RESPONSE OF AN ANIMAL MODEL TO MIXTURES OF ENDOTOXIN AND N-FORMYL-METHIONYL-LEUCYL-PHENYLALANINE (FMLP) AEROSOLS

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Abstract

Since both endotoxin and FMLP have been identified in cotton dust, it is important to examine how the interaction of these two agents may contribute to the biological response attributed to cotton dust. The object of this study was to determine how the simultaneous exposure to FMLP and endotoxin differed from the exposure to FMLP or endotoxin alone in the guinea pig animal model. Results showed that simultaneous exposure to the two agents caused a reduction in the pulmonary cellular response in terms of total cells, granulocytes and lymphocytes recovered from the lungs by broncho-alveolar lavage when compared with the response to endotoxin alone. The breathing rate response measured in 10% CO_2 in air of guinea pigs exposed to a mixture of endotoxin and FMLP was more like animals exposed to FMLP alone than to animals exposed to endotoxin alone. In contrast, the macrophages from animals simultaneously exposed to FMLP and endotoxin appeared to have been activated to a greater extent than macrophages from animals exposed to either FMLP or endotoxin alone. These results are consistent with the hypothesis that macrophages activated by a combination of FMLP and endotoxin are inhibited from either producing or releasing factors which contribute to the cellular infiltration into the lung that normally follows endotoxin exposure.

Introduction

It has been well established that the breathing and cellular response of small laboratory animals following exposure to cotton dust correlates well with the measured endotoxin level within the dust (Fischer *et al.*, 1986) and that animals exposed to endotoxin behave similarly to animals exposed to cotton dust (Burrell and Rylander, 1987; Robinson *et al.*,1993). These results have supported the conclusion that endotoxin may be the principal mediator of the acute inflammatory response to inhaled cotton dust. Several other biologically active substances, however, have been identified in cotton dust in addition to endotoxin. One such substance is FMLP (Fedan *et al.*, 1989), a chemoattractant that is formed by *Escherichia coli* (Voelkel *et al.*, 1992). The role of FMLP in the animal response to cotton dust has not yet been determined since guinea pigs exposed to FMLP alone do not demonstrate all of the physiological responses observed following an acute exposure to cotton dust (Burrell and Rylander, 1985; Frazer *et al*., 1992). These findings have suggested that it is unlikely that FMLP is primarily responsible for the acute response of the guinea pig animal model to cotton dust. Another very important role that FMLP may have in the cotton dust response, however, is that of activating or deactivating the cellular response associated with exposure to endotoxin. The object of this investigation was to examine this possibility and determine to what extent FMLP modifies the guinea pig response to endotoxin. These objectives were accomplished by exposing animals to FMLP alone, endotoxin alone or a combination of both endotoxin and FMLP and then comparing the biological responses of the guinea pig model to these aerosols.

Methods

Experimental Design

A block diagram outlining the procedures followed during the exposure and testing of groups of guinea pigs (N=6) exposed to either filtered air, FMLP, endotoxin or a combination of FMLP and endotoxin is illustrated in fig. 1. The breathing rate of animals in air and 10% CO_2 in air was measured prior to exposure, immediately post-exposure and 18 hrs post-exposure. Following the last breathing rate measurement, animals were sacrificed and pulmonary cells were harvested from the lung by broncho-alveolar lavage. The total number of cells and the relative number of granulocytes, lymphocytes, red blood cells and macrophages in the lavage fluid of each animal was determined using a Coulter Counter (model # ZBI) equipped with a cell sizing attachment. The degree of macrophage activation was estimated at rest and following zymosan stimulation by measuring chemiluminescence under the two conditions.

Animals

Specific pathogen free guinea pigs (English short hair) weighing between 300 and 400 gms were purchased from Harlan Research Laboratory. They were acclimated for at least 1 week in the animal quarters facility prior to use. The animals were divided randomly into four groups of six animals each .

Methods of Exposure

A diagram of the system used to expose guinea pigs to liquid aerosols is illustrated in fig. 2. A detailed description of the design and testing of this system has been given previously (Frazer *et al.*, 1992).

In this study guinea pigs were exposed to either filtered air, or aerosols of FMLP alone, endotoxin alone or a combination of FMLP and endotoxin. The FMLP was

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purchased from Sigma (#F3506), and lyophilized endotoxin was obtained from the Institute of Agricultural Medicine in Lublin, Poland, and prepared as we have described previously (Robinson *et al.,* 1993). The FMLP, endotoxin and combination of FMLP and endotoxin were suspended in buffered solutions of 0.05 M NaH_2PO_4 with the pH adjusted to 7.4. Liquid aerosols were generated from these solutions with an ultrasonic nebulizer (De Vilbiss, model# O94HD). During an exposure period, samples were collected gravimetrically inside the exposure chambers on filters changed at 30 min intervals. Endotoxin collected on the filters was measured using the LAL method we have previously described (Robinson *et al.,* 1993), and FMLP was detected using the method described by Siegal *et al.* (1994). Analysis of the filters showed that the average $\pm$ standard error of the concentration of the FMLP aerosol inside the exposure chamber was 321.8 ± 30 µg/m^3, the endotoxin concentration was 168 ± 40 EU/m^3, and the concentrations of the mixture of FMLP and endotoxin were found to be 348.8 ± 51 µg/m^3 and 280 ± 0.08 EU/m^3 respectively . The characteristics of the aerosols and the exposure system were similar to those described previously (Frazer *et al.,* 1992).

Measurement of Breathing Rate in 10% CO_2 in Air

Prior to exposure, immediately post exposure and 18 hrs post exposure, guinea pigs were placed in a glass chamber in which 10% CO_2 in air was allowed to pass through at a constant rate of 2 l/min. Prior to measurements in 10% CO_2, the animals were allowed to equilibrate for 3 min. Pressure fluctuations inside the chamber containing the animals, created by differences in temperature and humidity of the inspired and expired gas in addition to the compression of gas within the guinea pig thorax, were measured and analyzed to obtain the breathing rate of the animals. Details of the procedures and calculations were described previously in detail (Frazer *et al.,* 1989).

Lung Cell Analysis

Lungs from all animals underwent broncho-alveolar lavage 18 hrs post exposure. The alveolar cells were harvested using methods we have previously described in detail (Castranova *et al.,* 1990). Total and differential cell counts were made using an electronic cell counter with a cell sizing attachment. Granulocytes, lymphocytes, red blood cells and macrophages were distinguished by their characteristic volume distributions.

The release of reactive species was quantified at rest and after stimulation with zymosan (2mg/ml) in the presence of 0.008 mg% luminol by measuring the resulting chemiluminescence with a luminometer (EG&G, model #LB953) as described by Blackford *et al.,*1994.

Results and Conclusions

The same system used in this study to expose guinea pigs to a liquid aerosol of either FMLP, endotoxin or a combination of FMLP and endotoxin has been previously described in detail. The number and mass distribution of the liquid aerosols within the exposure chamber was examined using a true aerodynamic particle analyzer (APS), and the distributions of particles were not significantly different than those presented previously (Frazer *et al,* 1995).

The breathing rates of guinea-pigs inspiring 10% CO_2 in air, after a 3 min equilibrium period, were determined for the four groups of animals exposed to either filtered air, FMLP, endotoxin or a combination of FMLP and endotoxin. Breathing rate measurements were made pre-exposure, immediately post-exposure and 18 hrs post-exposure. Changes in the breathing rate response to FMLP were similar to results previously described (Frazer *et al.,* 1992). Those results showed that the breathing rate of guinea pigs exposed to FMLP increased immediately post-exposure and then gradually decreased toward control values at 18 hrs post-exposure. The average breathing rates $\pm$ the standard error (SE) of the measurement for the group of animals exposed to endotoxin alone and the group of animals exposed to a combination of endotoxin and FMLP are shown in fig. 3. The breathing rate of guinea pigs exposed to endotoxin alone peaked later than the breathing rate of guinea pigs exposed to FMLP alone. Animals exposed to a combination of FMLP and endotoxin had a breathing rate response that was more like animals exposed to FMLP alone than to animals exposed to endotoxin alone.

The pulmonary cellular response of control and exposed animals was measured 18 hrs post-exposure by broncho-alveolar lavage. The results are shown in fig. 4. It can be seen that the total number of cells recovered from lungs by lavage was greatest in animals exposed to endotoxin alone, the next greatest was in animals exposed simultaneously to FMLP and endotoxin, then FMLP alone and finally control animals. The same order was observed for the number of granulocytes, lymphocytes, and red blood cells recovered. There was no significant difference in the number of macrophages recovered among any of the four groups. Thus, it appears that pulmonary inflammation and damage of the alveolar air-blood barrier are reduced in response to endotoxin when the macrophages are simultaneously being exposed to FMLP.

When macrophage activation was estimated by measuring their ability to generate chemiluminescence in response to *in vitro* zymosan stimulation, it was found that macrophages from animals exposed simultaneously to FMLP and endotoxin were activated the most in terms of reactive species production (Fig.5) . Macrophages from animals exposed to FMLP appeared to be activated to the next greatest extent followed by animals exposed to endotoxin and finally control animals. Thus, in contrast to the breathing rate and cell recruitment response, release of reactive species by alveolar macrophages in response to endotoxin is enhanced in the presence of FMLP.

In summary, this study indicates that the activation of macrophages resulting from exposure to a combination of FMLP and endotoxin may play an important role in the reduction of breathing rate and the pulmonary cellular infiltration that normally occurs following exposure to endotoxin alone. It is possible that the balance between release of chemotactic factors and oxidant species from alveolar macrophages varies with the degree of cell activation. Another possibility is that excessive release of reactive species can oxidize and thus reduce the effectiveness of chemoattractants produced by macrophages. Since FMLP and endotoxin are both found in cotton dust, interactions between these two agents are likely to play an important role in the overall animal model response to cotton dust.

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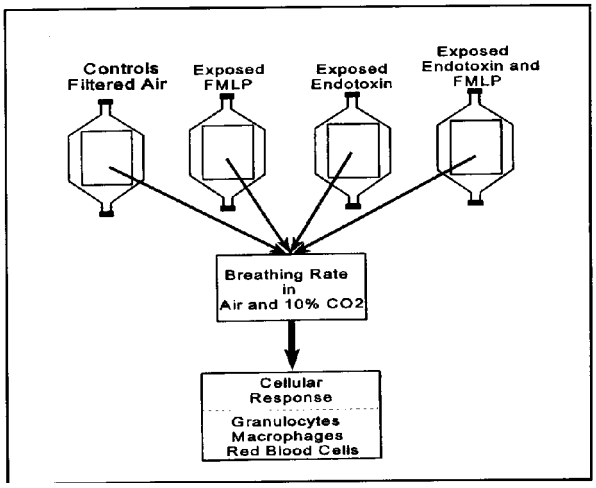

Figure 1. Experimental design used to study the response of guinea pigs to either filtered air, FMLP, endotoxin, or a combination of FMLP and endotoxin.

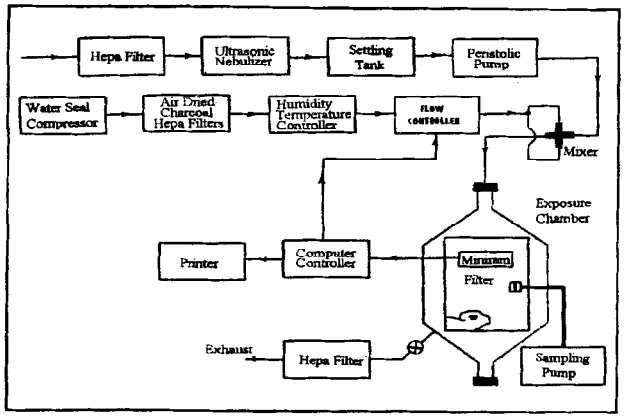

Figure 2. Block diagram of the exposure system used to maintain a constant concentration of either FMLP, endotoxin or a mixture of FMLP and endotoxin as liquid aerosols for small laboratory animal testing.

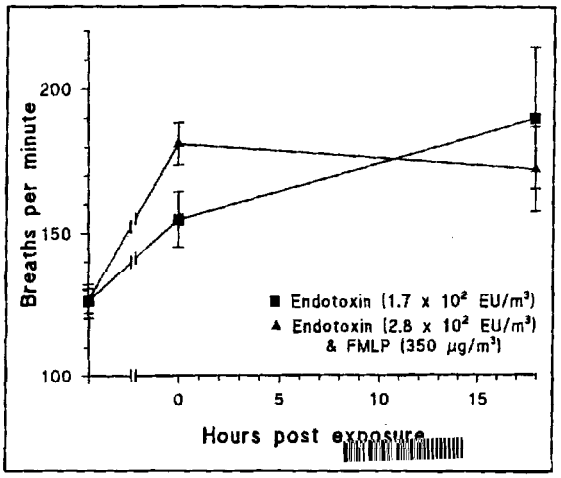

Figure 3. Breathing rate of CO_2 challenged animals measured pre-exposure, immediately post-exposure and 18 hrs post-exposure to filtered air, FMLP, endotoxin or a combination of FMLP and endotoxin.

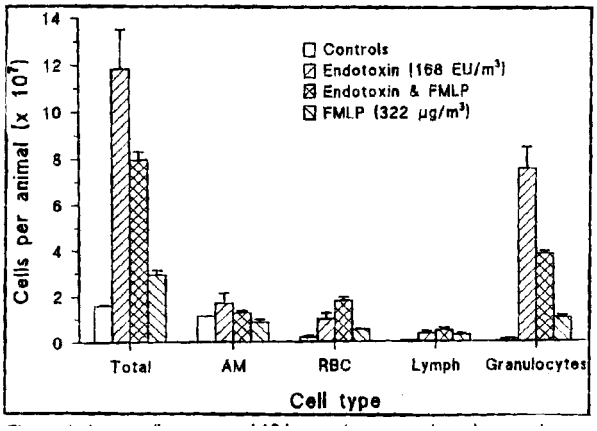

Figure 4. Lung cells recovered 18 hrs post-exposure by pulmonary lavage from guinea pigs exposed to filtered air, FMLP, endotoxin or a combination of FMLP and endotoxin.

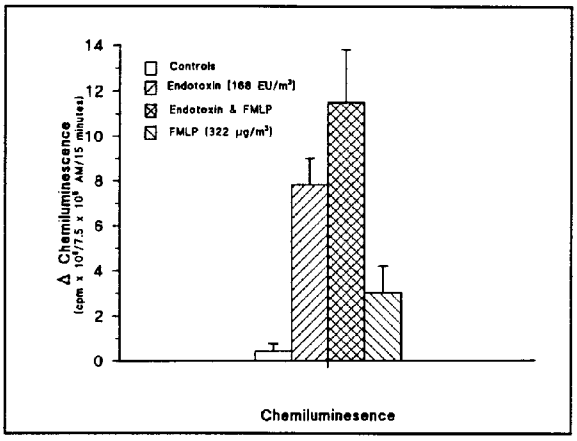

Figure 5. Difference between resting and zymosan-stimulated chemiluminescence generated by alveolar macrophages harvested at 18 hrs post-exposure from guinea pigs that had been exposed to either filtered air, FMLP, endotoxin or a combination of FMLP and endotoxin.