Evaluation of pulmonary alveolar epithelial integrity by the detection of restriction to diffusion of hydrophilic solutes of different molecular sizes

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ABSTRACT

The rate of transfer of a hydrophilic solute from the alveoli to pulmonary blood following inhalation as an aerosol depends on the molecular size of the solute and the permeability of the alveolar epithelium. The value of this measurement for assessing damage to the epithelium in lung disease is compromised by cigarette smoking, which accelerates clearance by unknown mechanisms. The rates of clearance of ^{99m}Tc-labelled diethylenetriaminepenta-acetic acid (DTPA) (molecular mass 492 Da) and ^{113m}In-labelled biotinylated DTPA (B-DTPA) (molecular mass 1215 Da) were monitored simultaneously by dynamic γ-radiation camera imaging following simultaneous inhalation, and compared between eight normal non-smoking subjects and nine habitual cigarette smokers. The clearance rates of DTPA were 0.95 (S.D. 0.39)%/min in non-smokers and 4.13 (1.06) %/min in smokers. These were about twice the clearance rates of B-DTPA, which in the corresponding groups were 0.41 (0.26) and 2.12 (0.72)%/min respectively. The ratio of the B-DTPA/DTPA clearance rates was, in all subjects, less than the ratio (0.74) of the cube roots of the molecular masses of the solutes, assumed to correspond to the ratio of their free diffusion coefficients in water, and was not significantly different between smokers and non-smokers. As alveolar permeability increased, the ratio of clearance rates in the entire population showed a significant trend to increase in a non-linear fashion towards the value corresponding to the ratio of the free diffusion coefficients. We conclude that the diffusion of at least the larger of these two solutes through the pulmonary alveolar epithelium is restricted (i.e. associated with a reflection coefficient greater than zero). Cigarette smoking, however, does not appear to cause a loss of this restriction, and may increase solute clearance by other mechanisms, such as reducing fluid volume within the alveolus, thereby raising the local radiotracer concentration, or increasing the number of pores available for solute exchange without affecting pore size. Conversely, if restriction was lost in lung disease, the ratio of the clearance rates of two solutes of dissimilar sizes could be used to detect disease in smokers as well as non-smokers.

Key words: alveolar permeability, lung aerosol clearance, ^{99m}Tc-DTPA, ^{113m}In-biotinylated DTPA.

Abbreviations: DTPA, diethylenetriaminepenta-acetic acid; B-DTPA, biotinylated DTPA.

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INTRODUCTION

Following inhalation as nebulized aerosol, hydrophilic solutes, such as ^{99m}Tc-labelled diethylenetriaminepentaacetic acid (DTPA), are cleared from the alveolus into pulmonary capillary blood and ultimately excreted in the urine. The blood–gas barrier in the lung consists of the vascular endothelium, the alveolar epithelium and the intervening pulmonary interstitium. Transfer of hydrophilic solutes across this barrier is thought to occur by passive diffusion through the respective intercellular junctions of the epithelium and endothelium [1–3]. Epithelial permeability is much lower than endothelial permeability, and is the rate-limiting factor in hydrophilic solute clearance across the blood–gas barrier [4]. Except for highly diffusible solutes, barrier permeability is independent of pulmonary blood flow and reflects alveolar epithelial integrity. Rates of solute diffusion across the alveolar epithelium are governed by the laws of physical diffusion in aqueous solution, and are broadly inversely proportional to the cube root of the molecular size [1–3].

As the alveolar permeability to ^{99m}Tc-DTPA is influenced so readily by lung damage [5,6], the rate of clearance of inhaled ^{99m}Tc-DTPA has become established as a means of detecting and quantifying the severity of diffuse interstitial pulmonary disease [1,2,5–7]. In normal subjects the half-life of clearance is approx. 80 min, corresponding to a fractional clearance rate of approx. 1%/min, but this falls to less than 20 min in several lung diseases. The great sensitivity of the technique is one of its drawbacks, because cigarette smokers with otherwise normal lung function have a markedly increased clearance rate [2,8,9], and in these subjects the test is uninterpretable. On the other hand, a normal clearance rate excludes the presence of pulmonary inflammation [10].

A potential solution applicable to smokers is the simultaneous use of two tracers. In the systemic circulation, a dual-tracer technique has been used to resolve the influence of capillary surface area on the rate of transfer of a solute across the vascular endothelium [11]. Restriction to diffusion is closely related to the hydrated radius of the solute in relation to the radius of the pore; if the pore radius were to increase, then restriction to diffusion of a given solute may be lost. If the increased clearance rate of a solute in smokers is not the result of an increase in pore radius, then restriction to diffusion of a solute of critical size may not be lost. The aim of the present work was to compare the clearance rate of ^{99m}Tc-DTPA, which has a molecular mass of 492 Da, with that of ^{113m}In-labelled biotinylated DTPA (B-DTPA), which has a molecular mass of 1215 Da. Both tracers are hydrophilic and are not significantly bound to protein. A higher ratio of the clearance rates of the larger to the smaller molecule in smokers compared with non-smokers would be consistent with a smoke-induced loss of restriction. If, on the other hand, the ratio of clearances was unchanged in smokers compared with non-smokers, then the problem of the higher clearance rate in smokers could potentially be resolved by the use of a dual-tracer clearance technique, provided that lung disease was associated with loss of restriction and could be distinguished from the effects of cigarette smoking.

THEORY

The permeability (P)–surface area (S) product (PS) of a solute in a specific vascular bed is related to blood flow (Q) and the solute extraction fraction (E) in the direction of blood to tissue, as follows:

$$PS = -Q \cdot \ln(1-E) \tag{1}$$

However, the actual transfer rate of the solute, J_s, is also governed by fluid filtration, J_v, and the reflection coefficient, σ:

$$\frac{J_s}{C_p} = \frac{J_v(1-\sigma)}{1-e^{-[J_v(1-\sigma)]/PS}}$$

where C_p is average capillary concentration.

When $PS \gg J_v(1-\sigma)$, J_s/C_p is virtually equal to PS, and this is generally the case for small hydrophilic solutes; however, for macromolecules $J_s/C_p > PS$.

For two solutes given simultaneously, Q and S cancel out in eqn (1), and:

$$P_1/P_2 = \ln(1-E_1)/\ln(1-E_2) \tag{3}$$

If P_1/P_2 is less than the ratio of the free diffusion coefficients of the solutes (where $P_2 > P_1$), then restricted diffusion is said to exist for at least the larger of the two solutes.

In the lung, provided that $PS \gg J_v(1-\sigma)$, the transfer rate, J_s, of solute from alveolus to blood can be defined in terms of the PS product of the alveolar epithelium and the solute concentration, C_a, in the alveolar fluid:

$$J_s = PS \cdot C_a \tag{4}$$

Dividing both sides of eqn (4) by M, the amount of solute present in the alveolus, gives:

$$J_s/M = PS \cdot C_a/M$$

J_s/M is the fractional clearance rate, k, of solute and C_a/M is equal to $1/V$, where V is alveolar fluid volume, so, assuming no back-diffusion from blood to alveolus:

$$k = PS/V \tag{5}$$

Assuming that the thickness, d, of the film of fluid lining the alveolus is small compared with alveolar radius, $V = d \cdot S$ [12], so:

$$k = P/d \tag{6}$$

On inhaling two solutes of different sizes and measuring k for both, d cancels out, so:

$$k_1/k_2 = P_1/P_2 \tag{7}$$

As with vascular endothelium, if P_1/P_2 is less than the

free diffusion coefficients of the two solutes, then restricted diffusion for the larger can be said to be present [13–16]. Saying a solute has restricted diffusion is equivalent to saying it has a reflection coefficient of greater than zero. The free diffusion coefficient of a solute is proportional to the radius of the hydrated molecule. This, in turn, is roughly proportional to the cube root of the molecular mass [16], so that the ratio of free diffusion coefficients of ^{113m}In-B-DTPA and ^{99m}Tc-DTPA will be approx. 0.74.

METHODS

Patients

We studied eight normal controls and nine subjects who had been habitual smokers for between 2 and 23 years, but had no evidence of lung disease or symptoms of chronic bronchitis. All subjects gave informed written consent to the study, which was approved by the Hammersmith Hospital and Royal Postgraduate Medical School Local Research Ethics Committee and by the Administration of Radioactive Substances Advisory Committee of the U.K.

Preparation of radioaerosols

^{99m}Tc-DTPA was prepared from a commercial kit (Amersham) in a standard fashion, and showed binding of $> 95\%$ as measured by TLC.

A total of 370 MBq of ^{113m}In was eluted from an ^{113}Sn generator (Amersham) with 0.04 M HCl. The eluate was immediately added to a 1 M solution of 3.8% sodium citrate containing 100 μg of DTPA-bis-biotin (Sigma). The DTPA-biotin compound was found to remain at the origin, while ^{113m}In chloride moved with the solvent front in a solution of 0.05 M EDTA when tested by TLC (Gelman, Ann Arbor, MI, U.S.A.). Instant TLC was performed with each experiment, and binding was routinely 95% or greater. A further 1.5 ml of 3.8% sodium citrate for injection was added at the end of the labelling procedure to raise the pH of the solution to 6–6.5. This solution was sterilized by 0.22 μm Micropore filtration (Millipore) and dispensed. The procedure was routinely completed in 25 min.

Administration of radioaerosols

The total volume of each aerosol was increased to 2.5 ml upon addition to separate acorn nebulizers (OEM, Richmond, VA, U.S.A.). Aerosols were generated in two separate 15 litre balloons immediately prior to inhalation; as a result of settling in the balloon, the inhaled particles had a median mass diameter of 1.8 μm (geometric S.D. 0.84 μm). A one-way valve was opened from each balloon simultaneously and dynamic imaging was performed for 12 min at a frame rate of 2 per min. The subject inhaled both aerosols for 120 s; after breathing the previously generated aerosol for 105 s, the circuit was changed to room air while the subject cleared the tubing of residual aerosol in the remaining 15 s. Aerosols generated in this fashion do not coalesce [17]. Imaging was continued for 10 min after the completion of aerosol inhalation.

Measurement of clearance rates

Lung clearance rates of inhaled ^{99m}Tc-DTPA and ^{113m}In-B-DTPA were measured simultaneously by dual-photon acquisition using a γ-radiation camera (IGE Starcam), fitted with a high-energy collimator, positioned over the posterior chest and on-line to an MDS A^2 computer. Data were acquired dynamically by dual-photon acquisition at a frame rate of 2 per min for 12 min from the beginning of aerosol inhalation. The photopeaks were centred on the ^{99m}Tc and ^{113m}In energies respectively, and appropriate corrections were made for spectral spillover ('cross-talk') between the two radionuclides.

Regions of interest were drawn around the whole lung fields, and time–activity curves were generated for both tracers. Least-squares regression analysis was applied to the natural logarithm of the lung activity as a function of time during a period of 7 min from the peak of the curve, thereby generating the fractional rate of clearance in units of percentage of residual tracer per min. Over such a limited period of data acquisition, background correction was not considered necessary and so no intravenous tracer was given. It is also necessary to make the assumption that there is negligible back-diffusion of tracer from blood to alveolar fluid over this period.

RESULTS

The mean clearance rate of ^{113m}In-B-DTPA in the eight non-smokers was 0.41 (S.D. 0.26)%/min, approximately half that of ^{99m}Tc-DTPA [0.95 (0.39)%/min]. The mean clearance rate of ^{113m}In-B-DTPA in the nine smokers was 2.12 (0.72)%/min, again about half that of ^{99m}Tc-DTPA, which was 4.13 (1.06)%/min (Figure 1).

There was a close linear correlation between the clearance rates of DTPA (x-axis) and B-DTPA (y-axis) in non-smokers ($y = 0.53x - 0.098\%$/min; $r = 0.8$), smokers ($y = 0.65x - 0.56\%$/min; $r = 0.95$) and the two groups combined ($y = 0.56x - 0.16\%$/min; $r = 0.98$). All data points were placed to the right of a theoretical regression line with slope 0.74, corresponding to the ratio of the cube roots of the molecular masses of the solutes (assumed to correspond to the free diffusion coefficients), indicating that clearance of B-DTPA was disproportionately slower compared with that of DTPA than would be expected from their molecular sizes (Figure 2). Negative y-axis intercepts were recorded for both smokers and non-smokers (implying ongoing DTPA clearance with no B-DTPA clearance), but they were not quite significantly different from zero.

The mean ratio of clearance rates of B-DTPA to DTPA in the non-smokers ranged from 0.019 to 0.65, with a median of 0.45. Two subjects had very low B-DTPA

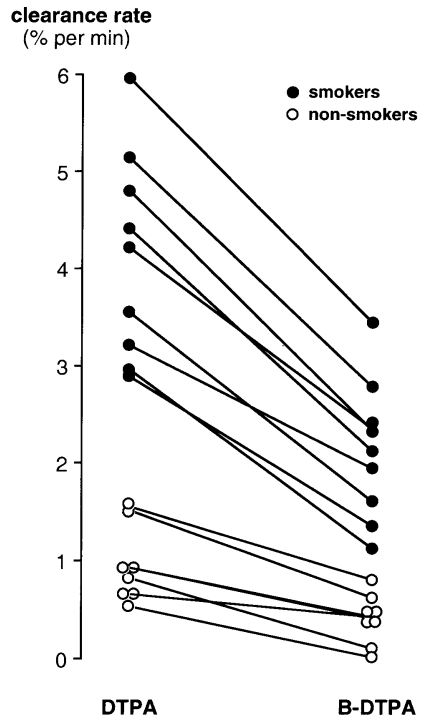

Figure 1 **^{99m}Tc-DTPA and ^{113m}In-B-DTPA clearance rates in non-smokers (○) and in cigarette smokers (●)**

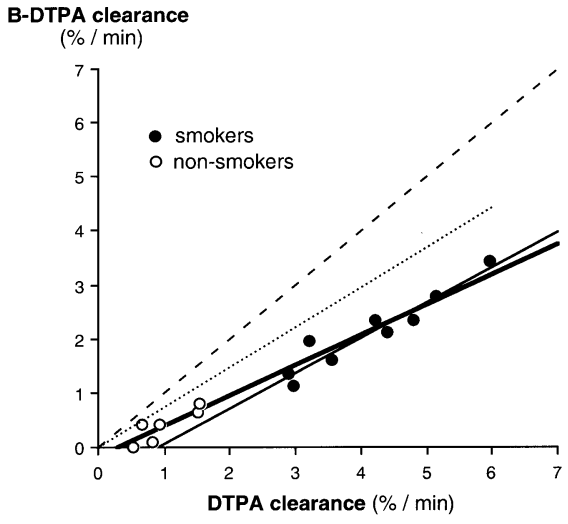

Figure 2 **Relationship between ^{99m}Tc-DTPA and ^{113m}In-B-DTPA clearance rates in smokers (●) and non-smokers (○)**

The overall linear regression (bold continuous line) is $y = 0.56x - 0.16$%/min ($r = 0.98$), while the relationship for the group of smokers (light continuous line) is $y = 0.65x - 0.56$%/min ($r = 0.95$). The dashed line is the line of identity. Note that neither regression line passes through the origin. The dotted line has a gradient of 0.74, which is the ratio of the cube roots of the molecular masses of the two solutes, and is therefore the regression that would be expected if the diffusion of neither solute was restricted (i.e. free diffusion for both solutes).

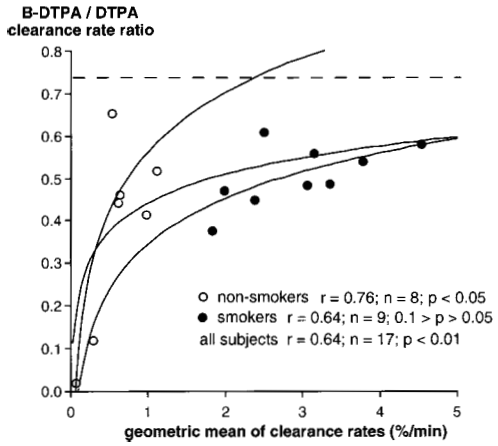

Figure 3 **Relationship between the geometric means of the ^{99m}Tc-DTPA and ^{113m}In-B-DTPA clearances (as a measure of permeability) and the ratio of ^{113m}In-B-DTPA to ^{99m}Tc-DTPA clearance rates in smokers (●) and non-smokers (○)**

The horizontal broken line is at 0.74, the ratio of the cube root of the molecular masses of the two solutes, and is assumed to correspond to the ratio of free diffusion coefficients. The curved lines are least-squares logarithmic fits to data for the smokers ($0.1 > P > 0.05$), the non-smokers ($P < 0.05$) and the two groups combined ($P < 0.01$).

clearances, giving very low clearance ratios. These low values are unlikely to be the result of measurement error, as they are close to the regression line of the relationship between DTPA and B-DTPA clearance rates in the whole subject population (Figure 2). The clearance ratio in the smokers ranged from 0.38 to 0.61, with a median of 0.49. There was no significant difference in the clearance ratio between non-smokers and smokers. All clearance ratios were lower than the ratio of the free diffusion coefficients, i.e. 0.74, assumed to reflect the cube roots of the respective molecular masses (Figures 2 and 3).

Within the entire population of 17 subjects, there was a significant ($P < 0.01$) positive non-linear correlation between alveolar permeability (based on the geometric mean of the DTPA and B-DTPA clearance rates) and the B-DTPA/DTPA clearance rate ratio, which, with increasing permeability, increased towards the value (0.74) corresponding to the ratio of the cube roots of the molecular masses (Figure 3). This association was also significant within the non-smokers ($P < 0.05$), but not within the group of smokers ($0.1 > P > 0.05$).

DISCUSSION

Several factors affect the rate of transfer of a hydrophilic solute from the alveolar fluid to pulmonary capillary blood. Although the mechanisms of transfer remain unclear, the most widely held view is that hydrophilic solutes diffuse through the epithelium via the intercellular junction pores [18]. Pore radius has a critical bearing on the transfer rate of a solute. A positive end-expiratory pressure (PEEP) and high lung volumes increase the

clearance rate of ^{99m}Tc-DTPA [19,20]; although several interpretations of the mechanism have been considered, an increase in pore size, as a result of 'stretching' of the epithelium, has been widely proposed. The electrical charge of a molecule also affects the transfer rate. Thus Barrowcliffe et al. [21] demonstrated in normal sheep that neutral dextran was cleared more quickly than either negatively or positively charged dextran of the same molecular mass. This differential rate of clearance disappeared following lung damage induced by intravenous oleic acid, suggesting that the damage to the epithelium was associated with loss of its normal electrical charge.

It is well known that cigarette smokers have an increased rate of clearance of ^{99m}Tc-DTPA compared with non-smokers, but the mechanism is not known. Earlier suggestions that the effects of smoking caused dissociation of the ^{99m}Tc-DTPA complex, with the smaller molecule, ^{99m}Tc, undergoing rapid diffusion, were discounted by the demonstration of a similarly rapid clearance of ^{111}In-DTPA, which is more stable than ^{99m}Tc-DTPA [22,23]. It can be appreciated from eqns (5) and (6) that smoking could increase solute clearance by several different mechanisms, including a decrease in alveolar fluid film thickness (d), an increase in alveolar surface area available for exchange [which would act indirectly through a decrease in d (eqn 6)] or an increase in epithelial permeability (P). Since hydrophilic solutes are unable to penetrate epithelial cells themselves, and must instead cross the barrier by diffusing through the pores created from incomplete epithelial cellular junctions [18], an increase in permeability could be achieved by increasing the size of individual pores or by increasing the number of pores. As it is dependent on the relationship between solute molecular radius and pore size, restriction of diffusion would be expected to be lost (or the reflection coefficient decrease) if the pore size increased, but not if the number of pores increased without an accompanying increase in pore size. One could speculate that ultrastuctural modifications at the interepithelial cellular junctions induced by cigarette smoking could result in an increase in pore numbers without an increase in the diameter of individual pores, so that while the rate of clearance would increase, 'sieving' would nevertheless be retained. On the other hand, if pores fused, say, so that pore size increased but overall pore surface area presented to a solute remained unchanged, then an increased clearance rate may be limited to a larger solute as a result of loss of restriction, as happens in the periperal microcirculation in response to histamine, which increases permeability to albumin but not to small solutes [24]. Weak evidence was recorded in the present study in favour of this, as shown by negative regression intercepts in the relationships between DTPA and B-DTPA clearances, hinting at heterogeneous pore size and the possibility that the clearance of the larger solute may be extremely slow while DTPA clearance continues, as was seen certainly in one, and probably in two, of our subjects. This suggests that it may be worthwhile to look at a solute larger than B-DTPA in pathology.

On the other hand, the rate of clearance of a large solute may be higher than predicted from its PS product, as a result of convective transport resulting from fluid filtration (J_v). Convective transport only becomes important when the PS product is low compared with the term $J_v(1-\sigma)$. The reflection coefficient, σ, is largely a property of pore size, while PS is a property of both pore size and number. Because of the relationship between J_v and PS, it is theoretically possible, if $J_v(1-\sigma)$ was high relative to PS under baseline conditions, for the clearance ratio of different sized solutes to remain unchanged in circumstances in which the ratio of PS product changed. Thus convection may contribute to the rate of transfer of the larger solute when PS is low, but not when there is increased rate of transfer as a result of an increase in PS product. Although this is important in the microvasculature, it is likely to be much less important in relation to the alveolar epithelium, since water transport from alveolus to interstitium is low, even in fluid-filled lungs, and energy-dependent [25,26]. Moreover, Higenbotham [27] found no increase in alveolar DTPA clearance when the alveolus was filled with fluid.

Since the free diffusion coefficient of a solute is approximately inversely proportional to the cube root of its molecular mass [2,12–14], the B-DTPA/DTPA free diffusion ratio should be approx. 0.74. The ratio of clearance rates was lower than this for both smokers and non-smokers, indicating that restriction to diffusion, to at least the larger of the solutes, is present in both subject groups. The failure of smoking to induce any change in the ratio of clearance rates therefore suggests that smoking is not associated with loss of diffusion restriction, and that it does not affect pore size. The simplest explanation for a proportionately similar increase in clearance rates of the two solutes, evident from eqn (6), would be that smoking reduces alveolar fluid thickness [12]. If, as seems the case, cigarette smoking does not affect diffusion restriction, then smokers could be distinguished from patients with lung disease in radioaerosol clearance measurements if a dual-tracer technique is employed and if the lung disease causes loss of restriction. Under such circumstances, the rate of clearance of a smaller solute in a smoker with superadded lung inflammation may not be increased much compared with that in healthy smokers; however, if the rate of clearance of a larger solute was increased disproportionately as a result of loss of diffusion restriction, then the ratio of clearance rates would be increased towards the ratio corresponding to free diffusion. Nevertheless, the situation may be more complicated than this in disease if fluid movements across the blood–gas barrier were to increase and bring 'solvent drag' into play.

A rather wide range of clearance values was recorded for both radiotracers, although their clearance ratios were less variable. The reasons for this are not obvious, although it is of interest that, as barrier permeability increased, the ratio tended to increase towards the ratio corresponding to free diffusion (Figure 3). This would be expected if increasing permeability was associated eventually with loss of diffusion restriction.

The clearance rate ratio of B-DTPA to DTPA in the present study is similar to that observed by Egan and co-workers [14,15] for sucrose and mannitol, which, although they are smaller molecules, have about the same molecular radius ratio. Restriction to these smaller molecules should have been less, and their clearance ratio should have been closer to their free diffusion ratio; the fact that they were not is explained by the lower overall clearance rates in sheep lung compared with normal human lung – the sucrose clearance rate in the study of Egan et al. [14] was only 0.15 %/min. In foetal sheep lung [13] and in fully inflated sheep lung [14], diffusion was almost completely unrestricted over a wide range of molecular sizes [13,14]. Lung inflation also significantly reduced restriction to sucrose in the dog lung [15].

The present study, although suggesting that smoking does not result in a significant loss of diffusion restriction, has not established the optimum sizes of the two tracers. This will depend on the relationship between an observed curve of molecular radius against clearance rate and the curve defining the same relationship for free diffusion, as described by Egan et al. [13–15]. Thus the two molecules used in the present study may not have the optimal size relationship, as can be appreciated from the relatively narrow angle in Figure 2 between the observed regression line and the line representing the ratio of free diffusion coefficients, above which data points, abnormal in comparison with normal smokers and non-smokers, cannot theoretically lie. Further work to define the optimal sizes and to explore dual-tracer clearance in lung disease is warranted.

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