Bronchial challenge of normal subjects with the endotoxin of Enterobacter agglomerans isolated from cotton dust

J P Jamison and R C Lowry

From the Respiratory Investigation Centre, Belfast City Hospital, and Department of Physiology, The Queen’s University of Belfast, Belfast, UK

ABSTRACT
Endotoxin produced by a culture of Enterobacter agglomerans isolated from cotton dust was inhaled by 12 normal subjects. No significant airway constriction was obtained in doses equivalent to those experienced in a workshift in a dusty mill. There was a statistically significant difference between this result and the bronchoconstriction that had occurred after flax dust inhalation in the same subjects. It is suggested that Enterobacter agglomerans endotoxin is not the causative agent of the acute bronchoconstriction that follows inhalation of textile dust.

Neal et al reported that inhalation of a sterile filtrate of a culture of Aerobacter cloacae by a volunteer resulted in fever, dyspnoea, and coughing.1 Pernis et al administered the endotoxin of Escherichia coli to three subjects and the endotoxin of Salmonella abortus equi to two of these subjects in doses of 15, 30, and 60 µg.2 Coughing, slight dyspnoea, and slight reduction in forced expiratory volume in one second (FEV₁) were reported along with fever, malaise, and a skin reaction. Cavagna et al found that two out of eight normal subjects inhaling 80 µg of E coli endotoxin showed a fall in FEV₁ of more than 10%.3 Van der Zwan et al found a 14% fall in FEV₁ in subjects with bronchial hyperreactivity after inhaling 4 mg endotoxin from Haemophilus influenzae.4

These experiments suggested that the bacterial endotoxin content of textile dusts may be the causative agent of the bronchoconstriction that is the response of normal subjects to these dusts. The effects were, however, not easily comparable with the equivalent dose of endotoxin in textile dusts. A further uncertainty is the evidence that endotoxins from different bacteria have differing biological activities.5–7

We have reported the ventilatory responses of a panel of 12 normal subjects to flax dust when we found a 30% reduction in this response after steam treatment which also reduced the endotoxin content by 50%.8 It is therefore of interest to compare the response of this panel of subjects with the direct inhalation of the appropriate endotoxin in the equivalent dose. Cotton and other organic dusts commonly contain Enterobacter agglomerans9 and this has also been found in flax dust (B Buick, personal communication). We have challenged our panel of normal subjects with the endotoxin produced by this organism, isolated and purified by the technique of Helander et al,6 and given in a dose guided by Limulus assay of the endotoxin content of flax dust.

METHODS

SUBJECTS
The same panel of 12 normal subjects who participated in the flax dust trial8 were studied. One subject was a smoker but did not smoke on trial days. The subjects gave informed consent and the experiments had the approval of the ethical committee.

ENDOTOXIN
The endotoxin was prepared in Helsinki by Helander et al.6 The organism Ent agglomerans was isolated and cultured from the cotton dust and the lipopolysaccharide extract prepared from the culture. The endotoxin was dissolved in 2 ml of saline and inhaled by ultrasonic nebuliser (Pulmosonic) over a period of 15 minutes.

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PROTOCOL
A crossover trial was carried out on the 12 normal subjects randomly divided into two groups of six subjects in each group; one group received 5 µg of endotoxin first followed in two weeks by saline whereas the other group received the saline first and then the endotoxin. A further trial was carried out using the higher dose of 12 µg. The subjects and the observer of lung function were unaware whether a saline control or endotoxin challenge was being administered. Three forced expiratory manoeuvres were recorded by a McDermott computerised spirometer and flow/volume curves constructed. These measurements were carried out immediately before and at one hour and four hours after the start of the bronchial challenge. Single breath carbon monoxide transfer factor was measured before and after the high dose of endotoxin using P K Morgan gas transfer equipment. This measurement was made on one group of six subjects.

ENDOTOXIN ASSAY OF FLAX DUST
The level of endotoxin in the flax dust was measured by a modified Limulus method (Rylander and Morey). The dust analysed was that used in the previous report on the effects of inhaled flax dust on these subjects.

STATISTICAL ANALYSES
Analysis of variance was used to compare mean expiratory volumes and flow rates after saline and after 5 µg endotoxin and to compare the means before and after 12 µg endotoxin. Analysis of variance was also used to compare the decreases after 12 µg endotoxin with the decreases after untreated flax dust found in the previous trial. A paired t test was used to compare the mean transfer factor before and after 12 µg of endotoxin.

Results

BRONCHIAL CHALLENGE

Forced expirations
The means of the three forced expirations in all 12 subjects before and after saline and endotoxin are shown in tables 1 and 2. The prechallenge means differed little, whether before saline or endotoxin, and these means were also similar to the control means obtained on these same 12 subjects in the trial of flax dust inhalation. After challenge with saline there were small increases at one hour in mean FEV, FVC, and forced mid-expiratory flow rate (MEF50) and at four hours either no change or small decreases.

After 5 µg endotoxin there were small decreases at both times, the largest decrease being 0·129 l in FVC at four hours. All p values comparing the means before and after saline and 5 µg endotoxin were greater than 0·22.

After 12 µg endotoxin (table 2) the changes in expiratory volumes showed slightly larger decreases at one hour and slightly smaller decreases at four hours, compared with the 5 µg dose. The mid-expiratory flows showed a smaller decrease at one hour and a slight increase at four hours. None of the postchallenge means was statistically significantly different from the prechallenge means, the smallest p value being greater than 0·18 for the FVC at one hour.

Gas transfer
The mean transfer factor fell from 12·18 to 10·8 mmol/min/kPa at four hours. There was no significant difference between the mean transfer factor at one hour and control, but the mean at four hours was significantly lower than control (p < 0·02). There was no significant change in mean alveolar volume, 6·1 l before and 6·2 l after four hours (p > 0·3). The transfer coefficient decreased significantly from 1·93 before to 1·75 mmol/min/kPa/l at four hours (p < 0·01).

Symptoms
One subject only (No 5) reported chest tightness after the higher dose of endotoxin. No fever, chills, malaise, or other symptom occurred in any subject.

Comparison with previously reported effects of dust
When the largest decreases after the high dose of

<table>
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<th>FEV1 (l)</th>
<th>FVC (l)</th>
<th>MEF50 (l)</th>
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<tr>
<td></td>
<td>Before</td>
<td>1 h</td>
<td>4 h</td>
</tr>
<tr>
<td>Saline</td>
<td>3·940</td>
<td>3·902</td>
<td>3·944</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>3·963</td>
<td>3·939</td>
<td>3·915</td>
</tr>
<tr>
<td>p &gt;</td>
<td>0·62</td>
<td>0·56</td>
<td>0·57</td>
</tr>
</tbody>
</table>
endotoxin, recorded at one hour, were compared with the decreases after inhaling flax dust for six hours there were statistically significantly smaller decreases with the present endotoxin challenge in FEV₁ (p < 0·03), FVC (p < 0·05), and MEF₅₀ (p < 0·02).

**ENDOTOXIN LEVELS IN FLAX DUST**

The two batches of untreated flax dust prepared for the two dust trials contained means (SEM, n = 6 analyses) of 111 (19) μg of endotoxin per gram of dust and 100 (17) μg/g for the second batch. The statistically significantly lower values (p < 0·05) of 16 (4) and 51 (9) μg/g were obtained for the dusts from the equivalent flax which had been steamed.

**Discussion**

**BACTERIA AND ENDOTOXIN SOURCE**

The *Ent. agglomerans* endotoxin used in this study was chosen as the most likely causative agent of the acute bronchoconstriction previously described when our panel of subjects inhaled flax dust. Large counts of *Enterobacter* have been reported in cotton and other organic dusts, and this organism has been found to be particularly effective in mobilising leucocytes to the lungs of guinea pigs. Tuffnell suggested that the commonest organisms in these dusts were of the genus *Bacillus* and he found occasional responses to their inhalation. Nevertheless, no leucocyte response to *Bacillus* was found by Rylander and Lundholm.

**DOSE OF ENDOTOXIN**

The dose of endotoxin used was chosen to be comparable with that given in the dust challenge experiments reported previously or in a working shift in a dusty mill. Assuming a resting ventilation rate of 7 l/min, respiration in an atmosphere containing 5 mg dust/m³ with 100 μg endotoxin/g of dust for six hours would be a total inhalation of 1·26 μg. Higher ventilation rates would occur in exercise. Cavagna *et al* estimated the endotoxin content of cotton dust by comparison with the dose of purified *E.coli* endotoxin that produced skin necrosis in rabbits. They found 1·4 mg/g of dust, which would be equivalent to a dose of 17·6 μg assuming a ventilation rate of 7 l/min as above. Cinkotai *et al* found variable endotoxin concentrations from 0·2 to 1·6 mg/g cotton dust. Pernis *et al* found 0·15 to 0·5 mg/g of cotton by a pyrogenic assay, although up to 10 mg/g of material was extracted. Antweiler calculated that a content of 0·5 mg/g of cotton card room dust is more than the maximum possible. The *Limulus* assay is thought to be both sensitive and specific for endotoxin. Using this assay, Rylander and Morey quote airborne concentrations of 0·5 to 2·5 μg/m³. The equivalent calculation would give a dose range of 1·26 to 6·3 μg. These comparisons are subject to various assumptions and uncertainties associated with the techniques of estimating endotoxin concentrations, but the doses of 5 μg and 12 μg do appear to be comparable with the doses to which our panel of subjects were exposed during dust inhalation.

The duration of exposure in the endotoxin challenge experiments was necessarily much shorter than with dust inhalation. This means that to administer the same total dose the airways are being exposed to a correspondingly higher concentration of endotoxin.

A further difference between the dust and endotoxin challenge is that the liquid droplets from the nebuliser may be distributed differently in the airways than dust particles. With the wide size ranges for both droplets and dust particles probably neither large nor small airways were unexposed to either material. The manufacturers of the nebuliser, DeVibiss Health Care UK Ltd, quote the mass median diameter of the liquid droplets as 2·5 μm which is smaller and therefore more likely to enter the lung than the dust particles in our previous trials whose mass median diameter was 4·5 μm.

**TIME OF MEASUREMENTS**

A bronchoconstrictor response at times other than one hour or four hours after the inhalation of endotoxin cannot be excluded but against this possibility is the lack of symptoms except in one subject, a smoker who showed constriction at both times of recording. Neal *et al* found responses about 45 minutes after inhalation. Cavagna *et al* found a maximal effect after endotoxin inhalation at 30 minutes to one hour, and lasting six hours. Pernis *et al* found that responses in animals appeared 30–50 minutes after
the end of 15 minutes of endotoxin inhalation. Buck and Bouhuys found a maximal bronchoconstriction at 90–120 minutes after a cotton extract aerosol was inhaled. Van der Zwan reported a bronchoconstrictor response maximal at half hour and lasting four hours after inhaling endotoxin. The time delays for these responses suggest that the measurements made at one hour and four hours in the present study should have detected any response to the endotoxin inhalation. Such delays in responses are also the most relevant to byssinosis.

Physiological responses
There was an 11% decrease in transfer factor after four hours. This measurement was made on six subjects, randomly selected, and the difference was statistically significant. It could not be explained by a decrease in alveolar volume, nor by bronchoconstriction. Inhalation of cotton dust has been reported to cause no change in transfer factor, although some suggestion of impaired ventilation/perfusion ratios has been made by the finding of a fall in the arterial partial pressure of oxygen after exposure of mill workers to hemp dust. Merchant et al also reported some fall in arterial oxygen pressure after cotton dust but the mean fall was small and not significant. Muitari et al reported a decrease in transfer factor at four hours after inhaling 0.8–4 µg endotoxin in a study of bath water fever. There is therefore some suggestion that endotoxins may cause impairment of gas transfer but this is not a typical acute reaction to textile dust.

A relation of endotoxin to mill fever is not necessarily excluded by the lack of appearance of symptoms suggestive of this condition in this study. This was also the finding of Cavagna et al, though pyrexia was found in the inhalation studies of Pernis et al. Tolerance to the pyrogenic effect of endotoxin develops with repeated exposure and this tolerance may last up to five weeks. The limited but definite exposure of our subjects to flax dust as managers in flax mills might account for their development of tolerance.

Airways
Only one of our subjects (No 5) showed any appreciable fall in ventilatory lung function after 12 µg of endotoxin and this fall was larger than he experienced with flax dust inhalation. Possibly the histamine releasing property of endotoxin accounted for this prolonged bronchoconstriction, particularly if this subject had bronchial hyperreactivity to histamine. His reaction, however, may have been due to some other factor as the amount of histamine released by endotoxin was found to be insignificant by Antweiler.

Whereas previous work has suggested a causative association between endotoxin and byssinosis, the results of inhalation tests have had less specific effects, not easily extrapolated to the disease. The lack of significant change in the mean ventilatory lung function tests of this group of subjects is not likely to be easily consistent with endotoxin being the causative agent of the bronchoconstriction which this group has previously shown to acute textile dust inhalation, although an interactive role with other agents has not been excluded.

We thank Professor R Rylander, University of Gothenberg, who supplied the endotoxin, assayed the levels in flax dust, and provided financial support. We also thank Mr B Buick for his technical help.

Requests for reprints to: Dr J P Jamison, Department of Physiology, Medical Biology Centre, 97 Lisburn Road, Belfast, Northern Ireland.

Appendix A
Mean individual changes after 5 µg endotoxin (decreases negative) in expiratory volumes (l) and mid-expiratory flow rate (l/s), three forced expiratory manoeuvres per subject

<table>
<thead>
<tr>
<th>Subject No</th>
<th>FEV₁</th>
<th>FVC</th>
<th>MEF₅₀</th>
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<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>4 h</td>
<td>1 h</td>
</tr>
<tr>
<td>1</td>
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<td>+0.154</td>
<td>−0.100</td>
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<td>−0.023</td>
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<td>+0.107</td>
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<td>−0.077</td>
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<tr>
<td>12</td>
<td>+0.077</td>
<td>−0.150</td>
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Bronchial challenge of normal subjects with the endotoxin of Enterobacter agglomerans isolated from cotton dust

Appendix B
Mean individual changes after 12 µg endotoxin (decreases negative) in expiratory volumes (1) and mid-expiratory flow rate (1/s), three forced expiratory manoeuvres per subject, and transfer factor (mmol/L/kPa/min)

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<tr>
<th>Subject No</th>
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<th>MEF₅₀</th>
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<td>+0.090</td>
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<tr>
<td>3</td>
<td>+0.030</td>
<td>+0.037</td>
<td>+0.070</td>
<td>+0.087</td>
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<td>-0.100</td>
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<td>-0.017</td>
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<td>+0.133</td>
<td>+0.067</td>
<td>+0.270</td>
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References
Branchial challenge of normal subjects with the endotoxin of Enterobacter agglomerans isolated from cotton dust.

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