Long-term occupational outcomes of endotoxin exposure and the effect of exposure cessation

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ABSTRACT

Objective To study the possible respiratory and haematological effects of endotoxin exposure to bacterial single-cell protein (BSCP) in workers during a follow-up period of 5 years including 4 years of exposure and 1 year without exposure.

Methods The study included 28 workers examined in 2002–2005 and 1 year after exposure termination in 2007. The arithmetic mean endotoxin exposure was 5800–11 000 EU/m³ among the high exposure group and 390 EU/m³ in the low exposure group. Assessment of lung function included spirometry and gas diffusion in 2003, 2004 and 2007. Rhinometry was performed in 2004 and 2007. Blood analysis included leukocyte cell count and measurement of the acute phase proteins: C-reactive protein, interleukin-6, eosi

INTRODUCTION

Among workers producing bacterial single-cell protein (BSCP), a temporary endotoxin-associated reduction in forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV1) (210 ml) and forced expiratory volume in 1 s (FEV1) (180–210 ml) (p=0.004–0.03) 1 year after the end of exposure. The number of leukocytes and eosinophilic cationic protein and D-dimer levels increased significantly with increasing endotoxin exposures and decreased significantly 1 year after exposure termination. Changes in acute phase proteins suggested exposure-related tolerance.

Results

What this paper adds

- The long-term effects of occupational exposure to bacterial single-cell protein (BSCP) endotoxin are mostly unknown.
- Occupational endotoxin exposure may have a lasting impact on pulmonary function and blood inflammatory parameters.
- This study shows what happens when such exposure ceases.
- Endotoxin exposure causes respiratory and inflammatory effects in BSCP workers.

CONCLUSIONS

An inflammatory tendency during an exposure period of 4 years seems to reverse 1 year after cessation of exposure to endotoxins from a single species. Lung function improved significantly among workers exposed to low levels of endotoxin but not among the highly exposed workers.
several days/weeks. The present follow-up by the National Institute of Occupational Health (NIOH) started in 2003. The characteristics of the workers are shown in Table 1. Those who reported being smokers in 2003 were considered to be smokers throughout the study period. None started smoking during follow-up. Three workers, all men, reported having physician diagnosed asthma. Nasal cavity dimensions were measured in 2004 and 2007.

Blood samples were collected by our group in 2003, 2004, 2005 and 2007. In 2003 and 2004, blood sampling was carried out before a work shift. The sampling in 2005 was done during work in a period when production was stable. Some workers were not available for blood collection at each occasion.

Exposure measurements were conducted over 1 week in July 2003 and March–April 2004 and 2005.

The study was approved by the Regional Ethics Committees for Medical Research in Trondheim and Oslo, Norway. All participants gave their written informed consent at the start and end of the study. The anonymity of the subjects was secured.

### Job groups
Downstream operators were process workers handling dry bioprotein during bag filling and spray drier surveillance. Other operators included upstream process workers working with culture fluid, maintenance workers, and development and laboratory engineers. The office workers were located in an office building close to the production plant. The production workers changed their clothes in the office building, and the manager regularly visited the production plant.

Information on jobs performed by the workers during the study period was collected by self-administered questionnaires completed in 2005 and 2007.

### Exposure
The exposure of each worker not on leave was measured once during each examination from 2003 to 2005. In 2001, a selection of the workers had been monitored by company staff. Personal samples of inhalable particles were collected for all workers as described previously.

Endotoxins were measured by the kinetic LAL method at the company laboratory as described previously, except for four samples collected in 2001 that were analysed at a commercial laboratory. Use of respiratory protection during sampling was recorded by questionnaire.

### Pulmonary function tests
Pulmonary function testing was performed using the Spirotrac 2160 spirometer (Vitalograph, Maids Moreton, UK). The same technician (MS) performed all tests according to American Thoracic Society (ATS) criteria. FVC, FEV1 and forced mid-expiratory flow rate (FEF25–75%) were measured.

The transfer factor for carbon monoxide (DLco) and the transfer per unit effective alveolar volume (Kco) were measured by the single breath holding method according to the ATS criteria.

### Acoustic rhinometry
Nasal cavity dimensions were measured by acoustic rhinometry (Rhin2100; Rhino Metrics, Lyng, Denmark) according to current standards. The repeatability of the measurements has been reported to be less than 5%.

In 2004, measurements were performed before and after a work shift. A final test was performed 15 min after decongestion with a spray of xylometazoline. In 2007, the workers were not exposed and measurements were performed before and after decongestion.

### Assessment of blood parameters
Leukocytes were analysed in 2003, 2004 and 2005 using Sysmex XE-2100® (Fürst Medical Laboratory, Oslo, Norway) within 48 h of blood collection in EDTA vacutainers. In 2007, the cells were analysed using the Sysmex XE-2100 haematology system (Sysmex Europe, Hamburg, Germany) at Oslo University Hospital, Ulleval, Norway. Serum and plasma were treated and stored according to the criteria of the laboratory.

For the quantitative determination of C-reactive protein (CRP) in human serum, an immunoturbidimetric assay (Tinaquant C-reactive protein (latex) high sensitive assay) on a Hitachi 917 (Roche Diagnostics, Mannheim, Germany) automated clinical chemistry analyser was used. The inter-assay variation (coefficient of variation, CV) was 5%.

The fibrinogen concentrations in citrate plasma samples collected in 2004 were measured at the University Hospital of Trondheim, Norway with a Fibrif-Prest Automate using the Clauss clotting method (Medinor ASA, Oslo, Norway). The inter-assay variation (CV) was 4%. In 2007, fibrinogen was analysed in citrate plasma samples using a clotting test on a STA-R Evolution workstation (Diagnostica Stago, Asnières sur Seine, France). The inter-assay variation (CV) was 4%. These blood analyses were performed at Oslo University Hospital, Ulleval.

As two different methods had been used, we calculated the per cent of predicted fibrinogen on the two occasions by taking the highest value of the reported reference range according to the laboratories as 100%.

D-dimer was analysed from plasma samples at the University Hospital of Trondheim, Norway in 2004 using the Tina-quant immunoturbidimetric method on a Hitachi 917. Reported inter-assay variation for the selected analysis were in the range 6–8% (CV). In 2007, the samples were analysed using an immunoturbidimetric method on the STA-R Evolution workstation. This analysis was performed at Oslo University Hospital, Ulleval.

The inter-assay variation (CV) was 5%. Since two different methods were used, we calculated the per cent of predicted D-dimer on the two occasions by taking the highest value of the reported reference range according to the laboratories as 100%.

The serum samples were analysed for acute phase proteins using a microsphere based multiplexing bioassay system with Xmap technology (Luminex, Austin, Texas, USA). The analyses of interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 α (MIP-1 α), chemotacttractant protein RANTES or CCL5 and platelet-derived growth factor BB (PDGF-BB) were performed according to the instructions of the manufacturer (Bio-Rad, Hercules, California, USA).

### Table 1 Characteristics of the study population in 2003 (n=28)

<table>
<thead>
<tr>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>34.2</td>
<td>6.5</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>176.9</td>
<td>11.6</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>84.2</td>
<td>27.5</td>
</tr>
</tbody>
</table>

*Four women. †One woman.
Eosinophilic cationic protein (ECP), collected both in 2004 and 2007, was analysed in serum samples at Fürst Medical Laboratory, using a fluoroenzyme immunoassay method (UniCAP 1000; Pharmacia, Uppsala, Sweden) with an inter-assay variation (CV) of 4%.

Statistical methods

Standard measures of central tendency and distributions (arithmetic means and SDs) were computed. Exposure data and the outcome variables PDGF-BB, RANTES, ECP, D-dimer and CRP were highly skewed and described by arithmetic means, geometric means (GM), geometric standard deviations (GSD) and range, and were log-transformed before statistical analysis with parametric methods. All tests were two-sided and a significance level of 5% was chosen.

Exposures of job groups were analysed by linear mixed effect models using different groupings and a dichotomous respirator use variable. Models were compared using ML estimation, while the final model was estimated using REML.

Lung function and inflammatory markers in blood were analysed with linear mixed models by a statistician (ØS) as repeated observations were available for each worker. The models take into account the dependency of the repeated observations by adding random effects for workers. In a first analysis, models were constructed using calendar year as predictor (fixed effect). The effect of year is expected to be mainly due to the endotoxin exposure that varied between years when workers changed job types and after closure of the plant. In a second analysis, we divided the workers into categories with low versus high endotoxin exposure, and then analysed the effect of calendar year for these two categories. As only a few endotoxin measurements had been performed before 2003, we excluded CRP, RANTES, IL-6, MIP-1α, PDGF-BB and MCP-1 from this second analysis. In both analyses, we chose 2007 (1 year after exposure ceased) as the baseline because endotoxin exposure then was assumed to be low. The associations were adjusted for gender, age, height, weight, smoking status and asthma. The two models can be represented formally by $y_{ij} = x_{ij}^T \beta + u_i + \epsilon_{ij}$.

Here, $y_{ij}$ is the observed lung function, or inflammatory parameter, of worker $i$ and replication $j$, $x_{ij}^T$ is a vector of regressors linking the observations to the fixed effects $\beta$. Furthermore, $u_i$ are independent and identically normal distributed random effects with mean 0 and variance $\sigma^2_u$, while $\epsilon_{ij}$ are

Table 2 Exposure to endotoxin (in 1000 EU/m$^3$) in a bacterial single-cell protein factory in 2001–2005

<table>
<thead>
<tr>
<th>Job type</th>
<th>No. of workers</th>
<th>Without respirator</th>
<th>With respirator</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>AM</td>
<td>Range</td>
</tr>
<tr>
<td>Office</td>
<td>2</td>
<td>5</td>
<td>0.14</td>
</tr>
<tr>
<td>Engineer/lab/maintenance</td>
<td>7</td>
<td>9</td>
<td>0.47</td>
</tr>
<tr>
<td>Upstream operator</td>
<td>10</td>
<td>12</td>
<td>0.33</td>
</tr>
<tr>
<td>Downstream operator</td>
<td>11</td>
<td>8</td>
<td>7.4</td>
</tr>
</tbody>
</table>

AM, arithmetic mean; GM, geometric mean; GSD, geometric SD; n, number of measurements; —, no data.

Eosinophilic cationic protein (ECP), collected both in 2004 and 2007, was analysed in serum samples at Fürst Medical Laboratory, using a fluoroenzyme immunoassay method (UniCAP 1000; Pharmacia, Uppsala, Sweden) with an inter-assay variation (CV) of 4%.

Figure 1 Total leukocyte cell counts, and levels of C reactive protein (CRP), eosinophilic cationic protein (ECP), interleukin-6 (IL-6), platelet-derived growth factor BB (PDGF-BB), chemotactarctant protein RANTES, monocyte chemotactarctant protein-1(MCP-1) and macrophage inflammatory protein-1 α (MIP-1α) in blood from workers in a bacterial single-cell protein (BSCP) factory (n=28). The horizontal line represents the mean. *p<0.05, **p<0.01, ***p<0.001.

identically independent distributed normal random variables with mean 0 and variance $\sigma^2$.

Nasal dimensions were compared by paired t tests and associations between changes in dimensions and exposure were adjusted for gender and smoking by multiple regression.

Analysis by linear mixed models was performed with the nlme package in R (http://www.r-project.org) and all other tests were performed with SPSS v 16.0 for Windows (SPSS, Chicago, Illinois, USA).

**RESULTS**

**Exposure**

The endotoxin levels during regular work from 2001 to 2005 are shown in table 2. Measurements with and without the use of respiratory protection are shown separately (table 2). Different groupings were attempted but due to Hessian matrices not being positive definite, only models with downstream operators and a group including all other job types could be modelled. These models showed that neither year nor respirator use were significantly associated with endotoxin exposure, and the model with only two job groups had the lowest Akaike's information criterion. The final model estimated the GM endotoxin exposure level of the downstream workers at 6700 EU/m$^3$ and of the other workers at 230 EU/m$^3$. Using the residual variance from the model, the arithmetic mean exposure levels were estimated to be 11 000 and 390 EU/m$^3$, respectively. Inclusion of the dichotomous job type variable in the model reduced the between-worker variance from 3.32 to 0.40 ($\pi^2/8$) and the within-worker variance from 1.11 to 1.03 ($\pi^2/10$).

**Health effects**

Lung function and blood parameters (including the pre-shift values of 2003 and 2004) are shown in figure 1 and tables 3 and 4.

**Airways**

Compared to 2007, FVC was significantly lower in 2003 and 2004 in the low exposed group by $\approx 30$ and $\approx 290$ ml, respectively (p=0.004–0.006; table 4). In the high exposed group, FVC in 2003 and 2004 was not significantly different from the 2007 level, and differences were smaller. Similar associations were found for FEV$_1$, which showing significant improvements in 2007 compared to 2003 and 2004 for the low exposure group of 210 and 180 ml, respectively (p=0.02 and p=0.03; table 4).

As for the annual loss in millilitres, using the equation $(\text{FEV}_1 - \text{FEV}_2) \times 12/48$ months where $\text{FEV}_2$ is $\text{FEV}_1$ in 2003 and $\text{FEV}_2$ is $\text{FEV}_1$ in 2007, we found that the total group had a mean annual increase in $\text{FEV}_1$ of 8 ml/year (SD 69). Accordingly, the low exposure group had a mean increase of 37 ml/year (SD 76), whereas the high exposure group had a mean loss of 14 ml/year (SD 56) with no significant difference between the groups (p=0.07).

No significant differences for the two exposure groups were found for $D_{15}$ or Kco during the follow-up period.

**Blood parameters**

The observed blood parameters of all workers are shown in figure 1 where significant calendar year differences are indicated.

Compared to the baseline year 2007 (1 year after exposure ceased), the leukocyte counts and ECP and D-dimer levels (results not shown) were significantly increased during the exposure period as compared to 1 year after exposure cessation. IL-6, PDGF-BB, RANTES, MCP-1 and MIP-1α were decreased in 2005 compared to 2007. However, these parameters were increased in 2002 compared to 2007. Furthermore, when comparing calendar year differences for the two exposure groups in 2007, leukocytes were significantly lower in the high exposure group (p = 0.02) as compared to all previous years of exposure, and ECP and D-dimer levels were decreased in both the high and low exposure groups in 2007 when exposure had ceased (p = 0.002; table 4).

**DISCUSSION**

We have found endotoxin exposure levels often exceeding 10 000 EU/m³ among former workers at a BSCP plant where co-exposure to other components that correlate with endotoxin exposure is lacking. The changes in FVC and FEV₁ were significantly associated with previous exposure to endotoxin but only among the lower exposed group, with an arithmetic mean exposure of 5800 EU/m³ and high (8800–17 000 EU/m³) levels of endotoxin exposure. Termination of exposure to endotoxins was associated with significant increases in FVC and FEV₁, but only in the lower exposure group, possibly reflecting sustained lung inflammation among workers with the highest exposure levels. An accelerated loss in lung function with an excess decline of 9 ml/year was also found among grain farmers, which is in the same magnitude as the total loss of 14 ml/year among the high exposure group in this study.\(^{10}\) However, the decline in lung function among endotoxin exposed workers has been shown to normalise once exposure ceases.\(^{11,12}\) Among cotton workers, cessation resulted in a 7% increase in mean per cent of predicted FEV₁, which is in agreement with the results in the present low exposure group.\(^{11}\)

We did not observe changes in diffusing capacity, which may be because the BSCP consisted of large aggregates affecting the eyes and not reaching the alveoli (Wijnand Eduard, unpublished results).\(^{1}\)

In 2007, the dimensions of the anterior part of the nose were increased compared to 2004. However, this may not indicate the reversal of mucosal swelling as no changes were observed in the interior part of the nose where the mucosal area is substantially larger.

Inhalation of endotoxins may induce a systemic inflammatory response by activation of macrophages and influx of neutrophils into the lung.\(^{5}\) In the present study, we found an increase of IL-6 in 2002, and the endotoxin-associated increase in blood leukocytes, ECP and fibrin D-dimer was reversed 1 year after the plant closed. Endotoxin was shown to be present in the blood of the workers, and thus direct stimulation of circulatory immune cells seems possible.\(^{2}\) Consequently, both mechanisms can result in an increase in acute phase proteins.

Tolerance, known to result in both impaired and augmented activities when cells are restimulated with lipopolysaccharide,\(^{13}\) might explain our results showing a reduction in the levels of IL-6, MIP-1α, MCP-1, PDGF-BB and RANTES in 2005 compared to both 2002 and 2007. In vitro studies, and in an occupational setting, restimulation with organic dust results in impaired

### Table 4: Lung function and inflammatory parameters in blood in 2003, 2004 and 2005 predicted by linear mixed models among workers exposed to low (130–540 EU/m³) and high (8800–17 000 EU/m³) levels of endotoxin (n=28)

<table>
<thead>
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</thead>
<tbody>
<tr>
<td>FEV₁</td>
<td>Low</td>
<td>−0.21*</td>
<td>0.02</td>
<td>−0.18*</td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>−0.01</td>
<td>0.9</td>
<td>0.05</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FVC</td>
<td>Low</td>
<td>−0.30*</td>
<td>0.006</td>
<td>−0.29*</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>−0.06</td>
<td>0.8</td>
<td>−0.03</td>
<td>0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEF₂₅−₇₅%</td>
<td>Low</td>
<td>−0.10</td>
<td>0.6</td>
<td>−0.37</td>
<td>0.06</td>
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<tr>
<td></td>
<td>High</td>
<td>−0.04</td>
<td>0.8</td>
<td>0.25</td>
<td>0.2</td>
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<tr>
<td>Kco</td>
<td>Low</td>
<td>−0.04</td>
<td>0.5</td>
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<tr>
<td></td>
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<td>−0.10</td>
<td>0.10</td>
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<tr>
<td>D₂co</td>
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<td>−0.11</td>
<td>0.8</td>
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<tr>
<td></td>
<td>High</td>
<td>0.03</td>
<td>0.9</td>
<td></td>
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<tr>
<td>Leukocytes</td>
<td>Low</td>
<td>0.43</td>
<td>0.5</td>
<td>1.15</td>
<td>0.07</td>
<td>0.98</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>1.31*</td>
<td>0.01</td>
<td>1.43*</td>
<td>0.02</td>
<td>1.71*</td>
<td>0.002</td>
</tr>
<tr>
<td>ECP†</td>
<td>Low</td>
<td></td>
<td></td>
<td>130%*</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>210%*</td>
<td>&lt;0.0001</td>
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<td></td>
</tr>
<tr>
<td>D-dimer†</td>
<td>Low</td>
<td>170%*</td>
<td>0.0003</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>100%*</td>
<td>0.002</td>
<td></td>
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<tr>
<td>Fibrinogen</td>
<td>Low</td>
<td>−0.15</td>
<td>0.5</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>−0.27</td>
<td>0.2</td>
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</tbody>
</table>

Differences compared to the 2007 level and the p value of the year variables are shown adjusted for gender, age, height, weight, smoking status, asthma (fixed effects) and individuals (random effect) in mixed effect models.

*Significant differences.
†Variables were log-transformed before statistical modelling, back-transformed and expressed as per cent change.
F₂₅, forced mid-expiratory flow rate; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; Kco, transfer coefficient.
macrophage defence capability demonstrated by reduced secretion of acute phase proteins.14–16

The highly exposed downstream workers experienced high endotoxin levels of up to 17 000 EU/m3 without respirator use and the exposure level of downstream workers was also high compared with other occupational settings where endotoxin exposure is often below 1000 EU/m3.17

In conclusion, the results obtained after a follow-up of 5 years among BSCP workers where co-exposure to components other than endotoxin is rare, show a change in previously detected inflammation in the lung,1 3 reflected by a significant improvement in lung function values 1 year after the termination of exposure among workers exposed to moderate levels of endotoxin from M. capsulatum. Lung function did not improve among the highest exposed workers. An increase in the anterior volume of the nose was detected. Furthermore, we found normalisation of the blood leukocyte count and the level of most acute phase proteins.14

REFERENCES

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