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The acute effect of cigarette smoking on the neutrophil elastase inhibitory capacity of peripheral lung lavage from asymptomatic volunteers

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ABSTRACT: Cigarette smoke-induced emphysema is thought to involve reduction of antielastolytic capacity, resulting in elevated elastase activity and lung tissue damage. Peripheral lavage collected from ten asymptomatic subjects immediately before and 20 min after smoking two high tar cigarettes was analysed for neutrophil elastase (NE) inhibitory capacity (IC), α_1-proteinase inhibitor (PI) function, elastolytic activity and immunoreactive levels of PI and bronchial inhibitor (BI). The only change found was a small fall in mol immunoreactive PI/mol albumin after smoking (~17%, $p<0.05$) which did not affect NEIC, since PI contributed less than 50% of the NEIC. There was often more NEIC than mol BI + functional PI, suggesting the presence of other NE inhibitors. Thoracic computerized tomography scans of eight of these subjects highlighted two with emphysematous regions of lung; lavage from these two subjects contained either undetectable BI or inactive BI and this suggests a protective role for BI in emphysema.

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Pulmonary emphysema is thought to develop as a result of increased elastolytic activity in the lung [1]. Neutrophil elastase (NE) has attracted most attention since a deficiency of α_1-proteinase inhibitor (PI), the major circulating inhibitor of NE, is associated with the premature development of emphysema [1, 2]. However, cigarette smokers, who account for most of those with emphysema, usually have normal levels of PI [3]. Consequently, it has been proposed that there is reduced functional PI within the lungs of smokers, possibly due to inactivation by tobacco smoke or phagocyte-derived oxidants or, alternatively, due to proteolysis of PI by macrophage-derived or other enzymes [1]. Although studies in man confirm that PI in bronchoalveolar lavage fluid (BALF) is partially inactive [4], and early studies showed lower levels of PI activity in BALF from smokers compared to nonsmokers [5, 6], more recent studies have shown that the degree of inactivation of PI is the same whether the subjects smoke or not [4, 7, 8]. In such studies there was nevertheless enough functional PI to counteract the level of immunoreactive NE present. Despite this, elastolytic activity, some of which appears to be neutrophil-derived, can be measured in BALF and it is chronically elevated in that from smokers suggesting that other mechanisms are also important in the control of NE activity [9–11].

A significant proportion of NE inhibitory capacity in BALF from both healthy subjects and patients is due to inhibitors other than PI and is stable in acid conditions [4, 11, 12]. A bronchial inhibitor (BI), synthesized and secreted by airways epithelium, accounts for some of the additional inhibitory activity, whilst other unknown inhibitors are thought to account for the remainder [4, 13]. Little is known about the role of the bronchial inhibitors in the protection of the lung from emphysema. In patients, lavage obtained from the peripheral lung (below the 7th airway generation) using a selective lavage technique contains BI [14, 15] and this, together with the observation that there is BI present in the secretory granules of the Clara cells in the terminal and respiratory bronchioles [16], where emphysema in smokers often begins, suggests that BI and possibly other inhibitors may play a significant role in the protection of peripheral lung tissue from elastolysis and emphysema.

The acute *in vivo* effect of cigarette smoking on BALF-PI is small [8] (10% decrease in activity 1 h after smoking two cigarettes) but may nevertheless be important in that it may represent extremely localized inhibition of functional PI, to the extent that NE is not inactivated in the immediate vicinity of the cell, which then forms a site for the production of focal damage [1]. Studies *in*

vitro show that bronchial inhibitors are also susceptible to oxidative inactivation [17] but to a lesser degree than PI; such inhibitors may provide a back-up to cigarette smoke inactivated PI as well as providing antiproteolytic protection in their own right. There are, however, no studies describing the acute *in vivo* effect of smoking on the total NEIC of BALF. In the present study we have utilized a previously described peripheral lavage technique [14] to examine NEIC and elastolytic activity of distal lung lavage samples obtained from the upper lobes (the area where most damage occurs) of asymptomatic subjects before and 20 min after smoking two cigarettes. Some of the results of this study have been reported in preliminary form elsewhere [18].

Methods

Subjects

Ten, asymptomatic smokers gave informed, written consent to take part in the study, the nature and purpose of which was explained by a physician who was not connected with the study. Permission was given by the hospital's Ethical Committee to study a maximum of ten subjects using this protocol. Details of age, sex, smoking history and respiratory function [19] of the subjects are given in table 1. No subject was on regular medication, and prior to the study all had fasted for 8 h and refrained from smoking for 24 h. All subjects completed the study and have subsequently remained well.

Table 1. – Details of subjects studied

No.	Age yrs	Sex	Cigarettes, pack-years*	FEV_1/FVC % pred	FRC % pred	Kco % pred	CT %
1	29	F	13	90	100	87	0.4
2	25	F	7	108	106	80	-
3	24	M	9	94	102	68	2.2
4	22	F	5	100	95	62	11.7
5	23	F	3	108	79	90	4.3
6	27	F	7	101	105	86	4.0
7	35	M	23	74	135	64	7.7
8	31	F	4	96	74	90	1.2
9	29	M	1	102	83	110	-
10	28	M	14	96	90	74	0.6
Median	26.5		7	98	97.5	83	3.1

*: pack-years is the product of the number of years of smoking and average number of packs of 20 cigarettes smoked per day; FEV_1: forced expiratory volume in one second; FVC: forced vital capacity; FRC: functional residual capacity; Kco: carbon monoxide transfer coefficient; % pred: % of predicted value; CT %: computerized tomography scan data, % of pixels in electromagnetic imaging number range -446 to -500. On the basis of pathological correlations at a lobar level a figure above 3% suggests the presence of emphysema [20].

Protocol

Subjects underwent bronchoscopy twice on the same morning, before and after smoking two cigarettes. They were premedicated with 1.0 mg of atropine and 10–15 mg of papaveretum intramuscularly 30 min before bronchoscopy. Mean alveolar carbon monoxide was measured using an Ecolyser 2000 (Energetics Science, Hawthorne, NY). A venous blood sample (10 ml) was taken to determine serum PI levels and phenotype. The mouth was anaesthetized using 4% Lidocaine spray, and the vocal cords and upper respiratory tract by transtracheal injection of 1–2 ml of 5% cocaine solution. An Olympus BF_1T bronchoscope was wedged at the third or fourth airway generation of the left upper lobe. A double lumen, 6F balloon-tipped catheter was passed through the biopsy channel and extended up to 5 cm beyond the tip of the bronchoscope to enable lavage of airways peripheral to the 7th generation as previously described [14, 15]. Following secure wedging of the inflated balloon, five separate 20 ml aliquots of warmed, sterile sodium chloride solution (0.15 M) were introduced and aspirated from the lower respiratory tract of a single bronchopulmonary segment. The balloon was then deflated and repositioned in an adjacent segment of the upper lobe and the procedure repeated. The sample was discarded if visible blood contamination had occurred and the lavage procedure repeated in another segment of the same lobe using a new catheter. The aspirated samples were collected and pooled for analysis.

The bronchoscope was withdrawn and the subjects then smoked two high tar (17 mg tar, 1.4 mg nicotine), unfiltered cigarettes through a cigarette holder. Subjects smoked each cigarette to a predetermined pattern which they had practised previously (5 s inhalation; 5 s breath-hold; 20 s normal breathing) until the lighted end of the cigarette reached the rim of the cigarette holder. There was a 3 min period of recovery between cigarettes. Immediately after cessation of smoking, measurement of the mean alveolar carbon monoxide level was repeated.

The bronchoscope was then reintroduced into the upper respiratory tract following supplementary topical anaesthesia of the vocal cords with 0.5–1.0 ml of 5%

cocaine solution. The bronchoscope was wedged in a segment of the right upper lobe and two separate bronchopulmonary segments lavaged as described above, within 20 min completion of smoking. The bronchoscope was withdrawn and a chest radiograph performed within 1 h of completion of the study.

Thoracic computerized tomography

Eight of the ten subjects underwent computerized tomography (CT) after completion of the study. Thoracic CT scans were performed with an electromagnetic imaging (EMI) 7020HR scanner as the subject breath-held for 20 s at functional residual capacity. Horizontal slices with a nominal slice width of 13 mm were obtained at 15 mm intervals from 15 mm above the sternal notch to below the diaphragm. The boundary of the lung was defined using a special computer programme which extracted the EMI number of pixels within the lung fields; these were then used to generate frequency distributions. The percentage of pixels with an EMI number range -446 to -500 was calculated for each subject and taken to represent the percentage of emphysematous lung tissue. This analysis was carried out by Dr A.T. Redpath, Dept of Medical Physics, Western General Hospital, Edinburgh, UK, as described previously [20].

Processing of lavage fluid

Lavage fluid was filtered through large gauge metal mesh and centrifuged at 300 g for 15 min at 4°C. The cell pellets were stored at -60°C until just prior to analysis when the cells were lysed by six cycles of freezing and thawing to room temperature. A 20 ml sample of the unconcentrated supernatant was kept at 4°C for immediate analysis, whilst the remainder was stored in aliquots at -60°C. All analyses were performed blind.

Antielastase activities

NEIC and functional PI activity in the lavage supernatant were assessed against pure NE and porcine pancreatic elastase (PPE), respectively. Both assays were performed with unconcentrated lavage fluid supernatant, immediately after processing to avoid loss of PI activity due to storage or extensive processing.

Determination of the relative activity of human neutrophil and porcine pancreatic elastase

The activity of human NE (a gift from Dr N.A. Roberts, Roche Products Ltd, UK) and PPE (Sigma Chemical Co., UK) was assessed by titration using substrates, substrate-enzyme concentrations and the buffer system described by NAKAJIMA *et al.* [21]; the NE substrate was methoxy-succinyl-dialanyl-prolyl-valyl-p-nitroanilide (AAPV) and the PPE substrate was N-succinyl-trialanyl-p-nitroanilide (SAPN), each dissolved in 0.1 M Hepes buffer at pH 7.5, containing 0.5 M sodium chloride (NaCl) and 10% dimethylsulphoxide to a final volume of 1.02 ml. The reaction was carried out at 25°C. The kinetic constants were, therefore, taken from the same source [21] to establish the activity of NE and PPE prior to the study.

Neutrophil elastase inhibitory capacity

Increasing volumes (2–200 μl) of lavage supernatant were incubated for 15 min at 37°C with 1.72 pmol active NE in 0.2 M Tris-HCl, pH 8, containing 0.016% v/v Triton-X-100 to a final volume of 1.25 ml. Residual enzyme activity was measured by the addition of 10 μl of the synthetic substrate AAPV (125 mM in N-methyl-pyrrolidone) for which NE is highly specific. After 1 h at 37°C, the reaction was terminated by the addition of 50 μl glacial acetic acid and the product of the reaction measured by its absorbance at 410 nm.

Alpha$_1$ proteinase inhibitor function

Increasing volumes (2–400 μl) of lavage supernatant were incubated for 15 min at 37°C with 7.3 pmol active PPE in the Tris buffer described above to a final volume of 1.25 ml. Residual enzyme activity was measured by the addition of 10 μl of the synthetic substrate, SAPN (125 mM in N-methyl-pyrrolidone). The reaction was terminated after 15 min at room temperature and the product released measured as described above. Any nonspecific effect of protein and normal saline was determined by adding equivalent volumes of bovine serum albumin (BSA; BDH Ltd, UK) in 0.15 M NaCl (250 $\mu g \cdot ml^{-1}$) to PPE and incubating in an identical manner to that for lavage samples.

Determination of supernatant inhibitory capacities

Conventional methods were used to determine the NEIC of lavage. Percent remaining elastase activity (y-axis) was plotted against volume of lavage fluid (x-axis) for each sample. The line of best linear fit was determined by least squares regression. Extrapolation of the line through the x-axis gave the volume of lavage fluid (containing known amounts of albumin and inhibitors) required to cause 100% inhibition of NE. Data were expressed as mol NE inhibited per mol albumin. The functional activity of PI against PPE was determined in a similar manner and the data expressed as mol PPE inhibited per mol PI and also as mol PPE inhibited per mol albumin.

Elastolytic activity

Elastolytic activity was assayed in unconcentrated lavage supernatant on the day of collection by the modified method of BANDA and WERB [22]. Elastolytic activity in the cellular component of lavage was assayed

during the following week. Lavage supernatant (0.5 ml) or lysed cell suspension (0.1 ml) was incubated for 24 h at 37°C with 0.22 mg tritiated elastin suspension in 100 mM Tris-HCl, 5 mM calcium chloride ($CaCl_2$), 0.02% (w/v) NaN_3, pH 7.8 to a final volume of 1 ml. Release of solubilized elastin fragments was determined as previously described [11]. Active site titrated NE was used as standard and activities expressed as mol NE equivalents/mol albumin and pmol NE equivalents·10^{-6} cells. Control assays consisted of lavage supernatant or lysed cell suspension and elastin substrate which were incubated independently and mixed in equivalent proportions to the test assay just prior to the termination of the assay.

Protein levels

Frozen lavage samples were used to measure protein levels. Total protein was assayed by the method of LOWRY *et al.* [23] standardized against bovine serum albumin (BSA). Levels of albumin (range of standards, 6–120 $\mu g \cdot ml^{-1}$) were measured by rocket immunoelectrophoresis (10 µl sample per well) as described previously [11] and PI levels were assayed by laser nephelometry [24]. Levels of α_2-macroglobulin (a_2M) were measured by a sandwich enzyme-linked immuno sorbent assay (ELISA) ([25]; range of standards, 1–26 $\mu g \cdot ml^{-1}$; 100 µl per assay). Standards for these assays were obtained from Behring Diagnostics (Behring Diagnostics, a Division of Hoechst UK, Ltd) and monospecific antisera was obtained from DAKO Ltd (UK).

Plasma albumin and PI levels were also measured as described above, except that the samples were diluted 1:500 for albumin and 1:50 for estimation of PI. In addition phenotyping of PI was carried out at the Supraregional Protein Reference Unit, Royal Hallamshire Hospital, Sheffield, UK.

In order to measure BI, a portion of each stored supernatant was concentrated tenfold as described previously [11], at least 90% of total protein being recovered in the concentrate. Levels of BI were measured in the concentrates using a previously described method of radial immunodiffusion [26]: BI purified from sputum in this laboratory was used as standard ([26]; 1.5–50 $\mu g \cdot ml^{-1}$; 10 µl sample per well).

The effect of cigarette smoke on the quantification of BI and PI.

The same brand of high-tar unfiltered cigarette was used as that employed in the *in vivo* study. Smoke was slowly drawn through a Millipore prefilter into a plastic syringe. Increasing volumes (1–4 ml) of smoke were immediately bubbled through 4 ml of: a) lavage fluid supernatant; b) sputum sol-phase; taking approximately 5 s per ml of smoke. Portions of test fluid were removed sequentially after each additional exposure to cigarette smoke. The BI and PI levels in the untreated and smoke-exposed samples were quantified side-by-side as described above. The experiment was repeated four times using samples from four of the lavages collected during the course of the study (before smoking) as well as other samples collected for other reasons.

Statistics

The data were not always normally distributed and were therefore analysed using non-parametric statistics. The Wilcoxon Signed Rank Test was used for paired samples. Where samples were not paired the Wilcoxon Rank Sum Test was used. A Spearman Rank Correlation Coefficient (r_s) was employed to test for correlation between variables. The significance level was taken as $p<0.05$ in a two-tailed test. Data are tabulated as median values together with the limits of the range, and refer to ten subjects unless otherwise stated.

Results

Evidence for airflow obstruction and emphysema in subjects under investigation

Details of the subjects' smoking history, lung function and extent of emphysematous lung tissue as determined by CT scanning are given in table 1. With the exception of subject 7, with the greatest cigarette exposure (23 pack-years), no subject showed evidence of airflow obstruction or hyperinflation of the lungs. Median transfer coefficient was 83% of predicted normal; subjects 3, 4 and 7 showed more serious reductions in transfer coefficient. Subjects 4 and 7 showed the largest percentage of pixels in the EMI number range -446 to -500 taken to represent the percentage of emphysematous lung tissue [20]. On the basis of a pathological correlation in patients undergoing lobar resection for bronchial carcinoma, an upper limit for normality of 3% of pixels with EMI numbers in this range has been suggested [20]. Subject 4 had cigarette smoke exposure of only 5 pack-years but showed the lowest transfer coefficient and the highest percentage (11.7%) of emphysematous lung tissue [20]. Although these tests revealed that there were some subjects with evidence of lung abnormality, they were included in the study since they were asymptomatic and may be the individuals at risk of developing clinically obvious emphysema.

Levels and phenotyping of serum PI

All subjects were PiM phenotype with a median plasma PI concentration of 2.5 $gm \cdot l^{-1}$ (range 1.9–3.5 $gm \cdot l^{-1}$). The median PI/albumin ratio (mol/mol) was 0.077, range 0.055–0.115.

Alveolar carbon monoxide level

Prior to smoking the alveolar carbon monoxide level was 5±3 ppm (mean±SD); immediately after smoking this had risen to 14±4 ppm (mean±SD).

Fluid recovery and cells

The percentage of instilled fluid recovered before smoking was approximately 30% and did not differ after cigarette smoke exposure (table 2). Similarly, the cell numbers and profiles were within the normal range for this laboratory [15], with the exception of one subject (subject 4) who had above normal levels of polymorphonuclear neutrophils both before and after smoking (7 and 11%, respectively). Eosinophils were detected in the second lavage of two of the subjects (1% and 2% of the total cell number; subjects 6 and 2, respectively). Red blood cells and epithelial cells were either absent or minimal, when peripheral lavage fluid (PLF) was examined microscopically.

Total albumin and protein

Albumin levels were measured in all supernatant samples and although the median increased, there was no significant difference after smoke exposure (table 3). Total protein levels were assessed in 17 of the 20 lavage supernatants (there was insufficient volume in the remaining three). There was no difference in protein concentration ($mg \cdot ml^{-1}$ PLF) or total protein (per lavage) following smoking (table 3). Consequently the albumin/protein ratio was also unaltered after smoking, albumin accounting for approximately one third of total protein (table 3).

Antiprotease levels

Addition of cigarette smoke to lavage fluid and sputum sol-phase made no difference to quantification of BI and PI (mean recovery:- BI 98%, range 92–105%; PI 96%, range 88–99%). Previous studies in this laboratory showed that when BI was mixed with BALF or NE the recovery was 96±13% and 95±17% (mean±SD), respectively [26, 30], which compares well with recoveries obtained by others [13]. BI (n=18) contributed (mol/mol) approximately one third of the measured antiproteases (median 28%, range 0–46, before; median 37%, range 0–56, after), while PI accounted for most of the remainder of the antiproteases assayed in the lavage supernatants (median 72%, range 53–100, before; median 63%, range 44–100, after) (table 3). Alpha$_2$-macroglobulin was present in only trace amounts, contributing less than 1% of the total antiprotease measured, except for one sample (subject 5, after smoking) in which a_2M contributed 3% of the total. There were no differences in the levels per lavage of these inhibitors after smoking (table 3), although standardization to albumin showed a statistically significant reduction in PI/albumin following smoke exposure ($0.02<p<0.05$, table 3). Thus, there was a positive linear correlation between the serum PI/albumin ratio (mol/mol) and pre-smoking PLF PI/albumin ($r_s=0.756$, $0.02<p<0.05$), but no correlation between the serum and PLF PI/albumin ratio after smoking ($r_s=0.562$, $p>0.10$). Neither mol a_2M/mol albumin nor mol BI/mol albumin altered in PLF obtained after smoking (tables 3 and 4).

Antielastase activity

Median NEIC per mol albumin did not significantly alter following cigarette smoke exposure (table 4).

Table 2. – Recovery of lavage fluid and cells before and after cigarette smoking

	Before smoking	After smoking
% recovery of instilled saline	34.3 (14.0–62.0)	29.5 (14.5–45.0)
Total cell number ($\times 10^6$)	25.2 (4.2–42.6)	22.1 (5.1–67.0)
Cell profile		
% alveolar macrophages	94.5 (81.0–100.0)	94.5 (84.0–99.0)
% polymorphonuclear neutrophils	4.0 (0.0–7.0)	3.0 (0.0–11.0)
% lymphocytes	2.0 (0.0–11.0)	1.5 (0.0–13.0)

Data are expressed as medians with ranges in parentheses.

Table 3. – Lavage albumin and protein levels and ratio of antiproteases to albumin before and after smoking

	Before smoking	After smoking
Total/lavage		
Protein mg**	14.38 (3.53–35.14)	13.17 (3.39–25.60)
Albumin mg	3.99 (0.32–11.83)	4.26 (0.74–8.38)
Albumin/protein**	0.332 (0.184–0.463)	0.332 (0.143–0.410)
Ratio to albumin mol/mol		
BI***	0.027 (0.000–0.074)	0.034 (0.000–0.093)
PI	0.092 (0.044–0.115)	0.076* (0.040–0.113)
a_2M ($\times 10$)	0.004 (0.002–0.071)	0.005 (0.001–0.027)

Data expressed as medians with ranges in parentheses. n=10, unless stated otherwise; **: n=9 before, 8 after; ***: n=9; *: significantly lower than before smoking, $0.02<p<0.05$. BI: bronchial inhibitor; PI: proteinase inhibitor; a_2M: α_2-macroglobulin

Table 4. – Protease inhibitor levels and neutrophil and pancreatic elastase inhibitory capacity in lung lavage before and after smoking

Subject		BI/Al ×10	NEIC/BI+ active PI	NEIC/ active PI	NEIC/Al ×10	%PPEIC /NEIC	PPEIC/Al ×10	PI/Al ×10	PPEIC/PI
4	B	0.00	4.42	4.42	0.84	23	0.19	0.91	0.21
	A	0.00	3.26	3.26	0.62	31	0.19	0.77	0.24
7	B	0.44	0.26	0.89	0.16	>100	0.18	0.66	0.29
	A	0.39	0.27	0.88	0.15	>100	0.17	0.40	0.41
1–10,	B								
median		0.27[$]	0.96	2.59	0.47	40	0.19*	0.92	0.23
range		0.00–0.74	0.26–4.42	0.81–4.53	0.16–1.07	22–100	0.13–0.31	0.44–1.15	0.19–0.32
1–10,	A								
median		0.34[$]	0.86	2.33	0.51	40	0.20*	0.76**	0.25
range		0.00–0.93	0.24–3.26	0.88–3.71	0.15–0.64	25–100	0.10–0.30	0.40–1.13	0.17–0.41

Data expressed mol/mol. B: before lavage; A: after lavage; Al: albumin; BI: bronchial inhibitor; NEIC: neutrophil elastase inhibitory capacity; PI: proteinase inhibitor; PPEIC: porcine pancreatic elastase inhibitory capacity; [$]: n=9; *: <NEIC/Al, $p<0.005$; **: A<B, $p<0.05$.

The functional activity of PI was low (~25%), both before and after smoking, with no significant change following smoke exposure (table 4). The mol PPE inhibited/mol albumin did not alter after smoking cigarettes even though there was a fall in the mol PI/mol albumin ratio, because there was a significant inverse relationship between the change in mol PI/mol albumin and the change in mol PPE inhibited/mol PI (r_s=-0.88; $p<0.001$). The resultant change in porcine pancreatic elastase inhibitory capacity (PPEIC)/albumin after smoking therefore contributed little to the overall change in NEIC, particularly since PPEIC/abumin was significantly lower than NEIC/albumin ($p<0.001$, all samples), before ($p<0.02$) and after ($p<0.01$), smoking (table 4). Only in subject 7 could PLF-NEIC be accounted for by the active PI present, as in the remaining subjects there was usually more than twice as much NEIC than could be attributed to active PI in PLF both before and after smoking (table 4).

Ideally, NEIC should be expressed in terms of functional inhibitors. Acid treatment of PLF to destroy other inhibitors causes dissociation of NE-BI complexes and prevents accurate assessment of functional BI in PLF. In the absence of these values we have expressed the data in terms of total BI (assuming 100% activity) and active PI. NEIC per mol BI plus mol active PI did not alter after smoking (table 4). There was frequently more NEIC than could be provided by the known level of inhibitors, *i.e.* the NEIC/BI + active PI was greater than 1.0 (table 4). This was true of subjects 1, 2, 4 and 6 before smoking and subjects 3, 5, 6 and 8 after smoking. In the remaining three subjects the value was always less than 1.0. There was no correlation between the change in BI/albumin and the change in NEIC/albumin after smoking (data not shown).

Elastolytic activity

Elastolytic activity (expressed as NE equivalents; MW NE=30 kDa) against insoluble elastin did not vary significantly after smoking (median 0.20: range 0.02–0.75 before smoking; median 0.14: range 0.00–0.50 after smoking; mmol NE equivalents/mol albumin; n=9), however the data were expressed. Elastolytic activity in lavage supernatants was low, the ratio to NEIC (mol/mol) being less than 0.01, with the exception of subject 1 in whom the ratio was 0.03 and 0.02 before and after smoking, respectively. There were no differences between cellular elastolytic activity before and after smoking (n=6 paired samples; median 2.2: range 0.7–10.2 before; median 1.8: range 1.0–9.0 after; pmol NE equivalents·10^6 cells). Cellular and acellular elastase activity was assayed in 14 PLF samples; cellular elastolytic activity accounted for a median of 79.2% (range 13.3–91.6) of the total activity measured.

Discussion

We have attempted to determine the acute effect of cigarette smoke on the NEIC of epithelial lining fluid below the large airways, sampled by saline lung lavage. The antielastase content of peripheral lavage from asymptomatic subjects was very heterogeneous, with wide variation in the levels of bronchial inhibitor and NEIC per unit albumin. It was notable that, in the two subjects who had regions of emphysematous lung tissue (measured by CT scanning), there was either no detectable BI, or the BI detected was inactive both before and after smoking. Although the PI/albumin ratio fell significantly

after smoking there was no concurrent fall in NEIC/albumin since: a) the fall in the level of PI tended to be compensated for by an increase in the proportion that was active; and b) the contribution of functional PI to the NEIC was usually less than that of other inhibitors (approximately 40%). Furthermore, NEIC could not always be accounted for by the level of BI and active PI present, supporting the suggestion that there are other inhibitors of NE in lung secretions [13].

The differences between the levels of PI before and after smoking might have been caused by the first bronchoscopy and lung lavage or may reflect normal differences between contralateral lobes; we could not investigate this as the volunteers were not prepared to undergo a sham smoking study. However, we think it unlikely as, in a previous study comparing peripheral and bronchoalveolar lavage from patients, we found no differences in the PI or BI to albumin ratio in lavage from contralateral lungs or between consecutive lavages [15]. PI is thought to diffuse freely across the pulmonary interstitium alongside albumin [27], which was by convention used to standardize this data [27]. The PI/albumin ratio in PLF before smoking correlated well with that in the serum suggesting free, equivalent diffusion of the two proteins, which have similar molecular masses. It is, therefore, unlikely that PI/albumin varies significantly between lobes of largely normal lungs. Thus, the significant fall in PI/albumin after smoking suggests a specific, consistent effect of smoking. Perhaps smoking stimulates clearance of inactivated PI (complexed to enzyme, oxidised or proteolysed) from the lung possibly by diffusion into the circulation due to increased lung permeability or by stimulating phagocytosis by alveolar macrophages. This would explain our observation of less immunoreactive PI per unit albumin which was proportionally more active in lung washings after smoking, resulting in only minimal changes in the overall functional PI per unit albumin.

The observation that PI is only ~25% active, and that there is more than twice as much NEIC than active PI in PLF, agrees with recent similar studies with BALF from healthy smokers [13, 28] and suggests that changes in the level or activity of PI may only partly explain the pathological effects of cigarette smoke in emphysema. Since this is the only published study on PI activity in peripheral lavage we cannot make direct comparisons even though these findings are highly comparable to studies where BALF has been analysed [28]. Early investigations of PI activity in lung washings suggested that PI was fully active [8] and accounted for all the measurable NEIC; however, later studies, possibly employing purer standards, active site titrated enzymes and more appropriate methods of processing and analysis of lavage, indicate that the PI in lung washings is partially inactive and that other inhibitors of NE exist [4, 12, 13, 28].

The BI/albumin ratios (mol/mol; median 0.031, median of data before and after smoking) are higher than those described by BOUDIER *et al.* [13] (mean 0.013), but lower than those described by ABRAMS *et al.* [29] (mean 0.137) for BALF from healthy subjects. Such inconsistencies may reflect measurement of different proteins or sub-units of the same protein [26, 30] or overestimation of BI when measured by radial immunodiffusion [31], as used in this study and that of ABRAMS *et al.* [29]. However, we have shown that the latter explanation is unlikely in this study (results; [26, 32]). A possible explanation for the low BI/albumin values obtained in this study compared to those obtained by ABRAMS *et al.* [29] is that peripheral lavage is not analogous to BALF, which contains proteins from large airways [14]. Thus, hypersecretion by the airways of some smokers could significantly elevate the levels of BI in BALF but not in PLF [14, 15]. The level of bronchial inhibitors measured in lung washings and sputum may also depend on the clinical status of the subject [29, 32] and it has been hypothesized that there may be genetic variability between individuals in the levels of bronchial inhibitors [34]. Sub-clinical and genetic factors may, therefore, account for the current observations as well as the findings of BOUDIER *et al.* [13] of a high degree of heterogeneity in the composition of the antielastase screen of apparently healthy subjects.

As mentioned previously, functional PI contributed little to the overall change in NEIC activity after smoking. However, in subject 7, NEIC was the lowest, did not alter after smoking and appeared to be entirely due to PI, suggesting that the relatively high level of BI was inactive. In contrast, subject 4 had no measurable BI but the highest NEIC/BI+active PI, which could not be accounted for by PI, suggesting the presence of other NE inhibitors. Both subjects had emphysematous regions of lung tissue on analysis by CT scanning, as well as impaired carbon monoxide transfer coefficient (KCO), implying that the presence of functional bronchial inhibitor may be critical in the control of emphysema. While subject 7 was the heaviest smoker in the group (23 pack-years), and might have been expected to have focal emphysema, subject 4 was young, had a smoking history of only 5 pack-years and yet had the highest percentage of abnormal lung tissue. The latter subject may fall into a group of individuals who are inherently susceptible to cigarette smoke-induced emphysema possibly due to absent or low levels of bronchial inhibitor.

The elastolytic activity measured in these samples was very low; studies in this laboratory [11, 35] and by others [9, 10] indicate that it is unlikely to be entirely due to neutrophil-derived enzyme. The subjects with emphysematous regions of lung tissue had less than half the lavage elastolytic activity measured in subject 1. A critical question, therefore, is whether elastase at the epithelial surface ever gets to, or is active within, the interstitium. Cigarette smoking induced a rapid increase in the level (not activity) of NE in BALF [36] but the proportion of NE to PI was minute, suggesting that large amounts of enzyme released during smoking is phagocytosed by macrophages [37] or passes rapidly into the lung tissue and into the blood stream. The latter suggestion is supported by the observation of elevated NE-PI complexes in serum immediately after smoking [38]. Whether NE complexed to PI or BI would subsequently be active in the interstitium is unknown, although *in vitro* studies

indicate that this is unlikely. Thus, if elastase released at the epithelial/air interface were to be important in interstitial connective tissue turnover, one would hypothesize that it reaches the interstitium in its functional state or in an uncomplexed form (oxidised?) which might easily be re-activated under conditions that exist in the interstitium.

In summary, the only consistent acute effect of cigarette smoke on the elastase:antielastase profile of epithelial lining fluid was a reduction in the PI/albumin ratio. Since this appeared to have been a ubiquitous effect, and emphysema in smokers is not, the exact relevance of this observation to the development of the disease is not clear but may, nevertheless, be important in association with other changes. For example, the observation of emphysematous regions of lung tissue in a young subject with undetectable levels of lavage BI who had only a short smoking history, and in another subject who was a heavy smoker but whose lavage BI was apparently inactive, implies that BI might be significant in preventing cigarette smoke-induced emphysema. A caveat to such a suggestion would be that the emphysematous regions might reflect damage that had occurred earlier in life and were not related to smoking or the observations made in the present study. Continuous monitoring of such subjects should establish whether or not this is so.

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References

1. Janoff A. – Elastases and emphysema. Current assessment of the protease-antiprotease hypothesis. *Am Rev Respir Dis*, 1985, 132, 417–433.
2. Eriksson S. – Studies in α_1-antitrypsin deficiency. *Acta Med Scand*, 1965, 177 (Suppl.), 1–85.
3. Bridges RB, Kimmel DA, Wyatt RJ, Rehm SR. – Serum antiproteases in smokers and nonsmokers. Relationship to smoking status and pulmonary function. *Am Rev Respir Dis*, 1985, 132, 1162–1169.
4. Boudier C, Pelletier A, Pauli G, Bieth JG. – The functional activity of α_1-proteinase inhibitor in bronchoalveolar lavage fluids from smokers and nonsmokers. *Clin Chim Acta*, 1983, 132, 309–315.
5. Gadek JE, Fells GA, Crystal RG. – Cigarette smoking induces functional antiprotease deficiency in the lower respiratory tract of humans. *Science*, 1979, 206, 1315–1316.
6. Carp H, Miller F, Hoidal JR, Janoff A. – Potential mechanism of emphysema: α_1-proteinase inhibitor recovered from lungs of cigarette smokers contains oxidised methionine and has decreased elastase inhibitory capacity. *Proc Natl Acad Sci*, 1982, 79, 2041–2045.
7. Stone PJ, Calore JD, McGowan SE, Bernado J, Snider GL, Franzblau C. – Functional α_1-proteinase inhibitor in the lower respiratory tract of cigarette smokers is not decreased. *Science*, 1983, 221, 1187–1189.
8. Abboud RT, Fera T, Richter A, Tabona MZ, Johal S. – Acute effect of smoking on the functional activity of α_1-protease inhibitor in bronchoalveolar lavage fluid. *Am Rev Respir Dis*, 1985, 131, 79–85.
9. Janoff A, Raju L, Dearing R. – Levels of elastase activity in bronchoalveolar lavage fluids of healthy smokers and nonsmokers. *Am Rev Respir Dis*, 1983, 127, 540–544.
10. Niederman MS, Fritts LL, Merrill WW, Fick RB, Matthay RA, Reynolds HY, Gee BL. – Demonstration of a free elastolytic metalloenzyme in human lung lavage fluid and its relationship to α_1-antiprotease. *Am Rev Respir Dis, 1984*, 129, 943–947.
11. Smith SF, Guz A, Cooke NT, Burton GH, Tetley TD. – Extracellular elastolytic activity in human lung lavage: a comparative study between smokers and nonsmokers. *Clin Sci*, 1985, 69, 17–27.
12. Stockley RA, Morrison H, Smith SF, Tetley TD. – Low molecular mass bronchial proteinase inhibitor and α_1-proteinase inhibitor in sputum and bronchoalveolar lavage. *Hoppe-Seyler's Z Physiol Chem*, 1984, 365, 587–595.
13. Boudier C, Pelletier A, Gast A, Tournier J-M, Pauli G, Bieth JG. – The elastase inhibitory capacity and the α_1-proteinase inhibitor and bronchial inhibitor content of bronchoalveolar lavage fluids from healthy subjects. *Biol Chem Hoppe-Seyler*, 1987, 368, 981–990.
14. Tetley TD, Smith SF, Burton GH, Winning AJ, Cooke NT, Guz A. – Effects of cigarette smoking and drugs on respiratory tract proteases and antiproteases. *Eur J Respir Dis*, 1987, 153 (Suppl.), 91–100.
15. Smith SF, Guz A, Winning AJ, Cooke NT, Burton GH, Tetley TD. – Comparison of human lung surface protein profiles from the central and peripheral airways sampled using two regional lavage techniques. *Eur Respir J*, 1988, 1, 792–800.
16. De Water R, Willems LNA, Van Muijen GNP, Franken C, Fransen JAM, Dijkman JH, Kramps JA. – Ultrastructural localisation of bronchial antileukoprotease in central and peripheral human airways by a gold-labelling technique using monoclonal antibodies. *Am Rev Respir Dis*, 1986, 133, 882–890.
17. Smith CE, Stack MS, Johnson DA. – Ozone effects on inhibitors of human neutrophil proteinases. *Arch Biochem Biophys*, 1987, 253, 146–155.
18. Tetley TD, Smith SF, Burton GH, Cooke NT, Winning AJ, Guz A. – Acute effects of cigarette smoking on the antiprotease-protease balance of the human lung surface. *Clin Sci*, 69 (Suppl.), 58P.
19. Cotes JE. – *In*: Lung function assessment and application in medicine. 3rd edn, Blackwell, Oxford, 1975.
20. Gould GA, MacNee W, McLean A, Warren PM, Redpath A, Best JJK, Lamb D, Flenley DC. – CT measurements of lung density in life can quantitate distal airspace enlargement - an essential defining feature of human emphysema. *Am Rev Respir Dis*, 1988, 137, 380–392.
21. Nakajima K, Powers JC, Ashe BM, Zimmerman M. – Mapping the extended substrate binding site of cathepsin G and human leukocyte elastase. *J Biol Chem*, 1979, 254, 4027–4032.
22. Banda MJ, Werb Z. – Mouse macrophage elastase. Purification and characterisation as a metalloproteinase. *Biochem J*, 1981, 193, 589–605.
23. Lowry OH, Rosebrough JJ, Farr AL, Randall RJ. – Protein measurement with the folin reagent. *J Biol Chem*, 1951, 193, 265–275.
24. Colley CM, Fleck A, Goode AW, Muller BR, Myers MA.

– Early time course of the acute phase protein response in man. *J Clin Pathol*, 1983, 36, 203–207.
25. Voller A, de Saigny D. – Enzyme linked immunosorbent assay (ELISA). *In*: Techniques in clinical immunology. 2nd edn. R.A. Thompson ed, Blackwell, Oxford, 1981, pp. 157–169.
26. Smith SF, Guz A, Burton GH, Cooke NT, Tetley TD. – Acid-stable low molecular mass proteinase inhibitors in human lung lavage. *Biol Chem Hoppe-Seyler*, 1986, 367, 183–187.
27. Gadek JE, Hunninghake GW, Fells GA, Zimmerman RL, Keogh BA, Crystal RG. – Evaluation of the protease-antiprotease theory of human destructive lung disease. *Bull Eur Physiopathol Respir*, 1980, 16 (Suppl.), 27–40.
28. Afford SC, Burnett D, Campbell EJ, Cury JD, Stockley RA. – The assessment of α_1-proteinase inhibitor form and function in lung lavage fluid from healthy subjects. *Biol Chem Hoppe-Seyler*, 1988, 369, 1065–1074.
29. Abrams WR, Fein AM, Kucich U, Kueppers F, Yamada H, Kuzmowycz T, Morgan L, Lippman M, Goldberg SK, Weinbaum G. – Proteinase inhibitory function in inflammatory lung disease. 1. Acute bacterial pneumonia. *Am Rev Respir Dis*, 1984, 129, 735–741.
30. Boudier C, Carvallo D, Roitsch C, Bieth JG, Courtney M. – Purification and characterisation of human bronchial proteinase inhibitor. *Arch Biochem Biophys*, 1987, 253, 439–445.
31. Tournier JM, Sautegeau A. – Effect of leukocyte proteinases and protein concentration on the immunological assessment of bronchial inhibitor in sputum and bronchoalveolar lavage fluids. *In:* Pulmonary emphysema and proteolysis: 1986. J.C. Taylor and C. Mittman, eds. Academic Press, London, pp. 357–358.
32. Smith SF. – Biochemical changes in extracellular lung surface components following tobacco smoke exposure. PhD Thesis, University of London, 1985.
33. Morrison HM, Kramps JA, Burnett D, Stockley RA. – Lung lavage fluid from patients with α_1-proteinase inhibitor deficiency or chronic obstructive bronchitis: anti-elastase function and cell profile. *Clin Sci*, 1987, 72, 373–381.
34. Dijkman JH, Kramps JA, Franken C. – Antileukoprotease in sputum during bronchial infections. *Chest*, 1986, 89, 731–736.
35. Hersh AD, Roberts NA, Guz A, Tetley TD. – Elastase profile of human lung lavage. *Clin Sci*, 1987, 72 (Suppl.), 6P.
36. Fera T, Abboud RT, Richter A, Johal SS. – Acute effect of smoking on elastase-like esterase activity and immunologic neutrophil elastase levels in bronchoalveolar lavage fluid. *Am Rev Respir Dis*, 1986, 133, 568–573.
37. White R, Janoff A, Gordon R, Campbell E. – Evidence for *in vivo* internalisation of human leukocyte elastase by alveolar macrophages. *Am Rev Respir Dis*, 1982, 125, 779–781.
38. Lacronique J, Braunstein G, Ferrer P, Labrune S, Lockhart A, Marsac J. – Peak of plasma immunoreactive neutrophil elastase after acute smoking in man. *Clin Sci*, 1987, 72 (Suppl.), 74P.

Effet aigu de la consommation de cigarettes sur la capacité d'inhibition de l'élastase neutrophilique dans les produits de lavage pulmonaire périphérique chez les volontaires asymptomatiques. T.D. Tetley, S.F. Smith, A.J. Winning, J.M. Foxall, N.T. Cooke, G.H. Burton, E. Harris, A. Guz.

RÉSUMÉ: Un des mécanismes proposés pour l'emphysème pulmonaire induit par la fumée de tabac, comporte la réduction de la capacité anti-élastolytique avec, par voie de conséquence, une augmentation de l'activité élastasique des neutrophiles (NE) et ainsi que des lésions tissulaires pulmonaires. Des études antérieures ont montré que l'effet aigu in vivo de la consommation de cigarettes sur un inhibiteur des protéinases (PI: l'agent sérique majeur anti-NE) dans les sécrétions pulmonaires, était faible (diminution de l'activité de 10%). L'inhibiteur bronchique dérivé de l'épithélium (BI) et d'autres inhibiteurs de NE, sont présents dans les sécrétions pulmonaires et contribuent à la capacité inhibitrice NE (NEIC). Le but de cette étude est de déterminer l'effet aigu de la consommation de cigarettes sur le NEIC (c'est-à-dire celui dû à tous les inhibiteurs NE), aussi bien que la fonction PI, BI et les niveaux de PI et l'activité élastolytique dans les lavages périphériques prélevés chez 10 sujets asymptomatiques immédiatement avant et 20 minutes après avoir fumé 2 cigarettes à contenu élevé en goudron (17 mg/cigarette). On n'a pas relevé de modification du NEIC médian (0.047 avant, 0.051 après; mol NE inhibited/mol albumin) ou BI (0.027 avant, 0.034 après; mol BI/mol albumin) ou d'activité élastasique (0.20 avant, 0.14 après; mol NE equivalents/mol albumin x 10^3) après consommation de tabac. PI s'avère actif à 25% avant et après le tabagisme, contribuant donc pour moins de la moitié à NEIC totale. Quoique le rapport mol immuno-réactif PI/mol albumin tombe après consommation de tabac (médiane 0.092 avant, 0.076 après; $p<0.05$), ceci n'influence pas NEIC, puisque les modifications dans mol immuno-réactif PI/mol albumin sont en relation inverse avec les modifications dans mol functional PI/mol albumin ($r_s=-0.88$; $p<0.001$). Il n'y avait pas de relation entre les modifications de NEIC et les modifications des niveaux de BI. NEIC ne pouvait pas toujours être prise en compte pour le niveau de BI + PI fonctionnel, suggérant la présence d'autres inhibiteurs de NE. L'analyse des scans thoraciques tomographiques computérisés chez 8 des sujets, a fait ressortir la présence de régions emphysémateuses pulmonaires chez 2 d'entre eux. Un de ceux-ci (femme de 22 ans, avec 5 années-paquet de consommation tabagique) n'avait pas de BI mesurable dans le lavage, alors que les deux lavages provenant de l'autre (homme de 35 ans, avec 23 années-paquet de consommation de cigarettes) contenaient du BI inactif suggérant un rôle de protection pour BI. A côté de la confirmation de l'hétérogénéité entre sujets dans les niveaux de NEIC et de BI des sécrétions pulmonaires, cette étude démontre que l'hétérogénéité se produit en périphérie par rapport aux voies aériennes centrales et de grand calibre chez les fumeurs asymptomatiques.
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