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Lack of Neutrophil Degranulation in Low-Dose Endotoxin Inhalation Based on a Novel Intracellular Assay

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SUMMARY To study the nature of endotoxin or lipopolysaccharide (LPS) induced inflammation, we developed a method of quantifying intracellular human neutrophil elastase (HNE) in lysed sputum polymorphs as a means to study the degranulation status of LPS-recruited neutrophils. Induced sputum, blood and exhaled nitric oxide (NO) were collected from 10 healthy non-atopic human subjects after inhaling a single 15 μg dose of *Escherichia coli* LPS in an open study. At 6 hours, LPS inhalation caused significant increase of sputum and blood neutrophils but without parallel increase in myeloperoxidase, HNE or interleukin-8 (IL-8) in sputum sol and blood, or exhaled NO. Intracellular HNE in lysed sputum polymorphs or purified blood neutrophils did not show any significant changes between inhaled LPS and saline, nor was there any appreciable change in percentage HNE release induced by N-Formyl-Met-Leu-Phe (fMLP) *in vitro*. We concluded that in healthy humans, the transient neutrophilic inflammation induced by a single dose of inhaled 15 μg LPS is mainly characterized by cell recruitment, not enhanced secretion of granular mediators or increased exhaled NO based on our experimental conditions.

Inhaled bacterial endotoxin is implicated in the pathology of pulmonary diseases such as organic dust lung diseases,[1,2] acute lung injury,[3,4] and worsening of asthma.[5,6] Studies using inhaled endotoxin or its pure derivative, lipopolysaccharide (LPS) in healthy subjects[7-9] and asthmatic patients[10-12] have shown that the inflammatory nature in lung is predominantly neutrophilic. Neutrophils are phagocytes that after internalizing foreign pathological particulate, such as LPS, generate a variety of toxic products and enzymes such as superoxide anion, nitric oxide and neutrophil elastase to digest the foreign invader. However these products can also be damaging to host tissue if such response is exaggerated or not counteracted by neutralizing enzymes.[13] Therefore what normally constitute a protective immune response may become pathological to host under certain conditions. This has been the hypothesis of lung damage for some neutrophil-driven pulmonary disease such as chronic obstructive pulmonary disease (COPD)[14] and adult respiratory distress syndrome.[15,16]

To further our understanding of endotoxin-induced lung injury, we developed a novel method of quantifying intracellular human neutrophil elastase (HNE) in lysed polymorphs obtained from induced sputum. Such quantification allowed a means of objectively assessing whether active degranulation had

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occurred. Several studies using the approach of induced sputum had suggested a lack of evidence for active neutrophil degranulation based on the observation that the levels of neutrophil granular mediators merely paralleled that of neutrophil numbers.[8,9] For the purpose of our study, parallel intracellular experiments were also carried out on autologous blood, and in addition, exhaled nitric oxide (NO), sputum sol and blood myeloperoxidase (MPO), HNE and interleukin (IL)-8 were also measured.

MATERIALS AND METHODS

Subjects

Ten healthy human subjects (mean age [range]: 24 yrs [19-36]; 5 males) were recruited through local contacts. The inclusion criteria were non-cigarette smokers for at least 6 months and if previously smoked, did not exceed 5 pack years; no physician-diagnosed respiratory disease; non-atopic as defined by negative skin prick reactivity to common aeroallergen; normal spirometry and methacholine PC_{20} of > 16 mg/ml. Urine cotinine was measured to confirm the history of non-cigarette smoking.

Study design

This was an open study where subjects attended three visits, each separated by one week. At each visit, induced sputum and blood were collected at 6 hours after inhaling either 0.9% isotonic saline (visits 1 and 3) or LPS (visit 2) via a breath activated dosimeter (Mefar MB3 dosimeter, Brescia, Italy). This time point was chosen based on the approximation to the onset of 'flu-like' symptoms in healthy human subjects from our earlier studies[17] and findings of neutrophilia at such time point.[9,12] Also at each visit, exhaled NO was measured before and hourly (up to 8 hours) after inhalation with LPS or isotonic saline. This study was approved by local hospital ethics committee and written informed consent was obtained from all subjects.

Inhalation of LPS

In an earlier dose-related study using 15 μg and 50 μg LPS (Sigma, Poole, UK) doses of ours[17] and others,[9] 'flu-like' symptoms induced by the lower dose was shown to be less severe and more tolerable compared to the higher doses. Furthermore, activated sputum CD14+ neutrophils had been shown to be present at this time point at inhalation level of 5 μg LPS,[12] providing the assurance that the study of neutrophil activation at this dose is feasible. The dose of 15 μg LPS was chosen primarily on these reasons. Each LPS solution was freshly made up prior to each inhalation using the powder formulation of *Escherichia coli* serotype 026:B6 (Sigma, Poole, UK) diluted with 0.9% pyogen-free normal saline. Based on a 10 μl volume delivered per inhalation via dosimeter, five inhalation of a 0.25 mg/ml LPS solution would deliver 15 μg LPS.

Sputum induction and processing

Hypertonic saline at concentrations of 3%, 4% and 5% was inhaled via an ultrasonic nebulizer (DeVilbiss Ultra-Neb 2000, Heston, Wollaston, UK) sequentially for 7 minutes each.[18] After each 7-minute period, subjects were asked to rinse and gargle mouth and throat to get rid of saliva and other debris before coughing into a sterile container. Monitoring of FEV_1 during each inhalation was performed for safety reason. We had intentionally omitted pre-treatment with short-acting inhaled β_2-agonist in order to avoid any possible modifying effect on LPS-induced airway inflammation.

The sputum was selected from saliva and processed within 2 hours.[19] Sputum was first homogenized by adding four volumes of freshly made 0.1% dithiothreitol (DTT) (Sputolysin, Calbiochem Ltd, Nottingham, UK) that was then added equal volume of Modified HBSS (without Ca^{2+}/Mg^{2+}) (Sigma, Poole, UK). The cell suspension was filtered through a 48 μm nylon gauze (BBSH Thompson, Scarborough, Ontario, Canada) and the filtrate centrifuged at 1,890 x *g* for 4 minutes at room temperature. The supernatant (sputum sol) was aspirated and stored at -70°C for future assay while the cell pellet was re-suspended with HBSS. Total cell count, cell viability and leukocyte count were determined using trypan blue exclusion and kimura stain. Nine aliquots of 20 μl sputum cell suspension at concentration of 2 x 10^6 polymorphs/ml were then collected for the later preparatory stages of measuring intracellular HNE. The remaining cell suspension was finally adjusted to 0.5 x 10^6 cells/ml and placed into cups of

Shandon III cytocentrifuge (Shandon, Inc. Pittsburgh, PA, USA) to make cytoslides. After air-dried, cytoslides were stained with Wright's Giemsa for differential cell count on at least 400 non-squamous cells. These cytoslides were counted by two independent observers. For these experiments, any vigorous handling of sputum samples such as vortexing was strictly prohibited in order to minimize the theoretical risk of causing neutrophil activation.

Myeloperoxidase (MPO), HNE and interleukin-8 (IL-8) in sputum sol and serum

MPO, HNE and IL-8 are well-documented as mediators released by neutrophil during the final stages of activation.[13] As such, they were used as indication for neutrophil degranulation and activation. MPO and IL-8 levels was measured by radioimmunoassay (Pharmacia & Upjohn Ltd, Milton Keynes, UK) and colorimetric enzyme immunoassay (R & D Systems, Abingdon, UK), respectively, according to standard protocols. Their sensitivity limits were 8 ng/ml and 31.2 pg/ml, respectively. HNE activity was estimated based on a colorimetric reaction assay where the rate at which HNE cleaved a nitroanilide substrate to form a coloured product was measured. The method has the sensitivity limits of 0.1 mOD/minute.

Separation of neutrophils from whole blood

Nine milliliters of venous blood was gently transferred into a 15 ml polypropylene tube containing 1 ml of 2.7% EDTA (Sigma, Poole, UK). The mixture was then added to 10 ml of dextran solution (Hespan®) in a 50 ml centrifuge tube. After leaving to sediment for 30-40 minutes at room temperature, erythrocytes were removed and then the white cell-rich supernatant collected and centrifuged at 693 x *g* for 7 minutes at 8°C, to minimize neutrophil activation. After this, supernatant was discarded and the remaining whole cell pellet was mixed with 5 ml of 55% Percoll (Sigma, Poole, UK). This suspension was carefully poured onto a gradient of 3 ml of 70% Percoll upon 5 ml of 81% Percoll and centrifuged at 1,543 x *g* for 25 minutes at 8°C to collect the neutrophil cell layer. These cells were washed once with modified HBSS (without Ca^{2+}/Mg^{2+}) at 926 x *g* for 6 minutes at 8°C and total cell count and viability determined with trypan blue exclusion and kimura stain. After another wash at 926 x *g* for 6 minutes at 8°C, the neutrophils was re-suspended with HBSS (with Ca^{2+}/Mg^{2+}) to make nine aliquots of 20 µl cell suspension at concentration of 2 x 10^6/ml ready for the preparatory stages of measuring intracellular HNE activity.

Intracellular HNE activity in sputum polymorphs and blood neutrophils

All 20 µl cell suspension aliquots were assayed in triplicates. For each sputum and blood, one set of triplicates was stored at -70°C without any further addition. These samples reflected the basal measurement of intracellular HNE level (i.e. without *in vitro* stimulation). Another two sets of triplicates for each sputum and blood preparation were added 50 µl of HBSS, 10 µl of 10 U/ml adenosine deaminase (Sigma, Poole, UK) and 10 µl of Cytochalasin B (50 µM) and then incubated in a shaking water bath at 37°C for 5 minutes. After this, one set was added 10 µl of 1,000 nM N-Formyl-Met-Leu-Phe (fMLP) while the other, 10 µl of 1% DMSO as control. They were then incubated for another 5 minutes and centrifuged at 700 x *g* for 6 minutes at 8°C. After this, the supernatant was removed and the cell pellets stored at -70°C pending measurement of intracellular NE activity.

Similar to estimating HNE activity in fluid phase described above (i.e. sputum sol and serum), a colorimetric reaction assay was used to determine the intracellular HNE level in sputum and blood neutrophils after cell lysis. The thawed samples were analyzed in triplicate in a 96-well microtiter plate. They were firstly incubated for 5 minutes at 37°C with 60 µl of 0.1% HTAB (Sigma, Poole, UK) to lyse cells and then added 20 µl of 30 mM neutrophil elastase substrate-I (CalBiochem Ltd., Nottingham, UK). The plate was then placed in the kinetic plate reader at 37°C, shaken for 5 seconds and the HNE activity rate measured at OD_{405nm} at intervals of 20 seconds for 5 minutes. As before, the corresponding V_{max} and the HNE endpoint (at the end of the kinetic run), were calculated by the computer software SoftMax Pro. For control experiments, triplicate of 20 µl HBSS and 20 µl neutrophil elastase substrate-I, used as blank and standard respectively were also ana-

lyzed in parallel. For intracellular experiment, we used HNE endpoints to express all results and they were expressed as standard HNE equivalents (ng) normalized to 1 x 10^6 polymorphs (PMN). For *in vitro* stimulation study, the results were expressed as percentage release of HNE induced by fMLP in cytochalasin B-primed neutrophils, compared to control (cytochalasin B alone).

Exhaled NO

Exhaled NO was measured by chemiluminescence analyzer (Model LR2000; Logan Research, Rochester, UK), with sensitivity from 1 ppb to 100 ppm of NO, accuracy ± 0.5 ppb, and response time of < 2 seconds to 90% of full scale. The sampling rate was 250 ml/minute for all measurements. Ambient air NO levels were recorded and the absolute zero were adjusted prior to all measurements. Subjects exhaled slowly from total lung capacity over 20 to 30 seconds with exhalation flow 5 to 6 l/minute, bypassing the analyzer and thus with minimal resistance to flow. NO was sampled from a side-arm attached to the mouth piece. The mean value of the last 100 measurements, acquired with 0.04 seconds intervals, was taken from the point corresponding to the plateau of end-exhaled (CO_2 reading 5 to 6%) to represent the lower respiratory tract sample. Results of the analyses were computed and graphically display on a plot of NO and CO_2 concentrations, pressure, and flow against time.

Data analysis

All results were expressed as mean and standard error. Significance of difference at different time points before and after LPS inhalation was studied using repeated measures analysis of variance (ANOVA). Where significant variation was found, Dunnett's multiple comparison was used to investigate the significance of change between time points. All statistical analysis and graphic representation of data were carried out on the GraphPad Prism™ graphic and statistical package (PC Windows® version). For all statistical tests, $p < 0.05$ was considered significant.

RESULTS

At 6 hours, LPS inhalation caused significant increase of sputum total leukocytes (mean [95% CI] increase: 12.2 [6.3 to 18.1] x 10^6 cells/ml; $p < 0.001$ and neutrophils (mean [95% CI] increase: 13.3 [6.2 to 20.4] x 10^6 cells/ml; $p < 0.002$), compared with inhaled isotonic saline (baseline visit 1). There were also a similar increase, albeit smaller, in blood total leukocytes (mean [95% CI] increase: 6.1 [4.8 to 7.4] x 10^6 cells/ml; $p < 0.0001$) and neutrophil (mean [95% CI] increase: 6.6 [5.1 to 8.0] x 10^6 cells/ml; $p < 0.0001$], compared to inhaled saline (baseline visit 1). These changes were resolved by one week to levels comparable to baseline (Fig. 1).

In sputum sol, there was no significant difference in MPO (mean [95% CI] difference = 139 [-253 to 533] ng/ml), HNE activity (2.5 [-1 to 6.3] mOD/minute) or IL-8 (201 [-1,668-2,071] pg/ml) between baseline and 6 hours after LPS inhalation. In serum, there was also no significant difference in MPO (mean [95% CI] difference: 95 [-32 to 222]

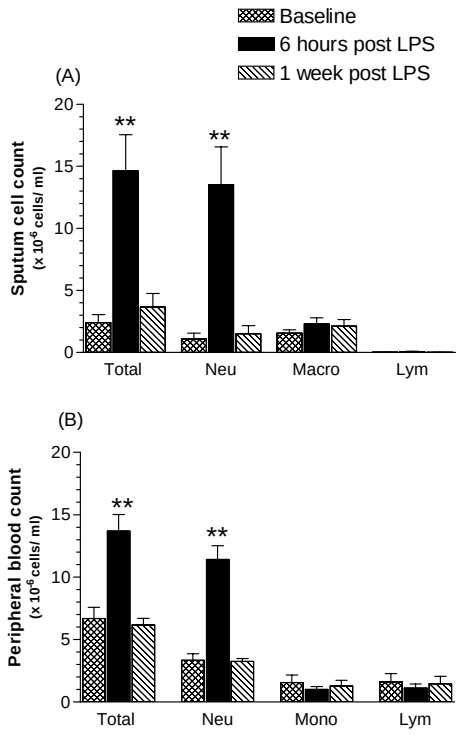

Fig. 1 Effects of inhaled LPS on (A) sputum total and differential cell counts and (B) peripheral blood total white cell and differential counts. Bars represent means and standard errors. $^{**}p < 0.01$ compared to baseline (i.e. one week prior). Total = total cells; Neu = neutrophils; Macro = macrophages; Lym = lymphocytes; Mono = monocytes.

ng/ml). All serum HNE activity and all serum IL-8 level (except two) were below quantifiable levels (BQL). There were also no significant changes in these parameters at one week when compared to baseline or 6 hrs after LPS inhalation (Fig. 2).

Compared to baseline, HNE in lysed sputum polymorphs collected at six hours (mean [95% CI] difference: 357 [-170 to 462] ng/10^6 polymorphs) and one week (184 [-279 to 372] ng/10^6 polymorphs) after LPS inhalation was not significantly changed. Similarly there was no significant difference in lysed purified blood neutrophils collected between baseline and 6 hours after LPS inhalation (mean [95% CI] difference: 376 [-195 to 521] ng/10^6 polymorph) and between baseline and one week after LPS inhalation (612 [-213 to 928] ng/10^6 polymorphs). Percentage release of HNE induced by fMLP in sputum and blood neutrophils primed with cytochalasin B also showed no appreciable changes at baseline, 6 hrs and one week after LPS inhalation (Fig. 3).

With regards to exhaled NO, there was no significant increase in the immediate period or one week after LPS inhalation, when compared with one-week prior where saline inhalation was used as control. The one exception was a statistically significant difference of exhaled NO recorded at 6th hour between the first visit and the ensuring two visits (Fig. 4).

DISCUSSION

We have shown that inhalation of a single dose of 15 μg LPS evoked significant but transient (i.e. not present after one week) neutrophilic inflammation in airway and to a lesser extent, blood in healthy human subjects. Based on the same study condition, others using inhalation doses between 5 and 50 μg LPS also documented such findings.[8-12] Regarding the release of granular mediators, Michel *et al.*[9] and Thorn *et al.*[8] reported a normal or reduced ratios of neutrophil mediators over neutrophil number, following inhaled LPS at doses up to 50 μg, suggesting that active degranulation had not occurred. We have similarly showed that at LPS dose of 15 μg, there were also no parallel increases of MPO, HNE or IL-8 in sputum sol or blood. On this aspect, we have corroborated these findings by showing that there were no appreciable changes in intracellular HNE with or without *in vitro* stimulation with fMLP, based on our novel method using lysed sputum polymorphs.

Employing the sputum method that measures cell numbers and soluble mediators during different stages of sputum processing,[8-10] the comparison of

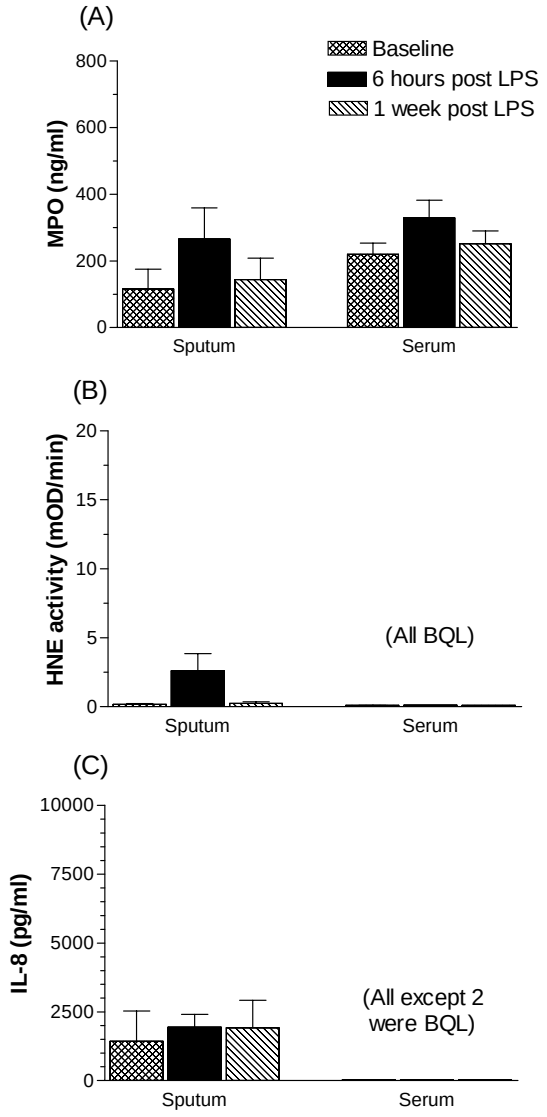

Fig. 2 Effects of inhaled LPS on sputum sol and serum (A) myeloperoxidase (MPO) (B) human neutrophil elastase (HNE) activity and (C) interleukin (IL)-8. Bars represent means and standard errors. Note that all serum HNE activity and all serum IL-8 level (except two) were below quantifiable levels (BQL).

cell and mediators may be inaccurate. For this reason, estimation of intracellular HNE by cell lysis in a specified number of neutrophils may provide a more accurate picture on the degranulation status of phagocytic cells. The assumption is that if significant increase in neutrophil degranulation had occurred, there would be a difference in intracellular HNE in neutrophils recruited by inhaled LPS, compared to those by inhaled saline. Any changes of basal intracellular HNE should also parallel to changes in terms of percentage release of mediators by stimulation with fMLP *in vitro*. The findings from our experiment on sputum and autologous blood suggest that they are phenotypically not dissimilar.

Experiments based on measurement of intracellular granular mediators in lysed sputum cells have previously been reported. Gibson *et al.*[20] described the measurement of eosinophil cationic protein in lysed sputum cells from asthmatic and COPD patients as an index of eosinophil numbers. To our knowledge, we are the first to describe a method of measuring intracellular HNE in lysed sputum polymorphs to study neutrophil degranulation status. HNE is chosen on the basis that it is specific for neutrophils, and this is especially relevant since we did not separate out neutrophils from the whole population of sputum granulocytes. Adenosine deaminase (AD) was added in the experiments to inactivate any naturally occurring adenosine, as these molecules are capable of modulating neutrophils in their superoxide anion production. Consequently, the addition of AD would ensure that any observed changes in intracellular HNE were attributable to the effect of fMLP alone. To further optimize the experiment, cytochalasin B was used to prime neutrophils to the effects of fMLP and that a high concentration of fMPL was used (i.e. 1,000 nM).

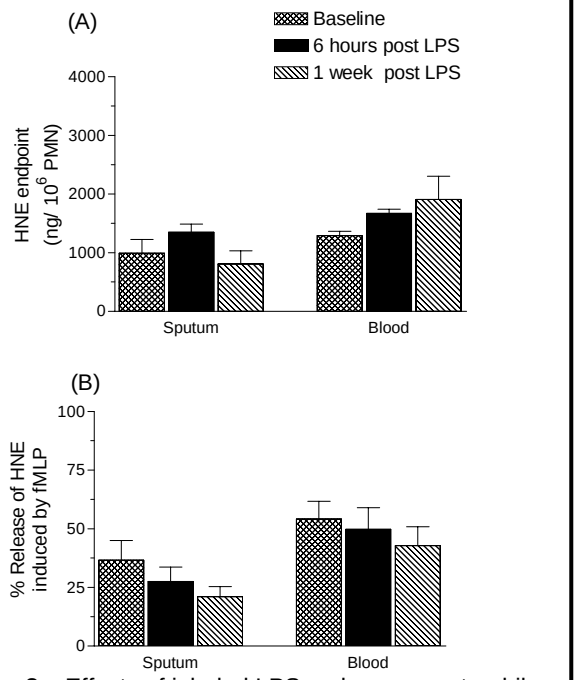

Fig. 3 Effects of inhaled LPS on human neutrophil elastase (HNE) in lysed sputum polymorphs and purified blood neutrophils (A) without and (B) with *in vitro* stimulation with fMLP. Bars represent means and standard errors. HNE activity endpoints were normalized to ng per 10^6 polymorphs (PMN). Results following *in vitro* stimulation are expressed as percentage HNE release induced by fMLP in cells primed with cytochalasin B, compared those with cytochalasin B alone.

The inherent weakness in our intracellular experiment is that we are unable to ascertain the degree of cellular activation that may have been inadvertently caused by the handling process. We specifically avoided any form of vigorous handling of sputum sample such as vortex, and kept low the speed and temperature for centrifugation. We made the assumption that these effects are negligible.

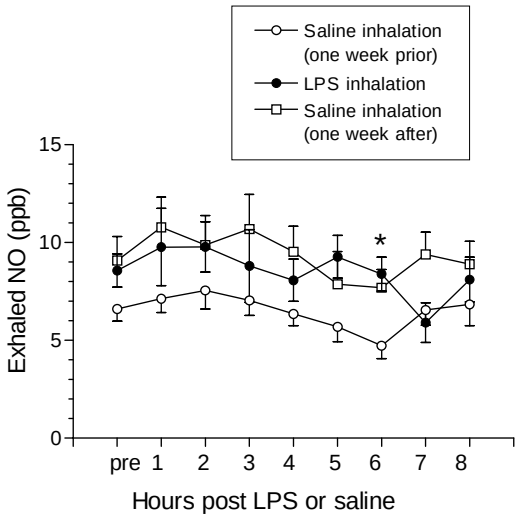

Fig. 4 Effects of inhaled LPS or saline on exhaled nitric oxide (NO) over an eight-hour period. Symbols represent means and standard errors. *$p < 0.05$ between saline inhalation (one week prior) *vs.* LPS inhalation.

DTT, a reducing mucolytic agent used to improve cell dispersion in sputum processing, can affect measurements of some mediators.[21] The concern that DTT treatment would significantly affect our experiments was largely dispersed when we showed that there were no significant differences of intracellular findings between parallel DTT- and saline-treated sputum specimens obtained from 7 subjects during our earlier validation experiments (data not shown).

The implications of our findings on LPS-induced inflammation are confined to that of 15 μg single LPS inhalation at 6 hours in healthy human subjects. A recent landmark study by O' Grady *et al*.[11] using segmental bronchial challenge with LPS or saline in health human subjects at different time points, demonstrated two distinct phases of inflammation. The early phase (from 2 to 6 hours) was characterized by an influx of neutrophils accompanied with a wide array of chemokines including IL-8. The later phase (from 24 to 48 hours) consisted of the addition of macrophages, monocytes, lymphocytes and a return to basal levels of the majority of the earlier mediators. This study elegantly illustrated a qualitative and temporal inflammatory profile in healthy human caused by LPS inhalation. Our inability to show any increase in airway IL-8 following inhaled LPS is most likely due to the method of induced sputum being a lesser efficient method to detect small increases of some mediators.

Regarding neutrophil activation, several issues are noteworthy. Firstly, our intracellular experiments primarily address the question of granular mediators release from cells. This generally represents the final stages of neutrophil activation and not other phases of neutrophil activation such as those in recruitment and priming.[13] Intuitively, this final phase of activation may be more clinical relevant since these granular mediators are proteolytic enzymes. Secondly, our findings that show a lack of evidence for degranulation could not be extrapolated to those of higher doses of inhaled LPS. Generally, the effects of inhaled LPS are dose-related[9,22] and it is very possible that active degranulation can occur at higher doses of LPS. Our decision to study LPS effect at dose of 15 μg has been explained earlier. Finally, our findings of inflammatory changes returning to basal levels at one week has important implication, because activated neutrophils have been shown to perpetuate further neutrophil recruitment, by a positive autocrine feedback loop mediated by chemokines such as IL-8, independent of initiating stimulus of the inflammatory response in disease state.[23] This view may partly explain the difficulty in correlating microbiologic findings and inflammation during the clinical episodes of infection.[24]

Exhaled NO has been advocated as a marker for airway inflammation.[25] Although studies had shown that exhaled and nasal NO are raised following inhaled LPS in subjects with airway hyperresponsiveness[26] and in healthy subjects underwent nasal challenge with endotoxin-containing dust from swine confinement,[27] we could not demonstrate any appreciable increase after inhaled LPS at 15 μg dose in normal healthy subjects. NO exhalation may be mediated by neutrophil during their respiratory burst, an event important in activation and destruction of ingested microbial products,[13] or alternatively, mediates neutrophils recruitment.[28] Our finding at 6 hours after saline inhalation in the first visit of abnormally low records of exhaled NO (Fig. 4) could not be fully explained except for technical causes.

To reiterate, our study addresses LPS-induced airway inflammation at a single 15 μg inhaled dose in subjects who are healthy. However, it remains to be researched as to what nature of endotoxin exposure in healthy human that is required to cause a disease state and whether there is a certain threshold of inappropriate LPS-induced neutrophil activation that would become pathological.

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