Effects in man and rabbits of inhalation of cotton dust or extracts and purified endotoxins

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Cavagna, G., Foà, V., and Vigliani, E. C. (1969). Brit. J. Industr. Med., 26, 314-321. Effects in man and rabbits of inhalation of cotton dust or extracts and purified endotoxins. The incidence of byssinosis in workers in a cotton card-room, where the airborne concentration of bacterial endotoxins was 7.2 μg./m.³, was 32%; and 47% in a hemp card-room where the endotoxin concentration was 8.7 μg./m.³; no cases were observed among workers exposed to only traces of endotoxins.

The effect of the inhalation by aerosol of purified Escherichia coli endotoxin on F.E.V₁₀ and F.V.C. was studied in normal subjects and in patients with chronic bronchitis. A significant reduction in F.E.V₁₀ lasting more than 6 hours was observed in two out of eight normal subjects, in one out of three subjects with chronic bronchitis inhaling 80 μg. endotoxin, and in one out of four subjects with chronic bronchitis inhaling 40 μg. endotoxin. These results show that the inhalation of bacterial endotoxin can produce, in some individuals, changes in F.E.V₁₀ similar to those experienced on Mondays by some card-room workers.

A study of the mechanism of pathogenesis of inhaled bacterial endotoxins was carried out on rabbits subjected for 20 weeks to aerosols of purified E. coli endotoxin (20 μg./day) and cotton extract (2 mg./day). This treatment produced patterns of bronchitis: i.e., an increase in the respiratory tract fluid with increased protein content and characteristic histopathological changes. The bronchitis occurred after the appearance of cross-reacting circulating antibodies against E. coli endotoxin and cotton extract. These antibodies were detected with the haemagglutination tests after the first three weeks of treatment, and in subsequent weeks reached progressively higher titres, up to a maximum of 1:512.

A challenging aerosol of 0.1 mg. E. coli endotoxin in two rabbits and 10 mg. cotton extract in another two of the rabbits treated as above produced a marked increase in pulmonary resistance lasting more than two hours. In control rabbits a challenging aerosol of 1 mg. endotoxin or 100 mg. cotton extract caused only a moderate increase in pulmonary resistance, which returned to normal in less than one hour.

It may be concluded that the repeated inhalation of endotoxins induces in rabbits a state of hypersensitivity and at the same time the appearance of inflammatory reactions in the bronchi and alterations in the mechanical properties of the lung. These changes may be significant in the pathogenesis of byssinosis.

Evidence has been accumulated that the acute diseases of the 'mill-fever' group are due to the inhalation of endotoxins derived from bacteria which contaminate vegetable dusts (Neal, Schneiter, and Caminita, 1942; Vigliani, Parmeggiani, and Sassi, 1954; Pernis, Vigliani, Cavagna, and Finulli, 1961). Further evidence has recently been provided by Mentesana, Deodato, Balsamelli, Curatolo, and Vaccaro (1968), who described an outbreak of 'mill-
fever' in workers of a cotton mill processing a Peruvian cotton heavily contaminated with bacteria of the Brevibacteriaceae group. However, it is still debatable whether endotoxins can play a part in the pathogenesis of a chronic disease such as byssinosis. Indeed, only the 'Monday feeling', which characterizes the early stages of byssinosis, may be explained by comparing it to the well-known phenomenon of lost endotoxin tolerance. Repeated daily exposures to endotoxins induce in the host a state of refractoriness which is lost if the exposure is interrupted: therefore the 'Monday feeling' may correspond to the loss of tolerance following discontinuation of exposure to endotoxins. On the other hand, it has been objected that the 'endotoxin hypothesis' cannot explain why there is no fever in byssinosis, why years of exposure to cotton or hemp dust are necessary to induce the onset of the disease, and why the early stages of the disease progress to a chronic bronchitis with emphysema.

We first approached the problem by trying to see (1) whether a relationship exists between the airborne concentration of endotoxins in textile mills and the prevalence of byssinosis; and (2) whether it is possible to reproduce a phenomenon like the 'Monday feeling' in man by the administration of endotoxins by inhalation.

Prevalence of byssinosis and airborne concentration of endotoxins

A study of the prevalence of byssinosis was carried out in different rooms of a cotton mill and of a hemp mill. Workers were medically examined and interviewed with the aid of the questionnaire for chronic bronchitis of the British Medical Research Council (1960), with additional questions to find out whether they suffered from chest tightness on Mondays. The one-second forced expiratory volume (F.E.V.₁₀) and the forced vital capacity (F.V.C.) were measured with a direct reading spirometer before the beginning and at the end of a Monday shift. The airborne dust concentration was measured repeatedly at different points using a M.S.A. electrostatic sampler.

Endotoxin-like substances were extracted in trichloracetic acid (Boivin, Mesrobeanu, and Mesrobeanu, 1933) from dust collected by means of a Soxhlet apparatus. The amount of endotoxins was measured in the extracts by determining, in rabbits, the minimum intravenous dose which induced a necrosis in epinephrine-injected skin sites, in comparison with the minimum dose of purified E. coli endotoxin (Thomas, 1956; Falk and Spink, 1967). Byssinosis was diagnosed in those subjects who complained of chest tightness on Mondays and who showed a significant reduction in F.E.V.₁₀ (more than 10%) after the Monday shift.

As shown in Table 1, the prevalence of byssinosis may correlate better with the airborne concentration of endotoxins than with the total amount of dust; byssinosis was present among workers exposed to 8-9 μg./m³, while no cases were observed among those exposed to only traces of endotoxins.

**TABLE 1**

<table>
<thead>
<tr>
<th>Type of mill</th>
<th>Rooms</th>
<th>No. of workers</th>
<th>Mean years of dust exposure (ranges in parentheses)</th>
<th>No. with byssinosis</th>
<th>Total dust conc. (μg./m³)</th>
<th>Endotoxin-like substances in dust (μg./g.)</th>
<th>Airborne conc. of endotoxins (μg./m³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotton</td>
<td>Card</td>
<td>71</td>
<td>13 (1-30)</td>
<td>23 (32%)</td>
<td>5-20</td>
<td>1-40</td>
<td>7-20</td>
</tr>
<tr>
<td></td>
<td>Spinning</td>
<td>24</td>
<td>20 (1-36)</td>
<td>—</td>
<td>0-46</td>
<td>0-11</td>
<td>0-08</td>
</tr>
<tr>
<td>Hemp</td>
<td>Card</td>
<td>21</td>
<td>21 (4-39)</td>
<td>10 (47%)</td>
<td>5-25</td>
<td>1-66</td>
<td>8-71</td>
</tr>
<tr>
<td></td>
<td>Roving</td>
<td>20</td>
<td>19 (7-33)</td>
<td>—</td>
<td>2-82</td>
<td>0-13</td>
<td>0-36</td>
</tr>
</tbody>
</table>

Effect of inhaled endotoxin on F.E.V.₁₀

Assuming that a textile worker at work has a pulmonary ventilation of 10-15 l/min., an endotoxin concentration of 8-9 μg./m³ means that 40-50 μg. of endotoxins are inhaled in the course of an eight-hour shift. Bearing this assumption in mind, the effect of a purified endotoxin on the respiratory function was studied by letting normal subjects...
inhale an aerosol containing 40-80 μg. of purified E. coli endotoxins in 2 ml. saline. F.E.V.1.0 and F.V.C. were determined with a direct reading spirometer before and 5 minutes, 30 minutes, 1, 3, 6, and 24 hours after the aerosol. White blood cell counts and body temperature measurements were also made every 2 hours until 8 hours after the aerosol.

As shown in Table 2, a significant reduction in F.E.V.1.0 (more than 10%) was observed in two out of eight normal subjects inhaling 80 μg. endotoxin. This reduction was substantial 5 minutes after the aerosol, reaching a maximum after 30 minutes to 1 hour, and could still be observed after 6 hours; after 24 hours the F.E.V.1.0 values returned to normal. From this experiment it appears that in normal subjects a first inhalation of endotoxins, in a range similar to that inhaled during a shift in a card-room, affects the respiratory function in only a limited number of cases.

The experiment was then repeated in subjects with chronic bronchitis, classified as grade 2 in accordance with the suggestions of the British Medical Research Council (1960), and a moderate impairment of the ventilatory function. As shown in Table 2, a reduction in F.E.V.1.0 after the inhalation of endotoxin occurred in a greater proportion of bronchitic patients than of normal subjects, was more pronounced, and occurred also at lower doses. The patterns of F.E.V.1.0 changes observed in the bronchitic patients are shown in Figure 1.

In no case, not even where the F.E.V.1.0 was reduced, were significant changes in white blood cell counts or any increase in body temperature observed.

The above investigations induced us to assume that the inhalation of bacterial endotoxins can produce changes in the F.E.V.1.0 in some individuals similar to those experienced on Mondays by some card-room workers. This finding supports our hypothesis that endotoxins are a possible causative agent of mill-fever and the so-called 'Monday feeling'.

If endotoxins play a part in the pathogenesis of first-stage byssinosis, the question arises how they can be implicated in the pathogenesis of the subsequent stages of the disease.

Some experimental results have provided evidence of the presence of two interdependent activities in the endotoxin molecule: (1) an intrinsic or primary toxicity which is responsible for phenomena such as pyrogenicity, tissue damage, etc., bound to the molecular fraction defined as 'Lipid A'; (2) a secondary toxicity responsible for phenomena such as skin hyperreactivity, Schwartzmann reaction, enhanced lethality, etc., and which is believed to be dependent on the acquisition of a hypersensitivity to a complex configuration of the endotoxin molecule which acts at an antigenic site different from the polysaccharide with somatic 'O' antigen specificity (Lee and Stetson, 1960; Schaedler and Dubos, 1961; Watson and Kim, 1963, 1964; Kim and Watson, 1966; Cavagna, Foà, Nichelatti, Calgaro, and Locati, 1966; Kováts and Végh, 1967). The endotoxins could therefore contribute to the pathogenesis of byssinosis through a mechanism of immunological sensitization. For a better understanding of this mechanism we carried out the following experiments, aimed at finding out

<table>
<thead>
<tr>
<th>TABLE 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Changes in F.E.V.1.0 observed in normal subjects and in patients with mild chronic bronchitis after an aerosol of purified E. coli endotoxin</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of subjects examined</th>
<th>Diagnosis</th>
<th>Endotoxin dose (mg.)</th>
<th>Subjects with F.E.V.1.0 reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>No. out of total</strong></td>
</tr>
<tr>
<td>2</td>
<td>Normal</td>
<td>0.04</td>
<td>0/2</td>
</tr>
<tr>
<td>4</td>
<td>Chronic bronchitis</td>
<td>0.04</td>
<td>1/4</td>
</tr>
<tr>
<td>8</td>
<td>Normal</td>
<td>0.08</td>
<td>2/8</td>
</tr>
<tr>
<td>3</td>
<td>Chronic bronchitis</td>
<td>0.08</td>
<td>1/3</td>
</tr>
</tbody>
</table>

FIG. 1. Patterns of F.E.V.1.0 in two patients with mild chronic bronchitis after inhalation of aerosols of 0.04 and 0.08 mg. E. coli endotoxin respectively.
whether repeated inhalations of purified endotoxin can produce pathological changes similar to the pattern of byssinosis.

Long-term inhalation of endotoxin and cotton extract in rabbits
Three groups of 10 rabbits each inhaled daily, from Monday to Friday, for 20 weeks from March to August, aerosols of (1) E. coli endotoxin (0111:B4 purified lipopolysaccharide Difco), 20 μg. in 2 ml. isotonic saline; (2) cotton extract, 2 mg. in 2 ml. isotonic saline; (3) isotonic saline only. The cotton extract was prepared from pooled raw cotton, collected in different cotton mills, by grinding 1 g. of finely cut material with 6 ml. distilled water and then freeze-drying it. In this way, from each gram of raw cotton we obtained 60-80 mg. of raw extract, which contained an average of 6 mg. proteins and 1-6 mg. hexoses; its endotoxin activity, assayed in rabbits by Thomas's method (1956), was about 1/100 of the purified E. coli endotoxin. Therefore 2 mg. of cotton extract are equivalent to 20 μg. of purified E. coli endotoxin.

Before the beginning of the experiment, rabbit sera were examined for antibody activity against E. coli endotoxin and cotton extract with the haemagglutination method (Neter, Bertram, Zak, Murdock, and Arbesman, 1952; Davies, Crumpton, Macpherson, and Hutchison, 1958) and the agar-gel double diffusion test. A few animals, in which the titre of haemagglutinating antibodies were more than 1:4, were discarded. Rabbit sera were then examined for antibodies each Monday in the first month and once a month subsequently. White blood cell counts and body temperature measurements were carried out in five animals per group each Monday, before the aerosol and every 2 hours afterwards for 8 hours.

At the end of the 20 weeks, respiratory tract fluid was collected by the method of Boyd and Boyd (1967) from three rabbits of each group, and the mechanical properties of the lung were studied in four animals which had inhaled E. coli endotoxin and in four controls, by simultaneous recording of air-flow rate and transpleural pressure. Lung compliance was calculated from the pleural pressure difference and the lung volume changes between two points at zero air flow. Pulmonary resistance was calculated from the difference between pleural pressure and flow rate at the same lung volume (Davidson, Wasserman, Lillington, and Schmidt, 1966). All animals were then killed and the lungs were examined histologically.

White blood cells and body temperature No significant changes in white blood cell counts or in body temperature were observed after the aerosols of endotoxin and cotton extract, on Mondays.

Respiratory tract fluids Rabbits treated with endotoxin or cotton extract had a fluid output averaging 0.39 ml./hour with a protein content of 6 mg./ml., while the controls had a mean fluid output of 0.2 ml./hour with a protein content of 3.5 mg./ml. A search for variations in the different classes of immunoglobulins (IgA, IgG, and IgM) in the fluid was made. The pattern of the three immunoglobulin classes in the treated rabbits showed no difference compared with the controls.

Antibody activities During the first three weeks of the experiment, sera of rabbits inhaling endotoxins or cotton extract showed no antibody activities against the inhaled antigens with either the haemagglutination test or the agar-gel double diffusion test. After four weeks, antibodies were detected with the haemagglutination test and, in subsequent months, reached progressively higher titres up to a maximum of 1:512 (Fig. 2). Sera from rabbits inhaling E. coli endotoxin showed antibody activity not only against the purified antigen, but also against the whole cotton extract; similarly, sera from rabbits inhaling cotton extract reacted with both cotton extract and purified endotoxin. This may be due to the presence of E. coli endotoxin in the cotton extract, but it could also be due to the cross reactivity which exists between different endotoxins (Lee and Stetson, 1960; Watson and Kim, 1963). Precipitating antibodies were never detected with the agar-gel double diffusion test.

Pulmonary function tests The rabbits which had inhaled endotoxins over a period of five months were examined on the Monday before the last aerosol was given: no significant changes were seen in the mechanical properties of the lung compared with the controls. When challenged with 0.1 mg. E. coli endotoxin or 10 mg. cotton extract per aerosol, however, the same animals showed a significant increase in pulmonary resistance and a decrease in lung com-

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**FIG. 2.** Patterns of antibody activity (haemagglutination test) of sera from rabbits inhaling purified endotoxin and cotton extract over a five-month period.
TABLE 3
CHANGES IN LUNG MECHANICS AFTER AN AEROSOL OF PURIFIED ENDOTOXIN IN A RABBIT PREVIOUSLY SUBJECTED TO DAILY INHALATION OF ENDOTOXIN OVER A FIVE-MONTH PERIOD

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Breathing rate (breaths/min.)</th>
<th>Tidal volume (ml.)</th>
<th>Lung compliance (cm. of H2O/cm. of water column)</th>
<th>Pulmonary resistance (cm. of H2O/cm. of water column/1/sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>42 (37-54)</td>
<td>23-2 (20-25-6)</td>
<td>6-3 (3-6-7-8)</td>
<td>24-7 (18-6-27-5)</td>
</tr>
<tr>
<td>t = 0</td>
<td>40 (31-8)</td>
<td>24-6</td>
<td>7-7</td>
<td>24-0</td>
</tr>
<tr>
<td>t = 5 min.</td>
<td>46 (32-6)</td>
<td>20</td>
<td>7</td>
<td>31-8</td>
</tr>
<tr>
<td>15 min.</td>
<td>54 (41-7)</td>
<td>22-6</td>
<td>6-9</td>
<td>34-5</td>
</tr>
<tr>
<td>30 min.</td>
<td>60 (47-8)</td>
<td>20-8</td>
<td>6-8</td>
<td>35-3</td>
</tr>
<tr>
<td>60 min.</td>
<td>65 (52-7)</td>
<td>19-3</td>
<td>6-5</td>
<td>36-3</td>
</tr>
<tr>
<td>90 min.</td>
<td>72 (59-8)</td>
<td>18-3</td>
<td>5-8</td>
<td>38-4</td>
</tr>
<tr>
<td>120 min.</td>
<td>70 (67-8)</td>
<td>17-3</td>
<td>6-4</td>
<td>32-7</td>
</tr>
</tbody>
</table>

Changes in respiratory function followed the inhalation of an aerosol of isotonic saline.

Histopathology A synopsis of the histological findings is reported in Table 4. The inhalation of purified E. coli endotoxin and cotton extract over a

TABLE 4
SYNOPSIS OF HISTOLOGICAL LESIONS OBSERVED IN LUNGS OF RABBITS INHALING AN AEROSOL OF PURIFIED E. coli ENDOTOXIN (0-02 mg.) AND COTTON EXTRACT (2 mg.) DAILY OVER A FIVE-MONTH PERIOD

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bronchial cells exfoliation</th>
<th>Endobronchial secretion</th>
<th>Peribronchial lymphocyte infiltrate</th>
<th>Interalveolar septal thickening</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. out of total A</td>
<td>No. out of total B</td>
<td>No. out of total A</td>
<td>No. out of total B</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>4/10</td>
<td>6/10</td>
<td>4/10</td>
<td>3/10</td>
</tr>
<tr>
<td>(10 rabbits)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cotton extract</td>
<td>4/10</td>
<td>6/10</td>
<td>4/10</td>
<td>3/10</td>
</tr>
<tr>
<td>(10 rabbits)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isotonic saline</td>
<td>3/10</td>
<td>—</td>
<td>2/10</td>
<td>3/10</td>
</tr>
<tr>
<td>(10 rabbits)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A = slight and scattered; B = marked and diffuse.
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FIG. 4. Rabbit subjected to aerosol of *E. coli* endotoxin (20 µg./day for 20 weeks). Marked endobronchial secretion and exfoliation of bronchial cells. H. and E. × 150.

FIG. 5. Rabbit subjected to aerosol of cotton extract (2 mg./day for 20 weeks). Peribronchial lymphocyte infiltrate and exfoliation of bronchial cells. H. and E. × 150.
five-month period induced in all rabbits the histological patterns of bronchitis and bronchiolitis, i.e., bronchial cell exfoliation, endobronchial secretion, and peribronchial lymphocyte infiltrates (see Figs. 4 and 5). In nearly all the animals, a thickening of the alveolar septa was also present. These histological patterns were clearly different from those observed in the controls, in which bronchial alterations were found only occasionally and were of a minimal degree.

Search for signs of bronchitis before the appearance of antibodies
Ten rabbits inhaled E. coli endotoxin and 10 cotton extract for three weeks, as previously described. After such a period, when no antibodies against E. coli endotoxin and cotton extract were found in the rabbit sera, the animals were killed and the lungs were examined histologically. The histological patterns were not different from those observed in the controls which had inhaled only isotonic saline.

Discussion
Our experiments show that the repeated and prolonged inhalation of E. coli endotoxin or cotton extract in rabbits produced bronchitis, as demonstrated by an increased protein-rich fluid in the respiratory tract and by the histopathological findings. Furthermore, these rabbits appear to have acquired a state of hypersensitivity to endotoxins: in fact they show a marked increase in pulmonary resistance after a challenging aerosol of 0.1 mg. E. coli endotoxin or 10 mg. cotton extract, while in control rabbits even a 10-fold dose of endotoxin or cotton extract had only a slight and transient effect on the pulmonary mechanics.

It may be asked what relationship exists between the above phenomena and the appearance of endotoxin-reacting antibodies. In our experiments, no histological signs of bronchitis were evident before the appearance of antibodies: it seems, therefore, that the bronchitis may be related more to a hypersensitivity-linked mechanism of the endotoxin than to their primary toxicity. In fact, it has been demonstrated that a state of hypersensitivity to the endotoxins may be induced in the host through the production of circulating antibodies (Spink and Vick, 1961; Spink, 1962; Stetson, 1964; Kim and Watson, 1966; Kováts and Végh, 1967).

The changes in mechanical properties of the lung induced by the endotoxin could be explained by a release of biogenic amines such as histamine and serotonin; in fact, the endotoxins are well-known histamine and serotonin releasers (Hinshaw, Jordon, and Vick, 1961; Davis, Bailey, and Hanson, 1963; Stein and Thomas, 1967). Rabbits, however, lack a histamine-sensitive system in their airways (Palecek, Oskou, and Aviado, 1967); in our experiments, therefore, we can consider only serotonin, which in rabbits produces marked constriction of airways and pulmonary vessels. The increase in pulmonary airway resistance we observed lasted too long to be attributed only to the effect of serotonin; thus it seems possible that an inflammatory response with more prolonged evolution is also involved.

The patterns of our experiments suggest that a state of hypersensitivity is necessary for endotoxins to produce changes in mechanical properties of the lung. In fact, there is evidence that an antigen-antibody reaction is involved in the production of serotonin release by endotoxins (Ream, Deykin, Greweich, and Wessler, 1965; Weiner and Zweifach, 1966; Spielvogel, 1967), just as an immune mechanism is recognized in the inflammatory tissue reactions to endotoxins (Stetson, 1964; Kováts and Végh, 1967).

Even though we cannot say that we obtained an experimental model of byssinosis, we can, however, conclude that repeated inhalations of endotoxins do induce in rabbits a state of hypersensitivity and at the same time the appearance of inflammatory reactions in the bronchial tree and changes in the mechanical properties of the lung. This may be significant in the pathogenesis of byssinosis; however, we are not unaware of the fact that cotton extracts contain other pharmacologically active substances. Therefore, we are far from saying that the endotoxins are the sole cause of byssinosis, but they may well be one of the causes. It is important to observe, in this connexion, that the endotoxins potentiate the effects of histamine (Weiner and Zweifach, 1966; Urbaschek, 1966) and that they produce a marked increase in the activity of the histidine-decarboxylase (Schayer, 1960), i.e., an increase in the production of histamine. It is therefore not unlikely that the endotoxins interfere with the effects of other histamine releasers possibly contained in the cotton dust.

References
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