J. Membrane Biol. 116, 177–184 (1990)

The Journal of
Membrane Biology

Maintenance of the Macromolecular Barrier at Cell Extrusion Sites in Intestinal Epithelium: Physiological Rearrangement of Tight Junctions

James L. Madara
Departments of Pathology, Harvard Medical School and Brigham and Women's Hospital, and Harvard Digestive Diseases Center, Boston, Massachusetts 02115

Summary. All epithelia slough dying cells but the consequences of this physiological process to epithelial barrier function is unknown. In mammalian small intestine absorptive cells are known to migrate from the villus base to the villus tip from which they slough. These villus tip extrusion zones are often envisioned as sites at which macromolecules could leak across the epithelium. However, only trace amounts of macromolecules cross this epithelium even though, based on known epithelial turnover rates, extrusion events occur millions of times daily. Here, we examine the characteristics of the epithelial barrier to macromolecular permeation at villus tip extrusion zones in rats and hamsters. Freeze-fracture, light and electron microscope studies reveal that extruding cells do not leave transient holes behind as they lift from the epithelium. Rather, as cells extrude, processes of adjacent cells extend under them. Moreover, tight junction elements proliferate between extruding cells and their neighbors and appear to move down the lateral margin of the extruding cell as it extends into the lumen. These observations suggest that newly formed junctional elements "zipper" the epithelium closed as extrusion proceeds thus preventing epithelial discontinuities from occurring. Correlative in vivo perfusion experiments using horseradish peroxidase as a macromolecular-tracer show that the above described dynamic alterations in tight junctions at extrusion sites are generally sufficient to prevent transepithelial leaks of macromolecules.

Key Words tight junction · tight junction regulation

Introduction

The major route for passive movement of hydrophilic solutes across small intestinal epithelium appears to be paracellular (for review *see* ref. [6, 13])—that is, around epithelial cells, rather than through them. The rate-limiting barrier for movement through this "paracellular pathway" is the intercellular tight junction [6, 13]. Although the passive movement of small solutes, such as ions, across this epithelium is substantial, only trace amounts of macromolecules leak across this epithelium [15]. The low rate of transfer of macromolecules across this epithelium is remarkable given the huge surface area of this organ. All the more remarkable is that this epithelium maintains this macromolecular barrier while individual cells are turning over at an exceedingly fast rate: absorptive cells migrate from the villus base up the 1-mm villus over 3–4 days and subsequently slough from the villus tips into the intestinal lumen [5]. During extrusion it has often been suggested that transient defects in the epithelial barrier are likely to appear. However, it has recently become clear that the tight junctions of this epithelium are highly dynamic structures which not only respond to pharmacologic stimuli [7] but also appear to be physiologically regulated [8]. Thus, it is conceivable that dynamic alterations in the junctional barrier at extrusion sites might prevent loss of barrier function.

Here, we ask whether the cell extrusion from villus tips necessarily requires that breaks be formed in intercellular tight junctions with subsequent leaks of macromolecules. We find that this epithelium has a surprising ability to discard individual cells without obvious loss of either morphologic confluency or macromolecular barrier function.

Materials and Methods

General

The majority of experiments utilized 200–250-g male Sprague-Dawley rats. To assure that findings were not species specific, subsets of additional experiments were performed using 200-g Syrian Golden hamsters. Thirty-eight animals were used. Animals were anesthetized by intraperitoneal injection of nebutol, 35 mg/kg. For morphologic studies, distal jejunal-mid ileal tissue was immediately and rapidly removed for processing for structural studies described below. In other experiments (12 animals),

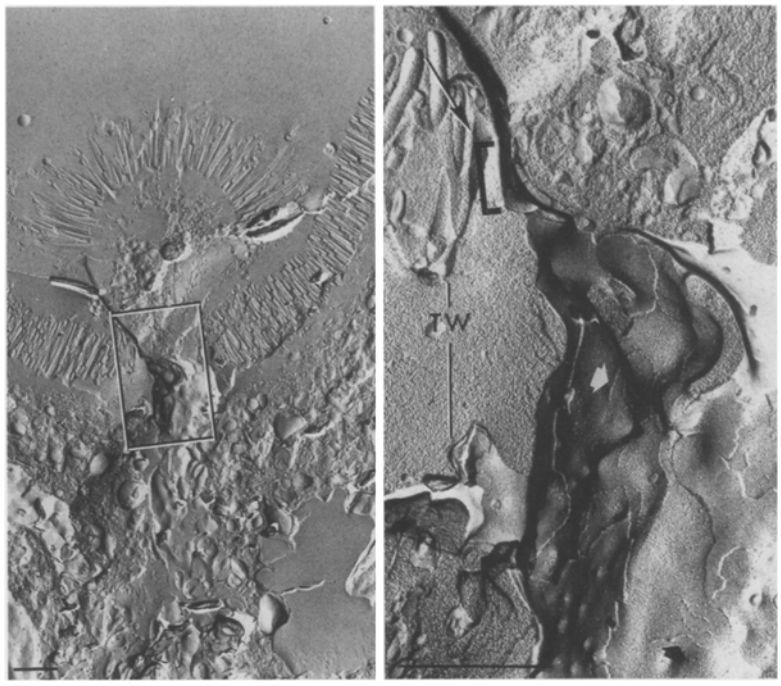

Fig. 1. *Left:* Freeze-fracture replica of extruding epithelial cell from an isolated villus tip. Recognition of such events in replicas relies, as in thin sections, on recognition of the classical image of a protruding cell with splayed microvilli. The narrow base of the extruded cell directly abuts neighboring cells (i.e., no space is left after extrusions). *Right:* Higher magnification of a plasma membrane fracture face associated with this extruding cell. The location of the expected tight junction is shown by the bracket. Due to the angle of fracture, the plasma membrane faces cannot be seen at this site. Further down the lateral membrane, formation of new and/or recruited tight junction elements is apparent (arrowheads). The cell extruding a surface projection a short distance up over the periphery of the extruding cell (arrow) and the terminal web (*TW*) is thickened in this area. Bars = 1 μm

isolated distal jejunal-proximal ileal loops were exposed in vivo for 60 min to a solution composed of Ringer's solution with 0.5% horseradish peroxidase, as previously described [10].

Morphology

Fixation and processing protocols for light and electron microscopy and localization of horseradish peroxidase reaction product were carried out using methods previously detailed by us [10]. For routine electron microscopy, one can prescreen, 1-μm sections and thus selectively sample specific sites, such as extrusion zones, for ultrastructural analysis even if they occur infrequently. Freeze-fracture studies pose more of a problem. The investigator has no control over the fracture plane when using this technique. Thus, when attempting to image a low-incidence event, such as extrusion zones, the yield is very low. To partially offset this technical problem, we effectively increased the density of extrusion zones in the mass of tissue to be fractured. To do this, prefixed normal tissues were sectioned by tissue-chopping into 200-μm slices along the axis of villi. Under a dissecting microscope the upper third of villi were severed using a razor blade. Aliquots of several hundred villus "tips" were thus isolated and packed into double replica hats (which allow replicas of several mm in cross diameter to be produced). Fracture of these tissues yielded replicas in which the frequency of imaging zones of active extrusion was increased although still low. Except for this step, the freeze-fracture replication process was carried out as previously described in detail [10]. Repetition of this procedure ultimately yielded approximately 30 high quality replicas. These replicas were broken into grid-sized fragments for subsequent electron microscope analysis [10].

Results

In comparing rat and hamster experiments, no substantial differences were noted between the structure or the functional behavior of tight junctions of extruding epithelial cells. Thus, we assume that the data described below are not species specific and are likely to relate to mammals in general.

Tight Junction Morphology at Extrusion Sites

In freeze-fracture replicas, extruding cells were identified by the classical appearance of rounded cells, with splayed microvilli, lifting off the epithelial sheet (Fig. 1). Frequently, as seen in Fig. 2, dilated, round structures, presumably the equivalent of the dilated cytoplasmic organelles often seen in routine electron microscopy of senescent cells, were present in extruding cells. Eighteen informative (i.e., large portions of well-replicated plasma membrane fracture face seen) villus tip extrusion sites were so identified in freeze-fracture replicas. The appearance of the tight junction at such sites was consistently distinctive; differing in morphology from that previously described for normal absorptive cells in this epithelium [11]. First, even

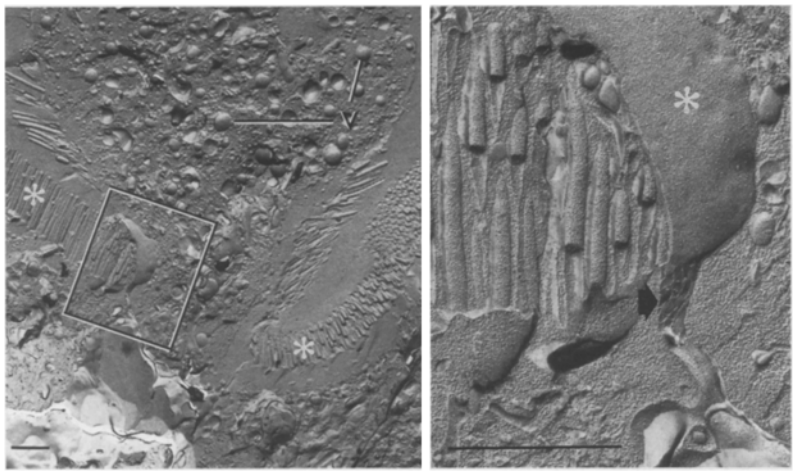

Fig. 2. Freeze-fracture images of extruding epithelial cell at villus tip. *Left:* Low magnification orientation view. The extruding cell has cytoplasmic vacuoles (*V*) indicative of a senescent cell. The microvillus borders of neighboring nonextruding cells are marked with asterisks. *Right:* Enlargement of the boxed area seen in the left-hand panel. Here the fracture and replication angle affords a view of the area of the junctional collar (arrowhead) not specifically seen in Fig. 1. In the flat lateral membrane surface, which now appears to be apical to this junctional collar (asterisk), no junctional elements are found. This image thus suggests membrane flow from the basolateral membrane to the apical pole of the cell, across the tight junction. In this particular extrusion zone, the membrane faces, below the junctional collar where new junctional strands are formed, are not imaged. Bars = 1 μm

when a large fraction of the total cell volume of the extruding cell jutted above the plane of the epithelium and into the lumen, a collar of tight junctional elements remained at the level of the apex of the adjoining nonextruding cells (Figs. 2 and 3). Commonly, adjacent cells appeared to lose perijunctional microvilli, display thickening of the terminal web in the zone of the perijunctional actomyosin ring, and extend perijunctional cell processes a short distance along the side of the extruding cell (Fig. 3). The junctional collar adjoining extruding cells with their neighbors often did not have the uniform net-like structure routinely found in absorptive cell-absorptive cell junctions in the baseline state. Rather, variations in depth and strand-strand crosslinking were seen (Fig. 3), and, focally, discontinuities in junctional structure were found (Fig. 3). The most remarkable feature of tight junctions of extruding cells was proliferation of junctional strand-like elements. This occurred along the portion of the plasma membrane of the extruding cell which had not yet projected into the lumen and therefore remained within the plane of the epithelium (Figs. 1 and 3). Thus, the architecturally aberrant apical collar of tight junction elements by which the extruding cell contacted its neighbors was accompanied by a basal proliferation of strand-like elements. Also remarkable was that, regardless of whether the fracture plane passed through the cytoplasm (Fig. 2) or cleaved the residual "basolateral" membrane (Figs. 1 and 3), cell processes of neighboring cells were always present underlying the basal pole of the extruding cell. That is, extruding cells did not appear to leave empty spaces below them. In addition, the brush borders of neighboring cells indented toward the basement membrane at sites of extrusion (Figs. 1–3). We attempted to determine whether the cell processes of neighboring nonextruding cells, which appeared to approximate underneath the extruding cell, formed new junctional contacts between them. However, due to the geometric complexity of this process, it was difficult to make this determination with certainty in freeze-fracture replicas. For this reason, we prepared thin sections of extrusion sites and found within them, images showing tight junction-like elements forming both on the lateral membranes of extruding cells and between the membranes of abutting cells underlying the extrusion zone (Fig. 4). In cells neighboring extruding cells, round membrane-bound structures contained tight junction strand-like elements (Fig. 5). Such elements are not readily found in vesicular structures of normal absorptive cells although they do become apparent in celiac sprue [9], a disease in which epithelial cell turnover is accelerated. It thus appeared that substantial movement and recruitment of tight junction elements accompanied the extrusion process.

Function of Extrusion Zone Tight Junctions

Were the dynamic structural rearrangements of tight junctions in areas of extrusion sufficient to retard passive transepithelial movement of macromolecules? To answer this question, we analyzed the ability of extrusion sites to impede diffusion of a macromolecular tracer applied from the mucosal side. For these studies, carried out in rats, 36 extrusion sites originating from 482 villus tips were examined. A less extensive sample was obtained from

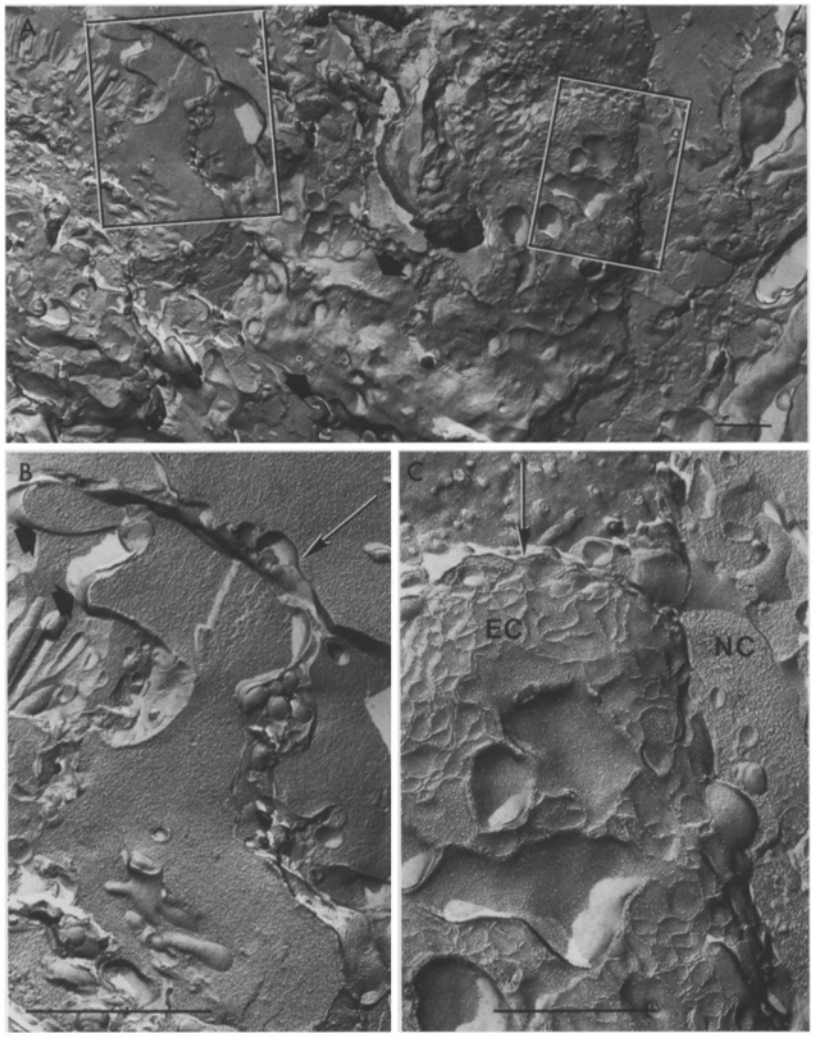

Fig. 3. Freeze-fracture images of extrusion zone. (*A*) Orientation image showing extruding cell which fortuitously displays a large portion of the fracture face of the plasma membrane. The box on the left is shown at higher magnification in *B* and the box on the right is shown at higher magnification in *C*. Even at this low magnification the distorted junctional collar and the marked underlying recruitment of new junctional strands can be faintly identified (arrowheads). (*B*) Enlargement of area designated *B* in *A*. A cell neighboring the extruding cell projects a cytoplasmic process, devoid of organelles (arrowheads), along the margin of the extruding cell (arrow). (*C*) Englargement of area designated *C* in *A*. An irregular collar of junctional elements (arrow) is present at the interface between the extruding cell (*EC*) and the neighboring cell (*NC*). Striking proliferation of tight junction strand-like elements is seen below this junctional collar. Bars = 1 μm

four experiments utilizing hamsters. The images obtained from hamsters yielded the same information as that obtained from rats.

By light microscopy it was confirmed that extruding cells did not leave behind open epithelial defects (Fig. 6). Rather neighboring cells appeared to migrate under the extruding cell as it progressively protruded into the intestinal lumen (Fig. 6). In 19 of the 36 analyzed extrusion sites, horseradish peroxidase was able to penetrate a short distance into the paracellular space surrounding the extruding cells—that is, into, but not completely across, the remodeling tight junction described above (Fig. 6). We never detected a macromolecular leak which successfully negotiated across the epithelium or extended to the basal lamina at the 36 extrusion sites studied. We [12] and others [2, 14] have previously shown that when the absorptive cell tight junction barrier is disrupted, comparable exposures to that reported here for this macromolecular tracer result in dramatic and widespread diffusion of the tracer throughout the paracellular spaces of the villus tip and into the lamina propia. Thus, the above data suggest at most that, if not all, extrusion sites extruding cells are able to maintain the junctional barrier to macromolecular permeation. As shown in Fig. 6, maintenance of the macromolecular barrier also occurred at sites in which two to three neighboring cells were extruding simultaneously (10 sites of multiple extrusion identified). Similar to the freeze-fracture findings, it was often noted that cells neighboring the extruding cell would project a process a short distance into the lumen, which closely associated with the body of the extruding cell (Fig. 6). Electron microscopy showed such processes to be free of organelles (Fig. 7) and packed with cytoskeletal elements dominantly microfilaments. These findings correlate with the lack of cross-fractured organelles seen at comparable sites in corresponding freeze-fracture images such as that shown

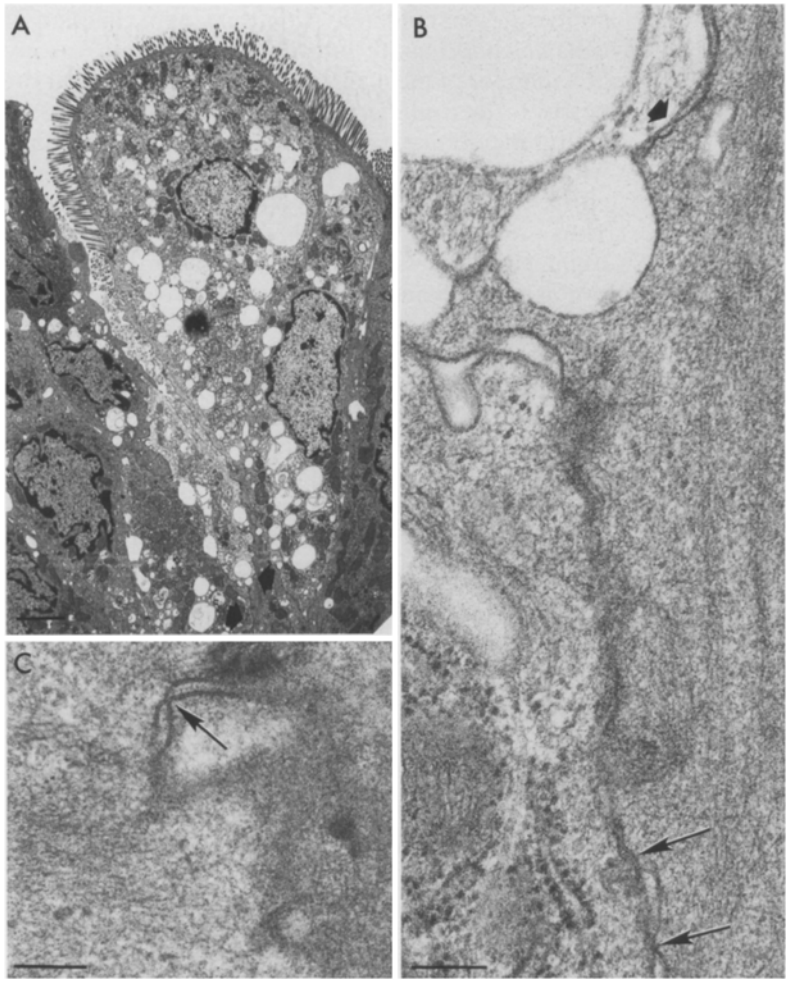

Fig. 4. Thin section electron micrographs of extrusion zones. (*A*) Low magnification orientation image. The areas marked with arrowheads are shown at higher magnification in *B*. (*B*) Tight junction-like structures (kisses) can be found underlying extruding cell and the nonextruding neighbor (arrow) and between the migrating underlying cells previously shouldering the extruding cell (arrowhead). (*C*) Example of focal tight junction kiss-like element (arrow) deep within the epithelium beneath another extruding cell. Bars = 1 μm (*A*), 0.1 μm (*B* and *C*)

in Fig. 3. Electron microscopy also confirmed that horseradish peroxidase could leak a short distance into the architecturally complex and expanded tight junction zone surrounding extruding cells but that such tracer leaks never extruded to the basal lamina and, in fact, were always seen localized in the superficial 1–3 μm of the epithelium depth (Fig. 7).

Discussion

Villus tip extrusion zones are widely cited as potential sites for transepithelial macromolecular leaks [15]. It is thus somewhat surprising that, to our knowledge, a detailed study of the junctional barrier during this physiological event has not been previously reported. Our data, in aggregate, suggest that tight junction elements undergo dramatic rearrangement and expansion on the basolateral surface at extrusion zones. More importantly, it appears such junctions are effective in preventing paracellular leaks of macromolecular at these sites. Based on the available static images, we wish to present a dynamic, speculative working model, of the extrusion process (Fig. 8). As shown in the first panel of the cartoon, the extruding cell initially rounds at its apex and the process of formation of new junctional strands begins on the basolateral membrane. As the extruding cell extends into the lumen (center panel), existing junctional elements sweep along the periphery, maintaining contact with neighboring cells at their apex. Formation of new junctional strands is also marked at this time. Although macromolecules are, at this point, often able to focally penetrate a short distance into the remodeling apical collar of junctional elements, they are unable to completely permeate the underlying dense web of newly formed junctional elements. Maintenance of the junctional seal may be aided by cell processes jutting from the neighboring cells which intimately interface with the plasma membrane of the extruding cell, sweeping along its periphery as it lifts from

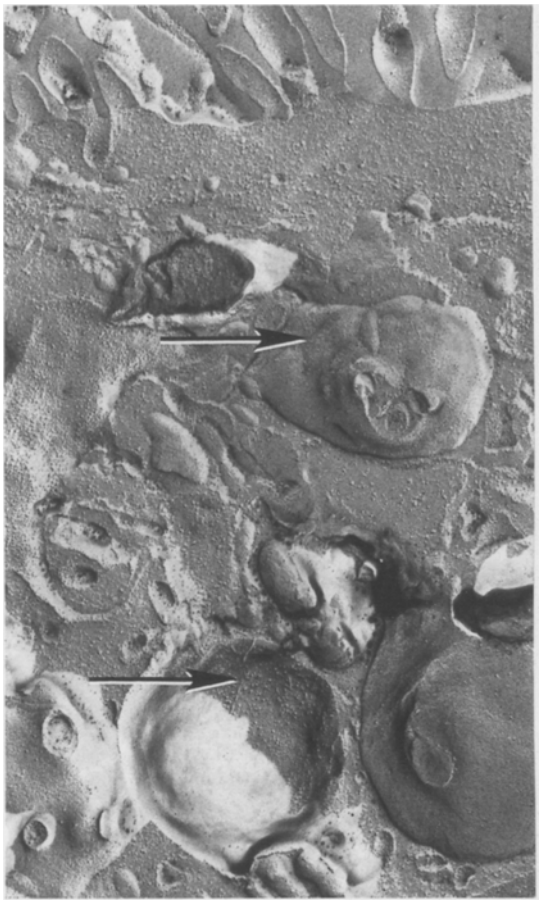

Fig. 5. Electron micrograph of fractured cell neighboring extrusion site. Lysosome-like membrane bound organelles are present in the cytoplasm and contain strand-like elements on their functional membrane surface (arrow)

the epithelium. Simultaneously, neighboring cells also extend basal processes to close the space vacated by the epithelial cell and establish junctional contacts. One could envision this approximation of cells neighboring the extruding cell to occur via any of a number of mechanisms; one possibly is that the newly formed junctional elements along the basolateral membranes, where extruding cells interface with neighboring cells, act, in zipper-like fashion, to close the space vacated as extrusion proceeds. Thus prior to final detachment of the extruding cell (right panel) new junctions have been formed between cells originally flanking the extruding cell.

From the viewpoint of a cell biologist, it is unfortunate that the extrusion event is of relatively low prevalence. For example, if active extrusion events were commonly visualized in tissues, they would be perfect models for studies of the sequence in which the development of cell-cell adhesion, desmosomes, intermediate junctions, and tight junctions occurs in a physiologic process in a natural epithelium. The extrusion process is also of potential interest from the point of view of the forces which must be generated for extrusion. For example, does this resealing event, which three dimensionally would amount to an iris diaphragm-like constriction of neighboring cells around the extruding cell, provide a portion of the force required to drive (or in this case, pull) cells along their migratory route up the villus? Lastly, the extrusion zone could represent a physiologic model for the study of epithelial cell motility.

The rapidity of the extrusion process is not known. It would not be surprising if it were rapid [3]—a view supported by the fact that cell turnover is known to be rapid yet the prevalence of morphologically detected extrusion events is relatively low at villus tips. Could it logically be expected that tight junction elements might be rapidly recruited and remodeled? We now know that absorptive cell tight junctions can remodel during physiological regulation within minutes [1, 8]. Additionally, it has

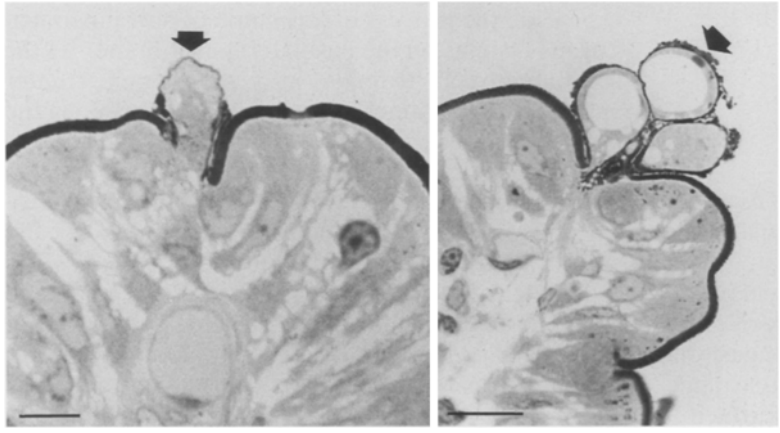

Fig. 6. Light micrographs of villus tip extrusion zones following luminal perfusion with the macromolecular tracer horseradish peroxidase. As shown by us [12], as well as others, when the tight junction barrier is broken, tracer is readily detected throughout the paracellular spaces and under the epithelium. In contrast, at sites of single (left) or multiple (right) cell extrusion (arrowheads), as occurs physiologically, the tight junction barrier to macromolecular permeation remains intact. Note that cells adjacent to extruding cells appear to appose under the lifting cell such that no space is left behind with extrusion. Bars = 10 μm

previously been shown, in other epithelia, that recruitment of tight junction-like elements can be exceedingly rapid. For example, rat prostate epithelial cells can be induced to expand the total of surface tight junction-like elements several-fold within minutes [4]. This striking recruitment of junctional elements is morphologically identical to the basolateral recruitment of strands in extruding cells described here. Previously, there existed no evidence that such recruited tight junction strand-like elements indeed behaved, as normal junctional strands do, as barriers to passive molecular movement. Thus, our finding that these proliferating strands block penetration of macromolecules across the epithelium is, to our knowledge, the first demonstration that recruited strands behave as the barriers they are assumed to be.

One of the most interesting experimental models for events which might occur at cell extrusion sites was reported by Hudspeth [3]. In these elegant experiments single cells in *Necturus* gall bladder epithelium were damaged with a micropipette and plucked from the epithelial surface. Even with this nonphysiologic approach, neighboring cells were seen extending processes into the denuded zone within 5 min and the site of the induced defect often became morphologically inapparent by 30 min. Although movement of macromolecules across the defect was not assessed, undoubtedly this would have occurred since a hole was visible at the site from which the cell was removed. However, the resistance to passive ion flow at these sites also returned to normal values within 30 min. Such observations suggest that the presence of the senescent cell is not required to induce the movement of neighboring cells which results in closure. In contrast, the presence of the senescent cell may be required to maintain the barrier during the short course of the extrusion event.

We have previously shown that absorptive cell tight junctions may be physiologically regulated by exposure to luminal glucose [1, 8]. Here, the structural plasticity of these junctions in the physiological state is further highlighted. The dynamic altera-

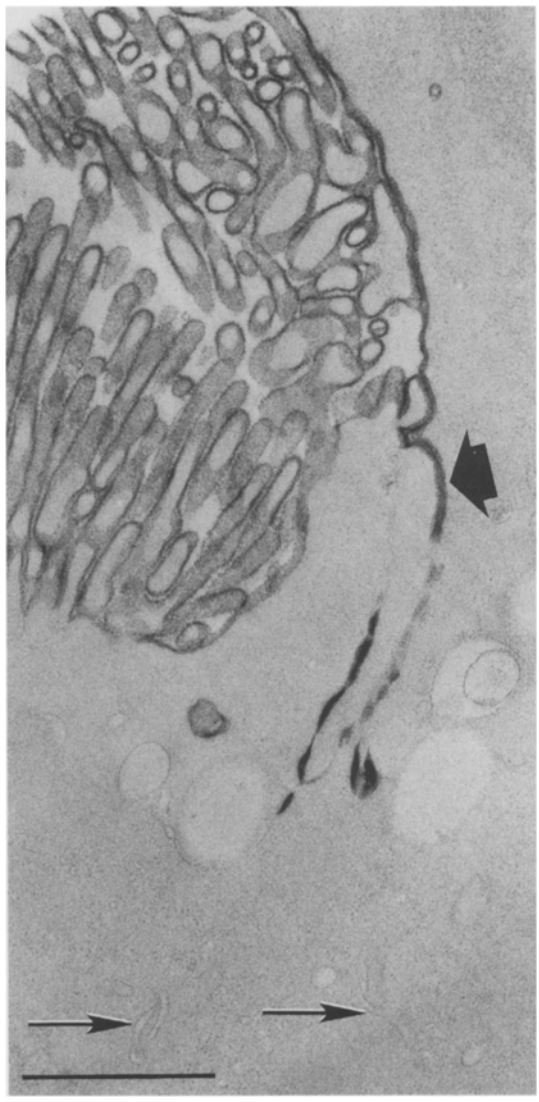

Fig. 7. Thin section of extrusion zone in animal perfused with horseradish peroxidase. Tracer leaks into the site at which the apical collar of irregular junctional strands is found (arrowhead) (*see* Figs. 2 and 3). However, as suggested by the light micrographs of Fig. 6, as shown here in detail, the tracer only penetrates a short distance into the paracellular space. The paracellular space below this site contains no tracer (arrows). Bar = 1 μm

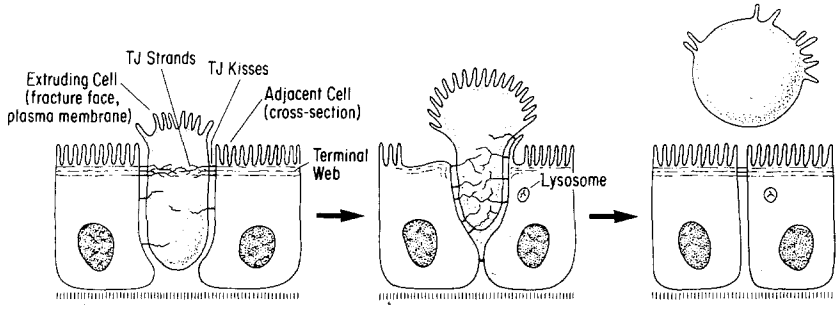

Fig. 8. Speculative model of cell extrusion from intestinal epithelia (*see* Discussion)

tions in tight junction structure at extrusion zones that we report likely serves as the basis for the remarkable conservation of macromolecular barrier function in the face of epithelial cell sloughing, an event which, based on known kinetic data [5], occurs in the intestine millions of times daily.

I am grateful for the expert technical assistance of Ms. Susan Carlson. I thank Ms. Virginia Hamel for preparing this manuscript. This work was supported by NIH grant DK35932. Portions of this work were carried out when receiving support from an American Gastroenterological Association/Ross Research Scholar Award.

References

1. Atisook, K., Carlson, S., Madara, J.L. 1990. Effects of phlorizin and sodium on glucose-elicited alterations of cell junctions in intestinal epithelia. *Am. J. Physiol.* **258:**C77–C85
2. Cooper, M., Teichberg, S., Lifshitz, F. 1978. Alterations in rat jejunal permeability to a macromolecular tracer during a hypersomatic load. *Lab. Invest.* **38:**447–452
3. Hudspeth, A.J. 1975. Establishment of tight junctions between epithelial cells. *Proc. Natl. Acad. Sci. USA* **72:**2711–2713
4. Kachar, B., Pinto da Silva, P. 1981. Rapid massive assembly of tight junction strands. *Science* **213:**541–543
5. Lipkin, M. 1987. Proliferation and differentiation of normal and diseased gastrointestinal cells. *In:* Physiology of the Gastrointestinal Tract. L.R. Johnson, editor. pp. 255–284. Raven, New York
6. Madara, J.L. 1989. Loosening tight junctions: Lessons from the intestine. *J. Clin. Invest.* **83:**1089–1094
7. Madara, J.L., Moore, R., Carlson, S. 1987. Alteration of intestinal tight junction structure and permeability by cytoskeletal contraction. *Am. J. Physiol.* **253:**C854–C861
8. Madara, J.L., Pappenheimer, J.R. 1987. Structural basis for physiological regulation of paracellular pathways in intestinal epithelia. *J. Membrane Biol.* **100:**149–164
9. Madara, J.L., Trier, J.S. 1980. Structural abnormalities of jejunal epithelial cell membranes in celiac sprue. *Lab. Invest.* **43:**254–261
10. Madara, J.L., Trier, J.S. 1982. Structure and permeability of goblet cell tight junctions in rat small intestine. *J. Membrane Biol.* **66:**145–157
11. Madara, J.L., Trier, J.S., Neutra, M.R. 1980. Structural changes in the plasma membrane accompanying differentiation of epithelial cells in human and monkey small intestine. *Gastroenterology* **78:**963–975
12. Marcial, M.A., Madara, J.L. 1981. Analysis of absorptive cell occluding junction structure-function relationships in a state of enhanced junctional permeability. *Lab. Invest.* **56:**424–434
13. Powell, D. 1981. Barrier function of epithelia. *Am. J. Physiol.* **241:**6275–6288
14. Rhodes, R.S., Karnovsky, M.J. 1971. Loss of macromolecular barrier function associated with surgical trauma to the intestine. *Lab. Invest.* **25:**220–228
15. Walker, W.A. 1981. Intestinal transport of macromolecules. *In:* Physiology of the Gastrointestinal Tract. L.R. Johnson, editor. pp. 1271–1286. Raven, New York

Received 13 November 1989