Hasiwa et al.:

Evidence for the detection of non-endotoxin pyrogens by the whole blood monocyte activation test

Supplementary Data

Cultivation of fungi

Filamentous fungi collected from environmental sources and expertly identified were grown at 23°C on malt extract agar (MEA) plates; yeasts were cultivated on saubauroud agar plates (Merck, Darmstadt, Germany). Fungi were plated on the surface of polycarbonate membrane filters with a pore diameter of 0.8 µm (Millipore, Billerica, MA, USA). After 3-6 days the filters with the fungal spores were removed and transferred to 50 ml falcons with 10 ml 0.9% saline (Berlin Chemie AG, Berlin, Germany). Vigorous mixing led to detachment of the spores from the membrane filters. Spores were separated from fruit bodies and remaining hyphae by polyamide filters (Franz Eckert GmbH, Waldkirch, Germany); filters with pore diameters of 20 or 40 µm were used, depending on the fungal spore size. Fungal spore purity was verified by microscope. Fungal spore counts were determined in a Neubauer cytometer.

Stimuli and inhibitor

Lipopolysaccharide (LPS) from *Escherichia coli* O-113 (National Institute for Biological Standards and Controls, Hertfordshire, UK), 100 pg/ml of *E. coli* 0-113 LPS is defined as one endotoxin unit (EU). Lipoteichoic acid (LTA) from *Staphylococcus aureus* was isolated by n-butanol extraction according to the protocol from Morath et al. (2001). Polymyxin B, Zymosan A, laminarin, lichenan, curdlan and mannan were purchased from Sigma, Deisenhofen, Germany.

Whole blood incubation

Differential blood cell counts were performed with a Pentra 60 (ABX Diagnostics, Montpellier, France) to exclude donors with acute infections. Heparinized whole blood was diluted tenfold in 0.9% saline (Berlin Chemie AG, Berlin, Germany) in polypropylene vials (Eppendorf, Hamburg, Germany) and stimulated with fungal spores, LPS, LTA or zymosan. After incubation for 20 h at 37°C in a humidified atmosphere with 5% CO_2, the vials were shaken and spun down. Supernatants were stored at -80°C until cytokine measurement.

Alveolar macrophages

The murine alveolar macrophage cell line MH-S (ATCC, CRL-2019), derived by SV40 transformation of an adherent cell-enriched population of mouse alveolar macrophages, was cultured in a humid atmosphere at 37°C and 5% CO_2 in RPMI 1640 with 2 mM L-glutamine (Cambrex, Vervies, Belgium) and 5 mM ultraglutamine 1 (Cambrex), adjusted to contain 1.5 g/l sodium bicarbonate (PAA, Cölbe, Germany), 4.5 g/l glucose (Sigma), 10 mM HEPES (PAA), 1 mM sodium pyruvate (PAA), 0.05 mM 2-mercaptoethanol (Sigma), 10% heat inactivated FCS and 1% penicillin/streptomycin. Cells were plated at 2.5 x 10^5 cells/well in 96-well culture plates (Greiner bio-one, Frickenhausen, Germany) for at least two hours to allow adherence before stimulation.

Vitality test

The Alamar blue reduction assay was employed to measure the activity of the cellular mitochondria as turnover of Alamar blue (Biosource, Camarillo, USA) to the fluorescent product resorufin, which can be detected at 544 nm excitation and 590 nm emission wavelengths. Blood cells from incubation vials were spun down and resuspended in 200 µl prewarmed 10% Alamar blue in RPMI 1640 (Cambrex, Verviers, Belgium). After 2 h incubation at 37°C in a humid atmosphere with 5% CO_2 the vitality was quantified in a fluorometer. 100 µl fresh blood cells were employed as positive control, and 100 µl blood cells were killed by incubation with 10% DMSO for 15 minutes to provide a negative control.

Coolant:

Samples of different fluid coolants were kindly provided by the Austrian Social Insurance for Occupational Risks (AUVA). The fluid coolants were subjected to gradual dilution (150 mM saline), in 3-fold steps up to 1:300,000. A series of diluted coolant samples were spiked with 100 pg/ml LPS from E. coli O-113 [1EU], in parallel with 10 µg/ml LTA from *Staphylococcus aureus* and also with with 10 µg/ml Zymosan from *Saccharomyces cerevisiae*. After 2 h spiking time the samples were treated according to the standard protocol of the in vitro pyrogen test (MAT). The margin of spike recovery was set corresponding to the LAL protocol, i.e., between 50% and 200% of the cytokine response compared to the spiked saline. In parallel to the MAT testing the cells were subjected to a Alamar blue viability test after the incubation.

Statistics

Statistical analyses were performed using GraphPad Prism program 4.01 (GraphPad Software, San Diego, USA). Statistics on 3 or more groups were performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. For two groups we used the unpaired t-test. Data are means ± SEM. A p-value ≤0.05 was considered significant; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. All statistical analyses are based on raw data. For calculation of the minimal cytokine inducing spore count or spore surface area, linear regression was performed on increasing mean data points from a dose response curve in Excel (Microsoft, Redmond, CA, USA).