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THE RESPONSE PHASE - THE FIRST SIX HOURS AFTER ACUTE AIRWAY INJURY BY SO_2 INHALATION: AN *IN VIVO* AND *IN VITRO* STUDY

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Abstract

We have identified an airway epithelial response following acute injury that cannot be termed 'repair' or 'regeneration'. It precedes these well characterized events and it is termed the 'response phase'. We tested the hypothesis that for the first 6 h following acute injury to the tracheal mucosa, the initial cellular events of the response phase will continue as *in vivo* even if the tissue is maintained *in vitro* in an Ussing chamber. The tracheal mucosa of anesthetized, intubated mongrel dogs was injured by the inhalation of SO_2 500 ppm for 1 h (7 dogs); controls (3 dogs) breathed filtered, compressed air for 1 h. 4 dogs were killed, in pairs, at 1 and 6 h after 500ppm of SO_2; their tracheas were removed and fixed for microscopic examination. 3 dogs were killed immediately after the SO_2 exposure, their tracheas were removed and epithelium isolated from the posterior-membranous sheath was mounted in Ussing chambers in oxygenated, Krebs-Henseleit buffer (8 per dog with aperature area of 1.5 cm^2). These tissues (and those from control dogs prepared identically) were fixed after 1 and 6 h incubation for microscopic examination.

Epithelial damage was not observed in any controls but was in all tissues exposed to SO_2. A wide spectrum of mucosal cell injury during the response phase was observed. The patterns of exfoliation noted were: individual cells, rows (several cells wide) of mucosal cells and entire regions (several hundred μm^2). At 1 h after exposure, in some lesions, the injury is difficult to assess because the tracheal surface was either blanketed in exfoliated cells or appeared in total disarray. By 6 h, the lesions were well defined and large flattened cells (130 μm^2 in surface area) covered the basement membrane in areas where mucosal cells had exfoliated. Some ciliated cells still remained attached at their base in these areas.

These were the findings whether the tissues were taken fresh from the animal or have been maintained in Ussing chambers for up to 6 h. These results show that cellular repair of the tracheal epithelium can be studied *in vitro* during the first 6 h after injury, even if the injury has occurred *in situ*.

Key words: Dogs, cells, cell death, trachea, toxicity, gases, sulfur dioxide.

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Introduction

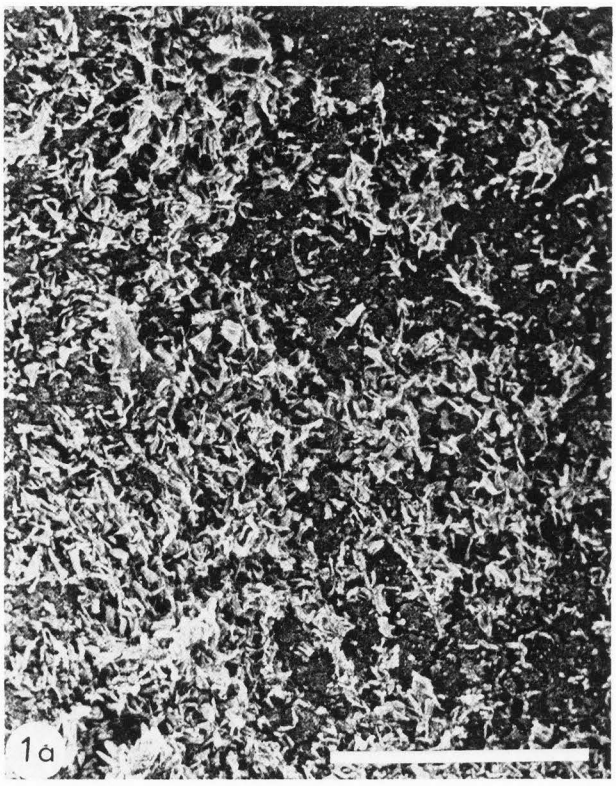
Our investigations have been focused on airway mucosal repair during the first 6 h after injury by cigarette smoke (10) or sulfur dioxide (17,19). Measurements of mucosal permeability to macromolecules (mannitol, horse-radish peroxidase) and the bioelectrical properties (differences in trans-epithelial electrical potentials, short-circuit current, and resistance) are abnormal immediately after these exposures, but rapidly progress towards normal over the next 6 to 12 h. This is the time period that precedes the proliferative waves of mitoses that follow cigarette smoke and SO_2 injury and has been termed the pre-mitotic repair phase (18). That terminology was developed to differentiate the reparative events immediately following acute injury from those events arising from cellular division several hours later. Unfortunately, the term is somewhat misleading. The events being described were not 'repair' as defined in pathological terms (the replacement of lost tissue by scar tissue). Indeed, it has been suggested (E.A.McDowell, personal communication) that the cellular responses during this time period are neither 'repair' nor 'regeneration', rather, they are pre-repair and pre-regeneration. We have termed this the 'response phase'.

In this paper we show that even if airway injuries are initiated *in vivo*, the cellular events of the response phase continue regardless if the tissues were left in the animal, or when they were removed and supported *in vitro*.

Materials and Methods

The dogs weighed between 20 and 30 kg and were free of clinically detectable respiratory disease. All of them were anesthetized with pentobarbital sodium (30 mg/kg body weight, iv) and intubated with the tip of the endotracheal tube at the glottis to maintain the patency of the airway. The dogs were kept anesthetized until death. They were killed by the induction of respiratory arrest with a large bolus of anesthetic and cardiac arrest with an iv injection of saturated KCl.

Our method for exposing dogs to SO_2 (500) ppm has been described in detail (17). Briefly, the dogs were anesthetized, intubated and inhaled SO_2 generated by mixing pure SO_2 with medical air using a Matheson dyna blender and flow controller. There are three groups of tissues that were studied. *In situ* tissues, fixed at the time of death, were obtained from 4 dogs killed in pairs 1.0 and 6 h after SO_2 exposure. *In vitro* tissues, incubated in Ussing chambers, were obtained from 3 dogs killed immediately after SO_2 exposure. The trachea was removed immediately after death. The posterior membranous sheath was exposed and 8 pieces of epithelium were mounted and incubated in Ussing chambers (1.5 cm^2 aperture) in oxygenated Krebs-Henseleit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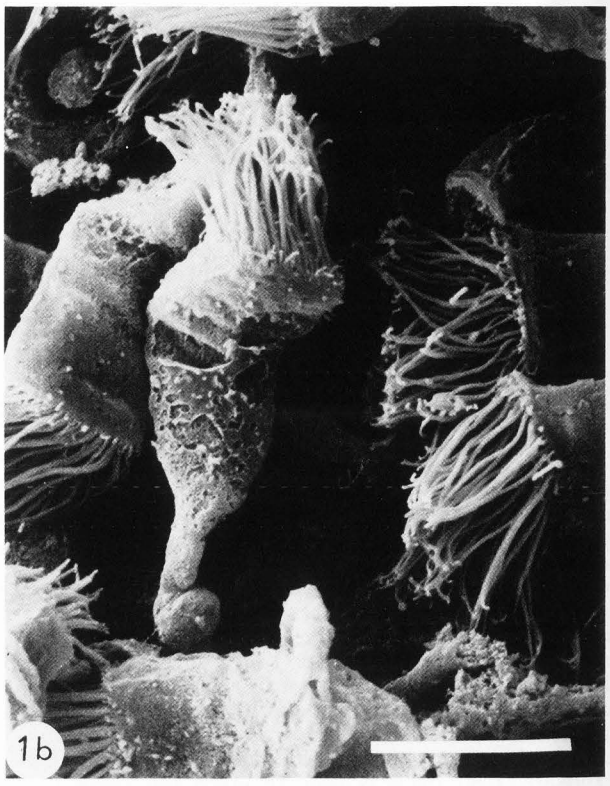
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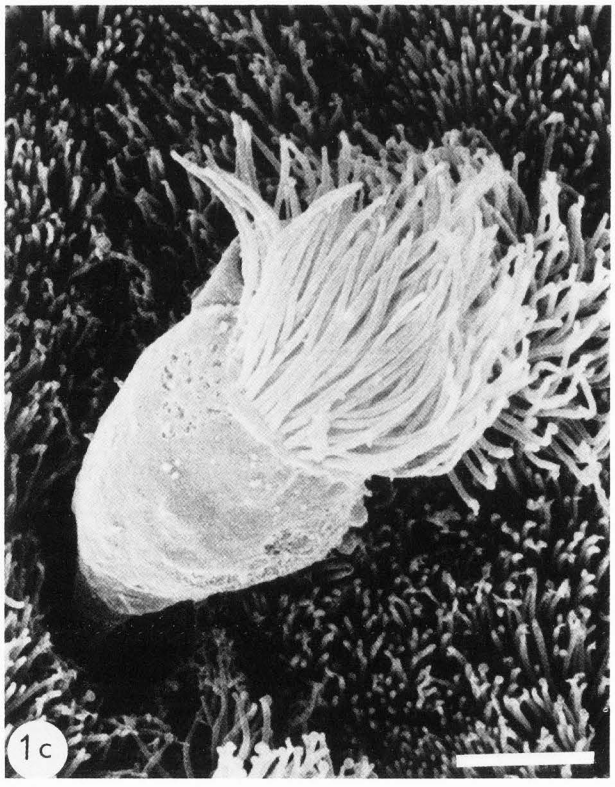
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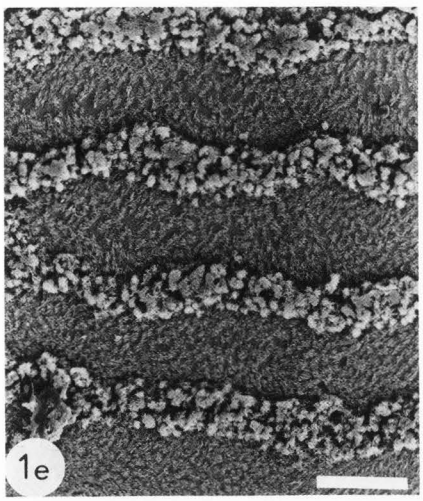

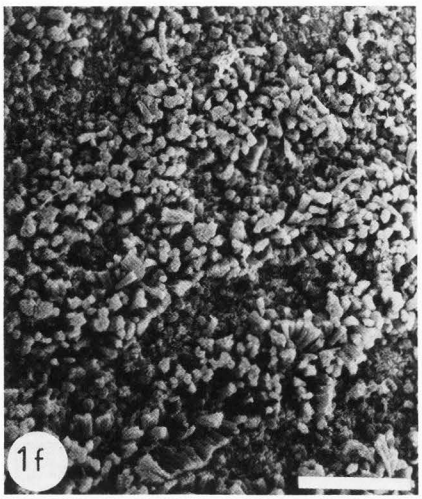

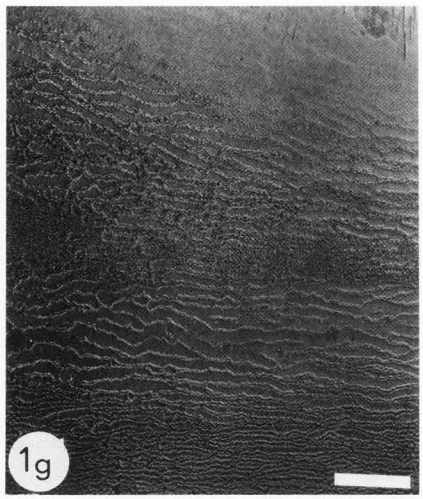

Figure 1a. The tracheal surface of a tissue obtained *in situ* 1 h after exposure to SO_2 500 ppm. The surface is blanketed with exfoliated cells. Bar = 200μm.
Figure 1b. The tracheal surface from a tissue obtained *in situ* 1 h after exposure to SO_2 500 ppm showing exfoliated cells. The lateral membranes are disrupted on several of the cells. Bar = 10μm.
Figure 1c. An individual ciliated cell exfoliating from the tracheal mucosa in a tissue obtained *in situ* 1 h after exposure to SO_2 500 ppm. Bar = 5μm.
Figure 1d. Clumps of exfoliated mucosal cells lying on the tracheal mucosa in a tissue obtained *in situ* 1 h after exposure to SO_2 500 ppm. Bar = 20μm.
Figure 1e. Rows of exfoliating mucosal cells on the tracheal surface in a tissue obtained *in situ* 1 h after exposure to SO_2 500 ppm. Bar = 100μm.
Figure 1f. Total devastation of the mucosa from a tissue obtained *in situ* 1 h after exposure to SO_2 500 ppm. Bar = 10 μm.
Figure 1g. Overview of the tracheal mucosa obtained from an *in situ* tissue 1 h after exposure to SO_2 500 ppm. This micrograph shows spectrum of responses like those in Figs. 1c to 1f and illustrates that these different responses can co-exist within millimeters of each other in the same tissue. The dark strips are non-ciliated cells of basal and superficial cell origin that have spread laterally, and the light strips are areas with ciliated cells intact. Bar = 10μm.

solution, at 37°C, and pH 7.4. At 1 and 6 h after mounting, pieces were removed and fixed by immersion. Finally, control tissues from 3 dogs that inhaled compressed air for 1 h were incubated in Ussing chambers as above; tissues were fixed by immersion after a 1 or 6 h incubation.

Tissue Processing

All tissues were processed while mounted on Lucite rings to prevent curling during dehydration and critical point drying. Tissues were fixed in ice-cold 3% glutaraldehyde containing 0.1% OsO_4 and 1 mM $CaCl_2$ in 0.1 M cacodylate buffer, pH. 7.4. Glutaraldehyde and OsO_4 were kept separately on ice and combined immediately before tissue fixation. After primary fixation, the tissues were washed briefly with ice cold buffer, re-fixed in ice-cold 1% OsO_4 in 0.1 M cacodylate buffer. Then, at room temperature, they were washed with distilled water, stained *en bloc* with saturated uranyl acetate in distilled water, washed again with distilled water and dehydrated in a continuous ETOH series (i.e., beginning with 30% ETOH and adding small volumes of 100% ETOH to increase its concentration in 2% steps). The tissues were dried to critical-point with CO_2, mounted on aluminum stubs with silver conductive paint, sputter coated with gold, and examined with a Cambridge 250 SEM. After SEM examination, the tissues were infiltrated with propylene oxide and embedded in LX112 (Ladd Industries). Thin sections were cut on a Porter-Blum MT-2 ultramicrotome using a Diatome diamond knife. They were stained with uranyl acetate and then lead citrate using an LKB automatic grid stainer, and viewed with a Philips 410 TEM.

Cell surface areas were measured on SEM micrographs with a Dapple Image Plus image analyzer. The analyzer was operated in the manual mode and the cell boundaries were traced using a mouse. Data were transferred on-line to a Macintosh Plus® with Hyperdrive 2000® and analyzed statistically with Stat View 512®.

Results

In situ Tissue

The appearance of the tracheal surface 1 h after exposure varied greatly between animals; indeed, some scattered areas of the trachea did not show any evidence of injury at all by SEM (see Fig.1g &2a). In others, however, low magnification views (Fig.1a) were often not interpretable because the number of individual exfoliated cells and their aggregations (Fig.1b) covered the tracheal surface. Note that the lateral membranes were disrupted on several of these ciliated cells. The extent of response we found is put into perspective in the next series of micrographs. We observed the exfoliation of individual ciliated cells (Fig.1c), clumps of mucosal cells (Fig.1d), rows of exfoliating mucosal cells (Fig.1e) and total devastation with entire areas undergoing desquamation (Fig.1f). Many of these features were observed on the same tissue (Fig.1g).

In tissues fixed 6 h after the exposure, the surface of the mucosa had been cleared of the exfoliated cells and the features of the lesions were more evident (Fig.2). There were bands of cells (Fig.2a) that appeared dark on SEM due to their relative lack of surface features (Fig.2b). The surface of these cells was characterized by villi that were drawn into ridges and an extensive percentage of the apical


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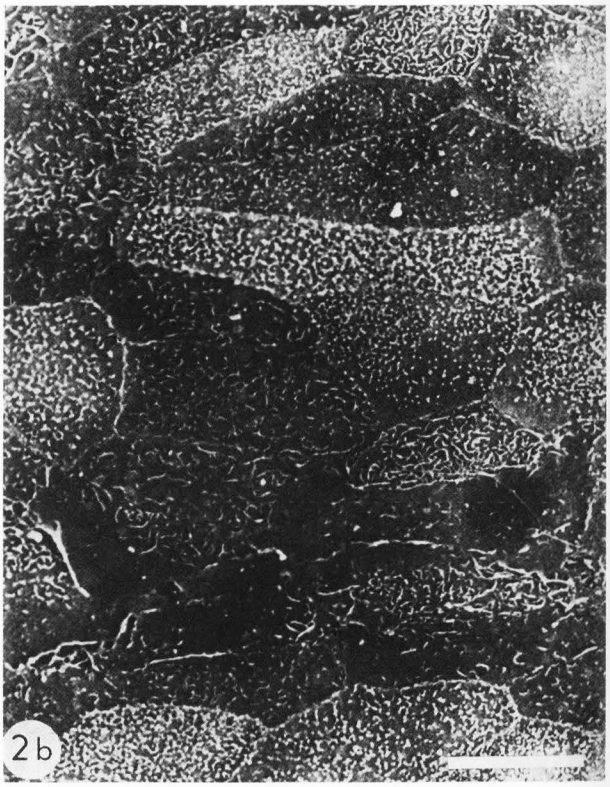
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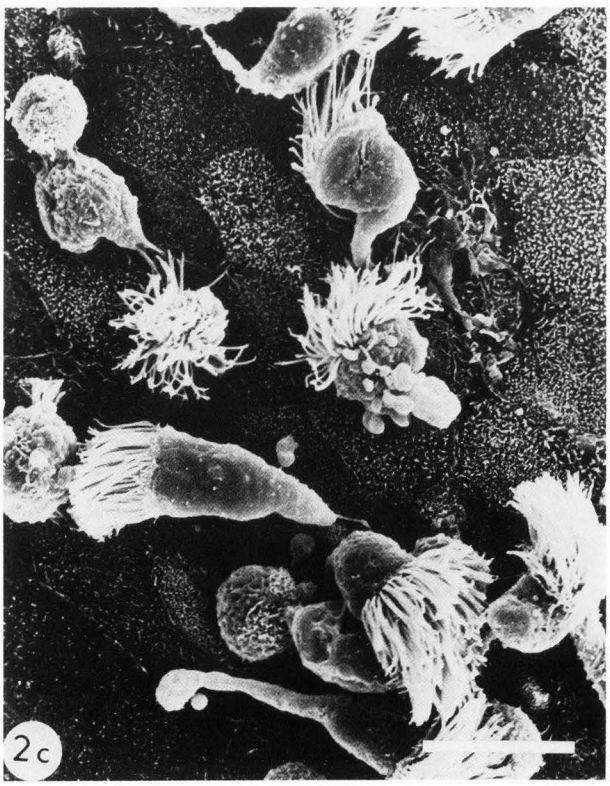
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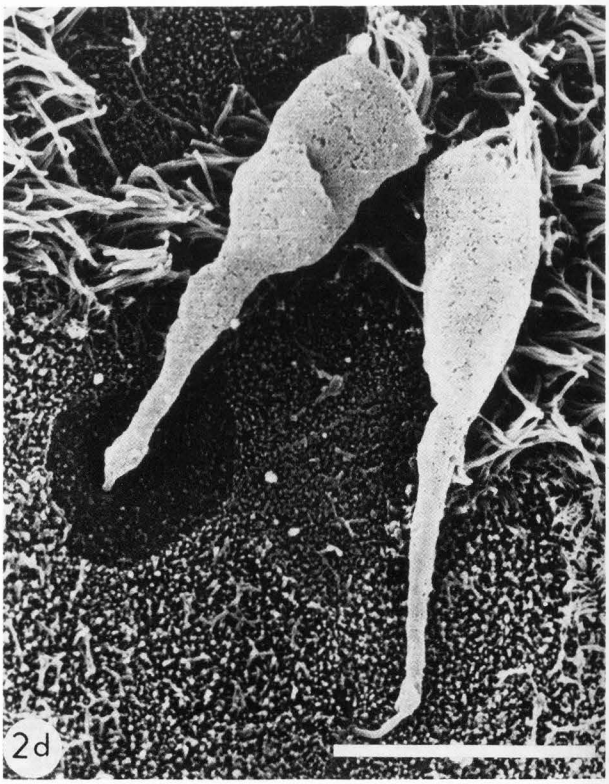
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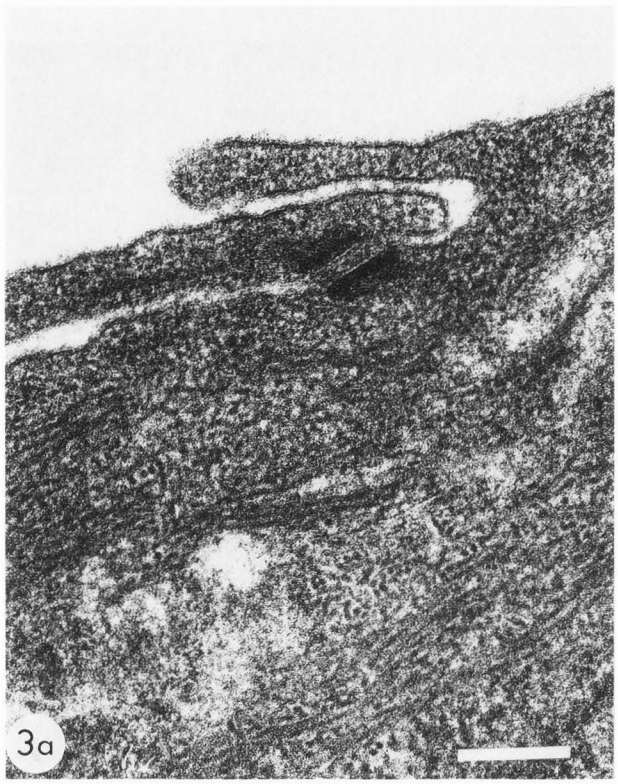


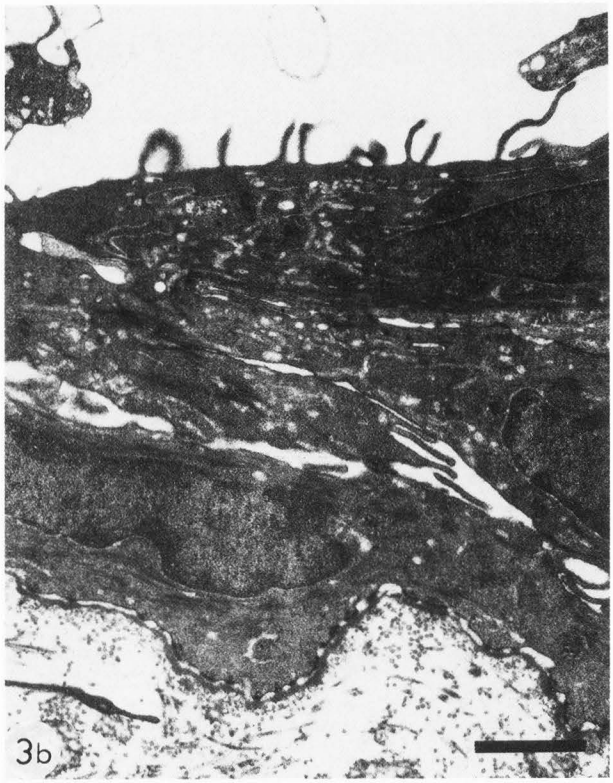


Figure 2a. Overview of the tracheal mucosa from an *in situ* tissue obtained 6 h after exposure to SO_2 500 ppm. The dark banded areas are regions in repair (see Fig. 2b). Bar = 1μm.
Figure 2b. The surface of the reparative cells from an *in situ* tissue obtained 6 h after exposure to SO_2 500 ppm. These cells have little surface relief as their microvillae have been stretched into micro-ridges leaving the majority of the apical membrane devoid of features. Bar = 10μm.
Figure 2c. At 6 h post inhalation injury by SO_2 500 ppm (*in situ* preparation), many ciliated cells that have exfoliated are still lingering attached to the epithelium by a cytoplasmic strand. In this field of view, two ciliated cells are exhibiting blebs and are in the initial sequences of exfoliation. Blebs are also present on some of the non-ciliated cells. Bar = 10μm.
Figure 2d. At 6 h post inhalation injury by SO_2 500 ppm (*in situ* preparation), many exfoliated cells remain physically attached to the epithelium at points either between cell boundaries or from within an individual cell boundary. Bar = 10μm.
Figure 3a. Cross sections of tissue obtained in situ 6 h after exposure to SO_2 500 ppm show that some cells interact at the apical membrane with desmosomes and that tight junctions have not formed. Further, the reparative layer is only 1 cell thick in many areas. Bar = 0.14μm.
Figure 3b. Cross sections of tissue obtained *in situ* 6 h after exposure to SO_2 500 ppm show several cell layers. The basal layer is comprised of basal cells anchored to the basement membrane by hemi-desmosomes, and the upper layers are secretory and non-ciliated non-secretory cells that are flattened. Bar =1.44μm.

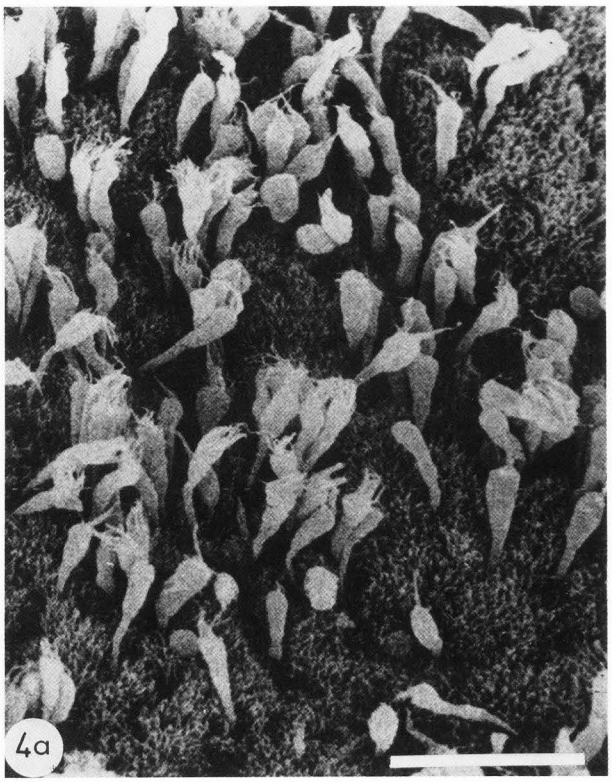
membrane was devoid of relief. At this time frame, a few cells were in the beginning of exfoliation whereas others linger attached to the mucosal epithelium (Fig.2c) by a slender process protruding from between cell boundaries as well as from within a cell boundary (Fig.2d). The disrupted areas 6 h after injury varied between 10% and 100% of the 1.5 cm^2 tissue piece, had no caudal/cranial relationship or consistent pattern of area affected within an individual. TEM examination of the areas showing repair varied in cellular composition from one (Fig.3a) to several (Fig.3b) cell layers deep. The basal layer is comprised of basal cells (with hemidesmosomes) and the upper layers are reparative cells of the response phase. Between many cells, the tight junction was absent and cellular adhesion was maintained by desmosomes (Fig.3a).

In vitro Tissue

Figure 4 shows tissues that had been incubated in an Ussing chamber for 1 h and were obtained from a dog that had inhaled 500 ppm SO_2 for 1 h. As in the specimens fixed *in situ*, there was a range of epithelial response observed, from the exfoliation of individual cells (Fig.4a) to total devastation (Fig.4b). Other lesions including rows and clumps of exfoliated cells as described above (Fig.1) were also observed.

At 6 h, most of the ciliated cells in these tissues had exfoliated and had been cleared from the tissue surface by the stirring action of the bathing fluid; however, some remained attached just like that observed in *in situ* (Fig.5). To confirm that the cells were sloughed during the incubation period, we filtered the mucosal bathing fluid through a Millipore filter immediately after mounting the tissues and again at 6 h; on the second occasion the filter was covered with exfoliated cells (Fig.6).

By SEM examination (Fig.7a), we found the lesions in these tissues were also characterized by large non-ciliated cells as shown in Fig. 1g. The number of cell layers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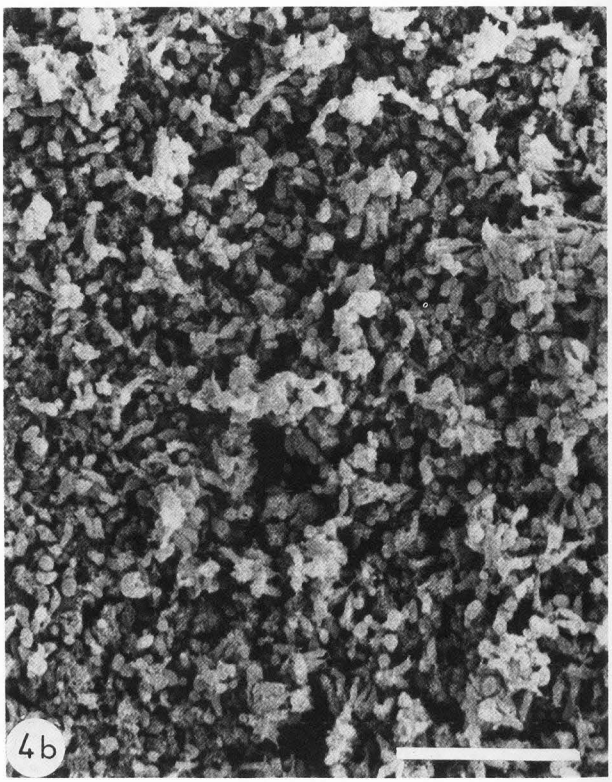
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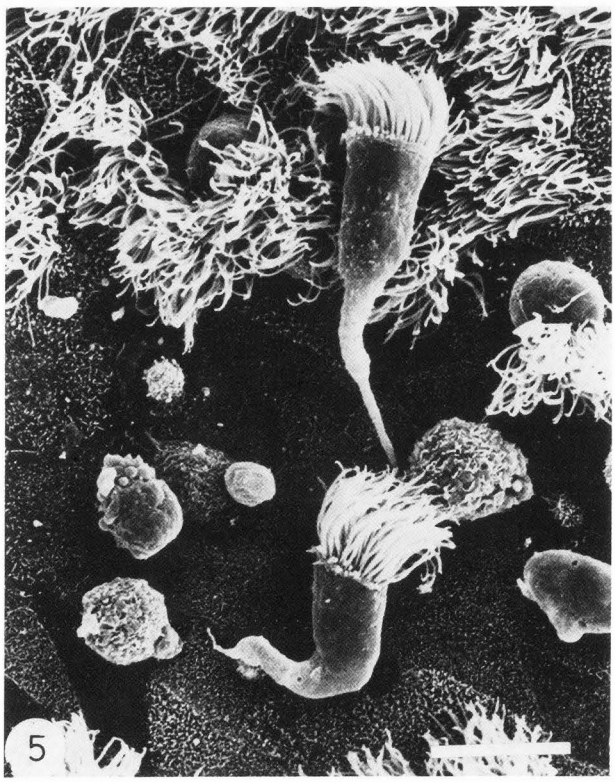
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6

involved varied from one to several (Figs.7b & 7c) and not all cellular apices were bounded by tight junctions (Fig.7b). Dense arrays of intermediate filaments were apparent in these early reparative cells. The apical surface area of the reparative cells shown in Fig.2b was 130.3 ± 5.6 μm^2 (n=412) and was 126.8± 12.2 μm^2 (n=360) for those shown in Fig.7a. These were not significantly different by analysis of variance (ANOVA).

Discussion

Though several studies have examined the acute effects of SO_2 on pulmonary tissue (1,14,15,16,21, 22,23,24), only Man et al. (17) have focused on the events within 6 h after SO_2 exposure. That physiological study did not address cellular morphology during repair. Based on observations made in that work and subsequently (19), we investigated whether *in vitro* techniques could be used to study the epithelial changes we observed *in vivo* following toxic gas injury that occurs within the first 6 h following exposure. These changes are neither repair nor regeneration and they do not depend upon mitotic activity. They occur prior to inflammatory cell infiltration, but during the exudative phase of an acute inflammatory response. They occur in response to acute injury and thus, we have termed this the 'response phase'.

There are many advantages of an *in vitro* experimental system to investigate the response phase following acute injury. They include measurements of epithelial function (trans-epithelial potential difference, short circuit current, paracellular permeability) during and following the addition of drugs (e.g., ionophores) or agents (e.g., free radical generators) capable of modifying cell function, and of course the subsequent correlation of these data with cellular and epithelial morphology. Moreover, large numbers of chambers can be obtained from each dog (up to 30) allowing serial type studies that can be prohibitive using other models either because of cost or numbers of individual animals required. Further, using the *in vitro* method, each dog serves as its own control; finally, it is logistically possible to fix tissues for examination at intervals only minutes apart and do this with accuracy. The SEM approach we have taken in this study was stimulated by the work of Dungworth et al. (6) who, in their recent review on morphological methods for evaluating pulmonary toxicity in animals stress the point that SEM should be used in the initial analysis because the airway mucosa develops focal lesions following oxidant gas exposure.

This study shows that the response phase initiated by SO_2 injury *in vivo* continues even if the tissue has been removed from the animal and incubated *in vitro*. The criteria used in this study focussed on the morphological features of the lesions and no physiological assessment was employed. From previous studies in the intact animal (10) and using *in vitro* methods (9,19), we can suggest that these criteria are sufficient to allow speculation that the physiological function of the epithelium during this time is probably similar in the *in situ* and *in vitro* conditions. That is, the mucosa is leaky and the bioelectrical properties are abnormal but that both these parameters are tending towards control levels in a time course related to the severity of the lesions induced. Figs. 3a & 7a show a lack of tight junction formation at some cellular apices 6 h post injury even though the lesions are covered with flattened cells (Figs.2b, 3a & b, 7a & b) supporting the notion that nonelectrolyte permeability and bioelectrical properties are likely not normal.

The frequent lack of tight junctions in thin section (Fig.4a) is consistent with the findings of Gordon et al., (8) who have shown that the re-adherence step of the tight junctions proceeds gradually during this time. This finding also suggests a hierarchy of junctional formation in repairing tissue with those cellular interactions providing adherence preceding those sealing the epithelium. Factors that modulate the final adherence step (tight junction formation and cytoskeleton), and affect cell adhesive molecules (e.g., L-CAM) (7), may exert significant control on the rate (and fate) of the response phase and thus the wound healing that follows. The response phase may occupy a key pivotal point influencing whether healing resolves with regeneration or repair.

Man et al., (19) showed that even at concentrations as high as 500 ppm, SO_2 injury is highly variable in severity from one area of trachea to another (Fig.1g), from one animal to another, and not in any consistent caudal/cranial-dorsal/ventral pattern. The focal nature of lesions following the inhalation of ozone has been reported in cats (3), but why these localized lesions develop is not clear. In our study, we examined the entire tracheal segment from the larynx to the carina after the inhalation of a constant concentration of 500 ppm SO_2 for 1 h. We can think of no factors or circumstances that would produce regional differences in either the distribution of the SO_2 (which is highly soluble in aqueous solution) or the ability of the airway lining layer to modify its penetration or activity that would contribute to the response we found.

In the response phase, the range of severity of injury is from individual ciliated cells exfoliating (Figs.1c), to bands of exfoliating cells (Fig.1e) to desquamation of the entire mature layer (Fig.1f). In a previous study, Wells and Lamerton (25) showed that the acute exposure to SO_2 (800 ppm for 2 h) resulted in a 50% decrease in the number of cells per field in rat trachea 6 to 12 h after the exposure. The mechanisms eliciting this difference in cellular responsivity within the same individual and among groups of cells only micrometers apart, are unknown. However, with regard to the mechanism of individual cell exfoliation, some features can be speculated upon and hypotheses can be developed that can be tested. The first step in this injury-response [once cellular membrane permeability is altered] is the down regulation of the nexus, zonula adherens, spot desmosomes and zonula occludents which results in cell detachment. The movement of the de-adhered cell out of the plane of the epithelium can occur 3 ways: pulled out by mucus, forced out by osmotically driven fluid and squeezed out by the remaining epithelial cells.

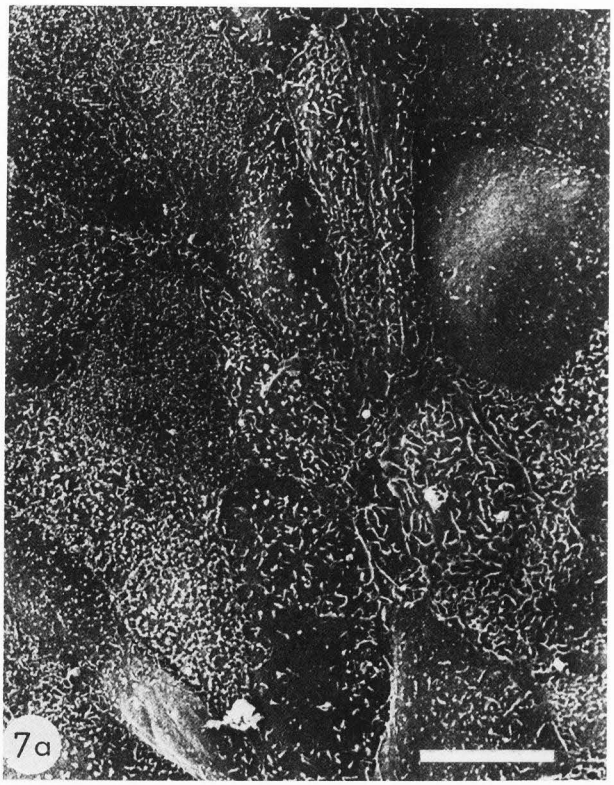
Our observation that ciliated cells were the

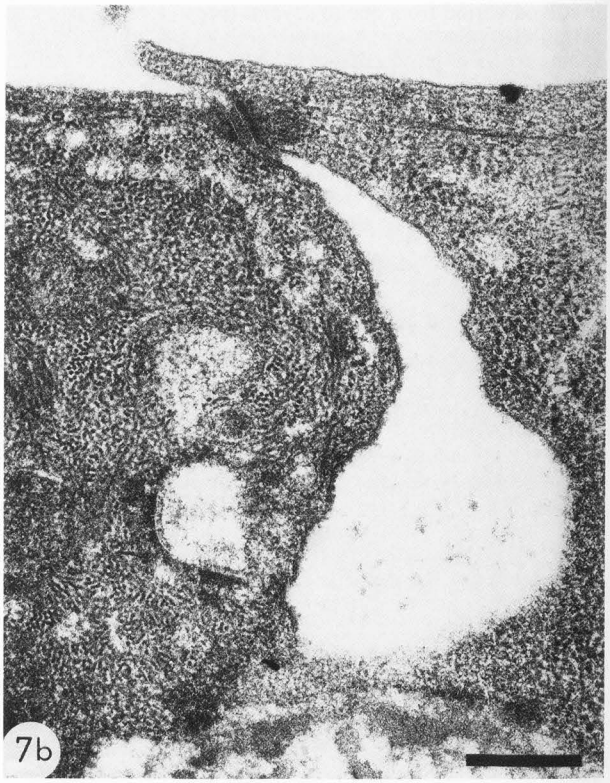
Figure 4a. Individual ciliated cells exfoliating from the tracheal mucosa in a tissue obtained *in vitro* 1 h after exposure to SO_2 500 ppm. Bar = 50μm.

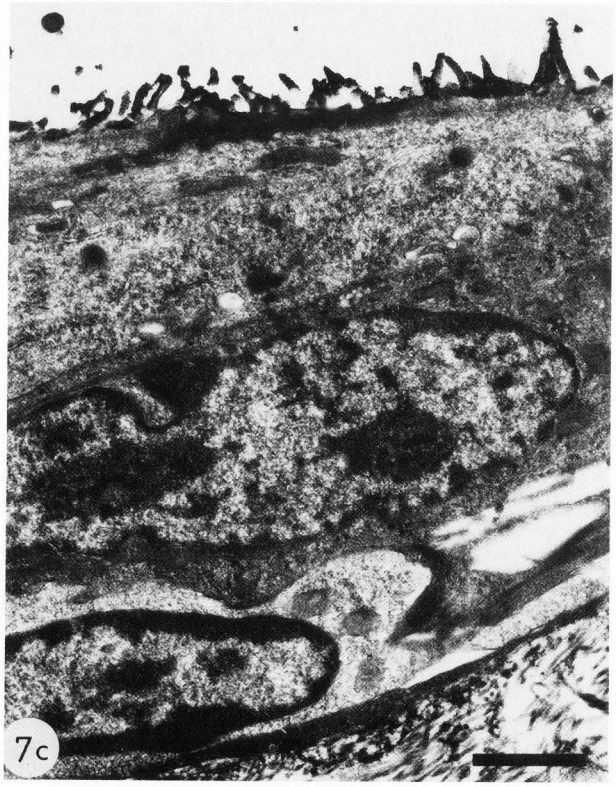
Figure 4b. The tracheal surface from a tissue obtained *in vitro* 1 h after exposure to SO_2 500 ppm. There is total devastation in this view. Bar = 100μm.

Figure 5. The time course of cellular exfoliation is not synchronized as indicated in this image of a tissue obtained *in vitro* 6 h after exposure to SO_2 500 ppm. Ciliated cells are still observed attached to the areas evidencing repair. Bar = 10μm.

Figure 6. The surface of a Millipore filter from a tissue obtained *in vitro* 6 h after exposure to SO_2 500 ppm at the end of the incubation period. The exfoliating cells and debris are trapped on the filter confirming that exfoliation is taking place *in vitro* in the Ussing chamber just like that occurring *in situ*. Bar = 50μm.







predominent cell observed exfoliating (Fig.1b) extends the previous observations on 0_3 (2,3,5) and $N0_2$ (4) injury to now include $S0_2$ inhalation injury where the lesion is characterized by eliciting a cellular response that can only arise if ciliated cells are more susceptible to injury than non-ciliated cells. It may be that this feature of the mucosal cells acts in defense by allowing injured, and easily infected cells (20) to be cleared 'en masse' from the mucosa. Also, as we have suggested (18), the transient loss of ciliated cells does not cause a major compromise in airway defense as the backup clearance mechanism, cough, is usually not affected. Moreover, as ciliated cells do not replicate, their loss does not impair regeneration of the epithelium. The gradual clearing of exfoliated cells may aid in the recruitment of the cellular components of an acute inflammatory response via the release of necrophilic factors that are highly chemotactic for neutrophils (26).

The dynamic nature of the response phase, initiated concurrent with de-adherence, is illustrated in micrographs like Fig.3d that capture the final attachment site of an exfoliating cell within the apical cell boundary of a flattened epithelial cell. This is a special feature of the response phase: the lateral spreading and shortening of the non-ciliated superficial cells and ciliated cells that remain intact. In cross-sections of control tissues, the mean apical diameter of the superficial non-ciliated cells was 4.0 $\pm$0.30 μm^2 (17), equivalent to an estimated mean apical surface area of about 12.6$\pm$3.0μm^2. The surface area of the flattened cells in this study was approximately 130μm^2 regardless of whether the measurements were obtained from *in situ* or *in vitro* tissues. Thus, in the first 6 h after acute inhalation injury, these cells increased their surface area roughly 10-fold whether they remained *in situ* or were incubated *in vitro*. Three events associated with this activity are: it may forcefully remove

Figure 7a. The surface of the reparative cells in tissues examined after 6 h *in vitro* incubation following SO_2 injury 500 ppm, are like that seen above (Fig. 3b). The apical membrane is characterized by a noticeable lack of topography and the villae that exist have been stretched, with the expansion of the cell, into micro-ridges. Bar = 10μm.
Figure 7b. Cross sections of the tissue obtained *in vitro* show the mucosal epithelium varying from 1 to several cell layers thick. Bar = 0.27μm.
Figure 7c. Cross sections of the tissue obtained *in vitro* showing that in focal spots, the epithelium may be only one cell layer thick; the cells being derived from the basal and superficial non-ciliated cells. Many of the cellular apices are not bounded by tight junctions at this time. Bar = 0.942μm.

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de-adhered cells from the epithelium, it may position adjacent membranes so that cell adhesive molecules may interact to form tight junctions and desmosomes, and (as a consequence), it ensures that the airway surface remains covered by cells, even when a significant surface area is lost in the form of exfoliating ciliated cells.

Whether the airway mucosa is injured by exposure to cigarette smoke (10) or SO_2 (26), or mechanically (11,12,13), mitotic activity becomes apparent 12 h post injury and usually does not peak until 24 to 48 h later. As indicated, the sequence of cellular events in the time preceding this proliferative phase is likely crucial to the resolution of the injury and perhaps expression of the cellular phenotypes generated by the mitotic activity. A major feature of the response phase is restoration of the mucosal barrier properties which is the essential first step to seal leaks in the epithelium that would otherwise allow the entry of noxious molecular species; indeed, increased paracellular permeability has been suggested to result in chronic broncholitis in rats exposed to SO_2 (24).

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Discussion with Reviewers

R.E.Gordon How do previously reported data on the early reparative event following mechanical injury compare and contrast to those observed following SO_2 exposure. Are the changes seen following SO_2 injury a general phenomenon or unique to this kind of injury?

Authors: There are several similarities and some distinct differences in the reparative activity seen following acute tracheal epithelial injury by the two methods. The lateral spreading activity by the reparative cells and the development of cell-to-cell and cell-to-substrate interactions are events that are programed to proceed once initiated regardless of the initial stimulus; however, the time course for full junctional formation in the SO_2 exposed tissues seems longer than for the tissues injured mechanically. Using several techniques [Gordon RE, Lane BP (1976) Regeneration of rat tracheal epithelium after mechanical injury. Am Rev Respir Dis,. 113, 799-807], it has been shown that the formation of tight junctions and the barrier function of the epithelium was complete by 6 h post mild mechanical abbrasion to rat trachea, by contrast, we find that desmosomes are present at the cellular apecies 6 h post SO_2 inhalation injury, but that tight junctions frequently are not.

It is possible that this delay reflects the different roles the lateral spreading activity by the reparative cells in the two models. In the mechanical injury model, the surface epithelial cells are denuded except for some secretory and non-secretory non-ciliated cells that somehow survive. The lateral spreading activity in this model has one principal function, to form membrane-membrane interactions between adjacent cells and the substratum and in doing so, cover the exposed basement membrane and create the epithelial barrier. By contrast, in addition to this function, the lateral spreading activity of the reparative cells in the toxic gas model must also squeeze (or force) the deadhered ciliated cells out of the epithelium - [if the cells are not moved out by osmotically driven fluid flows or 'pulled' out by mucous]. The time spent in this activity may account for the delay in tight junction formation as the membranes will not come into position until after the deadhered cells have exfoliated.

As indicated, the focus of activity by the reparative cells following toxic gas injuries is on squeezing deadhered cells out of the epithelium, forming new junctional contacts between cells (apices and basolateral membranes) and the substratum and restoring the physiological barrier. The unique component of toxic gas injury that is not a primary consideration in the mechanical injury model is the exploitation of differential susceptibility to injury by specific cell types; hense the additional role of lateral spreading in this model.

E.A.McDowdell The events described in this study constitute neither repair nor regeneration, rather they are pre-repair and pre-regeneration. Is it possible to use terminology that does not include the term 'repair' to describe these early reparative events?

Authors: The umbrella term 'pre-mitotic repair phase' was suggested (18) to encompass the dynamic sequence of cell de-adherence and exfoliation, restoring cell adhesion, communication and barrier function in the first few hours following acute injury - events occurring prior to mitotic activity that follows an acute injury. However, in pathological terminology, repair means the replacement of normal tissue with scar tissue. As it is not known whether the end point of the epithelial changes being examined at this time will be 'repair'(or 'regeneration'), this previously suggested terminology can be somewhat misleading.

In this manuscript, as a result of these reviewer comments, we suggest the term 'response' phase as appropriate in terminology and meaning. Neither pre-repair nor pre-regerative phase is appropriate as the end point is not known. The response phase refers specifically to that period of time, immediately following an acute injury, when the tissue is responding with a sequence of predictable coordinated events - what is not predictable is what the final resolution is.

The reparative events in the first 6 h following acute injury are usually ignored because most pathological studies generally look at sub-acute and chronic effects and the sequelae that follow through to resolution in one form or another. It is entirely possible to completely miss the response phase if the tissue is examined later than 6 h after the acute injury. By then, the massive exfoliation of individual cells is over and there may be no remaining clues to indicate this event has even happened. A decrease in the percentage of ciliated to superficial non-ciliated cells may be the only indicator, and this can easily be explained away as animal and tissue variability.

E.A.McDowell. The tracheal epithelium is pseudostratified. As all cells rest on the basement membrane, perhaps there is always only one cell layer, but more than one nuclear layer, depending on cell shape and configuration.

Authors: In the canine and guinea pig tracheal epithelium, not all cells rest on the basement membrane. We believe likewise that there probably is always one nuclear profile; additional profiles in areas evidencing repair are likely mucous cells as suggested by the reviewer, in-addition, there are probably some superficial non-ciliated non-secretory cells and intermediate cells also. Our TEM micrographs were selected on the nature of the cellular interactions more so than the number of profiles. We found most of the nuclear profiles varied between 1 and 3, with 4 or 5 layers rarely observed. A separate manuscript in preparation focuses on this aspect of acute airway injury. It documents the time course of cellular responses during and following individual cell exfoliation and banding exfoliation, and it will show the accompanying changes in epithelial height and cell numbers that occur following a massive loss of mucosal cells by toxic gas injury.

D.E.Schraufnagel. What are the reparative cells, and were some confirmed as basal cells on light microscopy?

Authors: Some of the reparative cells were identified as basal cells on LM and TEM examination, however others identified were mucous and non-ciliated non-secretory cells, the other two primary cell types in the canine tracheal epithelium inaddition to the ciliated cells.