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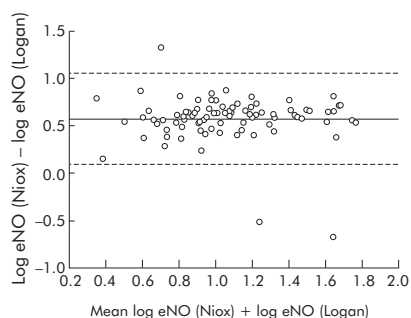
## LETTERS TO THE EDITOR

### Comparison of measured exhaled nitric oxide at varying flow rates

Altered levels of exhaled nitric oxide (FeNO) have been well documented in a number of conditions, although it is in asthma that this phenomenon has been most extensively investigated.<sup>1</sup> Raised FeNO levels in patients with asthma have been correlated not only with other markers of airway inflammation (including induced sputum eosinophil count), but also with airway hyperresponsiveness and response to inhaled corticosteroids.<sup>2</sup> Furthermore, the detection of a raised FeNO level has been shown to have a positive predictive value of up to 95% for the diagnosis of asthma.<sup>3</sup>

A number of factors can influence the production and measurement of FeNO including airway calibre, caffeine, smoking and, in particular, respiratory flow rate. A standardised flow rate of 50 ml/s has recently been adopted by both the European Respiratory Society and the American Thoracic Society; however, to date, there has been a discrepancy in the rates used by clinicians and researchers worldwide.<sup>4</sup> The Logan LR 2000 chemiluminescence analyser (Logan Research Ltd, UK) uses a mouth flow rate of 250 ml/s to measure FeNO while the Niox® Nitric Oxide Analyzer (Aerocrine AB, Sweden) uses a flow rate of 50 ml/s. Both analysers use online measurements to calculate FeNO, express the results in parts per billion (ppb), and have similar accuracies.

Few data are available to allow direct comparison between the two analysers and hence flow rates. This can make comparison of studies using the different methods difficult. We have prospectively analysed the FeNO from asthmatic (n = 63) and non-asthmatic (n = 29) adult patients with both devices in a head to head fashion. The geometric mean for the FeNO using the Niox analyser was 25.6 (95% CI 24.4 to 26.8) ppb for asthmatics and 16.8 (95% CI 15.6 to 18.0) ppb for non-asthmatics (p < 0.01). With the Logan LR 2000 the values were 6.8 (95% CI 5.6 to 8.0) ppb in asthmatics and 4.4 (95% CI 3.1 to 5.7) ppb in non-asthmatics (p < 0.01). The pooled data from the Niox and Logan LR 2000 were found to be closely correlated to



**Figure 1** Comparison of eNO values with the Niox and Logan analysers

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one another ( $r^2 = 0.62$ ,  $p < 0.001$ ). Altman-Bland plots of the data obtained support the suggestion that there is a high level of agreement between the two methods (fig 1). This agreement is retained when subgroup analysis of asthmatics and non-asthmatics is performed. The slightly better discrimination between asthmatics and non-asthmatics at lower flow rates (shown by the well separated confidence intervals) may be partly because of the improved FeNO plateau at this rate.

These results suggest that data obtained using either flow rate are valid, and the methods demonstrate a strong degree of correlation. This is an important confirmatory analysis as it facilitates comparison of results obtained by the two techniques, both previously and in ongoing clinical trials using flow rates which differ from that recently recommended.

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## Local IFN- $\gamma$ responses in TB

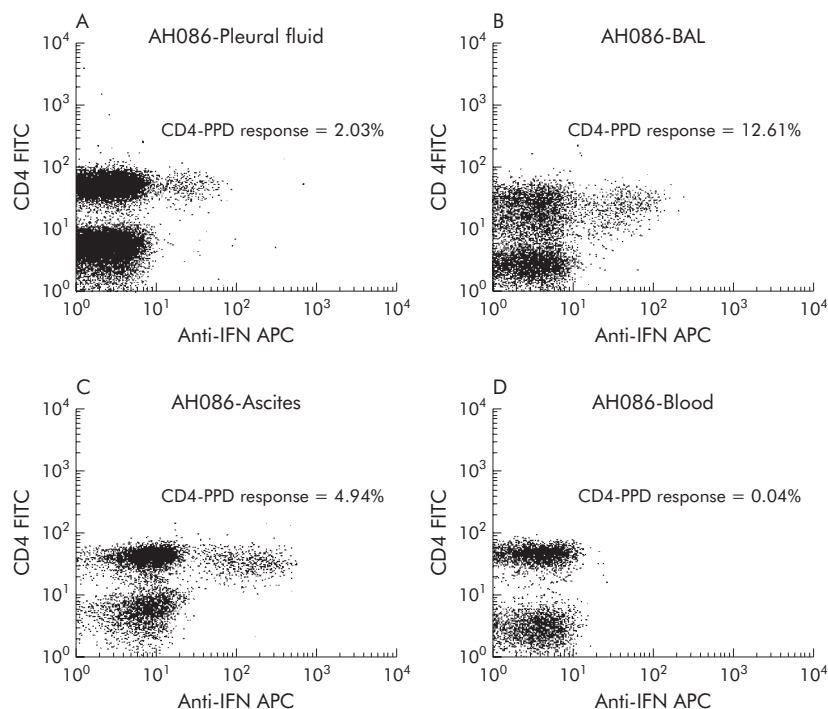
Globally, the tuberculin skin test (TST), smear microscopy, and culture remain central

to the diagnosis of tuberculosis (TB) because of the cost and ease of performance. However, TST has poor specificity and reduced sensitivity in settings including HIV and advanced TB,<sup>1</sup> smear microscopy lacks sensitivity, and TB culture (the diagnostic gold standard) takes weeks and is positive in only two thirds of treated cases.<sup>2</sup> TB pleuritis and peritonitis can be particularly difficult to diagnose due to paucity of bacilli and often need invasive or open procedures. In this setting, assays measuring interferon  $\gamma$  (IFN- $\gamma$ ) production by lymphocytes in response to TB antigens may be useful. While most studies have used blood based assays, more clinically relevant information may exist in local fluids such as bronchoalveolar lavage (BAL) fluid and pleural fluid in which much higher responses have been achieved.<sup>3-5</sup>

We investigated a 32 year old Somali man, resident in Britain for 2 years, who presented with a 3 week history of vomiting, diarrhoea, anorexia, and abdominal pain. On examination he was febrile (38.5°C), tachycardic, and tachypnoeic. No lymph nodes were palpable. A BCG scar was noted. He had signs of a right pleural effusion and ascites with peritonism.

A full blood count showed lymphopenia ( $0.26 \times 10^9/l$ ), normal neutrophils ( $3.6 \times 10^9/l$ ), hypochromic microcytic anaemia (Hb 10.7 g/dl), and a normal platelet count. Hyponatraemia (129 mmol/l (normal range 135-145)), hypoalbuminaemia (28 g/l (normal range 35-50)), and mild hepatitis (aspartate transaminase 121 U/l (normal range 5-40)) were noted. Inflammatory markers were increased as follows: C reactive protein 280 mg/l; erythrocyte sedimentation rate 75 mm/h. An HIV antibody test was negative. A tuberculin skin test was not performed. The CT scan showed a moderate right pleural effusion and small left pleural effusion, small bowel dilation with mesenteric induration, large volume ascites, and small (<1 cm) mediastinal but no abdominal lymph nodes. On abdominal paracentesis, a leucocytosis of 640 cells/ml (75% lymphocytes) with 55 g/l protein and 3.8 mmol/l glucose was found but no organism was identified. The pleural fluid had a protein level of 36 g/l and a glucose level of 6.4 mmol/l.

Bronchoalveolar lavage was performed. Auramine staining of sputum and ascitic, pleural, and BAL fluids was negative. Molecular assays (TB strand displacement assay) were negative from all sites. TB cultures were negative at 8 weeks. The pleural fluid, ascitic fluid, BAL fluid, and peripheral blood were examined for absolute leucocyte and lymphocyte numbers by flow cytometry, as well as lymphocyte phenotypes. The frequency of lymphocytes synthesising IFN- $\gamma$  in response to purified protein derivative of *Mycobacterium tuberculosis* (PPD) was then measured as described previously.<sup>4</sup> The percentage of lymphocytes in BAL fluid, ascitic fluid, and pleural fluid was 10.5%, 79.2%, and 91.1%, respectively. In CD3+ T cells the CD4/CD8 lymphocyte ratios in BAL fluid, ascitic fluid, pleural fluid, and blood were 1.5, 7.7, 2.7, and 2.1, respectively. In the CD4+ T cell populations the frequency of PPD specific IFN- $\gamma$  positive lymphocytes in BAL fluid, ascites, pleural fluid, and



**Figure 1** Proportions of interferon (IFN)- $\gamma$  synthetic CD4<sup>+</sup> T cells in response to purified protein derivative (PPD) after 16 hours of incubation in (A) pleural fluid, (B) bronchoalveolar lavage fluid, and (C) ascitic fluid of a patient with acute TB. These cells are virtually absent in the peripheral blood (D). In these flow cytometric assays the CD3<sup>+</sup> T lymphocytes are gated and shown in the histograms. Of these, the CD4<sup>+</sup> populations contain the IFN- $\gamma$  positive cells (upper left quadrants) while the CD4<sup>-</sup> (CD8<sup>+</sup>) T cells are IFN- $\gamma$  negative.

blood was 12.61%, 4.94%, 2.03%, and 0.04% (fig 1).

A presumptive diagnosis of tuberculous peritonitis was made. The patient was too unwell for exploratory surgery. Empirical antituberculosis treatment with rifampicin, isoniazid, ethambutol, and pyrazinamide was commenced with adjunctive corticosteroids which resulted in rapid resolution of his symptoms and signs. Corticosteroids were tailed off over a few weeks. He continues on rifampicin and isoniazid and remains well.

Although we were unable to obtain histological or microbiological confirmation of the diagnosis in this case, clinical and radiological evidence combined with the treatment response were highly suggestive of TB. The patient had marked lymphopenia which, in HIV negative TB patients, has been associated with advanced TB as well as decreased rates of smear positivity, an increased rate of extrapulmonary disease, and an attenuation of skin test reactivity.<sup>6</sup> Hence, in a setting where traditional diagnostic tools are least effective, blood based immune assays are also likely to be suboptimal due to low CD4 counts and diminished response to antigens. The demonstration of high frequency lymphocyte responses in BAL fluid, pleural fluid and ascitic fluid, despite a negligible response in the blood as observed in our patient, is therefore of great clinical importance. We believe that this case highlights the potential usefulness of immune assays in TB with tissue fluids other than blood. Investigation of a larger patient cohort is warranted to delineate the responses seen in different stages of infection (such as latent and treated TB) and to determine clinically relevant cut

off points which will allow these assays to be used as a diagnostic tool.

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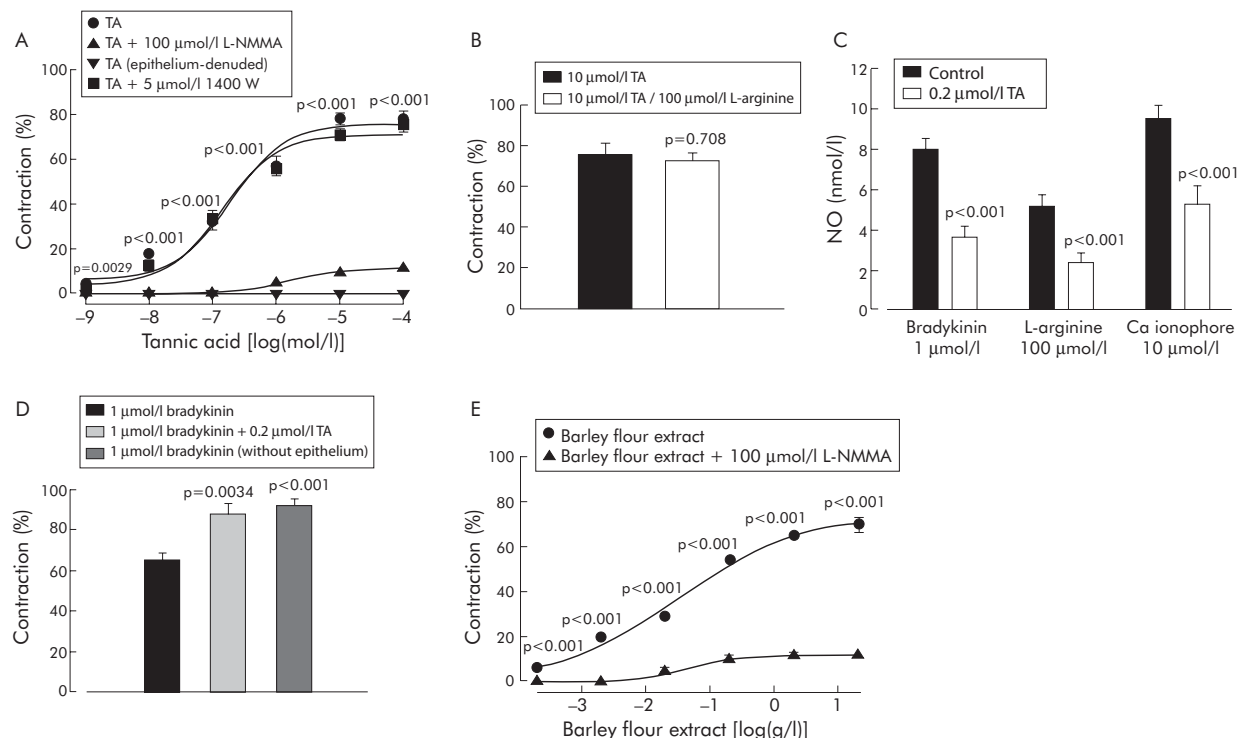
## Tannic acid in plant dust causes airway obstruction

Occupational or environmental exposure to plant dusts has been shown to increase the risk of obstructive lung diseases, primarily by non-immunological activity.<sup>1</sup> However, the causative agents and the underlying mechanisms have not been established. We have recently suggested that the polyphenolic fraction of hydrolysable tannins in plant derived dusts, with tannic acid (TA) as the main constituent, may contribute to airway constriction.<sup>2</sup> Here we present experimental evidence that TA causes acute airway obstruction by non-competitive inhibition of the constitutive endothelial isoform of nitric oxide synthase (eNOS) in the tracheobronchial epithelium, which is reported to provoke airway hyperresponsiveness and bronchoconstriction.<sup>3</sup>

Organ bath experiments were performed using the trachea and main bronchi of non-sensitised guinea pigs. The tracheobronchial tree was dissected out of CO<sub>2</sub> sacrificed guinea pigs of either sex weighing 300–450 g and cut into rings of 3–4 cartilage segments wide. Isometric contractions were recorded as described previously.<sup>4</sup> Briefly, individual rings were mounted in organ baths containing 10 ml carbogen aerated Tyrode solution (pH 7.4, 37°C), kept at a preload of 25 mN, left to equilibrate for 60 minutes, and precontracted by addition of 25  $\mu$ mol/l prostaglandin F<sub>2 $\alpha$</sub>  to 30–40% of their individual isometric maximum (100%).

NO release was determined in real time by an amperometric microsensor as described elsewhere.<sup>4</sup> Briefly, the tracheal and bronchial rings were opened longitudinally and kept in Hepes-Krebs solution (10 ml, pH 7.4, 25°C). The sensor was placed onto the luminal surface at a distance of 200  $\mu$ m. After 30 minutes of equilibration, individual NO reactivity was assessed by addition of 15 nmol/l substance P.

Tannic acid (penta-*o*-digalloyl- $\beta$ -D glucose; Fluka, Seelze, Germany) produced an immediate concentration dependent contraction of the tracheobronchial rings (lasting 30–60 minutes) with a mean EC<sub>50</sub> of 0.19  $\mu$ mol/l (95% CI 0.10 to 0.35) and a maximal response (E<sub>max</sub>) of 85.0% (95% CI 76.6 to 93.4). The threshold concentration of TA eliciting a significant contraction (p = 0.05) was 0.7 nmol/l (corresponding to 1.2 mg/m<sup>3</sup>). The contraction was completely abolished in epithelium denuded rings and by pretreatment with an unselective NOS inhibitor. It was not affected by the presence of an inhibitor of the neuronal and inducible isoforms of NOS (fig 1A), indicating that the TA mediated contraction in non-sensitised guinea pig was entirely due to inhibition of eNOS in the airway epithelium and that TA does not elicit direct effects on tracheobronchial muscle. The contractions were not blunted by addition of the NO synthase substrate L-arginine, which suggests a non-competitive eNOS blockade by TA (fig 1B). This finding also agrees with a biochemical



**Figure 1** Effects of tannic acid (TA) and barley flour extract on muscle tone and nitric oxide formation of guinea pig tracheobronchial rings. (A) Contraction following treatment with cumulatively increasing concentrations of TA after pre-incubation (15 min) with the non-specific nitric oxide synthase (NOS) inhibitor L-N<sup>G</sup>-monomethyl arginine (L-NMMA), after removal of the epithelium, and after pre-incubation (15 min) with the inducible NOS isoform specific inhibitor 1400 W. Contraction in response to a single 10 μmol/l dose of TA compared with subsequent treatment with 100 μmol/l L-arginine. (C) NO release in response to 1 μmol/l bradykinin, 100 μmol/l L-arginine, or 10 μmol/l calcium ionophore A23187 in the absence and after pretreatment (15 min) with 0.2 μmol/l TA, and after removal of the epithelium. (D) Contraction in response to 1 μmol/l bradykinin, after pre-incubation (15 min) with 0.2 μmol/l TA, and after removal of the epithelium. (E) Contraction following treatment with cumulatively increasing concentrations of aqueous barley flour extract (20 g/l) and after pre-incubation (15 min) with L-NMMA. Values represent mean (SE) of five independent experiments each. A p value < 0.05 (two tailed t test with Holm correction for multiple comparisons) was considered statistically significant. p values in (A) and (E) indicate the nominal significance levels of TA and barley flour extract induced contractions compared with the respective contractions in the presence of L-NMMA.

study which reported that TA non-competitively inhibits the eNOS enzyme with a 20–30 fold higher selectivity compared with inhibition of nNOS or iNOS isoforms.<sup>3</sup> A possible TA induced formation of spasmogenic prostanooids was ruled out because the cyclooxygenase inhibitor indomethacin (10 μmol/l) did not reduce vascular tone (data not shown). In keeping with these findings, the epithelial release of NO caused by receptor independent agents (L-arginine, calcium ionophore A23187) as well as receptor dependent eNOS activating agents (bradykinin) was reduced in tissue pretreated with TA (fig 1C). Moreover, the contraction response to stimulation with bradykinin (1 μmol/l) was significantly enhanced when the rings were pretreated with 0.2 μmol/l TA (the EC<sub>50</sub> for contraction). A similar effect was achieved after removal of the epithelium, indicating that the hyperresponsiveness to bradykinin was due to a lack of concomitant bradykinin induced eNOS activation (fig 1D).

We determined concentrations of total hydrolysable tannins in samples of barley flour, oak wood, and green tea dust of 8.7, 11.2 and 5.9 mg/g, respectively, by spectrophotometric detection after reaction with potassium iodate, as described.<sup>6</sup> Acute bronchoconstriction in previously unexposed subjects after challenge with grain dust

concentrations above 100 mg/m<sup>3</sup> may therefore be explained by direct exposure to contractile levels of hydrolysable tannins, while chronic obstructive respiratory symptoms associated with occupational exposure to average inhalable dust concentrations in the range of 5–20 mg/m<sup>3</sup> may result from tannin accumulation in the airway epithelium.<sup>7</sup> In support of the mechanism of dust induced bronchoconstriction proposed here, we found that an aqueous extract of barley flour caused contraction of guinea pig tracheobronchial rings that was blunted by pretreatment with L-NMMA (fig 1E). The threshold concentration for contraction (p = 0.05) was 58.9 μg/l extract, corresponding to 0.5 mg/m<sup>3</sup> hydrolysable tannins.

Other plant phenols (including non-hydrolysable tannins and the hydrolysable tannin monomers gallic acid and ellagic acid) did not alter smooth muscle tone or, conversely, caused relaxation,<sup>4</sup> indicating that acute contractions induced by plant dust are specific for the hydrolysable tannin fraction.

In contrast to our findings, Flesch *et al*<sup>8</sup> reported that TA produced endothelium dependent relaxations of rat aortic rings. In our model we could also record epithelial NO release accompanied by diminished contractions, but this effect required TA concentrations >10 mmol/l above any clinical relevance (data not shown) and was

probably caused by non-specific activities of TA towards the airway epithelium.

In conclusion, hydrolysable tannins may be aetiologically involved in the development of plant dust induced acute and chronic obstructive airway diseases by impairing the endogenous release of bronchoprotective NO.

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