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Tumor Necrosis Factor and Interleukin-1 Activities in Free Lung Cells after Single and Repeated Inhalation of Bacterial Endotoxin

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Bacterial endotoxins (lipopolysaccharides), important components of many organic dusts, are known to induce macrophages to produce the inflammatory mediators interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α). To investigate the role of these mediators in the early inflammatory responses in the lung, guinea pigs were exposed to an aerosol of bacterial endotoxin. A bronchoalveolar lavage (BAL) was then performed, and TNF-α and IL-1 in lysed BAL cells and in the supernatants from BAL cell cultures were studied. The effect of single and repeated LPS inhalation exposures on the activities of TNF and IL-1 was studied, as was the effect of LPS added to the cell culture medium. A single inhalation exposure to LPS caused an increase in the TNF-α and IL-1 activities in cell lysate and in the cell culture supernatant. After a second inhalation exposure, cell-associated and extracellular TNF-α activity could not be detected, whereas IL-1 activity was markedly enhanced. IL-1 activity was increased when LPS was added to the cell culture medium with or without a prior inhalation exposure. In contrast, TNF-α activity was not affected after a second exposure.

A variety of environmental agents can cause inflammation in the lungs when inhaled. The inflammatory response comprises a series of cellular reactions involving the secretion of mediators and invasion and activation of inflammatory cells, particularly alveolar macrophages.

In occupational settings, exposure to organic dusts causes the development of pulmonary diseases characterized by acute and chronic inflammation. These changes may lead to disability and early retirement owing to chronic bronchoconstriction, increased secretion of mucus (chronic bronchitis), or hyperreactivity (20).

There is accumulating evidence that bacterial endotoxins (lipopolysaccharides [LPS]) are important components of many organic dusts (18). Previous studies, chiefly employing in vitro techniques, have shown that endotoxin induces the production of a variety of mediators in macrophages, including the inflammatory mediators interleukin-1 (IL-1) and tumor necrosis factor (TNF) (1, 13, 15, 25). The latter causes tumor necrosis in vivo and in vitro (3, 11, 24).

To further investigate the role of these mediators in the early inflammatory response in the lungs, experiments in which animals were exposed to an aerosol of bacterial endotoxin were undertaken. After the aerosol exposure, the biological activities of TNF-α and IL-1 in lysed cells obtained by bronchoalveolar lavage (BAL) and in the supernatants from cultured BAL cells were measured. The effects of a second in vivo inhalation exposure were also investigated, as was the influence of LPS added to the cell culture medium.

MATERIALS AND METHODS

Reagents and media. Thiopental sodium (Pentothal) (5%; catalog no. 3321) was obtained from Abbott Laboratories AG, Zug, Switzerland. LPS refers to LPS from *Escherichia coli* O26:B6 (Difco Laboratories, Detroit, Mich.) (100 μg/ml) in pyrogen-free distilled water. DAB is Dulbecco's medium A plus Dulbecco's medium B (GIBCO Laboratories, Paisley, Scotland). PBS is cation-free phosphate-buffered saline (GIBCO). RPMI 1640, Dulbecco's modified Eagle's medium, and heat-inactivated (1 h at 56°C) fetal calf serum were also from GIBCO. NaCl (0.9%) is pyrogen-free saline (catalog no. FD 25556; Vifor, Geneva, Switzerland). Recombinant human TNF-α and recombinant human IL-1α were from Biogen SA, Geneva, Switzerland.

Cell lines. Dermal fibroblasts were obtained by proteolytic dispersion of human infant foreskin tissue (6). To assess TNF, we used the L929 murine TNF-susceptible cell line (17). All glassware was siliconized and sterilized at 200°C for 4.0 h. The plastic equipment used for cell culture was from Falcon, Becton Dickinson, Paramus, N.J. All glassware and chemicals used were endotoxin free.

Animals. Conventional 4-week-old Hartley strain guinea pigs of both sexes, weighing 350 to 400 g, were obtained from Kleintier Farm, Madorin, Basel, Switzerland, and the Geneva University Medical Center breeding unit, Arare, Switzerland. Groups of at least three animals were housed in a continuous-flow chamber for a 40-min exposure to LPS. Some groups of animals were given a second, identical 40-min exposure 4.0 or 24.0 h after the beginning of the first exposure.

The aerosol was generated by a Collison atomizer (19). All particles generated (molecular aggregates of LPS soluble in water) were less than 0.5 μm in diameter, as measured with a Royco optical particle analyzer. The concentration of LPS in the chamber was about 2.5 $\mu g/m^3$. According to calculations based on tidal volume and breathing frequency in guinea pigs, the minute volume (volume of air inhaled in 1 min) of a 400-g guinea pig was estimated to be 200 ml (21). With a respirable-particle fraction of 100% and an assumed deposition level of 50%, the lung dose was calculated to be about 50 ng per animal or 150 ng kg of body $weight^{-1}$.

Controls were unexposed animals maintained under the same conditions as the others. In our previous experiments, animals were exposed to pyrogen-free distilled water, and no

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changes in the number or distribution of cell types were observed in comparison with unexposed animals (19).

The animals were sacrificed at various times (0.7, 1.5, 2.0, 4.0, or 24.0 hours) after the beginning of the first or second exposure by an intraperitoneal injection of 3 ml of thiopental sodium.

BAL. Free cells from the airways of the guinea pigs were obtained by BAL. A volume of 100 ml of sterile NaCl solution was instilled in the lung by sequential injection and aspiration of 10-ml aliquots with a broad stainless steel 2-S 1-in (2.54-cm) cannula (model 2R2 Luer lock steel cannula; Urimed, Geneva, Switzerland) introduced into the trachea. The recovered fractions (about 70 ml) were pooled and centrifuged at 800 × *g* for 10 min at 4°C. The cells were washed once at 4°C in DAB, counted, and assessed for viability by trypan blue exclusion (85 to 93%). Approximately 20×10^6 BAL cells were obtained from each animal. Cell differention was performed with cytocentrifuge slides stained with May-Grunwald-Giemsa stain. About 75% of the cells in control animals were macrophages; after exposure to endotoxin, there was a large increase in neutrophils which reached 15- to 20-fold at 24.0 h.

Cell lysate preparation. BAL cells (20×10^6/ml) were resuspended in PBS (pH 7.4) containing 20 mM EDTA and 5 mM iodoacetamine and disrupted by four sonications on ice at 50 W for 165 s each time. The cell lysate was centrifuged at 40,000 × *g* for 10 min at 4°C, filtered through 0.22-μm-pore-size filters (no. SLG.S 0.25 BS; Millipore, Bedford, Mass.), and stored at −70°C (10).

Cell culture. BAL cells (2.0×10^6/ml) were plated into 5.0-cm-diameter Falcon petri dishes in RPMI 1640–10% heat-inactivated fetal calf serum and incubated for 24 h with or without 2.5 ng of LPS per ml at 37°C in a 5% CO_2 humid air atmosphere. Cell-free supernatants were harvested and stored at −70°C.

TNF-α and IL-1 bioassay. TNF-α bioactivity was measured in a cytotoxicity assay using a murine TNF-susceptible cell line (L929) in the presence of actinomycin D (1.0 g/liter) (14). All samples of supernatants and sonicated cells were tested at a 1:10 final dilution. The cytotoxicity was measured by vital staining with gentian violet (optical density at 570 nm). The calibration curve was obtained from a standard recombinant human TNF-α curve, and the limits of detection were 0.1 and 2.0 ng/ml (17). Each sample was assayed in quadruplicate.

IL-1 bioactivity in the same samples was measured at a 1:10 dilution of IL-1 in Dulbecco's modified Eagle's medium on fibroblasts obtained from human infant foreskins (5, 6). After 72 h, the fibroblast culture supernatants were removed and assayed for prostaglandin E2 (PGE_2). Levels of PGE_2 were determined by a radioimmunoassay using an antiserum to PGE_2 (kindly provided by L. Lewin, Brandeis University, Waltham, Mass.) (5). PGE_2 release by fibroblasts exposed to medium alone was 4.1 ± 0.7 ng/ml (mean ± standard error of the mean [SEM]), and PGE_2 release by those exposed to 250 pg of recombinant human IL-1 per ml was 146.2 ± 34.2 ng/ml. Each sample was assayed in triplicate.

Statistical tests. The results were expressed as mean and SEM. Comparisons between two means were made by the paired Wilcoxon sign rank test.

RESULTS

Cell-associated TNF-α and IL-1 after inhalation. The TNF-α and IL-1 activities in lysed BAL cells after a single inhalation are illustrated in Fig. 1. The TNF-α activity was increased as soon as 0.7 h after the exposure. The highest value was found at 1.5 h, and at 2 h no activity was detected. The IL-1 activity was also increased at 0.7 h, but the highest value was observed 2 h after the start of exposure. At 24 h, no TNF-α or IL-1 activity could be detected.

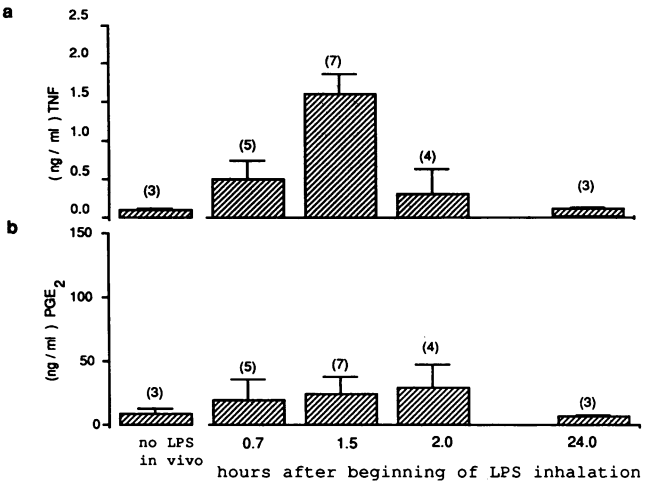

FIG. 1. TNF-α and IL-1 cell-associated activities at different times after the beginning of LPS inhalation. (a) TNF concentrations were assayed in the L929 cytotoxicity test. (b) IL-1 values are expressed as amounts of PGE_2 produced by fibroblasts exposed to IL-1 as measured by a radioimmunoassay. Error bars indicate SEMs. The number of animals per group is indicated in parentheses.

The TNF-α and IL-1 activities after a second inhalation of LPS given 4 h after the beginning of the first exposure are shown in Fig. 2. This second exposure did not induce TNF-α activity. At 1.5 h after the second exposure, the IL-1 activity was higher than that induced by a single exposure ($P < 0.01$).

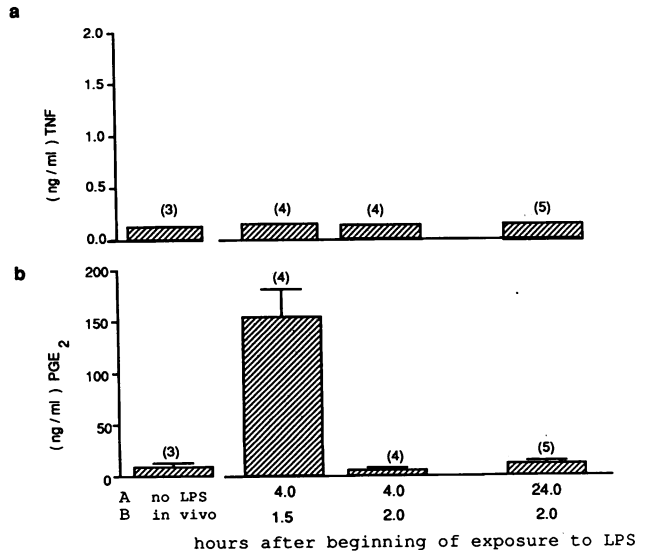

FIG. 2. TNF-α (a) and IL-1 (b) cell-associated activities at different times after a second inhalation of LPS (given 4 or 24 h after first inhalation). A, time after beginning of the first LPS inhalation; B, time after beginning of the second LPS inhalation. Error bars indicate SEMs. The number of animals per group is indicated in parentheses.

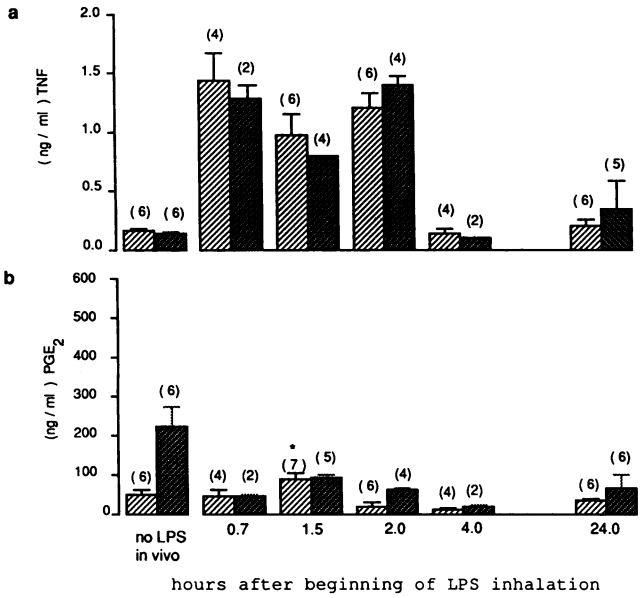

FIG. 3. TNF-α (a) and IL-1 (b) extracellular activity in supernatants of 24-h cultures of BAL cells obtained at different times after a single inhalation of LPS. The cells were incubated for 24 h without (light hatching) or with (dark hatching) 2.5 ng of LPS per ml. Error bars indicate SEMs. The number of animals per group is indicated in parentheses.

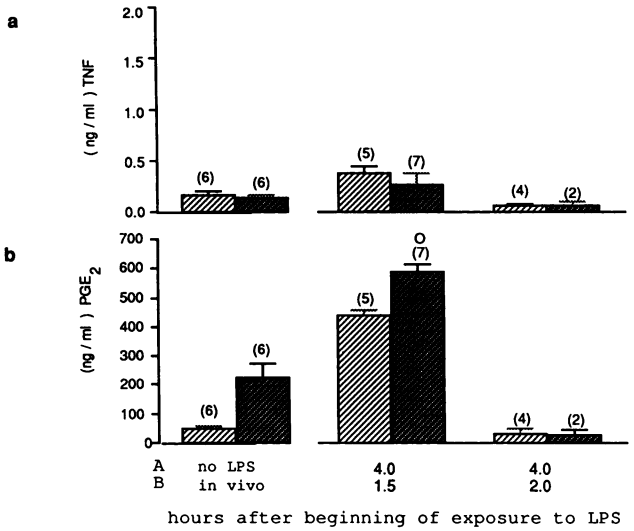

FIG. 4. TNF-α (a) and IL-1 (b) extracellular activity in supernatants of 24-h cultures of BAL cells obtained at different times after a second inhalation of LPS (given 4 h after first inhalation). The cells were incubated for 24 h without (light hatching) or with (dark hatching) LPS. A, time after beginning of the first LPS inhalation; B, time after beginning of the second LPS inhalation. Error bars indicate SEMs. The number of animals per group is indicated in parentheses.

At 2 h, no activity was detected. When the second exposure was given 24 h after the first, no TNF-α or IL-1 activity could be detected (Fig. 2).

TNF-α and IL-1 activities in cell culture supernatants. The TNF-α and IL-1 activities in BAL cell culture supernatants with and without LPS are shown in Fig. 3. In control animals not exposed to LPS by inhalation, LPS in the BAL cell culture medium did not induce TNF-α activity, whereas the IL-1 activity was significantly increased ($P < 0.01$).

When cultures were prepared from cells obtained from animals exposed to LPS by inhalation, the TNF-α activity was increased in cultures from cells harvested 0.7, 1.5, and 2.0 h after exposure. No activity was found 4 or 24 h after exposure. The presence of LPS in the medium did not influence the TNF-α activity. An increased level of IL-1 activity was found only in the cultures of cells harvested 1.5 h after the inhalation exposure ($P < 0.01$). The presence of LPS in the medium did not increase IL-1 activity.

The TNF-α and IL-1 activities in supernatants of cells from animals given a second inhalation exposure 4 h after the first are shown in Fig. 4. At 1.5 h after the second exposure, there was a tendency toward increased TNF-α activity in comparison with control animals, but this difference was not statistically significant.

The IL-1 activity level at 1.5 h after exposure was higher than that induced by a single exposure (Fig. 3; $P < 0.01$). In the supernatant from cells cultured in the presence of LPS, a further increase in IL-1 activity was observed ($P < 0.05$). No IL-1 activity was found in the culture supernatant from BAL cells harvested 2 h after the second inhalation exposure.

DISCUSSION

TNF-α and IL-1 activities were estimated by conventional methods. The test used was specific for TNF (10). The production of IL-1α and IL-1β could not be differentiated, as PGE_2 is stimulated by both types of mediators.

The activities of TNF-α and IL-1 were studied with BAL cell lysates (4) or supernatants from BAL cell cultures. Preliminary assays in BAL fluid could not demonstrate any activity caused either by dilution or by the presence of inhibitory factors in the airway fluid. The results from the two test systems were similar. Both the in vivo and in vitro exposures demonstrated that bacterial endotoxin can induce the production of TNF-α and IL-1. The duration of production is rather short, and additional mechanisms are probably required in order to cause the clinical symptoms of chronic inflammation observed after long-term exposure to organic dusts.

The concentration of the LPS chosen for cell cultures was based on experience from previous experiments in which the production of chemotactic factors was found to increase at this concentration (23). At higher levels of up to 1 μg/ml, a depressing effect was found, which could indicate a decrease in the production of other mediators as well. Differences in dose levels of LPS are probably the reason for the discrepancy between the present results and those previously published by Becker et al. (2). Those authors incubated human alveolar macrophages with 100 or 1,000 ng of LPS per ml and found that TNF-α and IL-1 were produced. The kinetics of that secretion, with a peak at 4 to 8 h and a duration of 42 h or more, were also different from those in the present study. A tentative explanation for these differences is that these high doses of LPS blocked the secretion of TNF and IL-1 inhibitors. We have recently observed the secretion of IL-1 and TNF inhibitors by human alveolar macrophages (7, 22).

The results of this study demonstrate important differences between TNF and IL-1 kinetics in the acute inflammatory response in the lungs. The cell-associated TNF-α activity was increased very soon after the inhalation expo-

sure, and the highest value was found at 1.5 h; by 2 h the activity had disappeared. The IL-1 activity increased more slowly and was present at 2 h. This difference between TNF and IL-1 was also reflected in the cell culture supernatants, in which extracellular TNF was detected in cells cultured very soon after the inhalation exposure, whereas the IL-1 activity was found later. This suggests that TNF-α induces IL-1 production (8, 26) and that IL-1 production is then self-induced by a positive feedback mechanism (9, 27). This relationship between TNF and IL-1 could, however, not be demonstrated in the in vitro experiments, suggesting that other cell systems, e.g., platelets, are necessary to induce the full response.

The differences between TNF-α and IL-1 were further illustrated by their activities after a second inhalation exposure. The TNF-α activity demonstrated tachyphylaxis, whereas the IL-1 activity increased. The practical importance of this result could be that primed and naive cell systems may induce different inflammatory responses to an environmental challenge. It is possible that this involves the first pathway of a sensitization process, but further experiments need to be undertaken to evaluate this hypothesis.

Differences between TNF and IL-1 activities were also found when cells were challenged with LPS in vitro. Although LPS in the cell culture did not elicit extra TNF-α activity in unexposed or exposed animals, the IL-1 activity was increased but only in cell supernatants from unexposed animals (Fig. 3). No additional IL-1 stimulation was detected in animals previously exposed to LPS by inhalation, suggesting a blocking of receptors or an exhausting of the supply of IL-1 precursor. In the reexposed animals, however (Fig. 4), the LPS in the cell culture caused a further increase in IL-1 production. The potential blocking mechanism was thus not effective in previously exposed (primed) cells.

TNF-α and IL-1 in combination could be the mechanism behind the migration of neutrophils from the blood into lung tissue and airways soon after exposure to endotoxin or organic dusts (23), an effect resembling that seen in the skin of rabbits (26).

The experimental model used here implies an acute inflammation induced by LPS. Several aspects of the model must be elucidated before the relationship to chronic inflammation induced by the environment can be established. We used an isolated preparation of LPS, but in nature this agent occurs bound to cells. Even though data from LPS challenges in humans do not suggest a major difference in the capacity to induce acute inflammation (18), differences may be present after long-term exposures.

The increase in TNF activity after an acute exposure to endotoxin may have clinical implications. In epidemiological studies of cotton workers, the standardized mortality ratios for lung cancer were found to be consistently lower than standardized mortality ratios for all sources (12). There was also an inverse relation in one study between the standard mortality rate for respiratory cancer and the degree of exposure and an inverse relation in another study between this rate and the duration of dust exposure. A study of cancer morbidity between 1970 and 1979 in Denmark demonstrated that the lung cancer incidence among females working in the textile industry (spinning and weaving) was the lowest among all occupational groups studied and lower than the average for the population at large (16).

However, an influence of TNF on lung cancer risk would require that TNF activity be maintained under chronic exposure conditions. There is thus a need to ascertain the production of TNF by lung cells in experiments involving a chronic exposure to isolated as well as cell-bound LPS.

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