Cigarette smoking influences cytokine production and antioxidant defences

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(Received 13 July/18 November 1994; accepted 8 December 1994)

INTRODUCTION

Overwhelming evidence indicates that cigarette smokers, when compared with lifelong non-smokers, are at a 2- to 3-fold increased risk of coronary heart disease (CHD) and cancer [1, 2]. The underlying biochemical mechanisms in these and many other pathological conditions associated with smoking are not fully understood. Cigarette smoke contains more than 1015 organic free radicals in the gas phase of each inhalation [3], these radicals cause widespread biological damage in lung, which includes inactivation of human α1-protease inhibitor, leading to emphysematous lesions and loss of lung elasticity [4].

Smoking also exerts an inflammatory stimulus on lung macrophages which may, like bacterial and viral infection, bring about the production of free radicals and the inflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor α (TNF) [5]. This might be an early event in the development of the disease states associated with smoking [6, 7]. Furthermore, IL-1 and TNF may exert damaging effects upon lung tissue [8]. Cytokine production is enhanced by free radicals and other oxidant molecules by activation of nuclear transcription factor κB (NFκB). NFκB activation increases transcription of cytokine and acute-phase protein genes [9]. In vitro studies indicate that synthetic antioxidants, such as butylated hydroxyanisole and N-acetylcysteine, can prevent up-regulation of cytokine production by free radicals [10, 11].

TNF displays a wide variety of metabolic effects, which include stimulation of acute-phase protein synthesis [12]. Increases in acute-phase protein production can thus be used as a measure of the degree of inflammation or tissue damage. Indeed, raised concentrations of the acute-phase proteins C-reactive protein and α1-acid glycoprotein have also been reported in smokers [13, 14]. However, an unexplained decrease in serum albumin in smokers has been reported [15] and was related to cigarette consumption [16]. Chronic cytokine production might contribute to the phenomenon [17]. The diets of smokers have been associated with low levels of antioxidant vitamins [18, 19]. As in vitro and in vivo studies have shown that the ability of free radicals to enhance the production of TNF and IL-1, IL-6 and IL-8 is suppressed by antioxidants [10, 11, 20], the extent to which smoking influences cytokine production by alteration of antioxidant status is unknown. Studies in rats have demonstrated that vitamin E deficiency is associated with
an increased number of polymorphonuclear cells in lung and enhanced production of α1-acid glycoprotein in response to an endotoxin challenge. The data indicated an enhanced responsiveness of animals with reduced antioxidant defences to the acute inflammatory challenge [21]. The smoking-related inflammatory response may stem from, or be exacerbated by, an imbalance in oxidant and antioxidant status. The low antioxidant status of cigarette smokers may predispose them to oxidant- and cytokine-inflicted tissue damage and disease, which may manifest itself as CHD, atherosclerosis and cancer [1, 2]. The oxidant-related damage and subsequent disease may necessitate an increased intake of specific antioxidant nutrients in this population.

Few studies have examined the relationship between antioxidant nutrients and cytokine production in conditions in which free radicals are present in enhanced amounts in vivo. To examine these possibilities, we investigated the ability of blood from non-smokers and smokers to produce TNF in response to an inflammatory challenge. We also determined plasma IL-6 and plasma acute-phase protein concentrations in these subjects. The relationship of these results to the dietary intake and blood concentrations of antioxidant vitamins was then examined.

METHODS

Subjects

A subset of 55 male and female smokers and 51 male and female non-smokers was randomly selected from subjects that were recruited for a similar study [22]. A further 20 male and female smokers and 27 male and female non-smokers of the same age range from among the students and staff of Southampton University volunteered for the study. Dietary information was estimated by a self-administered food frequency questionnaire [22]. The health data and subsequent subject selection were as reported previously [22]. The procedures followed were in accordance with the ethical standards of the Ethical Sub-Committee of the University of Southampton. Informed consent was obtained from the subjects.

Processing of blood samples

A 2-ml sample was incubated with 10 ng/ml endotoxin, at 37°C in a humidified CO2 incubator, to induce production of TNF. The remainder was centrifuged at 700 g for 15 min with as little delay as possible. Plasma was removed and the red blood cell mass was washed twice with 0.9% (w/v) saline and then diluted with 0.5 mol/l pyrogallol (1:1) to preserve red blood cell vitamin E, before analysis. A selection of acute-phase proteins (α1-acid glycoprotein, α2-macroglobulin, C-reactive protein, caeruloplasmin and albumin), antioxidants, blood components and IL-6 were quantitatively measured in plasma samples.

Plasma acute-phase proteins

Radial immunodiffusion (RID) was employed for the determination of plasma α1-acid glycoprotein (AGP), α2-macroglobulin (AM) and C-reactive protein (CRP) concentrations. A 2% (w/v) solution of agarose gel made up with a barbitone buffer was allowed to set in a RID plate. Wells of 2.5–3.0 mm diameter were cut in the gel and 5 µl of plasma added. A circular precipitate resulted, the diameter of which was related to the concentration of AGP, AM and CRP in the sample as calculated from a standard curve. Plasma caeruloplasmin was determined by measurement of its oxidase activity [23] and plasma albumin was determined using the Bromocresol Green method [24].

Determination of circulating antioxidants

Plasma and red blood cell vitamin E concentrations and the concentrations of plasma vitamin C and uric acid were determined as described in [25]. Red blood cell glutathione was assayed by the method of Tietz [26] and plasma sulphhydrlys by the method of Ellman [27]. The activities of antioxidant enzymes glutathione peroxidase and catalase were assayed in red blood cells by the methods of Beutler [28] and Aebl [29] expressed per mg of haemoglobin (Hb) [30].

Determination of TNF production by whole blood

TNF concentrations in blood after endotoxin (10 ng/ml) challenge were estimated by the cytotoxicity of this cytokine towards murine fibroblasts (L929 cells) as previously described [31].

Determination of plasma IL-6

The IL-6 concentration in the plasma of smokers and non-smokers was determined by an assay based on the proliferative effect of this cytokine on the IL-6-dependent murine hybridoma cell line B9 as previously described [31].

Statistical analysis

The skewed distributions of red blood cell vitamin E and plasma CRP were normalized by logarithmic transformation before analysis. One-way analysis of variance was used to assess the differ-
RESULTS

The mean age and sex of all subjects involved in the study is shown in Table 1. The daily intake of antioxidant vitamins, polyunsaturated fatty acids (PUFAs), saturated fatty acids (SFAs) and total energy in smokers and non-smokers is shown in Table 2.

Dietary intakes of the antioxidant vitamins and total energy were found to be similar in the two groups. The intake of PUFAs in smokers was lower than in non-smokers (P=0.04), although no significant difference in total fat intake was observed between the two groups.

The activities of red blood cell catalase and glutathione peroxidase and red blood cell glutathione concentrations were not significantly different between the two groups. No significant difference was observed in red cell vitamin E per g Hb between the two groups (Table 3).

Plasma vitamin A and E concentrations were unaffected by smoking (Table 4). Plasma vitamin C concentration in the smoking subjects was 21% lower than that of non-smokers (P=0.04). (Table 4).

Plasma concentrations of AGP and caeruloplasmin were significantly elevated by 39% and 28% respectively in smokers compared with non-smoking subjects. AM concentration was also higher in smokers (12%) than in non-smokers. Neither albumin nor CRP concentration was significantly different in the two groups (Table 5).

TNF production in blood from smokers exposed to 10 ng/ml endotoxin was elevated relative to that in non-smokers' blood by 38% (P=0.01). Plasma IL-6 concentration in smokers was 10% higher than in non-smokers, but this difference was not significant (P=0.07).

DISCUSSION

Systemic defense against the damaging effects of free radicals in biological systems results from a complex interaction between antioxidants derived from the diet and endogenously synthesized antioxidants and antioxidant enzymes. The interaction of free radicals, derived from cigarette smoke, with this system will have complex characteristics. The present study indicates that, in agreement with the findings of Duthie et al. [32], intakes of the anti-
Table 5. Measurements of cytokines and acute-phase protein concentrations in non-smokers and smokers. Results are expressed as means (±SD) for 20 smokers and 27 non-smokers, except *measurements conducted in 20 smokers and 20 non-smokers and †measurements conducted in 55 smokers and 51 non-smokers aged 18-55 years. One unit of caeruloplasmin activity will oxidize 1.0 μmol of D-diastilose dichloral-Child per minute at pH 7.0 and 25°C. P < 0.05 was taken to be a statistically significant difference between smokers and non-smokers. TNF, tumour necrosis factor; IL-6, interleukin-6, AGP, α1-acid glycoprotein; AM, α1-macroglobulin; CRP, C-reactive protein.

<table>
<thead>
<tr>
<th></th>
<th>Non-smokers</th>
<th>Smokers</th>
<th>P-value</th>
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<tbody>
<tr>
<td><strong>Cytokines</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>TNF (ng/ml)</td>
<td>2.5 (±1.1)</td>
<td>3.4 (±1.5)</td>
<td>0.01</td>
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<tr>
<td>IL-6 (ng/ml)*</td>
<td>2.58 (±0.35)</td>
<td>3.00 (±0.73)</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>Acute phase proteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caeruloplasmin (units/ml)†</td>
<td>177 (±91)</td>
<td>227 (±97)</td>
<td>0.001</td>
</tr>
<tr>
<td>Albumin (mg/ml)†</td>
<td>43.2 (±8.0)</td>
<td>40.0 (±7.4)</td>
<td>0.52</td>
</tr>
<tr>
<td>AGP (mg/ml)†</td>
<td>0.93 (±0.33)</td>
<td>1.02 (±0.35)</td>
<td>0.099</td>
</tr>
<tr>
<td>AM (mg/ml)†</td>
<td>2.16 (±0.48)</td>
<td>2.41 (±0.44)</td>
<td>0.008</td>
</tr>
<tr>
<td>CRP (log mg/ml)</td>
<td>3.2 (±0.3)</td>
<td>3.2 (±0.6)</td>
<td>0.22</td>
</tr>
</tbody>
</table>

oxidant vitamins (vitamin E, vitamin C and β-carotene) were similar in smokers and non-smokers; however, in contrast to the findings of other studies, smokers do not have a subnormal dietary intake of antioxidant vitamins [18, 19]. We suggest that differences in antioxidant vitamin status are manifested as a consequence of smoking. While no differences in the enzymatic antioxidants were observed, a reduced plasma vitamin C concentration was observed, suggesting that a component of antioxidant defence in smokers, which is directly derived from the diet, is sensitive to the oxidative challenge exerted by cigarette smoke. Indeed, in vitro studies have suggested that vitamin C may be the most important antioxidant in human plasma [33].

Ideally, the plasma vitamin E should be expressed on the basis of plasma lipid content, since a small difference in the fat content of the diet in the two groups was observed; however, we were unable to do this within the scope of this study.

Inadequate dietary antioxidant vitamin status, as a consequence of smoking, may lead to free radical-associated lipid peroxidation [32], increase in CHD incidence [32] and malignancy [2]. The finding that smokers have decreased vitamin C status suggests that smokers have a reduced capacity to withstand oxidative stress when activated polymorphonuclear cells release free radicals in response to cigarette smoke. To add strength to this assertion, an index of oxidative damage to lipids, proteins or DNA would need to be measured. Since this was beyond the scope of the present investigation it is hoped that this issue will be addressed in future studies. In the present study, the capacity of the subjects to produce cytokines was studied by measurement of TNF production by blood in vitro, in response to an artificial stimulus in the form of endotoxin. A similar situation may occur on each occasion that lung macrophages are exposed to cigarette smoke. Indeed, recent studies report raised levels of IL-1 and IL-6 production by alveolar cells after stimulation with a tobacco-derived antigen [6]. In our studies, higher plasma concentrations of a caeruloplasmin, AGM and AM were observed in smokers. These observations, together with the tendency towards a higher IL-6 concentration in plasma, indicate that chronic production of cytokines in response to smoking is a strong possibility. Since IL-1 can induce TNF gene expression, the observed increase in the capacity of smokers’ blood for TNF synthesis may, therefore, result from dual and synergistic effects of IL-1 and free radicals or from an increased sensitivity of neutrophils/monocytes to inflammatory stimuli or to increased numbers of cells, the latter finding having been reported previously [34]. Nonetheless, either event may contribute to the clinical and pathological states observed in many smokers.

We postulate that the following sequence of events may be occurring in smokers. The inflammatory stimulus of cigarette smoke activates alveolar macrophages to produce TNF and IL-6. IL-6 increases hepatic acute-phase protein production and TNF induces free radical release from polymorphonuclear cells. This may lead to a greater oxidative burden and depletion of antioxidant defences. Hence, an increased dietary intake, in particular of vitamin C, may be necessary to maintain antioxidant defences in smokers. Also, vitamin C supplementation reduces plasma lipoprotein peroxidation in smokers [35]. It has thus been suggested that recommended daily allowance for vitamin C should be increased [36]. The findings of the present study provide some new information into the important area of the influence of smoking on cytokine production and antioxidant defences of human subjects which in future may illuminate nutritional mechanisms of smoking toxicity.

ACKNOWLEDGMENT

We thank R. L. Thompson of the Department of Medicine, University of Southampton, Royal South Hants, Hospital, Southampton, for providing blood samples from smokers and non-smokers, and Professor A. Shenkin, Department of Clinical Biochemistry, Royal Liverpool University Hospital, for the gift of IL-6-dependent murine B9 hybridoma cells.

REFERENCES

Cytokines and antioxidants in smokers