GUINEA PIG RESPONSE TO ZYMOSAN AND A SERIAL EXPOSURE OF ZYMOSAN AND ENDOTOXIN

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Abstract

This study had two objectives. The first was to measure the response of guinea pigs exposed to either a low (1 mg/m^3), medium (6 mg/m^3) or high (23 mg/m^3) concentration of a zymosan aerosol and the second was to determine how the guinea pigs' response to endotoxin exposure ($10.7 \cdot 10^3$ EU/m^3) was altered by pre-exposure with a zymosan aerosol (23 mg/m^3). The animals' reaction to each exposure was evaluated by measuring both changes in breathing rate in 10% CO_2 in air and the pulmonary cellular response by broncho-alveolar lavage. Results showed that exposure to zymosan alone had very little effect on the guinea pigs' breathing rate response but did exhibit a dose response relationship with respect to the total number of cells and granulocytes recovered by lavage. In addition, the macrophages appeared to be activated by zymosan exposure as evidenced by their ability to produce reactive species when challenged by zymosan *in vitro*. Results of the second part of the study showed that when compared with the cellular response of animals exposed to endotoxin alone, there was a significant decrease in both the total number of cells and the number of granulocytes recovered in animals exposed serially to zymosan and endotoxin. The macrophages of serially exposed animals appeared, however, to be more activated than macrophages from animals exposed to endotoxin or zymosan alone.

Introduction

It has been shown that (1→3)-β -D-glucan is the macrophage activating component of the yeast cell wall fraction known as zymosan (Riggi and DiLuzio, 1961). Rylander *et al.* (1993) has described the effects of an acute exposure of inhaled (1→3)-β -D-glucan on the number of lung cells recovered 24 hrs post exposure by broncho-alveolar lavage. Results of those studies showed that exposure of guinea pigs to an insoluble form of (1→3)-β -D-glucan (curdlan), resuspended in a liquid aerosol generated by a Collision atomizer, did not greatly influence the number of cells recovered in the lavage fluid. It was found, however, that animals exposed simultaneously to endotoxin and (1→3)-β -D-glucan had a marked decrease in the number of cells recovered when compared to the number of cells recovered from lungs following endotoxin exposure alone. Since the guinea pig's acute response to cotton dust is of interest and cotton dust contains both endotoxin and (1→3)-β -D-glucans (Rylander *et al.*, 1993), interactions between multiple components of the dust are of great importance in determining the overall response to cotton dust.

Rylander and Bergstrom (1995) used a Limulus assay to show that glucans found in cotton dust are primarily in a non-soluble form. Since it is likely that glucans are dispersed as a dust rather than a liquid aerosol, we designed a self-feeding acoustical exposure system that used computer feedback to generate and hold the mass-concentration of an insoluable aerosol of glucan (zymosan) constant.

This study had two objectives. The first was to establish the dose-response relationship of aerosols of zymosan particulates in the guinea pig animal model following exposure to a low, medium or high concentration of zymosan dust. The second was to determine the effect of pre-exposure to zymosan particulates on the guinea pig response to an endotoxin aerosol. This was achieved by exposing guinea pigs to either filtered air, zymosan, endotoxin or zymosan immediately followed by exposure to endotoxin. The animals' response to these exposures was determined by comparing their breathing response in 10% CO_2 in air, their broncho-alveolar lavage cellular yield and the zymosan stimulated chemiluminescence of the recovered macrophages.

Methods

Experimental Design

During the initial set of experiments, groups of guinea pigs were exposed to either filtered air or to a low, medium or high concentration of (1→3)-β -D-glucan (zymosan) dust for 4 hrs. The breathing rate in air and 10% CO_2 in air was measured pre-exposure, immediately post exposure, and prior to sacrifice at 18 hrs post exposure. Lungs were broncho-alveolar lavaged and the total number of cells, as well as the relative number of granulocytes, lymphocytes, red blood cells, and macrophages recovered from each lung were determined. In addition, the chemiluminescence produced by *in vitro* zymosan stimulation of the macrophages was determined. A block diagram illustration of the exposure methods and testing procedures is given in fig. 1A.

In the second set of experiments, groups of guinea pigs were exposed to either filtered air, liquid aerosols of endotoxin, dry suspensions of zymosan, or a sequential exposure of zymosan immediately followed by an exposure to endotoxin as illustrated in fig 1B. Experiments were designed so that 4 guinea pigs were exposed at one time. First, 4 guinea pigs were exposed to zymosan for 4 hrs, the animals were removed from the chamber and 2 of the

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zymosan exposed animals along with two additional naive guinea pigs were exposed to endotoxin. This sequence was repeated on three occasions. Breathing rate and the pulmonary cellular response were measured in the same manner as the first set of experiments.

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 and then randomly divided into eight groups. Four groups were used for both the first set and second set of experiments.

Exposure System

Diagrams illustrating the system used to expose guinea pigs to zymosan are shown in figs. 2A and B. Zymosan particles were resuspended using the generator system shown in fig. 2A. A constant rate feeder mechanism was constructed from a modified syringe pump (Harvard Apparatus, model #901) which advanced a stainless steel tube inside a glass tube that had been uniformly packed with zymosan powder. The rate of advancement of the tube was controlled by the syringe pump gear box and worm drive. Airflow through the stainless steel tube impinged on the zymosan powder causing it to become entrained in the moving air. Zymosan particulates suspended in air were transported to the small acoustical generator. The generator chamber was formed from a cylinder 10.5 cm in diameter and 35 cm in length. It was constructed in a similar manner to that described previously (Frazer *et al*., 1987). The generator was used to disperse agglomerated particles before they entered the animal exposure chamber that is shown in fig. 2B. The complete exposure chamber and the use of computer feedback to control the mass concentration of the dust has previously been described in detail (Frazer et al., 1987). The system used to expose animals to liquid endotoxin aerosols has also been described previously (Frazer *et al*.,1995).

Aerosol Characterization

Characteristics of the zymosan aerosol generated from zymosan powder purchased from Sigma (Zymosan A, #Z4250) and resuspended in our exposure system were determined with an APS true aerodynamic particle analyzer (TSI model #3300). A typical particle number distribution is shown in fig. 3A and the equivalent mass distribution is illustrated in fig. 3B. It can be seen that the mass distribution formed a bimodal curve. The peak of the number distribution curve representing the largest number of particles is centered about 2.0 µm. Gravimetric samples were collected from the exposure chamber every hour during the four hour animal exposure period by drawing the aerosol through a Gelman 37 mm PVC filter, having a pore size of 0.5 µm at a flow rate of 1 l/m. Lyophilized endotoxin was obtained from the Institute of Agricultural Medicine in Lublin, Poland, (Milinowsky *et al.*, 1996) and prepared using the methods we have previously described in detail (Robinson *et al.*, 1993). A liquid solution of *E. agglomerans* extract was aerosolized with an ultrasonic nebulizer, and animals were exposed within chambers that were controlled to keep the mass concentration of the aerosol constant. The characterization of the endotoxin aerosol has been described elsewhere (Frazer *et al.*, 1995). Gravimetric filter samples were collected at half hour intervals during all animal exposure periods, and the amount of endotoxin on the filters was determined by LAL analysis (Robinson *et al.*, 1993).

Aerosol Concentrations

In the first set of experiments, groups of guinea pigs were exposed to either filtered air or a low (1 mg/m^3), medium (6 mg/m^3) or high (23 mg/m^3) concentration of zymosan dust. In the second set of experiments, groups of guinea pigs were exposed to either filtered air, zymosan alone (23 mg/m^3), endotoxin ($10.7 \cdot 10^3$ EU /m^3) alone or a serial combination of zymosan (23 mg/m^3) and endotoxin ($10.7 \cdot 10^3$ EU/m^3).

Measurement of Breathing Rate in Air and 10% CO_2

Prior to exposure, immediately post exposure, and at 18 hrs post exposure, each guinea pigs' breathing rate was measured in air and 10% CO_2 in air using methods we have previously described (Castranova *et al*., 1987).

Lung Cell Analysis

Lung cell analysis was performed on the broncho-alveolar lavage from each animal within the eight groups of guinea pigs at 18 hrs post exposure. The method used to perform the lavage has previously been described (Castranova *et al*., 1987). Total and differential cell counts were made using an electric cell counter equipped with a cell sizing attachment. Using this system the total number of cells was determined, then the fraction of macrophages, granulocytes, lymphocytes, and red blood cells was distinguished by their characteristic volume distributions (Castranova et *al.*, 1979; Jones *et al*., 1980; Miles *et al*., 1978). Release of reactive species from alveolar macrophages was measured at rest and after stimulation with unopsonized zymosan (2mg/ml) in the presence of 0.008 mg% luminol by quantifying the resulting chemiluminescence with a luminometer (EG&G, Modelm #LB 953) as described by Blackford *et al*., (1994).

Results and Discussion

It has been noted that when β-glucans are inhaled in the workplace environment, they represent a component of the fungal cell wall that is in all likelihood particulate in nature (Williams, 1993). In order to simulate workplace conditions, we exposed guinea pigs to a constant concentration of microparticulates of chemically pure, water insoluble, (1→3)-β -D-glucan in the form of zymosan dust. The dust was generated using a pneumatic dust feeder coupled to a computer controlled acoustical generator which formed a resuspension system that

provided a constant particle concentration for small animal exposure studies. The zymosan dust was characterized by a multi-modal mass distribution as shown in fig. 3. Although the count distribution of the dust indicates that most particles had aerodynamic diameters less than 3 µm, there was a small number of larger particles which were great in mass.

During the first part of this study in which guinea pigs were exposed to either filtered air or zymosan, only small changes in the breathing rate in 10% CO_2 in air were observed in any of the groups of animals exposed to zymosan. The breathing rate of animals exposed to 1 mg/m^3 and 6 mg/m^3 was not significantly different from the control group. The breathing rate of the group of animals exposed to 23 mg/m3 produced a minimal response which is shown in fig 5. In terms of the breathing rate response, zymosan appears much like a nuisance dust.

Results of the cellular response measurements determined by broncho- alveolar lavage are given in fig. 6. It can be seen that a dose relationship appears to exist in terms of total cells and the number of granulocytes recovered. Red blood cells and lymphocytes were significantly higher during the 23 mg/m^3 response while the number of macrophages recovered remained nearly constant for control and exposed animals. Fig. 4B shows that the chemiluminescence produced by macrophages in response to zymosan stimulation *in vitro* increased as the animal exposure concentrations of zymosan dust increased.

The breathing rate of guinea pigs inspiring 10% CO_2 in air, after a three minute equilibrium period, was determined for the four groups of animals that had been exposed to either filtered air, zymosan dust, a liquid aerosol of endotoxin, or zymosan dust immediately followed by an endotoxin aerosol. Results of the breathing rate measurements made prior to exposure, immediately post exposure and 18 hrs post exposure are shown in fig. 5. When compared to control animals, guinea pigs exposed to 15 mg/m^3 zymosan for 4 hrs tended to have a slightly elevated, but not significantly increased, breathing rate while breathing 10% CO_2 in air. The breathing rate in 10% CO_2 in air of animals exposed to endotoxin showed a significantly higher breathing rate than control animals at 18 hrs post exposure. Animals exposed to both zymosan and endotoxin tended to have a higher breathing rate in 10% CO_2 in air than animals exposed to endotoxin alone. There was a significant difference at zero hrs post-exposure but not at 18 hrs post exposure.

The results of pulmonary cellular response measurements at 18 hrs post exposure are shown in fig.6. The figure shows that the total number of cells recovered from the lung following the serial exposure to zymosan and endotoxin was less than the total number of cells obtained from animals exposed to endotoxin alone but greater than those from animals exposed to zymosan alone. A similar observation can be made for the number of granulocytes recovered. The number of lymphocytes and red blood cells was greatest in animals serially exposed to zymosan and endotoxin when compared to animals exposed to either endotoxin or zymosan alone. The number of macrophages was nearly the same for both the three groups of exposed animals and the control group of animals.

Fig.7 shows the zymosan-stimulated release of reactive species was greatest in animals serially exposed to zymosan and endotoxin, followed next by the group of animals exposed to endotoxin alone, then the group exposed to zymosan alone, and, finally, the smallest response as expected, was in the control group of animals. It has been suggested that the decrease in granulocyte recruitment following an acute serial exposure to zymosan and endotoxin may be due to a toxic effect of zymosan on macrophages that causes a decreased secretion of chemotactic substances which have been linked to the migration of neutrophils into the lung air spaces. This study suggests, however, that the ability of macrophages to release reactive oxidant species is not diminished by zymosan exposure. This is illustrated by the fact that macrophages from animals exposed to a serial exposure of zymosan and endotoxin are capable of generating large amounts of chemiluminescence in response to zymosan exposure *in vitro*.

In the past Hoffman *et al*. (1993) examined how β-glucans modulate the macrophage release of tumor necrosis factor-α (TNF-α) in response to endotoxin *in vitro*. They showed that high concentrations of β-glucans inhibited macrophage secretion of TNF-α that was normally induced by endotoxin. Interestingly, their study also showed that low concentrations of β-glucan (<500 µg/ml) stimulated TNF-α release while high concentrations of β-glucan (>500 µg/ml) suppressed TNF-α release from macrophages. The results of their studies with β-glucans at high concentrations did not appear to be due to a toxic effect since macrophage viability was not affected. Although the authors have suggested the use of caution in extrapolating their *in vitro* data to the intact animal, their findings are consistent with the effects that zymosan appears to have on the endotoxin-induced release of chemoattractants from alveolar macrophages that are reported in the present study. It appears likely, therefore, that the balance between cytokine and oxidant released from alveolar macrophages varies with the degree of macrophage activation *in vivo.*

In summary, this study shows first that guinea pigs exposed for 4 hrs to different concentrations of (1→3)-β -D-glucans in the form of zymosan particulates of up to 23 mg/m^3, caused little changes in their breathing rate in 10% CO_2 in air. It was found, however, that the total number of cells and granulocytes recovered by broncho- alveolar lavage, 18 hrs post exposure, was significantly increased in a dose dependent manner. In the second part of this study, it was shown that the breathing rate of guinea pigs equilibrated in

10% CO_2 in air tended to be greater in animals exposed serially to zymosan and endotoxin than when they were exposed to either zymosan or endotoxin alone. This result was in contrast to pulmonary cell recruitment measurements, made under the same exposure conditions, which demonstrated a significant reduction in the number of total cells and granulocytes recovered by lavage in animals exposed serially to zymosan and endotoxin when compared to animals exposed to endotoxin alone.. Machrophages from animals exposed to both zymosan and endotoxin appeared to produce more reactive oxidants, however, as illustrated by their chemiluminescence response to zymosan *in vitro*, than macrophages from animals exposed to either zymosan or endotoxin alone.

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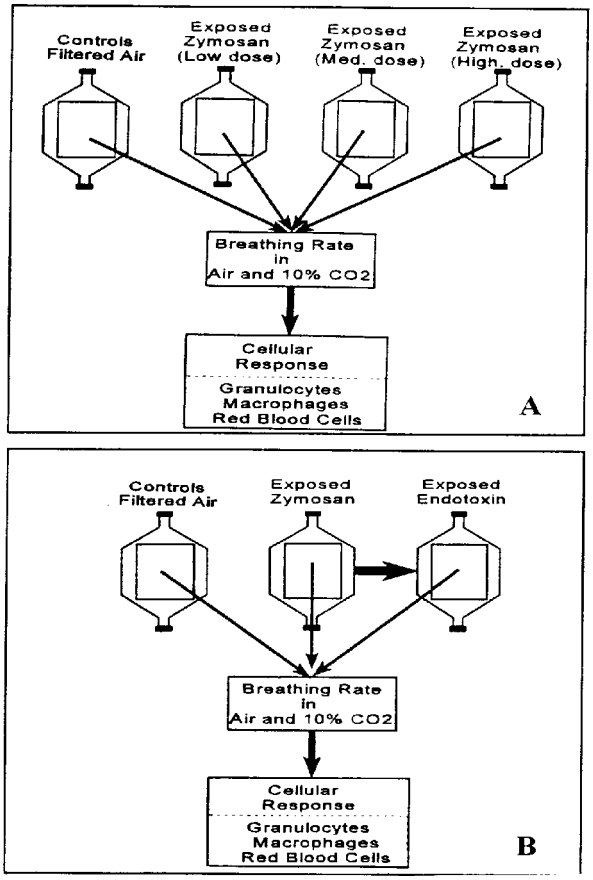


Figure 1. Experimental design used to study the response of guinea pigs to: A) Filtered air and three concentrations of zymosan particulates, or B) Filtered air, zymosan, endotoxin and a combination of zymosan and endotoxin.

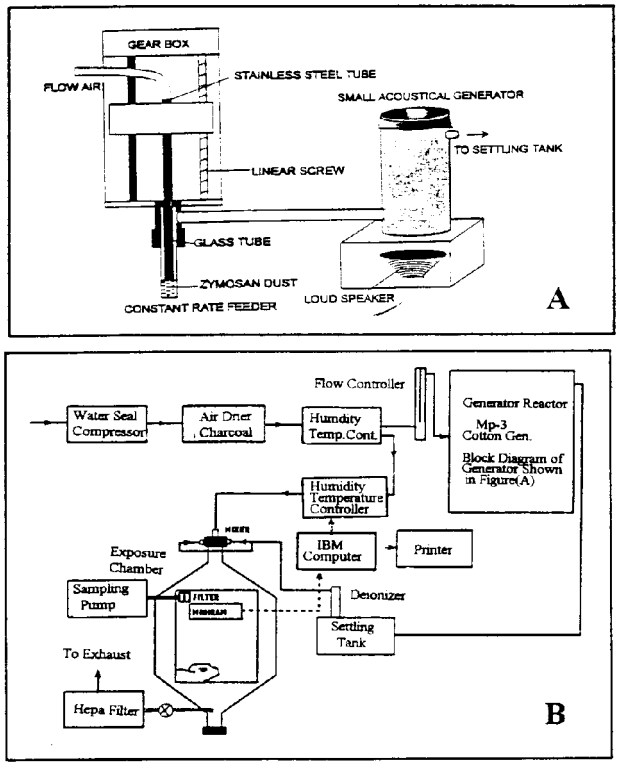
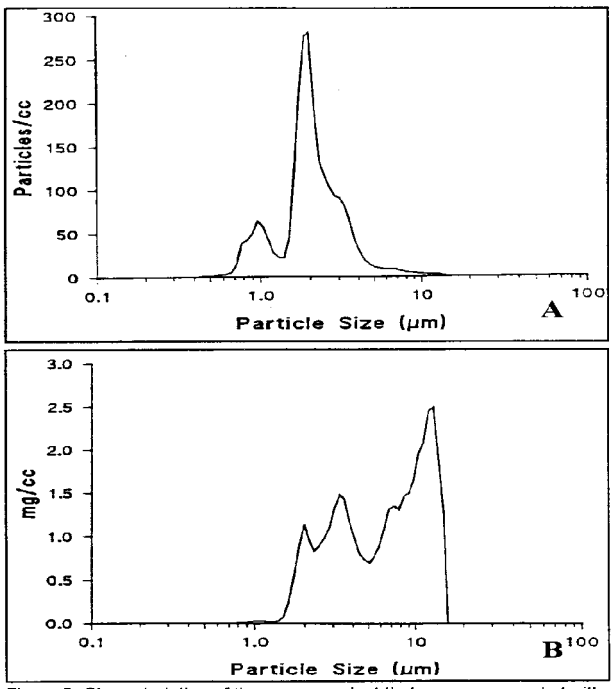


Figure 2. A) Diagram of the acoustical generator and the constant feed device. B) Block diagram of the exposure system used to maintain a constant exposure of zymosan dust



.Figure 3. Characteristics of the zymosan dust that was resuspended with the acoustical generator. Measurements were made in the exposure chamber with an aerodynamic aerosol analyzer. A) Number count distribution of the aerosol, B) Mass distribution of the same aerosol.

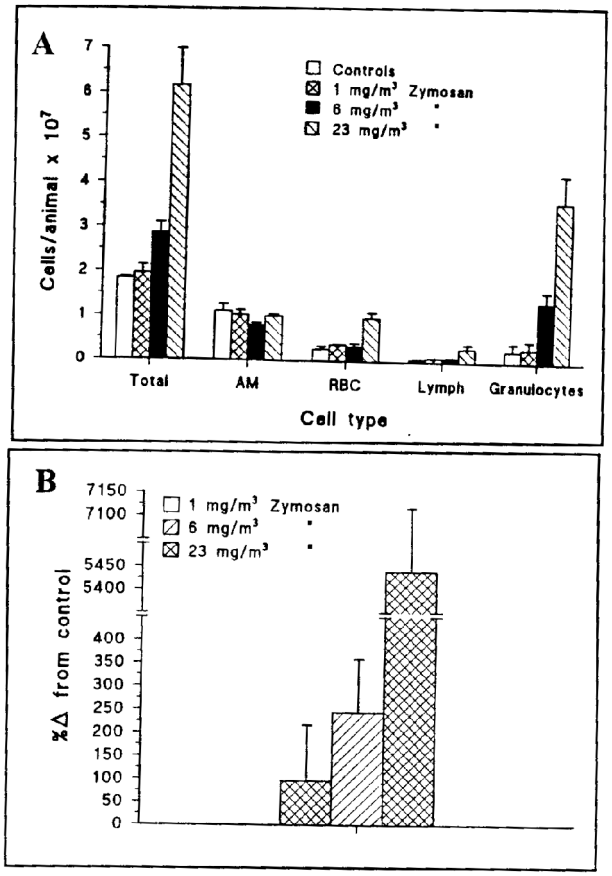


Figure 4. A) Lung cells recovered 18 hrs post-exposure by broncho-alveolar lavage from guinea pigs exposed to filtered air and a low (1 mg/m^3), medium (6 mg/m^3) and high (23 mg/m^3) concentration of zymosan dust. B) Percent increase of *in vitro* zymosan-stimulated chemiluminescence generated by alveolar macrophages from animals exposed to zymosan particulates when compared with macrophages from control animals.

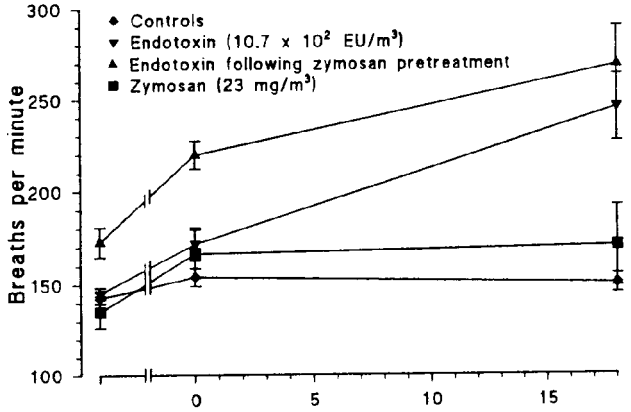


Figure 5. Breathing rate of CO_2 challenged guinea pigs measured pre-exposure, immediately post-exposure and 18 hrs post exposure to filtered air, zymosan, endotoxin or a serial combination of zymosan and endotoxin.

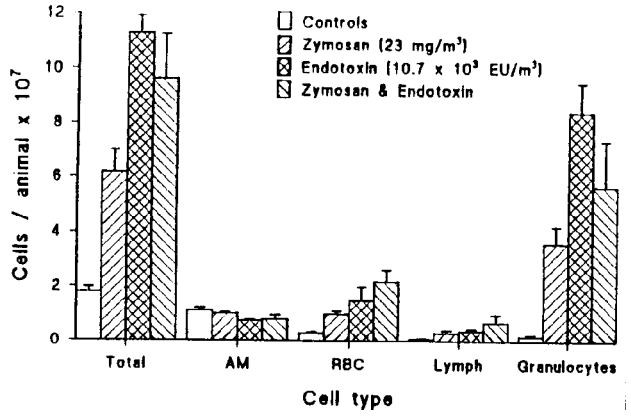


Figure 6. Lung cells recovered 18 hrs post-exposure by broncho-alveolar lavage of guinea pigs exposed to filtered air, zymosan, endotoxin or a serial combination of zymosan and endotoxin.

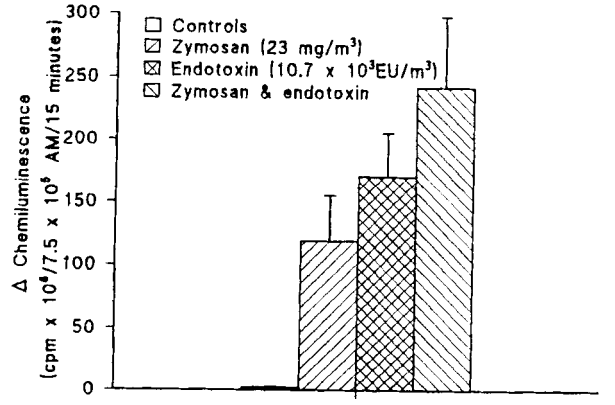


Figure 7. Difference between resting and zymosan-stimulated chemiluminescence generated from alveolar macrophages harvested at 18 hrs post-exposure from guinea pigs that had been exposed to either filtered air, zymosan dust, endotoxin or a serial combination of zymosan and endotoxin.