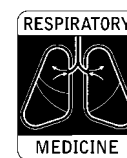



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T-cell activation by organic dust *in vitro*

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Inhalation of swine dust causes intense airway inflammation with a multifold increase of inflammatory cells and lymphocyte activation as assessed by bronchoalveolar lavage. To further investigate the mechanism for lymphocyte activation the present *in vitro* study focuses on the lymphocyte response to swine dust in whole blood.

Various concentrations of phytohaemagglutinin (PHA) (final concentrations: 3.16, 10.0, 31.6 and 100 $\mu\text{g ml}^{-1}$) and swine dust (final concentrations: 10.0, 31.6, 100 and 316 $\mu\text{g ml}^{-1}$) were added to heparinized whole blood from healthy donors. The blood samples were incubated in duplicate, using the homologous unstimulated blood as control, for 4, 24, 48 and 72 h in a water bath at 37°C. The cells were stained with fluorochrome conjugated monoclonal antibodies. For analysis of T-cell activation CD3 was doublestained together with the activation markers CD69, CD25 and HLA-DR. Cell count percentages were analysed by flow cytometry. Soluble IL-2sR α in plasma was analysed using commercial sandwich ELISA technique.

At baseline CD69, CD25 and HLA-DR were expressed in < 1%, approx 5% and < 1% of the T-cells respectively. We found a dose response relationship between swine dust exposure and the expression of all three T-cell activation markers which appeared at different time-points. Maximal expression of CD69 (8%, $P < 0.05$) and CD25 (15%, $P < 0.001$) was found after 24 h of activation. HLA-DR was significantly expressed after 48 h (8%) and maximally expressed after 72 h of activation (13%, $P < 0.05$). The soluble IL-2sR α in plasma was maximally expressed after 24–48 h (1200 pg ml^{-1} and 1500 pg ml^{-1} , respectively).

In conclusion, T-cells were activated by swine dust *in vitro*. Thus, our previous findings of T-cell activation following swine dust exposure, *in vivo* may be an effect of the dust either directly on T-cells or on other cells which in turn contribute to the T-cell activation.

Key words: lymphocytes; activation markers; whole blood; dust.

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Introduction

The T-helper lymphocytes are important mediators in 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 (1). Three hours of exposure to swine dust in a swine confinement building causes intense alveolar inflammation with a multifold increase of inflammatory cells, predominantly neutrophils, and a two to three-fold increase in the number of lymphocytes in the lungs as assessed by bronchoalveolar lavage (2).

The exposure leads to activation of the T-cells (3). T-cells are activated by interaction between the T-cell receptor

(TCR)/CD3 complex and the MHC molecule, together with processed antigen presented by an antigen presenting cell (APC). T-cell activation can also be achieved by interaction with bacteria produced superantigens which are capable of T-cell activation by direct cross-linking between the class II molecule and the V β region of the T-cell receptor (4). In addition, mitogens are polyclonal activators which non-specifically induce DNA-synthesis and cell division in a high percentage of B- and T-cells (5). Activation by mitogens may affect many clones of T- and B-cells irrespective of antigen specificity. Ionophores may also function as mitogens by inducing ion transport across the cell membrane (6). It is not clear by which of these mechanism(s) T-cell activation occurs following exposure to swine dust, and it is not clear which components of the dust are responsible for the activation of T lymphocytes. It could be a direct effect of the swine dust on T-cells but there is also a possibility that indirect mechanisms, mediated by other cell mediators, contribute to the cell activation. The aim of the present study was to investigate whether swine dust activates T-cells in heparinized whole blood *in vitro*.

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Materials and Methods

SUBJECTS

Blood was obtained from five (one female) non-smoking, healthy volunteers (mean age 44 years; range 27–57). None of the participants had a history of allergy, asthma or other chronic airway diseases. None had a history of respiratory infection during the last 2 weeks prior to the trial. Blood was collected using Vacutainer™ tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, U.S.A.) containing sodium heparin anticoagulant. All subjects gave their informed consent and the study was approved by the Ethics Committee of Karolinska Institute, Stockholm, Sweden.

Study design

Swine dust, collected in a swine confinement building approximately 1.5 m above the floor, was dissolved in RPMI 1640 (Gibco Laboratories, Paisley, U.K.), sonicated for 10 min and diluted in whole blood to final concentrations: 10.0, 31.6, 100 and 316 $\mu\text{g ml}^{-1}$. The polyclonal activator, phytohaemagglutinin (PHA), used as a positive control, was dissolved in RPMI 1640 and sonicated for 10 min. PHA was further diluted in whole blood to final concentrations: 3.16, 10.0, 31.6 and 100 $\mu\text{g ml}^{-1}$. Homologous whole blood containing RPMI 1640, was used as negative control. Blood samples were incubated with dust or PHA in a shaking water bath for 4 h, 24 h, 48 h and 72 h, at 37°C. After incubation EDTA was added to all samples to a final concentration of 5 mM. All concentration steps were run in duplicate (7).

MONOCLONAL ANTIBODIES (MABS) AND FLOW CYTOMETRIC ANALYSIS

Whole blood was characterized by different groups of fluorochrome conjugated monoclonal antibodies. For analysis of T-cell activation, doublestaining for CD3 together with the cell surface activation markers CD25 (interleukin-2 receptor-1), human leucocyte antigen-DR (HLA-DR) major histocompatibility complex (MHC class II), CYTO-STAT/COULTER CLONE (Coulter Corporation Miami, FL, U.S.A.) and CD69 early T-cell activation marker (Immunotech S.A., Marseille, France). Ten microlitres of monoclonal antibody was added to 100 μl aliquots of blood. The samples were incubated for 20 min in the dark, 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., Hialeah FL, U.S.A.). For background staining, isotypic control were performed considering immunoglobulin subclasses. Cell count percentages was analysed by flow cytometry using an EPICS Profile II (Coulter Electronics, Inc., Hialeah, FL, U.S.A.).

Soluble IL-2 receptor assay in plasma

Soluble interleukin (II)-2 receptor, (Human IL-2sR α) was analysed using commercial colorimetric sandwich ELISA (Quantikine™ R&D systems, Europe Ltd, U.K.) with a sensitivity 6 pg ml^{-1} . All samples were run in duplicate. A variation in duplicate measurements of less than 10% was accepted.

Statistics

Statistical analysis was performed using StatView® program, version 4.02 for Macintosh and Super Anova 1.11 (Abacus Concepts, Inc, Berkeley, CA, U.S.A.). Results are presented as medians (25th–75th percentiles). *P*-values <0.05 were considered statistically significant.

Results

Less than 1% of the unstimulated T (CD3) cells expressed CD69. PHA induced CD69 expression in a dose response manner and CD69 was expressed in >60% of the T-cells at a concentration of >31.6 $\mu\text{g ml}^{-1}$, after 4 h incubation and onwards (Fig. 1). Swine dust also induced a dose dependent CD69 expression although to a much lesser extent than PHA (Fig. 1). CD69 expression was maximally expressed by 8% of the T-cells, after 24 h incubation with swine dust and did not increase further with prolonged incubation (Fig. 1).

Five percent of the unstimulated T-cells expressed CD25. PHA induced a dose dependent increase in CD25 expression after 24, 48 and 72 h of incubation (Fig. 2) with no significant differences between the incubation times. Swine dust also induced increase expression of CD25 in a dose response manner after 24 h and onwards. At maximum 13% of the T-cells expressed CD25 at the highest dust concentration (Fig. 2).

Almost no unstimulated cells expressed HLA-DR. Both PHA and swine dust stimulated T-cells to express HLA-DR, which appeared later than the expression of CD69 and CD25 (Fig. 3). Fifty-five percent of the PHA stimulated T-cells expressed HLA-DR after 72 h of incubation at the highest concentration. The corresponding figure for swine dust was 15% (Fig. 3).

Plasma obtained from blood samples incubated with swine dust or PHA shows a dose and time dependent increase of the soluble IL-2sR α (Fig. 4). The pattern for IL-2sR α increase with regard to dose response differs from both CD69 and CD25 expression on the cell surface. For CD69 and CD25 there was a reduced expression after 48 h compared to 24 h and 72 h which was not the case for IL-2sR α . IL-2sR α increased from a baseline level of 700 pg ml^{-1} to 1400 pg ml^{-1} in samples incubated with swine dust at the highest concentration. For samples incubated with 31.6 $\mu\text{g ml}^{-1}$ PHA, the increase reached a maximum of 8000 pg ml^{-1} .

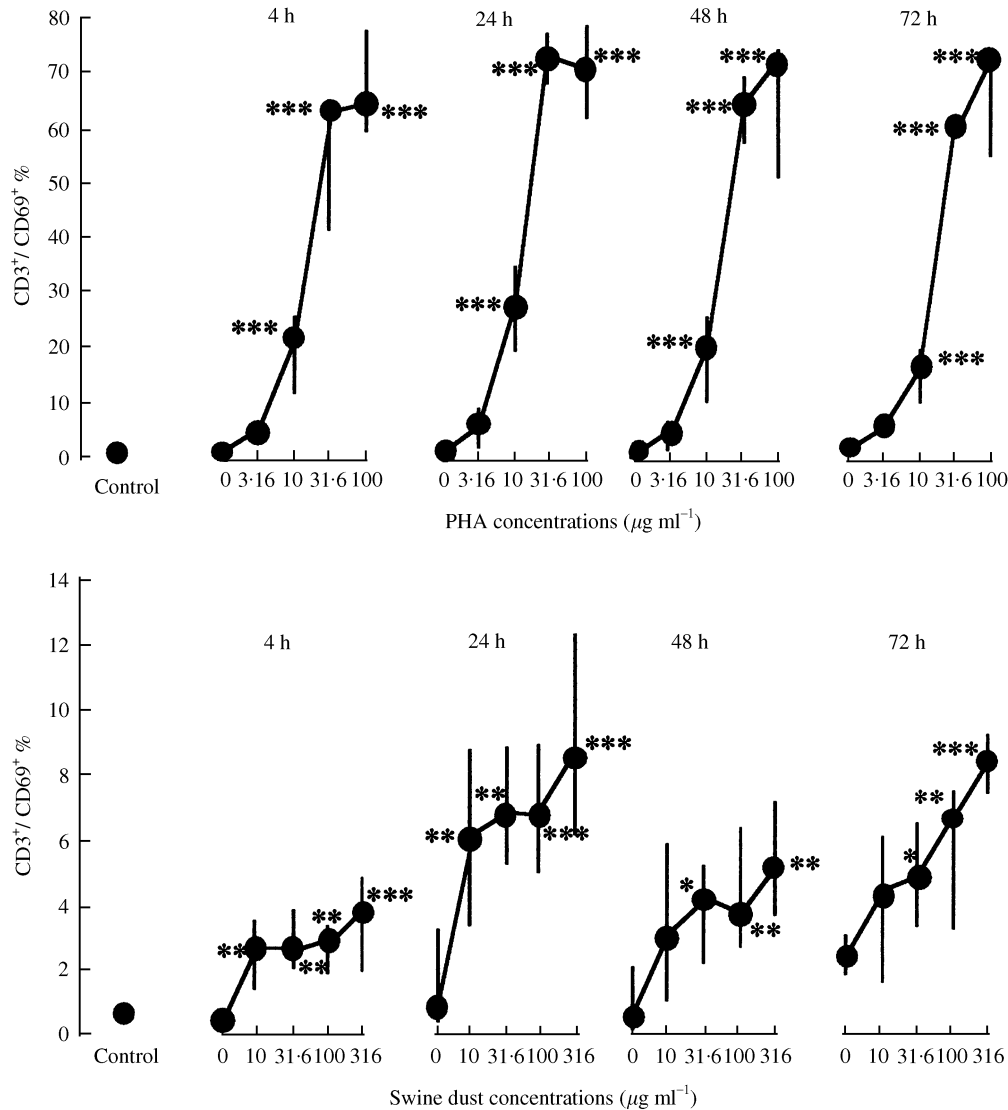


FIG. 1. CD69 expression on T-lymphocytes (CD3⁺) in peripheral whole blood incubated with PHA (upper panel) or swine dust (lower panel) in a dose-response manner for 4, 24, 48 and 72 h. Results are presented as median and 25th and 75th percentiles. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to unstimulated blood (time-point 0) at each incubation time-point.

Discussion

In the present study it has been demonstrated that swine dust activates lymphocytes in whole blood *in vitro* as expressed by the surface markers CD69, CD25 and HLA-DR. It is also shown that the maximal activation occurs at different time-points for the different markers, that the activation is dose dependent and that the time course is similar for dust and PHA. Furthermore, it has been shown that the soluble IL-2 receptor in plasma increases following exposure to swine dust.

The expression of CD69, the earliest activation antigen, requires interaction between the T-cell receptor (TCR/CD3) complex and a ligand. In previous studies CD69 has

been found to be increased 2–3 h after stimulation of the T-cell and to reach peak levels 18–24 h after stimulation (8–10). In the present study the expression of CD69 was thus measured at a time-point when it could be expected to be maximal. In the present *in vitro* study, CD69 was expressed on 70% of CD3⁺ cells after stimulation with PHA but only approximately 10% of the cells following 72 h of swine dust incubation. Although being a weaker activator than PHA it is clearly demonstrated that dust from swine confinement buildings significantly increases the T-cell expression of CD69, which is less than 1% in unstimulated cells.

It has previously been demonstrated that the increase of the IL-2 receptor and HLA-DR expression on T-cells

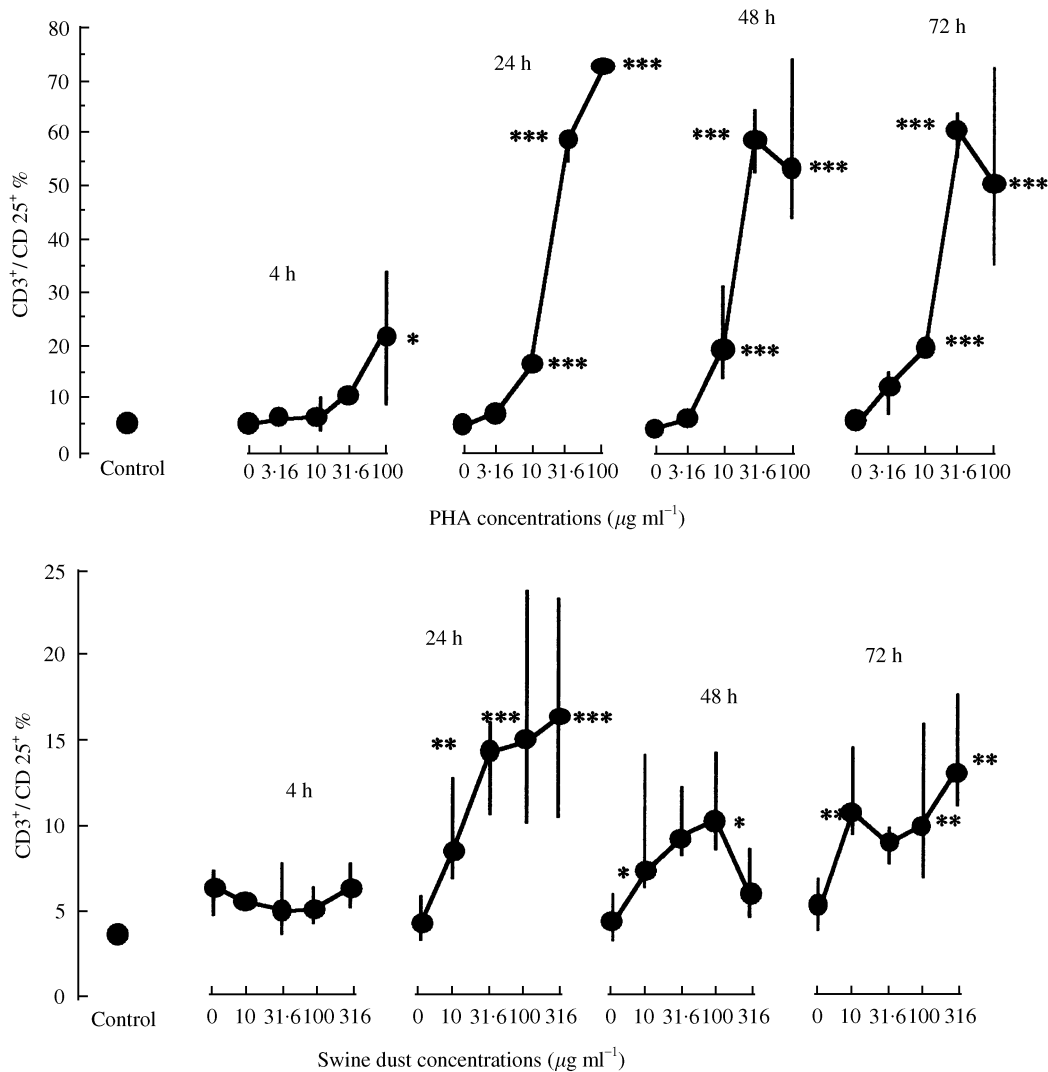


FIG. 2. CD25 (IL-2R) expression on T-lymphocytes (CD3+) in peripheral whole blood incubated with PHA (upper panel) or swine dust (lower panel) in a dose-response manner for 4, 24, 48 and 72 h. Results are presented as median and 25th and 75th percentiles. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to unstimulated blood (time-point 0) at each incubation time-point.

appears later than the CD69 antigen (9). The maximal appearance of the IL-2 receptor on the cell surface is observed approximately 24 h after antigen exposure (1,11). In the present study, it seems thus likely that the IL-2 receptor expression on T-cells (CD3) stimulated with swine dust for 24 h *in vitro*, reflect maximal, or near maximal activation. This conclusion is based on the assumption that the time course for lymphocyte activation by *in vitro* stimulated whole blood with swine dust is similar to that of antigen stimulation.

Although blood collecting and experimental conditions, such as heparinized blood collection tubes, oxygenation and incubation times, were identical in exposure and control blood we found that the T-cell activation assessed by CD69 and CD25 tended to be reduced expressed after

48 h of swine dust incubation while there was an increase, to the same level as 24 h, at 72 h incubation. Swine dust incubated T-cells might lose their expression of antigen receptors after 48 h which seem to be re-expressed on the cell surface after 72 h. We think that this, at least in part, can be explained by antigen receptor shedding. The analysis of blood for CD markers are made from the same sample tube, and HLA-DR expression on CD3 positive T-cells did not show the same time-response pattern as did CD69 and CD25. The assumption of receptor shedding is supported by the finding of a dose and time dependent, non-biphasic, increase of the soluble IL-2 sR α in plasma from blood samples incubated with swine dust. Thus we found no corresponding reduction of the IL-2-sR α levels after incubation with swine dust for 48 h compared to 24 and

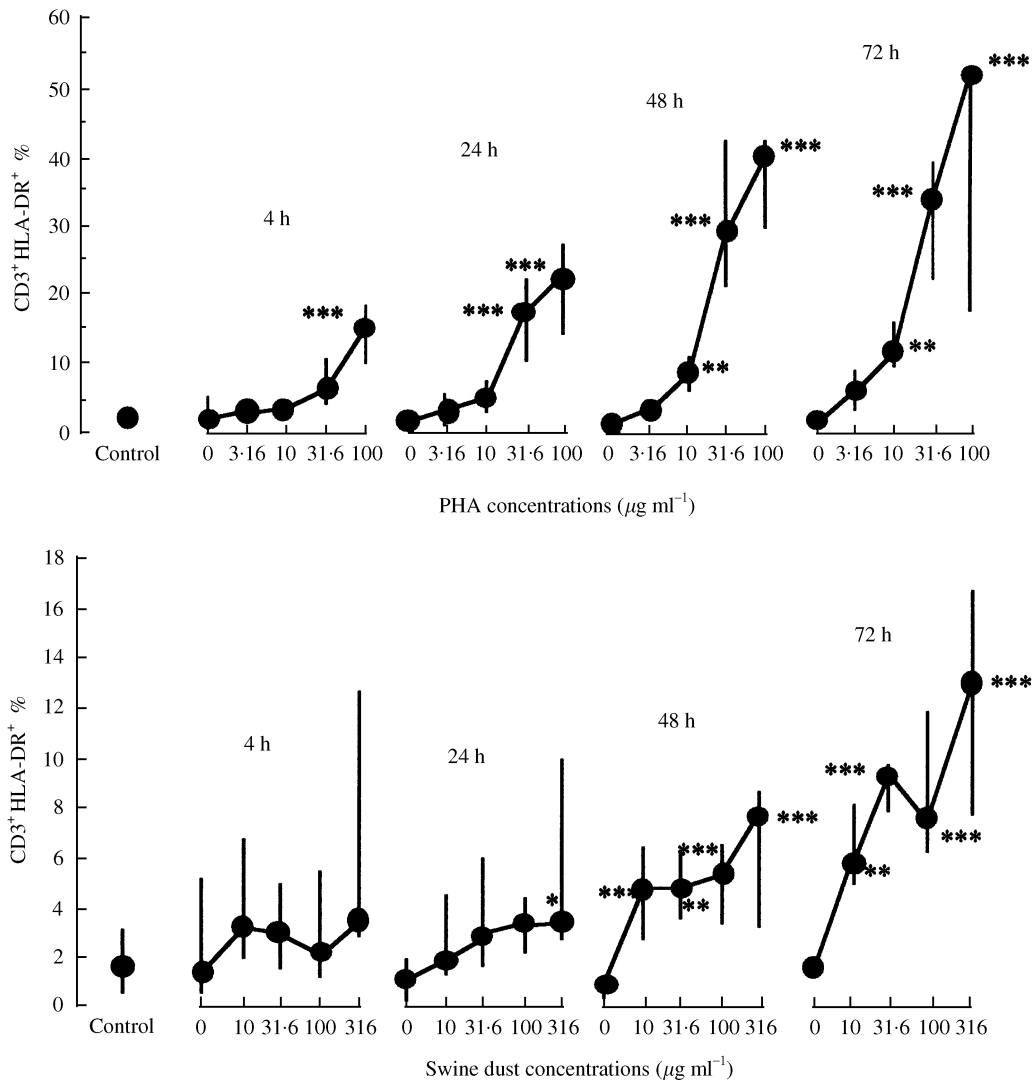


FIG. 3. HLA-DR+ expression on T-lymphocytes (CD3+) in peripheral whole blood incubated with PHA (upper panel) or swine dust (lower panel) in a dose-response manner for 4, 24, 48 and 72 h. Results are presented as median and 25th and 75th percentiles. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to unstimulated blood (time-point 0) at each incubation time-point.

72 h of incubation. The findings suggest that the attenuation of CD25 expression at 48 h of incubation may be due to receptor shedding.

HLA-DR molecules appear on the T lymphocyte surface after antigen stimulation and reach a maximum after several days (1). We detected a significant increase in the expression of HLA-DR, on approximately 15% of the swine dust stimulated T (CD3) cells after 48–72 h. The maximal HLA-DR expression would have occurred later than 72 h after both PHA and dust stimulation (1,12) and, therefore, in the present study we have probably detected the T-cell HLA-DR expression prior to its maximum.

The present results indicate that our previous findings of T-cell activation following swine dust exposure *in vivo* (3) is an effect mediated by the dust. Whether this is a direct effect

of the dust or an effect mediated by humoral factors is unclear. It is not known what agent(s) in the swine dust are responsible for the lymphocyte activation. The dust in swine confinement buildings contains micro-organisms, predominantly Gram-positive, but also Gram-negative, bacteria (13). Peptidoglycans and lipopolysaccharide (LPS), which are used as markers for Gram-positive and Gram-negative bacteria respectively, have been found in concentrations of approximately 6.5 and $4 \mu\text{g m}^{-3}$, respectively, in sampled air from swine confinement buildings (14). Airborne peptidoglycan concentration correlates with increase in body temperature and inflammatory indices following exposure to swine dust (14). Enterotoxins produced by Gram-positive bacteria are capable of T lymphocyte activation (4,15,16) and may thus be one of the

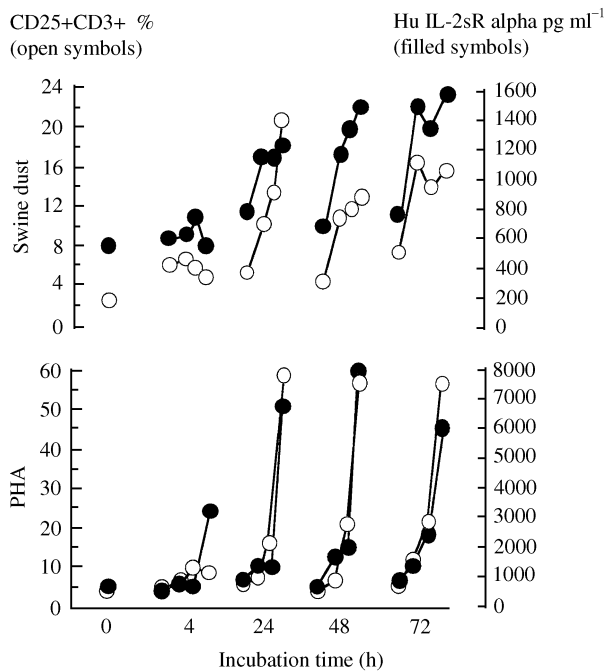


FIG. 4. Cell surface expression of the IL-2 receptor (CD25) in percentage of CD3 positive cells and soluble IL-2 receptor (pg ml^{-1}) in blood. Samples were incubated with swine dust (upper panel), and PHA (lower panel). Time 0 represents pre-incubation baseline values. At each time of incubation a dose response curve was performed for swine dust (0, 10 μg , 31.6 μg , 100 $\mu\text{g ml}^{-1}$) and PHA (0, 3.16 μg , 10 μg , 31.6 $\mu\text{g ml}^{-1}$).

responsible factors for the findings of the T-cell activation in the present study.

Following exposure to swine dust it is not clear whether the antigen presenting cell processes the antigen or if the T-cell activation is mediated by a superantigen (e.g. enterotoxin) which binds to the MHC molecule and the $V\beta$ -region of the T-cell receptor. Recent studies also provide other mechanisms for T-cell activation such as T-cell recognition of non-peptide antigens presented by the CDI molecule (17).

Inhalation of organic dust, such as dust from swine confinement buildings, induce secretion of pro-inflammatory cytokines, such as IL-1, IL-6 and $\text{TNF-}\alpha$ (18) and chemokines such as IL-8 (19) *in vivo*, probably from macrophages and epithelial cells. It is known that swine dust is a more potent activator than LPS to stimulate epithelial cells to synthesize IL-6 in cell cultures. IL-1 together with the MHC+ antigen are competence signals for T cell activation (5). It can not be excluded that our previous findings of *in vivo* lymphocyte activation caused by inhaled swine dust is an indirect effect as a consequence of the cytokine release cascade. Further studies attempting to characterize the lymphocyte response and the pro-inflammatory constituents of the dust are in progress.

Acknowledgements

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