Extracellular release of hydrogen peroxide by human alveolar macrophages: the relationship to cigarette smoking and lower respiratory tract infections

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(Received 20 December 1982/1 June 1983; accepted 23 June 1983)

Summary
1. It has been suggested that oxidants from pulmonary inflammatory cells may contribute to the development of emphysema by (i) direct tissue toxicity and (ii) inhibition of $\alpha_1$-antitrypsin, thus diminishing protection of the lung from proteolytic damage.

2. The extracellular release of hydrogen peroxide ($H_2O_2$) by human alveolar macrophages (AM) has been measured. AM were obtained by bronchoalveolar lavage and adherence from 24 smokers and 17 non-smokers.

3. Smokers' AM released significantly more $H_2O_2$ (3.83 nmol h$^{-1}$ µg$^{-1}$ of DNA; SEM 0.44) than those of non-smokers' (2.33 nmol h$^{-1}$ µg$^{-1}$ of DNA; SEM 0.40) ($P<0.05$).

4. AM from donors with a recent lower respiratory tract infection released increased quantities of $H_2O_2$ (3.22 nmol h$^{-1}$ µg$^{-1}$ of DNA; SEM 0.72; $P<0.01$) even when allowance was made for smoking habits.

5. These findings are consistent with the hypothesis that $H_2O_2$ of AM origin contributes to the development of emphysema in smokers.

Key words: alveolar macrophages, emphysema, hydrogen peroxide, respiratory tract infections, smoking.

Introduction
At present it is believed that emphysema develops as a consequence of protease-antiprotease imbalance in the lung. This proteolytic theory has evolved after the recognition of an association between severe deficiency of $\alpha_1$-proteinase inhibitor and the development of panacinar emphysema [1] and the observations that emphysema can be induced by instillation of elastolytic enzymes into the lower respiratory tract in animals [2, 3]. It is thought that the alveolar structures are normally protected from extracellular proteases, which can originate from inflammatory cells, by an anti-protease screen. At the alveolar level $\alpha_1$-proteinase inhibitor is the principal antiprotease [4] but it can be inhibited by oxidation of the methionine that is part of the molecular structure in or near the active site [5]. Thus, in the presence of oxidants, a functional deficiency of $\alpha_1$-proteinase inhibitor may occur in the lower respiratory tract, disturbing the protease-antiprotease balance in favour of protease activity. Cigarette smoking has been shown to induce functional antiprotease deficiency in the lower respiratory tract [6]. This may be due to a direct action of oxidants in cigarette smoke [7] but cigarette smoking also enhances the oxidative metabolic response of alveolar macrophages (AM) [8] and might, therefore, promote release from these cells of hydrogen peroxide ($H_2O_2$) and other oxidants that are important for the cells' microbicidal functions [9]. Such release of AM-derived oxidants might lead to...
functional inhibition of α1-proteinase inhibitor in the microenvironment of these cells. We have therefore measured the extracellular release of H₂O₂ from human AM and examined the influences of the macrophage donors’ smoking habits and pulmonary diseases.

Methods

Alveolar macrophages

These were obtained by bronchoalveolar lavage [10] from 41 patients (Table 1) during diagnostic bronchoscopy. Patients categorized as ‘recent lower respiratory tract infection’ had all had parenchymal shadowing on chest X-ray within the preceding 3 months. Two of these patients had bronchographically demonstrated bronchiectasis. Informed patient consent and approval of Hammersmith Hospital Ethics Committee were obtained. Lavage of the lingula or middle lobe was performed with two 120 ml volumes of sterile NaCl solution (154 mmol/l: saline) with immediate aspiration. For patients with unilateral lung disease (bronchial carcinoma or recent infection) the contralateral lung was lavaged. Cells were recovered from the lavage fluid by low speed centrifugation (60 g for 15 min), resuspended at 2 x 10⁶ cells/ml and AM separated from other cell types by adherence and maintenance in tissue culture as monolayers. AM were cultured in 35 mm plastic Petri dishes (Nunc, Gibco Europe Ltd, Paisley, Scotland, U.K.) for 72 h in medium 199 (Gibco Europe Ltd), supplemented by 10% autologous serum, 4% bovine embryo extract (Flow Laboratories, Irvine, Scotland, U.K.) and 1% liver fraction L (United States Biochemical Corp., Cleveland, OH, U.S.A.). Preliminary tests showed no change in H₂O₂ responses of AM between 24 and 72 h in culture.

Measurement of H₂O₂ release

H₂O₂ release was estimated by the oxidation of p-hydroxyphenylacetic acid, catalysed by horseradish peroxidase, to a fluorescent product [11]. After 72 h in culture AM monolayers were incubated, with and without stimulation by phorbol myristate acetate (PMA; 1 μg/ml), in 1 ml of medium 199 containing 2.4 μmol of p-hydroxyphenylacetic acid and 5 μg of peroxidase. Standards were prepared by inoculating cell-free Petri dishes with 2-20 nmol of H₂O₂ together with the test reagents in 1 ml of medium 199. After 60 min incubation, 900 μl of each supernatant was removed into 1 ml of ice-cold borate buffer (0.2 mol/l, pH 10.4) and the mixture was allowed to reach ambient temperature in the dark before fluorescence measurement. To allow for any minor variations in numbers of adherent AM in different monolayer preparations the H₂O₂ release was standardized to the DNA content of the monolayer. DNA was measured by an adaptation [12] of the method of Giles & Myers [13].

Statistical analyses

Comparison of H₂O₂ release by smokers’ and non-smokers’ AM was performed by using Student’s t-test for unpaired data. Comparison of H₂O₂ release by AM from donors with different diseases, taking into account the donors’ smoking habits, was performed by analysis of covariance.

Results

After 72 h culture Giemsa staining showed AM monolayers to be free of other cell types. The AM were >95% viable as judged by trypan blue exclusion.

<table>
<thead>
<tr>
<th>Disease</th>
<th>No. of subjects</th>
<th>Mean age (years)</th>
<th>H₂O₂ (nmol h⁻¹ μg⁻¹ of DNA)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Smokers</td>
<td>Non-smokers</td>
<td>Men</td>
</tr>
<tr>
<td>No active pulmonary disease</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Recent lower respiratory tract infection</td>
<td>7</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Bronchial carcinoma</td>
<td>9</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>Non-bronchial malignancies</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Sarcoidiosis</td>
<td>0</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Fibrosing alveolitis</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

* 1 x 10⁶ alveolar macrophages, 6.5 μg of DNA.
† P < 0.01, for comparison of these two groups against the other four.
‡ Ex-smokers of >8 years.
Alveolar macrophages and hydrogen peroxide

Unstimulated AM released minimal quantities of H$_2$O$_2$ ($<0.2$ nmol h$^{-1}$ µg$^{-1}$ of DNA). However, on stimulation with PMA this was markedly enhanced (0.2–8.73 nmol h$^{-1}$ µg$^{-1}$ of DNA). PMA-stimulated AM from smokers released more H$_2$O$_2$ (3.83 nmol h$^{-1}$ µg$^{-1}$ of DNA; SEM 0.44; $n = 24$) than PMA-stimulated AM from non-smokers (2.33 nmol h$^{-1}$ µg$^{-1}$ of DNA; SEM 0.40; $n = 17$). The difference was significant ($P < 0.05$). Age and sex of the AM donors had no influence in the quantity of H$_2$O$_2$ released. However, some diseases of the donors did affect the H$_2$O$_2$ release, even when allowance was made for smoking habits (Table 1; Fig. 1). AM from patients with a recent lower respiratory tract infection released more H$_2$O$_2$ (5.22 nmol h$^{-1}$ µg$^{-1}$ of DNA) on stimulation as did AM from patients with non-bronchial malignancies (4.70 nmol h$^{-1}$ µg$^{-1}$ of DNA). The difference was significant ($P < 0.01$) in both instances.

Discussion

Our results show that AM from cigarette smokers, when stimulated, released significantly greater quantities of H$_2$O$_2$ into the extracellular environment than AM from non-smokers. These results accord with those of other workers who, by measuring release of superoxide, have shown similar enhancements of AM oxidative metabolism for young asymptomatic cigarette smokers [8] and for smoke-exposed rats [14]. The unstimulated AM released minimal quantities of H$_2$O$_2$, indeed, for AM from all but three patients (all smokers) the basal levels of release were below the lower limit of sensitivity of our assay ($<0.2$ nmol h$^{-1}$ µg$^{-1}$ of DNA); thus it was not possible to determine whether unstimulated AM from smokers released greater quantities of H$_2$O$_2$ than the AM of non-smokers. Nevertheless, *in vivo*, AM will be subject to frequent phagocytic stimulation. We chose PMA as our receptor-mediated [15] phagocytic stimulus [16] in view of its degree of cellular stimulation, repeatability of results and convenience of use.

Our interpretation of the data for H$_2$O$_2$ release from stimulated AM is made cautiously since numbers of smokers and non-smokers in the various disease groups were not matched (Fig. 1). However, even if the disease group that contributes most to the high value for the smokers (recent lower respiratory tract infection) is removed from analysis, smokers' AM still release significantly greater quantities of H$_2$O$_2$ than the AM of non-smokers (3.1 ± 0.4 nmol h$^{-1}$ µg$^{-1}$ of DNA vs 2.3 ± 0.4). This implies that the effects of smoking and infection might be cumulative but whether infection would have an effect in non-smokers cannot be assessed from the data. The mechanisms by which the enhancement of release of H$_2$O$_2$ from smokers' macrophages occurs are not known. Clearly one or more components of cigarette smoke may directly affect the AM. A more intriguing possibility is that smokers' AM have been primed for enhanced responses by pulmonary lymphocytes or lymphocyte products as part of a cell-mediated immune response. In support of this hypothesis two of the five patients with non-bronchial malignancies (one Hodgkin's disease and one disseminated thyroid carcinoma) and one patient with bronchiectasis had significant elevation of the percentage of lymphocytes in their bronchoalveolar cells (24.0, 45.6 and 33.6% respectively). We have shown [17] that AM, *in vitro*, can be primed by lymphokines for an enhanced H$_2$O$_2$ release on stimulation. There would be ample opportunity *in vivo* for cell-mediated immunity to be developed against antigenic components of cigarette smoke or bacterial antigens. We suggest that *in vivo* priming of AM by pulmonary lymphocytes also explains the enhanced H$_2$O$_2$ release by cells from donors with recent lower respiratory tract infections or with non-bronchial malignancies (an heterogeneous group including disseminated nasopharyngeal and thyroid carcinoma and pulmonary lymphomas). In these groups the presumptive cell-mediated immunity would be in response to microbial and tumour antigens respectively.
Irrespective of the mechanisms underlying the macrophage activation the enhanced release of \( \text{H}_2\text{O}_2 \) may result in a functional deficiency of \( \alpha_1 \)-antiproteinase inhibitor in the microenvironment around AM, thus locally favouring uninhibited protease activity [18, 19]. In addition \( \text{H}_2\text{O}_2 \) may itself be directly tissue-toxic [20]. Thus our findings sustain the possibility that \( \text{H}_2\text{O}_2 \) of macrophase origin might contribute to the development of emphysema by smokers.

Acknowledgments

A.P.G. was in receipt of a Medical Research Council Training Fellowship and gratefully acknowledges this support. We thank Mr V.R. Aber for statistical analyses and Alma Dixon for secretarial assistance. We thank our colleagues for referral of patients in this study and in particular Dr N. B. Pride for his encouragement and support.

References