotoxin is proportionally less than with sublethal doses, so that only 20% is found in the liver after 15 minutes (51). A similar difference in distribution of radioactivity of lethal (0.75 mg) and sublethal (0.01 mg) doses has been noted in mice (51). Livers of normal mice trap in one hour more than 80% of LPS injected intravenously (628).

In mongrel dogs, 99.8 to 99.9% of endotoxin administered intravenously at doses of 5, 50 and 500 µg/kg disappeared from the circulation within five minutes. For 5 and 50 µg/kg there followed a slower clearance rate, with a mean half life of 21.4 and 10.3 minutes respectively. When 500 µg/kg was administered, rapid clearance was shown till 30 minutes. Then, from 30 minutes to three hours, it was slowly cleared, but after three hours there were no remarkable changes and approximately 50 to 500 pg/ml of endotoxin were still found after 12 hours (361). It should be noted here that, according to Berczi et al., a lethal dose of LPS for dogs is 4.0 mg/kg of body weight (31).

There is also a possibility that, in some individuals, hypersensitivity to endotoxin is determined genetically and is expressed as an excessive number of endotoxin receptors. McCuskey and colleagues established that the livers of an endotoxin-resistant murine strain C3H/HeJ contained 60% fewer phagocytizing Kupffer cells, and that these cells contained much less lysosomal enzymes, in comparison with endotoxin-sensitive C3HeB/FeJ mice (312). The authors' conclusion was that their results suggested that the insensitivity of C3H/HeJ mice to endotoxin might be related in part to a lysosomal enzyme deficiency and a paucity of phagocytic Kupffer cells in these animals. However, impaired phagocytosis in endotoxin-resistant mice seems to be a secondary phenomenon, rather than a cause of resistance. Impaired phagocytosis of endotoxin would result in prolonged circulation of endotoxin in the bloodstream. This, in normal conditions, could lead to a much higher activation of various endotoxin mediators, as compared to that in endotoxin-sensitive mice.

However, a hypothesis, that endotoxin-resistant mice lack endotoxin receptors, and that this deficiency is genetically determined, could satisfactorily explain

their resistance. As a result of the lack of active, specific receptors, endotoxin would not be able to attach to the cell surface and thus would not be able to mediate the release of various enzymatic systems. These systems are probably responsible for secondary biological effects of endotoxin (i.e. through host mediators as opposed to direct toxicity). However, this hypothesis cannot be supported by any experimental evidence, because so far very little is known about receptors for endotoxin. The present state of knowledge is reviewed by Bradley (50).

It should also be taken into account that our understanding of the biological effects of endotoxin is based on the administration of endotoxin as a bolus, rather than as a continuous release in small amounts over a period of hours or days, as probably occurs during an infection (36). Unfortunately, the bolus mode of administration is favoured by many workers, and in nearly all animal models endotoxin is given in a single dose. Results thus obtained may be of very little value for interpretation of the clinical situation.

However, it seems likely that when a bolus dose of endotoxin is administered to healthy individuals with normal sensitivity to endotoxin and unimpaired clearance of endotoxin, endotoxin is cleared so rapidly from the bloodstream that the whole system of mediators remains unaffected. In contrast, there are very sick patients, compromised by illness and/or treatment, who may be genetically hypersensitive to endotoxin or who may be rendered hypersensitive, and in whom there may be a continuous flow of endotoxin from the source of infection into the bloodstream. In these patients the clearance of endotoxin may be significantly impaired. All these factors promote the activation of the cascade of host mediators of response to endotoxin, and as a result the patient may die from

secondary effects of endotoxin. This may also explain the phenomenon which is sometimes observed in septic shock patients; they may die apparently in septic shock, depite the fact that the level of endotoxin at the time of their death may be quite low. However, in such patients, a peak of endotoxin might have occurred some time earlier and may even have gone undetected. The clinical picture of such a patient at death would be that of low endotoxin levels and high cardiovascular and respiratory indices, as observed in patient A.McD. (Fig 31). It is likely that in this patient, endotoxin levels were elevated prior to his admission to ITU some 48 hours after the onset of shock. However, his endotoxin levels were unremarkable during his stay in ITU, despite grossly elevated pulmonary shunt and alveolar arterial oxygen difference.

Conclusions at present suggest that there are probably no "clinically significant" levels of endotoxin in critically ill patients. Their sensitivity to endotoxin may depend on clinical factors and their genetic make-up. This would imply a wide range of sensitivity to endotoxin.

It is probably the sensitivity of the patient to endotoxin which determines the patient's response. Septic shock would then be one of the ways in which the individual may respond to the presence of endotoxin in his bloodstream.

The degree of hypersensitivity to endotoxin could determine the extent of the organism response to endotoxin by releasing the appropriate amount of mediators of septic shock. In this way, septic shock would depend on endotoxin only indirectly, and the severity of septic shock could depend on factors other than endotoxin levels present in the patient's bloodstream. This hypothesis, if proven, could have profound implications for the development of new ways of

treatment of patients with Gram-negative septic shock. There could be a possibility of blocking endotoxin receptors in such patients (with endotoxin analogues, for example), thus preventing the release of mediators of the host response to endotoxin.

The way in which endotoxin gets into the blood-stream is probably another important factor which may decide that the response of the individual to endotoxin takes the form of septic shock. Continuous levels of endotoxin in blood may not be high enough to cause death directly as a result of endotoxin toxicity, but they may be sufficient to trigger irreversibly a cascade of mediators of septic shock.

From the above discussion, it seems unlikely that assays for endotoxin will become a routine clinical test which could, in an unequivocal manner, determine the patient's diagnosis and/or prognosis. This is because the presence of endotoxin in the bloodstream seems to be a requisite but insufficient condition for the pathogenesis of septic shock. However, the question of the value of the LAL assay as a clinical test requires further investigation, as recently Schreibman and colleagues found the Limulus lysate index valuable in the early prediction of fatal sepsis (488).

Nevertheless, the LAL assay for endotoxin can be useful in clinical research on endotoxaemia and septic shock. This study may be regarded only as a pilot one, which leaves many questions unanswered, and indeed seems to pose many new questions. It is proposed that in future studies endotoxin assays should be carried out concurrently with assays for fibronectin.

Little work has been done so far to show the relationship between endotoxin and fibronectin levels in different groups of patients (551). The present study seems to confirm that there is good inverse correlation between them. The fibronectin assay could be a helpful auxiliary assay in studies on endotoxaemia, especially since the LAL assay is very prone to accidental contamination. Concomitant fibronectin assays could therefore help in excluding false-positive results.

Furthermore, even a sensitive assay for endotoxin, such as that described in this study, has a certain limit of sensitivity. This allows endotoxin in some samples to escape undetected.

The amount of endotoxin contained in a single Escherichia coli bacterium is 40 femtograms per intact cell (378, 634), which is well below the threshold of sensitivity of any known assay for endotoxin. However, one viable bacterium may ensure a positive blood culture. This may lead to an apparent paradox when blood cultures are positive due to a Gram-negative organism, and concomitant endotoxin assays are negative. In such a case fibronectin levels would be expected to be decreased, which if confirmed would lend greater credibility to some results of LAL assays.

A proportion of septic shock episodes are due to Gram-positive pathogens. Clinically, Gram-positive septic shock is indistinguishable from Gram-negative septic shock (198, 279, 512). Gram-positive septicaemia could in some cases be accompanied by endotoxaemia due to endotoxin of intestinal origin. Nevertheless, in Gram-positive septicaemia, fibronectin levels would be expected to be low. In such cases the fibronectin assay could be used as an aid in interpreting endotoxin results.

Endotoxaemia without concomitant Gram-negative bacteraemia is also probably a common result of appropriate antibiotic treatment of Gram-negative sepsis. It has been demonstrated that antibiotics can induce the release of endotoxin in vitro (10) and in experimental models in vivo (241, 510). Although unproven, the propensity of an antibiotic to liberate significant amounts of endotoxin from Gram-negative bacteria into the bloodstream is potentially of pivotal clinical relevance. The possibility that the use of antibiotics for Gram-negative bacteraemia can actually cause endotoxaemia and greatly increased mortality in thus-treated patients has been a serious consideration for many years now (58, 59, 165, 402, 443, 498, 544, 630). Two more recent studies suggested that bactericidal antibiotics were responsible for the release of large amounts of endotoxin, whereas bacteriostatic antibiotics did not appear to liberate significant quantities of enditoxin (179, 509). Furthermore, Shenep and colleagues demonstrated significant differences in the propensity of dissimilar classes of bactericidal antibiotics to liberate endotoxin, independently of the rate of bacterial killing (509). Monitoring fibronectin levels could also be useful in cases of endotoxaemia which occur as a result of antibiotic treatment and which may lead to septic shock, despite negative results of microbiological investigations.

The final argument for monitoring fibronectin levels concomitantly with endotoxin is that the detectable endotoxaemia seems to be present only during the relatively short, initial stages of development of septic shock (Fig 26). As a result, it often may be missed, while decreased levels of fibronectin may persist for a longer period. This is what might actually have happened to patient A. Mc D. (Fig 31). He had undergone intensive antibiotic therapy for 48 hours prior to his admission to ITU. This therapy by itself could have been responsible for endotoxaemia, leading to endotoxic shock, although his endotoxin levels were not

remarkable at the time of admission to ITU. On the other hand, it cannot be excluded that antibiotic treatment itself did not have adverse effects on this patient, but endotoxin which was present in the blood during the period of bacteraemia caused irreversible damage by triggering systems of mediators.

Patients P.H., J.W. and J.M. had moderate septic shock. In P.H., septic shock was of Gram-positive origin, which could explain his unremarkable endotoxin levels and moderately depressed fibronectin levels. In any case, his pulmonary shunt and alveolar-arterial oxygen differences were low (Fig 33). However, patients J.W. and J.M., although their septic status was similar to that of P.H., had moderately elevated cardiovascular and respiratory indices (Figs. 35 and 36). Their endotoxin levels at the beginning were higher than those of P.H.

Acute pancreatitis - preliminary trial

Patient J.D. (Fig 41) seemed to have endotoxaemia related to his severe pancreatitis. However, monitoring of his endotoxin levels started only 60 hours from the onset of clinical symptoms and furthermore the patient had been commenced on antibiotics two days prior to the start of endotoxin sampling. These factors make any definite conclusions very difficult. However, mild pyrexia on day four may be related to transient increase of endotoxin levels in this patient (Fig 41). Also, testing of his peritoneal fluid for endotoxin in parallel with his blood was not done. This could have provided additional valuable information on the role of endotoxin in pathogenesis of acute pancreatitis. Technically, obtaining peritoneal fluid from patients with severe acute pancreatitis does not present any difficulty in cases where these patients are commenced on peritoneal lavage on medical grounds, as many such patients are.

In another patient with severe acute pancreatitis, no significant levels of endotoxin were found, but this could be attributed to the delayed commencement of monitoring his blood for endotoxin. This delay was 96 hours from the onset.

Patients C.B. and N.C. had endotoxaemia unrelated to pancreatitis (Figs. 42 and 43). Peaks in their endotoxin levels could be attributed to surgical manipulations - intubation and extubation - which were carried out during the course of their treatment. It is interesting to notice that the rise in endotoxin levels in these patients was not accompanied by fever. Although, in patient N.C., a rise in temperature to 38°C was observed, it preceded the extubation of this patient and concomitant rise in endotoxin level in one instance, and in another instance, no parallel increase in endotoxin levels was observed.

In patient M.M., who had mild pancreatitis, no remarkable levels of endotoxin were observed. However, endotoxin levels were slightly elevated on day two, which could probably be attributed to the indwelling urinary catheter (Fig 44).

On the basis of the present preliminary trial, it seems that endotoxin may play some role in acute pancreatitis, but it is difficult to assess the extent of its role in the pathogenesis of this condition, and also its potential value in diagnosis and prognosis of acute pancreatitis. In conclusion, for future studies frequent monitoring of endotoxin levels from the very onset of acute pancreatitis is proposed, combined with measuring of endotoxin levels in peritoneal fluid, wherever feasible.

4.2.5. Non-clinical applications of the LAL assay

Extra-corporeal filter to remove endotoxin from human blood

The obtained results clearly demonstrated that the I-DEP filter is not capable of extracting endotoxin from human plasma in amounts which could be useful clinically. These results are contradictory to those obtained by Gerba and colleagues, which showed that the I-DEP filter would extract endotoxin from human plasma (171). However, the results obtained in the present study suggest that spontaneous extracorporeal endotoxin decay may present as efficient a method of endotoxin removal from human blood as any known extraction system.

Thus, further work was planned, which could also widen our knowledge about the kinetics of endotoxin decay in normal human plasma and in septic plasma. It was proposed to test the rate of decay of exogenous endotoxin in plasma from 20 normal volunteers at 0°C and 37°C. The results thus obtained could be compared with the kinetics of decay of native endotoxin in plasma from Gram-negative septic patients, and with the kinetics of endotoxin decay in plasma from Gram-negative septic patients after the addition of exogenous endotoxin. It was also planned to test the rate of decay of exogenous endotoxin in plasma of patients with Gram-positive sepsis in order to establish whether the kinetics of endotoxin decay in Gram-negative septic patients was peculiar to this condition or whether it was similar in other comparable conditions.

It could be expected that the rate of endotoxin decay in septic patients is severely impaired. If so, this could be one of the factors responsible for the development of septic shock. A further project on the kinetics of endotoxin decay in plasma from the same patient at different stages of sepsis or septic shock (in a group of septic patients) was also planned.

Alternative means of removal of endotoxin from the blood of severely ill patients with endotoxaemia also seem to warrant urgent investigation. It is certainly possible to remove endotoxin from circulating blood by perfusion through charcoal or resins (416), or by passage over membranes, to which anti-endotoxin antibody has been attached (156). However, the clinical usefulness of these models requires further investigation. Also, Detoxi-Gel (endotoxin removing gel) manufactured by the Pierce Chemical Company (Rockford, Illinois 61105, USA) may prove to be a viable means of endotoxin removal from human plasma. Its formula (which cannot be revealed due to a binding Secrecy Agreement) suggests that Detoxi-Gel can be efficient and, what is more, applicable in the clinical situation for extracorporeal removal of endotoxin from patients' plasma.

Endotoxin can also be removed by ultrafiltration (599). However, while ultrafiltration techniques work well with some solutions which contain only small molecules, they are not suitable for plasma. A further means of removing contaminating endotoxin from biological preparations is by absorption with the Limulus amoebocyte lysate (40, 448). However, this method, if applied on a large scale, would be unacceptably expensive and is clearly unsuitable clinically.

It has been suggested by different authors (see above) that, in many cases, endotoxin in clinical endotoxaemia could be of intestinal origin. Therefore, Wellman and colleagues employed a modified method of whole gut irrigation as antiendotoxaemia therapy to treat patients, suffering from inflammatory bowel disease with marked endotoxaemia, and who were unresponsive to conservative treatment (640). Following the lavage, the condition of all the patients improved. This was accompanied by a rapid decrease in endotoxin levels. At the same time, serum iron increased and in all patients who had been persistently pyrexial prior

to irrigation, the body temperature returned to normal by the day after lavage and did not increase therafter. It should be noted here that decreased levels of serum iron and increased body temperature seen in these patients could be attributed to endotoxaemia.

Another approach to endotoxaemia of intestinal origin was employed by the Urbaschek group (609) and by Shimanko and colleagues (511). In their laboratory model, Urbaschek et al. found that Bentonite, Kaopectate and Kaolin administered per os were able to prevent endotoxaemia completely. Similarly, Shimanko et al. found that, after oral administration of Enterodes and Enterosorb, an improvement in the clinical condition of patients with severe endotoxicosis was observed.

The mechanism of action of the above preparations lies in their ability to bind toxins in the gut. They may be useful on a wider scale in the prevention and treatment of clinical endotoxaemia.

Baffle plate material and dust sample.

In view of the fact that both baffle plate material and dust sample were found to contain relatively high amounts of endotoxin, and that respiratory hypersensitivity reactions to airborne endotoxin are known to occur (see "Introduction", subsection 1.2.3. Limulus amoebocyte lysate assay for endotoxin. Applications of the LAL assay. Non-clinical applications), endotoxin may be at least one of the factors responsible for the symptoms reported by the semiconductor factory workers and the archives librarian (see "Materials, methods and patients").

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