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Bronchoalveolar lavage fluid urea as a measure of pulmonary permeability in healthy smokers

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Bronchoalveolar lavage fluid urea as a measure of pulmonary permeability in healthy smokers. C. Ward, F. Thien, J. Secombe, S. Gollant, E.H. Walters. ©ERS Journals Ltd 2000.

ABSTRACT: The effects of cigarette smoking on blood to airway pulmonary permeability to the low-molecular-weight solute urea were investigated, in an attempt to evaluate its use as a dilution marker for bronchoalveolar lavage (BAL) studies.

Five healthy normal smokers who smoked a cigarette 10 min prior to undergoing a 3 × 60 mL bronchoalveolar lavage (BAL), and five nonsmokers who also underwent BAL but without cigarette smoke exposure were studied. Five minutes before bronchoscopy, 4 MBq ^{3}H-water and 1 MBq ^{14}C-urea were injected intravenously and biochemical urea assays and an indirect radiotracer method were used to evaluate permeability.

It was shown that the smoking group had less urea in their BAL supernatants compared to nonsmokers the results using the radiotracer method being significant ($p<0.005$). Using both methods, it was shown that levels of urea increased in sequentially aspirated aliquots in both groups. The median directly assayed levels of urea in the smokers rose as follows: aliquot 1 0.05 μmol·mL^{-1}, (range 0.03–0.14), aliquot 2 0.10 μmol·mL^{-1} (0.07–0.17), aliquot 3 0.12 μmol·mL^{-1} (0.06–0.23) ($p<0.05$). This led to significantly increased calculated levels of epithelial lining fluid in the sequential aliquots ($p<0.05$). In addition, there were large but variable amounts of labelled water detected in both subject groups indicating a complex interaction between the BAL procedure and the circulation.

Changing urea measurements during the bronchoalveolar lavage procedure confound the use of the urea (epithelial lining fluid) method for normalizing dilution factors. The use of epithelial lining fluid determinations in smokers ignores the additional and probably complex permeability changes. The present data suggest that acute exposure to cigarette smoke in smokers may decrease blood to airway permeability.

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An increasing body of literature suggests that the integrity of the pulmonary blood/air barrier is altered in a diverse range of circumstances. These include intense exercise in elite athletes [1] and smoking in otherwise normal subjects [2] as well as airway [3] and parenchymal [4] inflammatory disease.

The precise mechanisms underlying increased pulmonary permeability are not fully understood. However, evidence indicates that extravasation of solutes occurs largely through paracellular routes, and, initially, *via* the formation of gaps in the endothelium. These 0.1–3 μm gaps, formed at the borders between adjacent cells, can open up within seconds, for example following an inflammatory stimulus [5]. Their reversible formation leads to leakage of plasma constituents into the perivascular connective tissue, and then through deformable tight junctions in the airway epithelium, leading to the emergence of some plasma constituents into the airway lumen [6]. Thus, the term pulmonary permeability, as used in this paper, describes movement of solutes through a modulable barrier that comprises the pulmonary vascular endothelium, the respiratory epithelium and the intervening interstitium.

Pulmonary permeability can be estimated using a number of approaches. Respiratory clearance of inhaled radioaerosols has been widely used and the limits and usefulness of this technique have been extensively reviewed [2, 7–9]. However, this method estimates movement of solutes from the airway to the blood, whereas physiologically and pathologically, it is movement in the opposite direction, *i.e.* from the blood to the airway lumen, that is important. Thus approaches based on injecting radiolabelled molecules into the vascular compartment and then monitoring their appearance in the lung have been used. Experiments in animals [10] and more limited studies in humans [11] have been carried out, with some of the latter involving bronchoalveolar lavage [12–14]. The literature on the effects of smoking on pulmonary permeability has predominantly indicated an increase [2], with studies typically measuring respiratory clearance from the lung to the circulation. This finding is not uniform, however, [15,

16], with a reported decrease in diethylenetriamine pentaacetic acid clearance following passive smoking [17].

Understanding the relevance of changes in pulmonary permeability will also be helpful in the interpretation of the large and rapidly growing literature on bronchoalveolar lavage (BAL) [18]. A fundamentally important issue in such work, which remains controversial, relates to how BAL results should be expressed. Some continue to use BAL fluid denominators of dilution, using constituents such as albumin [19], total protein [20] or urea [21]. In particular, the urea method for calculating epithelial lining fluid (ELF) volume as a means of expressing results (amount of solute per millilitre ELF) remains in widespread use in a significant number of centres for diverse patient groups and analyses [22–24]. The continued use of the urea method, however, is in spite of literature indicating that the assumptions underlying its rationale are incorrect [25–27]. Many such BAL studies have included smokers, and smoking itself has a major effect upon calculated ELF volume, although the reason for this is not clear [28].

Intravenously injected urea and water labels were used to study pulmonary permeability from the blood to the air compartment in smokers. The work extends previous investigations into the applicability of the urea method for estimating ELF and the expression of BAL results.

Methods

Permission for carrying out the study was obtained from the Alfred Joint Ethics Committee. All volunteers gave informed consent.

Subject groups

All subjects studied were normal healthy volunteers with normal lung function, who underwent a full medical examination prior to study. Five of the subjects had never smoked and five were active current cigarette smokers. On bronchoscopy, no evidence of localized airway disease was found.

Two nonsmokers and one smoker were atopic to grass pollen, but were studied out of season and had no upper respiratory tract symptoms or signs of acute rhinitis.

The clinical and demographic characteristics of the subjects studied are detailed in table 1.

Experimental details

The experimental protocols used were similar to those previously reported [26]. In order to standardize their recent history, smoking participants were asked to smoke one cigarette of their regular brand 10 min prior to bronchoscopy. 4 MBq of ^{3}H-water and 1.0 MBq of ^{14}C-urea in 0.5 mL normal saline were injected intravenously 5 min before undergoing a 3×60 mL sequential BAL. BAL was performed using sterile pyrogen-free phosphate-buffered saline prewarmed to 37°C in a segment of the right middle lobe using an Olympus BF P10 bronchoscope (5 mm external diameter; Olympus, Tokyo, Japan). Aspiration of each injected aliquot was carried out separately and immediately, at a suction pressure of -6.65–-13.3 kPa (-50–100 mmHg) into individual glass vessels kept on ice.

Three venous blood samples (20 mL) were taken from the arm contralateral to that used for the isotope injection simultaneously with each BAL aspiration.

Following measurement of BAL return volumes, a total cell count was performed using a Neubauer counting chamber (Reichert, Buffalo, NY, USA). Samples were assessed for obvious bleeding at this stage with the intention of excluding any such samples from further analysis. In the event, this was not required for any of the samples. Cells were separated from the BAL supernatant, and plasma was separated from whole blood within 1 h by centrifugation at 4°C (20 min at $100 \times g$ and 10 min at $200 \times g$, respectively). BAL supernatants and matched plasma samples were subdivided into 1.8-mL aliquots and stored at -80°C until subsequent assay.

Isotope measurement

In previous work, it was shown that, in separate measurements of three plasma samples, the dilution volumes for ^{3}H in all subjects were equal to or exceeded estimates of total body water based on calculated values according to the subject's height and weight [26]. In addition, dilution volumes calculated from ^{14}C counts were in excess of the volume of extracellular fluid, indicating that the isotopes had equilibrated with their target compartments. Background-corrected ^{14}C and ^{3}H counts were obtained from BAL and plasma samples using a liquid scintillation counter (Packard Tri Carb 2200; Packard Meriden, CA, USA). A count time of 60

Table 1. – Clinical and demograhic characteristics of the subjects studied*

Subject No.	Smoking status	Smoking exposure pack-yrs	Age yrs	FEV_1 % pred	Atopy
1	Smoker	>10	32	110	Nonatopic
2	Smoker	68	42	106	Nonatopic
3	Smoker	6	30	102	Nonatopic
4	Smoker	82	53	118	Nonatopic
5	Smoker	14	28	114	Atopic
6	Nonsmoker	0	27	101	Nonatopic
7	Nonsmoker	0	21	109	Nonatopic
8	Nonsmoker	0	20	113	Nonatopic
9	Nonsmoker	0	20	96	Atopic
10	Nonsmoker	0	37	106	Atopic

*: all male. FEV_1: forced expiratory volume in one second.

min per sample and an appropriate quench correction curve were used to express results in mean disintegrations per minute (dpm).

Calculations

The amount of BAL fluid urea or water derived acutely by influx from the circulation was calculated by proportionality:

$$\frac{\text{dpm·mL BAL fluid}^{-1}}{\text{dpm·mL plasma}^{-1}} \times [\text{plasma urea}] \qquad (1)$$

Where [U] is the concentration of urea in BAL fluid derived from the circulation, and the dpm corresponded to ^{14}C-labelled urea.

Total urea assays

The supernatants from the three separate BAL aliquots were analysed in duplicate for total urea concentration using a commercial urease/ultraviolent (340 nm) kit (Sigma, St Louis, MO, USA). Plasma levels were measured using a COBAS-FARA II autoanalyser (Roche Diagnostics, Basle, Switzerland). All assays were performed in the Dept of Clinical Biochemistry at Monash Medical Centre, Clayton, Victoria by one of the authors (J. Secombe).

Statistical analysis

The generalized linear model test was used for comparison of data using Minitab software (release 11, Minitab, Coventry, UK). A p-value <0.05 was considered significant.

Results

The observed mean plasma urea levels were 6.3±1.0 μmol·mL^{-1} in the nonsmokers and 5.3±1.6 μmol·mL^{-1} in the smokers (NS, p=0.3). BAL fluid total cell counts from smokers were elevated: aliquot 2, median 270 $\times 10^3$ (range 100–630) *versus* 90×10^3 cells·mL^{-1} (45–130) ($p<0.05$); aliquot 3,300 (80–690) *versus* 70 cells·mL^{-1} (30–120) ($p<0.05$). BAL return volumes were similar (table 2). Table 2 includes the individual radioisotope activities in the BAL fluid and plasma together with the background levels observed. The coefficient of variation for plasma counts was ≤0.02 and for BAL supernatant samples ≤4%. The calculated radiolabelled urea and water flux in the different aliquots are summarized in figure 1 and table 2 respectively.

Overall, in all subjects, there was a significant increase in urea levels in sequential aliquots, using both the radiotracer method and the biochemical assay ($p<0.05$; figs. 1 and 2). This was so for both smokers and nonsmokers. This means that, inevitably, there was a significant increase in the calculated volumes of ELF in the sequential aliquots ($p<0.05$); table 2.

There were lower levels of BAL fluid urea in the smokers compared to the nonsmokers in all aliquots. This was significant using the radiotracer method ($p<0.005$). There was no suggestion that the grass pollen-sensitive atopic individuals were different with regard to BAL fluid urea levels (figs. 1 and 2).

Large but variable amounts of ^{3}H-water were detected in the BAL supernatants from both the smokers and nonsmokers (table 2). There were no correlations between ^{14}C-urea and ^{3}H-water levels in either subject group (r=0.15 for smokers, NS; r=0.17 for nonsmokers, NS).

Discussion

There remains considerable confusion regarding the expression of BAL data. Many centres continue to use the urea method in an attempt to standardize results from a diverse array of studies [22–24]. This study has clearly demonstrated that, during the course of a standardized BAL with minimal dwell time, there is a substantial rise in urea levels in sequential aliquots, with a consequent increase in the calculated levels of ELF. In addition, it was shown that there was a trend toward lower urea concentrations in BAL fluid from smokers taken 10 min after smoking a cigarette. Given that urea levels change during BAL and, in addition, may be different between smokers and nonsmokers, it is felt that the urea method for calculating ELF volume should not be used.

Within a year of the publication of the urea method for determining ELF volume, the observation was made by MARCY *et al.* [25] that urea sampled at BAL increased with prolonged dwell time, with probable simple diffusion of urea from the circulation into the urea-free saline. This led some workers to advocate the continued use of the method, but with minimized dwell time [4]. The present finding of increased levels of urea in sequential aliquots is consistent with diffusion taking place, but indicates that this occurs even in the context of a minimized dwell time.

Large but variable amounts of radiolabelled water were also detected in BAL fluid in this study, supporting previous studies indicating that BAL is probably accompanied by dynamic movements of water, with a net influx into BAL aspirates [29–31]. This study, therefore, confirms that complex interactions occur between BAL and the circulation. Unfortunately, the present results using radiolabelled water and urea cannot be used to indicate how much movement is due to diffusion and how much to hydrostatic mechanisms. Induced gradients for both are inherent to the normal BAL process, with diffusion of labelled water into BAL fluid, in particular, likely to be very fast.

The insult of smoking represents an additional factor in an already complex and perturbed system. The finding of decreased ^{14}C-labelled BAL urea levels in smokers following acute exposure to a standardized cigarette challenge was surprising, as it suggests a decreased permeability in the direction from the blood to the BAL compartment. The general finding in previous studies has been of increased pulmonary permeability in smokers [2], with the specific finding of BURKE *et al.* [28] of higher levels of BAL urea and ELF volumes in smokers. Unfortunately, this interesting latter study is difficult to interpret because of the heterogeneity of the smoking group, in which 24 of the 26 subjects also had a variety of connective tissue disorders. In addition, as for many other studies into pulmonary permeability and smoking, the timing of the last cigarette

Table 2. – Individual bronchoalveolar lavage (BAL) fluid and plasma 14C and 3H counts*, BAL return volumes and calculated water flux and epithelial lining fluid (ELF) volumes for all individuals studied

ID**	^{14}C urea BAL fluid dpm	^{14}C urea plasma dpm	$^{3}H_2O$ BAL fluid dpm	$^{3}H_2O$ plasma dpm	BAL return mL	Water flux *** mL	ELF mL
NS 1:1	39	445	3005	7294	15	6.2	0.4
NS 1:2	33	472	2651	7522	20	7.0	0.4
NS 1:3	46	390	3403	7019	16	7.8	0.6
NS 2:1	2	299	1694	5026	17	5.7	0.1
NS 2:2	7	314	2691	5241	36	18.5	0.3
NS 2:3	10	302	3585	5062	55	39.0	0.5
NS 3:1	13	280	582	3763	25	3.9	0.3
NS 3:2	36	273	1662	3660	43	19.5	1.4
NS 3:3	46	274	1867	3636	50	25.7	2.3
NS 4:1	12	208	2592	8617	17	5.1	0.1
NS 4:2	16	364	5041	6822	28	20.7	0.3
NS 4:3	16	157	4051	7039	25	14.4	0.3
NS 5:1	6	664	333	7259	5	0.2	0.1
NS 5:2	30	572	3355	6641	36	18.2	1.0
NS 5:3	31	430	2466	6423	50	19.2	1.3
SM 1:1	20	724	1644	8756	25	4.7	0.3
SM 1:2	29	770	2689	9735	40	11.0	0.8
SM 1:3	28	773	3216	9224	45	15.7	0.9
SM 2:1	8	665	662	6442	16	1.6	0.1
SM 2:2	20	572	2128	6294	37	12.5	0.6
SM 2:3	29	441	2874	6261	47	21.6	1.2
SM 3:1	12	581	969	7874	13	1.6	0.2
SM 3:2	20	581	2400	6437	44	16.4	1.0
SM 3:3	17	523	2438	6741	46	16.6	1.0
SM 4:1	24	559	728	4294	12	2.0	0.4
SM 4:2	37	652	1456	4800	20	6.1	0.7
SM 4:3	49	690	2295	4941	23	10.7	1.2
SM 5:1	8	564	2290	8339	16	4.4	0.1
SM 5:2	17	652	4715	7506	50	31.4	0.5
SM 5:3	16	690	4315	8658	52	25.9	0.6

*: all isotope counts are background-corrected (^{14}C: 5; ^{3}H:10) and expressed in quench-corrected disintegrations per minute (dpm); **: the number after the colon identifies the BAL aliquot. Hence, NS 1:1 is nonsmoker (NS) 1 aliquot 1; ***: derived value for labelled water calculated from the BAL and plasma ^{3}H counts. ELF: epithelial lining fluid; ID: identifier; SM: smoker.

relative to bronchoscopy was not stated, but presumably was not standardized and could have been hours or days before in the hospital setting. Smoking represents a complicated insult to the lung and it is possible that acute events may be different and seemingly paradoxical to chronic sequelae. Some *in vivo* studies of respiratory clearance (lung to circulation) have shown that acute exposure to tobacco smoke does not increase pulmonary permeability further in smokers [15, 16], and, indeed, exposure to sidestream smoke (passive smoking) can cause a significant decrease in pulmonary permeability [17]. It has been speculated that closure of epithelial tight junctions may occur acutely in response to some of the complex constituents of cigarette smoke, resulting in an initial decrease in permeability [17]. Subsequently, damage to the epithelium is accompanied by the increased permeability that is generally described [2].

In all of the subjects studied, the amount of urea calculated to be derived from the circulation by means of radioisotope labelling was greater than or equal to the amount assayed directly in the BAL supernatant. The large differences between plasma and BAL concentrations of urea mean that the use of an indirect "tracer" to estimate flux is relatively imprecise compared to the direct biochemical estimation. The authors feel that the methods used are likely to be robust enough to give confidence in the general conclusions of the present study, however. Indeed, similar trends were observed using both measurement approaches. The present design indicates that studies into the dynamics of pulmonary permeability from the

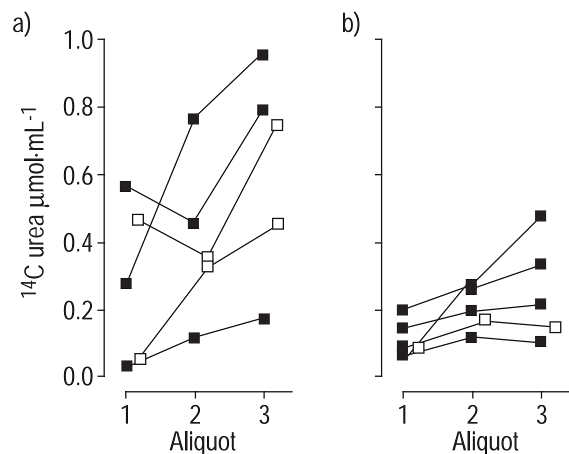

Fig. 1. – Concentrations of urea calculated as being due to movement of radiolabelled urea from the circulation between isotope injection and aspiration of bronchoalveolar lavage (BAL) fluid in three 60 mL BAL aspirates in: a) five nonsmoking; and b) five smoking normal healthy subjects. The results for each individual aspirated aliquot are shown. □: grass pollen-sensitive (atopic) individuals.

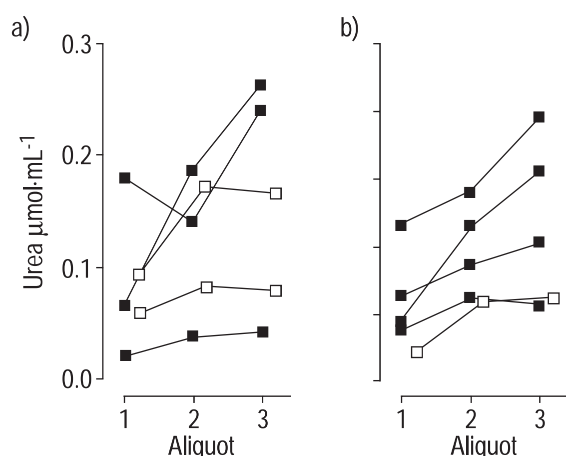

Fig. 2. – Biochemically measured concentrations of urea assayed in three 60-mL bronchoalveolar lavage (BAL) aspirates in: a) five non-smoking; and b) five smoking normal healthy subjects. The results for each individual aspirate are shown. □: grass pollen-sensitive (atopic) individuals.

blood to the airway compartment are practicable in humans.

In conclusion, the present work re-emphasizes the fact that urea cannot be used as a denominator of ELF volume. This is because BAL causes acute changes in the putative denominator being sampled, and because there are additional confounders to its use, which include smoking. These effects may not be uniform [16, 17, 28], and the present study would suggest that acute effects may be different to more chronic changes in permeability. Disease processes are also likely to affect permeability and such confounders may well include commonly occurring variables such as atopy.

Hence, the authors would support the view, outlined by the European Respiratory Society task force on measurement of acellular components in bronchoalveolar lavage, that results are most meaningfully expressed per millilitre of bronchoalveolar lavage aspirate [32]. Bronchoalveolar lavage fluid solute measurements remain a powerful and genuinely quantitative tool that will continue to yield valuable data, providing that known confounders are avoided.

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