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Assay of Pyrogens by Interleukin-6 Release from Monocytic Cell Lines

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Abstract—A novel in-vitro system has been developed for the detection and quantification of pyrogen in pharmaceutical products. The measured variable was evoked secretion of the pyrogenic cytokine interleukin-6 from MONO MAC 6 monocytic cells incubated with the product. The interleukin-6 was detected using a specific and sensitive ELISA developed for this purpose. The test system detected pyrogenic contamination in 3 batches of therapeutic human serum albumin which had caused adverse reactions in recipients. The contamination was not detected in conventional tests: the rabbit pyrogen test and the limulus amoebocyte lysate test.

Pyrogenic contamination in parenteral pharmaceuticals is detected in the rabbit pyrogen test (European Pharmacopoeia 1971) or in the Limulus amoebocyte lysate (LAL) test (United States Pharmacopoeia 1980; European Pharmacopoeia 1987). The former requires the use of experimental animals and is costly; the latter is sensitive but does not detect pyrogens other than endotoxin from Gram-negative bacteria and gives false negative results with certain products. Alternative pyrogen tests—'monocyte tests'—have been described in which test materials were incubated with human peripheral blood monocytes and the conditioned medium assayed for certain pyrogenic cytokines, e.g. interleukin-1 (IL-1) and tumour necrosis factor α (TNFα) (Duff & Atkins 1982; Dinarello et al 1984; Poole et al 1988).

While useful in detecting pyrogenic contamination, test systems that require monocytes freshly prepared from blood have limited applicability because their preparation is time-consuming and labour intensive; also, the sensitivity of monocytes to endotoxin varies with the individual sensitivity to endotoxin of the donors and the age of the buffy coat residues from which the cells were isolated (Arend et al 1989). A number of cell lines have been identified which retain monocytic characteristics, including the capacity to synthesize and secrete pyrogenic cytokines. The substitution of an appropriate cell line offers the possibility of a novel, in-vitro pyrogen test with greater applicability.

We have evaluated two established cell lines with monocytic characteristics, THP-1 (Tsuchiya et al 1980) and MONO MAC-6 (Ziegler-Heitbrock et al 1988), for their suitability for use in the 'monocyte test' for pyrogen. In addition to measuring endotoxin-evoked IL-1 production, IL-6 production was also measured since, unlike IL-1 and TNFα which remain largely intracellular, this pyrogenic cytokine is secreted into the (cell-line) conditioned medium making its complete estimation easier (Poole et al 1989a).

Materials and Methods

Recombinant human IL-6 produced in *E. coli* and with a specific activity of 5×10^6 units mg^{-1} (Hirano et al 1986) was a generous gift from Professors T. Hirano and T. Kishimoto, Institute for Molecular and Cellular Biology, Osaka University, Japan. The international standard for endotoxin for LAL gelation tests (preparation 84/650, *E. coli* 0113:H10:K-endotoxin, 1 pg=0·007 int. unit (Poole & Mussett 1989)), the British Standard for interferon gamma (IFN-γ, preparation 82/587), IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-8, TNFα, Granulocyte Colony Stimulating Factor (GCSF) and Granulocyte/Macrophage Colony Stimulating Factor (GMCSF) were provided by the National Institute for Biological Standards and Control (NIBSC). The preparations of human serum albumin (HSA) were protocol samples submitted to NIBSC as part of the batch release procedure required by the UK Medicines Control Agency. THP-1 cells (acute monocytic leukaemia, human) were obtained from the American Type Culture Collection, Rockville, MD, USA. MONO MAC 6 cells (monocytic leukaemia, human) were kindly provided by Dr H. Ziegler-Heitbrock, Munich, Germany. Other reagents and plasticware were purchased as sterile and pyrogen-free and glassware was baked at 250°C for 1 h.

Production of antiserum

A polyclonal antiserum to IL-6 was raised in a goat by intramuscular injection into four sites of 500 μg IL-6 emulsified in Freund's complete adjuvant. Boosts of 200 μg IL-6 in Freund's incomplete adjuvant were given intramuscularly into four sites at intervals of 5, 11, 24, 40 and 60 weeks after the primary injection. The goat was bled 11 days after the final boost.

Immunoaffinity purification of goat anti-IL-6 antibodies

Antibodies from the goat anti-IL-6 serum were purified by affinity chromatography on an Affi-Gel 15 (BioRad) column (1 mL) coupled to 1 mg of IL-6 (360 μg mL^{-1}) in phosphate buffered saline (0·1 M, pH 7·4, PBS). The purified antibodies were dialysed against PBS for use in coating wells or against borate buffer (0·05 M, pH 8·6) for use as a biotinylated tracer and stored at 1 mg mL^{-1} at −20°C.

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Biotinylation of goat anti-IL-6 antibodies

Forty μL of biotin-*N*-hydroxysuccinimide ester dissolved in dimethyl formamide was added to 1 mg of immunoaffinity purified antibody in a glass tube and the mixture incubated for 1 h at room temperature (20°C) with continuous agitation. Biotinylated antibody was purified on a Sephadex G25 column (120 × 15 mm) equilibrated with and run in PBS/0·1% BSA, pH 7·4. Fractions of 0·5 mL were collected and the void volume pooled for use in the ELISA.

Enzyme-linked immunoassay (ELISA) of IL-6

ELISA assays of IL-6 were carried out at room temperature in 96-well microtitre plates (Nunc-Immuno Plate MaxiSorp). Wells were coated with immunoaffinity-purified goat anti-IL-6 antibodies (1 μg mL^{-1} in PBS, 100 μL) by incubation overnight at 4°C. The plates were washed three times with assay buffer (0·01 M phosphate, 0·05 M NaCl, 0·1% Tween 20, pH 7·4). Samples or standards (100 μL) were added and incubated for 2 h. The plates were washed three times with buffer (250 μL) and incubated with biotinylated immunoaffinity-purified goat anti-IL-6 antibodies (0·014 μg/100 μL/well) for 1 h. The plates were washed three times with buffer and incubated with avidin-horseradish peroxidase (1/500, 100 μL, Dako Ltd) for 15 min. The plates were washed three times with buffer (250 μL) and incubated with *o*-phenylenediamine (OPD, 1 mM, Sigma, containing 0·4 μL of 30% v/v H_2O_2 mL^{-1}, 100 μL) for 15 min. The reaction was quenched with 150 μL 1 M sulphuric acid and optical densities (O.D.) at 490 nm determined.

Radioimmunoassay (RIA) of IL-1β

Intracellular immunoreactive IL-1β in MONO MAC 6 and THP-1 cells were measured by radioimmunoassay (RIA) as described previously (Poole et al 1989b).

Maintenance of cells and cell lines; preparation and stimulation of cells

THP1 cells seeded at 2 × 10^5 cells mL^{-1} were grown in RPMI 1640 containing 2 mM L-glutamine (Gibco or Flow Laboratories) + 5% heat-inactivated foetal calf serum (HIFCS, Gibco or Flow Laboratories). The culture medium was replaced twice weekly.

MONO MAC 6 cells seeded at 2 × 10^5 cells mL^{-1} were grown in RPMI 1640 containing 2 mM L-glutamine to which was added 5% HIFCS, 0·1 mM MEM non-essential amino acid solution (Sigma), 0·23 int. units mL^{-1} bovine insulin (Sigma), 1 mM oxalacetic acid (Sigma) and 1 mM sodium pyruvate (Sigma). The culture medium was replaced twice weekly.

Human blood mononuclear cells (MNC) were isolated from buffy coat residues of heparinized peripheral blood from healthy donors by density gradient centrifugation using Histopaque-1077 (Sigma). Buffy coat residues from the Blood Transfusion Service were diluted 1 : 1 with RPMI 1640 and 35 mL of this solution was layered onto 15 mL Histopaque-1077 in a 50 mL test-tube and centrifuged at 400 *g* for 30 min. The upper layer of plasma was discarded and the MNC layer at the interface of the plasma and histopaque was taken, washed twice in RPMI (400 *g*, 15 min) and resuspended at 5 × 10^6 or 10^7 cells mL^{-1} in culture medium (RPMI 1640) containing 2% HIFCS.

Monocytes (MC) were isolated from the MNC (10^7 cells mL^{-1}) by adherence to the plastic bottoms of 24-well tissue culture plates (Falcon 3047) or 2 mL flat-sided tissue culture tubes (Nunc 1-567758) for 1 h at 37°C. Non-adherent cells were removed by three washes with RPMI.

MC (adherent cells from 10^7 MNC mL^{-1}), MNC (5 × 10^6 cells mL^{-1}) THP-1 cells (0·5, 1·0 or 2·0 × 10^6 cells mL^{-1}) and MONO MAC 6 cells (0·5, 1·0 or 2·0 × 10^6 cells mL^{-1}) in 1·8 mL RPMI 1640 + 2% HIFCS were incubated with 0·2 mL RPMI 1640 containing endotoxin.

Preparation of IL-6 and IL-1β from cells

After 6, 16 or 24 h culture at 37°C, conditioned media were collected and stored at −70°C until assayed for IL-6. MONO MAC 6 and THP-1 cells were lysed with 300 μL water, made up to a final volume of 1 mL with 700 μL RPMI 1640 containing 2·9% HIFCS, to obtain intracellular IL-6 and IL-1β which were stored at −70°C.

Detection of pyrogen using IL-6 release from MONO MAC 6 cells

A 'monocyte test' for pyrogen in which evoked secretion of immunoreactive IL-6 from MONO MAC 6 cells as the measured variable was carried out on six preparations of human serum albumin (HSA) and aliquots of HSA preparations with endotoxin added 24 h previously. The results were compared with those from LAL tests (European Pharmacopoeia 1987) and rabbit pyrogen tests (European Pharmacopoeia 1971) on the same batches. MONO MAC 6 cells were centrifuged at 400 *g* for 10 min and resuspended at 2 × 10^6 cells mL^{-1} in 2 × concentrated RPMI + 4% HIFCS. Samples of cell suspension (0·5 mL) were dispensed into each well of 24-well tissue culture plates and 0·5 mL of a 1/5 dilution of HSA in PFDW was added. The cells were incubated overnight (16 h) in 5% CO_2 at 37°C and the conditioned medium assayed for IL-6. The standard curve was prepared in a reference preparation of HSA which contained low levels of endotoxin below the sensitivity (3 pg = 0·02 int. units mL^{-1}) of a LAL test and which was devoid of pyrogenic activity in rabbits and in human recipients.

Results

ELISA of IL-6

A typical standard curve of the ELISA of IL-6 is shown in Fig. 1. The sensitivity, or detection limit, was 8 pg mL^{-1}, a concentration of IL-6 which gave O.D. values ≥ two standard deviations above those obtained for 0 pg mL^{-1} IL-6. The rectilinear relation between log IL-6 concentration and log O.D. extended up to 1000 pg mL^{-1} IL-6. No cross-reactivity (< 0·01%) with IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-8, GCSF, GMCSF or TNFα was apparent in this assay.

Endotoxin-evoked IL-6 production

The ELISA of IL-6 was used to quantify endotoxin-evoked immunoreactive IL-6 production by monocytes, the mononuclear cell fraction of human peripheral blood, THP-1 and MONO MAC 6 cells. Concentrations of IL-6 secreted from cells incubated for 16 or 24 h with *E. coli* endotoxin are shown in Figs 2 and 3. Concentrations of intracellular and

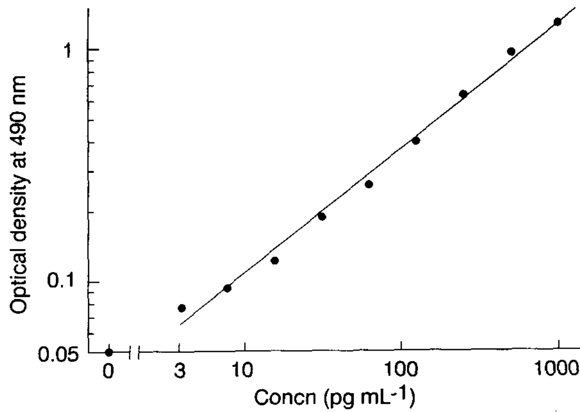

FIG. 1. Standard curve for ELISA of IL-6. Values are means of triplicates, s.e.m. (not shown) were <5% of means.

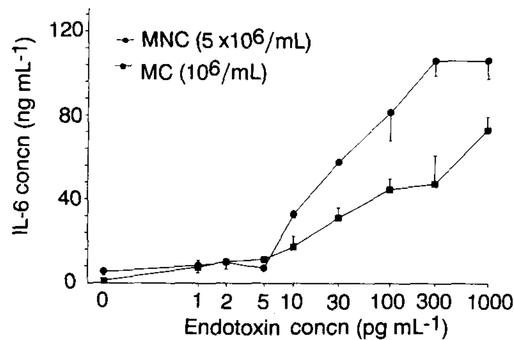

FIG. 2. The dose-response curves of immunoreactive IL-6 secretion by human peripheral blood mononuclear cells (MNC) and monocytes (MC) stimulated with *E. coli* endotoxin for 24 h. Values are means of triplicates ± s.e.m.

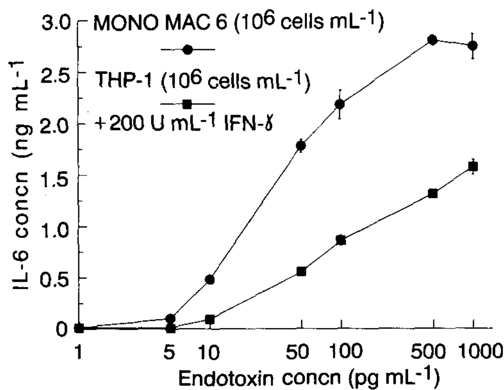

FIG. 3. The dose-response curves of immunoreactive IL-6 secretion by MONO MAC 6 and THP-1 monocytic cells stimulated with *E. coli* endotoxin for 16 h. Values are means of triplicates ± s.e.m.

cell-associated IL-6 from all cell types tested were <30 pg mL^{-1}.

MC and MNC responded to incubation with endotoxin (1–1000 pg mL^{-1}) for 24 h by secreting IL-6 (for 1000 pg mL^{-1} endotoxin: approx. 80 ng mL^{-1} and approx. 100 ng mL^{-1} IL-6, respectively) with dose-response curves generally steeper for MNC (Fig. 2). Addition of gamma interferon (IFN-γ) to the cells, either 1 h before or together with the endotoxin, did not significantly affect IL-6 secretion (data not shown). MONO MAC 6 cells and THP-1 cells also secreted IL-6 in a dose-dependent manner when incubated with endotoxin (1–1000 pg mL^{-1}) for 16 h (Fig. 3). For both cell lines the quantities of IL-6 secreted in response to a given dose of endotoxin were much less than those secreted by similar numbers of MC or MNC (for 1000 pg mL^{-1} endotoxin: approx. 2 ng mL^{-1} from MONO MAC 6 cells and approx 1 ng mL^{-1} from THP-1 cells). MONO MAC 6 cells generated a steeper dose-response curve than THP1-cells and this was unaffected by addition of IFN-γ (2000 units mL^{-1}) whereas THP-1 cells secreted IL-6 in response to endotoxin only in the presence of IFN-γ.

Stability of cell-lines

The MONO MAC 6 cell line was used for 40 generations (passages 30–70) without significant changes in its response to endotoxin. After this time, the threshold dose of endotoxin required to evoke IL-6 release remained 2·5 pg mL^{-1} but the quantities of IL-6 produced in response to larger doses of endotoxin were diminished. The THP-1 cell line was used for 100 generations without significant changes in its response to endotoxin.

Endotoxin-evoked IL-1β production

Concentrations of intracellular and cell-associated immunoreactive IL-1β in THP-1 and MONO MAC 6 cells incubated with *E. coli* endotoxin for 16 h are shown in Fig. 4. MONO MAC 6 cells and THP-1 cells responded to endotoxin by increasing production of intracellular IL-1β. MONO MAC 6 without added IFN-γ cells generated a steeper dose-response curve than THP-1 cells in the presence of IFN-γ (Fig. 4).

Detection of pyrogens using IL-6 release from MONO MAC 6 cells

MONO MAC 6 cells secreted detectable IL-6 (11·3 ± 0·8 pg mL^{-1}, mean ± s.e.m.) in response to a minimum concentration of 2·5 pg mL^{-1} endotoxin, yielding a test system for pyrogen with a sensitivity of 25 pg mL^{-1} endotoxin for products such as HSA tested at a dilution of 1/10. The dilution of 1/10 was chosen since this dilution of 5% HSA caused minimal shift in the dose-response curve (Fig. 5).

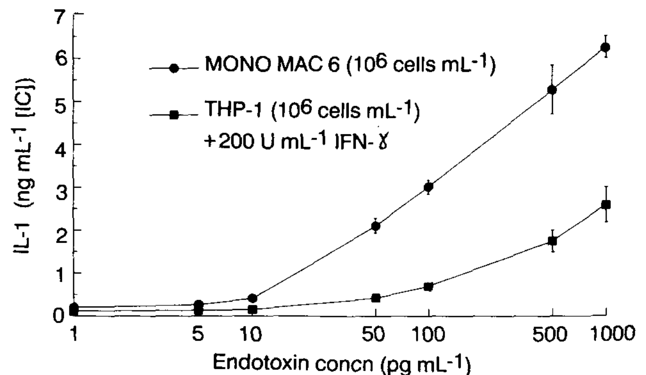

FIG. 4. The dose-response curves of intracellular and cell associated [IC] immunoreactive IL-1β produced by MONO MAC 6 and THP-1 monocytic cells stimulated with *E. coli* endotoxin for 16 h. Values are means of triplicates ± s.e.m.

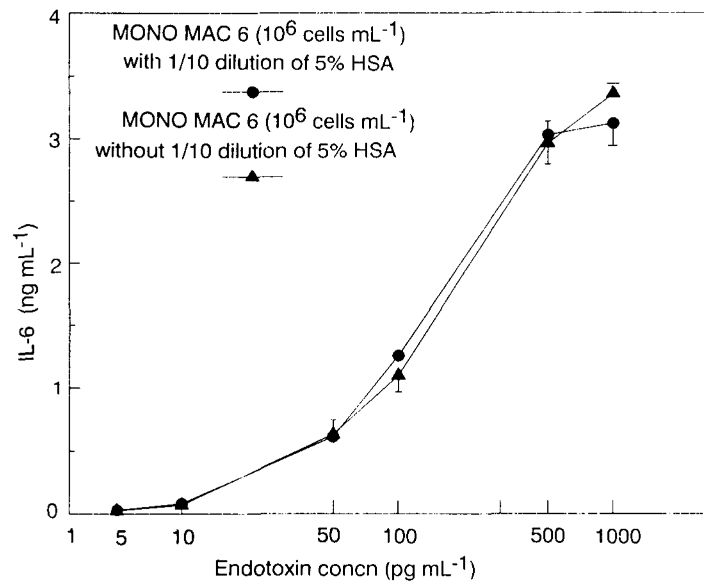

FIG. 5. The effect of a 1/10 dilution of 5% human serum albumin (HSA) on the dose response curve of immunoreactive IL-6 secretion by MONO MAC 6 cells stimulated with endotoxin for 16 h. Values are means of triplicates ± s.e.m.

Table 1. Detection of pyrogen in 6 batches of HSA by IL-6 release, LAL test and rabbit pyrogen test.

HSA (preparation)	Endotoxin by IL-6 release (pg mL^{-1})	Endotoxin by LAL (int. units mL^{-1})	Endotoxin by LAL (pg mL^{-1})[e]	Rabbit pyrogen test (pass or fail)
1[a]	97 ± 2·3[bc]	1·0–2·0	(140–280)	Pass
2[a]	30 ± 2·8[c]	2·4–3·2	(336–448)	Pass
3[a]	31 ± 2·3[c]	0·5–0·75	(70–105)	Pass
4	< 25[d]	< 0·24	(< 34)	Pass
5	< 25[d]	3·6–4·8[f]	(504–672)	Pass
6	< 25[d]	< 0·26	(< 36)	Pass

[a] Preparations which caused adverse reactions in recipients; [b] values are means ± s.e.m.; [c] values significantly different from subthreshold doses of endotoxin (< 2·5 pg mL^{-1}, $P < 0·001$); [d] values below the detection limit of the test system: preparations of HSA were tested at a dilution of 1/10 and 2·5 pg mL^{-1} endotoxin was the lowest dose of endotoxin tested which evoked a significant release of IL-6; [e] 1 int. unit = 0·14 ng for preparation 84/650; [f] false positive.

A comparison of the endotoxin content of six batches of human serum albumin (HSA, 5% w/v) as detected in a 'monocyte test' using MONO MAC 6 cells, a LAL test and a rabbit pyrogen test is shown in Table 1. Three of the batches (nos 1, 2 and 3) had caused adverse (pyrogenic) reactions in recipients despite having passed the rabbit pyrogen test and the LAL test: the pyrogenic contamination in these batches was detected in the monocyte test. One batch of HSA (no. 5), which was not pyrogenic in recipients, was negative in the monocyte test and in the rabbit pyrogen test but gave a false positive in a LAL test.

Discussion

We report the development, and application to batches of therapeutic HSA, of a novel in-vitro test—monocyte test—for pyrogen that does not use experimental animals. In the test, the end point is pyrogen-evoked secretion of IL-6 by the monocytic cell line MONO MAC 6. This end point is believed to represent a key event in the development of pyrexia. We evaluated two cell lines for their suitability for use in the 'monocyte test' by comparing their responses to endotoxin with those of fresh monocytes and mononuclear cells. MONO MAC 6 and THP-1 cells, while producing less IL-6 than freshly prepared monocytes, generated test systems for pyrogen which showed similar sensitivity to endotoxin (approx. 2·5 pg mL^{-1}).

Peripheral blood monocytes produce intracellular IL-1β in response to endotoxin concentrations as low as 0·1 pg mL^{-1} (Poole et al 1989b). The cell lines studied here also produced IL-1β in response to endotoxin, but only at doses of endotoxin above 50 pg mL^{-1}, making intracellular IL-1β a less useful endpoint than secreted IL-6. Also, the use of IL-1β as the measured variable has the disadvantage of requiring a cell-lysis step.

MONO MAC 6 cells offer advantages over THP-1 cells. They respond to lower doses of endotoxin, without co-stimulation with IFN-γ, with a steeper dose-response curve. The MONO MAC 6 cell line is stable and has been used for 40 generations (passages 30–70) in this study without significant changes in its response to endotoxin. Therefore IL-6 secretion from MONO MAC 6 cells was chosen as the basis of our in-vitro monocyte test for pyrogen. While sensitive to 2·5 pg mL^{-1} endotoxin, i.e. a similar sensitivity to that of the LAL test, in practice the sensitivity of the MONO MAC 6 'monocyte test' was limited by the need to dilute out interfering activity associated with the product (HSA) or excipients, a feature shared with the LAL test, which is also sensitive to interference by a variety of pharmaceutical products (Pearson et al 1985). Preliminary experiments had shown that a twofold dilution of HSA enhanced whereas a fourfold dilution inhibited endotoxin-evoked IL-6 release. The tenfold dilution step required gave an effective detection limit of 25 pg mL^{-1} endotoxin. For comparison, the sensitivity of the rabbit pyrogen test is approx. 1 ng mL^{-1} (Poole & Mussett 1989).

Preparations of HSA have been shown to be contaminated with pyrogens that were detected in an in-vitro 'monocyte test' (measuring IL-1 and TNFα release from peripheral blood monocytes) but not in a rabbit pyrogen test or LAL test (Poole et al 1988). In the present study the 'monocyte test', utilizing IL-6 release from MONO MAC 6 cells, detected pyrogen in three batches of 5% HSA that had proved to be pyrogenic in recipients although the pyrogen had not been detected in rabbit pyrogen tests and the batches contained low levels of endotoxicity in LAL tests. Also, the 'monocyte test' correctly identified as a pyrogen-free a batch of HSA that was not pyrogenic in rabbits or in human recipients but which was positive in a LAL test.

The novel test system described above thus represents an important alternative to the existing tests for pyrogen. While the simple, quick and relatively inexpensive LAL test will remain the choice for monitoring manufacturing processes and reagents, the 'monocyte test' would appear to be a more appropriate end product test for parenterals such as HSA which have been shown to contain pyrogens detected only with this test system.

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