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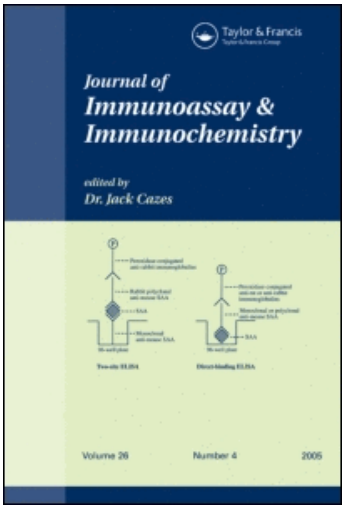

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The Detection of Pyrogens in Sera from Patients with Symptoms of Sepsis Using an *Ex Vivo* Whole Blood Culture Assay

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THE DETECTION OF PYROGENS IN SERA FROM PATIENTS WITH SYMPTOMS OF SEPSIS USING AN *EX VIVO* WHOLE BLOOD CULTURE ASSAY

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ABSTRACT

The aim of this study was to investigate whether the *ex vivo* whole blood culture (WBC) assay system can be used to detect pyrogens in blood from patients with symptoms of sepsis.

Blood samples from 35 patients with symptoms of sepsis were assayed for bacterial contamination using the radiometric blood culture assay. Serum from the same patients were screened for IL-6, C-reactive protein (CRP) and pyrogens using the whole blood culture assay. Serum samples from 26 patients tested positive for pyrogens. Of the 26 patients with pyrogenic serum, 15 had elevated serum IL-6 levels and 19 had elevated CRP levels. Only two of the samples had positive blood cultures as detected by the routine radiometric assay. Both of these patients had high serum CRP and pyrogen levels, while only one of them had an elevated serum IL-6 level. These results show that the WBC is very sensitive in detecting pyrogens in serum of patients. This technique can be a useful tool to quantitate pyrogens in sera from patients with symptoms of sepsis and to determine whether their clinical symptoms are caused by pyretic substances in their circulatory system.
(KEY WORDS: IL-6, CRP, pyrogen, sepsis)

INTRODUCTION

Systemic inflammatory response syndrome (sepsis) is a collective name for several microbial infections that affect the host and cause clinical abnormalities in the host (1,2).

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Some of these infections can be fatal if they are not diagnosed early. The main cause of sepsis in humans is bacterial, although other pathogens such as fungi can also be responsible. The bacteria and fungi release toxins in the blood of the patient and these are responsible for most of the symptoms associated with sepsis (3). Due to the fact that microbes often settle and grow on a structure, eg. a defective heart valve (bacterial endocarditis), low microbe concentrations in the blood stream make it difficult to detect these infections by conventional techniques (3).

Present methods to diagnose sepsis such as the radiometric blood culture assay, as well as the Limulus Amoebocyte Lysate (LAL) assay are known for underdiagnosing clinically important incidents of sepsis. This is due to the fact that the radiometric blood culture assay only detects live microbes in the circulatory system and does not detect toxic breakdown and/or secretory products of microbes attached to organs. The LAL assay has met with criticism due to non-specific reactions caused by blood proteins and also due to the fact that it cannot detect microbes other than gram negative bacteria (4,5,6).

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Previous studies done by ourselves and by other groups have shown that pyrogens such as bacteria, bacterial products and biological fluids contaminated with microbial breakdown products stimulate human blood cultures to secrete several cytokines such as interleukin-1, interleukin-2, tumour necrosis factor (TNF) and IL-6 (7, 8, 9, 10). The secretion of IL-6 is of particular importance for diagnostic purposes as it is known to stimulate the induction of most of the hepatic acute phase proteins such as CRP (11). The rate of cytokine secretion is dependent on the concentration of the pyrogen. This assay has proved to be a valuable method for the detection of whole microbes, its breakdown products, as well as its toxic secretory products i.e. the agents that cause the inflammatory response in bacterial and fungal sepsis.

The aim of the present study is to determine whether the WBC assay can detect the agents that induce the inflammatory response in sepsis and to compare results obtained for the WBC with those obtained for radiometric blood culture system, serum IL-6 and serum CRP.

METHODS

Selection of patients

Samples submitted to the Medical Microbiology laboratory at Tygerberg Hospital, South Africa from patients with symptoms of sepsis were used for this study. Blood samples are routinely taken for the radiometric culture assay as well as for C-reactive protein (CRP), a non-specific marker protein associated with sepsis. A sample of the plasma/serum used for the CRP assay was stored at -20 °C and was subsequently used for the WBC and also for serum/plasma IL-6 determination. The results obtained for the radiometric culture assay were compared to those obtained for the WBC, IL-6 and CRP.

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Radiometric blood culture assay

This is the technique commercially available from Becton Dickinson (Bactec 640) whereby culture bottles are aseptically inoculated with whole blood and cultured at 37 °C. Positive cultures generate ^{14}C-labelled CO_2. The radio-labelled CO_2 is detected by direct sampling of the gases from the culture bottles.

CRP assay

Serum CRP levels were determined on a Beckman nephelometer using specific antisera (Beckman) according to the manufacturer's guideline.

WBC for serum from sepsis patients

Assays were performed in 96 or 48 well culture plates. Serum samples from suspected sepsis patients or *E.coli* endotoxin standards (Bio-Whitaker) were added to the wells (25 µl/well). Each well then received 200 µl of RPMI medium. Finally 25 µl of whole blood collected from a healthy donor (healthy male personnel members, not on any medication, with normal white blood cell counts and between the ages 25 and 40 years) was added to each well. The plate was then sealed with plastic wrapping and incubated at 37 °C for at least 4 hours. Longer incubation periods (eg. 18 hours) increase the amount of IL-6 secreted but have no effect on the endotoxin level of the sample, provided that there are no living organisms present in the sample. At the end of the culture period the culture supernatants were assayed for IL-6 using an in-house ELISA assay as described previously (12). The pyrogen levels of the samples were determined by constructing a standard curve using the *E.coli* endotoxin standards run in parallel.

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RESULTS

The detection of pyrogens in serum from patients with symptoms of sepsis using the WBC assay

Some of the serum samples obtained from patients with symptoms of sepsis induce blood cultures from healthy donors to secrete IL-6, one of the cytokines associated with sepsis, without any additional stimulation. The pyrogen levels of these samples expressed as *E.coli* endotoxin units per ml (EU/ml) were determined by constructing a standard curve using the *E.coli* endotoxin standards run in parallel (Figure 1). When sera containing pyrogens are added to WBC, IL-6 is secreted and the level of IL-6 secreted is dependent on the concentration of sera added to the culture (Figure 2). Table 1 shows that 26 of the 35 serum samples obtained from patients with symptoms of sepsis contain

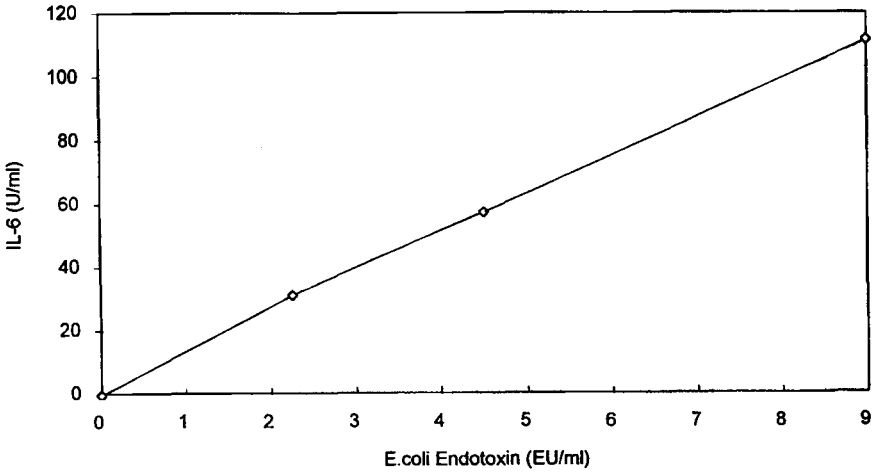

FIGURE 1: The effect of *E.coli* endotoxin concentration on IL-6 secretion by whole blood cultures.
Whole blood cultures were incubated in the presence of different endotoxin concentrations as indicated. The amount of IL-6 secreted after a 4 hour incubation at 37 °C was measured by ELISA.

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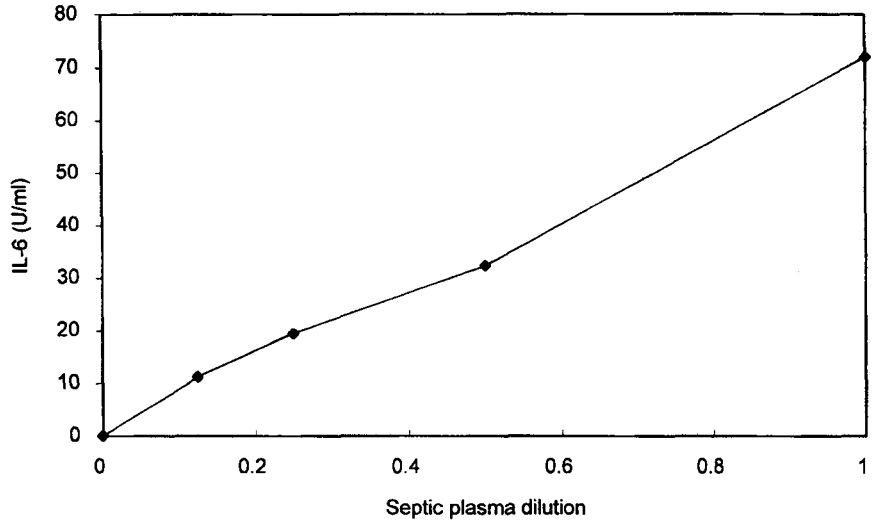

FIGURE 2: The effect of septic plasma concentration on IL-6 secretion.
Whole blood cultures were incubated in the presence of different septic plasma volumes as indicated. Plasma from healthy donors was added to the septic plasma to give a total plasma volume of 25 µl/assay. The total volume of the incubation mixture was 250 µl/assay. The amount of IL-6 secreted after a 4 hour incubation at 37 °C was measured by ELISA.

TABLE 1

The detection of sepsis using different assay systems.

Assay system	Positive	Negative
Radiometric Blood Culture	2	33
CRP	22	13
WBC	26	9
IL-6	15	20

Number of patients used = 35

pyrogens. Sera from a control group of healthy individuals (n=11 data not shown) failed to induce IL-6 secretion when added to WBC from healthy donors.

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Sensitivity of different assay systems to detect markers in blood of patients with symptoms of sepsis

The results obtained for the radiometric blood culture, WBC, IL-6 and CRP from the 35 samples are presented in table 1. Our internal cut-off for the CRP assay is 10 μg/ml and samples higher than this are presented as positive. At present there are no cut-off limits set for the WBC and IL-6. For comparative purposes a serum/plasma cut-off level of 1 EU/ml was used for WBC i.e. samples with an EU/ml level less than 1 are regarded as negative. A serum IL-6 level of greater than 25 U/ml (the upper limit for healthy subjects tested in our laboratory) is regarded as elevated and indicative of sepsis. The radiometric blood culture assay detected only 2 positive samples. This is in sharp contrast with the WBC which indicated 26 positive samples and several borderline cases if a cut-off of 1 EU/ml is used (data not shown). The CRP assay also showed a high number of positive samples. The IL-6 assay showed that 15 samples had elevated IL-6 levels. The

TABLE 2

Comparison between the various systems to detect sepsis. (a)Comparison between the WBC and the radiometric blood culture assay for sepsis patients. (b)Comparison between the WBC and the CRP for sepsis patients. (c)Comparison between the CRP and the radiometric blood culture assay for sepsis patients.

a

		Radiometric blood culture	
		Positive	Negative
WBC	Positive	2 (5.7%)	24 (68.6%)
	Negative	0 (0%)	9 (25.7%)

b

		CRP	
		Positive	Negative
WBC	Positive	19 (54.3%)	7 (20.0%)
	Negative	3 (8.5%)	6 (17.1%)

c

		Radiometric blood culture	
		Positive	Negative
CRP assay	Positive	2 (5.7%)	20 (57.1%)
	Negative	0 (0%)	13 (37.1%)

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serum IL-6 assay, contrary to the WBC and CRP, did not detect all the radiometric blood culture positive samples and was not used for comparative purposes.

Table 2a shows that only 2 of the 35 patients with symptoms for septicaemia had positive radiometric blood cultures. Serum samples from both of these patients were positive for pyrogens by the WBC. In addition the WBC detected a further 24 positive samples from patients with suspected sepsis. All the samples that tested negative in the WBC were also negative in the radiometric blood culture assay.

The CRP assay is a non-specific assay for sepsis whereas the WBC specifically detect microbes and microbial breakdown products and toxins. This must be borne in mind when comparing the results presented in Table 2b. This table shows that when these two methods are compared only 6 out of 35 samples from patients with symptoms of sepsis tested negative by both methods. The number of positive samples detected by either or both methods are 29 out of 35. The results for 71.4 % of the samples tested agree with one another (54.3 % positive by both methods plus 17.1 % negative by both methods). The WBC detects a further 20 % of samples not detected by the CRP while the CRP assay detects 8.5 % of samples missed by the WBC assay. The difference between the WBC and CRP assay results can be explained by the following: CRP only becomes detectable in plasma 12 hours after the start of an infection and can still be detected for up to 106 hours after an inflammatory event (13). The WBC assay on the other hand detects pyrogens in plasma at the time that the blood sample was drawn.

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Table 2c shows that only 2 of the samples collected from the patients with symptoms for septicaemia were detected by the radiometric blood culture assay. Both of these samples were also detected by the CRP assay. In addition the CRP assay detected a further 20 positive samples. All the samples that were negative for the CRP were also negative in the radiometric culture assay, indicating that the CRP assay, although not specific for sepsis, does not miss any radiometric assay positives.

DISCUSSION

Recent multicenter studies comparing different microbiology culture systems for sepsis detection have highlighted discrepancies in organism detection between two blood culture bottle types. These studies also showed that clinically important episodes of sepsis were missed by both microbiology blood culture bottle types investigated. The percentage of

positive cultures missed by the one culture bottle type was as high as 23 % of the total microbiology culture positives (14). Data like the above and also experience with endocarditis yielding negative results on various culture systems but responding to antibiotic therapy have resulted in the search for alternative assays for sepsis.

The culture system used for the WBC is a human whole blood culture *ex vivo*. Serum pyrogens detected by this system will most probably cause inflammatory reactions in humans. Previous studies using monocyte cultures have shown that sera from sepsis patients do not induce TNF synthesis, a cytokine that is associated with the inflammatory response (15). The data obtained for the above study shows that IL-6 secretion, contrary to TNF, is stimulated by the addition of serum samples from suspected sepsis patients to blood cultures from a normal donor. The above results show that the WBC is very sensitive to pyrogenic and inflammatory substances present in serum. This study also shows that a large number of patients with symptoms of sepsis contain pyretic substances other than live bacteria which are missed by conventional microbiology culture assays. Confirmation for the possible false negatives by microbiology culture methods were also seen when non-specific serum indicators of sepsis such as IL-6 and CRP were investigated.

Due to the relatively short assay time and the high sensitivity obtained with the WBC assay system, research is being carried out at present to establish whether this assay can also be used to differentiate between gram negative bacteria and other pyrogenic substances using antibiotic screening systems.

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