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SHORT COMMUNICATION

The potential of a monocyte cell surface marker as an indicator of endotoxin exposure

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Endotoxin is common in workplaces such as farms, grain processing plants and cotton mills, and exposure can lead to a wide variety of respiratory symptoms including organic toxic dust syndrome and chronic bronchitis. We developed an *in vitro* model to enable us to determine the use of lipopolysaccharide receptor (CD14) expression to act as a biomarker of endotoxin exposure. Whole blood was incubated with endotoxin, human serum albumin (HSA) or phosphate buffered saline (PBS) at 37 °C to determine the time course of CD14 expression following *in vitro* stimulus. Fluorescent-labelled antibodies were used to label CD14 on monocytes, and CD45 on monocytes and lymphocytes. Levels of CD14 and CD45 expression were measured by flow cytometry. Levels of expression were determined on eight different samples at the optimum time point and concentration of endotoxin. CD14 expression on monocytes was upregulated in response to endotoxin exposure ($p < 0{\cdot}0001$) and could be measured easily in whole blood samples using flow cytometry 4 h after exposure. CD45 upregulation in response to endotoxin was monocyte-specific ($p < 0{\cdot}0001$), there was no significant difference in expression of CD45 on lymphocytes between the PBS and HSA controls and endotoxin-exposed cells ($p = 0{\cdot}6$). We have shown that the expression of cell surface CD14 and CD45 was significantly increased following *in vitro* exposure to endotoxin, and that this response was specific for monocytes. We suggest that the measurement of CD14 on monocytes by flow cytometry may be a useful biomarker of endotoxin exposure.

Keywords: endotoxin, CD14.

Introduction

Endotoxin is a lipopolysaccharide (LPS) component of the cell walls of Gram negative bacteria. Inhalation of endotoxin is thought to have a major role in occupational respiratory diseases. Potential sources of endotoxin in the workplace include harvested products such as vegetables and cereals, and in cotton mills endotoxin is considered to be important in the aetiology of byssinosis (cotton mill workers lung). High levels of endotoxin have been found in the settled and airborne dust in cotton mills (1·7 μg m^{-3}) (Christiani and Eisen 1993), grain mills (8·8 μg m^{-3}) (Olenchock *et al.* 1990) and during potato processing (1894 μg m^{-3}) (Dutkiewicz 1986). During a typical working day, a worker may breathe in 10 m^3 air, which in the grain mill would result in a daily exposure to 88 μg of endotoxin via inhalation. The inhalation of endotoxin causes both short-term illness (flu-like symptoms, e.g. organic dust toxic syndrome) and long-term illness (e.g. chronic bronchitis, chronic obstructive pulmonary disease and long term decline in lung function) (Blainey *et al.* 1995).

Previous work has shown that a range of immune cell surface markers can be identified and measured by flow cytometry as biomarkers of immunotoxicological

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response (Curran *et al.* 1997a, b). Gordon *et al.* (1977) have shown activation of helper T-cells (CD4+CD25+) in the peripheral blood of bakers reporting work-related respiratory symptoms consistent with the changes observed in mild to severe asthmatics. However, workers with similar symptoms who were exposed to irritant chemicals did not show this pattern of phenotypic or inducible cell surface markers, reflecting an absence of airways inflammation in these individuals. In fact workers exposed to irritant fumes showed a significant decrease in total T-cells (CD3+). However, there are currently no objective markers to demonstrate a cause and effect relationship between endotoxin exposure and impairment of respiratory function.

CD14 is the functional cell activating receptor for lipopolysaccharide and is found on monocytes and neutrophils. Previous studies have shown that CD14 expression was increased on human alveolar macrophages following endotoxin exposure (Hopkins *et al.* 1995). We have developed an *in vitro* model of endotoxin exposure and investigated the immunologic consequences of endotoxin exposure on monocytes and lymphocytes.

Materials and methods

Blood samples

Nine volunteers provided a 6 ml venous blood sample for flow cytometric analysis, which was collected into EDTA containing Vacutainers™ (Becton Dickinson). All samples were processed on the day of collection.

Endotoxin

Pure endotoxin lipopolysaccharide (LPS) from *E. coli* 055-B5 (Sigma; cell culture tested) and human serum albumin (HSA) (Sigma) were used in all tests. Dilutions were made in PBS Dulbeccos buffer (Gibco; endotoxin tested); this PBS was also used as a control. Concentrations of endotoxin were chosen from previously described studies (Koyama *et al.* 1991, Hopkins *et al.* 1995).

Antibodies

The following labelled antibodies were used in the study to measure cell populations, phenotypic and inducible cell surface markers. Phycoerythrin (PE) conjugated mouse monoclonal anti-human CD14 (Coulter Electronics, UK) Dual Test CD14-PE/CD45-FITC (Coulter Electronics, UK). Appropriately labelled isotype control antibodies were purchased from Coulter Electronics (UK).

Antibody staining

Aliquots (100 μl) of whole blood were added to tubes containing different combinations of labelled antibodies. Tubes were gently mixed and left in the dark for 30 min. After this time the red cells were lysed and remaining cells were fixed using the Coulter Immunoprep system. Prior to analysis, 250 μl of Isoton II (Coulter Electronics, UK) was added to each tube to ensure good separation of lymphocytes from monocytes when visualized using forward and side scatter characteristics. If cells were not to be analysed immediately they were fixed with 250 μl of 1 % paraformaldehyde, stored in the dark at 4 °C and analysed within 24 h.

Flow cytometry

Cells were analysed on a Coulter Epics XL flow cytometer equipped for four colour analysis. Prior to analysis the instrument was calibrated for optical alignment, and fluorescence intensity using Immunocheck and Immunobrite fluorescent microspheres (Coulter Electronics, UK). Fluorochrome-labelled cells were excited with a 488 nm laser and FITC and PE emission spectra were identified with FL1 and FL2 channel detectors respectively, with suitable compensation based on isotype control samples. The lymphocytes and monocytes were identified by their forward and side scatter characteristics and by CD14CD45 backgating, and 2,000–10,000 events within this gate were analysed.

Time course assay

Whole blood was incubated with 1, 10 or 100 μg ml^{-1} of endotoxin or 1, 10 or 100 μg ml^{-1} of human serum albumin (HSA) or PBS. Aliquots were removed at 0, 0·5, 1, 2, 4, 5, 6 and 24 h, and labelled with CD14 or isotype control. A gating control of CD14/CD45 was carried out at 0 and 24 h to optimize the purity of the CD14+ CD45+ monocyte population in the cell sample investigated.

Endotoxin effect after 4 h

The experiment was then repeated on eight other blood samples using 10 μg ml^{-1} endotoxin, 10 μg ml^{-1} HSA or PBS, and incubated for 4 h. Gatechecks were carried out for every sample so that CD45 on monocytes and lymphocytes could be compared as well as the purity of the cells.

Statistical analysis

Significant differences between treatments were determined using paired Student's *t*-test. The C-stat statistical package was used for the calculations.

Results

CD14 time course

During exposure to LPS, CD14 expression on monocytes increased after 30 min (figure 1) with all three concentrations tested peaking between 1 and 4 h, after which levels of CD14 dropped. The PBS and HSA controls showed a slight increase in CD14 expression at 30 min but then levels then remained constant for 24 h. After 5 h, levels of monocyte CD14 expression in response to LPS dropped. Furthermore, the monocyte population identified by $CD14^{+}CD45^{+}$ staining of putative monocytes and lymphocytes disappeared after 24 h in the endotoxin-treated samples, but not PBS- or HSA-treated samples (figure 2).

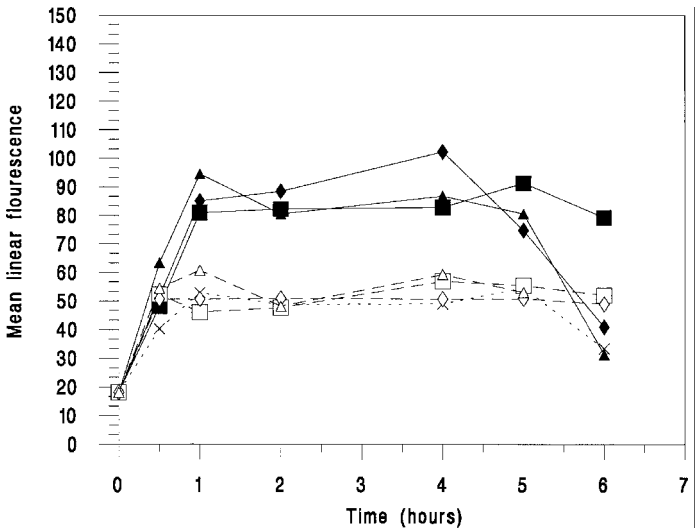

Figure 1. Time course of CD14 expression on monocytes in human whole blood after up to 6 h exposure to PBS, HSA or endotoxin. Monocytes were stained with PE-anti-CD14 monoclonal antibody and mean linear fluorescence was measured by flow cytometry. ×, PBS; ■, LPS (1 μg ml^{-1}); ♦, LPS (10 μg ml^{-1}); , LPS (100 μg ml^{-1}); □, HSA (1 μg ml^{-1}); ◈, HSA (10 μg ml^{-1}); △, HSA (100 μg ml^{-1});

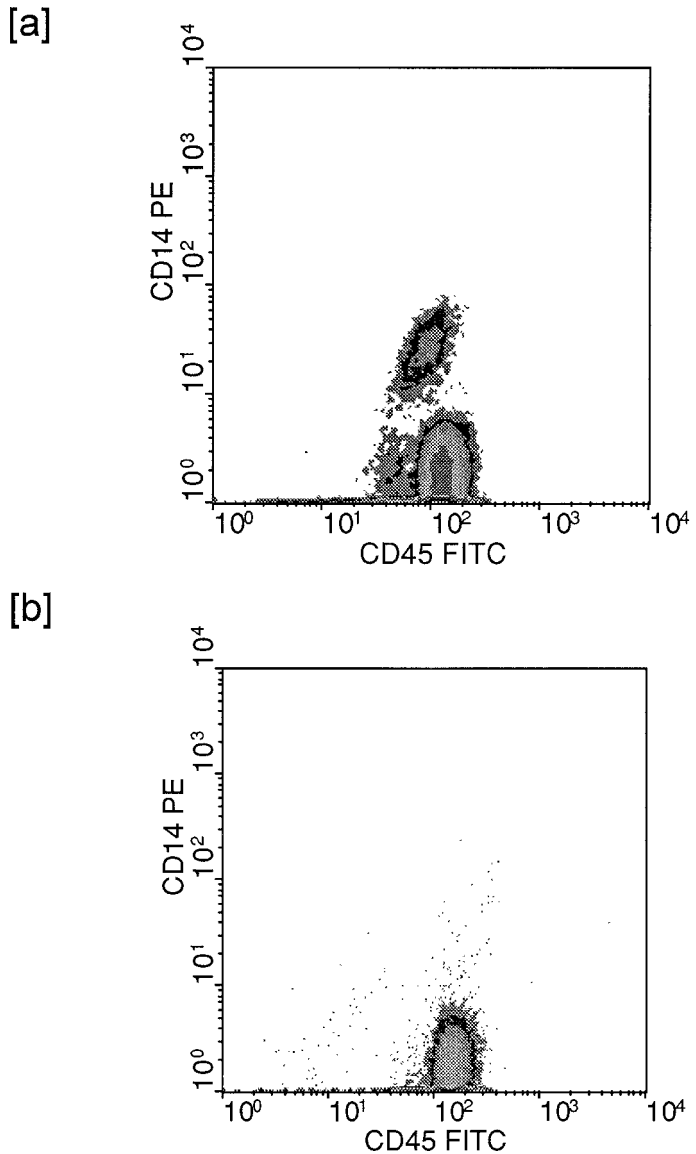

Figure 2. Density dot plot of $CD14^+CD45^+$ cells in human whole blood samples, cells were stained with Dual Test CD14-PE/CD45-FITC and a gate used to select $CD14^+CD45^+$ cells. a: Cells exposed to PBS 24 h, monocytes and lymphocytes. b: Cells exposed to LPS 24 h, the monocyte population is no longer present.

CD14 and CD45 expression after 4 h endotoxin exposure

The upregulation of CD14 on monocytes in response to endotoxin exposure was highly significant when compared with both PBS ($p = 0{\cdot}0002$) and HSA ($p < 0{\cdot}0001$) treated cells (figure 3). CD45 expression on monocytes was also significantly upregulated when compared with PBS ($p < 0{\cdot}0001$) and HSA ($p < 0{\cdot}0001$).

CD45 upregulation in response to endotoxin was monocyte specific, since there was no significant difference in expression of CD45 on lymphocytes following

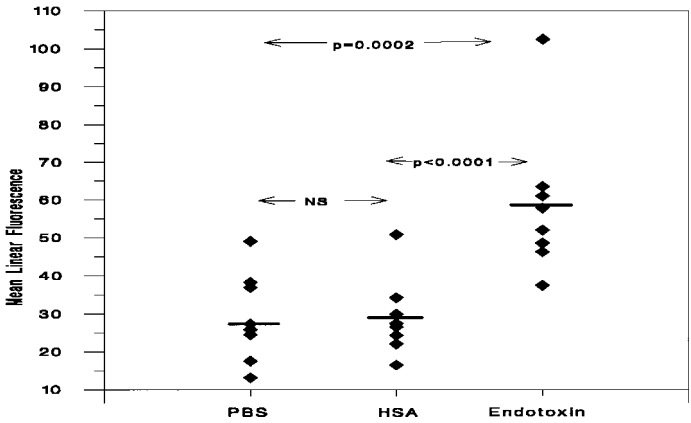

Figure 3. CD14 expression on monocytes in human whole blood after 4 h exposure to PBS, HSA or endotoxin. Monocytes were stained with PE-anti-CD14 monoclonal antibody and mean linear fluorescence was measured by flow cytometry. A bar indicates the mean of the mean linear fluorescence (MLF). The statistical significances of the results are shown by the p values determined using paired Student's t-tests. NS—no significance.

either PBS ($p = 0{\cdot}6$), or and HSA ($p = 0{\cdot}3$) treatment compared with endotoxin-exposed cells.

Discussion

We have developed an *in vitro* challenge model using whole blood and have shown specific upregulation of CD14 and CD45 on monocytes following endotoxin exposure.

During exposure to LPS, CD14 expression on monocytes increased after 30 min and peaked between 1 and 4 h, after which levels of CD14 decreased. Although this could be explained in part by the disappearance of the monocyte population, the levels on the remaining monocytes had dropped to basal levels. This suggests that the monocytes removed the endotoxin rapidly or internalized the receptors. The reasons for the disappearance of the monocyte fraction are not clear. The cells could have undergone apoptosis as a result of endotoxin processing and it may be that this effect could contribute to the long-term health effects caused by endotoxin exposure. The increase in CD14 expression on monocytes exposed to LPS was very clear compared with cells stimulated with control materials. We needed to ensure that LPS was not having a non-specific effect on all cell types so we also investigated CD45 expression on monocytes and lymphocytes. CD45 expression on monocytes also increased but CD45 expression on lymphocytes did not change in response to LPS exposure. This showed that the increase in CD14 and CD45 was a monocyte-specific response and not due to non-specific change in peripheral blood cells in response to LPS exposure. Unlike CD14, CD45 is not an endotoxin-specific marker. This increase in non-endotoxin specific markers such as CD45 could explain the symptoms of endotoxin exposure, e.g. organic dust toxic syndrome (ODTS). We cannot explain the disappearance of monocytes after 24 h of LPS exposure. The disappearance of monocytes after 24 h cannot be explained by adherence to the container walls since they were still present in both the PBS- and HSA-treated samples. Therefore their disappearance was a direct result of LPS exposure. The mechanisms for this effect cannot be determined from these studies.

Previous studies used human alveolar macrophages or purified monocytes to investigate monocyte CD14 expression. Landmann *et al.* (1996) looked at CD14 mRNA, membrane CD14 on monocytes and macrophages and soluble CD14. Serum factors were shown to accentuate the upregulation of CD14 by LPS when Hopkins *et al.* (1995) investigated the surface expression and regulation of CD14 on alveolar macrophages. They found very low levels of CD14 at 24 h which increased over 48 h. However, our *in vitro* challenge model mimics more accurately the events occurring *in vivo* after endotoxin exposure since the endotoxin is presented to the monocytes in a milieu of other cells, serum factors and intracellular messages. Marchant and Delville (1992) investigated CD14 expression on monocytes in whole blood and obtained similar results to those described here. However, these studies did not address the question of the specificity of the endotoxin response with respect to cell type. We have shown that endotoxin only altered the expression of monocyte surface markers since the upregulation of the pan-leucocyte marker CD45 was monocyte-specific and did not occur in lymphocytes.

Jagielo *et al.* (1996) compared physiological and inflammatory response following inhalation of corn dust extract and LPS solution in normal subjects. They found that at equivalent exposure levels of endotoxin (30–60 μg), LPS and corn dust extract result in similar symptoms. They suggest that the concentration of endotoxin in corn dust strongly influences the physiological and biological response and appears to be the principal component in grain dust causing acute airway injury. Other studies (Clapp *et al.* 1994, Schwartz 1996) also suggest that endotoxin may be the principal mediator responsible for the acute inflammation of the airway and development of airflow obstruction in response to inhaled grain dust.

Endotoxin is also found in house dust where it has been related to severity of asthma (Michel *et al.* 1996). Therefore some patients reporting asthma-like symptoms may in fact be suffering from an endotoxin-related respiratory problem causing non-specific respiratory symptoms. At present there is no objective test to distinguish true cases of asthma from non-specific respiratory symptoms caused by repeated exposure to endotoxin.

We propose that the use of flow cytometry to measure CD14 expression on monocytes may serve as a biomarker of response to endotoxin exposure *in vivo* in workers at risk of respiratory ill-health from exposure to endotoxin contaminated aerosols. This is currently under investigation. It may also be a valuable biomarker of response following domestic exposure to endotoxin.

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