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A comparative study of Mono Mac 6 cells, isolated mononuclear cells and Limulus amoebocyte lysate assay in pyrogen testing

Lise Moesby *, Susanne Jensen, Erik W. Hansen, Jens D. Christensen

The Royal Danish School of Pharmacy, Department of Pharmacology, 2 Universitetsparken, 2100 Copenhagen, Denmark

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Abstract

Pyrogen induced secretion of interleukin 6 (IL-6) in Mono Mac 6 (MM6) cells was measured. The ability of the MM6 cell culture to detect pyrogens was compared to the Limulus amoebocyte lysate (LAL) test and isolated mononuclear cells (MNC). The detection limit of MM6 for lipopolysaccharide (LPS) and *Staphylococcus aureus* was comparable to that of MNC. *Aspergillus niger* and *Candida albicans* induced IL-6 in isolated MNC, but not in MM6. The detection limit for *Salmonella typhimurium* in the MM6 assay was comparable to that of the LAL assay. As expected, *S. aureus* and *C. albicans* did not show any LAL activity. *A. niger* and *Influenza* virus showed some activity in the LAL test, but could not be detected by MM6 cells. In conclusion, the MM6 assay is a good supplement to the current pyrogen assays for detection of LPS, *S. aureus* and *S. typhimurium*, but the MM6 assay could not detect *A. niger*, *C. albicans* and *Influenza* virus.

Keywords: *Aspergillus niger*; *Candida albicans*; Interleukin 6; LPS; *Salmonella typhimurium*; *Staphylococcus aureus*

1. Introduction

Pharmaceutical products for parenteral administration must be free of pyrogens such as lipopolysaccharides (LPS), bacteria, fungi and virus. Sterile pharmaceuticals are normally tested for pyrogenic contamination by the rabbit pyrogen test or the Limulus amoebocyte lysate (LAL) test. Rabbits have the same sensitivity towards LPS as humans (Wolff, 1973) and the rabbit pyrogen test is able to detect several kinds of pyrogens (Probey and Pittman, 1945) but a major disadvantage of this test is the troublesome, costly and controversial use of laboratory animals. The LAL test is very sensitive in the detection of LPS but it does not detect pyrogens other than LPS (Wildfeuer et al., 1974; Devleeschouwer et al., 1985). Due to the disadvantages of the two pyrogen tests, alternative methods are needed to supplement the currently used methods.

In vitro stimulation of human mononuclear cells (MNC) with pyrogens results in secretion of

* Corresponding author. Tel.: +35-30-63-03; fax: +35-30-60-20.

E-mail address: lm@mail.dfh.dk (L. Moesby)

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cytokines and it seems that MNC are even more sensitive towards pyrogens than the rabbit pyrogen test (Hansen and Christensen, 1990). However, the use of freshly isolated human MNC must be restricted for research purposes only, for practical and ethical reasons.

Monocytes, macrophages and cell lines derived from MNC have been proposed as test systems to detect pyrogenic contamination (Poole et al., 1988; Hansen and Christensen, 1990; Taktak et al., 1991; Pennanen et al., 1995; Eperon and Jungi, 1996; Moesby et al., 1997). The monocytic cell line Mono Mac 6 (MM6) has proven to be sensitive to LPS of various origins (Eperon and Jungi, 1996; Eperon et al., 1997), but the sensitivity of MM6 to pyrogenic microorganisms is unknown.

In this study we examine the ability of inactivated *Staphylococcus aureus*, *Salmonella typhimurium*, *Candida albicans*, *Aspergillus niger*, *Influenza* virus and LPS to induce secretion of interleukin 6 (IL-6) in the monocytic cell line MM6. The sensitivity of MM6 towards these pyrogens is compared to that of the LAL test and freshly isolated human mononuclear cells.

2. Materials and methods

2.1. Reagents

Human recombinant IL-6 was purchased from Genzyme Diagnostics, Cambridge, MA, USA (specific activity according to the manufacturer 2.12×10^8 U/mg). Monoclonal anti-human IL-6 antibody, biotinylated polyclonal goat anti-human IL-6 antibody, monoclonal anti-human tumor necrosis factor α antibody, biotinylated polyclonal goat anti-human tumor necrosis factor α antibody and human recombinant tumor necrosis factor α (TNF-α) were obtained from R&D Systems, UK. The antibodies were recommended by the manufacturer as capturing and detection pairs for sandwich enzyme-linked immunosorbent assay (ELISA).

Standard solutions of lipopolysaccharides (LPS) were prepared from *Escherichia coli* 005:B5 (Whittaker Bioproducts, MD, USA) in pyrogen free water. Limulus amoebocyte lysate (LAL) obtained from Whittaker Bioproducts was reconstituted in pyrogen free water. The chromogenic substrate S2423 (Chromogenix, Mölndal, Sweden) was diluted in pyrogen free water to 2.2 mmol/l and kept at 4°C.

S. aureus ATCC 29213, *S. typhimurium* ATCC 14028, *C. albicans* ATCC 10231 and *A. niger* ATCC 16404 were purchased from OXOID, GB. *Influenza* PR8-34 approximately 10^8 infectious units/ml was kindly provided by Dr Jan Wilschut, University of Groningen, The Netherlands.

Europium labeled streptavidin (0.1 mg/ml), DELFIA® assay buffer, DELFIA® wash concentrate and DELFIA® enhancement solution were all obtained from Wallac (Turku, Finland).

All laboratory glassware to be used in the endotoxin handling were rendered pyrogen free through heating at 180°C for 6 h.

2.2. Maintenance of cell lines

The human monocyte cell line Mono Mac 6 (MM6) was originally provided by Dr Ziegler-Heitbrock, University of Munich, Germany (Ziegler Heitbrock et al., 1988). The cells were maintained in RPMI 1640 (Gibco BRL, NY, USA) which was ultrafiltrated (Ultrasart D 20, Sartorius) to become pyrogen free. RPMI 1640 was supplemented with 5% Myoclone Super Plus fetal bovine serum (Gibco BRL, NY, USA), 200 U/ml penicillin, 200 μg/ml streptomycin, 2 mM glutamine, 9 μg/ml insulin and non-essential amino acids (Gibco BRL, NY, USA). The cells were incubated in humidified atmosphere containing 5% CO_2 and 95% air at 37°C. The medium was replaced twice weekly and the cells were seeded at $1.5–2.5 \times 10^5$ cells/ml.

B9 hybridoma cells (Aarden et al., 1987) were grown in RPMI 1640 supplemented with 5% Myoclone Super Plus fetal bovine serum, 5 mM Hepes, 2 mM glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin. The medium was changed twice weekly and the cell concentration was standardised to not less than 1.5×10^5 cells/ml. The medium was supplemented with human IL-6 100 U/ml.

2.3. Preparation of freshly isolated mononuclear cells

Human mononuclear blood cells (MNC) obtained from healthy volunteers were isolated as previously described (Hansen and Christensen, 1990), resulting in cell suspensions of approximately 12% monocytes and 88% lymphocytes judged from α-naphthyl esterase staining. MNC were diluted in RPMI 1640 supplemented with 5% Myoclone Super Plus fetal bovine serum, 10 mM Hepes, 2 mM glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 10 μg/ml streptomycin and plated (10^6 cells/ml) onto petri dishes (Nunc, Roskilde, Denmark).

2.4. Preparation of S. aureus, S. typhimurium, C. albicans and spores of A. niger

S. aureus and *S. typhimurium* were grown at 37°C overnight in RPMI 1640 isolated by centrifugation ($2000 \times g$, 10 min) and washed twice with pyrogen free water. The washed bacteria were resuspended to approximately 10^9 cells/ml. Some of the suspension was serially diluted and spread on agar plates to determine the number of colony forming units (CFU) and the rest was exposed to ultraviolet (UV) light for 1 min to kill the bacteria. No CFU could be detected after spreading of the irradiated suspension on agar plates.

C. albicans was grown at 37°C overnight in RPMI 1640, isolated by centrifugation ($2000 \times g$, 20 min) and washed twice with pyrogen free water. The cell concentration was determined after serial dilution of some of the suspension and spreading on agar plates. The yeast concentration was standardised to 10^9 cells/ml and exposed to UV light for 1 min to kill the cells. No yeasts could be detected after spreading of the irradiated suspension on agar plates.

A. niger was grown at room temperature for 4–5 days on Sabouraud Dextrose agar plates. The spores were isolated by pouring pyrogen free water onto the plate and gentle stirring. The suspension was passed through filter paper, centrifuged ($2000 \times g$, 10 min) and washed twice with pyrogen free water. The spores were counted using a Bürker-Türk cell counter and diluted to approximately 10^7 spores/ml. The spores were killed by UV irradiation for 15 min. No viable spores could be detected after spreading of the irradiated suspension on Sabouraud Dextrose agar plates. The irradiated spore suspension was standardised to 10^6 spores/ml.

Influenza virus was diluted in pyrogen free water to 10^7 infectious units/ml and inactivated by UV irradiation for 5 min.

The irradiated suspensions of microorganisms were serially diluted and used for stimulation of MM6 cells.

2.5. Detection of pyrogens by kinetic chromogenic Limulus amoebocyte lysate assay

The LAL assay was performed as previously described (Pedersen et al., 1994). The proteolytic enzyme present in the LAL is activated by incubation with LPS and upon addition of the chromogenic substrate S2423 the activated enzyme hydrolyses S2423 to *p*-nitroaniline.

Standards and samples of pyrogens (100 μl) were dispensed into a sterile microtiter plate (Nunc, Roskilde, Denmark) and preheated for 30 min at 37°C. Then 50 μl LAL was added and incubated at 37°C for 10 min. The substrate S2423 was mixed with an equal volume of Tris–NaCl buffer pH 9.0, preheated at 37°C, and 100 μl of diluted S2423 was added. $OD_{405\,nm}$ was read every 30 s for 10 min using a MAXline Microplate reader (Molecular Devices Corporation, CA, USA). The MAXline reader was equipped with kinetic software to deal with the kinetics of enzymatic reactions and a thermostat to control the temperature of the chamber.

2.6. Stimulation of Mono Mac 6 and mononuclear cells by pyrogens

Mono Mac 6 cells were seeded at 4×10^5 cells/ml and incubated for 2 h. The culture were centrifuged and standardised to 2×10^6 cells/ml. Equal volumes of test solution and MM6 cell suspension were mixed and incubated for 20 h. The cell cultures were centrifuged ($1100 \times g$, 10 min), the supernatants were isolated and analysed in DELFIA.

Test solutions (100 μl) were added to 1 ml MNC 10^6 cells/ml and incubated for 20 h. The cultures were centrifuged ($4000 \times g$, 10 min), the supernatants were serially diluted and the IL-6 like activity was determined in the IL-6 bioassay.

2.7. Determination of IL-6 and TNF-α

The IL-6 and TNF-α concentrations were determined using non-competitive immunoassays involving time-resolved dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) as previously described (Moesby et al., 1997).

2.7.1. IL-6 immunoassay

The DELFIA assay (Moesby et al., 1997) was used with a few modifications. Briefly Fluoro-Nunc™ microtiter plates (Nunc, Roskilde, Denmark) were coated with monoclonal anti-human IL-6 antibody 2 μg/ml (100 μl/well). Standards and tests diluted in RPMI 1640 and water (1:1) were added to the wells (100 μl/well). A standard curve in the range 5–5000 pg/ml IL-6 was used. Biotinylated polyclonal goat anti-human IL-6 antibody (100 μl of 50 ng/ml) was added and incubated. Then 100 μl europium labeled streptavidin (100 ng/ml) was added to each well and incubated for 30 min. Finally, the europium was rendered fluorescent with DELFIA enhancement solution (100 μl). The fluorescence was determined in a 1234 DELFIA fluorometer (Wallac, Turku, Finland).

2.7.2. TNF-α immunoassay

The assay follows the same procedure as for IL-6 (Moesby et al., 1997). The following concentrations of antibodies were used: capture antibody anti human TNF-α 4 μg/ml and detection antibody biotinylated anti TNF-α 100 ng/ml. A standard curve in the range 5–5000 pg/ml TNF-α was used.

2.7.3. IL-6 bioassay

The B9 assay which has been described previously (Aarden et al., 1987; Pedersen et al., 1995) was used with minor modifications. The growth of B9 cells depend on the presence of IL-6 and the cell growth is proportional to the amount of NADH/NADPH present in the cell culture. B9 cells were harvested by centrifugation ($110 \times g$, 5 min), washed twice in IL-6 free and pyrogen free RPMI 1640 and seeded in 96-well microtiter plates (Nunc, Roskilde, DK). Briefly, 50 μl cell suspension (2.5×10^5 cells/ml) and 50 μl test or standard was mixed to give a total volume of 100 μl per well and supplemented with polymyxin B 5 μg/ml. Both tests and standards were run in triplicates. The cells were incubated for 72 h at 5% CO_2 and 37°C. The response of the B9 assay was quantified by addition of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (Sigma Chemicals, St Louis, MO, USA). After addition of 20 μl MTT 5 mg/ml in phosphate buffered saline pH 7.4 to each well the cells were incubated for 4 h; then 100 μl lysing buffer (10% sodium dodecylsulphate, 50% *N*,*N*-dimethylformamide) was added to each well and the microtiter plate was incubated for 24–48 h. The absorbance at 570 nm was determined using a MAXline Microplate Reader (Molecular Devices Corporation, CA, USA).

2.8. Statistical analysis

The detection limit is calculated as the minimum concentration of a substance inducing a IL-6 secretion greater than the mean of control + 3 S.D. Mean and S.D. of the control are calculated on the basis of all experiments.

3. Results

3.1. Stimulation of MM6 cells with LPS

Mono Mac 6 cells were incubated with solutions of *E. coli* LPS in the range 2.5–1000 pg/ml. The final LPS concentration was half the concentration of the test solution. The IL-6 secretion of three independent assays are shown in Fig. 1. The detection limit of the MM6 assay was 3.1 pg/ml LPS. LPS dose dependently increased the IL-6 secretion in the range 3.1–1000 pg/ml. The controls without LPS induced secretion of 51.7 ± 20.2 pg/ml IL-6 (mean ± S.E.M.). Maximal induction of IL-6 secretion was not reached using a test solution of 1000 pg/ml LPS.

The MM6 cell line did not secrete any detectable amounts of TNF-α when stimulated with LPS test solutions in the concentration range 10 pg/ml to 1 μg/ml (results not shown).

3.2. IL-6 secretion induced by S. aureus and S. typhimurium

Mono Mac 6 cells were incubated with the UV-killed Gram-positive bacteria *S. aureus* in the range 3×10^2–2×10^8 bacteria/ml. The secreted IL-6 was determined (Fig. 2). The detection limit of MM6 to *S. aureus* was 3×10^5 bacteria/ml. *S. aureus* dose dependently increased the IL-6 secretion in the range 3×10^5–4×10^7 bacteria/ml. Maximal induction of IL-6 secretion (30 ng/ml) was seen after incubation with 4×10^7 bacteria/ml.

Fig. 3 shows the IL-6 secretion after 20 h stimulation of MM6 with the UV-killed Gram-negative bacteria *S. typhimurium* in the range 70–7×10^6 bacteria/ml. The detection limit was 1.8×10^2 bacteria/ml. *S. typhimurium* concentration dependently increased the IL-6 secretion in the range 1.8×10^2–7×10^4 bacteria/ml. Maximal induction of IL-6 secretion (35 ng/ml) was seen after incubation with 7×10^4 bacteria/ml.

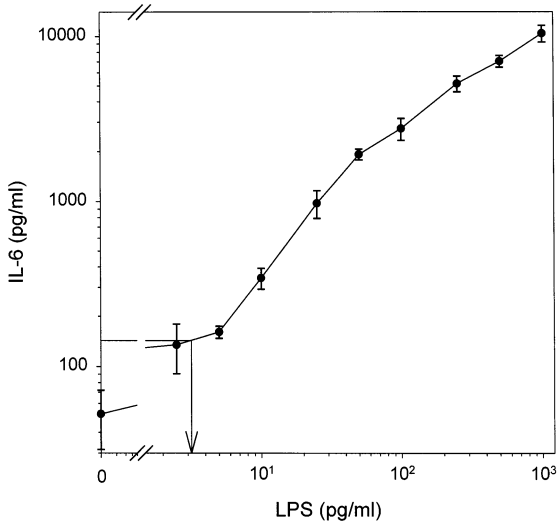

Fig. 1. Stimulation of MM6 with LPS. MM6 cells were incubated for 20 h with solutions of LPS (2.5-1000 pg/ml) and a control without LPS. The IL-6 secretion was determined by DELFIA. The results represent the mean ± S.E.M. ($n = 3$). The arrow indicates the detection limit.

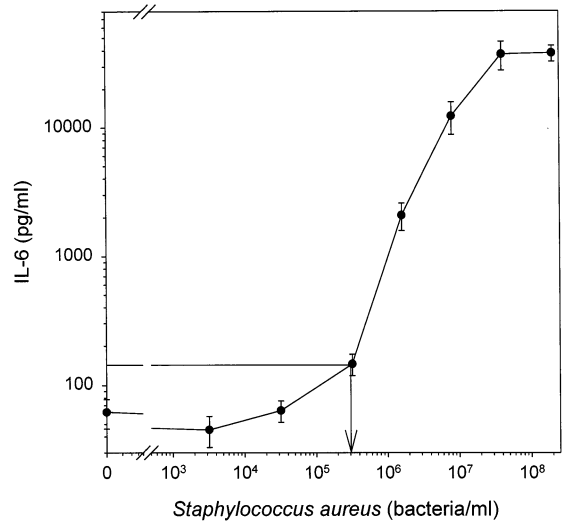

Fig. 2. Stimulation of MM6 cells with *S. aureus*. MM6 cells were incubated for 20 h with suspensions of UV-killed *S. aureus* (0–2×10^8 bacteria/ml). The IL-6 secretion was determined by DELFIA. The results represent the mean ± S.E.M. ($n = 6$). The arrow indicates the detection limit.

3.3. Detection of pyrogens and microorganisms with MM6 cells

Fig. 4 shows a summary of the sensitivity of MM6 towards various pyrogens after 20 h of incubation. The shown concentrations of pyrogens are the lowest concentration producing a significant IL-6 release as compared to the controls. In case of no significant increase in IL-6 secretion the highest concentration of pyrogen tested is shown. *A. niger* (10^6 spores/ml), *C. albicans* (10^6 yeasts/ml) and *Influenza* virus (10^6 infectious units/ml) did not induce significant IL-6 secretion compared to controls. *S. aureus* (3.2×10^5 bacteria/ml), *S. typhimurium* (7×10^2 bacteria/ml) and LPS (5 pg/ml) induced a significant IL-6 release.

3.4. Detection of pyrogens and microorganisms by the LAL test

Table 1 shows the results of the LAL assay of tested pyrogens. *S. typhimurium* had a LAL activ-

ity of 8.8 pg LPS per 10^3 cells. *A. niger* (10^6 spores) showed a LAL activity of 9.2 pg LPS. The LAL activity of *C. albicans* and *S. aureus* was below the detection limit, being <1 pg LPS per 10^6 yeasts and <1 pg LPS per 10^8 bacteria, respectively. The *Influenza* virus stock was tested for LAL activity mainly to verify that the stock was LPS free, since the virus was not originally grown in LPS free environment. The result was 9.5 pg LPS per 10^6 infectious units.

All the samples were spiked with 100 pg/ml LPS and a recovery in the range 90–140% was found indicating that no interference was caused by the samples.

3.5. Detection of pyrogens and microorganisms by freshly isolated human mononuclear cells

In Fig. 5 the stimulation of freshly isolated human MNC with a panel of pyrogens is shown. The cells were incubated for 20 h with the pyrogen or control and the secreted IL-6 was determined by the B9 bioassay. The shown concentrations of pyrogens are the lowest concentration inducing IL-6 release as compared to the controls. The tested pyrogens all demonstrate a significant secretion of IL-6 in isolated mononuclear cells. LPS (7.8 pg/ml), *S. aureus* (10^5 bacteria/ml), *A. niger* (10^6 spores/ml) and *C. albicans* (10^6 yeasts/ml) induced significant IL-6 secretion compared to the controls.

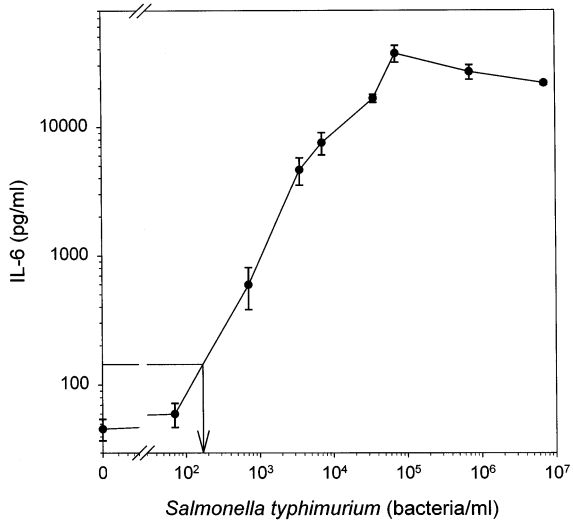

Fig. 3. Stimulation of MM6 by *S. typhimurium*. MM6 cells were incubated for 20 h with suspensions of UV-killed *S. typhimurium* ($0–7 \times 10^6$ bacteria/ml). The IL-6 secretion was determined by DELFIA. The results represent the mean ± S.E.M. ($n = 6$). The arrow indicates the detection limit.

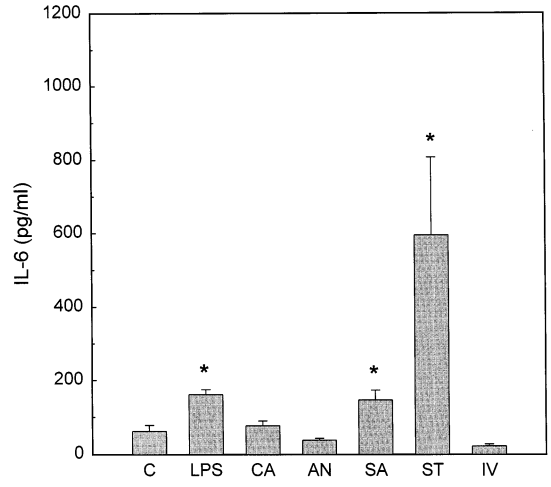

Fig. 4. Sensitivity of MM6 towards microorganisms and other pyrogens. MM6 cells were incubated for 20 h with suspensions of pyrogens. The IL-6 secretion was determined by DELFIA. The results represent the mean ± S.E.M. ($n = 6$). *IL-6 release significantly higher than the control. C, control; LPS, LPS (5 pg/ml); CA, *C. albicans* (10^6 yeasts/ml); AN, *A. niger* (10^6 spores/ml); SA, *S. aureus* (3.2×10^5 bacteria/ml); ST, *S. typhimurium* (7.1×10^2 bacteria/ml); IV, *Influenza* virus (10^6 infectious units/ml).

Table 1
Detection of various pyrogens by Limulus amoebocyte lysate (LAL) test

Pyrogenic microorganism	LAL activity[a]
C. albicans	<1 pg/10^6 yeasts
A. niger	9.2 pg/10^6 spores
Influenza virus	9.5 pg/10^6 infectious units
S. aureus	<1 pg/10^8 bacteria
S. typhimurium	8.8 pg/10^3 bacteria

[a] The LAL activity of the pyrogens is determined by comparison to a standard of *E. coli* LPS.

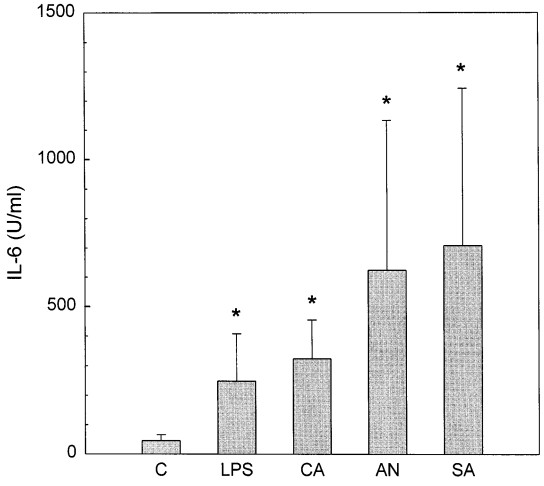

Fig. 5. Mononuclear cell sensitivity towards microorganisms and other pyrogens. Mononuclear cells were incubated for 20 h with different pyrogens. The secreted IL-6 was measured by a B9 bioassay. Results represents mean ± S.E.M. ($n = 3$-6).*IL-6 release significantly higher than the control. C, control; LPS, LPS (7.8 pg/ml); CA, *C. albicans* (10^6 yeasts/ml); AN, *A. niger* (10^6 spores/ml); SA, *S. aureus* (10^5 bacteria/ml).

4. Discussion

4.1. MM6 cells and LPS

The production of IL-6 by MM6 cells is a reliable indicator for LPS stimulation (Fig. 1). There is a dose–response relationship between LPS and IL-6 over a wide concentration range. The MM6 cells expose a day to day variation in their sensitivity to IL-6 inducing substances indicated by the error bars in Figs. 1–4. The MM6 cells show a basal release of IL-6 when grown in pyrogen free medium.

The MM6 cell line is a mature cell line as compared to other cell lines of human mononuclear origin (Ziegler Heitbrock et al., 1988; Taktak et al., 1991; Eperon and Jungi, 1996).

Eperon and Jungi (1996) describe a MM6 assay very similar to the one we use, only they use special subclones of MM6 primed with calcitriol to increase the number of CD14 (LPS receptor) on the cell surface hereby increasing the sensitivity towards LPS. In these primed MM6 subclones TNF-α is a better indicator for LPS than IL-6. A detection limit of approximately 5 pg/ml LPS was obtained, which is comparable to the detection limit of MM6 in our hands (3.1 pg/ml). In contrast to Eperon and Jungi (1996) the MM6 cell line in our hands does not secrete any detectable amounts of TNF-α when stimulated with LPS.

Pharmaceuticals for parenteral use must pass the test for pyrogens (rabbit pyrogen test) or the test for endotoxins (LAL assay). In accordance with Ph.Eur. the detection limits of these two tests are ≥ 50–100 pg/ml LPS. The MM6 assay fulfils this requirement.

4.2. Comparison of MM6 and freshly isolated mononuclear cells

Isolated MNC are sensitive to a wide range of pyrogens (LPS, *S. aureus*, *A. niger*, *C. albicans*) whereas MM6 cells under the present experimental conditions are sensitive to a more narrow spectrum (LPS, *S. typhimurium*, *S. aureus*). Mononuclear cells are a mixture of monocytes and lymphocytes whereas MM6 cells is a pure culture of monocytic cells. The cytokine response to some pyrogens may depend on cooperation between different leucocytes explaining that the MM6 cells do not respond to some pyrogens.

In the present experimental setup the detection limits for LPS and *S. aureus* in freshly isolated MNC and MM6 cells are comparable. MM6 cells lack the ability to detect *C. albicans*, *A. niger* and *Influenza* virus, whereas freshly isolated MNC are able to detect *C. albicans* and *A. niger*.

In agreement with this, peripheral human monocytes stimulated with *C. albicans* release several cytokines including IL-6 and TNF-α (Castro et al., 1996) whereas others have found that stimulation of peripheral human blood cells with *C. albicans* failed to produce inflammatory cytokines including IL-6 (Henderson and Rippin, 1995). The murine macrophage-like cell line RAW 264.7 secrete IL-6 when stimulated with a cell extract of *C. albicans* (Vazquez et al., 1996).

In conclusion, the MM6 assay using IL-6 as the parameter is a good alternative to freshly isolated MNC for detection of LPS, Gram-negative and Gram-positive bacteria, but the MM6 assay is at the moment not able to detect *A. niger*, *C. albicans* or *Influenza* virus.

4.3. Comparison of MM6 cells and the LAL test

MM6 cells shows a high sensitivity towards *S. typhimurium*. This is not surprising since the pyrogenic effect of Gram-negative bacteria is caused by LPS. The effect on MM6 cells of a suspension of *S. typhimurium* (10^3 bacteria/ml) equals the effect of 22.8 pg/ml of LPS standard (estimated from Fig. 1, Fig. 3). In the LAL test (Table 1) *S. typhimurium* (10^3 bacteria) showed a LAL activity of 8.8 pg LPS standard. The amounts of LPS found using the two different assays are of the same magnitude. One might have expected a difference due to the fact that the LAL test reacts to LPS on the surface of the bacteria only, whereas the MM6 cells probably phagocytose the bacteria and in this way are exposed to the total bacterial LPS.

The pyrogenic effect of *S. aureus* is not caused by LPS, but probably by muramyl peptide (Johannsen et al., 1994) or lipoteichoic acid (Himanen et al., 1993). For this reason the LAL test is not able to detect *S. aureus*. The bacteria are grown in pyrogen free media and no contamination with LPS was detected in a suspension of 10^8 bacteria/ml. However the MM6 assay is able to detect *S. aureus* (3×10^5 cells/ml). This is in accordance to Eperon et al. (1997) who have shown that lipoteichoic acid (a cell wall component of Gram-positive bacteria) can be detected by MM6 cells.

C. albicans did not show any activity in the LAL assay and could not be detected by the MM6 assay either. *A. niger* had a low LAL activity in the highest concentration tested, but it was not detectable in the MM6 assay. Even though the *Influenza* virus was not grown in pyrogen free media only a limited LAL activity was found in the samples. In spite of the LAL activity *Influenza* virus was not detectable in the MM6 assay.

If the LAL activity of *Influenza* virus and *A. niger* was caused by LPS one would expect *Influenza* virus and *A. niger* to be detected in the MM6 assay, since the LAL activity (9.5 pg LPS per 10^6 infectious units and 9.2 pg LPS per 10^6 spores) was above the detection limit for LPS found in MM6 cells. This leads to the conclusion that the LAL activity of *Influenza* virus and *A. niger* probably was not caused by LPS.

Surprisingly, *Influenza* virus did not induce a significant release of IL-6 even though the virus before inactivation was found to be able to infect MM6 cells justified as agglutination and lysis of cells (results not shown). Furthermore *Influenza* virus is well known to act as a pyrogen. The release of cytokines (IL-1β, IL-6 and TNF-α) from monocytes and macrophages has been shown to be dependent of infectious *Influenza* virus, whereas UV-killed virus only induced secretion of interferons IFN-α and IFN-β (Peschke et al., 1993). This is in agreement with the present results. Interestingly, Peschke et al. (1993) found that the *Influenza* virus stimulated release of the cytokines IL-1β, IL-6 and TNF-α could be triggered after subsequent exposure to low levels of LPS.

5. Concluding remarks

The LAL assay is the classical in vitro pyrogen test, with a well-known specificity for LPS. However, pharmaceutical preparations for parenteral use might be contaminated with pyrogens other than LPS. Therefore an alternative less specific assay might be of interest. Another well-known risk of employing the LAL test is that substances in the preparation unspecifically interfere with the assay (Schmidtgen and Brandl, 1995; Moesby et al., 1997). A simple in vitro assay which is less susceptible to unspecific interference and with sensitivity to various pyrogens comparable to that of the rabbit pyrogen test or even better to that of humans, would be preferable. The MM6 assay might be a good supplement to the LAL assay for detection of LPS, Gram-negative and Gram-positive bacteria. Another advantage of the MM6 assay compared the LAL assay is the ability of the cells to detect LPS in lipid based total parenteral nutrition (Moesby et al., 1997).

There are several possible explanations for MM6 cells lack of sensitivity to some pyrogens. The possible use of the MM6 assay as an alternative method for pyrogen testing will depend on whether the sensitivity can be obtained and in-

creased by using a simple sample preparation, using another parameter than IL-6 or changing the setup of the assay. One possibility would be to use MM6 cells in a combination with lymphocytes. Pilot studies in our laboratory indicate that this may increase the spectrum of detectable pyrogens. Further studies are needed to verify this.

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