

A clinically relevant *in vitro* pyrogen test using a human cell line that have the similar responsiveness to various pyrogens to that of human peripheral blood cells (hPBC)

Akihiko Yamamoto, Masaki Ochiai, Kazunari Kamachi, Michiyo Kataoka, Hiromi Toyozumi,
Yoshichika Arakawa and Yoshinobu Horiuchi

National Institute of Infectious Diseases

Corresponding author: Akihiko Yamamoto

Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases

4-7-1, Gakuen, Musashimurayama, Tokyo 208-0011, Japan

Phone: +(81)-42-561-0771, Fax: +(81)-42-567-0740, yama-aki@nih.go.jp

Abstract

It is crucial to control not only content but also *in vivo* toxic action of contaminated bacterial pyrogens for the safety of parenteral drugs. However, toxic activity of such pyrogens would vary depending on their source bacteria and even human peripheral blood cells (hPBC) show varied responsiveness to the pyrogens depending on source individual. However, the responsiveness of hPBC to each of the pyrogens was found to be consistent irrespective of source individual when evaluated in relative to that to Reference Standard Endotoxin. HPBC showed clear correlations among TNF- α , IL-6 and IL-1 β productions in response to a panel of pyrogens in terms of the relative responsiveness. When nine different human monocytoic cell lines were examined, MM6, THP-1 and 28SC cells showed dose-dependent IL-6 productions. However, MM6 and THP-1 failed to show consistency with hPBC in responding to the panel of pyrogens. 28SC cells showed appropriate consistency with hPBC not only in the responses to the pyrogens but also in detecting the effect of human interferons to augment endotoxin to induce IL-6.

Keywords: validation method, pyrogen, human reaction, human cell line

Introduction

It is crucial to control not only content but also *in vivo* toxic action of contaminated bacterial pyrogens for the safety of parenteral drugs (Ho, 2001). A test method for directly evaluating *in vivo* action of pyrogens including the *in vivo* synergism is necessary for the effective safety control of such drugs. *In vitro* substitutes of the pyrogen test using human whole blood or cell lines of human origin have been described by some workers (Moesby, 1999, Pool, 1988, Nakagawa, 2002, Eperson, 1996). However, toxic activity of such pyrogens would vary depending on their source bacteria and even human peripheral blood cells (hPBC) show varied responsiveness to the pyrogens depending on source individual. We are presenting the results of a precise examination of nine different human monocytoic cell lines by using a quantitative method of evaluation to select an adequate cell line that has consistent responsiveness with that of human peripheral blood cells including the capability to detect a synergistic effect between drugs and pyrogens.

Materials and methods

Pyrogens

1. Endotoxins:

Japanese National Reference Standard Endotoxin Lot 3 (RSE) extracted from *Escherichia coli* UKT-B strain (13,000 endotoxin units (EU) per vial) (Murai, 1997) and commercial endotoxins extracted from *E. coli* O111: B4, *E. coli* O55: B5, *E. coli* O127: B8, *Salmonella typhosa* O901, *Salmonella enteritidis* (Difco Laboratories, Mi., USA), *S. abortus equi*, *Pseudomonas aeruginosa* Serotype 10, *Klebsiella pneumoniae* and *Vivrio cholerae* Serotype Inaba 569B strain (Sigma Chemical Co., Mo., USA) were used. Endotoxins were measured by colorimetric method (Endospeccy, Seikagaku Corp., Tokyo, Japan) referring RSE.

2. Peptideglycans and β -glucan:

Commercial peptideglycans extracted from *Staphylococcus aureus* (Sa), *Enterococcus faecalis* (Ef), *Lactobacillus acidophilus* (La), *Saccharomyces cerevisiae* (Sc), *Streptomyces* spp. (Ss), *Micrococcus luteus* (Ml) and beta-glucans such as CM-Curdlan, Zymosan (*Saccharomyces cerevisiae*), *Euglena gracilis*

(Eg), Laminarin (*Laminaria digitata*), Sizofiran, Lentinan were used. Peptideglycans and β -glucan were measured by SLP reagent (Wako pure Chemical Industries, Ltd.) for Peptideglycan and BG star (Wako) for β -glucan.

Biological products

Five batches each of commercially available human interferon (hIFN)- α , - β and - γ injections (Sumitomo Pharmaceuticals Co., Ltd. Osaka, Ohtsuka Pharmaceutical Co., Ltd. Tokyo, Mochida Pharmaceutical Co., Ltd. Tokyo, Toray Industries, Inc., Tokyo, or Suntory Pharmaceuticals Inc. Osaka, Japan) were purchased.

Human monocytoid Cell lines:

THP-1 (ATCC TIB 202), P31/FUJ (JCRB0091), P39/TSU (JCRV0092), MD (ATCC CRL 9850), 90196B (ATCC CRL 9853), EL1 (ATCC CRL 9854), MM6 (DSMZ ACC 124; one of the candidates for *in vitro* pyrogen test recommended by ECVAM), 28SC (ATCC CRL 9855) and KMA (ATCC CRL 9856) were used.

In vitro human peripheral blood cell (hPBC) assay:

Peripheral bloods were collected from healthy donors. A 100 μ l-volume of heparinised fresh human peripheral blood was mixed with 900 μ l of a dilution of test samples or RSE to incubate at 37 °C. After 18 hr incubation, supernatants of the mixtures were isolated to determine IL-1 β , IL-6 and TNF- α concentrations by enzyme-linked immunosorbent assay methods (ELISA) (BD PharMingen, San Diego, USA).

In vitro IL-1 β , IL-6 and TNF- α induction assays in human monocytoid cell lines:

Each of the cell lines was cultured and adjusted the concentration to 1×10^6 cells/ml. A 100 μ l-portion of each of the cultured cells was distributed to wells of a 96-well tissue culture plate (Nunc D, Nalge Nunc International Co., New York, USA). The cells were stimulated with a 100 μ l-portion of a dilution of test samples 72 hr later. Culture supernatants were isolated 18 h after the stimulation to assay IL-1 β , IL-6 and TNF- α concentrations by ELISA systems.

Statistical analysis

Relative activity was estimated according to the parallel line assay method (Finney, 1978). $P=0.05$

Results

Cytokine-induction activity in hPBC from different donors stimulated with various sources of endotoxins

HPBC culture was stimulated with serially diluted Reference Standard Endotoxin (RSE) and the

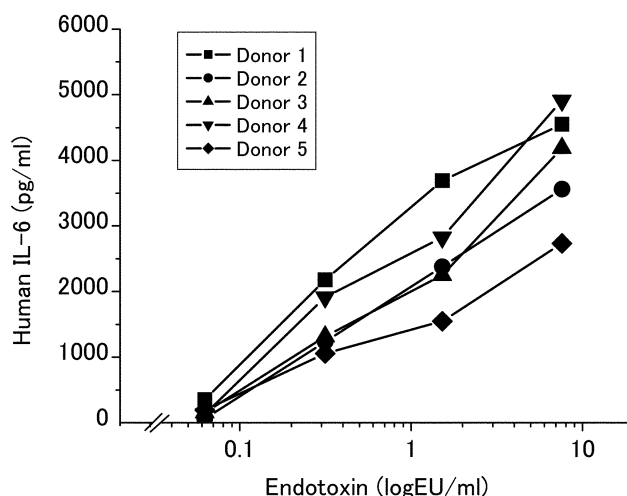


Fig. 1. HPBC show varied responsiveness to endotoxin HPBC collected from 5 individual donors were stimulated with graded concentrations of RSE to measure the concentrations of IL-6 in the culture supernatants 18 h later. HPBC showed IL-6 production in a dose-dependent manner. The production variance of IL-6 among 5 donors were up to 4 times.

supernatants were collected to measure IL-6 releases in culture fluid. The assay was repeated using hPBC from five different donors. HPBC from different donors showed considerable variation in production of IL-6 in response to RSE. HPBC show varied responsiveness to the pyrogens depending on source individual (Fig. 1).

We employed a panel of endotoxins of various sources for comparing test methods. The endotoxins were serially diluted with physiological saline at five-fold intervals from 300 to 0.48 EU/ml according to their endotoxin test results. HPBC culture was stimulated with the dilutions to measure IL-1 β , IL-6 and TNF- α releases in culture fluid to evaluate the cytokine-induction activities of each endotoxin relatively to those of RSE according to the parallel line assay method. Accordingly, the activities of endotoxins to induce IL-1 β , TNF- α and IL-6 were calculated as geometric means of the relative activities obtained in the repeated measurements. Induction profiles of IL-1 β and TNF- α in hPBC in response to endotoxins of various sources were found to be quite similar to that of IL-6. Although hPBC from different donors showed considerable variation in production of the cytokines in response to endotoxin, relative cytokine inductions by each of the donors referring to those by RSE were found to be highly reproducible between the repeated measurements. The responsiveness of hPBC to each of the pyrogens was found to be consistent irrespective of source individual when evaluated in relative to that to RSE (Fig. 2).

Fine correlations were seen between TNF- α and IL-6 ($r=0.945$ ($P<0.0001$)) and between IL-1 β and IL-6 ($r=0.914$ ($P=0.0002$)) inductions. The relationship of induction profiles between the

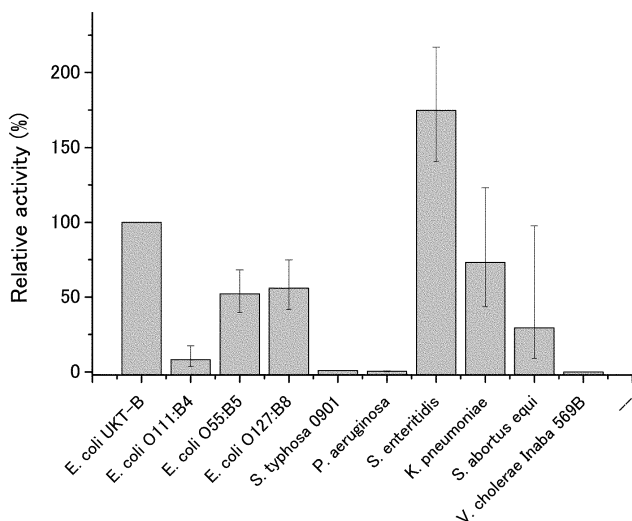


Fig. 2. Relative cytokine-induction activity in human peripheral blood cells of endotoxins of various different sources referring to that of RSE.

The endotoxins were serially diluted and were added to human peripheral blood cell culture to measure the production of IL-6 using an ELISA method. IL-6 induction activity of each endotoxin was calculated relatively to RSE according to the parallel line assay method. Although the levels of IL-6 production showed a considerable variation in the repeated measurements using blood from two different donors, the relative activity of each of the endotoxins was found to be quite reproducible. Accordingly, IL-6 induction activity of each endotoxin was shown as geometric mean and standard deviation of the repeated measurements of relative activity. The relative IL-6 induction activity of endotoxins showed a considerable variation in spite of comparison based on equivalent levels of LAL activity.

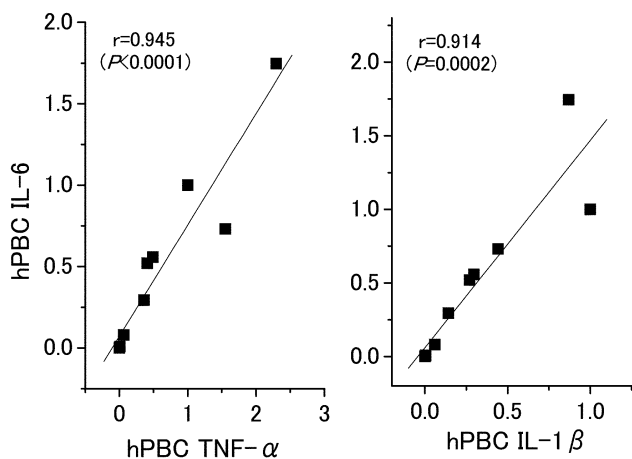


Fig. 3. Relationship between induction profiles of IL-6, IL-1 β and TNF- α by endotoxins of various sources in human peripheral blood cells.

Activities of the endotoxins to induce IL-6, IL-1 β and TNF- α in human peripheral blood cells were measured relatively to the activities of RSE similarly to Fig. 1. A particular characteristic of the responsiveness of human peripheral blood cells was shown to be the quite similar induction profiles of IL-1 β and TNF- α to that of IL-6 in response to endotoxins of various sources.

cytokines was used, hereafter, to compare with other assays as the characteristics of hPBC. hPBC showed clear correlations among TNF- α , IL-6 and IL-1 β productions in response to a panel of pyrogens in terms of the relative responsiveness (Fig. 3).

A cell line test system for endotoxin to have the responsiveness similar to hPBC

We searched for a cell line that exhibits the similar responsiveness to hPBC by detailed examinations using a panel of endotoxins. We examined IL-6 production in nine different monocytoid cell lines of human origin in response to the stimulation with graded concentrations of RSE to measure IL-6 in the culture fluid after incubating for 18 hr as shown in Fig. 4. Cell lines 90196B, KMA, P31/FUJ and P39/TSU showed no significant production of IL-6 over the control levels in response to the stimulation with RSE up to 5,000 EU/ml. Cell line MD was a spontaneous producer of a rather high level of IL-6 irrespective of stimulation. MM6, THP-1 and 28SC responded sensitively among those cell lines to produce IL-6 to graded concentrations of RSE. EL1 showed IL-6 production of a significant level over the control but of a much lower level than MM6, THP-1 or 28SC cells in response to RSE of over 40 EU/ml.

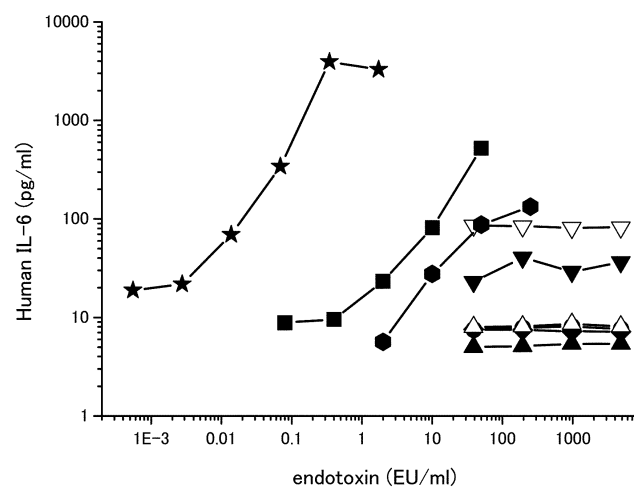


Fig. 4. IL-6 production of human monocytoid cell lines in response to graded concentrations of endotoxin.

Cultures of eight different human monocytoid cell lines were stimulated with graded concentrations of RSE to measure the production of IL-6 in culture supernatants 18 h later according to the parallel line assay method. Cells without RSE stimulation were also examined in parallel as the controls. Cell lines 90196B (\blacktriangle), KMA (∇), P31/FUJ (\blacktriangledown) and P39/TSU (\blacklozenge) showed no significant dose-dependent response to RSE to produce IL-6 over the control levels. Cell line MD (\square) spontaneously produced a significant level of IL-6 without stimulation and showed no dose-dependent change in IL-6 level. Although MM6 (\star), THP-1 (\bullet) and 28SC (\blacksquare) cells showed clear dose-dependent responses to RSE, 28SC cells were found to be the most sensitive cell line among those tested. EL1 cells (\triangle) produced IL-6 of a much lower level than THP-1 or 28SC but of a significant level over the control.

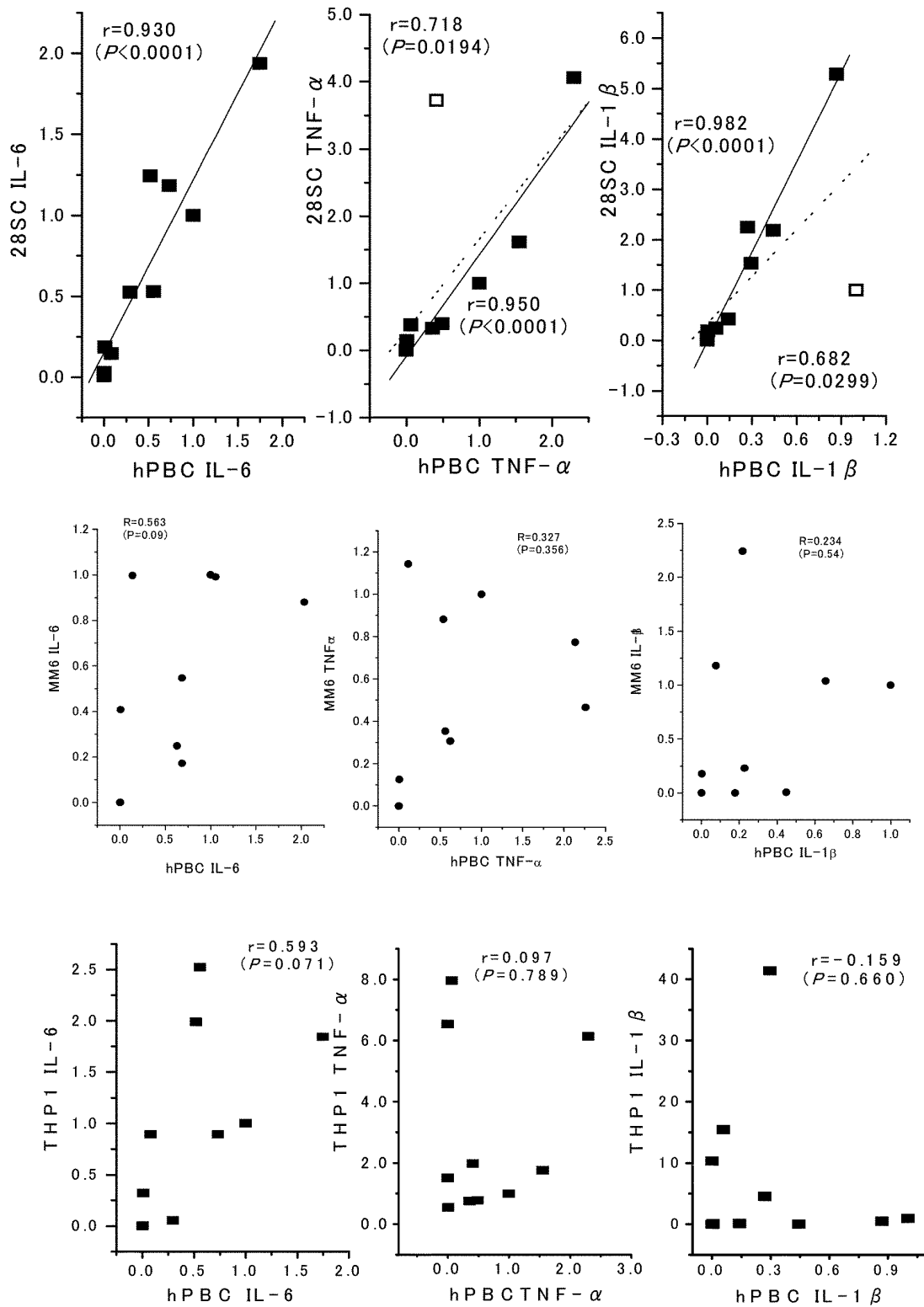


Fig. 5. Comparison of proinflammatory cytokine inductions by endotoxins of various sources in MM6, THP-1 and 28SC cells with those in human peripheral blood cells.

Relative activities of endotoxins of various sources to induce IL-6, TNF- α and IL-1 β in MM6, THP-1 and 28SC cells were measured referring to the activities of RSE. Profiles of the relative cytokine productions in MM6, THP-1 and 28SC cells in response to the endotoxins were compared with the responses of human peripheral blood cells. There was no consistency between responses of MM6, THP-1 and human peripheral blood cells. While 28SC cells showed clear correlations in productions of IL-6, TNF- α and IL-1 β with those of human peripheral blood cells to prove the consistent responsiveness with that of hPBC. Correlations between human peripheral blood cells and 28SC cells in TNF- α and IL-1 β productions could be improved considerably by excluding extreme results of *E. coli* O55:B5 (□).

We carried out further examinations of TNF- α and IL-1 β productions in MM6, THP-1 and 28SC cells according to the results shown in Fig. 3. MM6, THP-1 and 28SC cells were further evaluated their responses to endotoxins of various sources to produce IL-6, TNF- α and IL-1 β to compare with the responses of hPBC. Correlation coefficients between MM6, THP-1 cells and hPBC to produce IL-6, TNF- α and IL-1 β in response to the panel

of endotoxins were uncalculated (Fig. 5). While, correlation coefficients between 28SC cells and hPBC to produce IL-6, TNF- α and IL-1 β in response to the panel of endotoxins were 0.930 ($P<0.0001$), 0.718 ($P=0.0194$) and 0.682 ($P=0.0299$), respectively. The correlations of TNF- α and IL-1 β productions could be improved by eliminating the results of *E. coli* O55: B5 for TNF- α and RSE for IL-1 β significantly to 0.950 ($P<0.0001$) and 0.982 ($P<0.0001$), respectively.

Responses of MM6, THP-1 and 28SC cells to the panel of endotoxins to produce the cytokines were further compared with hPBC. When relative activities of endotoxins to induce IL-6, TNF- α and IL-1 β were measured referring to the activities of RSE, hPBC showed clear correlations between production profiles of the cytokines as shown in Fig. 2, while MM6 and THP-1 cells showed no such correlations between IL-6 and TNF- α and between IL-6 and IL-1 β productions in response to the endotoxins. On the other hand, 28SC cells showed clear correlations, as similarly to hPBC, between IL-6 and TNF- α ($r=0.907$ ($P=0.0003$)) and also between IL-6 and IL-1 β ($r=0.931$ ($P<0.0003$)) productions. Overall, MM6 and THP-1 failed to show consistency with hPBC in responding to the panel of pyrogens (Fig.6).

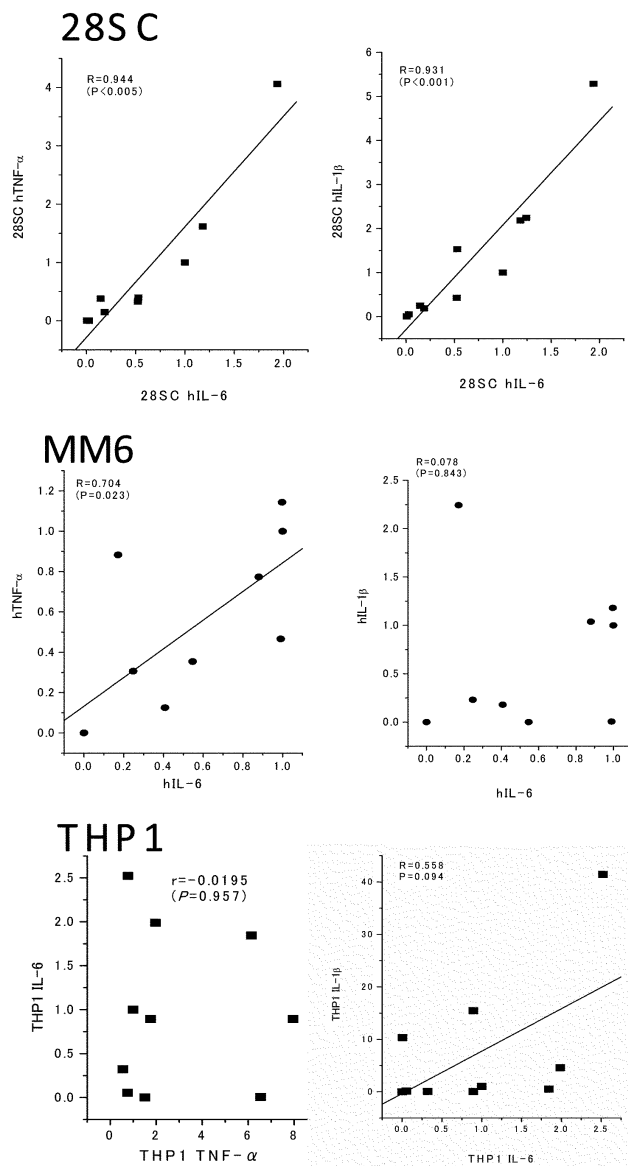


Fig. 6. Comparison of induction profiles of proinflammatory cytokines in MM6, THP1 and 28SC cells by endotoxins of various sources.

Activities of the endotoxins to induce IL-6 and TNF- α were assayed in MM6, THP-1 and 28SC cells relatively to the activities of RSE. Different from human peripheral blood cells, no consistent relationship was seen between IL-6 and TNF- α productions by the endotoxins in MM6 and THP-1 cells. On the other hand, 28SC cells showed a clear correlation between IL-6 and TNF- α productions in response to the endotoxins to suggest consistency of the responsiveness with that of human peripheral blood cells shown in Fig. 2.

Detection of synergistic effect between hIFNs and endotoxin

Effect of human IFN (hIFN) injections on endotoxin activity was examined in hPBC and 28SC cells. Serial concentrations of hIFN- α , hIFN- β or hIFN- γ were mixed with graded concentrations of RSE at five fold intervals and added to the cultures of hPBC and 28SC cells to examine IL-6 induction. The same dilutions of RSE without mixing with hIFNs and 10^4 U/ml of the hIFNs without RSE were also examined in parallel as the controls. IL-6 in the culture supernatants was determined by an ELISA after incubating at 37 °C for 18 hr. Doses of RSE used for stimulating hPBC were 12.5, 2.5, 0.5 and 0.1 EU/ml and those used for 28SC cell were 50, 10, 2 and 0.4 EU/ml. IL-6 induced by the graded doses of the control RSE in hPBC were 702.6, 431.2, 263.6 and 23.6 pg/ml, respectively, and that induced by 10^4 U/ml of the control hIFN- α , hIFN- β and hIFN- γ without RSE were 8.3, 9.1 and 9.4 pg/ml, respectively. However, 12.2 pg/ml of IL-6 was detected in the supernatant of hPBC culture without stimulating with RSE or hIFNs. Therefore, the IL-6 detected after stimulating with hIFNs alone could be considered as negligible. The graded concentrations of RSE induced 521.7, 84.4, 23.2 and 9.5 pg/ml, respectively, of IL-6 in 28SC cells, while no excess IL-6 over the detectable limit of 4.6 pg/ml was induced by any of the hIFNs without endotoxin even at 10^4 U/ml. IL-6 induced by the mixtures of dilutions of hIFNs and RSE was compared relatively to that induced by the control RSE to estimate the enhancing

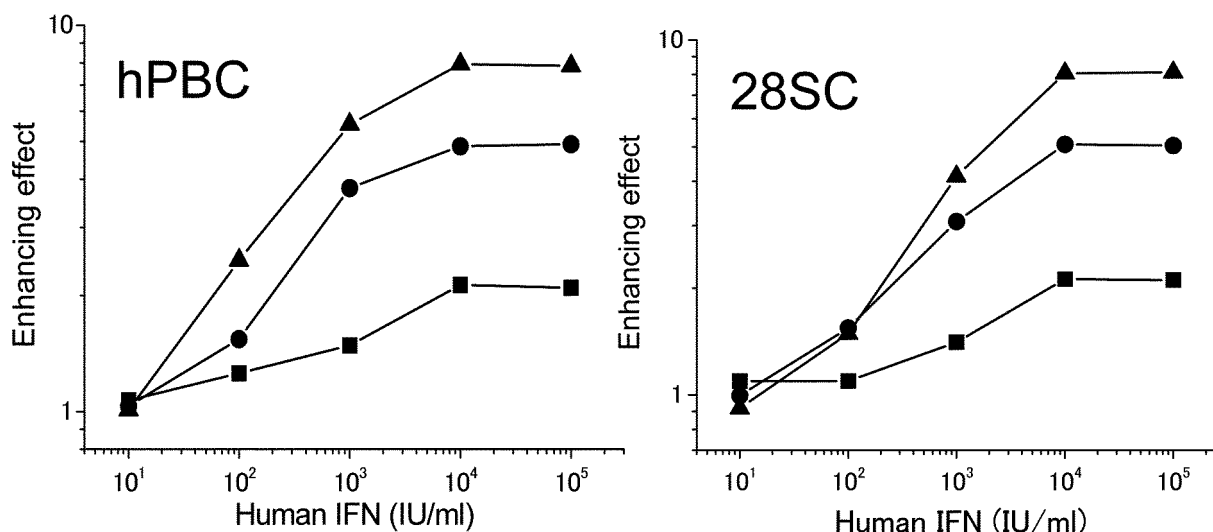


Fig. 7. Dose dependent effect of hIFN- α , hIFN- β and hIFN- γ to augment endotoxin to induce IL-6 in human peripheral blood cells and 28SC cells.

Human peripheral blood cells and 28SC cells were stimulated with serial dilutions of RSE ranging from 12.5 to 0.1 EU/ml or from 50 to 0.4 EU/ml, respectively. The cells were also stimulated with a mixture of a dilution of RSE and varied concentrations of IFNs as indicated in the figure or 10^4 U/ml of hIFNs without RSE to measure IL-6 production. Enhancing effects of IFNs were calculated relatively to the response to RSE alone. IFN- α (●), IFN- β (■) and IFN- γ (▲) augmented IL-6 induction of RSE both in hPBC (a) and 28SC cells (b) in a highly consistent manner.

effect quantitatively. The hIFNs mixed with RSE enhanced IL-6 induction in the both cells in a dose-dependent manner as shown in Fig. 7. Furthermore, the responses of hPBC and 28SC cells to hIFN- α , hIFN- β and hIFN- γ were shown to be quite consistent with each other to suggest again that 28SC cell line is an appropriate indicator of the responsiveness of human blood cells.

It is crucial for the safety control of interferon injections to regulate not only amount of contaminated endotoxin but also the adverse reactivity in humans. As 28SC cell were found to have consistent responsiveness with that of hPBC, we attempted to make a quantitative evaluation of the enhancing effect of hIFNs on the endotoxin activity to induce IL-6 using 28SC cells. We applied the method for evaluating the enhancing effect of five batches each of commercial hIFN- α , hIFN- β and hIFN- γ

injections on IL-6 induction by RSE. The results were compared with that in hPBC for a validation and summarized in Table 1. The batches of hIFN- α were shown to enhance the activity of RSE to induce IL-6 by approximately 2.2 fold with only a little batch-to-batch variation in the enhancing activity. The extent of enhancement was almost similar to that in hPBC as shown in the table. Batches of hIFN- β and hIFN- γ injections showed the enhancing effect of approximately 5.1 and 7.9 folds, respectively, and also showed a little batch-to-batch variation. The enhancing effects in 28SC were again shown to be consistent with those in hPBC.

Discussion

There are parenteral drugs that augment *in vivo* biological activities of endotoxin (Ho, 2001). Effective safety control tests for endotoxin contamination in such drugs require not only quantifying endotoxin but also regulating overall *in vivo* action of contaminated endotoxin. Particularly, drugs that show a species specificity in the synergistic enhancement would require a special caution for interpreting results of control tests (Terrell, 1993). Both the current pyrogen test and the endotoxin test are not adequate for predicting clinical safety of such drugs if not validated appropriately by taking the synergistic effect into consideration (Yamamoto, 2002). It is, therefore, assumed crucial to establish an assay method that can effectively predict the overall response of humans including the augmentation to contaminated endotoxin in pharmaceuticals.

Fennrich S. et al. and Pool E. et al. reported assay

Table 1.

Sample	Cell	Enhancing effect*	$\pm 2SD$ †
Control endotoxin		1.000	
Endotoxin + IFN- α	28SC	2.173	(1.980 - 2.367)
	hPBC	2.126	
Endotoxin + IFN- β	28SC	5.227	(4.874 - 5.580)
	hPBC	5.097	
Endotoxin + IFN- γ	28SC	7.938	(7.509 - 8.367)
	hPBC	7.942	

*: Calculated relatively to the control endotoxin

†: Measured on 5 batches

methods using peripheral human blood. However, an assay using human blood might have difficulties relating to safety and ethics. Other than human blood, human monocytoid cell lines were also examined their potential of indicator cells for testing pyrogenic substances in medical products (Moesby, 1999, Pool, 1988, Nakagawa, 2002, Eperson, 1996). Although the cell line assays were reported to be effective to detect pyrogens, validation procedures employed by the authors were not adequate enough for proving consistency of responsiveness of the cell lines with that of humans. There is a human cell line named MM6 that is recommended as a candidate of *in vitro* pyrogen test method by ECVAM (Thomas, 2006).

We examined nine different human monocytoid cell lines using the panel of endotoxins of various sources to identify 28SC cells to have the quite consistent responsiveness to endotoxins with that of hPBC. Furthermore, 28SC cells were shown to be effective in predicting the effect of interferons to augment endotoxin to induce IL-6 in hPBC. Accordingly, the IL-6 production in 28SC cell was selected as the most appropriate indicator cell line assay for controlling endotoxin in parenteral drugs.

Among the cell lines examined, only 28SC but not MM6 and THP1 was found to have maintained the characteristics of hPBC for the cytokine productions in response to the panel of endotoxins. Although actual reason is not yet known, it is interesting whether the difference between 28SC and MM6, THP1 is reflecting difference in endotoxin receptors or difference in transduction mechanisms.

The IL-6 induction assay in 28SC cells could be used for an effective validation of the current endotoxin limits for biological products. The regulation should be set to regulate the endotoxin content to below the level at which no any practically available endotoxin would show excess IL-6 induction in 28SC cells over the acceptable level for RSE alone.

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