Invited Review

Mucociliary and Cough Clearance as a Biomarker for Therapeutic Development

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Abstract

A workshop/symposium on "Mucociliary and Cough Clearance (MCC/CC) as a Biomarker for Therapeutic Development" was held on October 21–22, 2008, in Research Triangle Park, NC, to discuss the methods for measurement of MCC/CC and how they may be optimized for assessing new therapies designed to improve clearance of airway secretions from the lungs. The utility of MCC/CC as a biomarker for disease progression and therapeutic intervention is gaining increased recognition as a valuable tool in the clinical research community. A number of investigators currently active in using MCC/CC for diagnostic or therapeutic evaluation presented details of their methodologies. Attendees participating in the workshop discussions included those interested in the physiology of MCC/CC, some of who use *in vitro* or animal methods for its study, pharmaceutical companies developing muco-active therapies, and many who were interested in establishing the methods in their own clinical laboratory. This review article summarizes the presentations for the *in vivo* human MCC/CC methods and the discussions both at and subsequent to the workshop between the authors to move forward on a number of questions raised at the workshop.

Key words: mucociliary clearance, cough clearance, aerosol deposition, particle clearance

Introduction

On October 21–22, 2008, experts on the physiology of mucociliary and cough clearance (MCC/CC) in health and disease met in Research Triangle Park, NC, to discuss the methodologies for *in vivo* assessment of MCC/CC and its utility as a biomarker for therapeutic development. The workshop was organized by the International Society for Aerosols in Medicine (ISAM) and the University of North Carolina, and sponsored by pharmaceutical industries interested in new therapies for improving MCC/CC in airways disease. Specifically, the goals of the symposium/workshop were to (1) present the current understanding of the structure and function of the MCC/CC apparatus, (2) describe current and proposed aerosolized therapeutics for MCC/CC dysfunction as well as the practical considerations for their clinical use, and (3) compare the various methodologies used to measure *in vivo* MCC/CC and discuss further how/which methods may be standardized among investigators.

The focus of this review is to summarize the last aim of the workshop, that is, presentations on the state-of-the-art of MCC/CC methodologies and discussions on how these methods may be improved for use as a biomarker for therapeutic development. For the purposes of this review and our discussions at the workshop, we used the definition of biomarker provided by Mayer-Hamblett et al.[1] as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic process, pathogenic process, or pharmacologic responses to a therapeutic intervention." They further suggest that not all biomarkers will necessarily be proven to be markers of clinical efficacy, but as measures of biological activity, they may enable early proof-of-concept studies that can help screen potential drug candidates and identify therapeutic targets. As part of our discussions we

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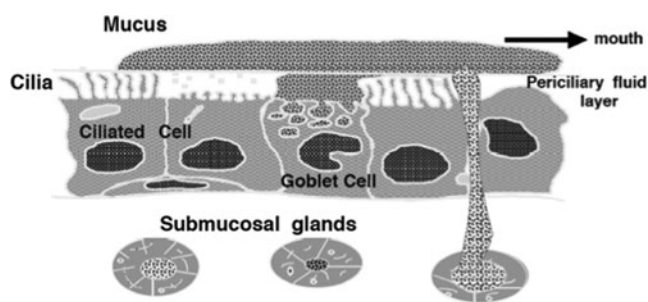[6]Activaero GmbH, Gemuenden, Germany.

FIG. 1. Airway epithelial components of the mucociliary clearance system.

consider how well current measures of MCC/CC serve as a biomarker for airways disease and its treatment.

The MCC apparatus is a well-coordinated system consisting of airway secretory cells and submucosal glands that produce a sol and gel (or mucus) fluid layer on the airway surface, and ciliated cells that propel the mucus out of the lung toward the mouth[1] (Fig. 1). In health, this system is effective at clearing mucus and associated bacteria and toxins from our lungs. However, in a variety of airway diseases [e.g., cystic fibrosis (CF), chronic bronchitis (CB), and asthma] this apparatus becomes dysfunctional, which may lead to further exacerbation of airway inflammation and an increasing reliance on CC to remove secretions from the airways. Eventually, CC may also become compromised, leaving patients with little defense to the accumulation of bacteria on airway surfaces. Consequently, there are continuing efforts to develop therapeutic aerosols that may be delivered to the airways to enhance both MCC/CC in compromised patients.

MCC/CC rates can be measured in humans by assuming that a nonpermeating, inhaled marker depositing on the airway surface moves out of the lung at the same rate as the airway secretions in which it is immersed. The most common technique is to use inhaled, radiolabeled (Tc99m) particles, aqueous or dry, that upon deposition in the lung can be followed by a gamma camera to determine their rate of egress from the lung (Fig. 2). After inhalation of these markers, retention of activity in the lung (as a percent or fraction of initial deposition) is monitored as a function of time over a period of up to 24 h to determine clearance rates.
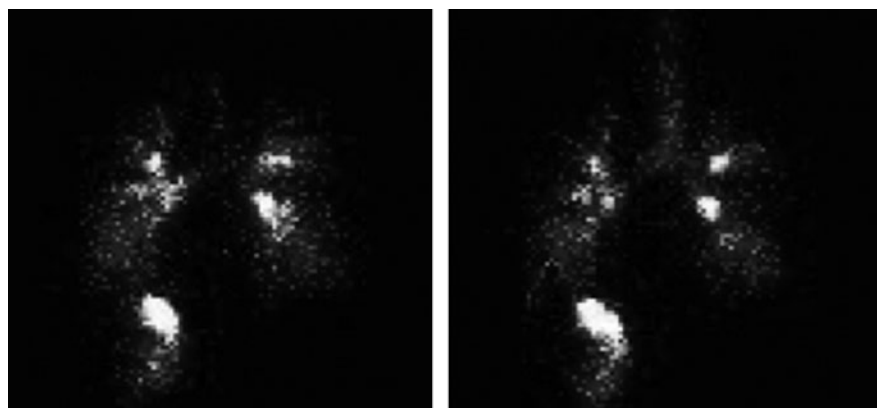
State of the Art

Many recent studies of MCC rely upon the nebulized delivery of Technetium sulfur or albumin colloid aerosols.[2–6] These radiopharmaceuticals are easy to label and handle (labeling efficiencies of >98%), having been developed principally for liver scanning by i.v. injection [e.g., CIS-Sulfur Colloid Kits (CIS-US, Inc., Bedford, MA)]. The colloidal particles are submicronic in size and dispersed in isotonic saline for nebulization, forming larger polydisperse aerosol particles (3–6 μm MMAD) that are inhaled. There is minimal observed Tc99m penetration into the bloodstream,[7] suggesting that the colloidal particles stay dispersed in the airway surface fluid for measures of MCC over the time of observation (0–24 h). For many years a few laboratories have used solid Tc99m, polystyrene or iron oxide particles generated by spinning disks[8–11] to measure MCC. These methods allow for strict control of monodisperse particle size and tight binding of the label to the particles. Unlike the colloidal particles, these particles are delivered as large (3–6 μm) *dry* particles during inhalation. These methods have been particularly useful over the years for assessing lung clearance for a range of particle sizes and for understanding regional deposition in the lung.[12,13]

Although most centers use Tc99m, longer-lived isotopes of I[123] and In[111] have also been used. Regardless of isotope used to tag the particles, it is important to check for potential leaching of the label from the particle. For example, Venticoll (colloidal albumin) leakage in vitro is <0.1%/24 h, while *in vivo*: 7% was recovered in urine within 24 h.[14] Most likely the *in vivo* leaching derives from the gastrointestinal (GI) tract because earlier studies using Tc-human serum albumin have shown 3% blood uptake from the lungs but 15% uptake from radiocolloid via the GI tract.

Controlled inhalation of the radiolabeled aerosols is recognized as an important factor for targeting the bronchial airways and improving intrasubject repeatability of MCC measures. The breathing patterns employed by different laboratories are quite varied, but each strives to produce sufficient airway versus alveolar deposition to provide for measure of MCC over a 24-h period. In each case the subject is trained to perform the breathing maneuvers usually by following a visual and/or audio signal. Some laboratories have subjects breathe the radioaerosol continuously as subjects control their tidal flow and rate of breathing, for ex-

FIG. 2. Left: initial gamma camera deposition scan. Right: 20-min postdeposition scan illustrating clearance of particles up the trachea.

ample, fixed rates of 500 mL/sec and 25 breaths/min.[2] Daviskas et al.[4,15,16] uses a closed breathing circuit[17] and controls the tidal volume (450 mL), inhalation rate (1 sec) and exhalation rate (2 sec) that are measured and reproduced. Others have subjects perform single breath maneuvers at controlled volume and flow, e.g., Hasani et al.[8,18,19] has subjects inhale single, small tidal volumes (450 mL) followed by a short 3-sec breathhold, and Scheuch et al.[10,11] uses bolus technology to deliver shallow boluses of aerosol during a single breath also followed by a breath old. Mortensen et al.[5,6] does not control inhalation flow rates or volumes per se, but rather have subjects perform slow inhalations followed by a forced exhalation, the latter likely providing good intrasubject reproducibility and efficient airway deposition.

Once sufficient radiolabeled particles are deposited in the lungs, subjects either sit against or lie beneath/above a gamma camera to measure clearance from the lungs. Early studies employed single-crystal detectors positioned over the subjects' torso to monitor particle movement from the lungs.[12,13] But most investigators now use a gamma camera and planar imaging to characterize both regional deposition and clearance of the inhaled particles. Most investigators use a single-head gamma camera that allows them to take posterior or anterior planar images. However, due to the structure of the respiratory tract, a more accurate measurement of the transport of the radiolabeled mucus, which corrects for the movement of mucus over time away or closer to the detector, is done by taking dynamic anterior and posterior images and then creating a geometric mean image of the two.[4,15,16,20] This requires either a rotating single head[15,16,21,22] or a dual-head gamma camera.[4] The dual-head gamma camera has the advantage that dynamic anterior and posterior images are taken simultaneously while the subject lies in the supine position.

It is recognized that the measured clearance from the lung is highly dependent on regional deposition in the lung (Fig. 3), that is, the more proximal the deposition of particles occurs in the bronchial airways the more rapidly particles will leave the lungs. Controlling particle size characteristics and inhalation patterns in patients is an attempt to minimize intrasubject variability in regional particle deposition. On the other hand, intersubject variability in regional deposition is affected by variation in airway and lung sizes between individuals. For either case, intra- or intersubject comparisons, it is recognized by most that an index of initial, regional deposition [e.g., central/peripheral (C/P) ratio as illustrated in Fig. 3] is required to include as a covariate in analyses of particle clearance from the lungs. To accomplish this a clear definition of the lung is required to prepare regions of interest (ROIs) (Fig. 3) that can be overlayed on the deposition image to calculate regional deposition. Currently, investigators use transmission scans of the lung[4,15,16] or radioisotopic gas ventilation scans (Fig. 4) (either Xenon 133 or Krypton 81m)[3,6,23] to outline the lung regions, and in some cases normalize deposition images to lung area/volume. The lung, usually the right, is divided into either two regions (central and peripheral)[2] or three regions (central, intermediate, and peripheral).[4,6,24]

Using the ROIs defined by transmission or gas images, retention as a function of time is determined from total lung counts in sequential images (usually 1–2 min acquisitions) acquired from 1–6 h. To avoid interference from stomach counts, many times only the right lung is used for retention versus time measures. Many investigators also obtain a static image the following day as an estimate of 24-h retention. Because the Tc99m has a half-life of only 6 h the static image at 24 h is acquired for a longer period (e.g., 10–30 min), depending on the initial activity, to obtain better counting statistics relative to background radiation. Later time points of acquisition are generally too noisy by planar imaging to be reliable. Finally, some investigators either incorporate voluntary coughing maneuvers[3,25] or attempt to assess the effect of spontaneous cough for separate measures of cough clearance (CC) during the period of monitoring lung retention.[4]

There are clearly potential advantages of using three-dimensional (3D) imaging to measure clearance, especially in better definition of regional airway deposition/clearance. Although the feasibility of using both single photon emission computed tomography (SPECT)[26,27] and positron emission tomography (PET)[28] has been demonstrated, there has been
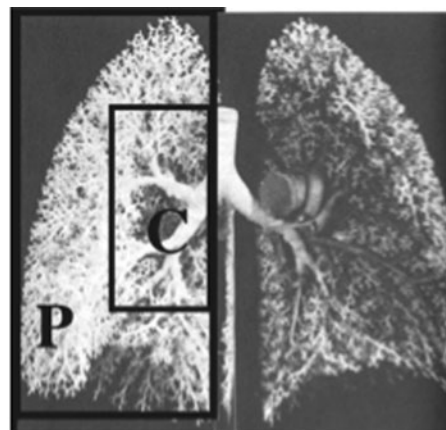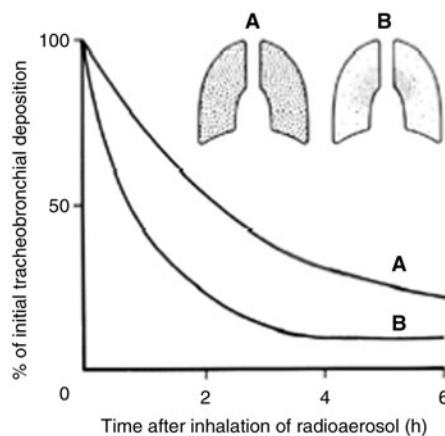

FIG. 3. Left: schematic illustrating faster clearance for peripherally (**A**) versus centrally (**B**) deposited particles. Right: lung morphometry and regions of interest: central (**C**), which includes the largest bronchial airways, and peripheral (**P**), excluding those largest airways.
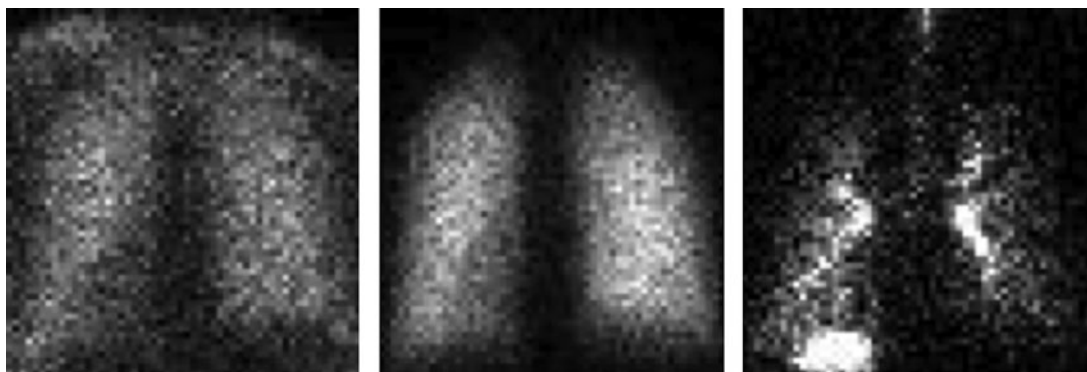
FIG. 4. Transmission (from a Tc99m flood source), Xenon133 equilibrium, and deposition image (aerosol of Tc99m-sulfur colloid) in the same healthy, nonsmoking adult.

little work following up these proof-of-principle studies, and the advantages of 3D data have yet to be fully explored. One disadvantage of these 3D methodologies is the increased radiation dose to patients compared to 2D methods. The total effective doses estimated by investigators for 2D measures of MCC using Tc99m labeled insoluble markers is in the range of 0.02–0.7 mSv (or 2–70 mRem) associated with depositing from 2–20 MBq (or 50–500 uCi) in the lungs. These effective doses are considered acceptable by comparison (1) to other radiological procedures, for example, as low as a single chest X-ray and much less than a chest CT scan, and (2) to the average annual radiation dose that subjects receive from natural sources (about 2.5 mSv). SPECT imaging is performed with a deposited activity of between 20 and 100 MBq of Tc99m, giving rise to effective doses estimated between 0.3 and 1.6 mSv. If low resolution CT is also performed then an additional dose of 0.8 mSv is delivered.

Moving Forward

On the second day of the workshop the attendees discussed the following series of questions that need to be addressed to better understand, improve, and standardize MCC/CC methodologies.

1. Does the choice of inhaled radiolabeled marker affect the measured clearance?

It has been a topic of some research and controversy whether, once deposited on the airway surface, the geometric size, shape, or solubility of the radiomarkers influences their rate of clearance by MCC. Obviously, if solutes are too small (e.g., DTPA) their clearance rates will also be affected by their ability to pass through the airway epithelium.[29] But solutes as small as albumin (m.w. 66 K, 0.6 nm molecular size),[30] and, at the other extreme, 5–6 μm solid particles of polystyrene[19,23] and Teflon[30,31] have been used for measures of MCC. In between these extremes are the colloidal particles of sulfur or albumin that are in the range of 50 nm to 0.5 μm[3,4,6,15,16] and the more dense 2–3 μm geometric diameter iron oxide (Fe_3O_4) particles.[11] Particle size dependence for MCC has been studied with ambiguous results between laboratories. Some data have suggested that there is a slow cleared fraction of deposited particles from the tracheobronchial airways that increases with decreasing particle

size.[10] This seems especially evident for dry, ultrafine particles; for example, Moller et al.[32] showed approximately 75% retention of ultrafine particles (100-nm median diameter) deposited in the tracheobronchial region by shallow bolus methodology at 24 h postinhalation. However, for larger particles, recent experimental evidence from Smith et al.[33] showed no difference in tracheobronchial clearance among humans for particles with geometric sizes of 1.2 versus 5 μm, but the same d_{ae} (5 μm) so as to deposit similarly in the TB airways. Very fine and ultrafine colloidal particles in solution (e.g., sulfur colloid and albumin)[6,34] appear to clear at similar or faster rates as larger dry particles[10–12,30,31] and do not have the high 24-h retentions observed with dry carbon or metallic ultrafine particles.[32,35] On the other hand, there have not been direct *in vivo* clearance comparisons between these various types of particles. Sulfur colloid particles (mean size 200 nm) and nonaggregated albumin (0.6-nm molecular size) delivered by microspray nozzle in a small volume (6 μL) of saline solution to large airways of dogs shows rapid clearance,[36] with sulfur colloid completely clearing the airways by 24 h.[7] There were some differences observed in 1-h clearance kinetics between the sulfur colloid particles and molecular albumin, with the latter having a larger slow-clearing phase. It appears that small solutes may disperse more completely in the airway surface fluid and be retained for longer periods than particles that may associate more readily with the mucus. It is also not clear what role airway macrophages may play in particle clearance from the airways. Sulfur colloid particles have been found associated with macrophages in induced sputum samples following inhalation,[37] about 30% of those collected, but it is not clear whether this association affects their rate of clearance from the airways.

So the question remains as to what the appropriate particle is to be used for MCC measures, one that mimics bacterial/viral clearance, tracks mucus movement, or tracks the clearance of the airway surface fluid as a whole? With new particle engineering processes (e.g., nanotechnology) it may be possible to develop particles of controlled size and shape that when deposited on airway surfaces, (1) accurately track the movement of mucus from the lung and (2) allow for multimodal imaging [e.g., SPECT, PET, or magnetic resonance imaging (MRI)] provided that they can be labeled appropriately for each technique). Although we approach

methodologies to improve on markers for MCC measure, we have to be realistic as to what we can have patients safely inhale for such measurements. Thus we should make similar efforts towards comparisons of particles currently used and approved for these measures. Some suggestions for making crosslaboratory comparisons are presented further below.

2. How do we best target deposition to the airways of interest with inhalation patterns and aerosol size?

All agree that deposition in bronchi and bronchioles airways should be maximized with minimal deposition in the alveoli where MCC is not present. But (1) how do we best achieve that goal and (2) do we care what the distribution of deposition is along the bronchial tree? As discussed previously, each investigator has different approaches to maximizing airway versus alveolar deposition. Ultimately, comparison of deposition indices (described further below) and clearance rates through 24 h between laboratories would be useful for assessing the optimum approaches for targeting the airways. Each laboratory should also determine intrasubject repeatability of their measures of MCC, an important element for its use as a biomarker of therapeutic effects.

Based on mean retentions at 2 h in healthy adult subjects, it appears that the technique of Mortensen et al.,[6,38] incorporating rapid forced exhalations, may provide the most large airway deposition, that is, deposition at sites of flow limitation during exhalation. The greatest control is probably provided by the method of Scheuch et al.[10,11] where absolute volumes, flows, and bolus depths normalized to lung volumes are controlled by positive pressure ventilation of the subject with single breaths. The AKITA technology (Activeaero) provides a potential commercial tool to achieve this controlled breathing and aerosol delivery. One disadvantage of the device, however, may be its limitation on inhaled flow rates ($\leq$250 mL/sec) and the fact that exhalation is uncontrolled. By incorporating breathholds at end inhalation with this device, there is little aerosol left to deposit in the airways, but breathholding will also enhance deposition in the smallest airways where particles reside.

The second question, which airways are most important to target, assumes that we might affect the distribution of particle deposition along the airway tree with breathing patterns or particle size. One of the more innovative approaches to specifically target the small airways of the lungs is that of Anderson et al.,[30] where by using large particles (6 μm) and extremely slow inhalations (40 mL/sec) they predict particles will escape deposition by impaction in the upper airways and large bronchi but penetrate to the smaller bronchioles for deposition by sedimentation. Model predictions also suggested that the slow flow would allow near complete deposition in the small airways before particles could reach the alveolar region. Their experimental comparisons with tidal breathing of similar size particles confirmed that significantly greater clearance of particles was observed beyond the 24-h retention measurement for the slow inhalation versus tidal breathing approach. Bennett et al. (unpublished observations) have been making similar comparisons of MCC as measured after rapid shallow breathing (500 mL/sec at 30/min) of 5 μm particles versus slow inhalation (80 mL/sec) of 9.5 μm nebulized particles.

Initial results confirm those of Anderson et al.,[30] but also show enhanced MCC during the first few hours of observation post deposition, suggesting enhanced large airway deposition as well for the slow inhalation method. Targeting deposition of the radiolabeled particles to the small bronchiole airways of the lungs may be especially advantageous for studying MCC in patients with early lung disease as it is generally agreed that many airway diseases begin in these airways (e.g., CF) bronchiolar region of the lung. If large airway deposition dominates in such patients, MCC may appear to be relatively normal,[39] while the MCC defect in small airways may be disguised, as may the effect of the experimental therapy on this region of the lung. On the other hand, there may be therapies or situations where MCC in the larger airways is of primary interest, for example, a drug that stimulates or inhibits glandular secretions. In these cases a targeting strategy such as that of Mortensen et al.[6,14,38,39] that targets the largest airways may be best.

Finally, regardless of targeting methodology sufficient activity should be deposited with inhalation to provide for accurate 24-h retentions, by which time Tc99m will have decayed four half-lives. Moreover, the accuracy of this measure is critical for being able to calculate the tracheobronchial clearance or retention where 24-h retention is assumed to primarily represent alveolar deposition (i.e., subtracting 24-h retention from previous retention values). This assumption may not always be valid, however, especially in airways disease such as CF or non-CF bronchiectasis where the bronchial airways have not completely cleared by 24 h.[40,41] It is agreed that longer static acquisitions are required (up to 30 min) at 24 h to provide better counting statistics for comparison to background radiation.

3. Characterization of initial deposition pattern (C/P, PI, Skew, other)?

All investigators recognize that the measured clearance is a function of initial deposition pattern in the lung (Fig. 3) and consequently measure some index of regional deposition that will reflect airway versus alveolar deposition. Figure 5 shows data from Mortensen et al.[6] illustrating the correlation of % retention at 2 h with penetration index (PI, which is a ratio of activity in peripheral to central region normalised for volume differences, inverse of C/P in Fig. 3) in a large group of healthy adult subjects.[6] Are the various indices comparable and/or should there be a standard index that can be used to compare clearance between different studies and laboratories? Because the actual borders of the lungs are likely to be underestimated with the deposition scan alone, the first step in assessing regional deposition is defining the lung boundaries. As discussed above, this can be done with either a gas or transmission scan (Fig. 4). Equilibrium scans with Xe133 are more likely to define the whole lung than short half-life (13 sec) Kr 81m scans, which provide a measure of ventilated lung. Although either may suffice in the healthy lung, poorly ventilated regions in the diseased lung may make gas scans less reliable. In such cases, a transmission scan is probably better at defining the edges of the whole lung. Figure 6 illustrates how even a Xenon 133 equilibrium scan in a mild CF patient may tend to underestimate the edges of the lung compared to a transmission scan in the same individual; in this case, the upper right lung is poorly
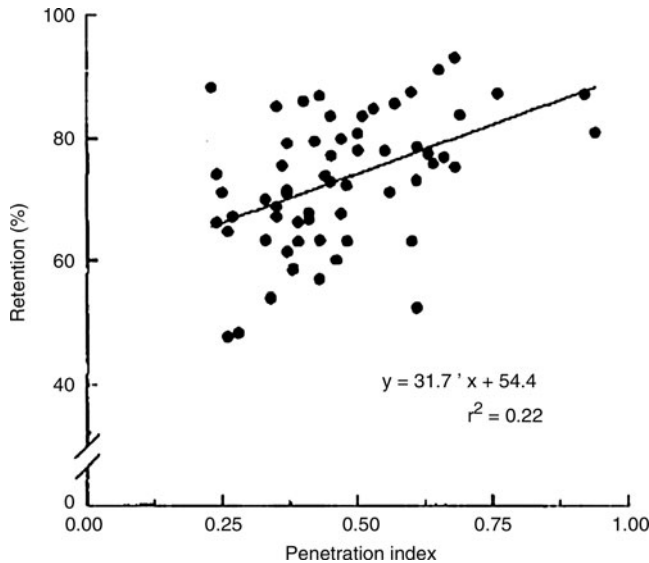
FIG. 5. The association between penetration index and lung retention at 2 h, analyzed by simple linear regression ($n = 62$) (6).

ventilated. On the other hand, the transmission scan is less likely to normalize for volume differences between the different regions of the lung in the way an equilibrium gas scan will if the lung is fully ventilated. A good comparison between these two methods in both healthy subjects and patients with airways disease would be useful to better define the limitations/advantages of each. But if all laboratories could measure a C/P or PI based on a transmission scan of the lung (the easiest and most accessible method for defining the lung), and define the right lung regions the same way, then crosscomparisons between laboratories (or within laboratories for different patient cohorts) of retention as a function of C/P or PI by analysis of covariance (e.g., Fig. 5) could be easily made.[6] For instance such a comparison would then allow us to determine if there are differences in MCC measures associated with particle type as discussed above, for example, sulfur colloid[3,4] versus polystyrene[8,9] versus iron oxide.[10–12] Ilowite et al.[42] reported such an analysis to determine if the retention vesus C/P relationship differed for different particle types used within the same laboratory. The definition of size and location of regions, that is, C versus P, should be standardized for such comparisons to be made across laboratories.

Regional deposition as defined by specific regions may be useful as an index of large bronchial airways versus small airways and alveoli, but other distribution indices that are not dependent on specific regions of interest may provide more sensitive measures of airway deposition and its homogeneity. For example, the coefficient of skew for the histogram distribution (counts/pixel vs. number of pixels)[43] within the whole lung ROI increases with increased frequency of "hot spots" in the lung. These hot spots are presumed due to increased deposition within bronchial airways throughout the lung so that skew is independent of the specific region within the lung (e.g., C vs. P). To determine skew, frequency distribution histograms are constructed from the deposition images, with the number of pixels with a given count value (expressed as a percentage of total pixels) on the y-axis and the count values on the x-axis. These histograms are then analyzed for skew (a measure of histogram symmetry, the third moment about the mean of histogram). Heterogeneity of deposition increases with increasing skew (i.e., more pixels with high counts/pixel) and, like C/P or PI, has been found to correlate with retention, that is, decreased retention at any time with increasing skew.[43]

Finally, three-dimensional multimodality imaging enables more accurate characterisation of the initial distribution of aerosol.[44] It provides both an improved description of the spatial deposition of aerosol and the possibility of estimating deposition by airway generation. Associated 3D lung volume scans from transmission SPECT or CT are also frequently obtained in these studies. All these data should help in interpreting clearance rate measurements.

4. Period of MCC/CC observation/imaging?

The period for assessing MCC/CC postradioaerosol deposition depends on the aims of the study, the isotope used, and the initial activity deposited. The latter is limited by radiation safety, that is, minimum levels of activity are desired to reduce radiation doses to subjects. As discussed above, most all laboratories use the short half-life Tc99m as the radiolabel but some have utilized Indium 111 (half-life of 2.2 days)[30,31] to allow measures of MCC over a longer period of time, assuming that sufficient activity is still residing in ciliated airways at these later time points. Although none
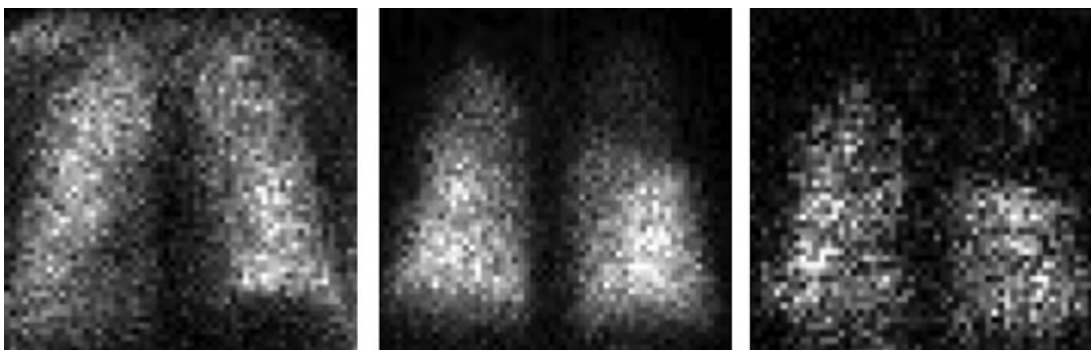

FIG. 6. Transmission (from a Tc99m flood source), Xenon133 equilibrium, and deposition image (aerosol of Tc99m-sulfur colloid) in the same CF adult.

of such studies to date have utilized gamma camera imaging but rather whole-body counting, due to the low activities deposited (again, to minimize radiation dose for the longer lived In 111 isotope). It is generally agreed that whole right lung clearance in the first 1–2 h of measurement represents clearance from the largest bronchial airways. For many drugs to be tested this initial period is likely sufficient to determine if a drug acutely stimulates MCC. But for drugs that are longer acting or when observed effects are expected in the smaller airways, longer periods of measurement of up to 6 h may be required.[45]

All agree that despite the period of the initial measurement, a later retention measurement (>6 h) is beneficial to assess residual small airway/alveolar retention. Twenty-four-hour retention measurements are common, and combined with regional deposition indices provides additional information on the degree of initial airway versus alveolar deposition. How much of the 24-h retention measurement reflects airway versus alveolar deposition is still a debatable question that likely depends on the inhalation maneuver and the patient type. For example, the very slow inhalation–large particle inhalation method[30] showed continued significant clearance beyond 24 h, suggesting retention of particles in small, ciliated airways for such long periods. Similarly, Regnis et al.[40] showed that cystic fibrosis patients have prolonged retention in the airways at 24 h compared to healthy subjects by comparing the 24-h ret versus C/P relationships between the two groups. Donaldson et al.[46] were able to show that aerosolized hypertonic saline treatment in CF was able to reduce 24-h retention of particles residing in the CF airways. Similarly, Daviskas et al.[41] were able to show not only that inhaled mannitol reduced the 24-h retention in patients with bronchiectasis but that clearance with mannitol over 2 h was equivalent to clearance over 24 h without mannitol.

As discussed previously, the variability (signal-to-background) of 24-h retention measures is surely dependent on the initial deposited activity in the lung because this initial activity will have decayed four half-lives by this time point. But even laboratories using relatively low deposited activity (2 MBq) have been able to show significant effects of therapy at this time period postdeposition.[46] However, it is important to acquire the image for long enough (at least 30 min) to ensure that the statistical errors in the lung count after subtraction of background are sufficiently small. The level of deposited activity associated with the variability in 24-h retention measurements might be compared between laboratories by determining the intrasubject variation in repeat measures of 24-h retention. The deposited activity required to provide good signal background at these later time points then needs to be weighed against the risks of radiation doses to the study subjects.

5. How to measure cough clearance (CC)?

For some patient groups and therapies the primary airway clearance mechanism of interest may be that of coughing. When MCC becomes dysfunctional in patients with airways disease, CC may become the primary means by which secretions are removed from their lungs. On the one hand, spontaneous cough during imaging of clearance may be a confounder of MCC measures. Because some patients may not be able to avoid coughing during clearance measure-

ments, the number and timing of spontaneous coughs should be recorded. Then, in the study design the number of spontaneous coughs might be considered as a covariate in the analysis to ensure that frequency of these coughs does not overly influence any differences due to therapy effects. Some investigators have included a separate study arm with voluntary coughs equal to the number of spontaneous coughs in the treatment arm as a "cough" control measurement,[16,22,41,47] that is, that can be compared to the other arms to correct for spontaneous cough effects.

On the other hand, although uncontrolled coughing can confound measures of MCC, measuring CC by incorporating controlled, voluntary coughs during the measures of particle clearance may provide a more sensitive indicator of rheological changes in airway secretions.[25,48] In fact, CC is the primary endpoint of interest in patients with primary ciliary dyskinesia (PCD), an inherited syndrome characterized by nonfunctioning cilia.[5,48] In other patients where both MCC and CC may be affected by therapy (e.g., CF) it may be useful to incorporate controlled coughing during the measurement period. Patients can be asked to perform voluntary, single coughs after tidal inhalation through a peak flow meter to periodically record cough efforts.[46] However, if the primary aim is to assess therapeutic effects on MCC, the coughs should not be incorporated until a later period (e.g., 60–90 min) so as not to confound early measures of MCC.[4,25] Even during this later period, clearance is a function of both cough and mucociliary action and changes in aerosol distribution during the first hour must be considered for its effect on subsequent clearance during the cough period (e.g., C/P at 1 h prior to coughing could be a covariate for the CC measure). Waiting too long in the period of clearance measurements likely diminishes any chance of seeing effects on CC, that is, it is generally believed that cough is most effective in the larger bronchial airways that likely clear during the first 1–2 h of observation. There was disagreement on the degree to which voluntary, controlled cough clearance should be incorporated in the clearance measurements on a routine basis, but it was agreed that CC should be considered in the context of the potential drug's effects, the patient population, and the limitations on number of treatment/control arms associated with each study design.

6. Which variables of MCC/CC are the most robust, or how do we best analyze retention versus time data?

The primary data from the MCC/CC measures are retention (as either fraction or % of initial deposition image) versus time (Fig. 7). Over the years, investigators have chosen several ways to characterize these data. The simplest method is to report retentions at a given time point (Fig. 7a). The disadvantage of this approach is that one point on a curve is not very descriptive of the rate of clearance, and if not chosen appropriately, can give a false impression of the data. For example in Figure 7c retention at 6 h would be similar for curves A and B, whereas the rate of clearance over earlier time periods is clearly faster for A versus B. Thus, it is best to incorporate all data points in the characterization of MCC. One approach is to fit a simple regression line through the data, the slope of which represents a clearance rate in %/min or h. For nonlinear clearance curves an area under the curve analysis (Fig. 7d) may be more appropriate, where
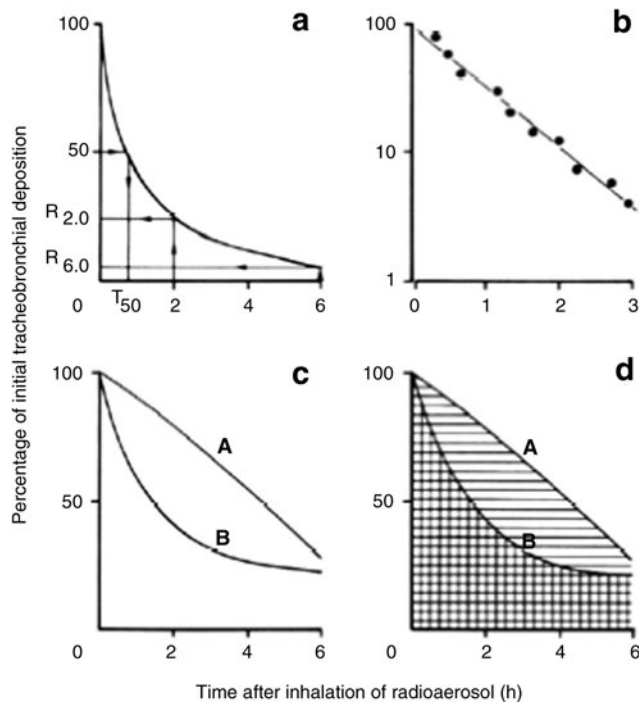
FIG. 7. Methods for analysis of retention versus time data. (**a**) Time taken to clear 50% (T_{50}) or amount retained [e.g., $R_{2.0}$ at a given time (e.g., 2 h)]. (**b**) Regression analysis to determine slope (rate) of decrease in retention versus time. (**c, d**) Area under curve or average retention over the time of interest.

essentially an average retention value is obtained over the period of interest. In some cases (e.g., Fig. 7c, curve B) it may be possible to fit multiple regressions (linear or exponential) to the data to obtain both rates and sizes of different compartments of clearance. The most sensitive approach for comparing acute drug effects may be to determine "break points" in the curves with rates only determined to that time point.[49] This approach eliminates bias from later time periods where clearance is slowed, probably reflecting retention of particles in more peripheral airways. On the other hand, such an approach also biases clearance comparisons to the most proximal airways that presumably clear in this early phase. More robust statistical models that allow inclusion of all retention versus time data points, rather than rates/slopes, along with covariates for regional deposition may also be more sensitive for detecting differences between treatment days. Finally, some investigators have attempted to reduce the bias associated with flat portion of the curve (e.g., alveolar deposition) by subtracting the 24-h retention from retentions at earlier time points, and renormalizing to 100%, to get a measure of "clearable" activity or as they define it "tracheobronchial clearance."[6,8,9] Although this may be valid in a particular patient group or for repeat measures in stable subjects, there may be situations in which the 24-h measure is not purely a reflection of alveolar deposition (i.e., nonclearable activity).

Comparisons of these various approaches should be made in several data sets from different labs to determine which may be the most sensitive measure of MCC. As discussed above the relationship between the MCC variable of interest (e.g., slope or average retention) and regional deposition could then be compared across laboratories to assess similarity of various approaches (e.g., particle type). In addition, repeat measures of these variables in healthy and diseased populations would be useful for determining appropriate sample sizes for therapy studies.

Generally the right lung has been used for analysis of whole-lung retention to avoid stomach activity that may be contiguous with the left lung. Alhough whole-lung retention versus time is usually of primary interest, regional clearance, for example, from central and peripheral regions in Figure 3, may prove more sensitive and of greater value in determining drug effects.[39,41,51,52] While imaging over time, the subject's body position might shift slightly on the camera, especially over long periods when subjects may take breaks from imaging. This probably has little effect on measures of whole-lung clearance, but subject movement may affect accurate measures of regional clearance. Most investigators attempt to maintain subject's position in front of the camera during periods of MCC measurements with laser light/mirror feedback[46] or by imaging in the supine position.[5,16,22,41,47] Point markers on the subject's torso (Co57 or Tc99m) outside the lung view have been used to allow realignment of images based on their positions.[5,6,16,40] More sophisticated analysis software may allow for an automatic algorithm to align images postacquisition based on the similarity between sequential images. This may be affected, however, by the stomach activity requiring it to be masked out manually, as this may vary between images and can bias the alignment.

3D imaging approaches again offer a potential advantage in regional analysis. As they are able to provide better measures of regional deposition compared to planar imaging,[44] they should be able to track changes due to MCC more accurately. In addition, these methods will better allow analysis of the left lung, due to the improved separation of lung and stomach counts in 3D.

Current methods of measuring MCC from imaging rely on measuring the rate at which the amount of aerosol in a region of interest reduces with time. The result is usually presented as the percentage cleared per unit time. The process of MCC describes the movement of aerosol particles along airways, with the rate being measured as a speed (mm/sec). This means that the conventional imaging measurements are really only indices of the actual mucociliary movement. 3D radionuclide imaging (SPECT and PET), particularly used in combination with aligned anatomical imaging, has the potential to be able to make more accurate measurements of the change of distribution within the lung over time. By combining this improved spatial data with mathematical modeling it might be possible to estimate mucociliary velocities. The mathematical model would have to describe variation of concentration of aerosol in different regions of the lung with time C_i (t) as a function, F, of the mucociliary speeds, s_j in different generations, j,

$$C_i(t) = F[C_i(0), s_j, l_j]$$

where s_j and l_j are the mucociliary speed and length of each generation.

This equation would provide the potential of estimating the mucociliary speed in each generation. A useful definition of the different regions of the lung might be the three dimensional shell approach.[53] This divides the lung into volumes on the basis of their position from the center to periphery of the lung and may therefore be well suited to describe the mucociliary movement, which is essentially in a radial direction from periphery to the hilum of the lung.

7. Timing of drug treatment/therapy relative to MCC/CC measurement?

The general approach/timing to assess the acute effect of a treatment on MCC/CC has been to treat after delivering the radioaerosol, based on the rationale that the regional deposition will then be unaffected by treatment versus control. However, more recently, this approach has been challenged by (1) therapies that might induce considerable, uncontrolled coughing, for example, hypertonic saline,[46] (2) placebos that are acutely active (e.g., normal saline from efficient nebulizers),[54] and (3) therapies designed to have durable or later onset of action. In some of these cases, it has been deemed necessary to pretreat with therapy at various times prior to inhalation of radioaerosol. These studies have still considered the potential effect of the pretreatment on regional deposition of aerosol by including the index of deposition (C/P, PI, or skew) as a covariate in the statistical analysis of treatment versus placebo MCC/CC comparisons,[46,49] that is, to test that drug had an effect independent of any possible differences in deposition pattern. Such analyses will also need to be employed to study the chronic (weeks or months) effect of therapies[46] as changes in lung function may alter regional deposition between pre- and posttreatment conditions. Study designs and timing between drug and radio-aerosol may provide the greatest challenge for future therapies but should be treatment-specific based on the drug's actions on lung function, cough frequency, and duration of action.

8. Evidence that MCC/CC is a good biomarker for airway disease and its treatment?

Finally what evidence is there that MCC/CC is a good biomarker[1] of airways inflammation/disease and, if little evidence exists, how do we best assess that? It seems reasonable to suggest that slowing of MCC/CC may enhance susceptibility to airway infection, exacerbation of airways disease, and further decreases in airway function. Early studies by Goodman et al.[55] showed progressive slowing of tracheal clearance with increased smoking history. Others studies have also indicated a slower whole lung clearance in healthy smokers versus. healthy never-smokers and healthy ex-smokers versus never-smokers.[6,38] But no studies have been able to show a clear correlation between rates of MCC/CC and lung function by spirometry in these chronic bronchitics or smokers. Robinson et al.[56] in a retrospective review of one laboratory's experience in normal ($N = 17$ subjects/22 studies) and CF ($N = 59$ subjects/184 studies) found that MCC was markedly impaired in CF subjects, in comparison to the normal cohort. But interestingly, the observed reductions in clearance appeared to be independent of baseline lung function, and even a subgroup of CF subjects with normal FEV_1 measurements ($N = 17$) manifested

markedly abnormal clearance rates. MCC has been shown to be mildly impaired in stable asthma[57,58] and severely during acute exacerbations with patients hospitalized for acute exacerbations having essentially static MCC within 3 days postadmission.[59] In the same study, MCC was improved to near normal rates with clinical improvement after treatment and discharge from the hospital. Monitoring MCC/CC along with other clinical outcomes in CF and chronic bronchitic patients over time may help to determine which biomarkers are most sensitive at predicting long-term outcome of patients. The importance of MCC for health status may be best observed in model disease for MCC dysfunction, PCD. We know that PCD results in infections and impairment of lung function and in a few patients even lung transplantation. It would further strengthen the concept if we could link genotypes with the different phenotypes of PCD and variability in MCC impairment between patients, and between time of diagnosis/institution of treatment with long-term outcome.

As discussed previously, MCC measurements have been utilized in a number of small clinical trials, but generally these have been acute in nature, that is, single treatment effects, and have not shown a correlation with lung function changes associated with treatment. Most recently, however, Donaldson et al.[46] found that 2-week treatment with aerosolized hypertonic saline (HS) in CF patients resulted in a sustained improvement in both their MCC and lung function, although the two were not correlated in the small group of patients studied. A larger 12-month trial of HS saline treatment in CF showed similar improvement in lung function and a decrease in pulmonary exacerbations requiring antibiotics, although the long-term effect of HS on MCC/CC was not measured in this cohort of CF patients.[60] It may be that exacerbation frequency is a better correlate with changes in MCC/CC with long-term therapy than lung function by spirometry, a robust but relatively insensitive measure of lung changes. The fact that spirometry measures resistance to airflow and lung compliance suggests MCC/CC measures may not have strong correlations. More recent studies have shown an improvement in lung function and quality of life when inhaled mannitol was administered twice daily over 2 weeks in patients with CF.[61] The improvement in lung function correlated with the increase in hydration and decrease in surface tension in the sputum properties of the same CF patients.[62] These studies in CF suggest that an acute effect of a treatment on MCC/CC is a good indication for potential long-term clinical benefits in these patients.

Conclusions

The development of methodologies for measuring MCC/CC in human studies has progressed during the past 20 years to the point where there is general agreement that controlled inhalation of nonabsorbable, tightly labeled radioaerosols must be used to accurately assess MCC/CC endpoints. The marker of choice for most laboratories is many times dictated by availability, ease of use, and acceptability by the regulatory agencies of that site. Despite these differences, the measured MCC/CC may not be dramatically different between laboratories when corrected for regional differences in lung deposition that may occur due to variability in breathing maneuvers and aerosol size. Although most all

laboratories use planar imaging to assess MCC/CC, the future of the methodology may lie in 3D imaging that should allow better resolution of airway regions. Meanwhile, the similarity of gamma camera acquisition and analysis methods should allow laboratories to share and compare data to address many of the questions raised in this review and, in doing so, better refine our methodologies for improving on their accuracy.

The utility of MCC/CC as a biomarker for disease progression and therapeutic intervention is gaining increased recognition as a valuable tool in the clinical research community. The methodology has clearly been valuable for assessing acute responsiveness of the mucociliary apparatus to therapies designed to improve clearance of secretions in patients with airways disease. The most recent example of this success has been with the application of inhaled, hyperosmotic agents (HS and mannitol) to improve clearance of secretions and lung function in CF patients. The MCC/CC biomarker may also become an important tool for assessing new therapies for other airway diseases, for example, chronic obstructive pulmonary disease (COPD) and asthma, as there becomes increasing awareness of the role that insufficient MCC/CC plays in these diseases. Finally, the greatest challenge for the utility of the methodology may lie in relating the endpoints of MCC/CC with other clinically accepted endpoints of lung health over time (e.g., lung function, exacerbation frequency), either in tracking disease progression or assessing the chronic effectiveness of new therapies.

Author Disclosure Statement

No conflicts of interest exist.

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