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Production of a neutrophil chemotactic factor by endotoxin stimulated alveolar macrophages *in vitro*

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Summary. Alveolar macrophages (AM) isolated from normal guinea-pigs and from those chronically exposed to endotoxin (LPS) were cultured in the presence of various concentrations of LPS (from 0.5 ng to 5 μg/ml). The presence of a neutrophil chemotactic factor (NCF) in culture supernatants was tested in migrations chambers. Contamination of all reagents has been tested using LAL test (Limulus Amoebocyte Lysate). The results indicate a production of NCF with low LPS concentrations (0.5 and 5 ng/ml) within the first 6 h of incubation: when larger doses are used the response decreases and a significant inhibition is observed with 5 μg LPS/ml ($P \leqslant 0.05$). When contaminated medium was used, all responses observed were three times higher than with LPS-free medium ($P \leqslant 0.01$). However, the response pattern was the same. AM from chronically exposed animals exhibit the same response patterns: the magnitude of NCF production was higher than with normal AM but not significantly. The data suggests that initial conditions of AM *in vitro* or *in vivo* with reference to LPS contamination have to be determined as they are of importance when AM NCF production has to be tested.

Keywords: neutrophil chemotactic factor (NCF), alveolar macrophage (AM), endotoxin (LPS), *in vitro* stimulation

In vivo exposure to airborne endotoxin (LPS) induces an inflammatory reaction in the airways of guinea-pigs. The reaction is characterized by an influx of neutrophils which reaches a maximum 12 to 24 h after the inhalation (Snella & Rylander, 1982).

It has been suggested that neutrophil migration is a consequence of the secretion of a chemotactic factor by alveolar macrophages (AM). We have recently demonstrated that free lung cells, harvested by lung lavage from guinea-pigs exposed to an aerosol of LPS, induce a migration of neutrophils *in vitro* (Snella & Rylander 1985).

The production of neutrophil chemotactic (NCF) factor by AM stimulated *in vitro* by other agents such as zymosan, immune complexes (Gadek *et al.* 1980), silica dust (Lugano *et al.* 1981) and manganese dioxide (Snella 1985), has also been demonstrated.

In the present study, AM were isolated from bronchoalveolar lavage fluid by adherence on petri dishes. After culture in the presence of different concentrations of LPS in the medium (from 0.5 ng–0.5 μg/ml), the production of NCF was tested in migration chambers. The contamination by LPS of fetal calf serum (FCS) and medium was checked,

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and its influence on NCF production tested. A series of experiments was performed with AM from chronically exposed to LPS animals in order to determine the influence of subclinical inflammation on AM NCF production.

Material and methods

Animals. Cells were taken from male and female non-inbred guinea-pigs, weighing 300–500 g. Animals were obtained from the Institute of Zuchthygiene, University of Zürich. In order to investigate the chemotactic activity of LPS-pre-treated AM, guinea-pigs were exposed to an aerosol of LPS 100 $\mu g/m^3$ (*E. Coli* 026 B6, Difco Lab) for a period of 40 min, three times a week for 4 months. This exposure corresponded to a dose of approximately 0.3 μg per animal. Subsequently, AM were harvested and cultured by the same methods as used for AM from non-exposed animals.

Alveolar macrophages. Animals were killed by overdose of sodium pentothal i.p. (Abbott Lab). Lungs were removed and washed with 10 ml aliquots of Gey's balanced salt solution (Gey's BSS) heated to 37°C. The collected cells in lung lavage fluid were washed and resuspended in Gey's BSS containing 10% of heat inactivated FCS Seromed) to give 1.6×10^6 AM per ml (about 70% of total cells). After cytocentrifugation, cells were stained for differentiation with Diff-Quick (Merz & Dade).

Two ml of the cell suspension was poured into a 35 mm diameter culture petri dish (Falcon) and incubated for 1 h at 37°C in 5% CO_2. Non-adherent cells were removed by washing the monolayer three times with Gey's BSS at 37°C.

The nature and number of adherent cells were determined as follows: cells which did not adhere were removed after 1 h in Gey's BSS containing 10% FCS and added to those from the three washings. These were then cytocentrifuged, stained with Diff–Quick and differentially counted. The nature and number of cells remaining in the petri dishes was determined by subtracting the number in the washing from the number of lung lavage cells present before incubation. Adherent cells were more than 96% AM as determined by the esterase staining method described by Diesselhoff–den Dulk *et al.* (1981).

AM with or without LPS were incubated for 2, 6, 12 and 24 h in Gey's BSS containing 2% human serum albumin (HSA, Behringwerke). Several concentrations of LPS, ranging from 0.5 ng to 5 μg per ml, were tested.

Following incubation, the supernatants were removed, centrifuged for 20 min at 1000 *g* and stored at 4°C. The chemotactic activity was tested within 24 h.

The viability of AM was checked by the Trypan blue exclusion test. In each experiment, the percentage of viable cells (at the end of incubation time) was over 96%.

Neutrophils. Blood was obtained from normal guinea-pigs by cardiac puncture. Two ml of heparinized blood, diluted with 6 ml of saline, was poured gently on to an Hypaque–Ficoll gradient (10 parts 32.8% Sodium Metrizoate, Nygaard and Co., and 24 parts Ficoll 400, Pharmacia). After centrifugation for 40 min at 1550 r/min, the supernatant was removed and the cell pellet was mixed with 1.0 ml of heparinized saline and 0·4 ml of Dextran 500 (Pharmacia). The mixture was allowed to sediment for 40 min at 4°C. The supernatant containing leucocytes and lymphocytes was removed and washed three times, and a suspension of 10^6 neutrophils per ml prepared in Gey's BSS 2% HSA.

Chemotactic activity. The chemotactic activity of culture supernatants was measured using a migration chamber as previously described (Snella, 1985). Culture supernatant (250 μl) was placed in the lower compartment of the chamber. Two millipore filters, with a pore size of 0·45 and 8·0 μm respectively, were placed over this compartment, which was then closed. Blood neutro-

phil suspension (250 μl) was placed in the upper compartment. After the chambers were incubated in a water bath at 37°C for 2 h, the filters were fixed, exposed to Weigert nuclear stain and processed for embedding in xylene mounting medium. The neutrophils which had migrated on to the lower surface of the 8 μm filter and on to the upper surface of the 0.45 μm filter were counted. Twenty-five microscope fields ($\times$ 500) were counted on each filter and a mean value was calculated. Each experiment was done in triplicate. The migration of neutrophils was tested against the medium (negative control) and against the 20% zymosan activated guinea-pig serum (ZAS) (positive control).

Neutrophil migration against the negative control was less than 0·5% of that against positive control. The migration against AM supernatants was expressed as a percentage of the migration against ZAS.

In a control experiment, fMLP 10^{-6} M (Sigma) was used as test chemoattractant. The results were also expressed as percentages of ZAS chemotactic activity.

Detection of endotoxin contamination. The Limulus Amebocyte Lysate test (LAL, Associate Cape Cod) was used to detect bacterial lipopolysaccharide (LPS) contamination in all sera and culture media. The sensitivity of LAL was 50 pg/ml. Positive batches were discarded. FCS was tested after a 1 : 3 dilution and 10 min heating at 100°C to avoid the influence of inhibitors on the Limulus reaction (Cooperstock *et al.* 1975).

Statistics. Non-parametric tests were used to assess differences between responses of normal and pretreated cell populations (Mann & Whitney's test) and differences due to different treatments or incubation times for the same cell population (Wilcoxon's test) (Gremy & Salmon 1969).

Results

Figure 1 shows neutrophil migration against AM culture supernatants. Different concentrations of LPS (range 0.5 ng to 5 μg/ml) were added to the medium for 2 h.

The migration induced by supernatants of culture pre-incubated in LPS-contaminated FCS (open circle) was about three times higher for the control and for all concentrations tested than the migration induced by supernatant of culture pre-incubated in LPS-free FCS (solid circles) ($P \leqslant 0.01$).

In both series an increase in neutrophil migration was present at the lowest concentrations of LPS. As the LPS concentration in the medium increased, migration decreased and inhibition was seen at 0.5 and 5 μg/ml ($P \leq 0.05$).

A control experiment used 10^{-6} M fMLP as test chemoattractant in order to determine the influence of LPS in supernatant on the neutrophil migration (Table 1). No significant differences were found with or without LPS (at three different concentrations).

Normal AM and those from chronically exposed animals were cultured for 2, 6, 12, and 24 h with 5×10^{-3} μg/ml and 5×10^{-4} μg/ml of LPS respectively. Results are shown in Fig. 2. Normal AM in culture produce NCF. The production reaches a peak at 6 h and remains at this level for at 12 and 24 h. The results after incubation with 5×10^{-4} μg LPS/ml indicate an increase in NCF production which reaches a maximum at 6 h followed by a significant inhibition at 24 h ($P < 0.05$). The higher dose (5×10^{-3} μg/ml) provokes an insignificant amount of NCF production within the first 2 h and then a decrease, all results being lower than control, (though not significantly). All three figures show that AM, pretreated with LPS by *in vivo* exposure, exhibit a higher response than normal AM; however this is not significant except after 24 h of incubation with 5×10^{-4} ($P < 0.05$) μg.

Discussion

The results demonstrate that AM produce NCF *in vitro* when a very low concentration of LPS is present in the culture medium (500

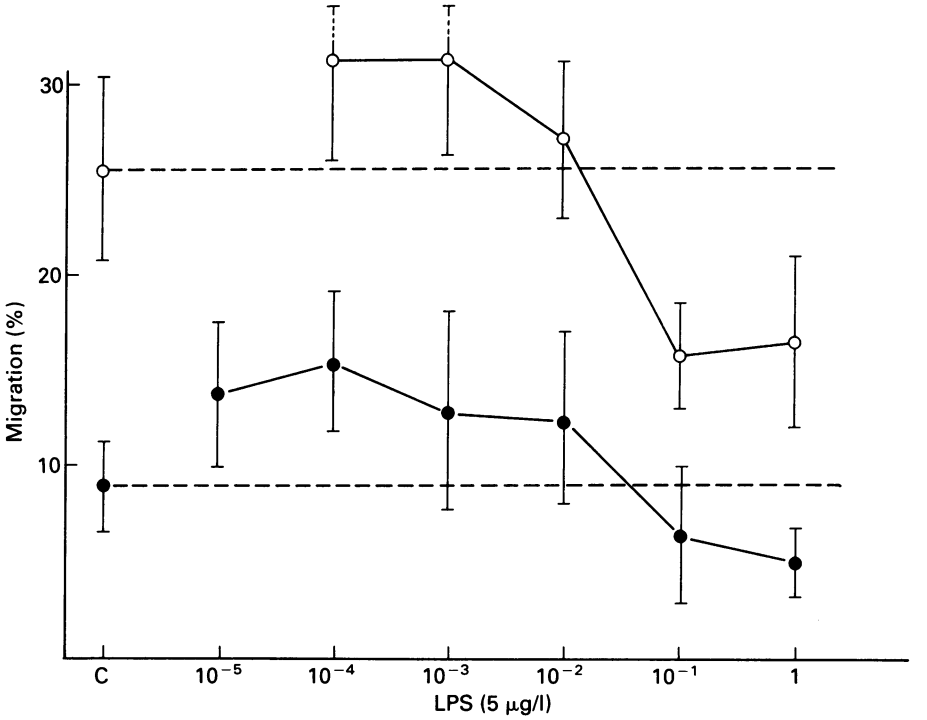

Fig. 1. NCF in 2 h-AM cultures with various concentrations of LPS. Results are presented as percentages of migration against 20% ZAS. ○, pre-incubation in LPS-contaminated FCS; ●, pre-incubation in LPS-free FCS; horizontal bar = control. Each point represents the mean of 6–10 experiments. Vertical bars represent standard error.

pg/ml). With circa 3×10^6 AM per 2 ml suspension in the petri dish, the amount of LPS per AM was approximately 3×10^{-4} pg. This is consistent with *in vivo* experiments which show that inhalation of LPS by guinea-pigs, resulting in a deposited dose of about 1.2 μg/animal (3 μg/kg), provokes an influx of neutrophils in the airways. In these *in vivo* conditions, the amount of LPS per AM is estimated to be less than 4×10^{-2} pg (Snella & Rylander 1985).

Table 1. Migration of blood neutrophils against fMLP 10^{-6} M

LPS (μg/ml)	Migration (%)
None	11.9 (3.2)
5	11.2 (4.0)
0.05	10.3 (3.6)
0.0005	8.8 (1.9)

Various concentrations of LPS were added top the chemoattractant.

Results are given as percentages of migration against 20% ZAS.

Mean values were obtained from four experiments.

Standard errors are in parenthesis.

As the dose of LPS per ml increased, the amount of NCF production decreased and migration was inhibited. The control experiment using fMLP as chemotactic factor (Table 1) indicates that the inhibition was not due to the presence of LPS in the supernatant. Lugano *et al.* (1981) also described an inhibition of migration in the supernatant of AM culture with silica dust and latex particles. They observed an optimal

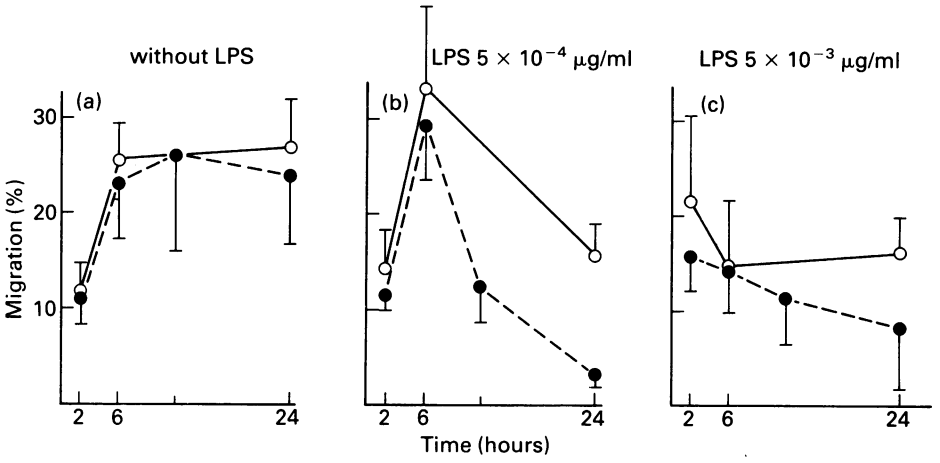

Fig. 2. NCF production by normal AM (●) and chronically exposed AM (○) at different incubation times. Results are given as percentages of migration against 20% ZAS. *a*, Control cultures; *b*, 5×10^{-4} μg LPS/ml; *c*, 5×10^{-3} μg LPS/ml. Each point represents the mean of five experiments. Vertical bars represent standard error.

effect when a 1:2 dilution was tested and explained it by the presence of an inhibitor or enzymes secreted by AM, which inactivate the chemotactic factor. In our case, dilution of the supernatant did not increase the migration (data not shown).

In a study of NCF production by rat AM harvested 3 h after an i.p. injection of a large dose of endotoxin (2.5 mg/kg) and cultured for 2 and 15 h, Rinaldo and Dauber (1984) described an inhibition of neutrophil migration when supernatants were tested in Boyden chambers. They concluded that 3 h after an i.p. injection of LPS, AM secrete an inhibitor of neutrophil chemotaxis. The present study suggests that this inhibition is not only time dependent but could also be due to the large dose of endotoxin used in their study.

A dose-dependent inhibitory effect of LPS on neutrophil chemotaxis in migration chambers has been reported (Dahinden *et al.* 1983). In that study, endotoxin was added to both sides of the chamber, not only to the lower compartment. Dahinden's study (1983) showed that if random migration is depressed, the chemotaxis level is still significantly elevated and the direction-finding mechanism still operated.

Recently, Donabedian (1985) has described the production of neutrophil chemotaxis inhibitor by blood monocytes exposed to bacteria.

Davis *et al.* (1980) showed a decrease in the phagocytosis, adherence and spreading of macrophages when the LPS dose in the medium was increased. The concentrations used in that study (10–100 μg/ml) were much higher than those used here.

When adherence of AM on the petri dish was obtained with 2.5 ng/ml LPS-contaminated FCS, a concentration often found in commercial preparations (Fumarola & Jirillo 1979; Weinberg 1981), all migration values were significantly higher than when FCS was LPS-free. However, the same dose response pattern was found: a slight decrease achieved a significant inhibition at the highest concentration.

The LPS concentration at which a response is observation depends also on the type of activity tested. For the production of NCF, a very low dose is sufficient, which is consistent with *in vivo* observations (Snella &

Rylander 1985). This supports a report by Weinberg (1981), which has shown that LPS in quantities of pico- to nanograms per ml have potent effects on macrophage functions, including interleukin 1, secretion, tissue factor elaboration, tumor cytotoxicity, and modulation of neutral proteinase secretion.

Moore *et al.* (1980) have similarly demonstrated that a dose of LPS over 0.01 μg/ml inhibited macrophage colony formation, showing that this was not due to cytotoxicity of LPS and was not mediated through prostaglandin synthesis. They also demonstrated that minute concentrations of LPS (10^{-7} μg/ml) significantly enhance macrophage colony formation. They attributed both effects to lipid A as they were inhibited by addition of polymyxin B in the medium.

When incubated with the lowest doses for various periods of time, NCF was produced by AM: within 2 at 5 ng of LPS/ml, and within 6 h at 0.5 ng/ml. This supports our earlier studies showing that AM harvested at different times after *in vivo* stimulation (by inhalation of LPS) and immediately tested *in vitro* produced NCF at 1 and 4 h but beyond that there is inhibition (Snella & Rylander 1985). A rapid and transient macrophage activity has also been described by Harvell and Spinantly (1983); endotoxin induces release of interferon by bone marrow derived macrophage. This was compared to the endotoxin effect *in vivo* where a maximum is reached at 2–3 h and the response stops at 6 h.

Increases or inhibitions observed in all supernatants of chronically exposed AM were similar to those observed in AM preincubated in FCS-contaminated serum. The experiment with AM from chronically exposed animals produced the same response pattern as did normal AM. However, all responses of pre-exposed cells were greater than those from normal cells. This confirmed the observations of Fidler *et al.* (1981) who studied the influence of host conditions on macrophage reactions and concluded that it is imperative that the starting population should be non-reactive.

Another conclusion is proposed by Taramelli *et al.* (1980) who studied the activation of macrophages by lymphokines using a microcytotoxicity assay *in vitro*. They observed that when LPS-free medium and FCS were used, small amounts (10 ng/ml) were required for significant macrophage activation.

The present study confirms the conclusions of the pre-cited studies. The presence of LPS in the medium or the reactants used for *in vitro* studies can significantly influence the observed responses. The presence of a subclinical inflammation stimulates NCF production. In order to evaluate the importance of an AM activation it is necessary to determine the presence of LPS in all reagents and to know the inflammatory status of the cell donor.

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