



Carbon loading of alveolar macrophages in adults and children exposed to biomass smoke particles

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Abstract

Exposure to carbonaceous particles from biomass burning is associated with increased respiratory morbidity in both women and children in the developing world. However, the amount of carbon reaching lower airway cells has not been determined in these populations. Alveolar macrophages (AM) remove inhaled particulate matter (PM), and are implicated in the pathogenesis of PM-induced lung disease. In this study, we aimed to compare AM carbon loading in women and children exposed to biomass PM in Gondar, Ethiopia, with individuals exposed to fossil-fuel PM in the developed world (Leicester, UK). To achieve these aims, we sampled AM from Ethiopian mothers and children, and from UK adults and children using induced sputum (IS). AM were imaged under light microscopy, and the total two-dimensional surface area of carbon within each AM determined by image analysis. AM containing carbon were detected in all subjects. The total surface area of carbon per AM was higher in Ethiopian women ($n=10$) compared with UK adults ($n=10$, median 9.19 vs. 0.71 $\mu\text{m}^2/\text{AM}$, $p=0.0002$). Similarly, the total surface area of carbon per AM was higher in Ethiopian children ($n=10$) compared with UK children ($n=10$, 3.32 vs. 0.44 $\mu\text{m}^2/\text{AM}$, $p=0.0002$). However, loading in Ethiopian children was lower than paired maternal levels (3.32 vs. 9.19 $\mu\text{m}^2/\text{AM}$, $p=0.011$). We conclude that analysis of AM obtained by induced sputum is a practical way of quantifying natural exposure of the lower airway to carbonaceous particles from the burning of biomass fuels.

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1. Introduction

For the world's poorest populations, the burning of biomass fuels such as wood, animal dung, and crop

residues, results in levels of exposure to inhalable carbonaceous particulate matter (PM) that are an order of magnitude above the health-based guidelines of the developed world (Ezzati and Kammen, 2001). This widespread exposure to PM from biomass smoke is estimated to cause two million excess deaths per year (Bruce et al., 2000), a major proportion of these in young children (Smith et al., 2000). To date, quantification of the amount of carbon particles

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reaching lower airway cells in these vulnerable individuals has not been reported.

Particles that evade pulmonary mucociliary defences are removed by airway macrophages (AM), the major phagocyte in the bronchi and alveoli (Geiser, 2002). Since AM reside exclusively in the lower airway, and particles of carbon are not formed in vivo, black material within the cytoplasm of AM must be derived from inhaled PM. Indeed, we have previously used the ability of AM to engulf and retain inhaled material to prove that carbonaceous PM penetrate the lower airway of healthy UK infants exposed to low levels of fossil-fuel derived PM (Bunn et al., 2001). The amount of phagocytosed particles in AM also provides insights into the level of exposure to ambient PM. For example, light microscopic analysis of AM from Mexican dogs has demonstrated that those living in areas of high ambient PM $<10 \mu\text{m}$ (PM_{10}) have an increased proportion (%) of AM containing particles compared with those living in less polluted areas (Calderon-Garciduenas et al., 2001).

Not only do AM remove PM, they may have an important role in the pathogenesis of biomass PM-induced respiratory disease. For example, phagocytosis of particles by AM impairs their ability to function as a major component of the pulmonary innate immune system (Yang et al., 2001). Although direct measurement during biomass burning has shown high levels of carbonaceous PM (Balakrishnan et al., 2002), it is unclear whether this translates into significantly increased exposure of cells (including AM) in the lower airway.

In this study, we aimed to define the level of carbon loading of AM in a population using biomass as the major fuel source, and to compare this with the levels of loading seen in a UK population exposed to low levels of fossil-fuel derived PM. To achieve this aim, we applied a simplified method of processing to AM sampled using induced sputum (IS), and an image analysis methodology for measuring the surface area of cytoplasmic carbon in AM.

2. Methods

The study was conducted at the Gondar Institute of Medical Sciences, Ethiopia, and at the University of Leicester, UK. Ethical approval was obtained

from Leicestershire Health Ethics Committee and in Gondar and informed consent was obtained from individuals or parents and guardians. Women and children in Gondar were recruited from administrative area *Kelebe* 16, who lived in mud huts and cooked using only biomass fuels. The inclusion criteria for Gondar women and children were: (i) had not spent more than 5 days away from home in the preceding 3 months, (ii) nonsmoking households, (iii) unremarkable clinical histories, (iv) no respiratory illness within the last 3 weeks, and (v) exclusive use of biomass fuels. Subject age, height, weight, and size of their family were recorded, along with details of household cooking (e.g. position of the cooking site, fuel used, and the members of the family who were involved in cooking). Adults and children recruited in UK had same inclusion criteria, except that adults could be both male and female and could not be exposed to biomass smoke.

Sputum induction was performed using ultrasonic nebuliser (Sonix nebuliser, Clement Clarke International, UK) and sequential 5-min inhalations of 4.5% saline (Cataldo et al., 2001). Spirometry was not available in Gondar, and the peak flow rate (Mini-Wright peak flow monitor, Clement Clarke International) was therefore used to detect significant bronchial obstruction ($>10\%$) during the procedure. In the UK, the forced expiratory volume in 1 s (FEV_1) (Vitalograph 2120 spirometer, Vitalograph, England) was used to monitor lung function. All subjects were pretreated with salbutamol (400 μg inhaled via metered dose inhaler and Volumatic™ spacer). Induced sputum samples were processed using a modified technique, which did not require cytocentrifugation (Kulkarni and Grigg, 2003). Sputum plugs were selected and 0.1% Dithiothreitol (DTT, Sigma Aldrich, USA), at four times the weight of sample, was added and mixed with a plastic pipette. The sample was placed at room temperature for 15 min to allow homogenisation, and then filtered with 48 μm gauze (Sefon, UK), microcentrifuged at 10,000 rpm for 10 min, and the cell free supernatant discarded. We had previously determined that this centrifugation speed did not alter AM morphology (Kulkarni and Grigg, 2003). A cell smear was then made by placing the cell pellet between two microscope slides and drawing them

apart, thus evenly distributing the cells and avoiding the formation of clumps. Slides were then air dried, and stained with Wright's stain in Ethiopia to rapidly assess smear quality (poor quality slides were repeated). Unstained air-dried slides were transported to UK and both the UK and Ethiopian slides were stained with Diff-Quik (Dade Behring, Switzerland). The leukocyte differential count (% AM, neutrophils, lymphocytes, eosinophils) was obtained from >300 cells (de Blic et al., 2000).

Image analysis of all slides was performed in the UK. Digital colour images of 50 randomly chosen AM per subject with an intact cell wall were obtained using a Olympus BX50 microscope (Olympus Optical UK), at 1000 \times magnification under oil immersion. We had previously ascertained that 50 cells produced a reliable estimate of the median surface area of carbon (data not shown). An image of a stage micrometer graticule (S-12S stage micrometer, 0.1 mm/50 division, Pyser-SGI Limited, UK) was obtained at the same time using the same magnification. Analysis for cytoplasmic carbon was performed blinded to the country of origin using the Scion image grabber and software (Scion image, Scion, USA). The analysis steps are illustrated in Fig. 1A and B. Each AM image was initially processed using Jasc Paint Shop Pro software (Paint Shop Pro 7, Jasc Software, MN, USA). First, the nucleus was removed from the image since the stained nucleus was identified as a "large aggregate" by the image analysis

Table 1

Individual data of Ethiopian women

Subject number	Kitchen type ^a	Biomass fuel used ^b	Baseline peak flow (l/min)	Median (IQR) area of carbon ($\mu\text{m}^2/\text{AM}$)
1	I	D, W, E	430	16.95 (10.34 to 28.83)
2	II	W, D	430	7.10 (1.59 to 15.49)
3	I	W, D	430	3.72 (1.81 to 7.91)
4	II	W, Ch	360	3.81 (1.5 to 8.52)
5	I	W, D, Ch	400	11.28 (6.24 to 11.28)
6	I	D, W	470	12.22 (8.03 to 24.56)
7	I	D, W	430	4.19 (2.12 to 9.56)
8	II	W, E	370	11.32 (6.36 to 23.44)
9	II	W, D	370	14.24 (7.11 to 25.54)
10	I+III ^c	W, D, Ch	430	6.20 (1.32 to 12.85)

IQR—interquartile range.

^a I=cooking outside in the open air, II=cooking in a 'kitchen building' outside the house, III=cooking inside the living area.

^b D=cow dung, W=wood, E=eucalyptus leaves, Ch=charcoal, order given is the priority of fuel use.

^c Kitchen type III was used for cooking the main meal once every 3 days (Injura).

software. Second, Scion image software was used to calculate carbonaceous particle area. Software scaling was calibrated using the image of the stage micrometer graticule (246 pixels=20 μm). The "density slice" command was adjusted to obtain the "best fit" for carbon that was visible on the colour image. If the software selected nonblack areas (usually areas of intense blue staining), these were manually excluded from the analysis. The individual areas of carbon were

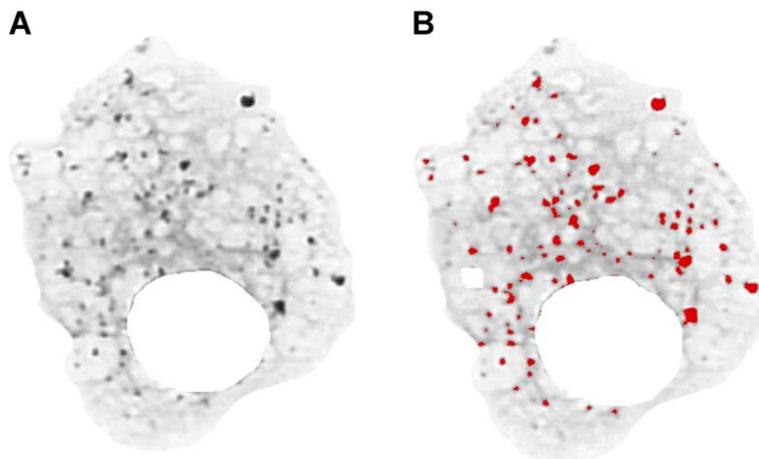


Fig. 1. Image analysis of alveolar macrophages (AM) for cytoplasmic carbon. (A) Digital image of AM obtained under oil immersion light microscopy ($\times 1000$). The black material in the cytoplasm is phagocytosed carbon. The AM nucleus has been cut using the image analysis programme. (B) Areas of carbon particles detected by the Scion image analysis software.

added together to produce the total two-dimensional surface area of carbon for each AM. The median area of carbon per AM per subject (the primary measure), and the percentage of AM containing one or more area of carbon per subject were calculated from 50 cells.

3. Statistics

Data are summarized as median and interquartile range and compared using Mann–Whitney *U*-test. Correlations were determined by Spearman rank correlation (R_s). The Minitab (Minitab release 13.1, PA, USA) statistical package was used for data analysis; *p*-values are given along with the confidence interval (CI) for the median difference. Significance was defined as a *p*-value <0.05.

4. Results

Induced sputum samples containing AM were obtained from all Ethiopian women and children, UK adults and UK children ($n=10$ for all groups). Although matched for age, Ethiopian children were lighter (mean 30 vs. 48 kg, $p<0.01$) and smaller (mean 140 vs. 155 cm, $p<0.01$) than UK children ($n=10$). Ethiopian mothers used a variety of biomass fuels, often in combination, and most families cooked outside in an outdoor fenced off area. Four mothers cooked in a separately constructed cooking hut (Table 1).

Carbon was visible in the cytoplasm of a proportion of AM in all subjects. AM representative of high and low carbon loading are shown in Fig. 2A and B. Carbon in some AM exhibited marked heterogeneity of colour and shape compatible with particle aggregation (Fig. 3). Ethiopian women had the highest level of

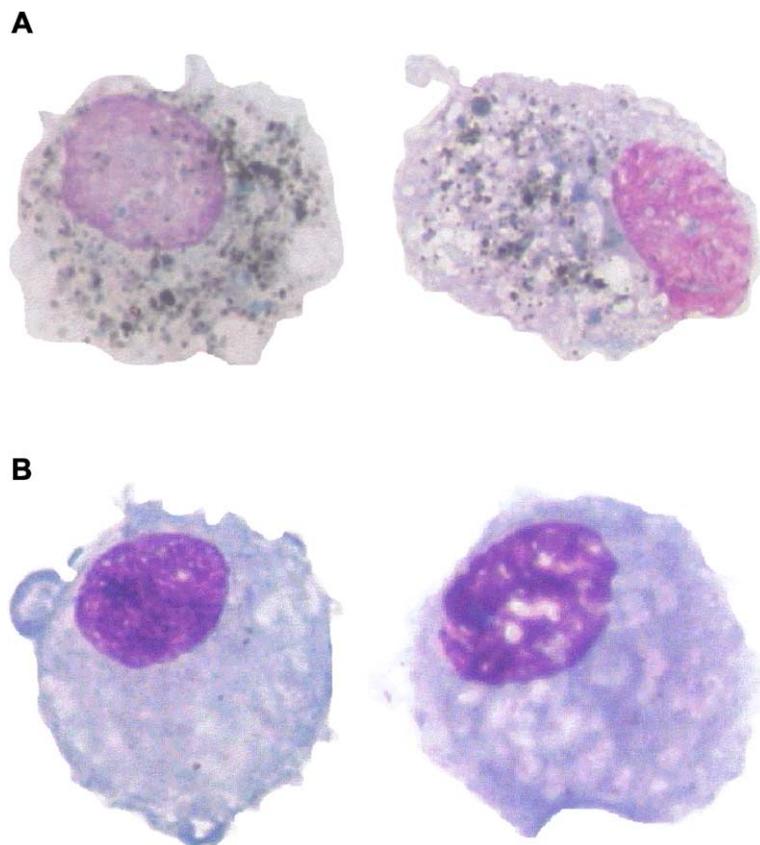


Fig. 2. Alveolar macrophages (AM) imaged under oil immersion and light microscopy ($\times 1000$) showing (A) high carbon particle loading of cells from an Ethiopian woman (left) and child (right). (B) Low levels of carbon loading from UK adult (left) and child (right).



Fig. 3. An area of carbon (arrow) within an alveolar macrophage with heterogeneity of contour and colour compatible with aggregation of smaller particles.

cytoplasmic carbon loading, with markedly increased levels compared with UK adults (median 9.19 vs. 0.71 $\mu\text{m}^2/\text{AM}$, $p=0.0002$, CI 3.3,11.5; Fig. 4A). Ethiopian children had lower levels of loading compared with maternal levels (3.32 vs. 9.19 $\mu\text{m}^2/\text{AM}$, $p=0.011$, CI 1.1, 9.1), with no association between loading in maternal–child pairs (R_s , $p=\text{NS}$). AM carbon loading in Ethiopian children was, however, higher than UK children (3.32 vs. 0.44 $\mu\text{m}^2/\text{AM}$, $p=0.0002$, CI 1.57, 6.77; Fig. 4B).

For all subjects ($n=40$), there was a significant correlation between the median area of cytoplasmic carbon, and the median percentage of AM containing any carbonaceous area ($R_s=0.81$, $p=0.01$). The median percentage of AM containing carbon in

Gondar women was therefore significantly higher than UK adults (100% vs. 86%, $p=0.002$) and Gondar children also had an increased percentage positivity compared to UK children (96% vs. 82%, $p=0.001$).

Twenty-three individuals had adequate numbers of leukocytes (>300) for a differential count. There was a trend for an increased proportion of neutrophils in Gondar women compared to UK adults (median neutrophils; 79.0% ($n=4$) vs. 32.6% ($n=8$), $p=0.05$, CI=-8.52, 78.51). There was no difference in the proportion of neutrophils between Gondar and Leicester children (13.6% vs. 10.4% $p=\text{NS}$).

5. Discussion

In this study, we have demonstrated that it is feasible to noninvasively assess carbon loading of AM in the developing world, and that loading is approximately 13 times higher in women exposed to biomass smoke than adults from a UK city where the majority of inhalable particles are from the combustion of fossil fuels. These data suggest that animal studies of biomass PM-induced disease should aim to achieve a median surface area of carbon of 9.0 μm^2 per AM, with 100% of cells containing at least one area of carbon. There are no animal models, to date, to examine the cellular and molecular mechanisms underlying the association between biomass-smoke exposure and increased vulnerability to lower respiratory tract infection: a major threat to global

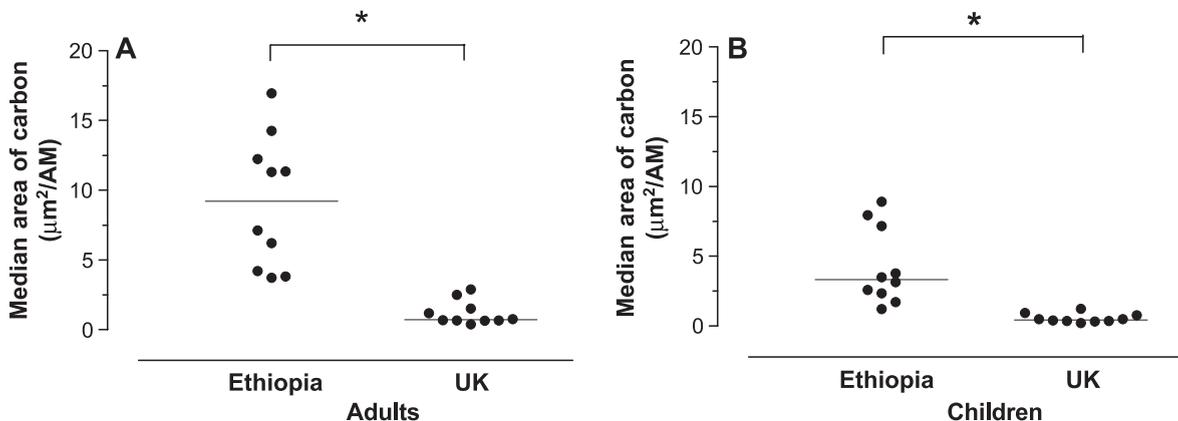


Fig. 4. Dotplot of comparison of carbon loading of AM ($\mu\text{m}^2/\text{AM}$) between Ethiopia and UK. Data are compared using the Mann–Whitney U -test. (A) Ethiopian (Gondar) women vs. UK (Leicester) adults $p=0.0002$, (B) Ethiopian children vs. UK children, $p=0.0002$. Bars represent medians.

respiratory health (Bruce et al., 2000). It is therefore unclear whether carbon loading of AM in vivo directly increases vulnerability to bacterial or viral infection, or is a surrogate marker for more significant exposures of other airway cells. However, phagocytosis of carbonaceous particles by AM alters a range of antibacterial functions, including attenuating phagocytosis of other non-opsonized particles (Renwick et al., 2001; Monn et al., 2002), impairing release of superoxide radicals (Kleinman et al., 2003), and downregulating pulmonary inflammatory responses to viral infection (Becker and Soukup, 1999).

There are several potential limitations of this study. First, carbon loading may not represent loading in the smaller airways since induced sputum selectively samples cells from the large central airways (Lechapt et al., 2003). However, central airway AM on the mucociliary escalator originate from the bronchioles and alveoli (Lehnert, 1992). Thus, 48 h after experimental particle exposure, loading of central AM and distal AM is identical (Lay et al., 1998). Second, phagocytosed particles are concentrated and aggregated by AM (Bunn et al., 2001), and the “areas” of carbon identified by image analysis (Fig. 3) do not represent the size distribution of inhaled PM. Third, we assessed carbon in a single focal plane, whereas loading in vivo is distributed in three dimensions. However, all digital images were obtained with the nucleus in focus, which ensured uniformity of focal plane and maximised the surface area of intracellular carbon. AM particle loading in three dimensions can be measured using confocal microscopy, but would require fluorescent labelling of biomass-PM prior to inhalation: an impractical option.

Notwithstanding the above limitations, is carbon loading of AM a valid way of assessing individual exposure? We did not measure ambient PM levels to validate our data. However, PM₁₀ levels have been reported during biomass cooking, with peak levels of 2000 µg/m³, and mean 24 h levels of 231 µg/m³ (Balakrishnan et al., 2002), i.e. 10 times greater than the annual 24 h mean PM₁₀ in Leicester (19 µg/m³ in 2003): a difference compatible with the 13-fold increase in loading seen in Ethiopian women. Whether AM carbon reflects short-term peaks of exposure (e.g. cooking in the last 3 days), or long-

term exposure (e.g. over several months/lifetime), or a combination of both, is unclear. Lay et al. (1998) instilled a single dose of iron particles into the lower airways of human volunteers, and found particle-containing AM 24 h post-instillation, and a fifth of AM containing particles at 90 days. Thus AM carbon loading is most likely an integral of short- and long-term exposure. Could we have used a less technologically demanding way of assessing loading (e.g. presence/absence of carbon)? Indeed, after nebulisation of 1 µm latex spheres, the proportion of AM with one or more ingested particle increases with increasing dose (Suarez et al., 2001). We chose image analysis to measure total surface area of carbon since it has a very good intra- and interobserver variability (data not shown), and total surface area provides an additional quantitative dimension over the presence/absence of carbon alone; but the significant correlation between median total surface area of carbon and the proportion of AM containing any carbon seen in the present study does suggest that there may be less time-consuming alternatives. Do our data provide any practical insights into ways of reducing biomass-PM exposure? We were surprised to find that carbon loading of AM in Ethiopian children was significantly less than their mothers but, on questioning, these children spent very little time near the outside fire. In contrast, infants (the age group most vulnerable to adverse effects of biomass smoke; Bruce et al., 2000) were strapped to their mothers backs during cooking. Moving infants away from the fire to areas used by older children may therefore attenuate exposure.

Sputum induction has been used in developing countries in the diagnosis of pulmonary infections (Zar et al., 2000; Hartung et al., 2002), and hemosiderin-laden AM have been described in spontaneously produced sputum samples from traffic policemen in India (Roy et al., 2001). The absence of a cytocentrifuge and ice in Ethiopia meant that we had to modify the standard sputum processing methodology (Djukanovic et al., 2002). Recently, a similar cytocentrifuge-free technique has been reported for processing of induced sputum samples (Saraiva-Romanholo et al., 2003). Induced sputum processing may therefore offer a new approach to the study of respiratory disease in the developing world. Indeed, our data suggest that airway neutrophils are

increased in healthy, asymptomatic, women who are chronically exposed to biomass smoke. Since neutrophils are implicated in the pathogenesis of cigarette smoke-associated chronic obstructive pulmonary disease (COPD) (Cosio Piqueras and Cosio, 2001), this may be a factor in the early pathogenesis of non-cigarette smoke associated COPD in women in the developing world (Varkey, 2004).

In summary, we have demonstrated high levels of carbon loading of AM after biomass-smoke exposure in mothers and children. Analysis of AM in a larger cohort of biomass exposed women may (i) provide insights into the major determinants of exposure, (ii) inform exposure-reduction interventions, and (iii) guide cellular and whole animal models of the association between biomass-smoke and increased vulnerability to infection.

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