Nitrogen dioxide depletes uric acid and ascorbic acid but not glutathione from lung lining fluid

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The aim of this study was to determine the kinetics of the reactions between the gaseous free-radical pollutant, nitrogen dioxide (NO_2), and the water-soluble antioxidants present in respiratory tract lining fluid (RTLTF). Samples of RTLTF, recovered from 12 subjects (mean age 54.1 ± 16.3 years; eight male, four female) as bronchoalveolar lavage (BAL) fluid were exposed *ex vivo* to NO_2 [50–1000 parts per billion (ppb)] for 4 h. For comparison, similar exposures were carried out with single and composite solutions with relevant RTLTF antioxidant concentrations. Ascorbic acid (AA), uric acid (UA), GSH depletion, and GSSG and malondialdehyde (MDA) formation were determined with time. In the three models, UA and AA were consumed in a time- and NO_2 -concentration-related fashion. In addition, their rate of depletion correlated positively with their initial concentration (UA, $r = 0.92$, $P < 0.05$; AA, $r = 0.94$, $P < 0.05$). Little difference was found between the rate of loss of AA (2.2 ± 0.2 ; 1.9 ± 0.5 ; $1.4 \pm 0.3 \text{ nmol} \cdot \text{l}^{-1} \cdot \text{h}^{-1} \cdot \text{ppb}^{-1}$), and that of UA (2.4 ± 0.2 ; 2.1 ± 0.6 ; $1.3 \pm 0.2 \text{ nmol} \cdot \text{l}^{-1} \cdot \text{h}^{-1} \cdot \text{ppb}^{-1}$) in the

three RTLTF models examined (single, composite, BAL fluid respectively). GSH loss from BAL fluid (0.2 ± 0.1) was significantly less than that seen in either single (1.4 ± 0.3) or composite ($1.2 \pm 0.5 \text{ nmol} \cdot \text{l}^{-1} \cdot \text{h}^{-1} \cdot \text{ppb}^{-1}$) antioxidant solutions. In all cases, GSH consumption was significantly less than AA or UA. As model complexity increased, the rate of individual antioxidant loss decreased, such that in BAL fluid, AA, UA and GSH consumption rates were significantly less ($P < 0.05$) than in the pure or composite antioxidant mixtures. In BAL fluid, little GSSG production was observed at any NO_2 concentration. MDA concentration, determined as a measure of lipid peroxidation, did not change following exposure to 50, 150 or 400 ppb NO_2 , but increased MDA was seen in BAL fluid from 8/12 subjects following exposure to 1000 ppb NO_2 for 1 h or more. In conclusion, NO_2 , at environmentally relevant concentrations, depletes BAL fluid of the antioxidant defences, UA and AA, but not GSH.

INTRODUCTION

With the increase in the number of motor vehicles in use, concerns are being expressed about the health effects of exhaust emissions which contain, among other pollutants, the nitrogen-centred free radical, nitrogen dioxide (NO_2). Hospital admissions due to asthma attacks and respiratory complaints increase during episodes of increased NO_2 concentrations [1]. Animal studies have shown that high concentrations of NO_2 can damage the lung [2], and cell-culture experiments indicate that NO_2 exposure increases cell permeability and injury [3]. In a series of elegant studies using a combination of environmental chamber exposure and fibre-optic bronchoscopy with bronchoalveolar lavage (BAL), Sandström and colleagues have shown that both single [4] and multiple [5] exposures of humans to occupational concentrations of NO_2 results in a time-dependent inflammatory response in the lung.

It is not at present appreciated how NO_2 elicits these changes in inflammatory cell influx into the lung; however, due to its free-radical properties, NO_2 is a powerful oxidizing species and this may represent its main mechanism of action. The observation that NO_2 exposure depletes antioxidants, such as glutathione, ascorbic acid (AA), uric acid (UA) and α -tocopherol, from human plasma [6], led us to question whether similar events occur in respiratory tract lining fluid (RTLTF), a thin layer that bathes the respiratory epithelium. RTLTF contains a complex array of antioxidant defences [7–11] that may protect the lung from toxic gases such as NO_2 .

To establish the hierarchy of utilization of antioxidant defences in RTLTF and to determine their consumption kinetics by NO_2 , RTLTF, obtained as BAL fluid, was exposed *ex vivo* to a range of environmental (50, 150, 400 ppb) and occupational (1000 ppb) concentrations of NO_2 . Aliquots were removed from the exposure chamber after 0, 0.5, 1, 2 and 4 h exposure to NO_2 , and the concentrations of glutathione (GSH and GSSG), AA and UA were determined by established HPLC methods. In addition, malondialdehyde (MDA) was determined as an index of lipid peroxidation. To further clarify the relative hierarchy of UA, AA and GSH with NO_2 , solutions of these three antioxidants, either alone or in combination, at concentrations that reflect undiluted RTLTF, were also exposed to NO_2 .

EXPERIMENTAL

Chemicals

All chemicals used were obtained from Sigma (Poole, Dorset, U.K.) or BDH (Poole, Dorset, U.K.) and HPLC-grade solvents were obtained from Rathburn Chemicals (Walkerburn, Scotland).

Subjects and BAL

BAL fluid was collected by fibre-optic bronchoscopy from 12 patients (eight male, four female) under routine investigation for a variety of conditions, including cancer of the lung. Samples were selected for study on the basis of recovery ($> 50\%$) and the

Abbreviations used: RTLTF, respiratory tract lining fluid; BAL, bronchoalveolar lavage; ppb, parts per billion; AA, ascorbic acid; UA, uric acid; MDA, malondialdehyde.

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absence of blood contamination alone. Bronchoscopy was performed as described previously [12]. Briefly, after topical anaesthesia of the upper respiratory tract with 2% (w/v) lignocaine, an Olympus BF Type 20 bronchoscope was wedged in the right middle or left lingula lobe of the lung at the third or fourth airway generation. Warmed (37 °C) sterile saline was introduced into the lower respiratory tract, in four sequential 50-ml aliquots, which were gently aspirated individually into a sterile trap, pooled and kept briefly at 4 °C. The lavage was filtered through coarse (0.74 mm) plastic mesh to remove mucus. The filtered lavage fluid was centrifuged at 300 *g* for 10 min at 4 °C to separate the BAL cells from the acellular BAL fluid. The BAL supernatants were stored in 10-ml aliquots at -40 °C for up to 6 weeks before antioxidant measurement and subsequent challenge with NO₂. Pilot studies had indicated that there was no appreciable deterioration of antioxidants during storage over this time.

Antioxidant solutions

Solutions of the three major water-soluble antioxidants associated with RTLF, AA, UA and GSH/GSSG, were prepared based on their reported concentrations: 200 $\mu\text{mol}\cdot\text{l}^{-1}$ AA [13,14], 412 $\mu\text{mol}\cdot\text{l}^{-1}$ GSH and 17 $\mu\text{mol}\cdot\text{l}^{-1}$ GSSG [15], and 200 $\mu\text{mol}\cdot\text{l}^{-1}$ UA [13,14], either as single antioxidant solutions, or as a composite mixture of the above moieties at the prescribed concentrations. Solutions were prepared by dissolving each antioxidant in 0.9% (w/v) saline: approx. 150 $\text{mmol}\cdot\text{l}^{-1}$ NaCl (Baxter Healthcare Ltd., Thetford, Norfolk, U.K.) to which EDTA and desferoxamine mesylate were added to a final concentration of 0.1 $\text{mmol}\cdot\text{l}^{-1}$. This precaution was taken to minimize autoxidation reactions, especially of AA, in the presence of transition metals. These solutions were then adjusted to pH 7.4, to reflect the pH of normal human airway secretions. Single and composite solutions were prepared in bulk and then divided into four batches corresponding to each of the exposure conditions employed. These samples were stored at -70 °C and thawed immediately before exposure.

Exposure protocol

Exposure of BAL fluid or antioxidant solutions were carried out in a 5.6-litre purpose-built Perspex chamber [16], flushed with NO₂ at 50, 150, 400 or 1000 ppb at a flow rate of 3 litres/min. The exhaust air from the chamber was monitored using a Dasibi 2108 chemiluminescence NO_x detector (Dasibi Environmental Corp., Glendale, CA, U.S.A.). Deviations from the desired chamber NO₂ concentration were compensated for automatically by adjusting the NO₂ supply. This positive feed-back mechanism ensured that steady-state NO₂ concentrations were maintained within the exposure chamber. NO₂ concentrations varied by less than 5 ppb under all of the exposure protocol conditions. The chamber was maintained throughout the exposure period at 37 °C and the whole apparatus was mounted on an orbital shaker, which facilitated mixing in the exposure wells. The orbital shaker was set at an operating frequency of 128 oscillations/min to ensure adequate sample mixing.

Sample treatment

NO₂ exposures were carried out over 4 successive days, i.e. one NO₂ concentration per day. Briefly, an aliquot (12 ml) of each subject's BAL fluid or single and composite antioxidant solution was thawed at 09:00 h, and the pH of each sample adjusted to 7.4. EDTA and desferoxamine mesylate were added to a final concentration of 0.1 mmol/l . A series of five 1.0-ml

aliquots of each sample were placed in duplicate into 24-well tissue-culture plates within the exposure chamber. These samples were allowed to equilibrate to 37 °C, at which point a zero control sample was removed and frozen in liquid N₂. The chamber was then sealed and the exposure protocol commenced. At set intervals throughout the exposure, 0.5, 1, 2 and 4 h, the chamber was opened briefly, samples were removed and snap-frozen in liquid N₂ before storage at -70 °C. Antioxidant analyses were carried out within 72 h of exposure to NO₂.

Analysis of antioxidants

Determinations of AA and UA were carried out by HPLC as described previously [17]. Glutathione (GSH and GSSG) concentrations were determined using the enzyme recycling method described by Tietze [18], adapted for use on a microplate reader [16].

Determination of MDA

The HPLC determination of MDA was based on the method of Chirico [19]. Lavage samples [3 ml with 10 μl of 0.2% (w/v) butylated hydroxytoluene] were concentrated by freeze-drying and reconstituted in 350 μl of water. To this 50 μl of 25% (v/v) H₃PO₄ was added, mixed and allowed to stand at room temperature for 10 min. A 100 μl aliquot of 0.67% (w/v) thiobarbituric acid was then added to the acidified sample, which was mixed and then transferred to a 0.8 ml HPLC vial, sealed and incubated for 20 min at 95 °C. Following the immediate cooling of the sample, aliquots of 20 μl were analysed. MDA standards (50–500 nmol/l), prepared from 1,1,3,3-tetraethoxypropane in the same manner described above for the samples, were also routinely run. As the acid hydrolysis is always incomplete, the final MDA concentration is determined from its molar absorption coefficient at 532 nm (153000).

MDA analysis was performed using a Gilson model 234 autosampler. A 100 mm $\times$ 250 mm Spherisorb ODS2 5 μm column and a mobile phase of 50 mM KH₂PO₄ (pH 6.8)/methanol at a ratio of 65:35 (w/v) and a flow rate of 0.9 ml/min was used for elution of the thiobarbituric acid-MDA adduct. The model 980 fluorescence detector (ABI Applied Biosystems) with an excitation of 515 nm and emission filter of 550 nm was used for detection, with the photomultiplier tube signal set at 1.48.

Data handling

Antioxidant consumption data were analysed using a two-way analysis of variance with the Bonferroni correction for repeated measures. Antioxidant (UA, AA, GSH) and NO₂ concentrations (50, 150, 400 and 1000 ppb) were the factors used for analysis of the data set. The Student-Newman-Keuls procedure was used to compare all paired group means. Correlations were performed using the Spearman's rank order correlation test. In cases where individual correlations indicated a potential spurious interaction between variables, the true predictor was determined using partial correlation analysis. Statistical significance for all comparisons of means and correlations was accepted at $P < 0.05$.

RESULTS

Baseline antioxidant and protein concentrations were determined in BAL fluid samples obtained from the 12 subjects recruited to the study (Table 1). UA was detected in BAL fluid recovered from all 12 subjects and was present in the range 0.8–6.1 $\mu\text{mol/l}$. GSH was also present in all BAL fluid samples in the range 0.6–2.6 $\mu\text{mol/l}$. GSSG was present in the majority (10/12) of

Table 1 Initial BAL fluid antioxidant concentrations

nd, not detected.

Subject no.	Protein (mg/ml)	GSH ($\mu\text{mol/l}$)	GSSG ($\mu\text{mol/l}$)	AA ($\mu\text{mol/l}$)	UA ($\mu\text{mol/l}$)
1	0.3	0.7	0.1	0.8	4.6
2	0.5	1.1	0.2	nd	4.3
3	0.4	2.6	0.7	nd	2.0
4	0.3	1.2	0.6	2.0	4.1
5	0.6	1.0	0.3	nd	6.1
6	0.5	2.4	0.1	4.2	5.7
7	0.5	0.8	nd	2.2	1.9
8	0.4	1.4	nd	2.9	3.3
9	0.5	1.9	0.2	5.8	6.0
10	0.1	0.8	0.1	nd	0.8
11	0.3	0.6	0.2	nd	4.7
12	0.3	0.8	0.2	6.2	2.1

Figure 1 BAL fluid UA consumption by NO_2

(a) BAL fluid was exposed for 4 h to NO_2 at: A, 50 ppb; B, 150 ppb; C, 400 ppb; D, 1000 ppb. Samples were removed for UA analysis at 0, 5, 1, 2 and 4 h. Data are representative of 12 individual experiments. (b) Correlation between initial concentration of UA in BAL fluid and its rate of consumption by NO_2 ($r = 0.92$, $P < 0.05$; $n = 12$). Rate of UA consumption is in units of $\text{nmol} \cdot \text{l}^{-1} \cdot \text{h}^{-1} \cdot \text{ppb}^{-1}$ of NO_2 .

individuals and it represented a significant fraction of the total glutathione pool (range 4.0–33.2%). AA was detected in BAL fluid from 7/12 subjects in the range 0.8–6.2 $\mu\text{mol/l}$, and BAL fluid protein concentrations varied between 0.1 and 0.6 mg/ml.

Figure 2 BAL fluid AA consumption by NO_2

(a) BAL fluid was exposed for 4 h to NO_2 at: A, 50 ppb; B, 150 ppb; C, 400 ppb; D, 1000 ppb. Samples were removed for AA analysis at 0, 5, 1, 2 and 4 h. Data are representative of seven individual experiments. (b) Correlation between initial concentration of AA in BAL fluid and its rate of consumption by NO_2 ($r = 0.92$, $P < 0.05$; $n = 7$). Rate of UA consumption is in units of $\text{nmol} \cdot \text{l}^{-1} \cdot \text{h}^{-1} \cdot \text{ppb}^{-1}$ of NO_2 .

Exposure to 50 ppb NO_2 resulted in little loss of UA from BAL fluid (Figure 1). However, exposure to higher concentrations of NO_2 resulted in the depletion of UA in a linear fashion, which was dependent on the concentration of NO_2 (Figure 1a). Regression analysis showed that the rate of depletion of UA was positively correlated to NO_2 concentration ($r = 0.99$, $P < 0.05$; results not shown). Furthermore, as was noted, the depletion rate of UA varied between samples, and correlated with the initial concentration of UA in the BAL fluid ($r = 0.92$, $P < 0.05$; Figure 1b).

Following exposure to NO_2 , AA was also depleted in a linear fashion, which related to both NO_2 concentration ($r = 0.98$, $P < 0.01$; Figure 2a) and the initial AA concentration of the BAL sample ($r = 0.96$, $P < 0.01$; Figure 2b). The rates of AA depletion were very similar to those seen for UA, but no interaction was apparent between the rates of AA and UA consumption, suggesting that their consumption was independent of each other. Likewise, no correlation was seen between AA and either GSH or GSSG.

The rate of GSH depletion from BAL fluid was low, as only approx. 25% of the initial GSH was lost over the 4 h exposure period and this loss was independent of the NO_2 concentration (Figure 3a). There was little change in the concentration of

Figure 3 BAL fluid GSH consumption and GSSG production by NO₂

BAL fluid was exposed for 4 h to NO₂ at: (▲), 50 ppb; (●), 150 ppb; (■), 400 ppb; (◆), 1000 ppb. Samples were removed for (a) GSH and (b) GSSG analysis at 0.5, 1, 2 and 4 h. Data are representative of 12 individual experiments.

GSSG throughout the exposure period (Figure 3b). Low levels of MDA were detected in BAL fluid obtained from all subjects (range 0.00–22.4 nmol/l). Exposure to 50, 150 and 400 ppb NO₂ did not change the concentration of MDA in BAL fluid at any of the time points considered. However, following 1 h exposure to 1000 ppb, an increased MDA concentration was seen in 8/12 subjects, and further increases were seen with time of exposure (Table 2).

Exposure of the single antioxidant solutions resulted in the clear NO₂-concentration- and time-dependent substrate consumption. The loss of each antioxidant could be described by linear regression, suggesting that the rate of consumption was unaltered as the substrate concentration fell (results not shown). Likewise, when the combined antioxidant solution was examined, consumption of each antioxidant again occurred in a time- and NO₂-dependent manner (results not shown). From these data, and those described above, we calculated the overall rate of consumption for each antioxidant and compared these with those obtained for these antioxidants in BAL fluid (Table 3). Little difference was found between the rate of loss of AA or UA in any of the three RTLF models examined. In all cases, however, GSH consumption was significantly less than AA or UA consumption. As model complexity increased, the rate of individual antioxidant loss decreased, such that in BAL, AA, UA and GSH consumption rates were significantly less ($P < 0.05$) than in the pure or composite antioxidant mixtures. In particular, the rate of

Table 2 MDA concentrations in subjects exposed to 1000 ppb NO₂

Subject	[MDA] (nmol/l)					
	Time (h)	0	0.5	1	2	4
1*		6.7	4.8	7.1	5.2	5.7
2		12.6	11.2	16.3	19.4	28.6
3		9.4	10.9	14.7	24.3	31.6
4*		0.00	1.6	4.8	3.3	2.8
5		14.1	13.6	17.1	23.4	27.8
6		3.1	5.6	14.7	28.9	37.8
7		0.00	1.2	9.6	19.2	24.7
8*		0.4	0.7	1.8	2.6	1.7
9		0.8	2.4	6.7	10.9	20.1
10		14.6	11.1	17.4	27.3	39.4
11*		8.4	4.9	5.6	4.8	4.4
12		7.7	9.9	21.6	27.9	31.6

* Samples in which MDA concentrations did not change appreciably.

Table 3 Overall calculated antioxidant consumption rates in single and composite antioxidant solutions and BAL fluid

Values represent means $\pm$ S.D. *Significant difference between UA, AA and GSH consumption rate within each exposure model. †Significant difference between the rate of antioxidant consumption between the composite solutions. In all cases significance was assumed when $P < 0.05$.

Antioxidant	Overall consumption rates (nmol·l ⁻¹ ·h ⁻¹ ·ppb ⁻¹)		
	Pure antioxidant solutions	Composite antioxidant solutions	BAL fluid
UA	2.37 $\pm$ 0.21	2.13 $\pm$ 0.64	1.30 $\pm$ 0.23†
AA	2.22 $\pm$ 0.23	1.94 $\pm$ 0.53	1.41 $\pm$ 0.30†
GSH	1.42 $\pm$ 0.31*	1.17 $\pm$ 0.46*	0.19 $\pm$ 0.07*†

GSH loss from BAL fluid (0.2 ± 0.1 nmol·l⁻¹·h⁻¹·ppb⁻¹) was markedly lower than that seen when GSH either alone (1.4 ± 0.3 nmol·l⁻¹·h⁻¹·ppb⁻¹), or in combination with UA and AA (1.2 ± 0.5 nmol·l⁻¹·h⁻¹·ppb⁻¹), was exposed to NO₂.

DISCUSSION

This study was designed to improve our understanding of the interactions between NO₂ and the antioxidants present in the complex milieu of RTLF and to explore the biological basis of individual responses to NO₂. As Postlethwait and colleagues [20–22] have demonstrated similar NO₂ interfacial transfer kinetics by the intact lung and isolated lung lining fluids, we utilized three models of RTLF and an *ex vivo* exposure system to examine antioxidant–NO₂ interactions. The results obtained demonstrate that exposure to NO₂, at environmentally relevant concentrations, will result in the depletion of low-molecular-mass antioxidants, UA and AA, from RTLF. In the three models of RTLF utilized, UA and AA were depleted in a linear fashion, and in a NO₂-concentration- and time-dependent manner, although their rate of depletion did decrease as the model complexity increased. This in itself is not a surprising finding. However, as the more complex the model, the more potential targets it will have for NO₂ to react with, individual reaction rates should be lower.

An important finding of the present study was the low rate of GSH loss from BAL fluid exposed to NO₂. Results from both the

single and the composite RTLF antioxidant model confirm that NO_2 reacts with and depletes GSH. Therefore in BAL fluid either this reaction proceeded at a much lower rate, or thiol radicals arising from the reaction of GSH and NO_2 were reduced to GSH, possibly by AA and UA. It has been suggested that the role of GSH in the intracellular environment is to maintain other antioxidants in a reduced state [23]. Hence similar events may also occur extracellularly in RTLF.

In humans and higher primates, UA is the final breakdown product of adenine and guanine purines. Until recently, UA was thought of as a waste product; however, following filtration by the kidney, the majority is reabsorbed into plasma, indicating that it has a physiological role [24]. In 1981, Ames and co-workers [25] demonstrated that UA, present in human plasma, is a powerful inhibitor of lipid peroxidation. Indeed, these workers found that UA acid was as effective as AA in this respect. UA is present in the respiratory epithelial lining fluid of both the upper and lower airways. Interestingly, fluid lining the upper airways has significantly higher concentrations of UA than that in the lower airways. Recent studies have shown that UA in nasal lavage fluid is an efficient scavenger of another gaseous pollutant, ozone [13], indicating that the upper airways may be important in scrubbing polluted air and thus diminishing the oxidant burden to the lower respiratory tract.

The rates of both UA and AA consumption were correlated with their initial concentrations in BAL fluid, such that the higher the initial antioxidant concentration, the greater the rate of depletion of UA and AA. This finding agrees well with the concept of 'reactive absorption' as outlined by Postlethwait [22], who suggests that the rate of NO_2 absorption (or its disappearance) is dictated by the availability of reactive substrates in the lung fluid layer. It therefore follows that individuals with high concentrations of UA in their lung lining fluid should be able to remove NO_2 , at a faster rate and to a greater extent, from inspired polluted air than those with low UA concentrations.

Ascorbate is present in lung epithelial lining fluid of animals and humans [9,14] and it has been suggested to be the most important antioxidant in extracellular fluids [26]. A beneficial role of AA in RTLF was seen in the present study, as it was found to react with NO_2 in a concentration-dependent manner. As the amount of AA (and UA) in BAL fluid differs considerably between individuals, this may explain, in part, the increased sensitivity of certain individuals to oxidant gases [14,27]. Further support for this concept comes from studies in AA-depleted guinea pigs. These animals, like humans, cannot synthesize AA and are thus dependent solely on dietary sources. Exposure of guinea pigs, in which lung AA concentrations were depleted by 80% to 5 or 10 ppm NO_2 for 4 h, causes a significant increase in pulmonary microvascular permeability, confirming the importance of this antioxidant [28].

AA was depleted at a similar rate to UA in all three RTLF models in the present study. This observation differs from that of Halliwell and colleagues [6], who found that exposure of plasma to 14 ppm NO_2 resulted in a faster rate of depletion of AA than UA. This difference may arise from either the different protein and antioxidant composition of blood plasma and BAL fluid or the different reaction kinetics between NO_2 and reducing agents above 10.5 ppm [20,21].

No change in MDA content was observed following exposure to 50, 150 or 400 ppb NO_2 . In BAL fluid from 8/12 subjects exposed to 1000 ppb NO_2 , increased levels of MDA were seen following 60 min exposure, implying that lipid peroxidation was prevented until a sufficient decrease in antioxidant defences had

occurred. This observation agrees with similar findings by Halliwell and colleagues using blood plasma [6] and by Sagai and colleagues in animals [29]. BAL fluid lipid concentrations were not determined in the present study; hence it is conceivable that those subjects in which no increase in MDA was seen was due to low BAL fluid lipid concentration.

Although public health in general appears to be at risk from air pollution, some groups of people appear to be particularly susceptible, such as the elderly and those suffering from cardiovascular and respiratory diseases. From the results of the present study it appears that the antioxidant composition of RTLF could influence the amount of NO_2 reaching the underlying pulmonary epithelial cells. Hence individual sensitivity to air pollution may be due, in part, to the antioxidant status of this layer of fluid which covers the pulmonary epithelium. Studies are therefore warranted to address the antioxidant status of lung lining fluids in these susceptible groups.

We thank Matthew Cotgrove for technical assistance in these studies and the Special Trustees of St. Thomas' Hospital for financial support.

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