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The combined effects of endotoxin and dust on the lung. Final report on HSE project 1/LMD/126/240/88

Cullen RT, Cowie HA, Beattie J

WORLD HEALTH ORGANISATION
COLLABORATING CENTRE
FOR OCCUPATIONAL HEALTH

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The Combined Effects of Endotoxin and Dust on the Lung

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INSTITUTE OF OCCUPATIONAL MEDICINE

THE COMBINED EFFECTS OF ENDOTOXIN AND DUST ON THE LUNG

by

RT Cullen, HA Cowie, J Beattie

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Report No. TM/92/01

INSTITUTE OF OCCUPATIONAL MEDICINE

THE COMBINED EFFECTS OF ENDOTOXIN AND DUST ON THE LUNG

by

RT Cullen, HA Cowie, J Beattie

SUMMARY

Endotoxin, a common component of organic dusts in agriculture and in industrial environments such as waste treatment plants, and grain, cotton, and wool mills, has been implicated in the aetiology of respiratory diseases associated with the inhalation of such dusts. The inhalation of endotoxin can reproduce many of the symptoms seen in these diseases. However, some studies have been unable to correlate the endotoxin content of organic dusts with respiratory symptoms, and others have implicated other components of the dusts in the disease processes. Separating the relative contributions of endotoxin and dust to disease has, therefore, been extremely difficult.

This project set out to study the interactive effects in the lungs of rats between lipopolysaccharide (LPS), the main active component of endotoxin, and a defined organic particulate (OVA-beads): polystyrene beads conjugated with ovalbumin to render them antigenic. In addition, we studied the effects of LPS on the lung responses to two inorganic dusts, the pathogenic quartz DQ12 and the relatively inert titanium dioxide (TiO_2).

Intratracheal instillation of OVA-beads resulted in an inflammatory response, characterised by increased total cell and neutrophil numbers recovered by bronchoalveolar lavage, which peaked within 24 hours and resolved by day 7 post-instillation. The percentage of lymphocytes recovered in lavage from these animals increased with time, peaking at day 35 post-instillation.

The inclusion of 80 ug LPS in the inoculum of OVA-beads produced an enhanced, synergistic neutrophil recruitment, which had also largely resolved by day 7. The LPS co-treatment greatly enhanced the weak serum IgG antibody response to ovalbumin resulting from the lung instillation of OVA-beads.

Intratracheal instillation of 0.01, 0.1, or 1 mg quantities of TiO_2 or quartz also resulted in an initial dose-dependent inflammatory response which, with the exception of the 0.1 and 1 mg doses of quartz, also resolved within a few days. In animals receiving the two

higher doses of quartz there was a slight reduction in cell numbers at day 3, from the initial peak at day 1, but macrophage and neutrophil numbers then increased through day 7 and remained greatly elevated in the 1 mg group and above normal levels in the 0.1 mg group. From earlier studies it is known that this chronic inflammation due to the 1 mg dose persists even at 3 months post-treatment. The inclusion of 10, 40, or 80 ug LPS to the 0.01 mg dust inocula resulted in enhanced, synergistic, but short-lived, inflammatory responses. Thus a strong acute response by the lung to an insult is not by itself sufficient to produce the chronic inflammation seen with the 1 mg dose of quartz; a minimum amount of quartz is necessary to overload the lung's clearance mechanisms. These results imply that, in this model, that minimum amount lies between 0.01 and 0.1 mg.

Cells recovered by lavage from lungs instilled with the inorganic dusts were cultured overnight for the spontaneous production of the pro-inflammatory cytokine TNF. TNF activity peaked at day 3 for PBS and TiO_2 groups and was still elevated at day 7 when inflammation had subsided. Cells from LPS or quartz-treated animals gave peak responses on day 7. However, activity was greatly reduced at day 21 in the 0.1 and 1 mg quartz animals, despite continuing inflammation. Co-instillation with LPS and the 0.01 mg doses of TiO_2 or quartz reulted in peak TNF activity at day 7 but there was no synergistic effect. Thus the relationship between TNF production *in vitro* and inflammation *in vivo* is obviously a complex one.

Although the inflammatory-enhancing effects of LPS were short-lived in our model, it is possible that in a working environment where people will be exposed on a regular basis, that the effects might have important long-term consequences. The overall conclusion of this study was that co-exposure of workers to endotoxin and dusts, whether organic or inorganic, could result in greater inflammatory reactions within the lung than would result from the dusts alone. Workers inhaling organic dusts containing antigenic material would be at further risk from endotoxin enhancement of potentially damaging immune responses.

1. INTRODUCTION

1.1 Background

Endotoxin, a lipopolysaccharide-protein complex from the cell walls of gram-negative bacteria, is often a significant contaminant of the organic dusts implicated in respiratory disease syndromes such as byssinosis (Berry *et al.* 1973) and extrinsic allergic alveolitis (Fink, 1984). Industrial environments where airborne endotoxin has been recorded include cotton mills (Cinkotai *et al.* 1977), wool mills (Ozesmi *et al.* 1987;Love *et al.* 1988), waste treatment plants (Mattsby and Rylander, 1978), grain mills (Dutkiewicz, 1978), and poultry and livestock confinement buildings (Clark *et al.* 1983). Levels of endotoxin in these occupational settings measured using the limulus amoebocyte lysis assay have ranged from 0.02 to 8.03 ug/m^3.

Animal studies on the effects of endotoxin inhalation show a rapid increase in numbers of macrophages and neutrophils in the lungs (Hudson *et al.* 1977; Venaille *et al.* 1989). Repeated exposure to inhaled or instilled endotoxin can lead to changes in the airways suggestive of chronic bronchitis (Snell, 1966) and emphysema (Rudolphus 1992).

There are some reports of inhalation testing in man. Symptoms observed include chest tightness, dry cough, fever, malaise and decreased FEV_1 (Rylander, 1986). In the cotton, flax, and hemp industries, workers can develop byssinosis, a condition characterised by chest tightness, declining ventilatory capacity, and fever, on return to work after a weekend or holiday (Berry *et al.* 1973; Pernis *et al.* 1961). There is also an increased prevalence of bronchitis (Bouhuys *et al.* 1977). The similarity of these symptoms to the effects seen with endotoxin exposure is clear and indeed several studies have attempted to correlate the clinical findings with airborne endotoxin in the cotton industry (Rylander *et al.* 1985) and a wool carpet factory (Ozesmi *et al.* 1987). In a study of cotton workers in Shanghai, current endotoxin exposure correlated with chronic bronchitis and FEV_1 measured before work but not with the change in FEV_1 measured over the workshift (Kennedy *et al.* 1987). The results of this study, and of another (Rask-Andersen *et al.* 1989), suggest that other etiologic factors in organic dusts may be important in accounting for the observed respiratory effects.

Another group of respiratory diseases associated with inhalation of organic dusts are the allergic alveolitides (also known as hypersensitivity pneumonitides) the classical example of which is Farmers' Lung caused by inhalation of microorganisms growing on vegetable matter such as hay. Symptoms, which may ensue 4 to 8 hours following exposure, include fever, chills, malaise, chest tightness, dry cough and dyspnoea without wheeze. Although endotoxin is undoubtedly present in many of the dusts associated with this group of diseases, its role in the aetiology of alveolitis has been largely ignored, most studies concentrating on the known specific

sensitisation to antigens in the offending dust (Fink, 1984).

1.2 Experimental models

The complex nature of organic dusts makes it difficult to determine the relative contributions of endotoxin and dust components to respiratory disease in exposed workers. It is also quite likely that endotoxin, being such a potent modulator of immune and inflammatory processes (Morrison and Ryan 1979), may also influence any responses to other components of the dusts . Thus, for example, several in vitro studies have found synergistic interactions between mineral dusts and endotoxin (Lepe-Zuniga and Gery, 1984; Oghiso and Kubota, 1986) and endotoxin is a well-known adjuvant (Johnson, 1985).

Considering the problems, outlined above, associated with studying organic dusts, we have used an animal model to study the interactive effects in the lung of lipopolysaccharide (LPS), the main active component of endotoxin, with an artificial particulate organic dust: ovalbumin conjugated to 3 um polystyrene latex beads. In addition, we have studied the effect of endotoxin on the lung responses to two mineral dusts: quartz DQ12, a pathogenic dust which produces silicosis, and titanium dioxide (TiO_2) a relatively non-pathogenic dust used extensively as a whitening agent in the manufacture of such goods as paints and ice-creams. The inclusion of these mineral dusts provided particulate controls and also enabled us to study the pulmonary effects of endotoxin/dust combinations in the absence of complicating immune responses to ovalbumin.

1.3 The effects of quartz and titanium dioxide on the lung

The fibrotic changes occurring in the lungs following exposure to quartz dust are well documented although precise mechanisms have not been established (Davis, 1986). Through its interaction with quartz particles, the alveolar macrophage appears to play an important initiating and regulatory role through the release of inflammatory and fibrogenic factors such as interleukin-1 (Schmidt *et al.* 1984), tumour necrosis factor (TNF) (Dubois *et al.* 1989), fibroblast growth factors (Brandes and Finkelstein, 1990), and interferon (Elias, 1988). However, it is also known that macrophages can inhibit fibroblast activity through the release of prostaglandin E_2 (Brown *et al.* 1988). Neutrophils from quartz-inflamed lung have also been shown to produce the fibroblast-regulating and immunomodulatory cytokine interleukin-1 (Kusaka *et al.* 1990). Thus the generation of silicotic lung lesions may reflect an imbalance in fibroblast regulation, favouring fibrosis. Production of the cytokine TNF was selected for measurement in our study, of the plethora of possible factors which might be important in determining the outcome of dust inhalation, since it appears to play a key role in silica-induced fibrosis (Piguet *et al.* 1990).

Previous studies at our Institute have established that instillation of 1mg quartz into rat lungs leads to chronic inflammation and lung damage (Brown *et al.* 1989). The study described here examines the

relationship between acute lung injury and the subsequent development of chronic inflammation using the same model, but with the inclusion of low doses (0.01 and 0.1mg) of dust, and studying the additional influence of endotoxin (LPS). Although endotoxin is ubiquitous, it is less likely to be as significant a component of air contaminated with inorganic dust, for example in mines or quarries, than it is in agricultural environments. However, mine or quarry-workers with bronchitis could be exposed to LPS through respiratory infection with bacteria such as *Haemophilus influenzae* (Seaton *et al.* 1989).

2. OUTLINE OF THE STUDY

We have used a rat model to study the interactive effects in the lung of endotoxin (LPS) and an artificial dust - ovalbumin conjugated to 3 um diameter polystyrene beads. Inflammatory effects and immune responses to ovalbumin were recorded following intratracheal instillation of the beads with or without LPS. We also studied the effects of endotoxin on the lung responses to two mineral dusts, the relatively non-pathogenic titanium dioxide and the pathogenic quartz DQ_{12}. Preliminary experiments were conducted in order to select a dose of quartz which would not obscure the effects of added LPS.

These studies were carried out using the following procedures and assays:

1. Cells were recovered from lungs by lavage at various times (from 1 day to 49 days) post-instillation of dusts and/or LPS and the cell numbers and cell types recorded.

2. Production of the pro-inflammatory cytokine, tumour necrosis factor (TNF), by cells recovered in lavage, was also measured.

3. Lungs from animals treated with ovalbumin-latex and ovalbumin-latex plus LPS were examined histologically.

4. Immunoglobulin G responses to ovalbumin were assayed using an enzyme-linked immunoassay (ELISA).

3. MATERIALS AND METHODS

3.1 Animals

Specific pathogen-free PVG female rats from the I.O.M. animal breeding unit were used for the first 18 months of the study. At this point the I.O.M. breeding unit closed and so, for the remainder of the study, rats derived from the same original breeding stock as used for the I.O.M. rats were obtained from a commercial supplier (Bantin and Kingman, Hull, UK). There were no differences in the baseline numbers of cells in lavage nor in the responses to the instilled test agents between animals from the two suppliers. All rats entered in the study weighed in the range 150-180g.

3.2 Intratracheal administration of test agents

Animals were anaesthetised using a combination of diazepam (Roche, Welwyn Garden City, UK) intraperitoneally (5.5mg per kg body weight) and "Hypnorm" (Janssen, Oxford, UK) intramuscularly at 250 ul per kg body weight. A small area of the trachea was then exposed by cutting the overlying skin and muscle. Test agents, suspended or dissolved in 0.5ml phosphate-buffered saline(PBS), were injected with a hypodermic syringe and blunted needle into the lung through a small incision between two cartilage rings made with a sterile 21 gauge needle. Wounds were closed with two Michel clips. Naloxone hydrochloride (Sigma, Poole, UK) was then injected intramuscularly at 0.33mg per Kg body weight to promote recovery of the animals from the effects of Hypnorm.

3.3 Bronchoalveolar lavage cells

Animals were killed by intraperitoneal injection of an overdose (70mg per Kg body weight) of sodium pentobarbitone (Nembutal; Sanofi Limited, Watford, UK). Lungs were removed and the bronchoalveolar space lavaged with 4 x 8ml volumes of warm (37^{o}C) saline. Bronchoalveolar lavage cells (BAC) were recovered by centrifugation and resuspended to 1x10^{6} in Ham's F10 medium (Gibco, Paisley, UK) supplemented with 1% bovine serum albumin (Sigma). This medium contained less than 0.1 ng/ml endotoxin (Coatest; Kabivitrum, Stockholm, Sweden). Differential cell counts were made on stained cytospin preparations. One million cells were cultured overnight in 35mm plastic Petri dishes (Sterilin Ltd, Hownslow, UK) at 37^{o}C in an atmosphere of 5% CO_2. Cell-free culture fluid from these dishes was prepared by centrifugation and stored at -70^{o}C until required for cytokine assay.

3.4 Endotoxin

The principal active component of endotoxin, lipopolysaccharide (LPS) was used in this study. LPS from *E.coli* strain 0111.B4 (phenol extraction) was obtained from Sigma (catalogue number L2630).

3.5 Dusts

An artificial organic dust (OVA-beads) was produced by conjugating ovalbumin (Sigma) to 3 um diameter carboxylated polystyrene beads (Polysciences Limited, Northampton, UK) using the method described below. Each batch of conjugated beads was tested for immunogenicity by intraperitoneal injection into rats and subsequent assay of serum antibodies to ovalbumin by the method described below.

Two mineral dusts, the pathogenic quartz (DQ12 standard) and the relatively non-pathogenic titanium dioxide (TiO_2 ; rutile; Tioxide, Stockton-on-Tees, UK) were used to provide a model of lung inflammation on which to observe the effects of endotoxin in the absence of any complicating immune response to the dust itself.

3.6 Conjugation procedure for polystyrene beads (OVA-beads)

Five hundred ul volumes of carboxylated, 3 um diameter polystyrene beads (Polysciences) in a 1.5 ml plastic blood collection tube (Sarstedt Limited, Leicester, UK) were washed 3 times by centrifugation at 3000 rpm for 10 minutes with carbonate/bicarbonate buffer (0.1M, pH 9.6). Beads were then washed 3 times in phosphate buffer (0.02M, pH 7.5) and finally suspended in 0.625 ml phosphate buffer. An equal volume of 2% 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide HCl freshly prepared in phosphate buffer was then added dropwise to the beads which were mixed by end-over-end rotation for 3-4 hours. Beads were then washed 3 times by centrifugation in borate buffer (0.2M, pH 8.5). The bead pellet was resuspended in 1.2 ml borate buffer and 16 ul of ovalbumin (10 mg/ml in borate) added. Tubes were rotated overnight at room temperature. Next day 20 ul of 0.5M ethanolamine was added to block any unconjugated sites on the beads. After mixing for 60 minutes beads were washed 3 times in borate buffer and finally resuspended in 70% ethanol for storage at $4^{o}C$. Conjugated beads were enumerated using scanning electron microscopy and used at final concentrations of about 2×10^7/ml (ie. each rat received about 1×10^7 beads).

3.7 Measurement of serum IgG antibodies to ovalbumin

An enzyme-linked immunoassay was developed to measure IgG serum antibodies to ovalbumin (OVA). One hundred ul volumes of OVA at 10 ug/ml in phosphate-buffered-saline (PBS) were added to wells of Nunc immunoplates (Gibco, Paisley, UK) and incubated overnight at $4^{o}C$. Unbound antigen was removed from the wells by 2 washes with PBS

containing 0.05% v/v polyoxethylene sorbitan monolaurate (Tween 20; Sigma Chemical Co., Poole, UK). PBS/Tween was used for all subsequent wash steps and was also used to dilute all sera and immunoglobulin test reagents. Uncoated areas of wells were "blocked" by incubation for 30 minutes with 150 ul of a TRIS-buffered, protease-degraded gelatin preparation (ELISA blocking reagent; Boehringer Mannheim UK Ltd, Lewes, UK). Following 4 washes, 100 ul test sera diluted 1/50, 1/150 and 1/450 were added to wells, and plates incubated for 1 hour at 37°C in a humid CO_2 incubator. Excess serum was removed with 4 washes and OVA-bound immunoglobulin was detected by the addition of biotinylated, sheep anti-rat immunoglobulin, immuno-affinity purified to ensure species specificity (Amersham International plc, Amersham, UK). Plates were incubated for a further hour at 37°C, washed 4 times, and peroxidase-conjugated streptavidin (Amersham International plc) added to detect bound biotinylated antibody. Streptavidin, a protein derived from the bacterium *Streptomyces avidinii*, has 4 high affinity sites with which to bind the low molecular weight (244 Daltons) vitamin biotin and, thus, this system provides a convenient amplification of the assay's ability to detect bound antibody. Plates were incubated 30 minutes at 37°C and washed 4 times. Bound streptavidin was detected by the addition of o-phenylene diamine (OPD; Sigma Chemical Co, Poole, Dorset) dissolved to 0.4 mg/ml in 0.05M citric acid/phosphate buffer (pH 5.0) containing 0.4 ul/ml 30% hydrogen peroxide (Sigma) which, on reaction with bound peroxidase, produces a yellow colour in the wells. Finally, the reaction was stopped by adding 50 ul 2M sulphuric acid to each well. Colour intensity was determined by reading optical density with a Dynatech MR650 plate-reader (Dynatech Laboratories Ltd, Billingshurst, UK) using a test wavelength of 490 nm and reference wavelength of 630 nm.

3.8 Tumour necrosis factor (TNF) assay

TNF activity was determined using the mouse L929 fibroblast lysis assay (Flick and Gifford, 1984). L929 cells were plated in 96-well microtitre plates at 2.5×10^4 cells per well in Eagle's minimal essential medium (Gibco, Paisley, UK) and cultured for 20 hours at 37°C. Serial dilutions of test supernatants were then added in medium containing 1 ug/ml actinomycin D (Sigma) but free of all other antibiotics. Eighteen hours later remaining cells were stained with crystal violet (0.5% in 20% methanol) and the absorbance of wells read at 540 nm using a Dynatech MR650 plate-reader (Dynatech Laboratories Ltd). TNF activity was standardised using a human recombinant TNF alpha preparation (1st International Standard; NIBSC, Potters Bar, UK).

3.9 Statistical analysis

Extensive preliminary examination of the data was carried out in a series of tables and graphs. Further statistical modelling was done using Poisson log-linear models to investigate the effects of quartz, TiO_2, or OVA.beads singly, or in combination with various

concentrations of LPS (endotoxin), on counts of total cells, macrophages and neutrophils and on the production of TNF. Details of the statistical analyses are given in the Appendix. Analyses were carried out using version 5 of the Genstat software package (Genstat 5 committee, 1987).

4. RESULTS

4.1 Cell recruitment to the lung following instillation of test agents

4.1.1 Quartz and Titanium dioxide

Bronchoalveolar lavage of 17 untreated rats gave a mean total number of cells recovered of 2.95 (s.d. 0.78) million and only four of these lavages contained neutrophils (0.2% on average).

Intra-tracheal instillation of quartz or TiO_2 resulted in a rapid, dose-dependent, inflammatory response in the lung as shown by increased total cell numbers (Figure 4.1a,b) and increased neutrophils (Figure 4.2a,b) recoverable by lavage. With the exception of animals receiving the 0.1 and 1 mg doses of quartz, these inflammatory responses were transient, peaking at or before 24 hours and resolving by day 7. PBS also elicited a small, transient increase in total cell and neutrophil numbers (dotted lines, Figures 4.1 and 4.2).

The responses to the two higher doses of quartz differed markedly from those arising from all other treatments. With both doses there was a reduction in total cell and neutrophil numbers on day 3 followed by an increase through day 7 to day 21. The relative proportions of the different cell types recovered in lavage for the various treatments 21 days post-instillation are given in Table 4.1. Neutrophils comprised on average 61.5% and 46.3% of lavaged cells in the quartz 0.1 mg and 1 mg groups respectively, contrasting with the low numbers of neutrophils in other treatment groups. The results from the three doses of quartz indicate that, in this model, a minimum dose, between 0.01 and 0.1 mg of quartz, must be exceeded for inflammation to persist.

4.1.2 The effect of including LPS in the dust inocula

The effect of mixing LPS with the lowest (0.01 mg) doses of quartz and TiO_2 was also studied. LPS on its own produced an intense, dose-dependent inflammatory response peaking on or before 24 hours which resolved quickly, cell numbers and neutrophil content returning to normal levels by day 7 (Figures 4.3a,b).

Compared to LPS alone (= PBS in the tables), the addition of 0.01 mg quartz to the various LPS treatments produced an enhanced response. Full results for total cells, macrophages, and neutrophils are given in Tables 4.2 to 4.4 and summaries of statistical analyses are given in Tables A1 to A3 in the Appendix. The results of the statistical analysis showed that there was evidence of a pattern of synergistic interaction between quartz and LPS across all timepoints. Examination of the data showed that total cell and neutrophil numbers in lavage were particularly increased at day 1 for all combined treatments and, with the exception of quartz plus 80 ug LPS, also at day 3. The

increases in total cell and neutrophil numbers indicate a synergistic interaction between quartz and LPS which is statistically significant in the case of neutrophils. The effect was most marked at day 1 and this is illustrated in Figure 4.4 which shows that the combined treatment produced a greater neutrophil influx than that seen with either agent alone or, indeed, with the higher doses of quartz seen in Figure 4.1b.

With the exception of the 10 ug LPS group where there was a reduction, the level of neutrophil recruitment to the lung arising from the TiO_2/LPS combinations at day 1 was slightly higher than that for LPS alone and suggested an additive effect of the two agents rather than a synergistic one (Figure 4.5). Full results are given in Tables 4.2 to 4.4. When all the neutrophil data was considered in an analysis of deviance there was a significant interaction between TiO_2 and LPS showing that there is some synergism. Summaries of this analysis are given in the Appendix, Tables A4-A6.

4.1.3 Recruitment of cells to the lung following treatment with OVA-beads or OVA-beads plus LPS

Intra-tracheal instillation of OVA-beads resulted in cellular infiltrates similar to those seen following treatment with PBS, or the 0.01 mg dose of quartz or TiO_2. Results are given in Table 4.5 and compare total and percentage cell-types recovered in lavage following treatment with OVA-beads, LPS, or OVA-beads plus LPS. As with the mineral dusts, the inclusion of LPS in the OVA-beads inoculum resulted in a greatly enhanced, synergistic neutrophil infiltrate peaking on or before 24 hours. Results of the analysis of deviance are given in Tables A7-A9 in the Appendix.

Table 4.5 also shows that the numbers of lymphocytes recovered in lavage have been significantly increased by the combined treatment at day 7.

4.2 In vitro production of tumour necrosis factor by lavaged cells

In addition to the changes in cell types and numbers recovered by lavage we also measured the in vitro production of the inflammation promoting cytokine TNF. Results have been expressed both as TNF units per ml culture fluid (equivalent to the production of 1 million cells) and as this concentration multiplied by the total number of macrophages recovered per lung (TNF.MAC). This latter measure gives an indication of the potential in vivo lung activity of TNF.

4.2.1 TNF production by cells from untreated rats

Cells (99% macrophages) from untreated rat lungs produced, on average, TNF concentrations of about 3 units per ml. This was equivalent to about 10 units per total macrophage number (TNF.MAC).

4.2.2 Effect of quartz dose on TNF production

The effect of the various *in vivo* quartz doses on *in vitro* TNF production is given in Figure 4.6. TNF activity varied greatly from rat to rat resulting in large standard errors of the mean. TNF production on day 1 was inversely proportional to quartz concentration. In other words, the greater the inflammatory response the lower the in vitro TNF production. This may simply have been due to there being much fewer macrophages, the principal producers of TNF, in the lavage cell populations from animals receiving the higher doses of quartz. TNF was greatest at day 7 for all quartz treatments. It is interesting that TNF was high for the 0.01 mg dose at this time point since inflammation had largely resolved by then. This contrasts with the reduced TNF activity for the higher quartz doses at day 21, at which time inflammation was still present. Thus the relationship between inflammation, as determined by the presence of neutrophils in lavage, and TNF production is a complex one. Analysis of deviance of TNF data showed large differences between number of days post-instillation and between quartz concentrations (Table A10). Further examination of the data showed that the differences between the day 7 and day 21 results were statistically significant.

Presenting the data as TNF.MAC (Figure 4.7) did little to alter this pattern of results with the exception that the highest TNF production was with the two higher doses of quartz at day 7. However, by day 21 there was, again, reduced TNF output. For TNF.MAC data, only time post-treatment was a significant factor (Table A11).

4.2.3 The effect of TiO_2 dose on TNF production

Results for the TNF and TNF.MAC variables following treatment with TiO_2 are shown in Figures 4.8 and 4.9. The patterns are similar for both variables. As for quartz, TNF production was inversely proportional to TiO_2 concentration at day 1, again perhaps reflecting reduced numbers of macrophages resulting from the higher dust concentrations. However, in contrast to quartz, peak production for all TiO_2 groups occurred at day 3; TNF concentration at day 21 tended to increase with TiO_2 concentration. Thus, as with quartz, TNF levels did not always reflect the degree of inflammation.

From analysis of deviance, interactions between TiO_2 concentration and time post-treatment were significant at the 5% level for TNF and at the 6% level for TNF.MAC, showing that differences between TiO_2 concentrations varied with time post-treatment (Appendix Tables A12 and A13).

4.2.4 Effect of including LPS in the dust inocula on TNF production

The effect of including LPS in the quartz and TiO_2 treatments on TNF production was also examined and results are summarised in Tables 4.6 and 4.7 for TNF and TNF.MAC respectively. The patterns of results were similar for both TNF and TNF.MAC with differences between LPS concentration and days post-instillation. In general, TNF activity was highest on day 7 and, in the presence of LPS, lowest on day 1. On days 7 and 21 for the quartz/LPS groups TNF concentrations were inversely related to LPS concentration. There was no evidence of a synergistic interaction between quartz and LPS with regard to TNF production (Appendix Tables A14 and A15).

Treatment with LPS alone also produced peak TNF activity on day 7, with an inverse relationship between the amount of LPS administered and TNF production at this time point. TNF activity was also highest on day 7 for the TiO_2/LPS combinations, although concentrations were lower than for either LPS alone or TiO_2 groups; synergism was, therefore, not apparent (Appendix tables A16 and A17).

4.2.5 Associations between TNF concentration and type and number of cells recovered in lavage

TNF data (log scale) was plotted against neutrophils or macrophages recovered in lavage at each time point; TNF values of zero were recoded as 0.1 units before log transformation. At day 1 (Figure 4.10), TNF was found to be positively correlated ($r = 0.702$) to the percentage of macrophages. A relationship was seen between macrophage number and TNF but this was less strong. These relationships were not found at later time points (days 3, 7, and 21; data not shown). TNF was also found to be negatively related ($r = -0.592$) to number (rather than percentage) of neutrophils on day 1 (Figure 4.11). There was no relationship at later time points (data not shown). When all TNF data was plotted against neutrophil number, a negative correlation was obtained (Figure 4.12; correlation coefficient, $r= -0.530$).

4.2.6 Re-analysis of TNF data using neutrophil number as a co-variate

Because of the negative correlation between neutrophil number and TNF, neutrophil number was used as a co-variate in analyses of deviance to investigate whether the synergism found between LPS and dusts described above also affected TNF production. However, the only results to be affected were those showing an interaction between TiO_2 concentration and day post-treatment: the statistical significance of the interaction was reduced from the 5% to the 7% level for TNF and from 6% to 9% for TNF.MAC.

4.2.7 Confirmation that the active agent in supernatants was TNF

In order to confirm that the lytic agent in the test supernatants was indeed TNF, two representative supernatants from each treatment group at days 3 and 7 were assayed for TNF activity in the presence of anti-TNF antibody. With the exception of 4 supernatants, the presence of anti-TNF produced 89%, or greater, reduction in TNF activity. The four exceptions were day 7 supernatants containing high TNF concentrations and were from the following treatment groups: (a) 0.1 mg TiO_2 (82% reduction); (b) 0.01 mg quartz (76% reduction); (c) 10 ug LPS (85% reduction); (d) TiO_2 plus 10 ug LPS (86% reduction). From these results we are confident that, by far, most of the lytic activity in test supernatants was due to TNF. It is likely that further dilution of the four exceptional supernatants would have given greater reductions.

4.3 Lymphocyte infiltration to the lung following instillation of OVA-beads

On days 1 and 3 following intra-tracheal instillation of OVA-beads the proportions of lymphocytes in lavage were similar to those resulting from treatment with PBS or the 0.01 mg doses of quartz or TiO_2. However, from day 7 to day 35 there was a marked rise in the percentage of lymphocytes recovered in lavage. Figure 4.13 compares the total cell, total lymphocyte, and percentage lymphocytes recovered in lavage resulting from treatment with OVA-beads or latex beads alone. Ovalbumin-conjugated beads produced a statistically significant increase in lymphocyte number compared to unconjugated beads ($p=0.007$ by analysis of variance). The increase in lymphocyte numbers reflects the higher percentage of lymphocytes recovered in lavage rather than an increase in total cell number.

4.4 IgG anti-ovalbumin response in rats treated with intra-tracheal OVA-beads

IgG antibodies to ovalbumin were measured in the sera of rats treated with one trans-tracheal instillation of OVA-beads. Results for three serum dilutions are given in Figure 4.14 and show that OVA-beads administered in this way were antigenic, although the antibody levels were very low when compared to a positive control serum which gave 0.58 OD units at a dilution of 1/7200 (not shown in figure). Antibody response peaked on day 21.

4.5 Effect of LPS on OVA antibody response

Figure 4.15 shows that the inclusion of 80 ug LPS in the OVA-beads inoculum caused a marked increase in anti-ovalbumin antibodies measured on day 7, the only time-point for which there was sufficient data. There was a statistically significant increase for each serum dilution.

4.6 Histology of lungs instilled with OVA-beads

Lungs examined 1 or 3 days post-instillation of OVA-beads showed no obvious differences from lungs instilled with PBS. However, by day 7 3 out of 6 lungs treated with OVA-beads showed some perivascular and peribronchial mononuclear infiltrate. By day 21, 6 out of 8 lungs examined had perivascular and peribronchial infiltrates with some bronchial walls showing thickening with goblet cell and epithelial cell hyperplasia. Focal mononuclear alveolitis and bronchiolitis were also seen. Areas of bronchus-associated lymphoid tissue (BALT) were enlarged in all lungs treated with OVA-beads compared to lungs from untreated control or PBS-treated animals.

4.7 Persistence of OVA-beads in alveolar macrophage population

During differential counting of cells, the percentages of macrophages containing OVA-beads were recorded. At any one time point only a minority of lavaged macrophages contained OVA-beads. The numbers of these bead-associated macrophages gradually declined with time but could still be found at day 49. The percentage and total number of bead-positive macrophages are shown in Figures 4.16a and 4.16b respectively.

5. DISCUSSION

It has been difficult to confirm the aetiological roles of the many components, including endotoxin, of the organic dusts associated with respiratory disease in workers in agriculture and the grain, wool, and cotton industries. This project set out to examine the effects on the rat lung of an artificial organic dust, OVA-beads, with or without the influence of LPS, the component of endotoxin accounting for most of its biological activity.

Intra-tracheal instillation of OVA-beads, PBS, TiO_2, or LPS, resulted in a transient rise in numbers of neutrophils recovered by lavage. A similar, short-lived inflammation is also seen with other particulate antigens such as sheep red blood cells (SRBC; Bice and Shopp 1988). Cell recruitment to the lung was synergistically enhanced by adding LPS to the inoculum of OVA-beads, although cell numbers quickly returned to normal; a similar effect was seen with the combinations of LPS with TiO_2 and quartz (discussed further below).

Histological examination of lungs treated with OVA-beads showed peribronchial and perivascular infiltration with mononuclear cells at day 21. Perivascular infiltrates have also been reported in dog lungs treated with SRBC (Bice *et al.* 1987; Brownstein *et al.* 1980). In these dog studies, and in non-human primates, the numbers of lymphocytes recovered by lavage increased with time, peaking between 7 and 14 days. This rise in lymphocyte numbers is not usually seen in rodents (Bice and Shopp 1988). In our study there was a late increase in the percentage of lymphocytes in lavage which peaked around day 35. The reason for this late change is not clear but seems to parallel the histopathological changes seen at day 21 and may be related to the persistence of OVA-beads within the lung.

The lymphocytes seen in lavage from animals treated with OVA-beads were not characterised but from a number of published studies of other models both specific T and B cells would be expected to appear in alveolar spaces and interstitium (reviewed by Kaltreider 1984). In dogs and rodents, antibody forming cells (AFC) appear in the hilar lymph nodes draining the lung within 5 to 7 days following deposition of SRBC. However, in contrast to dogs, very few AFC appear in the lungs or blood of rodents injected with SRBC (Bice and Shopp 1988). This may have been due to insufficient antigen doses as significant numbers of AFC are seen in mouse lungs immunised with a high dose of SRBC (Kaltreider *et al.* 1987).

The source of specific antibody in blood following a primary immunisation in the lung is probably the AFC in the hilar nodes. Levels of serum anti-OVA antibody were low in our study and peaked around day 21. As expected from the large literature on the adjuvant effects of LPS (Johnson 1985), the addition of LPS to the inoculum of OVA-beads led to a significant increase in antibody to ovalbumin.

The adjuvant effect of LPS is probably mediated by a multi-faceted process involving direct proliferative action on B cells, stimulation of T helper cells, macrophage activation leading to enhanced IL-1 release and antigen processing (Johnson 1985). The timing of antigen injection relative to LPS treatment is crucial: LPS injected together with, or at least within a few hours of, antigen, gives enhancement, whereas LPS given 1 or 2 days before antigen leads to a marked depression of antibody response (Behling and Nowotny 1977). Thus the immunological outcome for someone inhaling antigen contaminated with endotoxin could be affected by the relative amounts of antigen and endotoxin and their temporal relationship.

Another phenomenon that could also influence the outcome of endotoxin inhalation is that of tolerance in which the inflammatory and pyrogenic responses to subsequent exposures to endotoxin are greatly reduced (Johnston and Greisman 1985). This refractoriness consists of an early phase, developing within a few hours but waning within a matter of days and due to the inability of macrophages to release further inflammatory mediators, and a lesser, late phase developing a few days after LPS administration and which is mediated by antibodies to the O-polysaccharide of endotoxin (Johnston and Greisman 1985).

The effects of OVA-beads plus LPS were compared to those of very low doses (0.01 mg) of quartz or TiO_2. This dose was selected following preliminary experiments because, for quartz, it provided a level of inflammation which would not obscure any effects due to LPS.

The results arising from these quartz experiments were among the most interesting to come out of the study. Persistence of inflammation with the 0.1 and 1 mg doses as opposed to rapid resolution of inflammation with the 0.01 mg dose suggests that a threshold dose of quartz must be exceeded for the chronic inflammatory state to develop in this model. This chronic state has been shown to extend for at least 12 weeks for a 1 mg load resulting in marked alveolar lipoproteinosis and hyperplasia in alveolar septa (Brown *et al.* 1989). In addition, the combination of LPS with the lowest dose of quartz produced a strong acute response but no chronic inflammation. Thus the transformation from the acute to the chronic state does not relate simply to the magnitude of the acute inflammation, a minimum amount of quartz would appear to be necessary to achieve overload of the lung's clearance mechanisms (Bolton *et al.* 1983; Vincent and Donaldson, 1990). This project provides experimental evidence as to what that threshold, or minimum amount of quartz, would be in an instillation model, namely between 0.01 mg and 0.1 mg. The concept of "overload" as a factor in determining the outcome of dust exposure was originally mooted by Bolton and colleagues (1983) but has only comparatively recently attracted more widespread interest (McClellan, 1990). Although a single definition of overload encompassing all conditions of exposure for all dusts is difficult to achieve, there is agreement that, in an overload situation, harmful dusts will overwhelm the lung's clearance mechanisms leading to persistence (build-up) of dust at doses much lower than those required for a less harmful dust (McClellan 1990; Warheit *et al.* 1990). Even a relatively inert dust such as TiO_2 can produce chronic inflammation and fibrosis at sufficiently high doses

(Lee *et al.* 1985; Vincent and Donaldson 1990; Driscoll *et al.* 1990b).

Dust clearance from the alveolar region is believed to be accomplished mainly through the action of macrophages phagocytosing dust particles and then transporting them to the mucociliary apparatus, the principal means of removal of particles from the airways (Lehnert *et al.* 1990). While this is the main route for the elimination of dust from the lung, a proportion of free particles, or particles contained in macrophages, are removed via the lymphatic system to the hilar lymph nodes (Ferin and Feldstein 1978; Vincent *et al.* 1987).

Other aspects affecting the retention of particles in the lung include uptake and retention by alveolar epithelial cells and the interstitium (Heppleston and Young, 1974; Adamson and Bowden, 1981) and particle dissolution (Lundborg *et al.* 1984).

The activities of alveolar macrophages are considered to be extremely important in mediating the pathogenic effects of harmful dusts, principally through their release of chemotactic factors (Lugano *et al.* 1981; Streiter *et al.* 1990) and inflammogenic and fibrogenic factors such as interleukin-1 (IL-1), TNF, interleukin-6 (IL-6), leukotrienes, prostaglandins, and fibroblast growth factors (review: Brandes and Finkelstein 1990). IL-1 and TNF have a similar range of effects and both are released by alveolar macrophages in response to quartz exposure (Driscoll *et al.* 1990a) and both have been implicated in a rat model of acute immune alveolitis (Warren 1991; Warren *et al.* 1989). TNF has also been shown to play a major role in three animal models: bleomycin-induced pneumopathy and fibrosis (Piguet *et al.* 1989), silicosis (Piguet *et al.* 1990), and hypersensitivity pneumonitis (Denis *et al.* 1991). In addition, mRNA's for both IL-1 and TNF are induced within the lungs and alveolar macrophages of rats instilled with LPS (Ulich *et al.* 1991b). Moreover, the intratracheal instillation of IL-1 and, to a lesser extent TNF, produced a similar inflammatory response to that obtained with LPS (Ulich *et al.* 1991b). These workers went on to show that both IL-6 and transforming growth factor beta (TGFß) inhibited the inflammatory response to intratracheally-instilled LPS (Ulich *et al.* 1991a).

Other mediators which can be released in response to LPS or dust instillation include; cyclooxygenase and lipoxygenase metabolites of arachidonic acid, proteases, anti-proteases, reactive oxygen metabolites (Brigham and Meyrick, 1986; Davis 1986).

As with other studies (Driscoll *et al.* 1990a; Ulich *et al.* 1991b) we have shown that intra-tracheal instillation of LPS or mineral dusts results in enhanced *in vitro* production of TNF by cells recovered by bronchoalveolar lavage. Driscoll *et al* (1990a) found that TNF production by alveolar macrophages was only raised following instillation of relatively high amounts (10 and 20 mg) of TiO_2 or quartz. They also found that TNF production was significantly greater following quartz than with TiO_2 and remained raised throughout the experiment (28 days) whereas TNF production in the TiO_2 group had returned to normal levels by day 28. Instillation of 2 mg quartz produced an increase in TNF at day 1 post-instillation. The reasons

for this difference between our results and those of the Driscoll study are not known but may reflect differences in rat strains (they used Fischer 344 rats) and the sensitivities of the TNF assays. Probably the major difference between the two methodologies was that they removed non-adherent cells from their lavaged cells before culturing overnight. Their culture medium was RPMI 1640 containing 0.2% bovine serum albumin (BSA) whereas we used HAM's F10 supplemented with 1% BSA. Both culture media contained small amounts of endotoxin (less than 1 ng/ml).

Our TNF results differed from those of Driscoll in a number of other ways. In particular, the potential of lavageable bronchoalveolar cells to produce TNF does not always correlate with the level of inflammation as reflected by neutrophil recruitment. At day 1, taking all data, there was an inverse relationship between numbers of neutrophils and TNF, probably due to low numbers of macrophages, the main TNF producers. For all quartz treatments TNF production was highest at day 7, at which point neutrophils had returned to baseline levels for the 0.01 mg dose. Thus TNF levels for the higher doses of quartz were higher at day 7 than at day 21 when inflammation was greater. Even when data was expressed as a product of TNF concentration and macrophage number (TNF.MAC), TNF was still lower at day 21. The results for TiO_2 differed from those of quartz mainly in the time of peak activity, day 3 for TiO_2 as opposed to day 7 for quartz.

The reason for the drop in TNF activity at day 21 in the higher dose quartz groups, despite continuing inflammation, is not known. Possible explanations are that TNF production by the day 21 cells is being down-regulated by macrophage products such as prostaglandin E_2 (Kunkel *et al.* 1988), TGFß (Dunham *et al.* 1990) or IL-6 (Ulich *et al.* 1991a) and/or there are TNF inhibitors present in the cultures. A 33 KDa TNF inhibitor having anti-inflammatory activity has been isolated and characterised from the urine of febrile patients (Seckinger *et al.* 1989, 1990) and which may be a soluble form of the TNF receptor (Seckinger *et al.* 1990b). Thus it is conceivable that TNF receptor derived from cells, such as neutrophils, in the bronchoalveolar lavage could play an inhibitory role in our TNF assay.

In addition, TNF secretion *in vitro* by human peripheral blood mononuclear cells was suppressed by a variety of serine protease inhibitors (Scuderi 1989; Suffys *et al.* 1988). This indicates that TNF secretion may be dependent on the activity of one or more proteases. Rat alveolar macrophages have been shown to produce anti-proteases such as alpha-1-proteinase inhibitor and alpha-2-macroglobulin (White *et al.* 1981). Stimulation of macrophages by agents such as LPS leads to enhanced production of both collagenase and a collagenase inhibitor (Cury *et al.* 1988; Shapiro *et al.* 1991). Prostaglandin E_2 appears to play a regulatory role since the prostaglandin inhibitor indomethacin blocks collagenase release (Wahl and Mergenhagen 1988). High concentrations of PGE_2 have also been found to inhibit collagenase production (Wahl and Lampel 1987). If TNF inhibition is occurring in cultures, then presumably the nature of the lavaged cells has changed from day 7 to day 21. In this context,

another study from our laboratory found that protease activity in the supernatants of leukocytes recovered by lavage from rats treated intra-tracheally with 1 mg quartz peaked at day 3, was lowest on day 15 and then was high again at day 30 post-instillation; no data was obtained at day 21. Thus, this study also indicates a change in functional activity of lavaged cells with time post-treatment. It would be of interest to know if supernatants from day 15 rats contained higher levels of anti-proteases.

If, as argued above, TNF plays a crucial role in the development of silicosis, then an alternative explanation for the fall in TNF output at day 21 is that the cells producing TNF are, at this time, mainly within the interstitium and hence not recoverable by lavage.

The presence of LPS in the 0.01 mg dust inocula tended to reduce TNF production at days 1 and 3, for all treatments, and even on day 7 when levels were highest, TNF production in the dust/LPS groups tended to be lower than for the LPS alone groups; there was no synergistic effect.

In agriculture, and in industries handling fibres and dusty material of animal or vegetable origin, the risk of inhaling significant amounts of endotoxin is considerably greater than for workers exposed to inorganic dusts such as coal, asbestos, and quartz. However, the ubiqitous nature of endotoxin and the possibility of exposure to it through bacterial infection, suggests that, even in diseases due to inorganic dusts, endotoxin may be an aetiological factor. Endotoxin exposure, by inhalation or infection, either simultaneously or within a few hours of dust exposure, could lead to an intensified inflammatory response. In addition, enhancement of immune responses by endotoxin could play a role in dust diseases believed to have an immune component in the disease process. Thus in an agricultural or industrial setting, where this co-exposure could be occurring repeatedly over long periods of time, exposed workers may be at greater risk than those inhaling endotoxin-free dust. The extent to which endotoxin tolerance would affect any synergistic interaction would depend on the doses of endotoxin and the relative timing of endotoxin and dust exposures.

6. SUMMARY OF MAIN FINDINGS AND RECOMMENDATIONS FOR FURTHER WORK

6.1 The main findings of the project were:

1. The pulmonary cellular responses to TiO_2 and quartz were dose-dependent. The inflammatory response to the lowest dose of quartz (0.01 mg) was transient, whereas the responses to 0.1 and 1 mg were still evident at day 21. A threshold dose of quartz is therefore required to induce a chronic inflammatory reaction.

2. The inclusion of LPS (endotoxin) in the dust inocula synergistically enhanced the initial inflammation produced by OVA-beads and the 0.01 mg doses of quartz and TiO_2. This effect was particularly strong for quartz. However, LPS did not affect the kinetics of the inflammatory responses, total cell and neutrophil numbers returning to normal levels within the same time-span as for the dust alone treatments.

3. LPS co-treatment with OVA-beads enhanced the subsequent antibody response to ovalbumin.

4. The kinetics of the production of the cytokine tumour necrosis factor (TNF) by lavaged cells was complex and did not always correlate with neutrophil recruitment to the lung. The addition of LPS to the 0.01 mg quartz and TiO_2 inocula had no synergistic effect on TNF production.

5. The percentage of lymphocytes recovered in lavage from rats treated with OVA-beads increased with time peaking 35 days after instillation.

6. Histology of OVA-bead-treated lungs showed increasing pathological change with time post-instillation.

6.2 Recommendations for further work

1. Further investigation of the low TNF production by cells from the 1 mg quartz group at day 21. In particular, the lavage cell cultures should be examined for the presence of TNF inhibitors and mediators of down-regulation. TNF production at later time points should also be determined.

2. *In vivo* production of interleukin 1 and TNF. This could be studied using histochemistry and/or molecular biological techniques.

3. Further examination of the "threshold" dose of quartz required to trigger chronic inflammation. It would be of interest to see if the intermediate dose (0.1 mg) of quartz eventually produced fibrosis.

4. Pathological changes in the lung in response to the OVA-beads. This merits further study using other doses and later time points.

5. Enhancement of inflammation due to dust by LPS. This was a short-lived effect and, thus, it would be of interest to study a model incorporating repeated instillations, or inhalations, of LPS.

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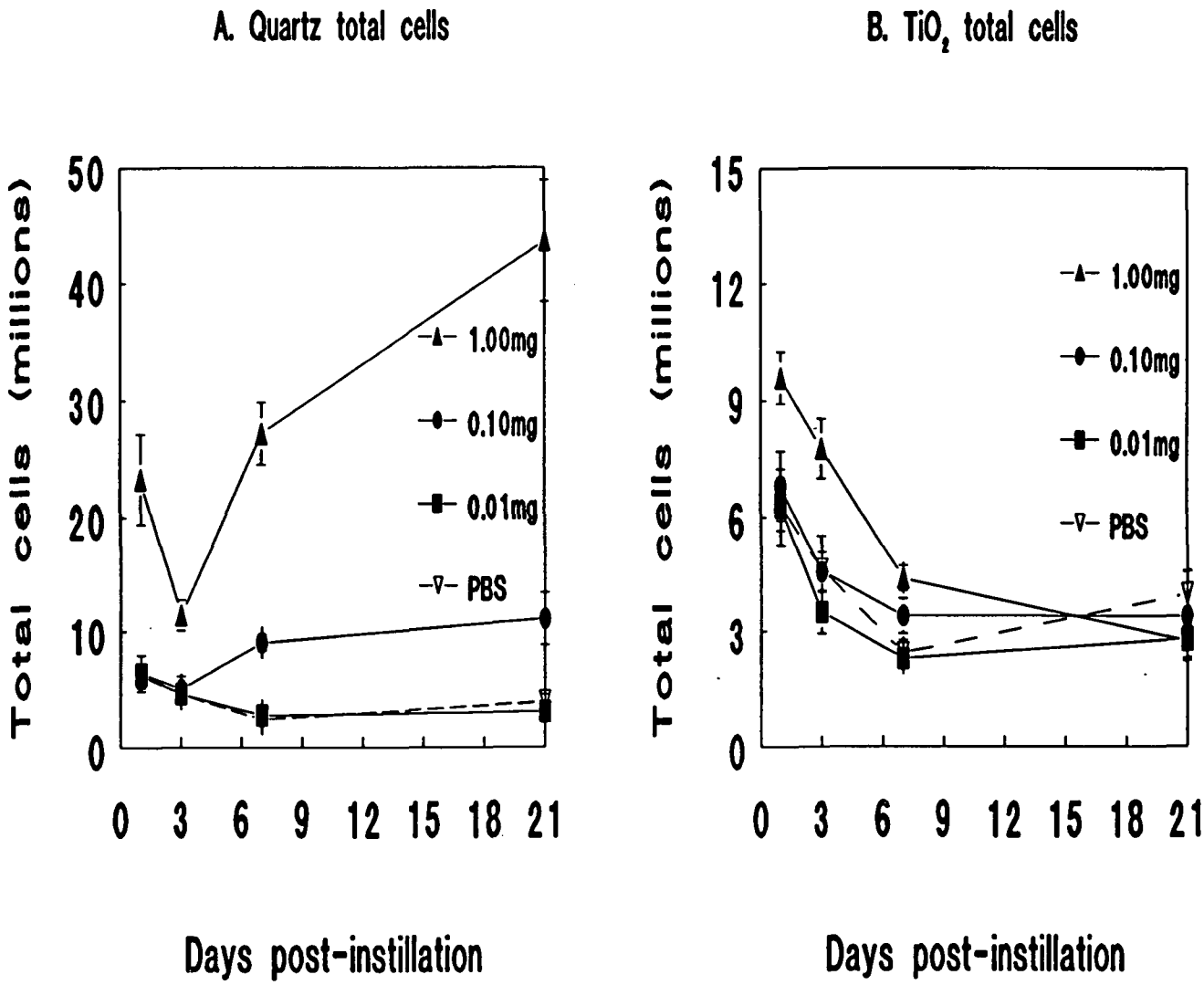

Figure 4.1 Total cell numbers recovered by bronchoalveolar lavage following instillation with phosphate-buffered saline (PBS) or various concentrations of (A) quartz or (B) TiO_2. Bars represent standard deviations from the mean.

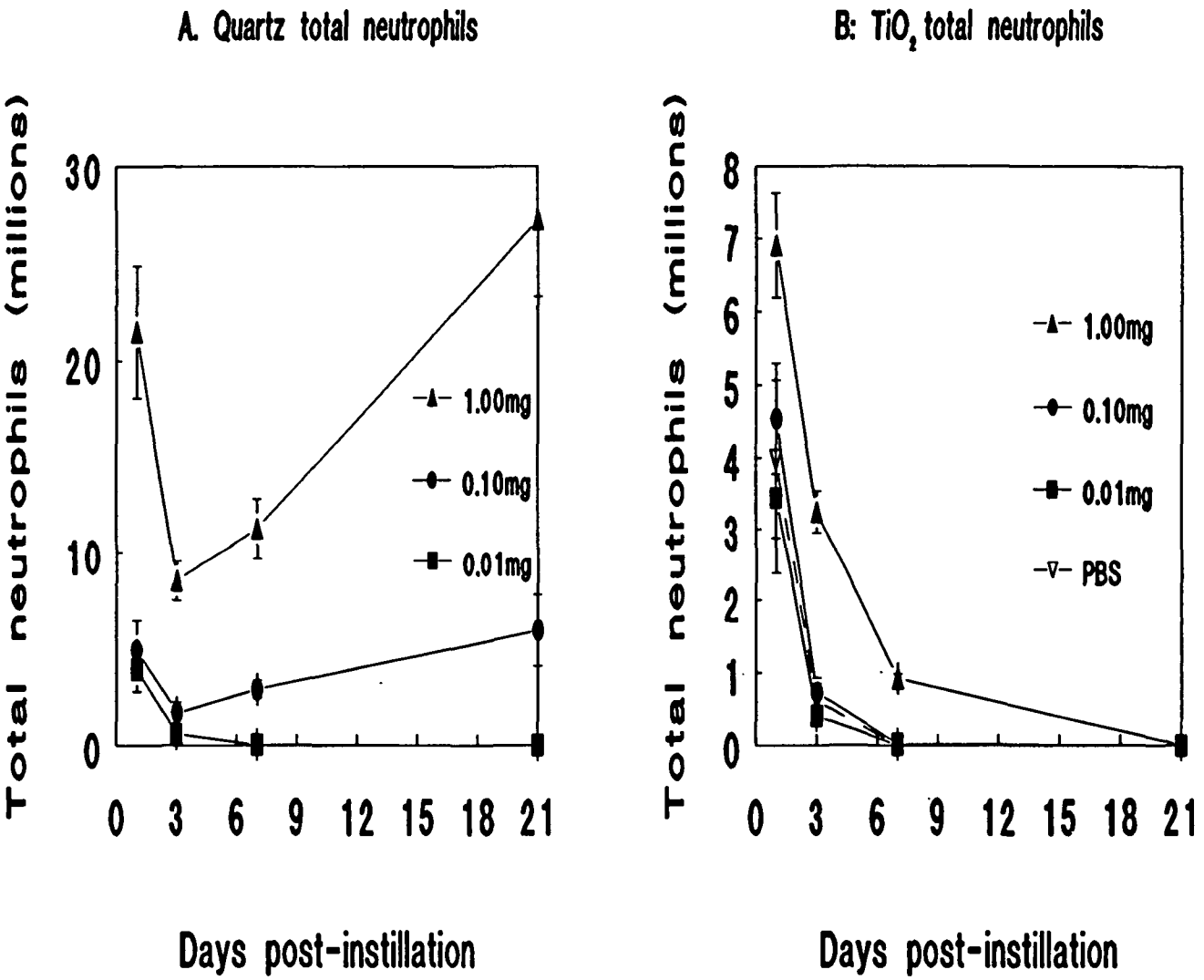

Figure 4.2 Total numbers of neutrophils recovered by bronchoalveolar lavage following instillation of phosphate-buffered saline (PBS) or various concentrations of (A) quartz or (B) TiO_2. Bars represent standard deviations from the mean.

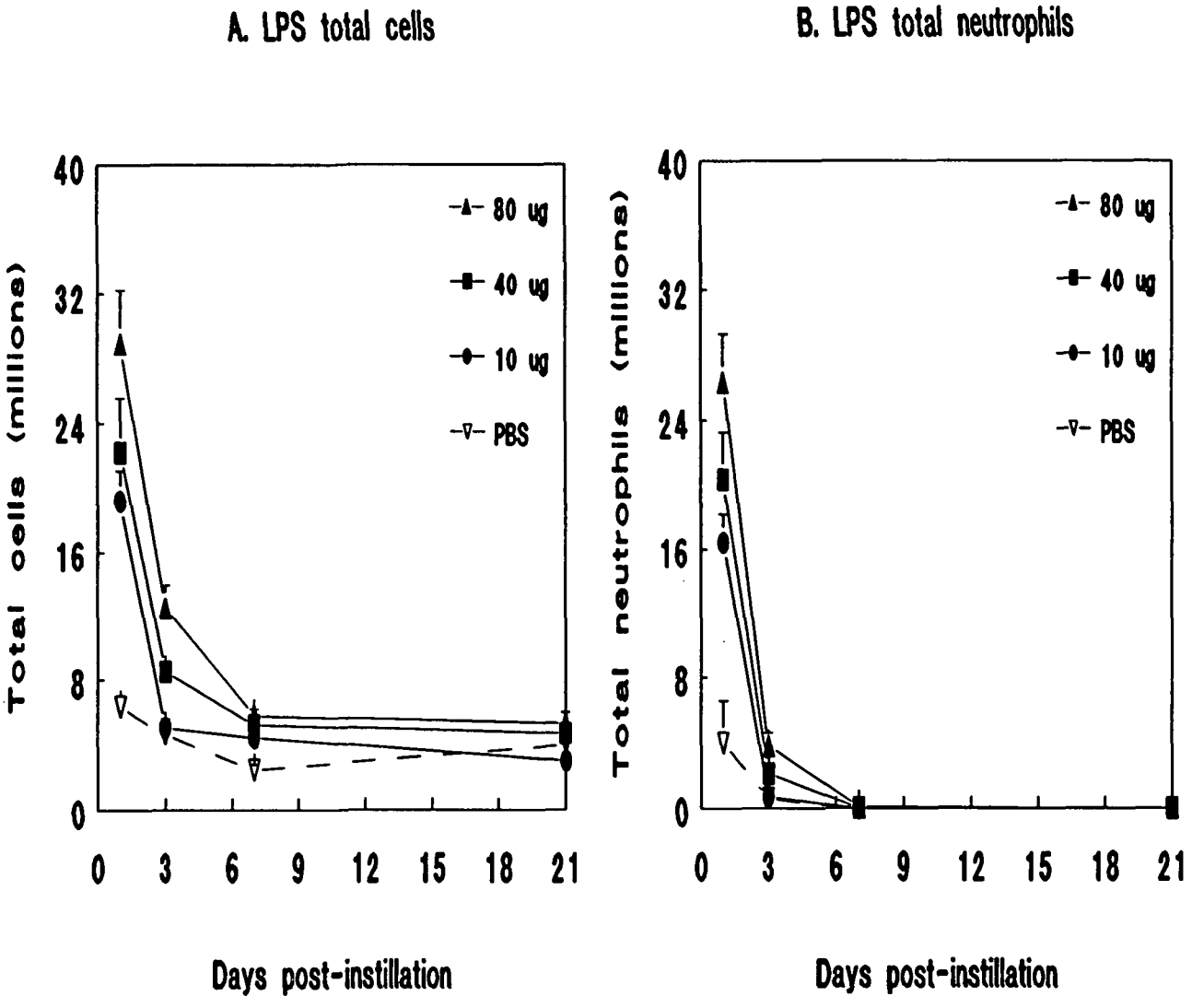

Figure 4.3 Total cell and total neutrophil numbers in lavage at various time points following lung instillation of 10, 40, or 80 ug LPS. Bars represent standard deviations from the mean.

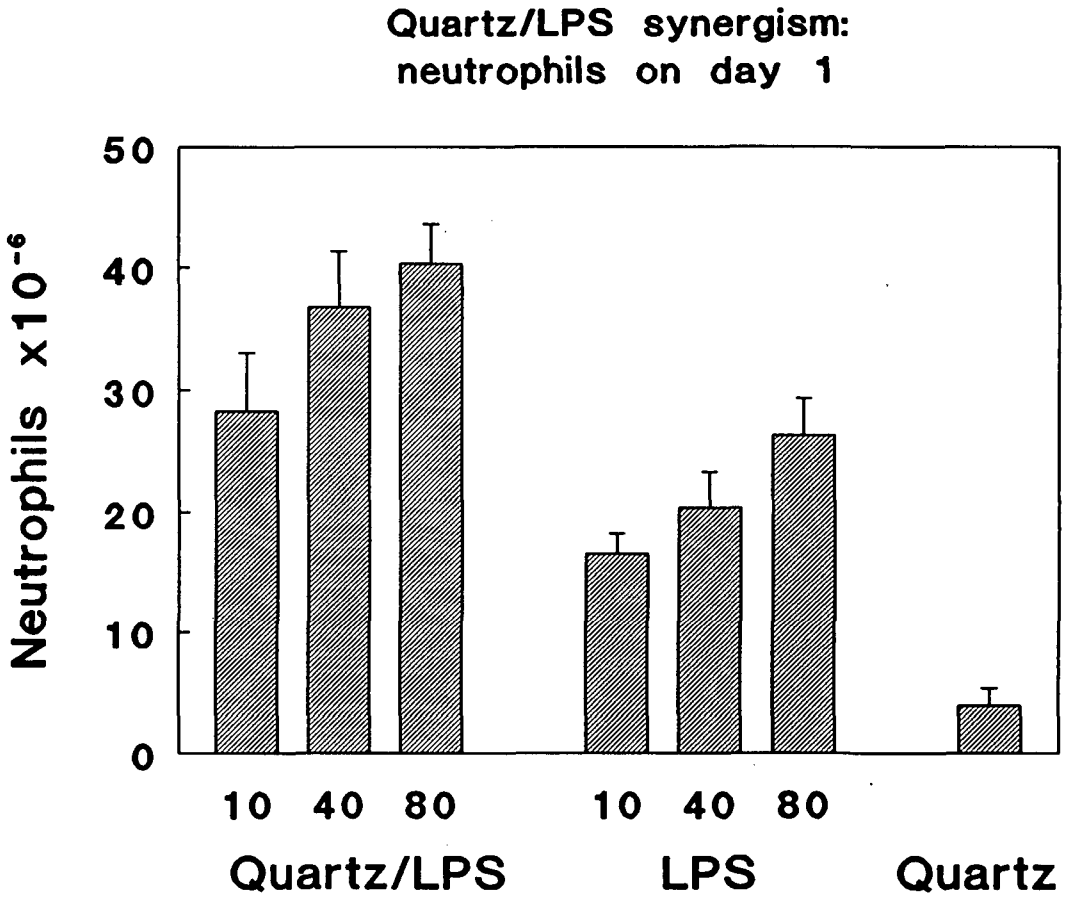

Figure 4.4 Synergistic effect of quartz/LPS combined treatment on mean (s.d.) number of neutrophils recruited to the lung one day post-treatment.

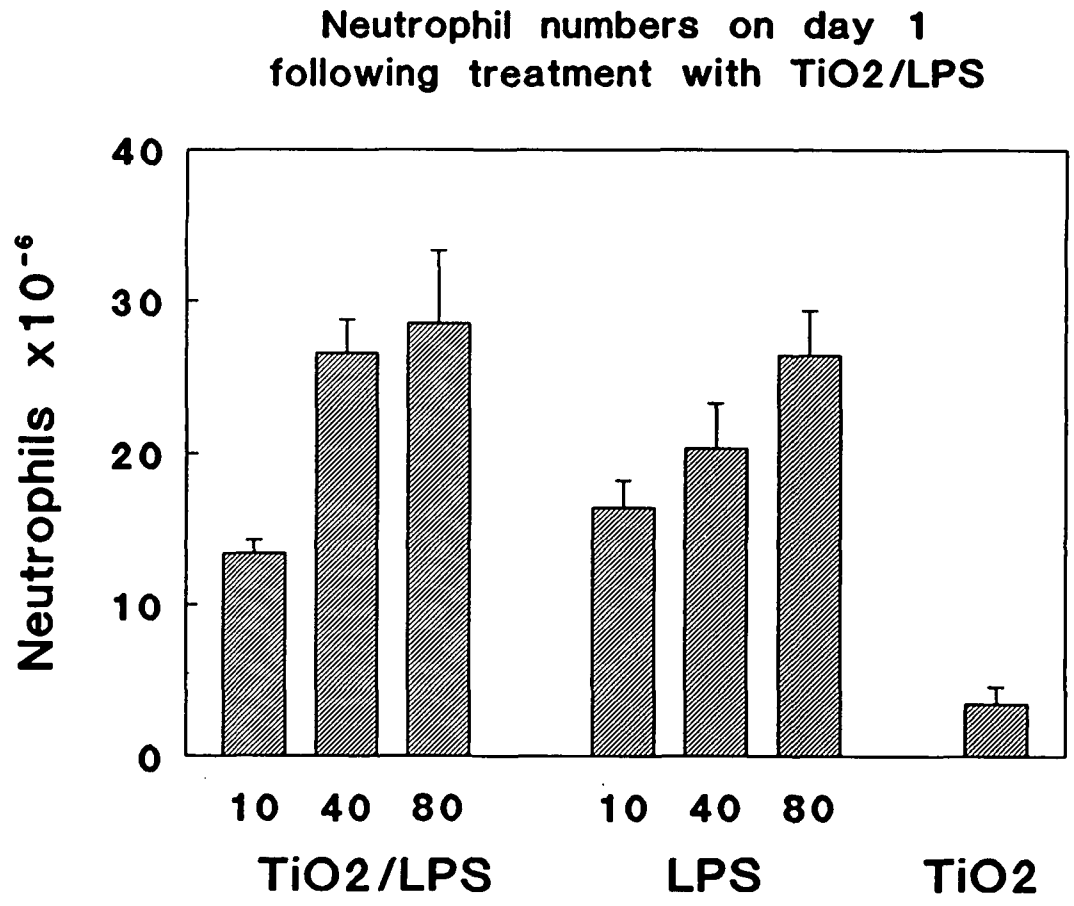

Figure 4.5 Effect of combined TiO_2/LPS instillation on mean (s.d.) number of neutrophils in lavage one day following instillation.

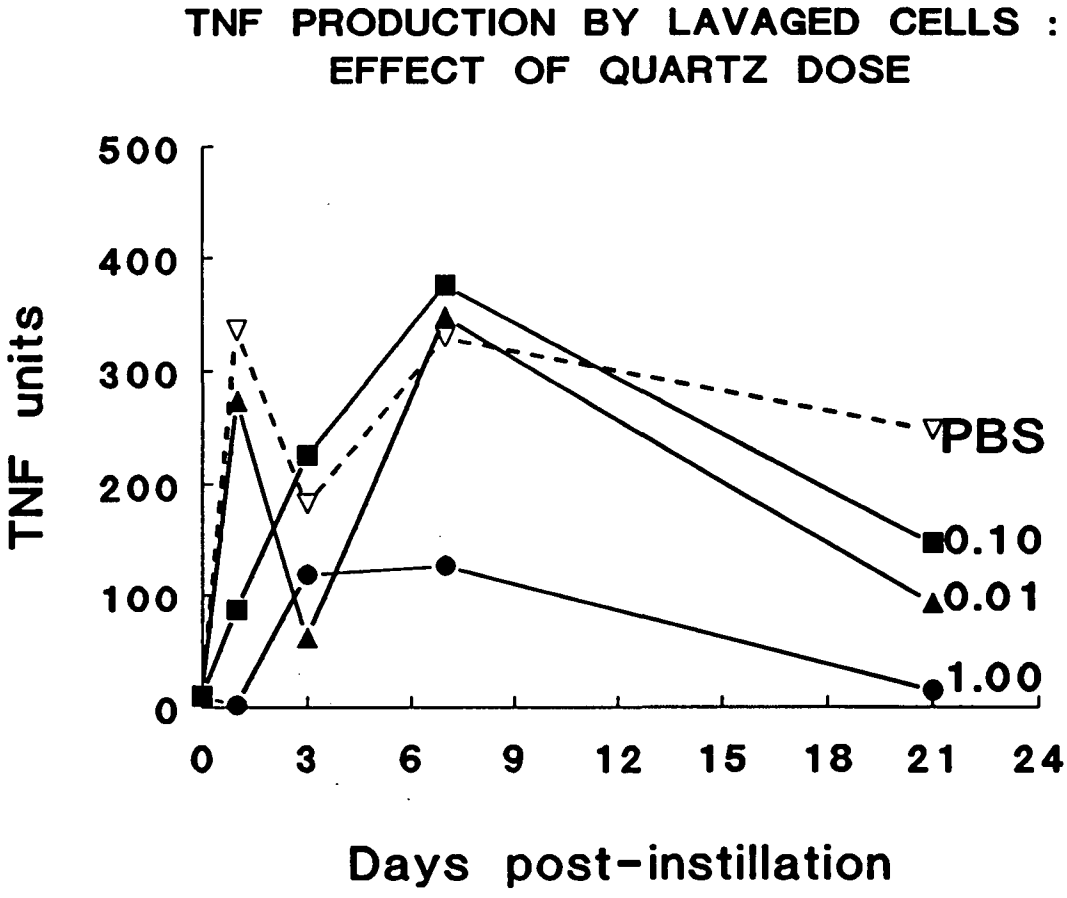

Figure 4.6 Effect of instillation of 0.01, 0.1, or 1 mg quartz on the production of TNF by cells recovered in lavage at various times post-instillation.

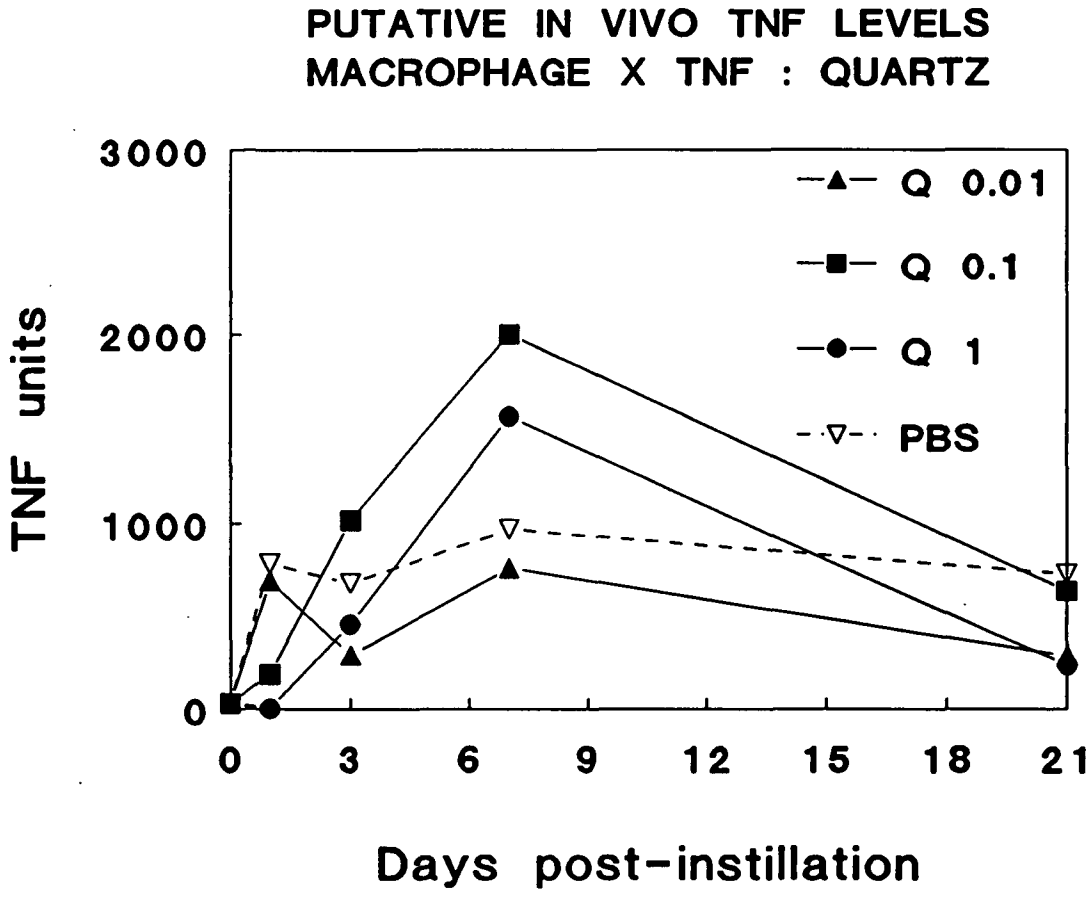

Figure 4.7 Putative *in vivo* TNF concentrations (TNF x macrophage number): Effect of instillation of 0.01, 0.1, or 1 mg quartz.

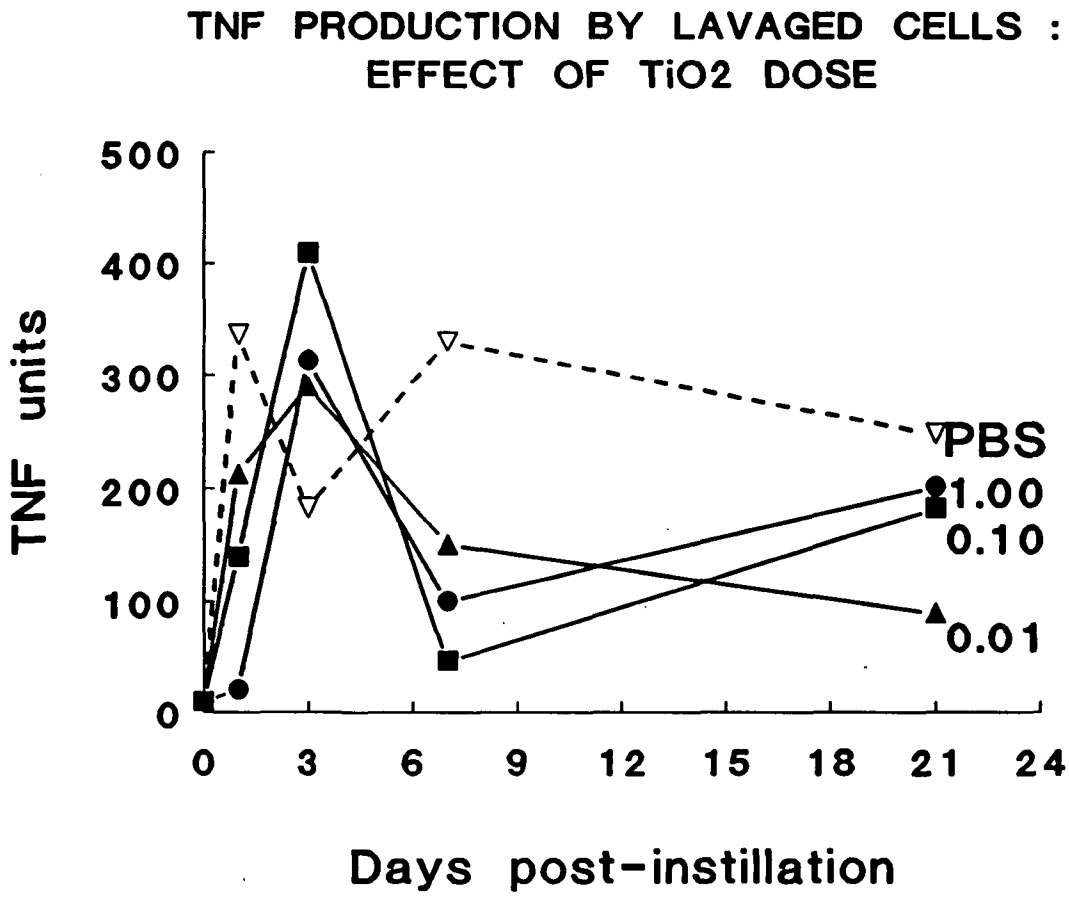

Figure 4.8 Effect of instillation of 0.01, 0.1, or 1 mg TiO_2 on the production of TNF by cells recovered in lavage at various times post-instillation.

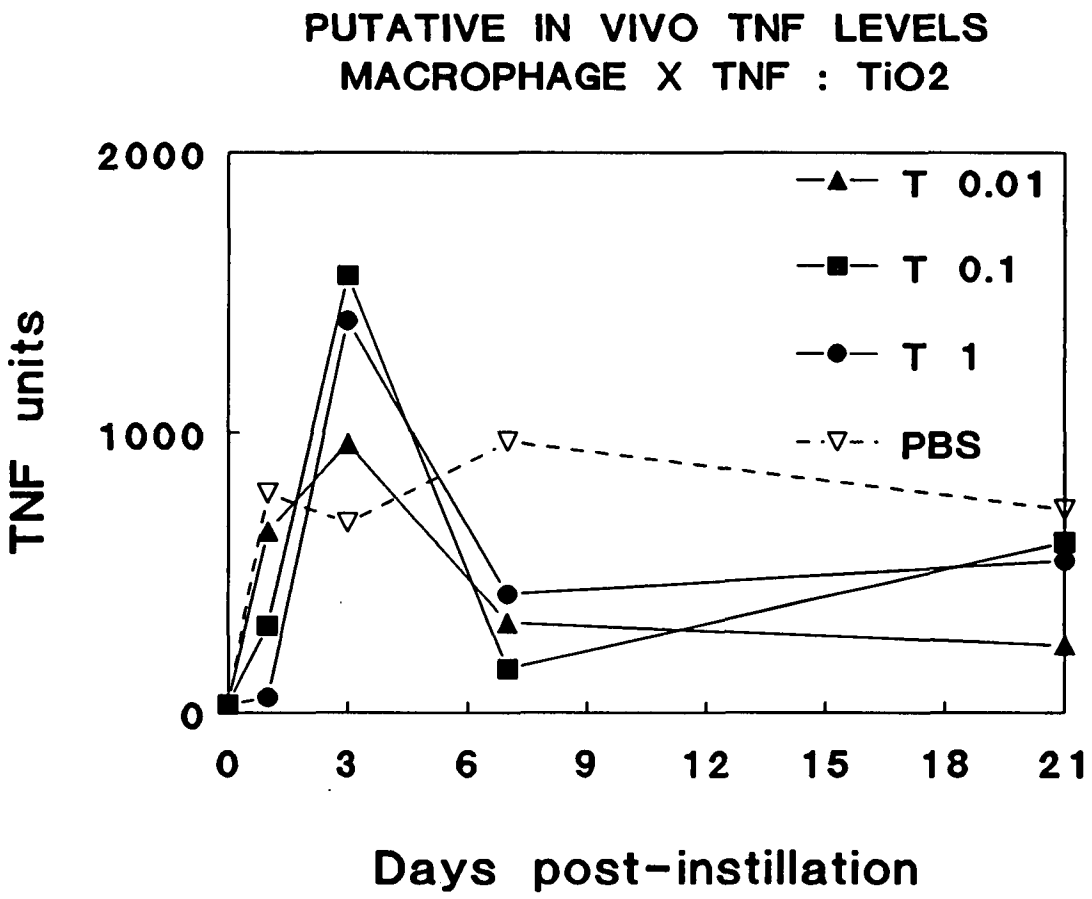

Figure 4.9 Putative *in vivo* TNF concentrations (TNF x macrophage number): Effect of instillation of 0.01, 0.1, or 1 mg TiO_2

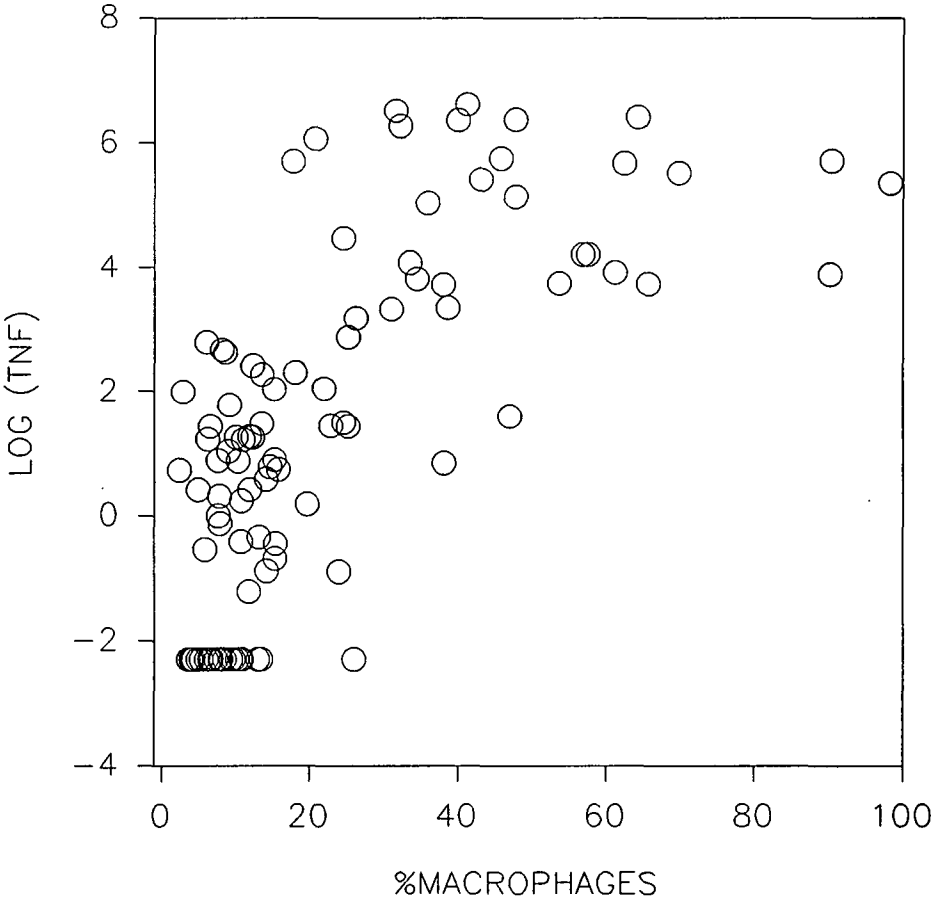

Figure 4.10 Positive correlation between log TNF concentration and percentage macrophages (pooled data from all treatment groups) at day 1 post-treatment; correlation coefficient r= 0.702.

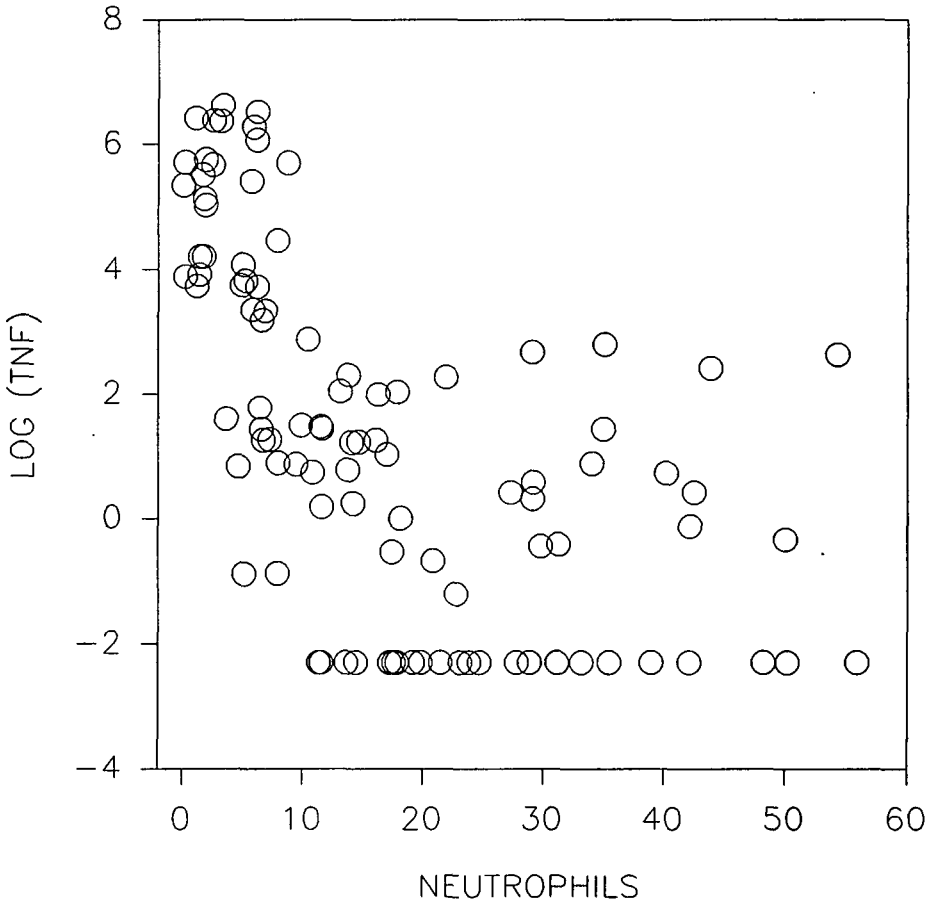

Figure 4.11 Negative correlation between log TNF concentration and neutrophil numbers (pooled data from all treatment groups) at day 1 post-treatment; correlation coefficient r= -0.592.

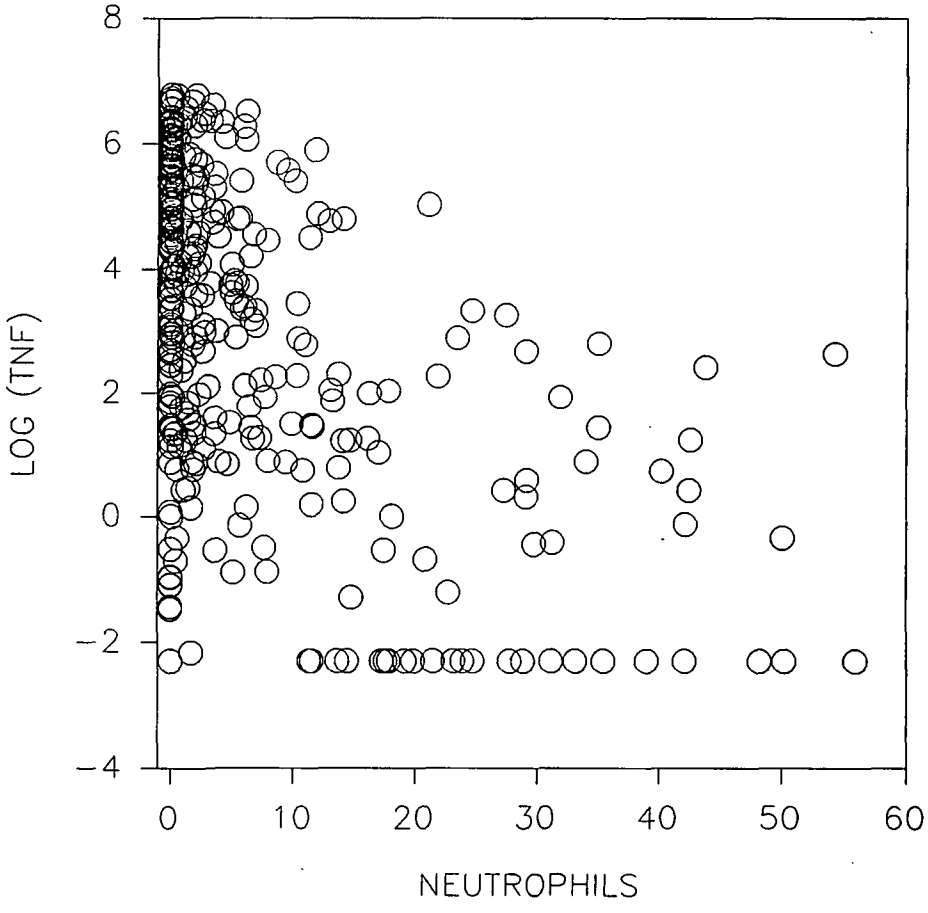

Figure 4.12 PLot of log TNF concentration against neutrophil numbers: data pooled for all time points and all treatment groups; correlation coefficient r= -0.530.

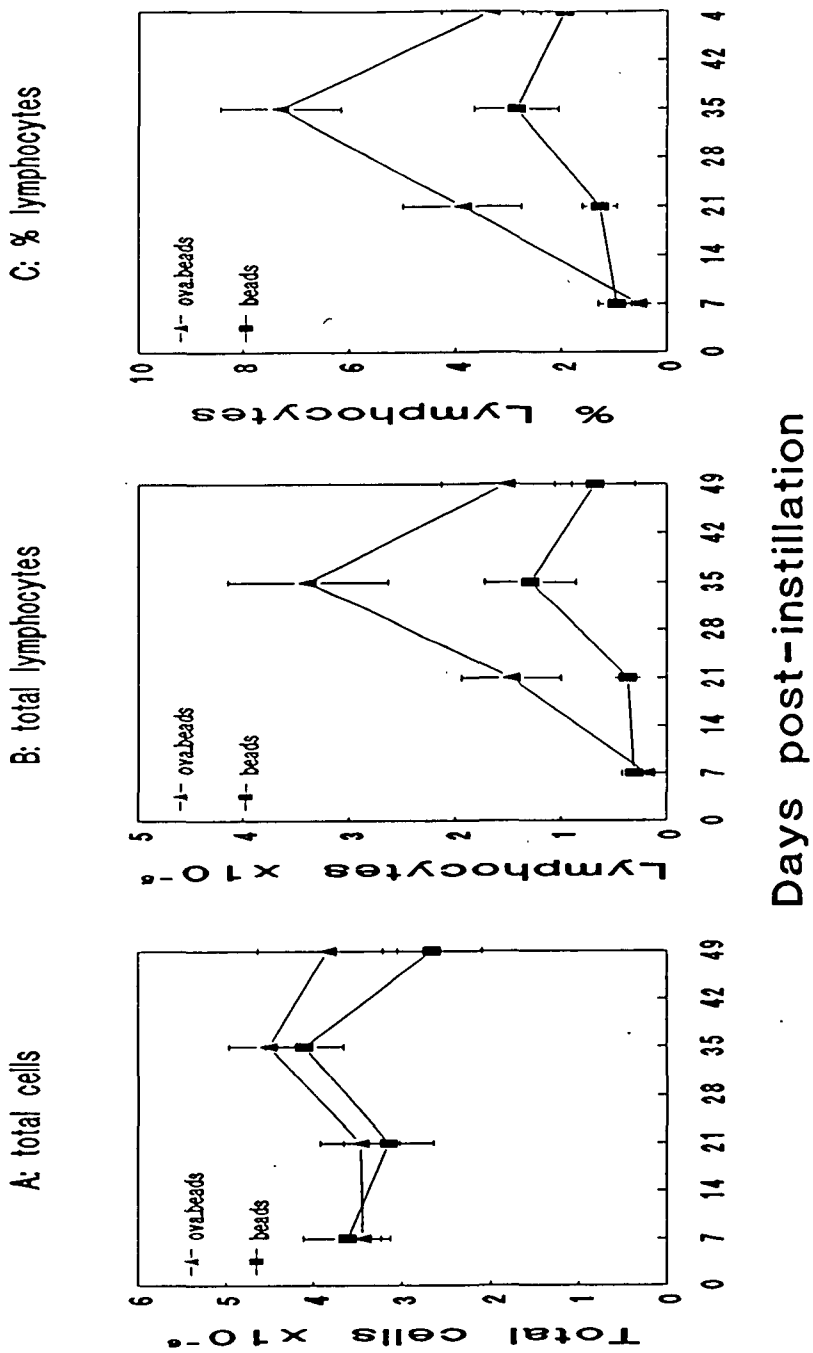

Figure 4.13 Mean (s.d.) of total cell (A), total lymphocyte (B), and percentage lymphocytes (C) recovered by lavage following lung instillation with OVA-beads or unconjugated beads.

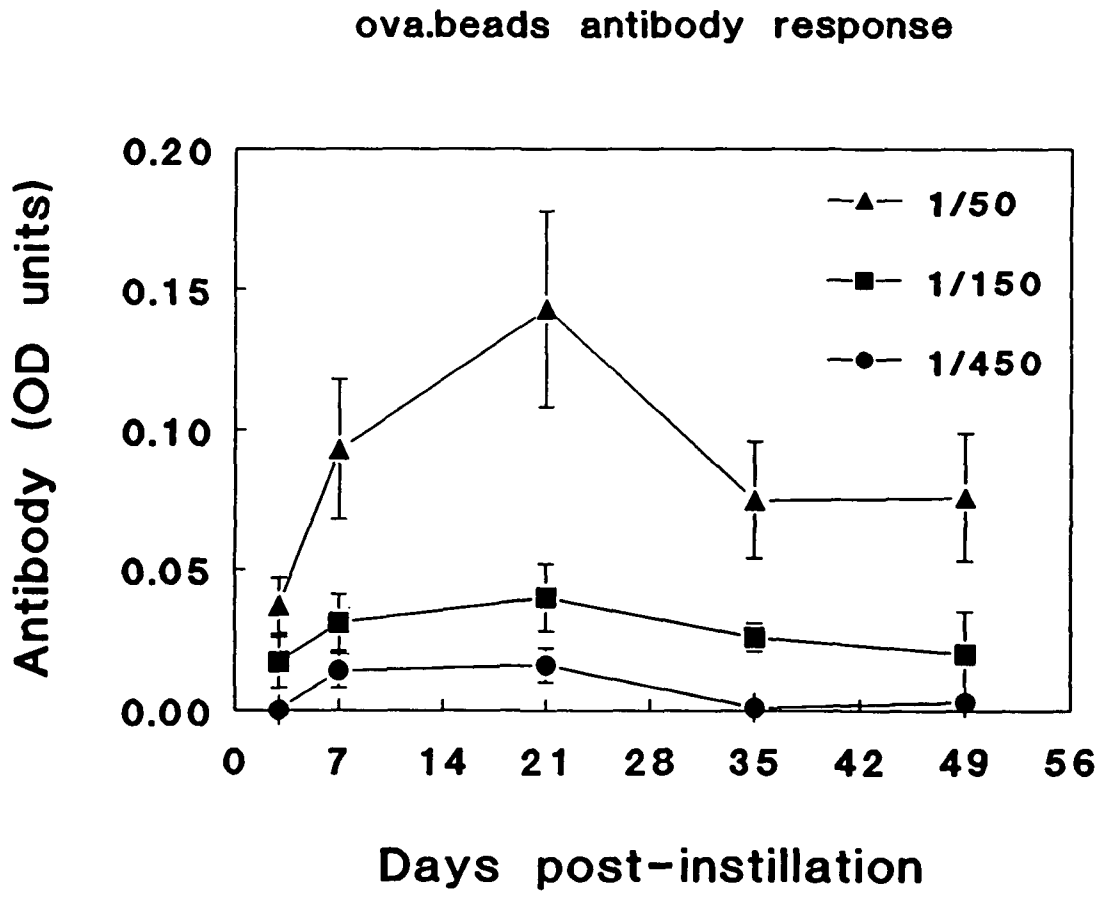

Figure 4.14 Mean (s.d.) serum antibody titres, as optical density units, to ovalbumin at various times post-instillation of OVA-beads. Results from three serum dilutions; 1/50, 1/150, 1/450.

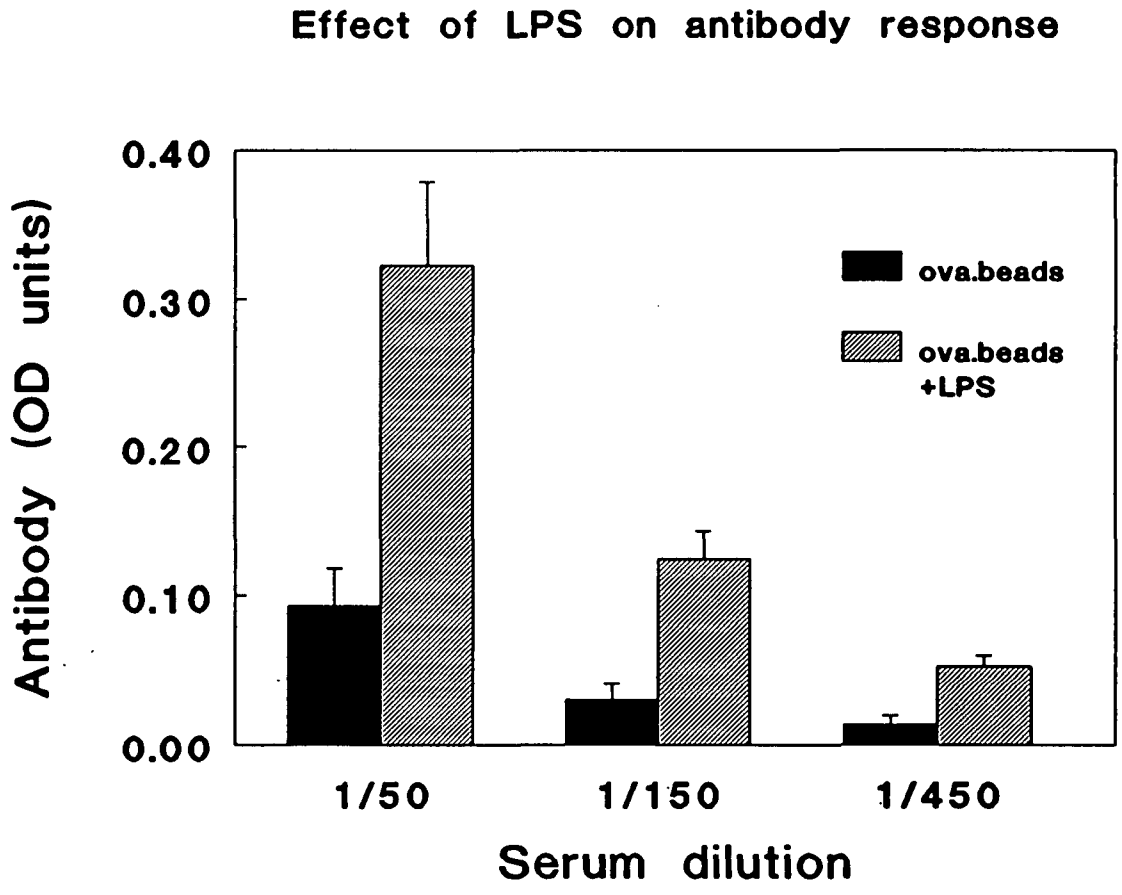

Figure 4.15 The enhancing effect of combining 80 ug LPS with the OVA-bead lung instillation on the antibody titre to ovalbumin on day 7; mean (s.d.) optical density units.

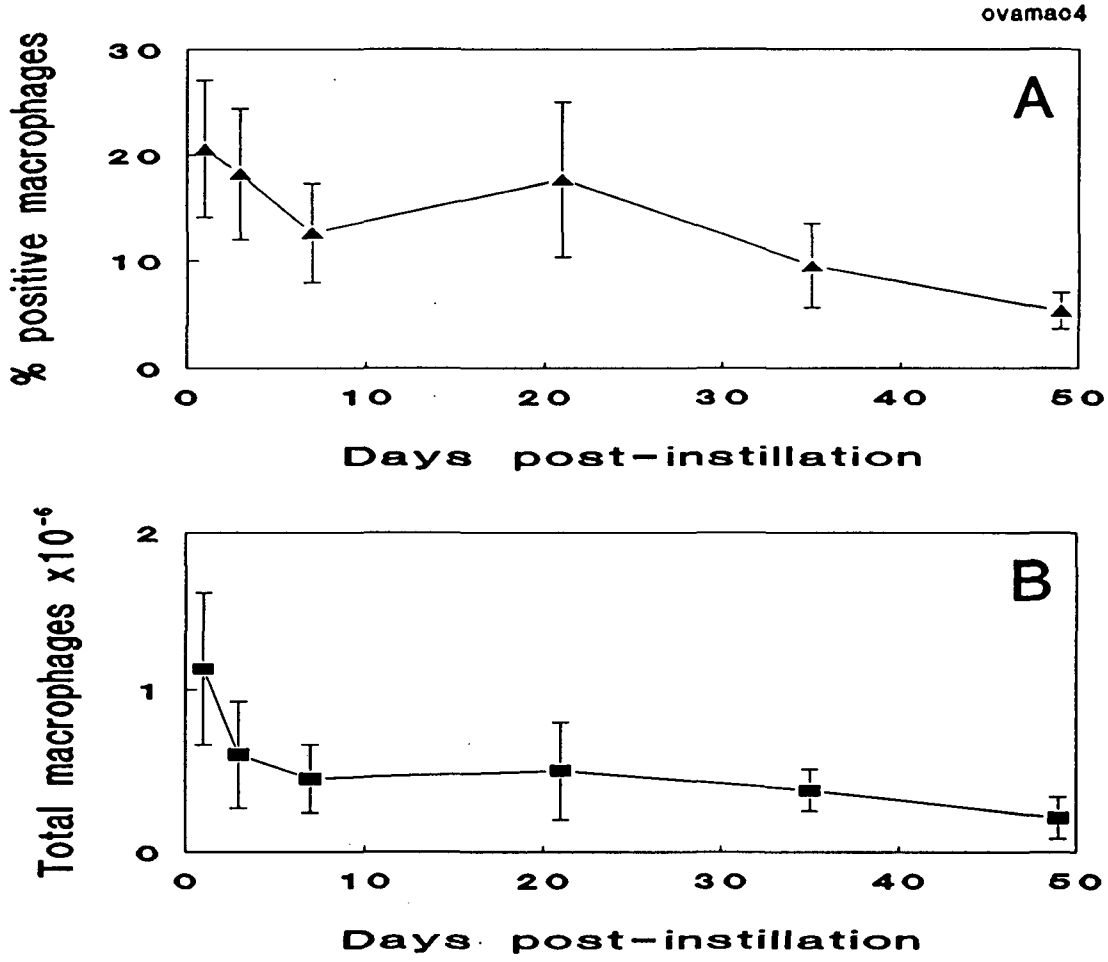

Figure 4.16 Relative numbers of alveolar macrophages containing OVA-beads at various times post-instillation; mean (s.d.).

Table 4.1 Percentage cell types recovered in lavage on day 21

DUST	Mean (standard deviation) percent cell types MACROPHAGE	NEUTROPHIL	LYMPHOCYTE
Quartz 1mg	37.09(5.47)	61.45(4.66)	1.46(1.36)
Quartz 0.1mg	52.42(18.69)	46.34(18.30)	1.24(0.93)
Quartz 0.01mg	98.23(0.77)	0.44(0.55)	1.33(1.15)
TiO_2 1mg	98.27(1.25)	0.44(0.65)	1.29(0.76)
TiO_2 0.1mg	98.25(0.53)	0.17(0.29)	1.58(0.33)
TiO_2 0.01mg	99.14(1.29)	0.00(0.00)	0.86(1.29)
PBS	98.43(1.87)	0.10(0.17)	1.47(1.82)

Table 4.2 Total cells recovered by lavage following instillation of 0.01 mg dust plus LPS

		Days post-instillation			
LPS	Dust	1	3	7	21
none	PBS	6.34(1.8)[a] n=7	4.64(2.3) 9	2.45(0.9) 9	3.99(1.6) 7
	TiO2	6.26(2.6) 7	3.50(1.7) 9	2.29(0.6) 6	2.82(0.8) 6
	quartz	6.18(3.1) 9	4.62(1.7) 8	2.79(1.0) 6	3.09(0.5) 6
10ug	PBS	19.19(4.6) 6	5.03(1.8) 10	4.43(2.1) 9	3.00 1
	TiO2	15.43(1.0) 6	6.88(4.1) 9	4.52(1.5) 7	1.49(0.2) 2
	quartz	32.34(13.7) 6	7.13(1.4) 11	4.37(1.3) 6	3.70(1.2) 6
40ug	PBS	22.20(8.3) 6	8.49(2.3) 7	5.21(1.5) 8	4.67(1.2) 6
	TiO2	29.19(5.1) 6	12.96(5.6) 8	4.76(2.5) 7	3.22(2.7) 2
	quartz	41.64(12.9) 6	9.68(4.1) 7	4.91(1.6) 7	3.03(0.9) 6
80ug	PBS	28.92(8.1) 6	12.48(4.0) 8	5.78(1.0) 6	5.32(0.9) 2
	TiO2	30.23(11.9) 6	16.58(1.6) 4	4.68(1.6) 6	4.37(1.0) 3
	quartz	43.70(8.9) 6	12.32(3.0) 6	6.29(1.0) 6	4.65(2.7) 4

a: mean (standard deviation) total cells in millions, with numbers of rats below.

Table 4.3 Total number of macrophages recovered by lavage following instillation of 0.01 mg dust plus LPS

LPS	Dust	Days post-instillation 1	3	7	21
None	PBS	2.35(1.0)[a] n=7	3.99(2.1) 9	2.40(0.9) 9	3.94(1.7) 7
	TiO_2	2.76(0.8) 7	3.03(1.4) 9	2.27(0.6) 6	2.80(0.8) 6
	quartz	2.21(0.8) 9	3.95(1.4) 8	2.70(1.0) 6	3.04(0.4) 6
10 ug	PBS	2.66(0.8) 6	4.25(1.3) 10	4.38(2.1) 9	2.99 1
	TiO_2	1.97(1.1) 6	5.43(3.2) 9	4.48(1.5) 7	1.49(0.2) 2
	quartz	3.99(2.3) 6	4.93(1.1) 11	4.13(1.1) 6	3.63(1.2) 6
40 ug	PBS	1.73(1.2) 6	6.27(1.9) 7	5.08(1.5) 8	4.62(1.2) 6
	TiO_2	2.52(1.0) 6	8.78(3.6) 8	4.71(2.5) 7	3.18(2.6) 2
	quartz	4.79(5.0) 6	6.72(1.9) 7	4.80(1.5) 7	2.97(0.9) 6
80ug	PBS	2.55(1.4) 6	8.38(2.3) 8	5.61(0.9) 6	5.29(0.9) 2
	TiO_2	1.77(0.7) 6	11.67(2.7) 4	4.62(1.6) 6	4.34(1.0) 3
	quartz	3.37(1.2) 6	9.04(3.3) 6	5.96(1.1) 6	4.46(2.6) 4

a: Mean (standard deviation) total number of macrophages in millions, with numbers of rats below.

Table 4.4 Total number of neutrophils recovered by lavage following instillation of 0.01 mg dust plus LPS

LPS	Dust	Days post-instillation 1	3	7	21
None	PBS	3.97(2.6)[a] n=7	0.61(0.6) 9	0.02(0.0) 9	0.00(0.0) 7
	TiO_2	3.44(2.8) 7	0.41(0.4) 9	0.01(0.0) 6	0.00(0.0) 6
	quartz	3.92(3.5) 9	0.61(0.6) 8	0.05(0.0) 6	0.01(0.0) 6
10 ug	PBS	16.45(4.3) 6	0.71(0.6) 10	0.01(0.0) 9	0.00 1
	TiO_2	13.44(2.1) 6	1.34(1.0) 9	0.01(0.0) 7	0.00 2
	quartz	28.28(11.7) 6	2.05(0.6) 11	0.19(0.2) 6	0.02(0.0) 6
40 ug	PBS	20.35(7.2) 6	2.10(0.7) 7	0.03(0.1) 8	0.01(0.0) 6
	TiO_2	26.54(5.4) 6	3.99(3.2) 8	0.00(0.0) 7	0.00 2
	quartz	36.76(11.2) 6	2.87(2.4) 7	0.01(0.0) 7	0.03(0.0) 6
80ug	PBS	26.35(7.3) 6	3.80(2.2) 8	0.10(0.1) 6	0.00 2
	TiO_2	28.47(11.8) 6	4.80(2.1) 4	0.02(0.0) 6	0.00 3
	quartz	40.33(8.1) 6	2.99(1.4) 6	0.15(0.2) 6	0.15(0.2) 4

a: Mean (standard deviation) total number of neutrophils in millions, with number of rats below.

Table 4.5 The effect of LPS on cell recruitment following instillation of OVA-beads

Treatment	Cells	Days post-instillation 1	3	7
OVA-beads	total	6.29(3.09)[a]	4.75(1.88)	3.45(0.67)
	macrophage	2.82(1.39)	3.90(1.54)	3.44(0.69)
	%	44.8(18.3)[b]	81.3(14.2)	98.3 (2.1)
	neutrophil	3.45(1.70)	0.85(0.34)	0.04(0.08)
	%	54.7(18.8)	17.7(13.9)	1.2 (1.9)
	lymphocyte	0.03(0.02)	0.05(0.02)	0.02(0.00)
	%	0.5 (0.9)	1.1 (1.1)	0.5 (0.4)
LPS	total	28.92(8.13)	12.48(4.03)	5.78(0.98)
	macrophage	2.51(0.70)	8.56(2.74)	5.16(0.88)
	%	8.7 (3.4)	68.5 (9.1)	97.3 (2.0)
	neutrophil	26.39(7.40)	3.66(1.17)	0.09(0.01)
	%	91.3 (3.3)	29.3 (8.9)	1.6 (1.9)
	lymphocyte	0.03(0.00)	0.29(0.09)	0.06(0.01)
	%	0.1 (0.1)	2.3 (1.8)	1.1 (0.5)
OVA-beads +LPS	total	44.50(9.78)	19.67(7.28)	14.07(1.89)
	macrophage	3.47(0.76)	7.66(2.84)	13.68(1.84)
	%	7.8 (3.1)	38.9(18.1)	97.0 (1.7)
	neutrophil	40.98(2.86)	11.86(4.39)[c]	0.07(0.01)
	%	92.1 (3.1)[c]	60.2(17.7)	0.5 (0.4)
	lymphocyte	0.09(0.02)	0.18(0.07)	0.35(0.05)[d]
	%	0.2 (0.3)	0.9 (0.8)	2.5 (1.9)[d]

a: Mean (standard deviation) cell number in millions.
b: Mean (standard deviation) % cell type.
c: Synergistic increase in total neutrophil numbers arising from the combined treatment.
d: Total and % lymphocytes in the combined treatment group significantly greater at day 7 than either the LPS or OVA-beads groups (t-test p=0.032).

Table 4.6 TNF production following instillation of 0.01 mg dust plus LPS

		Days post-instillation			
LPS	Dust	1	3	7	21
none	PBS	336.1(308.8)[a]	182.6(191.4)	330.4(321.5)	247.7(220.0)
	TiO2	213.4(142.7)	290.4(161.0)	151.2(131.8)	89.9(109.7)
	quartz	274.1(283.2)	62.3(40.2)	348.0(425.8)	92.9(77.7)
10ug	PBS	3.6(3.8)	11.8(22.2)	522.9(249.2)	not done
	TiO2	4.6(7.0)	96.3(89.6)	142.2(44.2)	not done
	quartz	3.7(5.2)	10.0(8.4)	455.4(313.1)	257.8(129.1)
40ug	PBS	1.3(1.4)	9.4(13.2)	394.2(279.2)	0.3(0.1)
	TiO2	0.1(0.2)	27.5(33.6)	96.0(51.5)	not done
	quartz	0.2(0.4)	110.9(38.6)	247.2(206.9)	120.7(129.8)
80ug	PBS	1.5(1.8)	30.7(59.5)	201.5(154.7)	0.3
	TiO2	1.0(1.1)	5.9(2.1)	109.7(88.1)	not done
	quartz	0.2(0.4)	88.7(21.5)	147.9(75.7)	52.9(58.1)

a: Mean (standard deviation) TNF concentration in units/ml

Table 4.7 TNF production times macrophage number (TNF.MAC) following instillation of 0.01 mg dust plus LPS

		Days post-instillation			
LPS	Dust	1	3	7	21
none	PBS	786(711)[a]	682(794)	969(968)	730(758)
	TiO2	650(531)	962(620)	323(264)	241(276)
	quartz	695(722)	291(242)	762(918)	288(262)
10ug	PBS	11(13)	40(71)	2557(1272)	not done
	TiO2	11(24)	487(425)	629(259)	not done
	quartz	14(17)	60(65)	1893(1420)	1003(566)
40ug	PBS	3(3)	47(57)	2019(1834)	1(0)
	TiO2	0(1)	206(234)	531(429)	not done
	quartz	1(1)	861(177)	1196(1231)	452(579)
80ug	PBS	4(5)	286(574)	1136(1025)	2(0)
	TiO2	2(3)	66(20)	589(670)	not done
	quartz	1(1)	785(260)	965(586)	179(189)

a: Mean (standard deviation) TNF.MAC arbitrary units

APPENDIX

Initial investigation of the lavage cell data showed that the within group variation appeared to increase as the mean increased, indicating that the data should be transformed to the log scale. However, such a transformation over-compensated for the inequality of the variances in this data set. The main statistical method used, therefore, was the Poisson log-linear model which assumes that the variance of the data is proportional to the mean by a constant amount. Such models are particularly appropriate when the variables to be analysed are in the form of counts, as is the case with the data here (McCullagh and Nelder, 1989)

The results of these analyses can be summarised in an 'analysis of deviance' table which can be interpreted in a similar way to an analysis of variance table. The ratio of mean deviances has an approximately F-distribution with degrees of freedom determined by the terms included in the ratio. Examination of these F-ratios indicates which terms are significantly related to the response variate, while subsequent examination of the fitted model and the table of means of the data shows the way in which they are associated.

The cell number data was thus analysed using the Poisson log-linear model. The results of these analyses were summarised in analyses of deviance tables which have been set out below.

The principle aims of these analyses were to investigate: (1) whether there was evidence of synergism between endotoxin and the three types of dust - quartz, TiO_2, OVA-beads - resulting in greater numbers of cells of various types or of tumour necrosis factor (TNF) production; (2) to investigate the effect of different concentrations of quartz and TiO_2 on TNF production.

Analysis of deviance tables

Table A1 : Quartz and Endotoxin - Total Cells

Variable	d.f.	deviance	mean deviance	mean deviance ratio	
Day	3	1149.572	383.191	369.91	***
Quartz (vs PBS)	1	32.175	32.175	31.06	***
Quartz.Day	3	21.411	7.137	6.89	***
LPS	3	421.248	140.416	135.55	***
LPS.Day	9	80.462	8.940	8.63	***
Quartz.LPS	3	5.321	1.774	1.71	
Residual	192	198.895	1.036		

Table A2 : Quartz and Endotoxin - Macrophages

Variable	d.f.	deviance	mean deviance	mean deviance ratio	
Day	3	56.3969	18.7990	29.56	***
Quartz (vs PBS)	1	0.7851	0.7851	1.23	
Quartz.Day	3	6.9727	2.3242	3.66	*
LPS	3	47.3371	15.7790	24.81	***
LPS.Day	9	13.8462	1.5385	2.42	*
Quartz.LPS	3	0.5787	0.1929	0.30	
Residual	192	122.0915	0.6359		

Table A3 : Quartz and Endotoxin - Neutrophils

Variable	d.f.	deviance	mean deviance	mean deviance ratio	
Day	3	2491.7129	830.5709	944.15	***
Quartz (vs PBS)	1	45.9431	45.9431	52.23	***
Quartz.Day	3	2.1597	0.7199	0.82	
LPS	3	486.2675	162.0892	184.25	***
LPS.Day	9	9.4538	1.0504	1.19	
Quartz.LPS	3	7.0186	2.3395	2.66	*
Residual	192	168.9035	0.8797		

*** = p<0.001 ** = p<0.01 * = p<0.05

Table A4 : TiO2 and Endotoxin - Total Cells

Variable	d.f.	deviance	mean deviance	mean deviance ratio	
Day	3	736.917	245.639	229.67	***
TiO2 (vs PBS)	1	1.162	1.162	1.09	
TiO2.Day	3	6.870	2.290	2.14	
LPS	3	340.221	113.407	106.03	***
LPS.Day	9	45.956	5.106	4.77	***
TiO2.LPS	3	7.282	2.427	2.27	
Residual	178	190.379	1.070		

Table A5 : TiO2 and Endotoxin - Macrophages

Variable	d.f.	deviance	mean deviance	mean deviance ratio	
Day	3	95.5894	31.8631	47.89	***
TiO2 (vs PBS)	1	0.0118	0.0118	0.02	
TiO2.Day	3	5.3748	1.7916	2.69	*
LPS	3	50.7239	16.9080	25.41	***
LPS.Day	9	28.6078	3.1786	4.78	***
TiO2.LPS	3	1.4330	0.4777	0.72	
Residual	178	118.4415	0.6654		

Table A6 : TiO2 and Endotoxin - Neutrophils

Variable	d.f.	deviance	mean deviance	mean deviance ratio	
Day	3	1864.9788	621.6595	805.62	***
TiO2 (vs PBS)	1	2.0162	2.0162	2.61	
TiO2.Day	3	1.7688	0.5896	0.76	
LPS	3	368.7843	122.9281	159.30	***
LPS.Day	9	10.7856	1.1984	1.55	
TiO2.LPS	3	7.0651	2.3550	3.05	*
Residual	178	137.3551	0.7717		

*** = p<0.001 ** = p<0.01 * = p<0.05

Table A7: Ovalbumin and Endotoxin - Total Cells

Variable	d.f.	deviance	mean deviance	mean deviance ratio
Day	3	348.3092	116.1031	127.26 ***
Ovalbumin (vs PBS)	1	26.9185	26.9185	29.51 ***
Ovalbumin.Day	3	12.1134	4.0378	4.43 **
LPS	1	465.2499	465.2499	509.96 ***
LPS.Day	3	25.4560	8.4853	9.30 ***
Ovalbumin.LPS	1	8.8949	8.8949	9.75 **
Residual	93	84.8472	0.9123	

Table A8: Ovalbumin and Endotoxin - Macrophages

Variable	d.f.	deviance	mean deviance	mean deviance ratio
Day	3	42.0439	14.0146	25.74 ***
Ovalbumin (vs PBS)	1	4.3835	4.3835	8.05 **
Ovalbumin.Day	3	20.7390	6.9130	12.70 ***
LPS	1	70.9947	70.9947	130.41 ***
LPS.Day	3	15.5880	5.1960	9.54 ***
Ovalbumin.LPS	1	1.1478	1.1478	2.11
Residual	93	50.6297	0.5444	

Table A9: Ovalbumin and Endotoxin - Neutrophils

Variable	d.f.	deviance	mean deviance	mean deviance ratio
Day	3	960.7891	320.2630	345.45 ***
Ovalbumin (vs PBS)	1	25.9268	25.9268	27.97 ***
Ovalbumin.Day	3	7.8729	2.6243	2.83 *
LPS	1	474.6166	474.6166	511.95 ***
LPS.Day	3	0.6611	0.2204	0.24
Ovalbumin.LPS	1	4.0582	4.0582	4.38 *
Residual	93	86.2188	0.9271	

*** = p<0.001 ** = p<0.01 * = p<0.05

Table A10 : Quartz concentration - TNF

Variable	d.f.	deviance	mean deviance	mean deviance ratio	
Day	3	1963.6	654.5	2.82	*
Quartz concentration	3	2699.9	900.0	3.88	*
Quartz conc.Day	9	2749.1	305.5	1.32	
Residual	82	19007.7	231.8		

Table A11 : Quartz concentration - TNF*macrophages

Variable	d.f.	deviance	mean deviance	mean deviance ratio	
Day	3	15903.6	5301.2	6.06	***
Quartz concentration	3	3061.9	1020.6	1.17	
Quartz conc.Day	9	11957.3	1328.6	1.52	
Residual	82	71733.3	874.8		

Table A12 : TiO2 concentration - TNF

Variable	d.f.	deviance	mean deviance	mean deviance ratio	
Day	3	682.3	227.4	1.27	
TiO2 concentration	3	864.4	288.1	1.61	
TiO2 conc.Day	9	3510.0	390.0	2.18	*
Residual	65	11614.7	178.7		

*** = p<0.001 ** = p<0.01 * = p<0.05

Table A13 : TiO2 concentration - TNF*macrophages

Variable	d.f.	deviance	mean deviance	mean deviance ratio	
Day	3	5581.8	1860.6	3.15	*
TiO2 concentration	3	1191.1	397.0	0.67	
TiO2 conc.Day	9	10734.1	1192.7	2.02	
Residual	65	38421.9	591.1		

Table A14 : Endotoxin and Quartz - TNF

Variable	d.f.	deviance	mean deviance	mean deviance ratio	
Day	3	10984.3	3661.4	24.87	***
Quartz	1	16.0	16.0	0.11	
Quartz.Day	3	160.1	53.4	0.36	
LPS	3	5094.4	1698.1	11.53	***
LPS.Day	9	8529.7	947.7	6.44	***
Quartz.LPS	3	324.7	108.2	0.74	
Residual	143	21055.5	147.2		

Table A15 : Endotoxin and Quartz - TNF*macrophages

Variable	d.f.	deviance	mean deviance	mean deviance ratio	
Day	3	55573.4	18524.5	31.20	***
Quartz	1	11.8	11.8	0.02	
Quartz.Day	3	2368.8	789.6	1.33	
LPS	3	4288.8	1429.6	2.41	
LPS.Day	9	37213.2	4134.8	6.96	***
Quartz.LPS	3	3284.8	1094.9	1.84	
Residual	143	84912.7	593.8		

*** = p<0.001 ** = p<0.01 * = p<0.05

Table A16 : Endotoxin and TiO2 - TNF

Variable	d.f.	deviance	mean deviance	mean deviance ratio	
Day	3	6001.02	2000.34	20.11	***
TiO2	1	1588.10	1588.10	15.96	***
TiO2.Day	3	2507.43	835.81	8.40	***
LPS	3	6167.57	2055.86	20.66	***
LPS.Day	8	8024.68	1003.09	10.08	***
TiO2.LPS	3	55.55	18.52	0.19	
Residual	138	13730.20	99.49		

Table A17 : Endotoxin and TiO2 - TNF*macrophages

Variable	d.f.	deviance	mean deviance	mean deviance ratio	
Day	3	38547.0	12849.0	27.66	***
TiO2	1	7833.5	7833.5	16.86	***
TiO2.Day	3	9985.5	3328.5	7.17	***
LPS	3	6492.7	2164.2	4.66	**
LPS.Day	8	32575.3	4071.9	8.77	***
TiO2.LPS	3	76.3	25.4	0.05	
Residual	138	64099.3	464.5		

*** = p<0.001 ** = p<0.01 * = p<0.05

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