Attenuation of fever and release of cytokines after repeated injections of lipopolysaccharide in guinea-pigs

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1. The effects of repeated injections of bacterial lipopolysaccharide (LPS) at 3 day intervals on abdominal temperature and systemic release of tumour necrosis factor α (TNF)-like and interleukin-6 (IL-6)-like activity were measured in guinea-pigs.
2. After the third injection of LPS the fever response was significantly attenuated.
3. TNF-like activity (peak 1 h after LPS injection) and IL-6-like activity (peak 3 h after LPS injection) in plasma changed correspondingly, both being significantly reduced after the third and subsequent injections of LPS.
4. The increase of IL-6-like activity in plasma after LPS injection correlated to the febrile change in body temperature. This correlation remained manifest throughout the whole time course of the development of endotoxin tolerance.
5. The reduced production of TNF-like activity after repeated injections of LPS correlated to the attenuation of the fever index, the integration of the thermal response after LPS application.
6. The results support the hypothesis that one component of the development of endotoxin tolerance is reduced production and release of cytokines in response to repeated injections of the same amount of LPS.

In man or experimental animals, injection with lipopolysaccharide (LPS), a component of the outer membrane of most gram-negative bacteria, results in fever. It has been known for about 45 years that repeated administration of LPS results in progressive attenuation of the febrile response, a phenomenon called endotoxin tolerance (Beeson, 1947; Atkins, 1960). Many studies have been performed to investigate the mechanisms responsible for the development of endotoxin tolerance. Thus, there is evidence that the initial clearance of radioactively labelled endotoxin from the circulation is more rapid in tolerant animals than in non-tolerant animals (Cooper & Cranston, 1963). Other investigators reported a neutralization of LPS by its binding to plasma lipoproteins in tolerant animals (Warren, Knights & Siber, 1986). Central nervous mechanisms participating in endogenous antipyresis have even been suggested as contributors to endotoxin tolerance. It has been observed that the activity of antipyretic vasopressinergic pathways from the hypothalamic paraventricular nucleus to the limbic septal area seems to be increased in guinea-pigs tolerant of the pyrogen Poly I:Poly C (Cooper, Blähser, Malkinson, Merker, Roth & Zeisberger, 1988), and intraseptal injections of a vasopressinergic V_1 receptor antagonist enhanced the thermogenic response to LPS in endotoxin-tolerant animals (Wilkinson & Kasting, 1990).

Interestingly, not only is fever attenuated by repeated or continuous administration of LPS, but so are other components of the so-called acute-phase response, such as the fall in serum iron concentration (Goelst & Laburn, 1991) or endotoxin-induced anorexia (O'Reilly, Vander & Kluger, 1988). Nowadays there is agreement that the whole array of signs of the acute-phase response are mediated by the actions of cytokines, which are produced and released by cells of the immune system in the infected organism (Blatteis, 1990; Kluger, 1991). The role of cytokines as endogenous pyrogens in the development of fever, which is the most manifest and best-known sign of infection, has been reviewed recently (Kluger, 1991). Our experiments in guinea-pigs showed that tumour necrosis factor (TNF)-like and interleukin-6 (IL-6)-like activity can be measured in increased amounts in plasma after injection of LPS (Roth, Conn, Kluger & Zeisberger, 1993). In these experiments the

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highest TNF-like activity was detected in the early phase of the fever response and IL-6-like activity in plasma showed a very close correlation with the febrile development of body temperature. Similar observations were made in rats (LeMay, Vander & Kluger, 1990) and even in man (Nijsten, DeGroot, TenDuis, Klasen, Hack & Aarden, 1987), supporting the hypothesis that IL-6 is a circulating endogenous pyrogen (Kluger, 1991).

The aim of the present study was to investigate the systemic release of TNF- and IL-6-like activities after repeated injections of LPS in relation to the febrile development of body temperature. These experiments should enable us to state whether tolerance to LPS, with all its physiological consequences, is related to the systemic production of cytokines.

METHODS

Animals

This study was performed on a group of nine male guinea-pigs with a mean body weight of 390 ± 8 g at the beginning of the experiments. The animals were housed in individual cages at 22 °C with a 12:12 h light–dark cycle (light off at 19.00 h).

Surgery and sampling of blood plasma

At least 1 week before the start of the experimental procedure, the animals were chronically implanted with intra-arterial catheters. Guinea-pigs were anaesthetized with 100 mg kg^{-1} ketamine hydrochloride and 4 mg kg^{-1} xylazin. Polyethylene catheters were inserted through the left carotid artery until they reached the aortic arch. The distal ends of the catheters were tunnelled subcutaneously into the interscapular region of the back where they emerged through the skin according to the method described by Shrader & Everson (1968). After implantation, the catheters were flushed with sterile heparinized saline and closed by heating. During the acute experiments single blood samples (0·5 ml) were slowly (within 1 min) drawn into sterile syringes, put into chilled polypropylene tubes and immediately centrifuged. The blood plasma was stored at −70 °C for later determination of cytokines. Before the experiments, the animals were accustomed to the blood sampling procedure, which then did not cause excitement or any measurable change in body temperature during experiments.

Fever induction and measurement of body temperature

Fever was induced by intramuscular injections of bacterial lipopolysaccharide (LPS from *E. coli*, 0111:B4, 20 μg kg^{-1}; Sigma Chemical Co., St Louis, MO, USA). Abdominal temperature was measured by use of battery-operated biotelemetry transmitters (VM-FH-discs; Mini-Mitter Co., Sunriver, OR, USA) implanted intraperitoneally after placement of the intra-arterial catheter. Output (frequency in Hz) was monitored using a mounted antenna placed under each animal's cage (RA 1000 radioreceivers; Mini-Mitter Co.) and multiplexed by means of a BCM 100 consolidation matrix (Mini-Mitter Co.) to an IBM personal computer system. A Dataquest IV data acquisition system (Data Sciences Inc., St Paul, MN, USA) was used for automatic control of data collection and analysis. Body temperature was monitored and recorded at 5 min intervals. For analysis and graphical documentation temperature data collected at 15 min intervals were used.

Experimental procedure

Injections of 20 μg kg^{-1} LPS were repeated 4–5 times at 3 day intervals at 10.00 h. Between the third and fourth injection of LPS one animal tore out its catheter. The same happened with three more animals during the two days before the fifth injection of LPS. These animals were not used for further injections of LPS. Blood samples were taken 1 h before and 1 and 3 h after each LPS injection. These times corresponded to the non-febrile stage, the stage when body temperature rose and the stage of maximum fever. Some pilot studies had shown that there are interindividual differences in the production and release of IL-6-like activity after the first injection of LPS with peak values ranging from 50000 to about 400000 u ml^{-1}. Peak values of TNF-like activity after the first injection of LPS ranged from 45 to 950 u ml^{-1}. Therefore, TNF- and IL-6-like activities during the development of endotoxin tolerance were analysed and evaluated in those animals in which repeated blood sampling could be performed in at least four of the repeated-injection experiments (Expts 1–4; $n = 6$). All plasma samples collected from the same animals were analysed in one assay.

Bioassay and enzyme immunoassay for TNF

Determination of TNF was based on the cytotoxic effect of this cytokine on the mouse fibrosarcoma cell line WEHI 164 subclone 13 (Espevic & Nissen-Meyer, 1986). The cells were kindly provided by Dr Anders Waage (University of Trondheim, Norway). Plasma samples to be tested for TNF were serially diluted in assay medium consisting of 1% fetal calf serum, 2 mM L-glutamine, 100 u ml^{-1} penicillin and 100 μg ml^{-1} streptomycin in RPMI 1640 (Gibco, Long Island, NY, USA), and 100 μl volumes were added in triplicate to 96-well tissue culture plates. Cells were washed and resuspended at a concentration of 500000 cells ml^{-1} in growth medium consisting of 10% fetal calf serum, 2 mM L-glutamine, 100 u ml^{-1} penicillin and 100 μg ml^{-1} streptomycin in RPMI 1640, supplemented with 10 u ml^{-1} sterile heparin and 0·5 μg ml^{-1} actinomycin D for the assay; 100 μl of this cell suspension (50000 cells) was added to each well. The plates were incubated for 20 h at 37 °C, in room air with 5% CO_2 at 98% humidity. Then 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg ml^{-1}, Sigma) was added to each well to determine the number of living cells (cytotoxicity). MTT is a tetrazolium salt that will form dark crystals when combined with metabolizing cells. After incubation for an additional 4 h, 150 μl of supernatant was removed from each well and 100 μl of 99% isopropanol–0·04 M HCl was added. The plates were then wrapped in aluminium foil to protect them from light and left at room temperature for another 20 h. Plates were read at 570 nm (and 630 nm for reference) using an EL 312 Bio-Kinetics reader (BIO-TEK Instruments Inc., Winooski, VT, USA). The units of TNF were calculated based on a purified recombinant human (rh) TNF standard run in the same assay with a specific activity of 1 u (0·037 ng)$^{-1}$ in the WEHI assay (Cetus, Emeryville, CA, USA). From this standard curve, a best-fit line was used to calculate TNF activity in the plasma samples. We considered any value greater than two standard deviations above media containing no TNF to be the limit of sensitivity of the assay.

For TNF this value approximated 1 u ml^{-1} (for further details see Long, Kunkel, Vander & Kluger, 1990).

In a group of five guinea-pigs, blood plasma samples were taken 60 min before and 60 min after injection of LPS. In these samples the WEHI assay (see above) and an enzyme immunoassay were used for determination of TNF. In the enzyme immunoassay (British Bio-technology Ltd, Abington, UK) a murine antibody specific for TNF was coated onto the wells of a polystyrene microtitre plate. Standards containing rh-TNF α in concentrations of between 0 and 1000 pg ml^{-1}, as well as the samples, were pipetted in duplicate into the wells at a volume of 200 μl and incubated for 2 h. A horseradish peroxidase-linked antibody specific for TNF was added to each well at a volume of 200 μl followed by further incubation for 2 h. After aspiration and washing, a substrate solution was added to the wells and colour developed in proportion to the amount of TNF. The colour development was stopped after 20 min by addition of 50 μl 2 N H_2SO_4 and the intensity of the colour was measured using a kinetic enzyme immunoassay reader (Milenia, Fa. Biermann GmbH, Bad Nauheim, Germany) at 450 nm (and 570 nm for reference). The minimum detectable concentration of TNF was 7·5 pg ml^{-1}. In the samples taken 60 min prior to LPS no TNF immunoreactive material was detectable (lower than 7·5 pg ml^{-1}). In three of the five samples taken 60 min after injection of LPS, TNF-like material in amounts of between 10·3 and 24·4 pg ml^{-1} were detected by the enzyme immunoassay; in the other two samples the amount of TNF immunoreactive material was below the detection limit (< 7·5 pg ml^{-1}). Bioactivity of TNF ranging from 265 to 578 u ml^{-1} could be measured in all plasma samples taken 60 min after injection of LPS. Two factors may be responsible for the variance between bioassay and immunoassay. First, guinea-pig TNF, which has not yet been sequenced according to our knowledge, may have a substantially lower cross-reactivity to the antibodies than the rh-TNF standard. Second, the presence of some undiscovered cytokine in the plasma samples causing cytotoxicity in the WEHI assay cannot be excluded. Because of the second possibility and since we did not neutralize the measured TNF activity in the plasma samples, we refer to this activity throughout the manuscript as TNF-like activity.

Bioassay and enzyme immunoassay for IL-6

Determination of IL-6 was based on the dose-dependent growth stimulation of IL-6 on the B 9 cell line, an IL-6-dependent mouse B-cell hybridoma (Aarden, De Groot, Schaap & Landsdorp, 1987). The B 9 cells were kindly provided by Dr Lucien Aarden (University of Amsterdam, The Netherlands). Plasma samples to be tested for IL-6 were serially diluted in 10 % fetal calf serum, 2 mM L-glutamine, 100 u ml^{-1} penicillin and 10 μg ml^{-1} streptomycin in Iscove's modified Dulbecco's medium (Sigma); 100 μl volumes were added in triplicate to 96-well tissue culture plates. Cells were washed and resuspended at a concentration of 50000 cells ml^{-1} in growth medium supplemented with 10 u ml^{-1} sterile heparin, and 100 μl cell suspension (5000 cells) was added to each well. The plates were incubated for 72 h at 37 °C, in room air with 5 % CO_2 at 98 % humidity. Then 20 μl of MTT (see above) was added to each well to determine the number of living cells (cell proliferation). After incubation for an additional 4 h, 150 μl of supernatant was removed and 100 μl of 99 % isopropanol–0·04 M HCl was added to each well. The plates were protected from light and left at room temperature for another 20 h before being read at 570 and 630 nm (for reference) using an EL 312 Bio-kinetics reader (BIO-TEK Instruments Inc.). Units were then calculated based on a purified rh-IL-6 standard run in the same assay with a specific activity of 10^9 u mg^{-1} in the B 9 assay (Dr G. Wong, Genetics Institute, University of Los Angeles, CA, USA). From this standard curve, a best-fit line was used to calculate the IL-6 activity in the samples. One unit is defined as the amount of IL-6 found to cause half-maximal cell proliferation in the assay (for further details see LeMay *et al.* 1990).

In a group of five guinea-pigs blood plasma samples were taken 60 min before and 60 min after injection of LPS. In these samples the B 9 assay (see above) and an enzyme immunoassay (British Bio-technology Ltd) were used for determination of IL-6. In the immunoassay a murine antibody specific for IL-6 was coated onto the wells of a polystyrene microtitre plate. Standards containing rh-IL-6, in a concentration of between 0 and 300 pg ml^{-1}, as well as the samples, were pipetted in duplicate into the wells at a volume of 100 μl. After incubation for 2 h at room temperature, aspiration and washing of the wells, a horseradish peroxidase-linked antibody specific to IL-6 was added to each well at a volume of 200 μl followed by further incubation for 2 h. After aspiration and washing, a substrate solution was added to the wells and colour developed in proportion to the amount of IL-6. The colour development was stopped by addition of 50 μl 2 N H_2SO_4 after 20 min and the intensity of the colour was measured by a kinetic enzyme immunoassay reader (Milenia, Fa. Biermann GmbH) at 450 nm (and 570 nm for reference). The minimum detectable concentration of IL-6 was 0·35 pg ml^{-1}. Bioactivity in the plasma samples taken 60 min after injection of LPS ranged between 8400 and 24900 u ml^{-1}. In plasma samples taken prior to injection of LPS no immunoreactive IL-6-like material was detected by the enzyme immunoassay. In the samples taken 60 min after injection of LPS, low amounts of IL-6-like material ranging from 1·65 to 5·75 pg ml^{-1} were detected by the enzyme immunoassay. Two factors may be responsible for the quantitative variance between bioassay and enzyme immunoassay. First, guinea-pig IL-6, which has not yet been sequenced according to our knowledge, may have a substantially lower cross-reactivity to the antibodies than the rh-IL-6 standard. Second, the presence of some undiscovered cytokine in the plasma samples causing proliferation of the B 9 cells cannot be excluded. Because of the second possibility and since we did not neutralize the measured IL-6 activity in the plasma samples, we refer to this activity throughout the paper as IL-6-like activity.

Evaluation and statistics

The fever responses were plotted as abdominal temperature–time curves starting 2 h before and ending 6 h after injection of LPS. Fever responses to the repeated LPS injections were compared by an analysis of variance (ANOVA) followed by Scheffe's *post hoc* test. Cytokine-like activities measured before and after four repeated LPS injections were compared with the corresponding cytokine activities recovered in the first injection experiment by t tests for paired observations in the same group of animals ($n = 6$). Correlations between logarithmic values of IL-6-like activity and the febrile changes in abdominal temperature after LPS injection were calculated from data of the single experiments or from data collected within the time course of all repeated-injection experiments. The mean fever indices, the area between the

thermal response of febrile and normothermic animals were calculated and expressed in degrees celcius hours (taken over 6 h). The mean value of all temperatures measured at 15 min intervals during the two hours prior to the injection of LPS was calculated and taken as the baseline temperature. The correlation between logarithmic values of TNF-like activity measured 60 min after injection of LPS and the corresponding fever indices was calculated from data collected in all repeated-injection experiments. All statistical evaluations were carried out on an Apple Macintosh computer using the software package Statview 512 (Abacus Concepts, Berkeley, CA, USA).

RESULTS

The febrile changes of abdominal temperature after repeated intramuscular injection of 20 $\mu g\,kg^{-1}$ LPS at 3 day intervals are summarized in Fig. 1*A* and *B*.

In Fig. 1*A* and *B* the thermal responses to the repeated LPS administrations are compared with the first fever reaction. The first injection of LPS resulted in the typical febrile response of guinea-pigs lasting about 6 h with a peak (change in body temperature, $\Delta T_b = 2{\cdot}0$ °C) occurring 3 h after pyrogen application. After the second injection of LPS (upper panel of Fig. 1*A*) the fever was slightly reduced, but not statistically different from the first febrile response. After the third LPS injection, however, the febrile increase in body temperature was significantly lower compared with the first experiment, 60–330 min after pyrogen application ($P < 0{\cdot}001$, ANOVA). The febrile response to the fourth injection of LPS was significantly lower than the responses to the first two LPS injections but showed no significant reduction compared with the third LPS-induced fever. The febrile response to the fifth injection of LPS, 30–210 min after pyrogen application, was significantly lower than the third and fourth febrile response ($P < 0{\cdot}01$, ANOVA). Taking all the results from Fig. 1*A* and *B* together, the repeated LPS injections resulted in progressive attenuation of the febrile response in guinea-pigs, which is in agreement with observations made in other species (see Introduction).

In the same six guinea-pigs, blood plasma samples were collected 1 h before, as well as 1 and 3 h after, each of the first four repeated injections of LPS and assayed for TNF- and IL-6-like activities. These selected time points corresponded to the non-febrile stage, the rising phase of fever and the peak of fever (cf. Fig. 1). TNF-like activities in plasma after LPS injections during the development of endotoxin tolerance are summarized in Fig. 2.

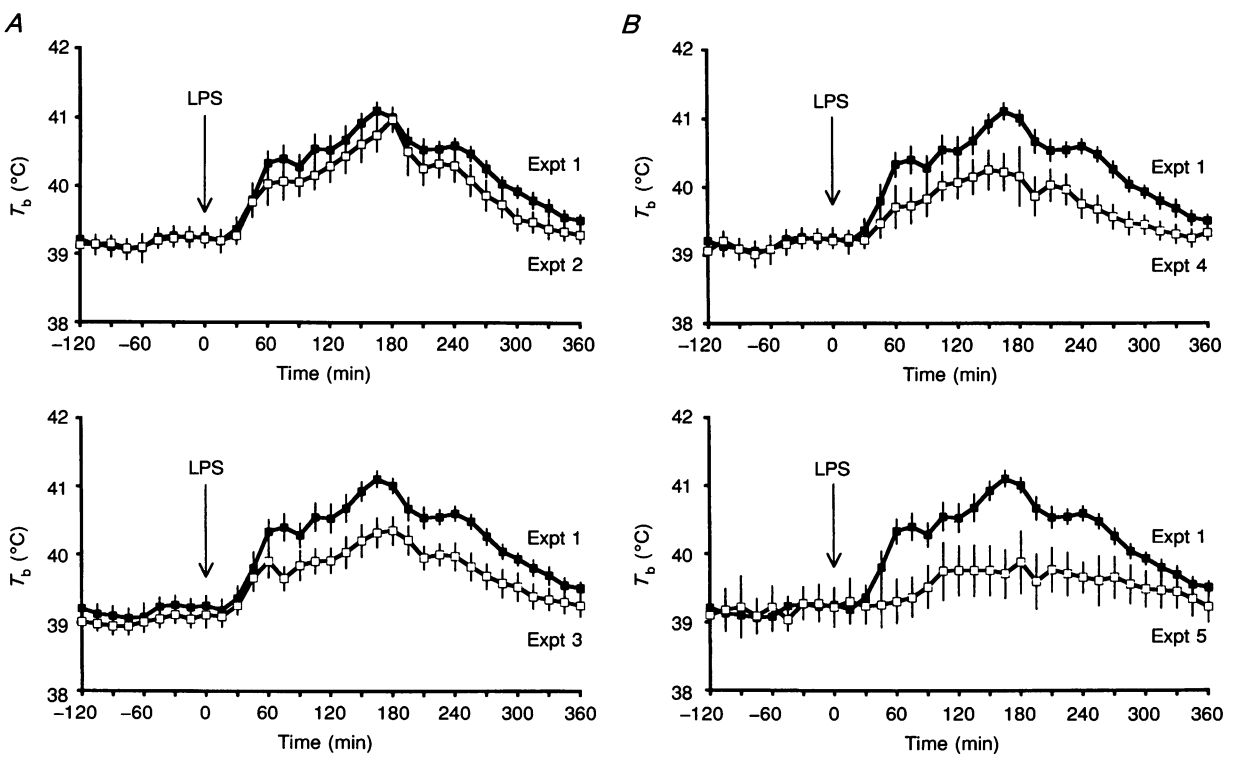

Figure 1. Fever response to repeated injections of LPS
A, upper panel: thermal response of guinea-pigs to the first (■, $n = 9$) compared with the second (□, $n = 9$) injection of LPS expressed as body temperature (T_b) over time. The symbols at each time point represent mean values with error bars indicating S.E.M. Lower panel: thermal response of guinea-pigs to the first (■, $n = 9$) compared with the third (□, $n = 9$) injection of LPS. *B*, upper panel: thermal response of guinea-pigs to the first (■, $n = 9$) compared with the fourth (□, $n = 8$) injection of LPS. Lower panel: thermal response of guinea-pigs to the first (■, $n = 9$) compared with the fifth (□, $n = 5$) injection of LPS.

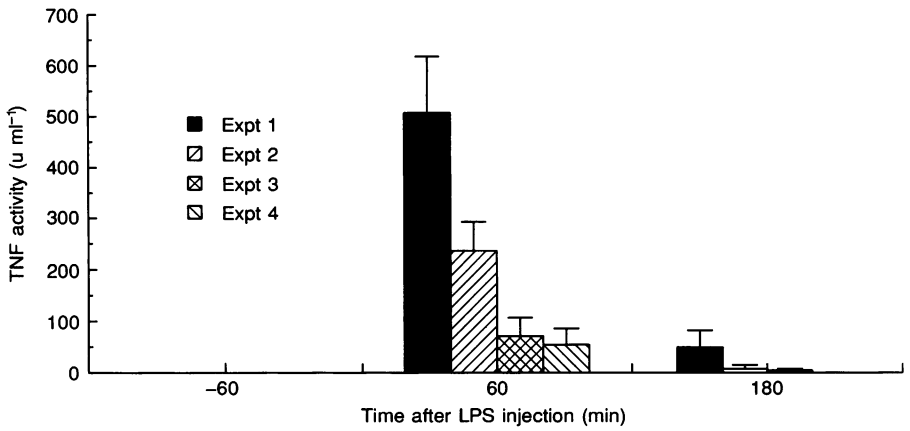

Figure 2. TNF-like activity during the development of endotoxin tolerance
TNF-like activity in plasma detected 60 and 180 min after each of four repeated injections of LPS in the same group of guinea-pigs ($n = 6$). Columns represent means ± S.E.M.

TNF-like activity was not detectable in any of the four repeated-injection experiments 1 h before LPS injection (lower than 1 u ml^{-1}). The highest levels of TNF-like activity became detectable 1 h after each of the intramuscular injections of LPS, and 3 h after LPS injection TNF-like activity declined, with the exception of the first experiment, to almost undetectable values. TNF-like activity in blood plasma 1 h after each of the four repeated LPS injections was progressively attenuated. This value declined from 505 u ml^{-1} in the first experiment to 235 u ml^{-1} in the second experiment ($P < 0{\cdot}05$). After the third and fourth injections of LPS the TNF-like peak activity further declined to 68 and 51 u ml^{-1}, respectively, both values being significantly lower than those observed in the first ($P < 0{\cdot}001$) and the second ($P < 0{\cdot}01$) experiments. In three of the six animals blood plasma samples could be collected at the selected time points before and after a fifth injection of LPS.

One hour after pyrogen application a mean TNF-like activity of only 6 u ml^{-1} was detected in blood plasma; 3 h after injection of LPS no TNF-like activity was detectable.

IL-6-like activities in plasma after LPS injections during the development of endotoxin tolerance are summarized in Fig. 3.

In all of the four repeated experiments a baseline IL-6-like activity of 200–450 u ml^{-1} could be detected in blood plasma without any significant differences or tendency to change on the different days of the experimental procedure. IL-6-like activity in blood plasma increased 1 h after LPS injection to 37019, 27052, 12664 and 5631 u ml^{-1} in the first, second, third and fourth experiments, respectively. The values observed in the third ($P < 0{\cdot}05$) and fourth ($P < 0{\cdot}01$) experiments were significantly attenuated in comparison with the IL-6-like activity in plasma, measured 60 min after the first and second injections of LPS. At the peak of the fever, 3 h after LPS injection, the highest IL-6-like activities were measured in all experiments. At this stage of the fever response IL-6-like activity of 257389 u ml^{-1} could be detected in the first experiment, which tended to decrease to 157658 u ml^{-1} in the second experiment and was

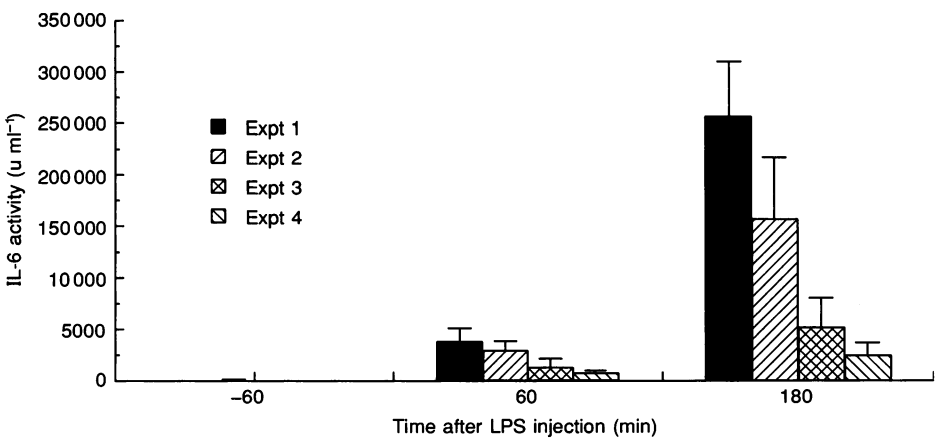

Figure 3. IL-6-like activity during development of endotoxin tolerance
IL-6-like activity in plasma detected 60 and 180 min after each of four repeated injections of LPS in the same group of guinea-pigs ($n = 6$). Columns represent means ± S.E.M.

significantly attenuated to 53605 u ml^{-1} in the third and to 26670 u ml^{-1} in the fourth experiment ($P < 0{\cdot}001$ compared with the first, $P < 0{\cdot}05$ compared with the second experiment). In three animals where blood plasma was collected before and after a fifth LPS injection, IL-6-like activity in plasma occurred 60 min after pyrogen application, reduced further to 786 u ml^{-1} and at the fever peak to 2638 u ml^{-1}.

As mentioned in the introduction, and as becomes obvious from Fig. 3, IL-6-like activity in plasma seems to increase parallel to the febrile rise in body temperature. Roth *et al.* (1993) reported a close correlation between logarithmic values of systemic IL-6-like activity and the febrile development of body temperature in guinea-pigs. Therefore, the correlation between the increase in body temperature after LPS injection (the difference from the baseline temperature) and the logarithmic values of IL-6-like activity in plasma after pyrogen application was calculated using all the individual data obtained in each of the repeated experiments 1 and 3 h after LPS injection. In the first experiment the simple regression between logarithmic IL-6-like activities (x = log IL-6 activity) and febrile increase of body temperature ($y = \Delta T_b$) showed a close correlation between these two variables ($r = 0{\cdot}802$, $P = 0{\cdot}017$, $n = 12$). This correlation remained manifest in all of the repeated experiments except the second, in which the two variables did not correlate as well. ($r = 0{\cdot}566$, $P = 0{\cdot}0551$, $n = 12$; $r = 0{\cdot}881$, $P = 0{\cdot}0002$, $n = 12$; $r = 0{\cdot}902$, $P = 0{\cdot}0001$, $n = 12$; $r = 0{\cdot}834$, $P = 0{\cdot}0392$, $n = 6$; second, third, fourth and fifth experiments, respectively.)

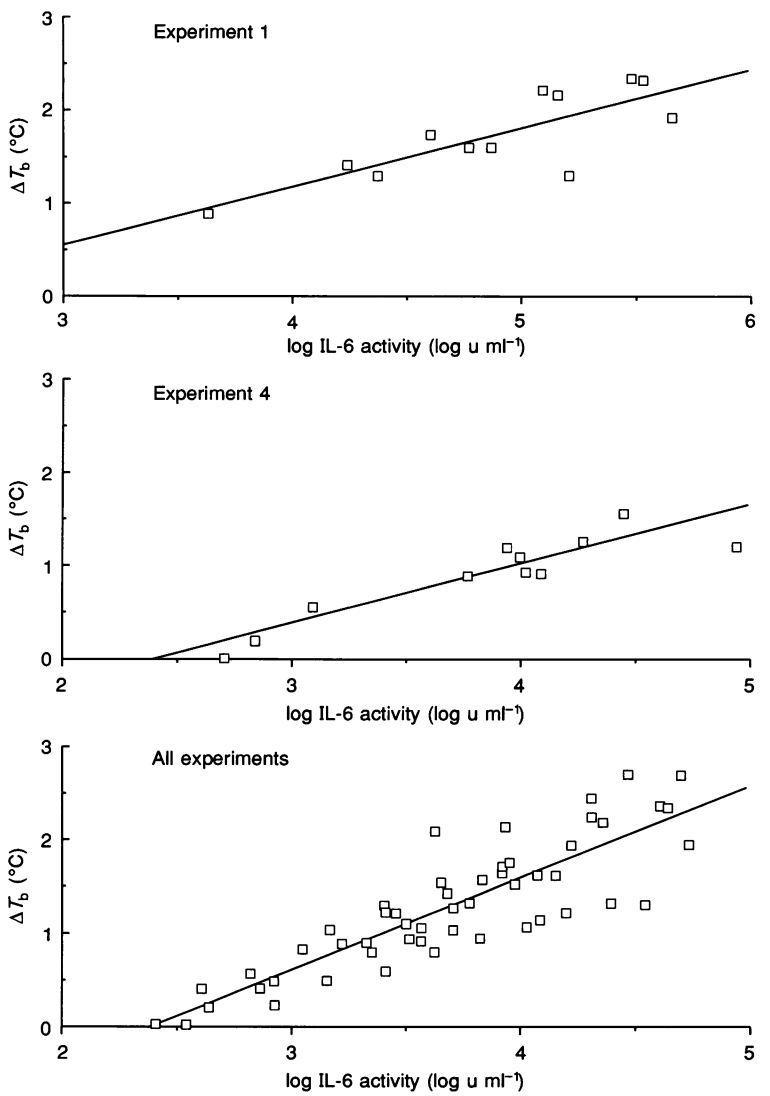

Figure 4. Correlation between IL-6-like activity and febrile temperature increase during development of endotoxin tolerance

Logarithmic values of all individual IL-6-like activities in plasma 60 and 180 min after injection of LPS *versus* the corresponding individual increases of abdominal temperatures from the baseline temperature measured in the first experiment (upper panel, $n = 12$), in the fourth experiment (middle panel, $n = 12$), and for all of the five repeated experiments (lower panel, $n = 54$, two points with identical pairs of values).

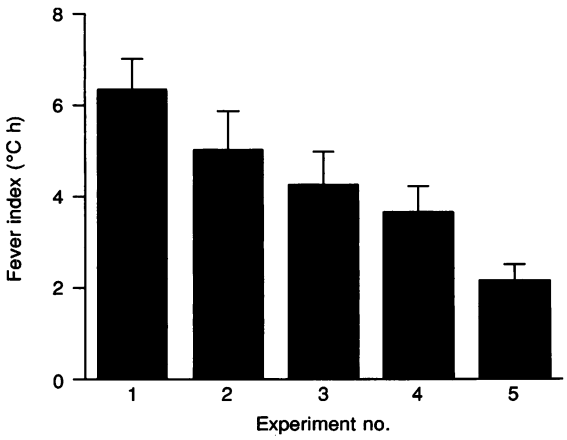

Figure 5. Integrated febrile response during development of endotoxin tolerance
Mean integrated areas of the febrile responses (fever indices) from the curves shown in Fig. 1*A* and *B* (columns represent means ± S.E.M.).

To illustrate the results of these calculations the individual values measured in the first and fourth experiments are shown in the upper and middle panels of Fig. 4.

In the lower panel of Fig. 4 all individual logarithmic IL-6-like activities measured 1 and 3 h after LPS injection in all of the five repeated experiments are plotted *versus* all corresponding individual differences between the febrile and baseline body temperatures. For these data collected during the whole time course of tolerance to bacterial endotoxin development, the close correlation between these two variables remained manifest ($r = 0{\cdot}862$, $P = 0{\cdot}0012$, $n = 54$).

There is no correlation between TNF-like activities in plasma and the changes in body temperature since the highest TNF-like activities can be observed in the early stage of fever rise. Systemic release of TNF-like activity and the magnitude of the febrile response were, however, both attenuated after repeated injections of LPS as shown in Figs 1 and 2. We therefore evaluated the magnitude of the febrile responses as fever indices, the integrated areas of the thermal responses 6 h after each of the pyrogen applications. The mean fever indices from the experiments in Fig. 1*A* and *B* are shown in Fig. 5.

The fever index of the first febrile response was $6{\cdot}29 \pm 0{\cdot}69$ °C h and was attenuated to $5{\cdot}02 \pm 0{\cdot}86$, $4{\cdot}27 \pm 0{\cdot}73$, $3{\cdot}66 \pm 0{\cdot}58$ and $2{\cdot}15 \pm 0{\cdot}41$ °C h in the second, third, fourth and fifth experiments, respectively. We tried to correlate all individual TNF-like activities, measured 60 min after each of the repeated injections of LPS to the corresponding fever index, as shown in Fig. 6.

A simple regression between logarithmic values of TNF-like activities (x = log TNF activity) and the fever indices (y = FI) showed a close correlation between these two variables ($r = 0{\cdot}832$, $P = 0{\cdot}001$, $n = 27$).

All results taken together indicate that endotoxin tolerance developing after repeated injections of the same amount of LPS is accompanied by reduced systemic production and release of cytokines.

DISCUSSION

In this study, it has been shown that the attenuation of fever in response to repeated injections of LPS from *E. coli* is associated with a corresponding reduction of systemic TNF- and IL-6-like activities. The reduction of systemic release of cytokines may be due to more rapid clearance of LPS from the circulation of endotoxin-tolerant animals

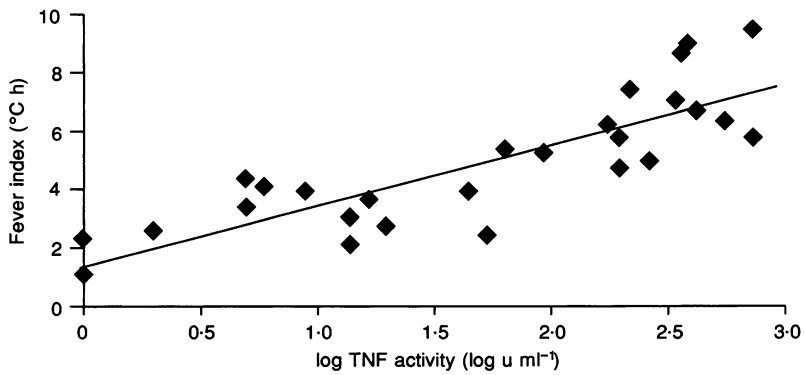

Figure 6. Correlation between TNF-like activity and fever indices during development of endotoxin tolerance
Logarithmic values of all individual TNF-like activities in plasma 60 min after injection of LPS *versus* the corresponding individual fever indices from all of the five repeated experiments ($n = 27$).

(Cooper & Cranston, 1963) or by binding and neutralization of LPS to plasma proteins in tolerant animals (Warren, Knights & Siber, 1986), as mentioned in the Introduction. Regarding the molecular events occurring due to the binding of LPS to its membrane receptor on macrophages, there might be an alternative, or at least an additional, possibility to explain the observed attenuation of systemic release of cytokines. The complex effects of bacterial endotoxins on the mammalian immune system include non-specific activation of the immune system, activation of the complement cascade and induction of the characteristic shock syndrome (Dinarello, 1991; Rackow & Astiz, 1991). Many effects of endotoxins on animals are secondary to the overproduction of cytokines, such as TNF (Van Zee, Kohno, Fischer, Rock, Moldawer & Lowry, 1992) or IL-1 (Fischer *et al.* 1992). The initial biochemical events that activate macrophages to synthesize cytokines (Raetz, Ulevitch, Wright, Sibley, Ding & Nathan, 1991) are, therefore, of special interest and can be briefly described as follows. Interaction of LPS alone or coupled to the lipopolysaccharide binding protein with specific receptors on macrophage membranes results in an activation of the macrophage cell nuclei to produce mRNA for cytokines and subsequently the production and release of cytokines themselves. Interestingly, it has been reported in rabbits that in response to two intravenous injections of LPS spaced 5 h apart, TNF in plasma rises only after the first LPS injection (Mathison, Virca, Wolfson, Tobias, Glaser & Ulevitch, 1990). This phenomenon was due to a strong decrease in LPS-induced TNF-mRNA without a change in TNF-mRNA half-life. This desensitization of rabbit macrophages to LPS was therefore discussed as the basic mechanism for the development of endotoxin tolerance by the authors of this paper. In addition it has been reported recently that daily injections of LPS in rats resulted in a measurable release of TNF and IL-6 within the first few days only and was abrogated thereafter (He *et al.* 1992); anorexic effects of LPS were also diminished and abolished over a similar time course. Our experiments in guinea-pigs support the results of these studies with regard to the attenuation of systemic cytokine release and the clearly corresponding reduction of fever as the most manifest component of the acute-phase responses, after repeated intramuscular injections of LPS.

Less clear from the results of our study is the controversially discussed question – which cytokine might be responsible for the generation of febrile signals to be transduced into the brain? Of all cytokines released into the peripheral circulation after administration of LPS, the time course of IL-6-like activity correlates best with the febrile changes of body temperature. The strong correlation between IL-6-like activity in plasma and the febrile increase of body temperature remained manifest during the development of endotoxin tolerance in guinea-pigs. In another study (Blatteis, Quan, Xin & Ungar, 1990) intravenous injection of guinea-pigs with an activity of IL-6 that might have approximated the bioactivity of IL-6-like material detected after the first injection of LPS, caused fever and synthesis of acute-phase proteins. IL-6 therefore fulfils two of the five criteria that should be met in support of the hypothesis that IL-6 is a circulating endogenous pyrogen in guinea-pigs (Kluger, 1991). However, as long as specific neutralizing antibodies to guinea-pig IL-6 are not available to be tested under the conditions of simultaneous or subsequent injection of LPS, the role of circulating IL-6-like activity remains questionable. In addition, it might be possible that the increased body temperature during fever is a stimulus for release of IL-6-like activity. Thus, the correlation between febrile body temperature and IL-6-like activity in plasma alone does not prove which is the cause and which is the effect.

In the experiments of this study as well as others (Long, Kunkel, Vander & Kluger, 1990) TNF-like activity showed no correlation with the febrile increase of body temperature, because its peak was detected at a very early stage of the fever response. The evaluation of the integrated areas of the febrile responses during the development of endotoxin tolerance showed, however, that the decreasing fever indices correlate well with the corresponding TNF-like peak activities measured 60 min after injection of LPS. This observation could support the hypothesis that TNF, released in the early stage of the febrile response, is relevant to the induction of fever. Injection of rats with TNF-antiserum, which was able to neutralize TNF-like bioactivity, 2 h prior to injection of LPS attenuated the short initial phase of the febrile response but significantly enhanced the longer second phase of the bimodal fever (Long, Kunkel, Vander & Kluger, 1990). These data suggested that TNF may indeed be involved in the initiation of the first phase of fever, but might then provide a signal for limitation of the second phase of the febrile response. In another study in rabbits small amounts of TNF ($1\ \mu g\ kg^{-1}$) caused a rather short monophasic fever, and larger amounts of TNF ($10\ \mu g\ kg^{-1}$) caused a much longer biphasic elevation of body temperature (Dinarello *et al.* 1986). The authors of this study attributed the second peak of the bimodal febrile response to IL-1-like activity induced by TNF. This again supports the hypothesis that TNF itself provides the febrile signal just for the short initial phase of the fever response and may participate in the induction of other cytokines, for example IL-6 (Dinarello, 1991).

The best known endogenous pyrogen, claimed to mediate the fever caused by exogenous pyrogens like LPS, is the β-form of IL-1 (Dinarello, 1991; for review). In many cases, attempts failed to measure plasma concentrations of bioactive IL-1 and to compare them with the magnitude of LPS fever (Kluger, 1991, for review). This phenomenon might be due to endogenous inhibitors, but does not exclude a role of IL-1 in the induction of the febrile response. Indeed, pretreatment of rats with antiserum to IL-1β not only blocked a significant portion of the fever in

response to injected LPS, but the antiserum to IL-1β also significantly attenuated the rise in plasma IL-6-like activity (LeMay, Otterness, Vander & Kluger, 1990). These data supported *in vitro* studies indicating that IL-1 can induce the production of IL-6, a property which has also been ascribed to TNF (Dinarello, 1991).

Taking together all these observations, it seems that a network of cytokines, rather than one cytokine alone, is responsible for the development of the febrile response to exogenous pyrogens like LPS. An important component of endotoxin tolerance is a down regulation of the cytokine network in response to repeated injections of LPS, as documented in our experiments for IL-6-like and TNF-like activities.

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