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Increased number of activated lymphocytes in human lung following swine dust inhalation

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Increased number of activated lymphocytes in human lung following swine dust inhalation. C. Müller-Suur, K. Larsson, P. Malmberg, P.H. Larsson. ©ERS Journals Ltd 1997.

ABSTRACT: Inhalation of swine dust causes intense alveolar inflammation, with recruitment of inflammatory cells, predominantly neutrophils, but also alveolar macrophages and lymphocytes. The present study focuses on the lymphocyte response to inhaled swine dust.

Twenty four healthy, nonsmoking, nonallergic subjects were exposed to swine dust for 3 h in a swine confinement building. Bronchoalveolar lavage (BAL) was performed before and 24 h after the start of exposure, and blood samples were drawn before, and at 7 and 24 h after exposure. Total and differential cell counts were carried out. Monoclonal antibodies recognizing T-cells, T-cell subsets, T-cell activation markers, and B-cells were analysed by flow cytometry.

The number of granulocytes increased more than 50 times and alveolar macrophages and lymphocytes increased two- to three-fold in BAL fluid. The exposure did not alter the proportion of T-cells but increased the number of activated T-cells in BAL fluid. The interleukin-2 (IL-2) receptor (CD25), human leucocyte antigen-DR (HLA-DR) major histocompatibility complex (MHC) class II and the early activation marker CD69 were expressed by 8.4% (25–75th percentiles 6.4–9.6%), 9.9% (8.2–21.6%) and 22.0% (18.1–24.3%) of the lymphocytes prior to exposure, and 11.6% (9.0–16.4%) ($p<0.01$), 18.8% (12.9–30.4%) ($p<0.01$) and 42.1% (38.4–47.3%) ($p<0.05$), respectively, after the exposure. In peripheral blood, the concentration of T-cells decreased after exposure and B-cells increased slightly but significantly. The ratio naive/memory T-cells (CD45RA/RO) did not change in blood.

In conclusion, 3 h of swine dust inhalation led to an influx of lymphocytes into the lower airways and increased expression of lymphocyte activation markers on the cell surface in previously unexposed subjects. The finding suggests a role for T-cells, in conjunction with other cells, in the inflammatory response to inhaled swine dust.
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A few hours inhalation of swine dust causes airways inflammation [1] and bronchial hyperresponsiveness [2]. The cellular reaction to swine dust, as assessed by bronchoalveolar lavage (BAL), is dominated by neutrophils but there is also a recruitment of macrophages and lymphocytes. Thus, an acute inhalation of swine dust has been found to induce a threefold increase in lymphocytes in BAL fluid [1]. Products of bacteria, including endotoxin and grain dust, have been suggested to be responsible agents in the swine dust [1, 3].

T-lymphocytes have a central role in many types of inflammatory response. Helper T-lymphocytes (CD4+) are important mediators of the inflammatory response and have the capacity to produce cytokines, which are believed to induce differentiation, recruitment, and activation of specific granulocyte effector cells at the mucosal surface [4]. It could, therefore, be anticipated that the lymphocytes may also play a role in the response to inhaled swine dust. Except for the increase of the lymphocyte number in BAL fluid, the lymphocyte reaction to acute exposure to swine dust has, however, not been studied. The aim of the present study was to characterize the lymphocytic response by investigation of lymphocyte numbers, subtypes, and lymphocyte activation markers in peripheral blood and BAL fluid following 3 h exposure to airborne dust in a swine confinement building.

Materials and methods

Study population and study design

Twenty four healthy, nonsmoking, volunteers (15 males and 9 females), mean age 32 (range 22–50) yrs, were exposed to swine dust for 3 h while weighing swine in a swine confinement building. None of the subjects had a history of asthma or other allergic manifestations and none of the participants were previously exposed to swine dust. Venous blood was collected before and at 7 and 24 h after exposure. BAL was performed more than 14 days (mean 22 (range 16–30) days) before exposure and 24 h after exposure. Oral temperature was registered before and several times after exposure, and symptoms were recorded. All subjects gave their informed consent, and the study was approved by the Ethics Committee of Karolinska Institute, Stockholm, Sweden.

Bronchoalveolar lavage

BAL was performed under local anaesthesia according to the procedure described previously [5]. Briefly, a total amount of 250 mL of sterile saline solution (37°C) was instilled in 50 mL aliquots *via* a fibreoptic bronchoscope, which was wedged in a subsegmental bronchus in the middle lobe. After each installation, the fluid was gently aspirated and collected in a siliconized plastic bottle kept on ice. After straining the fluid through a single layer of gauze, which, according to our experience, does not influence the cellular composition of the BAL fluid, cells were pelleted by cytocentrifugation at 400×g for 5 min at 4°C. The pellet was resuspended in Tris-Hank's balanced salt solution at pH 7.4, and the cells were counted in a Bürker chamber and prepared for flow cytometry. Smears for differential counts were prepared by cytocentrifugation after staining with May-Grünwald-Giemsa. Four hundred cells were counted.

Reagents and phenotypic evaluation

Ten microlitres of Cyto-Stat/Coulter Clone monoclonal antibody (Coulter Electronics Inc., FL, USA) were added to 100 µL aliquots of ethylenediamine tetra-acetic acid (EDTA) blood or BAL fluid and incubated for 10 min at room temperature. Lysing of red blood cells, fixation and stabilization of white blood cells was achieved by using Coulter® Multi-Q-prep (Coulter Electronics Inc.). Monoclonal antibodies from other companies, Becton and Dickinson, (Immunocytometry Systems, San Jose, CA, USA), Immunotech (S.A, Marseilles, France), and Ortho Diagnostic (Ortho Diagnostic Systems, Raritan, NJ, USA) were diluted and incubated according to the instruction for each reagent. For background staining pattern, isotypic controls were performed considering immunoglobulin subclasses. Analyses were carried out by flow cytometry using an Epics Profile II (Coulter Electronics Inc.,).

BAL cells and EDTA blood were characterized by different groups of fluorochrome conjugated monoclonal antibodies. Monoclonal antibodies recognizing T-cells, T-cell subsets and B-cells (Cyto-Stat/Coulter Clone, Coulter Corp., Miami, FL, USA) were used: CD2 (T11), CD3 (T3), CD4 (T4), CD8 (T8), and CD19 (B4). For recovery and purity of lymphocytes, a cell differential count was performed using CD14-CD45 (Mo2-RD1/Kc56-Fitc). The cell surface activation markers CD25 (interleukin-2 receptor-1 (IL-2R1)), human leucocyte antigen-DR (HLA-DR) major histocompatibility complex (MHC) class II) [I3] and CD69 (Leu-23; Becton Dickinson) were studied. To distinguish between naive and memory T-cells in blood, CD4-CD45RA (Ortho Diagnostic), and CD4-CD45RO (Immunotech) were used.

Statistics

Results are presented as medians (25–75th percentiles). Comparisons were performed by Wilcoxon Signed Rank test. A p-value of less than 0.05 was considered significant. The StatView® programme, version 4.02 was used for statistical analysis (Abacus Concepts Inc., Berkeley, CA, USA).

Results

Seven hours after the start of exposure, all subjects experienced malaise, and 19 out of 24 subjects reported slight to moderate chills, nausea or headache. Eighteen subjects experienced slight muscle pain. Exposure induced an increase in oral temperature in all but two subjects. The median increase was 0.9 (0.5–1.6)°C. The highest postexposure temperature was 38.8°C.

BAL fluid analyses

The recovery of BAL fluid was 72 (68–76%) before and 70 (72–76%) after exposure. Swine dust inhalation induced a more than 50 fold increase in the number of granulocytes in BAL fluid, from 1.9 (1.0–2.4) to 107 (50–180) $\times 10^6$ cells·L^{-1} (fig. 1). This increase was dominated by neutrophils, although a statistically significant increase in eosinophils was also observed (0.0 (0.0–0.4) to 1.5 (0.3–4.0) $\times 10^6$ cells·L^{-1}, p<0.001). The number of alveolar macrophages was approximately doubled (90

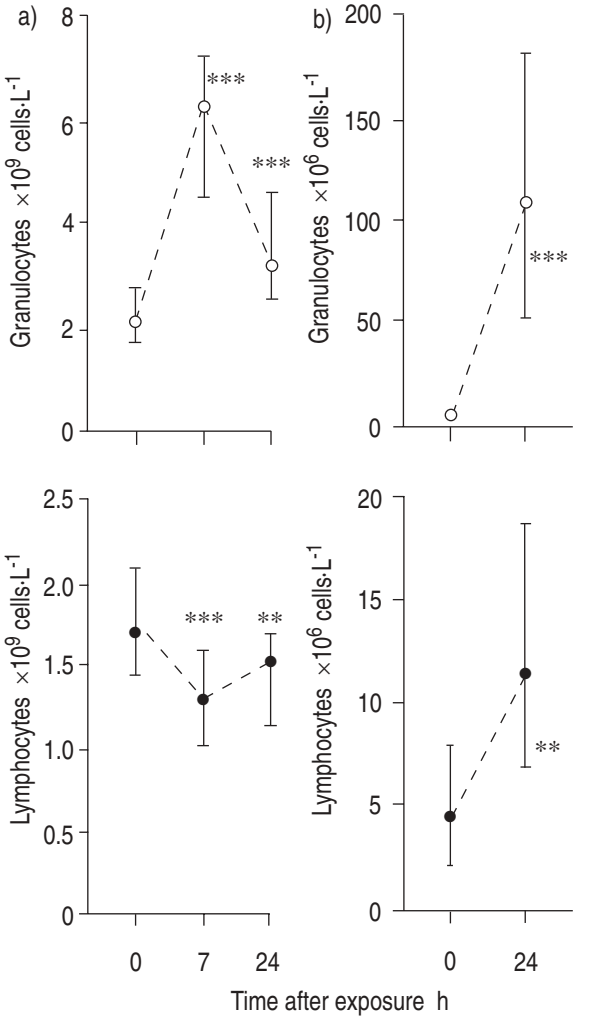

Fig. 1. – Granulocyte (—○—) and lymphocyte (—●—) numbers in: a) periperal blood; and b) bronchoalveolar lavage fluid (BAL) fluid before and after exposure to swine dust. Results are presented as median, and 25th and 75th percentiles. **: p<0.01; ***: p<0.001, compared with pre-exposure value.

Table 1. – Markers for lymphocytes and lymphocyte activation in BAL fluid before and after exposure to swine dust

	Before exposure	After exposure	Difference
CD2	93.5 (89.8–94.4)	94.1 (92.8–95.0)	0.4 (-1.0–4.0)
CD3	82.6 (80.3–85.9)	85.6 (81.1–88.4)	3.0 (-3.4–6.7)
CD4/CD8	1.5 (1.08–2.46)	1.55 (1.09–2.35)	0.0 (-0.5–0.3)
CD19	0.6 (0.3–1.3)	1.6 (0.7–2.1)	0.7** (0.4–1.3)
CD25+ (IL-2R)	8.4 (6.4–9.6)	11.6 (9.0–16.4)	3.3** (0.4–9.0)
CD3+ HLA-DR+	9.9 (8.2–21.6)	18.8 (12.9–30.4)	5.9** (1.4–9.4)
CD3+ CD69+	22.0 (18.1–24.3)	42.1 (38.4–47.3)	20.8* (10.1–27.0)

The figures denote the percentage of lymphocytes expressing the marker(s) (CD2, CD3: T-cells; CD4: T-helper cells; CD8 T-suppressor/cytotoxic cells; CD19: B-cells; CD25, HLA-DR and CD69: lymphocyte activation markers. Values are presented as median, and 25–75th percentile in parenthesis. BAL: bronchoalveolar lavage; HLA-DR: human leucocyte antigen DR. *: p<0.05; **: p<0.01, compared with pre-exposure values.

(68–10) to 190 (136–307) $\times 10^6$ cells·L^{-1}), and lymphocytes increased from 4.9 (3.0–9.0) before to 11.6 (6.6–18.0) $\times 10^6$ cells·L^{-1} after exposure (p<0.01) (fig. 1).

Swine dust exposure did not significantly alter the proportion of T-cells in BAL fluid, but increased the B-cell proportion significantly (p<0.01) (table 1). All markers for lymphocyte activation (IL-2R1 (CD25+), CD3+/HLA-DR+ and CD3+/CD69+) increased significantly following exposure (table 1).

Blood analyses

The granulocyte concentration in venous blood increased from 2.3 (1.9–2.8) before to 6.3 (4.6–7.3) $\times 10^9$ cells·L^{-1} 7 h after exposure (p<0.001), and was still significantly increased 24 h after exposure (p<0.001) (fig. 1).

The lymphocyte concentration in peripheral blood decreased from 1.7 (1.5–2.1) before to 1.3 (1.0–1.6) $\times 10^9$ cells·L^{-1} 7 h after exposure (p<0.001), and was still significantly decreased 24 h after exposure (1.5 (1.2–1.7) $\times 10^9$ cells·L^{-1}; p<0.01) (fig. 1). The proportion of T-cells (CD2+ and CD3+) in percentage of total lymphocyte count and the ratio helper/cytotoxic T-cells (CD4+/CD8+) were significantly decreased 7 and/or 24 h after exposure (table 2). The proportion of B-cells (CD19+) increased significantly following exposure to swine dust (table 2).

The proportion of T-cells (CD3+) expressing the IL-2 receptor (IL-2R1) increased slightly, but significantly (p<0.05), following exposure (table 2). The proportion of naive and memory T-cells (CD45RA/RO+) did not change significantly in peripheral blood (table 3).

Table 2. – Lymphocyte markers and markers for activation in venous blood before and after exposure to swine dust

	Before exposure	7 h after exposure	24 h after exposure
CD2	85.6 (80.6–87.1)	79.9*** (75.7–85.3)	81.9** (79.6–85.0)
CD3	77.5 (71.7–81.5)	73.0 (70.6–78.8)	74.3** (66.8–80.6)
CD4/CD8	1.75 (1.28–2.16)	1.73 (1.42–1.97)	1.48*** (1.12–1.77)
CD19	9.6 (7.8–15.0)	11.9*** (10.6–16.3)	12.2* (10.1–13.7)
CD25+ (IL-2R)	3.7 (2.5–8.4)	6.5* (3.9–10.0)	6.6 (3.3–8.9)
CD3+ HLA-DR+	4.0 (1.4–5.9)	4.2 (1.9–7.1)	3.8 (2.1–11.2)

With the exception of CD4/CD8 ratio the figures denote the percentage of lymphocyte expressing the marker(s). For definitions of markers see legend to table 1. Values are presented as median, and 25–75th percentile in parenthesis. HLA-DR: human leucocyte antigen DR. *: p<0.05; **: p<0.01, compared with pre-exposure values.

Table 3. – The CD45RA and CD45RO marker in blood lymphocytes before and after exposure to swine dust

	Before exposure	7 h after exposure	24 h after exposure
CD45 RA	64.4 (55.3–66.5)	61.6 (58.7–66.9)	63.1 (60.2–68.4)
CD45 RA+ CD4+	24.3 (19.3–30.8)	21.2 (17.9–28.7)	20.9 (18.3–27.4)
CD45 RO	28.0 (17.6–30.2)	24.7 (21.0–31.9)	23.8 (20.8–25.7)
CD45 RO+ CD4+	16.3 (11.6–19.6)	17.4 (14.1–21.6)	15.7 (14.0–16.0)

The figures denote the percentage of lymphocyte expressing the marker(s). Values are presented as median, and 25–75th percentile in parenthesis. No significant changes were found.

Discussion

In the present study, it has been demonstrated that a 3 h exposure to airborne swine dust induces a massive influx of inflammatory cells into the lower airways in healthy subjects. There was an increase in the numbers both of T- and B-cells in BAL fluid, while the proportion of T-cells (of all lymphocytes) was unaltered. It was also shown that the exposure caused not only an increased lymphocyte influx into the alveolar space but also an increased number of activated T-cells. The slight, but significant, decrease in the proportion of T-cells in peripheral blood is difficult to interpret but may reflect an increased recruitment of these cells to the airways [6–9].

The expression of the T-cell surface activation markers, IL2-R1, HLA-DR and CD69, increased on T-cells obtained by BAL. It has previously been demonstrated that the expression of CD69 (early-activating antigen), which requires interaction between the T-cell receptor (TCR)/CD3 complex and a ligand, increases within 2 h after stimulation of the T-cell and reaches peak levels

18–24 h after stimulation [10, 11]. In the present study, the expression of CD69 was, thus, measured at a timepoint (24 h after exposure) when it could be expected to be near maximal. CD69 was expressed on 42% of the BAL CD3+ cells following exposure. *In vitro* stimulation of T-cells with phorbol myristate acetate (PMA) has been shown to induce expression of CD69 in 80% of the cells [10]. Although comparisons between *in vitro* and *in vivo* data are hazardous, we assume that the exposure in the present study is fairly potent. Prior to exposure, approximately 20% of the T–cells obtained by BAL expressed CD69. In T-cells from the airways, it could be anticipated that CD69, being a marker of activation, is normally expressed, since cells in the airways may be continuously exposed to agents capable of inducing cell activation [12]. We did not study the CD69 antigen on peripheral blood cells, since it has been shown by others that this protein is normally expressed on circulating T-cells only weakly or not at all [11–13].

We found an increase of T-cells expressing CD3+/HLA-DR+ and the IL-2 receptor (CD25) in BAL fluid. It has been demonstrated that the increased IL-2 receptor expression on T-cells appears somewhat later than the early CD69 antigen [14]. The IL-2 receptor appears on the cell surface within a few hours and reaches a maximum 15–30 h after antigen exposure [15]. In the present study, it thus seems likely that the IL-2 receptor expression on T-cells, obtained from BAL fluid 24 h after exposure, reflects maximal or near maximal activation, assuming that the time course for lymphocyte activation by inhaled swine dust is similar to that of antigen. The increased appearance of cell surface IL-2 receptor is small in the present study, and not of the same magnitude as that found in patients with atopic asthma [16] or following antigen exposure *in vitro* [15]. Class II MHC molecules appear on the T-lymphocyte surface after antigen stimulation and reach their maximum after several days [4]. Although we could detect a significant increase in the expression of HLA-DR on CD3+ cells, we were probably only able to demonstrate this at an early phase. It is conceivable that maximal expression would have occurred later [4, 17].

It is not known what agents in the swine dust are responsible for the lymphocyte activation and influx into the airways. It is known that dust in swine confinement buildings contains bacteria of different kinds, mainly Gram-positive bacteria [18]. In investigations similar to the present study, exposure measurements have revealed airborne total dust concentration of approximately 20 $mg \cdot m^{-3}$. Lipopolysaccharide (LPS) and peptidoglycan (muramic acid), as markers for Gram-negative and Gram-positive bacteria, were found in concentrations of approximately 4 and 6.5 $\mu g \cdot m^{-3}$ sampled air, respectively [19]. Gram-positive bacteria are capable of T-lymphocyte activation [20–22], and, thus, may be the (or one of the) responsible factor(s) causing the reaction to swine dust. Although the subjects were not previously exposed to swine dust, an earlier sensitization due to exposure to agents present in swine dust may have occurred. If bacteria are the agents responsible, this explanation seems plausible.

Bacteria are also capable of activating T-cells *via* superantigens, which bind simultaneously to class II MHC molecules and the V_β-region of the T-cell receptor [21], and which are not processed prior to presentation by antigen-presenting cells [21, 23]. Toxins from bacteria such as *Streptococcus pyogenes, Staphylococcus aureus, Mycoplasma arthritidis* and possibly *Pseudomonas aeruginosa* can act as superantigens [20, 23]. It has previously been shown that activation of human T-cells by Staphylococcus enterotoxin B leads to increased expression of the IL-2 receptor, HLA-DR and the CD69 antigen [22]. Swine dust contains Gram-positive bacteria [18], which may support the hypothesis that superantigen may be a lymphocyte activator in the present study.

Cytokines play an important role in the recruitment of inflammatory cells to the site of inflammation. Thus, tumour necrosis factor-α (TNF-α) upregulates adhesion molecules on the endothlial cells, leading to cell recruitment [24]. We have recently demonstrated that the concentration of TNF-α increases in blood [25] and BAL fluid [26] following swine dust exposure. Interleukin-1 (IL-1) has a functional role in T-cell activation and may act synergistically in combination with antigen [27] but also other cytokines, such as interleukin-6 (IL-6) [28], and by inducing production of IL-2 and IL-2 receptors [29]. IL-1 and IL-6 increase considerably (8 and 20 fold) in BAL fluid following exposure to swine dust [26]. Interleukin-8 (IL-8) is a chemotactic factor for neutrophils and T-lymphocytes [30], and we have found that inhalation of swine dust causes an increase in interleukin-8 (IL-8) in BAL fluid (Larsson *et al*., submitted). A low concentration of IL-8 recruits mainly T-lymphocytes, whilst higher concentrations predominantly recruit neutrophils [31, 32]. *In vitro,* we have shown that swine dust, Gram-positive bacteria and LPS from bacterial endotoxin stimulate epithelial cells to release IL-8 [3]. It is, thus, plausible that T-cell recruitment and the increased expression of activation markers on these cells following swine dust exposure are regulated by cytokines released by cells such as alveolar macrophages and epithelial cells. This indicates the possibility that the T-cell recruitment and activation is mediated by nonspecific, *i.e*. non-antigen-dependent, mechanisms.

In conclusion, we have found that acute exposure to inhaled swine dust leads to an influx of lymphocytes into the alveolar space, and that these lymphocytes have an increased expression of surface activation markers. Further studies are in progress, attempting to characterize the inflammatory response to swine dust and to identify the proinflammatory constituents in the dust.

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