

Review article

Epithelium, microcirculation, and eosinophils – new aspects of the allergic airway *in vivo*

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Current research paradigms may give the impression that gross airway mucosal processes *in vivo* in allergy, inflammation, and repair are already well known. The widely acknowledged reductionist *in vitro* and cellular approaches thus appear to be very justified. Reflecting this development, reviews on every aspect of airway “inflammation” are now crowded with molecules and *in vitro* references, whereas critical discussions of events occurring in the complex living mucosa are dwindling. As a further corollary, major concepts of the mechanisms of asthma and allergic rhinitis, and their future therapy, are extrapolations from results obtained in clearly defined *in vitro* and cellular test systems.

This review develops a different theme. It is written to arouse some distrust in so-called established notions of *in vivo* functions of the airway mucosa. We also hope to encourage work that primarily explores events occurring in the complex biosystems, especially under proper disease-like conditions, not least because such research may

produce those essential, “unexpected” discoveries upon which significant medical progress depends (1, 2). Success in this work will also provide the reductionist approaches with increasingly accountable *in vivo* paradigms, without which modern airway research can merely explore phantom phenomena and predict phantom therapies (2).

This review deals with one inflammatory cell, the eosinophil, and two airway end organs, the epithelium and the microvasculature. Three events in particular are in focus: epithelial shedding-restitution processes, microvascular-mucosal exudation of bulk plasma, and generation of free extracellular eosinophilic granules in airway tissue. The new aspects that will be underscored are as follows:

- demonstrations and hypotheses of mucosal tightness in allergic disease
- roles for basal cells, ciliated cells, and other factors in the epithelial repair process *in vivo*
- columnar cell sacrifice as a well-functioning airway defense

- epithelial damage repair as a cause of asthma-like airway pathophysiology and disorder
- the multipotent bulk plasma exudate as a first-line defense on the surface of an intact airway epithelium
- plasma-derived proteins-peptides as determinants of the *in vivo* biology of the lamina propria, the basement membrane, the epithelium, and the mucosal surface
- nonapoptotic death as the ultimate activation mechanism of eosinophils in asthmatic and rhinitic airways.

We will make brief comments in the text about the balance between disease, defense, and repair, the three parts of any inflammatory mechanism. The corresponding requirements on the selectivity in action of anti-inflammatory therapy, such as the inhaled steroids, will also be mentioned.

Epithelium

Cell-culture phenotypes of epithelium may produce a wide variety of molecules: mediators, cytokines, adhesion molecules, and so forth. Hence, the cellular lining of the airways is now conceived of as being made up of effector cells that potentially orchestrate mucosal allergy, inflammation, and repair (2). In addition, based on the general assumption that asthmatic airways are denuded, an entire research field has developed dealing with studies of smooth-muscle responsiveness in isolated airways where the epithelium has been stripped off (3). Lack of *in vivo* confirmation, or lack of prior determination of *in vivo* relevance, appears to matter little to adopters of these *in vitro* research paradigms. The epithelial research field may thus provide prime examples of modern medical research in which reductionist approaches are favored at the expense of the exploratory research that observes and assesses basic *in vivo* phenomena.

One undisputed role of the epithelium is its function as a barrier. As will be discussed below, maintaining a barrier structure *in vivo* involves complicated mechanisms and dynamic processes both at baseline conditions and, particularly, when disruption has been inflicted. Although exceedingly functional in defense and repair, these processes, when exaggerated and chronically repeated, may be seen as a cause of airway diseases such as asthma.

Tightness of the airway mucosa in allergic disease

The composition of inhaled air, together with epithelial perviousness, determines the true airway load of environmental noxae. This is an important

consideration because epithelial permeability may vary greatly already in the normal airway mucosa (unpublished observations). The tightness around the apical pole of columnar cells, or other epithelial junctions, would thus determine how many of the (hydrophilic) molecules, deposited on the airway surface, will penetrate and come into contact with antigen-presenting dendritic cells, sensory nerve fibers, and the great variety of stationary and trafficking inflammatory cells of the epithelial and subepithelial layers. Just beneath the epithelial lining, there is also a profuse network of microvessels. Among the many roles of this superficial microcirculation is regulation of the dwelling time of solutes in the lamina propria. Hence, absorption of mucosally applied molecules into the bloodstream may well indicate the functional tightness of the airway epithelial lining cells.

The common concept of airway inflammation includes, if not grossly denuded airways, a severely disrupted epithelium (4). Although histologic evidence may support this notion, it cannot be ignored that epithelial derangement is readily inflicted both when biopsies are taken and at later stages of handling and at sectioning (5). Illustrations of completely denuded airways may, therefore, represent artifact phenomena. This possibility is supported by the work of eminent pathologists who, a century ago, observed a maintained airway epithelial lining in severe, lethal asthma (reviewed by Persson [6]). In addition, during the last 60 years, it has been repeatedly reported that molecules deposited on the mucosal surface in inflammatory airway diseases may not be absorbed at an abnormally increased rate (7). Indeed, by improved physiologic *in vivo* techniques, we can now demonstrate that allergic eosinophilic airway disease may be associated with decreased absorption rates (8, 9), reflecting an improved mucosal barrier function. These data not only call into question the established notions of increased perviousness, but they also suggest that functional tightness of the mucosa may be important. Perhaps the opposite, an increasing perviousness from continued exposure to allergens, might aggravate the allergic airway disease at an exponential rate. Furthermore, these data provide a novel paradigm that should prompt exploratory research into what may actually take place in and on the mucosa *in vivo*. From analyses of sputum and other airway surface samples, it is well known that there is epithelial shedding in the inflammatory airway diseases (3, 10). Rather than viewing this as a static picture linked with airway denudation, we should probably ask ourselves about the epithelial restitution processes that ought to be set in motion as soon as epithelial cells are shed.

Restitution of barrier structure at loss of columnar epithelial cells

The epithelial lining cells of the nasal and tracheo-bronchial airway may be grossly subdivided into basal cells and columnar cells. The basal cells are anchored to the basement membrane by hemidesmosomal welding spots (11). The basal cells and their neighboring columnar cells are linked by desmosomal attachments (11). Since the columnar epithelium lacks a structure that binds directly to the basement membrane, it is likely that complete denudation will result at those sites where basal cells are being shed. It is also quite possible that columnar cells can be shed selectively, leaving a layer of basal cells. Indeed, this selective loss of columnar cells, single cells, or clusters of cells may be the most common form of shedding. This view is consistent with findings of columnar cells in the lumen material of asthmatic airways (12). However, the studies carried out so far are not entirely conclusive because the number of free basal cells in the asthmatic bronchi (sputum or lavage) has not been determined.

We recently set out to study what effects the loss of columnar cells might have on the remaining basal cells (13). First, several aspects relating to specificity needed attention: how could the columnar cells be removed with sufficient efficiency, practically complete selectivity, and without undue trauma to the basal cells? Also, how could we ensure that the remaining cells were basal cells? Previous workers had attempted *in vivo* investigations in this field; however, because of variable trauma, variable loss of different epithelial cells, variable rates of loss of columnar cells, and poor identification of basal cells (14–16), the results were not conclusive. We chose to examine freshly excised intact airway walls under as *in vivo*-like conditions as possible. By developing a technique for gluing the mucosal surface, we could gently and selectively remove the columnar cells. The immediate result was an airway covered by a sheet of cobbled, apparently little disturbed, basal cells. This selective columnar cell removal was equally successful in guinea-pig tracheal, human nasal, and human bronchial specimens (17). Moreover, the layer of cobbled, nontight basal cell structure looked the same in the three types of tissue. By the next examination time, merely 20 min after the loss of the columnar cells, the basal cells had undergone dramatic changes. They had flattened very markedly. Furthermore, they had established extensive contact with each other by overlapping and by creating close embracings (13). This basal cell response alone, which has been corroborated by preliminary *in vivo* studies (unpublished observations),

may explain why epithelial shedding diseases do not exhibit an increased absorption perviousness. Moreover, the flattened cells would provide less paracellular stretches per unit surface area than the normal epithelium, thus potentially contributing to an even increased tightness.

Columnar cell sacrifice as a defense mechanism and a new role for the airway basal cells in health and disease

The extreme readiness of basal cells to create a barrier structure means that clusters of columnar epithelial cells may be shed without undue sequelae. We suggest, therefore, that this epithelial sacrifice may have to be regarded as a well-functioning airway defense mechanism (13). Such a defense would be of particular value and utility when severely damaging, toxic, or infectious agents enter the airways. It is conceivable that the severely affected columnar cells have well-developed mechanisms for separating themselves from the basal cells at a proper point in time, effectively warding off the insult and preventing its propagation further into the airway tissue. One may also view this separation as an ability of the basal cell to release columnar cells. These would be new roles for basal cells: a release of columnar cells in airway defense and in inflammatory airway disease, and an immediate taking over of the barrier structure that previously was upheld by the tight apical connections between columnar cells (13) (Fig. 1). In fact, the general ultrastructural properties of the airway epithelium make it well suited for rapid shedding-restitution events.

Epithelial restitution at denudation

Erjefält et al. (18) have recently developed a novel technique by which a well-defined denudation zone can be created *in vivo* with minimal additional trauma. They thus devised a special probe that was inserted orally into the tracheal lumen of anesthetized guinea pigs. By gentle strokes along the dorsal, noncartilaginous trachea, a denuded zone, 800 μm in width and 30 mm in length, was created. Complete shedding-like denudation was effected; no damage to the basement membrane was inflicted and no bleeding occurred. Furthermore, the method produced sharp edges between the intact epithelium and the denuded zone (18). This feature was particularly important because it provided excellent opportunities to examine the very first reactions of those epithelial cells that bordered on the denuded basement membrane.

Already by 15 min, poorly differentiated and flat cells had migrated to cover a substantial portion of

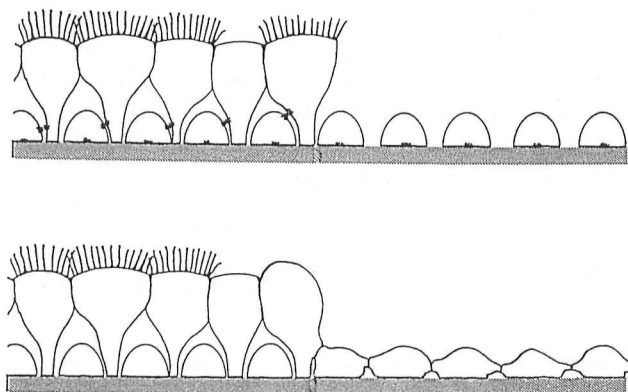


Fig. 1. Appearance, immediately after loss of their columnar neighbor cells (to which they had been attached by desmosomes), of basal cells agrees with observation in intact epithelium (top panel). Within 20 min after selective removal of columnar epithelium, basal cells underwent dramatic shape changes involving flattening and establishment of extensive cell-to-cell contact (lower panel).

the denuded membrane (18). The immediate response at the border could be well observed with respect to participation by secretory cells and ciliated cells (equal participation by basal cells is by no means excluded). By releasing their secretory granules and by "internalizing" their cilia, these columnar cell types dedifferentiated into flat migrating cells. There was no lag time. The repair thus started immediately by generation of the poorly differentiated cells that also migrated both promptly and at breathtakingly high speed ($\sim 2\text{--}3\text{ }\mu\text{m}/\text{min}$). Cell-culture experiments in this field have provided a very different picture of "repair events", and so has the previous *in vivo* work that employed more traumatic and less controlled experimental conditions than Erjefält et al. (18), particularly concerning the initial repair process (19–21).

The novel observations on the promptness and the rate of migration, and on the participation of both secretory and ciliated cells as a source of restitution cells would mean that patchy areas of denudation *in vivo* would not last long before being covered by a new, poorly differentiated epithelium. This suggestion is supported by experiments demonstrating that allergen challenge-induced patchy and crater-like epithelial damage may expand apically when the crater floor already (probably almost from the start of damage) has received a new cell cover (5). This floor of cells may in part be flattened basal cells (as suggested by staining with alkaline phosphatase) and in part dedifferentiated columnar cells. Together, these repair cells would effectively close a small denuded area.

Once there is complete cover of the basement membrane with repair (restitution) cells, proliferation starts in the new epithelium, the old

epithelium, and the subepithelial cells (see below). Reinnervation with tachykininergic sensory nerves proceeds at this stage. After the creation of a large denuded zone ($0.800 \times 30\text{ mm}$), a fully differentiated, normal epithelium can be seen *in vivo* after 3–5 days (18).

A new role for ciliated cells

The recent observations on shedding-restitution events indicate that the ciliated cell is not an end-stage cell. In contrast to previous contentions, ciliated cells, like secretory cells, can dedifferentiate into flattened, migrating repair cells (18). Considering the abundance of ciliated cells in the normal mucosa, it appears both functional and economical that these cells can fully contribute to swift repair of denuded airway sites (Fig. 2).

Epithelial shedding-restitution may cause asthma-like airway disorder and pathophysiology

When shedding-restitution processes are examined *in vivo*, one should probably expect a complex scenario with involvement of various airway end organs, leukocytes, other cells, and a dynamic soup of biologically active molecules. In this context, we have studied the activity of the secretory epithelium, the distribution and activation of eosinophils and neutrophils; and the proliferation of epithelium, fibroblasts, smooth muscle, and regional lymph nodes. The microcirculatory responses involving the creation of a biologically active mucosal tissue milieu of plasma-derived molecules (22) will be discussed below (under "Subepithelial microcirculation").

Even a single event of shedding-like denudation will cause a series of airway effects which, intriguingly, may be linked to the characteristics of inflammatory airway disease conditions. Among

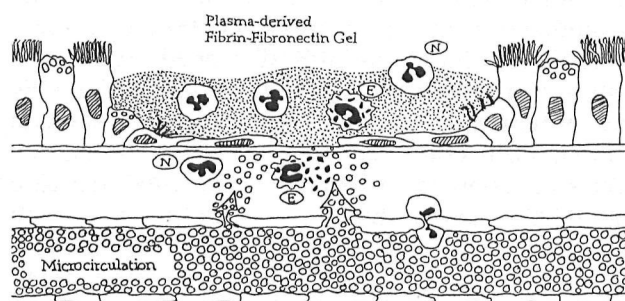


Fig. 2. Diagram showing that, after denudation, epithelial restitution occurs promptly and speedily under provisional cover of plasma-derived and leukocyte-rich gel (N=neutrophils; E=eosinophils). Equal participation of ciliated and secretory cells in dedifferentiation into migrating repair cells is indicated.

the acute effects of denudation is a secretory effect. Thus, secretory cells within 10–20 mm of the denuded zone promptly lose their PAS-positivity, which gradually is regained during the following several hours (18). Eosinophils, which normally reside in quite large numbers at the base of the guinea-pig tracheal epithelium, promptly move toward the lumen, and many eosinophils appear in the plasma-derived gel that covers the damage-repair zone (23). More dramatically, the eosinophils become ultimately “activated”, releasing free eosinophil granules in the lamina propria and the epithelium (23) (see also later under “Eosinophils”). Within the first 15 min after denudation, recruitment of neutrophils from the microcirculation beneath the repair site is in full progress. As shown by transmission electron microscopy (TEM) analysis, the neutrophils in the area of interest are active.

The plasma-derived adhesive gel and the activated leukocytes are dynamically maintained on the surface until the previously denuded basement membrane has become tightly covered by poorly differentiated epithelial repair cells (23). This is also the point in time when proliferation becomes markedly increased. There is obviously a need for more epithelial cells both in the old epithelium, which has been thinned out by acting as a source for repair cells, and in the new epithelium. However, the shedding-restitution process also propagates signals into the airway tissue where rather sustained increases in the proliferation of fibroblasts and smooth-muscle cells occur (18). A prominent additional feature of repeated shedding-restitution processes is enlargement of the epithelial basement membrane and of the regional lymph nodes (24). Hence, the shedding of airway epithelium by itself evokes a cascade of effects which, if found in human airways, we recognize as prominent signs of allergic and inflammatory diseases (25) (Fig. 3).

Erjefält et al. (5) have demonstrated that despite an even exposure of the airway surface to a defined concentration of allergen, extremely patchy epithelial damage sites are induced. Interestingly, these patches and their immediate surroundings are also the sites of accumulation of activated leukocytes (eosinophils and neutrophils) and plasma-derived adhesive proteins. Consistent with the notion that repair occurs promptly and at exceedingly high speed, there is increased proliferative activity in the epithelium and in subepithelial cells soon after the allergen challenge. These observations agree with the possibility that the pathophysiology, the leukocyte disorder, and the remodeling occurring in allergic airway disease are, to an unknown – but potentially great – extent, caused by epithelial shedding-restitution processes (25).

Defense-repair disease and treatment aspects

Single epithelial cells may escape without notice, the cell space becoming closed during the escape. Clusters of columnar cells may also be lost (actively released by basal cells?) in major defense operations and in disease without noticeable effects on the airway epithelial barrier (13). Even occasional denudation of small mucosal areas may occur without more than a brief period of increased perviousness in its wake. (In fact, this kind of transient disruption may be the *modus operandi* of detergents and other agents employed as airway absorption enhancers in certain inhaled drug formulations [7].) However, when epithelial damage repair becomes exaggerated and chronically repeated, this epithelial event itself may mount to a multipotential pathogenetic factor of significance in asthma and in other allergic airway diseases (25).

Two aspects of airway therapeutics emerge as being particularly important. One concerns the requirement that drugs not impede the prompt and speedy processes of epithelial restitution that follow from various shedding events. The other concerns the potential importance of epithelial “cytoprotection” in airway diseases. Although *in vitro* cell researchers might have predicted otherwise, guinea-pig *in vivo* studies now suggest that topical airway steroids do not disturb the swift restitution of epithelium over a denuded basement membrane (26).

Incidentally, this observation agrees with clinical experience, including histologic evaluations, which show a normalizing of the epithelial lining cells at chronic exposure to inhaled steroids (27, 28). This latter clinical observation may, in addition, reflect drug-induced protection of the airway epithelium from damage and shedding. In view of the observed sequelae to shedding alone, this second aspect may be developed into a major antidisease property of drugs (25). We thus propose that airway epithelial cytoprotection deserves prime consideration as a goal for future airway treatments (29). Among the current antiasthma and antirhinitis drugs, both steroids and chromones (30) may be promising agents to produce epithelial protection. It is further proposed that all classes of airway drugs, particularly those that are (to be) administered directly on the mucosa, urgently need to be studied in relation to epithelial damage-restitution *in vivo*. The bad news may emerge that some old, novel, or future drugs impede the intricate epithelial restitution process. The good news may include the discovery of chemically induced airway epithelial protection as a lead and basis for development of new classes of efficacious antiasthma and antirhinitis drugs.

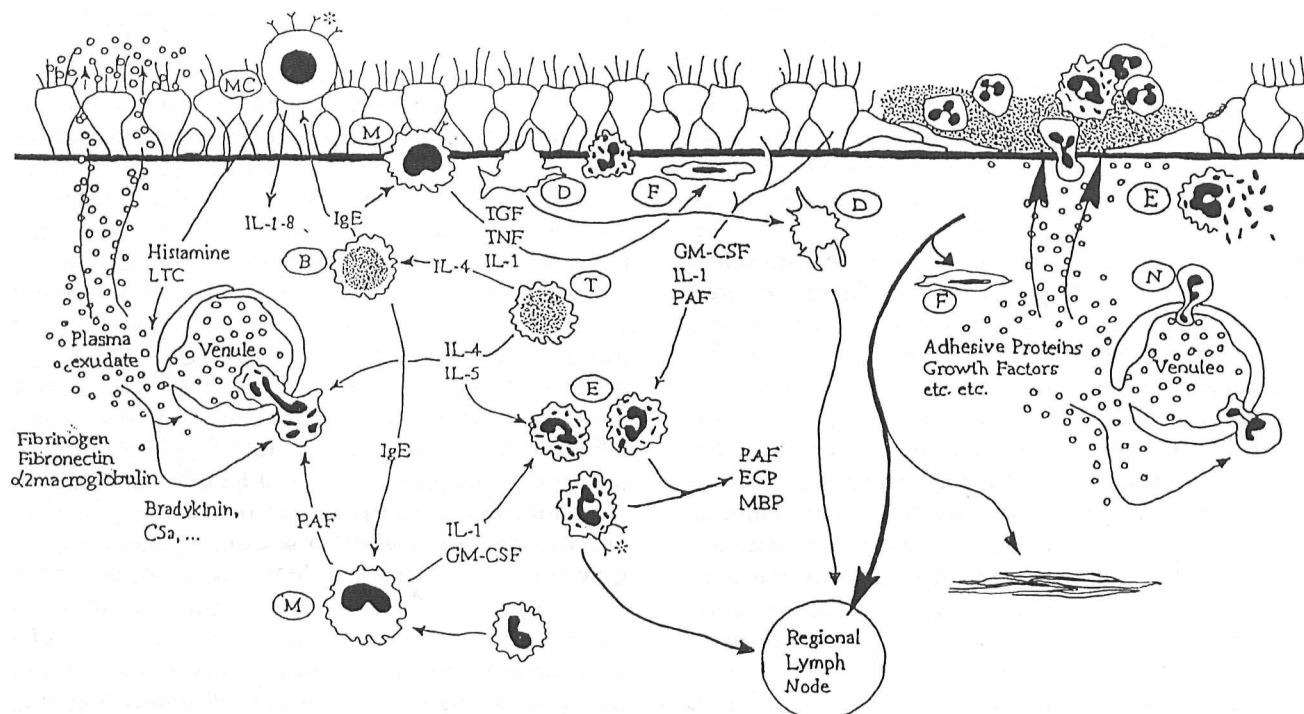


Fig. 3. Schematic drawing of airway mucosa where exudative inflammation goes on either with epithelium intact (left) or with shedding-restitution as prominent feature (right), latter event potentially causing pathophysiology, leukocyte disorder, and remodeling of inflamed airways. In both kinds of inflammatory conditions, plasma-derived adhesive proteins and other plasma-derived effector solutes contribute significantly to molecular milieu of lamina propria, epithelium, and mucosal surface.

Subepithelial microcirculation

The microcirculation is also now the subject of reductionist approaches, especially in dealing with endothelial cell cultures (31). As with epithelial cells, the *in vivo* relevance of endothelial cell-culture data – for example, in permeability mechanisms – may not always be well established. Hence, this paper deals exclusively with *in vivo* observations. Our selective approach may further be required by the present emphasis on airway distribution and potential roles of plasma-derived molecules.

Just beneath the epithelial lining of the human airways (the nose and the tracheobronchial tree), there is a profuse microvascular bed (32). This microcirculation readily reacts to inhaled factors. Some of the major responses take place in the wall of postcapillary venules. This is the site where extravasation of bulk plasma is regulated. This is also the vascular wall across which leukocytes move into the tissue. Both plasma and leukocytes move between endothelial cells but by different mechanisms. Thus, leukocytes may pass while maintaining a protein-tight seal. Extravasation of bulk plasma appears to occur through small round holes presumably created by actions of perme-

ability agents directly on the venular endothelial cells (22, 33) (Fig. 4). The profuseness of the superficial microcirculation makes processes such as plasma extravasation insensitive to vasoconstrictor agents (34) (in contrast to the case in the poorly perfused skin). The ability of the mucosal microcirculation to mount a plasma-exudation response should not be completely suppressed because of its importance in airway defense. On the other hand, it may be crucial to reduce exudation in disease in order to create a less reactive biologic milieu in the airway mucosal tissues.

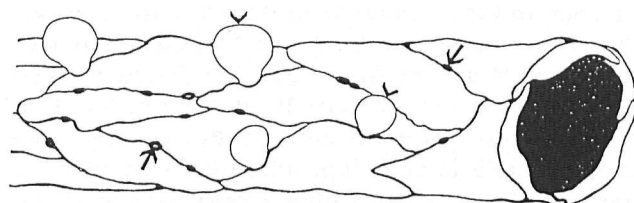


Fig. 4. Schematic drawing of silver-stained postcapillary venule actively involved in extravasation of plasma and leukocytes. Morphologic correlates to increased vascular permeability are distinct sites of endothelial cell separation (represented by small dark dots [arrows]). At separate interendothelial sites, leukocytes are adhering and moving across venular wall (arrowheads).

Extravasation, lamina propria flooding, and luminal entry of bulk plasma exudate as a finely tuned process

It has long since been experimentally established *in vivo* that bulk plasma may be moved out of venules by finely regulated and fully reversible endothelial mechanisms. In contrast, its further passage across the "tight" epithelial lining has been regarded only as a result of epithelial damage, although the damage in part has been thought to be caused by the exudation process. However, plasma proteins on the airway surface cannot be viewed as merely a sign of mucosal damage. We have thus demonstrated that also the intact airway epithelial lining will let through plasma (35), by an almost unsieved process (36). The acute luminal entry of bulk plasma disturbs neither the structural integrity of the epithelium nor the epithelial function as a tight absorption barrier (37, 38). These aspects, which now have been confirmed in human airways (39), together with other potent features, identify plasma exudation as a major first-line defense mechanism of the airway mucosa (40).

The bulk plasma that is extravasated from superficial venules is first widely distributed in the entire lamina propria (41). Within 15–30 s, the bulk exudate moves across the epithelial basement membrane and up between and all around epithelial cells in the challenge area. The exudate does not stop here but gently seeps through the epithelial lining by ubiquitous paracellular routes. (Paracellular stretches of about 40 nm would be available per cm² of mucosal surface.) No fountain is observed, and an even distribution of the plasma exudate is found on the mucosal surface in the area of interest (42). The unsieved nature of microvascular-epithelial exudation of plasma in the airways is clearly demonstrated by a maintained concentration ratio between macromolecules of very different sizes and charges in the circulation and on the mucosal surface that has been challenged by allergen or bradykinin (36). After histamine challenges of the human nose, the 60-kDa albumin and the 700-kDa α_2 -macroglobulin may thus move equally swiftly across all barriers and through all the intricate passages that exist between the venular compartment and the mucosal surface (unpublished observations). It also seems clear that the acute response to allergen challenge in human bronchi is best measured as luminal entry of the large proteins such as fibrinogen (43) and α_2 -macroglobulin (44). Indeed, the levels of albumin may not rise significantly in the postchallenge bronchoalveolar lavage (BAL) liquids because the bronchoalveolar surface already at baseline accumulates high and variable amounts of this small protein (43, 44).

From our observations on exudative epithelial mechanisms (see below) and actual observations in human allergic bronchi, we have proposed that the large plasma proteins are superior indices of plasma exudation in the lower airways (43–45). It is gratifying to see that fibrinogen is increasingly determined in sputum and BAL samples and that its postulated increase is confirmed in asthma at baseline (46, 47) and after mediator challenge (48). However, in these reports (46–48), the authors fail to comment upon the epithelial passage of this large and complicated molecule.

Luminal entry appears to be the main escape route for the extravasated plasma. It must be a swift route because it is rapidly engaged and because it extends fully down to threshold exudative stimuli (36, 49). Furthermore, the luminal entry occurs without prior buildup of edema in the mucosa. (The lack of edema at airways plasma extravasation responses led us to the discovery that extravasated plasma swiftly traversed the normal epithelial lining [35].) Finally, the acute extravasation of plasma in challenged airways may not lead to increased lymph plasma macromolecule transport (50). These observations prompt questions about the mechanism of epithelial transmission of bulk plasma exudates.

We hypothesized that the epithelial passage might be executed by the extravasated plasma itself (41). This emerged as one of the few possibilities compatible with the cumulated *in vivo* observations. The hypothesis was testable. It required a system where the epithelial barrier could be examined separately from the microcirculation, but otherwise the mucosa would remain as *in vivo*. Such a test system was achieved in the carefully isolated tracheal tube preparation maintained in an organ bath and mounted so that the serosal and mucosal surface solutions were separated. Furthermore, either solution could be raised separately to allow studies of the influence of a hydrostatic pressure load on the epithelial barrier. The whole setup required extreme care to avoid edge trauma and still provide full control over hydrostatic pressure changes and sampling procedures (51, 52).

When a macromolecular tracer (reflecting bulk plasma) was added to the serosal fluid, the growing luminal liquid levels of that tracer indicated a very low but stable epithelial passage, which was in good agreement with *in vivo* observations. When the serosal to mucosal hydrostatic pressure difference was raised from 0 to 5 cm H₂O, luminal entry of macromolecules increased promptly and continued to move at a significantly elevated rate until zero pressure was restored (51, 52). Then the low baseline flux became re-established. The procedure could be repeated with the same result in perfect

agreement with repeat challenge-induced luminal entry of plasma *in vivo* (51, 52). Another similarity to *in vivo* conditions was the finding that the serosal pressure increases had no effect on the absorption barrier of the mucosa, which thus maintained its normal tightness throughout the experiment (52).

The asymmetry of the epithelial lining barrier was expressed in three ways in these experiments:

- 1) The baseline passage of the macromolecules occurred swifter in the mucosal-luminal than in the luminal-serosal direction (52).
- 2) A small hydrostatic pressure load on the basolateral aspects of the epithelial lining cells readily moved macromolecules into the lumen, whereas the same load, when applied to the luminal aspect, was without effect (52).
- 3) The hydrostatic pressure-driven luminal entry of plasma was not associated with, nor was it followed by, an increased absorption permeability of the mucosa (52).

We were surprised to note that physiologists had not previously discovered this hydraulic mechanism for luminal entry of macromolecules across columnar respiratory epithelia. Less surprising was the recent report that hydrostatic pressure loads up to 20 cm H₂O on the basolateral aspect of cultured epithelial cells were associated with severe cell disruption (53). In our intact-tissue experiments, structural or functional damage to the epithelial barrier was not inflicted. We could also demonstrate in the intact airway tube indications that agents producing microvascular-epithelial exudation of bulk plasma *in vivo* influenced neither the baseline passage of macromolecules into the lumen of the tracheal tube nor the pressure-induced high rate of passage (51, 52). We concluded that the extravasated bulk plasma that moves up between the columnar epithelial cells needs to increase the local hydrostatic pressure by only a few centimeters of H₂O to create paracellular passage routes into the lumen, and that this process operates without contributions from any pharmacologic effects of mediators directly on the epithelial barrier. This hydraulic "plasma exudate-epithelial" mechanism exhibits reversibility, repeatability, and direction selectivity in full agreement with the *in vivo* observations on inflammatory stimulus-induced airway luminal entry of bulk plasma (Fig. 5).

Plasma-derived proteins-peptides as determinants of in vivo biology of the inflamed airway mucosa

It is infrequently realized that the entire lamina propria, the epithelial basement membrane, the epithelium, and the mucosal surface of the inflamed airway may be endowed with multipotent

protein systems that normally, in inactivated or less activated forms, would be constrained to the intravascular compartment. The ignorance here is due in part to a widespread unawareness of the airway plasma exudation process, its occurrence, its nature, and its distribution. The fact that *in vitro* studies now provide us with more bioactive molecules than can be fitted into readable schemes of the mechanisms of airway "inflammation" is a poor excuse for neglect of proteins-peptides contained in airways plasma exudates.

There is no reason to doubt the attraction of the specific information about cells and molecules that can be obtained best under *in vitro* conditions. What is surprising, though, is the current lack of *in vivo* perspective and the poor discussion of how the *in vitro* source and *in vitro*-produced molecules may fare in the *in vivo* soup of active agents; that is, if the *in vitro* observations are valid for the relevant *in vivo* conditions. ("In vivo" is used here to indicate living microcirculation-supplied tissues, although we are aware that, today, even studies involving isolated whole cells may be called *in vivo* experiments.) It is conceivable, but little recognized, that the plasma exudate-derived, dynamic molecular milieu of the inflamed allergic airway may be a major obstacle to the translation of *in vitro* cell data to proper *in vivo* function.

Although almost completely eclipsed by the current focus on *in vitro* cell-produced adhesion molecules, it is evident that the extravasated plasma always harbors adhesive and leukocyte-activating proteins such as fibrin(ogen) and fibronectin (22, 54). For example, these plasma-derived molecules are some of the key ingredients of the dynamic gel that is created on the denuded basement membrane and in which epithelial restitution proceeds at high speed (22). Plasma-derived adhesion molecules are also laid down along the important pathways for leukocyte trafficking in the intact mucosa, including the paracellular epithelial pathways for luminal entry of cells (55). Fibrin degradation peptides with intense biologic activities are also produced, and some of them are growth factors with no less potency than their more famous classmates (56). Other examples include the complement and the kinin families of biologically active compounds, and, not least, still unidentified peptide products. Immunoglobulins, proteases, antiproteases, and other plasma-derived molecules also have an important distribution and participation thanks to the airways exudation mechanisms.

The circulating plasma contains cytokines that, despite low concentrations, may exert biologic activity in airways flooded with exudate (57). Other components of plasma, such as the multifaceted

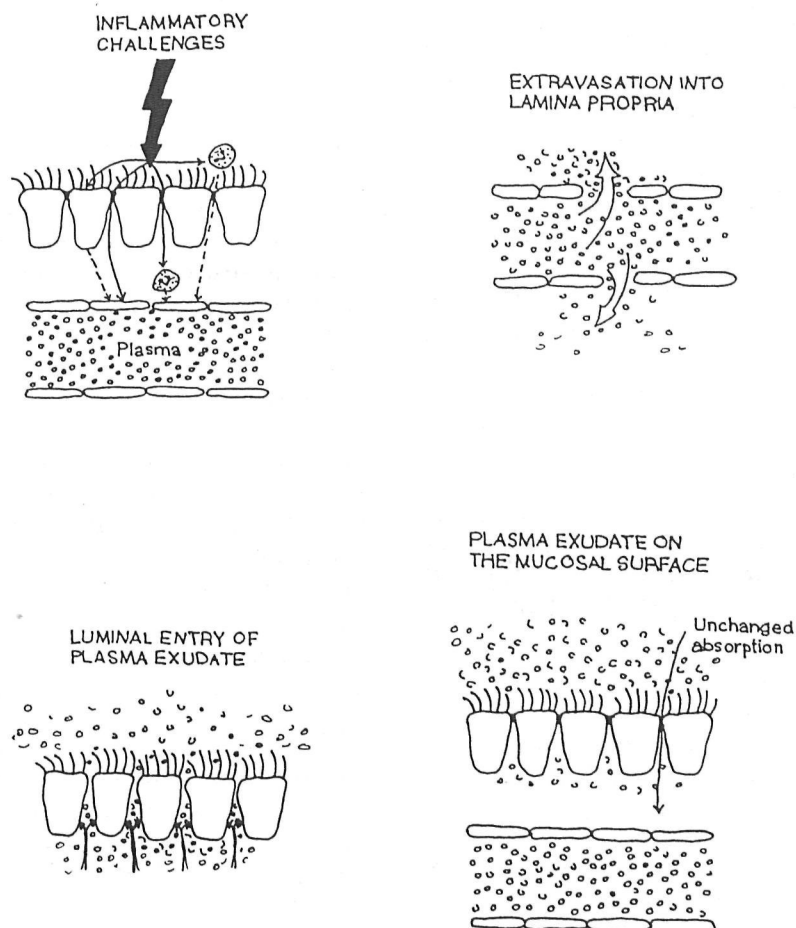


Fig. 5. Challenge with allergen, leukotriene-type mediators, or several other proinflammatory factors produces dose-dependent extravasation, lamina propria distribution, and luminal entry of "bulk" plasma. This process may occur without causing significant edema, without disrupting epithelial lining, and without increasing absorption ability of airway mucosa. Thus, in defense and inflammation, all plasma protein systems, irrespective of molecular size, appear in airway mucosa and on surface of intact epithelium. Epithelial passage appears to involve self-sustained (by exudate) hydraulic mechanism. Hence, only small increases in hydrostatic pressure load on basolateral aspects of epithelial cells are required for unidirectional movement of macromolecular solutes into airway lumen.

α_2 -macroglobulin (58), may have profound influences on airway mucosal cytokines. This molecule may bind, carry, and target most of the known cytokines (58). Since plasma proteins, including α_2 -macroglobulin (700 kDa), moves readily across an intact epithelial lining into the airway lumen (44, 59), a dramatic difference in the mucosal surface cytokines may be expected due to the presence or absence of plasma exudation in the airways. We thus hypothesized, and could demonstrate, that the exudation process offered piggyback riding to the surface of interleukin-6 in allergic airways where acute exudation was evoked by histamine challenges (60). Interesting molecules other than cytokines – for example, eosinophil cationic protein (ECP) – may also be moved from the subepithelium to the airway surface by the plasma-exudation process (61). This aspect may have to be considered in studies on airway surface material which draw

conclusions about the release and distribution of cellular products (62).

Defense-repair disease and treatment aspects

By furnishing the mucosal tissue components with a dynamic soup of effector solutes, the microvascular-epithelial exudation mechanism may critically contribute to airway defense, repair, inflammation, and disease (62). For both desired and less desired roles, there are additional physical considerations. For example, a tissue and surface rinse produced by the exudate may be an important defense (62), whereas the other side of the coin, edema and exudate (-mucous) plugs, are less attractive features that may be seen in chronic disease (63).

The central roles of plasma exudation strongly indicate that this process must not be inhibited indiscriminately. In animal studies, several classes

of airway drugs, including β_2 -agonists (64), xanthines (65), and chromones (66), exhibit direct microvascular antipermeability effects. However, this action may not be very pronounced in animal airways, and it remains to be demonstrated in human airway disease. Whether a vascular tightening effect of these drugs contributes to the therapeutic result, or attenuates defense and repair processes in the airways, is thus subject to conjecture. This uncertainty tallies with our ignorance of many other aspects of the true mode of action of most established antiasthma drugs, as recently exemplified by the failure to reduce the therapeutic efficacy of theophylline to either phosphodiesterase inhibition or adenosine antagonism (1). (Apparently, researchers and audiences alike do not appreciate the distinction between soft, pedagogic notions of antiasthma drug mechanisms and the rare, scientifically valid therapeutic mechanisms that have sufficient solidity to serve as a basis for development of new classes of efficacious drugs [1, 67].)

The only drugs that have been convincingly demonstrated to produce antiexudative actions in asthma and allergic rhinitis are the steroids (68–70). In human airways, this action may not reflect direct vascular actions (71, 72). Hence, steroid treatment may not prevent the airway mucosa from exuding plasma when needed in acute events such as epithelial repair (26), acute viral infections (73), or acute exposure to occupational chemicals (74) (including challenge with histamine-type mediators). The antiexudative action of steroids is believed to be a good measure of the global anti-inflammatory efficacy of these drugs, including effects on a whole range of inflammatory cells in the airways (75) (although the crucial antiasthma mechanisms of these drugs cannot [as yet?] be pinpointed). A neurogenic site of action can be excluded because the reputed neurogenic exudative inflammation, as archetypically induced by capsaicin (nicotine, substance P, or other tachykinins) in rodent airways, may not occur at all in human airways (76–78). Furthermore, there is no known mechanism by which acetylcholine and its analogs may exert microvascular plasma-exudation responses in the airways (36). A direct epithelial tightening effect of steroids can also be excluded. Indeed, such an action would not be desirable in any airway drug because it would cause airway edema.

Eosinophils

Eosinophils and their granule proteins – ECP, major basic protein (MBP), eosinophil-derived neurotoxin, and eosinophil peroxidase (EPO) – may have major roles in several human diseases including asthma and allergic rhinitis (79). Although clinical investigations

of various aspects of eosinophils continue to be carried out, the focus now is on eosinophil mechanisms *ex vivo* and on cell-culture systems showing cytokine-mediated inhibition of eosinophil apoptosis (80). This latter action would allow a sustained presence of eosinophils in diseased tissues, where they could repeatedly release fractions of their potent granular mediators (to produce epithelial damage and other effects). Underscoring the potential importance of this possibility, the pharmacology of airway steroids includes both antirelease effects and proapoptotic actions, as determined in eosinophil test systems *ex vivo* (81, 82). Apoptosis is clearly at the cutting edge of experimental and conceptual research activities (80, 83, 84). In contrast, the nonapoptotic death of eosinophils has attracted no interest. We find this surprising, particularly in view of the *in vivo* relevance of the latter mechanism (85).

Ultimate activation of mucosal eosinophils in vivo: rapid generation of clusters of free eosinophil granules by immune and nonimmune provocations

The tracheal mucosa of guinea pigs is rich in eosinophils that reside in particularly great numbers at the epithelial basement membrane (23, 55). The potent and eosinophil-selective CC chemokine, eotaxin, was also recently discovered in post-allergen challenged BAL lavage liquids obtained from guinea pigs (86). Korsgren et al. (87) have now demonstrated constitutive expression of eotaxin in guinea-pig large airway epithelium in accordance with the complex distribution of eosinophils in these airways. The latter characteristic feature makes the normal or sensitized guinea-pig large airway well suited for exploring which mucosal provocations may acutely activate the eosinophil. The responses to provocations such as epithelial removal (23) and exposure to toxic occupational chemicals (toluenediisocyanate) or allergen (in sensitized animals) (55) include prompt movement of the leukocytes from a basal to an apical position in the epithelium. However, other much more dramatic events also take place. Thus, within 1 h after provocation, a large number of eosinophils have died by nonapoptotic, lytic mechanisms, producing clusters of free extracellular eosinophil granules in the mucosal tissue (23, 55) (Fig. 6).

To avoid sampling and sectioning artifacts, it was important for the free eosinophil granules to be observed and counted in nonsectioned, large-tissue samples. To this end, a whole-mount technique combined with deep tissue staining by eosinophil peroxidase was developed (23). The analysis was complemented by TEM, which demonstrated lysis of the eosinophils and provided details of the

appearance and the location of the free granules (23, 55). By these controlled and quantitative animal observations, we could establish that clustering of free eosinophil granules (Cfegs) in the airway tissue is a real phenomenon, not an artifact caused by the handling of the tissue, and not just a reflection of chance observations. Furthermore, Cfegs were induced by common "asthma-like" provocations such as epithelial shedding-restitution (23) and allergen exposure (55). Most importantly, these observations allowed us to interpret the already published graphs of Cfegs in asthmatic airways that, so far, had been completely ignored in discussions of the roles and mechanisms of eosinophils in inflammatory and allergic disease (79, 85).

Thus, nonapoptotic death or "necrosis" of eosinophils *in vivo* has been eclipsed as an important area of investigation by an overwhelming interest in mechanisms of apoptosis (80, 83, 84) and other cell biology events which are under intense *in vitro* investigation (79). In a scholarly review, Majno & Joris (84) argue that cell necrosis actually refers to changes secondary to cell death by any mechanism, and favor the term "accidental cell death" for the nonapoptotic kind of death. However, eosinophils are not discussed. Awaiting the advent of a more specific term for the mechanism of Cfegs production, we have used "nonapoptotic death" interchangeably with "eosinophil lysis" in the present paper.

Nonapoptotic eosinophil death and free extracellular granules may characterize the airway mucosa in asthma and allergic rhinitis

Cfegs were first seen in the 1800s and later by Felix Marchand, who, in 1916, carefully examined the eosinophilia of the respiratory tract in severe asthma (85). He regarded the free granules as a result of both cell death and tissue handling ("z.T. künstlich z.T. Zugrundegehen der Zellen"). Electron microscopists no doubt observe Cfegs both in asthma (88–91) and in allergic rhinitis (92) but may not always report them, perhaps due to lack of guidance by an acknowledged research paradigm. Similarly, when Cfegs are observed in animal models of asthma, it may, at best, lead to a brief note in passing (93). The unanimous caution is justified because biopsy and sectioning artifacts have not been excluded by the investigators. The authors may also have considered the fact that they have examined only a very minute portion of the tissue (by TEM), raising additional doubts as to how representative the observations were.

Filley et al. (89) observed "a sea of eosinophilic granular debris" in the airways of patients who had died of status asthmaticus. Jeffery et al. (88)

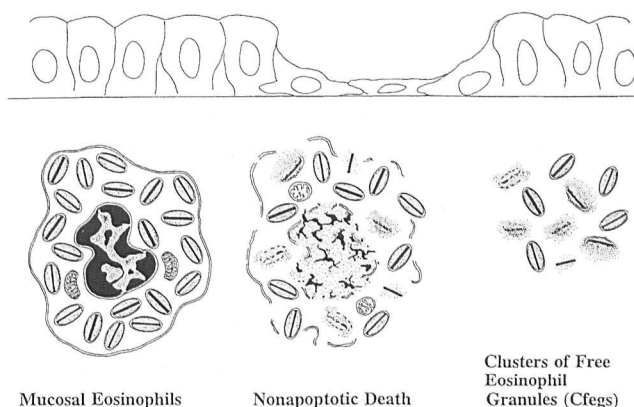


Fig. 6. *In vivo* in airways of subjects with asthma or allergic rhinitis, mucosal eosinophils are lysed (nonapoptotic death) to release clusters of free eosinophil granules (Cfegs) in lamina propria and epithelium. In guinea-pig airways *in vivo*, Cfegs are promptly produced by provocations such as allergen challenge and epithelial damage-restitution. Free extracellular granules exhibit variable degrees of dissolution, suggesting effective release from Cfegs of granule proteins-mediators into mucosal tissue close to target cells and target end organs.

reported that mild allergic asthma presented with highly elevated numbers of Cfegs compared to control. Inhaled steroid treatment reduced the eosinophilia, but, most clearly, Cfegs were reduced. Patients with more severe asthma had received daily steroid treatment for at least 6 months but still exhibited varying degrees of Cfegs in their bronchial mucosa. These intriguing data aroused no interest in Cfegs in asthma, probably because the phenomenon was not recognized as being different from classical "degranulation" (85).

In guinea-pig trachea and asthmatic bronchi, free eosinophil granules may be seen close to neutrophils (23) and lymphocytes (88). The free granules also reside in or close to airway end organs such as the epithelium (23, 55), the subepithelial microvasculature, the smooth muscle (88), and the regional lymph nodes (94). Dvorak et al. (95), examining skin tissue in the hypereosinophilic syndrome, have further demonstrated variable densities of membrane-bound free eosinophil granules. Similarly, free eosinophil granules, with or without membrane, exhibit variable degrees of dissolution (density) in the airways (23, 55, 92). Leakiness of the extracellular granules is also clear from early demonstrations of tissue toxicity caused by i.v. injection of eosinophil granules (96). Hence, the generation of Cfegs would promptly expose the tissue to the entire pathogenic potential of the eosinophil cell.

The currently proposed ultimate eosinophil-activation process (85) may be viewed grossly as a two-step event; first, the release of granules, and then the release of granular products (Fig. 6). This

process may occur widely in the airway mucosa, but may not deplete the tissue of eosinophils, since a profuse subepithelial microcirculation could readily deliver new leukocytes. Antiapoptotic actions of cytokines (80), if valid *in vivo*, could synergize with this lytic activation process by increasing the number of eosinophils residing in the airway mucosa.

Defense-repair disease and treatment aspects

The eosinophil granule proteins are considered culprits in airway diseases, where they are thought to be involved particularly in causing epithelial fragility and shedding (79). Accordingly, the generation of Cfegs may be regarded as a major mechanism of airway diseases. This notion appears to be strongly supported not only by the widespread occurrence of Cfegs in asthma (85) and allergic rhinitis (92), but also by the demonstration of a highly significant association of Cfegs with allergen challenge-induced patchy damage sites (55). Nevertheless, it cannot be excluded that, beside their important roles in the pathogenesis of asthma and allergic rhinitis, and their role in combating parasites, eosinophils may contribute to airway repair processes. The latter suggestion is prompted by the observation that epithelial restitution cells migrated rapidly, particularly where leukocytes, including eosinophils, had accumulated at the moving front (18, 23). Furthermore, the rapid restitution of a new epithelium *in vivo* occurred "despite" the presence of Cfegs in the area (23).

The swift induction of Cfegs *in vivo* by different kinds of provocation suggests that mechanisms involved in eosinophil lysis may well be subject to physiologic and pharmacologic regulation. This area should now challenge investigators willing to embark on *in vivo* studies. Guinea-pig and human airways are suited for this research, whereas the proliferating mouse "models of asthma" appear less helpful. Although allergic mice readily develop pulmonary eosinophilia, these leukocytes may neither be activated nor reside in the airway epithelium (97). (This particular allergic eosinophilia appears to be independent of IgE since it occurs and is no less pronounced in the lungs of B-cell-deficient mice that lack all classes of immunoglobulins [98].) Once we know more of the *in vivo* paradigms of Cfegs generation, complementary *in vitro* approaches may be devised. This exploratory work holds promise of finding both novel cell-activation mechanisms and novel eosinophil-active drugs. Inhibition of eosinophil lysis in the allergic airway diseases may thus be a major asset of future antiasthma and antirhinitis drugs.

Concluding remarks

As discussed in the present review, *in vivo* activities of the epithelium, the subepithelial microcirculation, and the mucosal eosinophils, such as can be evoked by harmful environmental factors, potentially initiate and may critically perpetuate the disease process in asthma and allergic rhinitis. Furthermore, these disease processes may result in rather unexpected functional *in vivo* changes, one of which is an improved absorption barrier.

There are two biases in this review, the first being an almost exclusive focus on *in vivo* observations, a bias which may be inexcusable since most novel data are now being generated *in vitro* and in cell-culture test systems. The second bias, which is inexcusable, is a heavy use of self-references. We cannot defend ourselves by suggesting that one bias may outweigh the other. However, the message of this review is that exploratory *in vivo* approaches are highly worthwhile (and badly needed in studies of the balance between mechanisms and processes involved in airway defense, repair, disease, and effects of treatment, respectively). We hope that this is evident from the various recent discoveries that we try to comprehend and conceptualize within the present paper.

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