

## *Invited Review*

# **Epithelial integrity, cell death and cell loss in mammalian small intestine**

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**Summary.** In recent years, the different mechanisms of epithelial cell loss which occur in mammalian and avian small intestine have been re-investigated. Information is now available for a variety of mammalian types and mechanisms can be divided into two major classes: [i] those preserving epithelial integrity by maintaining intercellular tight junctions throughout early-to-late stages of cell extrusion; and [ii] those which compromise integrity by introducing breaches in epithelial continuity. Both classes are associated with the activity and/or proximity of non-epithelial cells (mainly lymphocytes and mononuclear phagocytes) located in the epithelium or underlying lamina propria. Intraepithelial lymphocytes may be involved in enterocyte targetting and killing whilst lamina propria (LP) macrophages sequester cell debris. Where epithelial integrity is maintained, two types of loss can be identified. In the first (type 1), complete cells are extruded into the lumen. In the second (type 2), only anucleate apical cell fragments pass into the lumen. There are two variants of type 2 loss distinguishable by the fate of the nucleated basal portions of cells. One variant (type 2a) creates large intercellular spaces extending from the preserved apical cap to the basal lamina and containing enterocyte debris for phagocytosis. The second (type 2b) involves the gradual shrinkage of individual cells (which become more electron-dense) and in situ degeneration of their nucleated subapical portions in increasingly narrower intercellular spaces between adjacent healthy enterocytes. The mechanism of removal of these fragments is unclear but may be via macrophages or surrounding enterocytes. Apoptosis has been implicated in both type 1 and type 2 extrusion. In contrast, type 3 loss involves morphological changes in enterocytes which are reminiscent of those seen in necrosis and is accompanied by breaks in epithelial continuity following cell swelling, a decrease in cell electron density and total

or subtotal degradation of organelles and membranes. It ends in loss of either an abnormal cell apex (with subsequent exposure of the degraded cell contents and their spillage into the lumen) or a complete cell remnant (extruded into the lumen before total disintegration of plasma membranes).

**Key words:** Small intestine, Enterocytes, Extrusion mechanisms, Tight junctions, Apoptosis, Necrosis, Intraepithelial lymphocytes, Mononuclear phagocytes

## **Introduction**

The lumen of mammalian small intestine is lined by a highly polarised epithelium in which the principal epithelial cell types are tall columnar absorptive enterocytes and goblet cells although many intraepithelial lymphocytes (IELs) are also present. The epithelium displays two fundamental characteristics which are potentially conflicting: it is a *continuously renewing epithelium* and also a *tight epithelium*. As a continuously renewing epithelium (Eastwood, 1977; Leblond, 1981; Potten and Loeffler, 1987) it is replaced at regular intervals of roughly every 2-6 days depending on species. Cells are born in the bases of crypts (the proliferative compartment) and then migrate onto villi (functional compartment). During this migration, cells differentiate and become functionally mature before finishing their journey at or near the tips of villi whence they are extruded into the lumen (Brown, 1962; van Dongen et al., 1976; Pothier and Hugon, 1980; Leblond, 1981; Stenling and Helander, 1981; Zoubi et al., 1994). It is classified as a tight epithelium by virtue of the fact that there is a seal between the luminal contents on the one hand and the enterocytes and their underlying connective tissues on the other. The structural and physiological basis of this seal is the intercellular tight junction (Hirsch and Noske, 1993; Anderson and van Itallie, 1995). Intact enterocytes are bound to each other by these junctions which surround each cell at its apex, partition its plasma membrane into apical and basolateral

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domains and help to regulate transepithelial transport. Some luminal solutes are constrained to move across the epithelium by the transcellular route and others by the paracellular route (Powell, 1981; Madara, 1990; Madara and Trier, 1994).

This review summarises current views on how these conflicting properties of the epithelium might be reconciled. It identifies the principal cell players (IELs, LP macrophages) and the modes of cell death (apoptosis, necrosis) involved in identifying enterocytes for removal whilst preserving, for the most part, epithelial continuity and tight junctional integrity.

### Tight junctions and transepithelial transport

Two important functions of small intestine are vectorial transport (secretion and absorption) and forming a seal (to constrain the passive transfer of nutrient and other solutes across it). Most solutes cross the epithelium by transcellular or paracellular routes. Adult small intestine is permeable to small molecules such as ions, amino acids and monosaccharides but is considered to be relatively impermeable to large molecules (Powell, 1981; Madara, 1990; Madara and Trier, 1994). However, it is known that some macromolecules manage to leak across. Despite the continuous and relatively fast turnover of epithelium, only trace quantities of macromolecules manage to do so under normal conditions. Hitherto, this has been attributed to leakiness at the enterocyte extrusion zone at or near the villous tip (Walker, 1981).

The paracellular route comprises an apical tight junctional complex and an underlying lateral intercellular cleft between the apposed membranes of adjacent enterocytes. Both components help to regulate passive flow of hydrophilic solutes but it is the tight junction which is rate-limiting (Hirsch and Noske, 1993; Anderson and van Itallie, 1995). In freeze-fracture replicas, tight junctions reveal a band-like network of interconnected strands representing the punctate approximations of plasma membranes in transmission electron microscope (TEM) ultrathin sections (Claude and Goodenough, 1973). Although the basic structure is qualitatively similar in different regions and epithelia, quantitative variations may correlate with functional differences. The number of strands shows a negative correlation with electrophysiological estimates of epithelial permeability (Claude and Goodenough, 1973). Tight junctions in human and monkey jejunum tend to be narrower and contain fewer strands than those in the ileum and there are differences along the crypt-villus axis, with crypt mitotic activity and in control versus fasted intestines in other species (Tice et al., 1979; Madara et al., 1980; Madara and Trier, 1994). The relationship between macromolecular permeability and tight junctional dynamics is less clear-cut. Where permeability has been altered, distortions of tight junction ultrastructure have been reported.

Clearly, the epithelium of small intestine can

undergo short-term alterations in tight junction structure and function and some of this may be regulated by intracellular signalling events involving junction-related and junction-specific proteins such as ZO-1, ZO-2, occludin, cingulin and cytoskeletal microfilaments (Madara, 1989; Hirsch and Noske, 1993; Anderson and van Itallie, 1995). In addition, IELs produce interferon- $\gamma$  which affects the barrier function of intestinal epithelial cell monolayers in vitro (Madara and Stafford, 1989). This opens up the possibility that IELs can regulate tight junctional permeability directly by secreting interferon- $\gamma$ . Recent evidence suggests that mouse enterocytes may be the principal producers of an interferon- $\gamma$ -inducing factor, interleukin-18" (Takeuchi et al., 1997). Finally, it is conceivable that intestinal permeability characteristics, including permeability to macromolecules, alter passively as a consequence of non-epithelial cells invading the epithelium via intercellular clefts (Nash et al., 1987).

It follows that there are functional implications to the possible occurrence of cell extrusion mechanisms which influence epithelial and/or tight junctional integrity. As if to reinforce this, the bulk of recent studies on mechanisms of enterocyte extrusion have emphasised how intact tight junctions and epithelial continuity are retained despite cell loss. Only more recently has the presence of mechanisms compromising this integrity been re-emphasised. All these studies have demonstrated that, whilst cell extrusion shares certain features (notably the non-epithelial cell players), details vary with species.

### Mechanisms of cell loss

Most textbooks of microscopical anatomy continue to perpetuate the notion that cell extrusion occurs by a simple process of sloughing of single cells or cell clusters (Leblond, 1981). It is envisaged that whole cells detach from the underlying basal lamina, and from neighbouring cells, and pass into the lumen. The disadvantage of such a process resides in the fact that it implies loss of epithelial continuity and persistent leakiness. However, it is now known that cell extrusion from the villus can occur without leaving temporary discontinuities. In fact, more recent studies have demonstrated that several varieties of cell extrusion are possible (Table 1, Fig. 1).

In order to clarify their salient features, it is necessary to distinguish processes which preserve structural and functional integrity of the tight epithelium from those which do not. In addition, species differences in the nature and activity of IELs and LP mononuclear phagocytes are implicated in the different types of cell loss (Iwanaga et al., 1992, 1993, 1994a,b; Han et al., 1993b; Iwanaga, 1995; Shibahara et al., 1995; Takahashi-Iwanaga et al., 1995; Suzuki et al., 1997; Kagnoff, 1998). Investigations into the precise roles played by these cells are continuing.

**Table 1.** Summary of currently known mechanisms of enterocyte loss in small intestine and their species distribution (see text for references)

TYPE OF LOSS	DISTINGUISHING FEATURES	KNOWN DISTRIBUTION
Type 1	Tight junctions and epithelial integrity maintained Loss of whole cell (apoptotic) into lumen IELs and LP lymphocytes and macrophages implicated LP macrophages scattered and not so activated	Hamster, human, mouse, rat
Type 2a	Tight junctions and epithelial integrity maintained Loss of apical cell fragment into lumen Phagocytosis of (apoptotic) basal nucleated fragment from large space IELs and LP lymphocytes and macrophages implicated LP macrophages activated/aggregated at strategic sites, e.g. villous tips	Cattle?, guinea pig, horse, monkey, reindeer
Type 2b	Tight junctions and epithelial integrity maintained Loss of apical cell fragment into lumen In situ shrinkage and degeneration of enterocyte Phagocytosis of (apoptotic) basal nucleated fragment? from narrow space IELs and LP lymphocytes and macrophages implicated LP macrophages may or may not appear activated	Reindeer, seal
Type 3	Breaches of tight junctional and epithelial integrity In situ swelling and total/subtotal degeneration of enterocyte Degeneration by a necrosis-like process IELs and LP lymphocytes and macrophages implicated LP macrophages may or may not appear activated	Chicken, mouse?, pig, rat, reindeer, seal

### *Mechanisms maintaining epithelial integrity*

At least two mechanisms of cell extrusion do not compromise epithelial tightness because intact tight junctional complexes are retained. They involve the shedding into the lumen of complete cells or apical cell fragments only (Fig. 1).

#### Type 1 loss - extrusion of entire cells

This is now known to occur in hamster, human, mouse and rat small intestines (Han et al., 1993a; Iwanaga et al., 1994a,b; Iwanaga, 1995; Shibahara et al., 1995) and seems to involve "zipper-like" migration of tight junctions along the apposed lateral borders of adjacent healthy enterocytes (Madara, 1990). As extruding cells detach from the basal lamina, tight junctions migrate over the lateral plasma membranes staying at, or beneath, the apical brush borders of neighbouring intact cells. Processes of the surrounding enterocytes then extend below the extruding cell(s), become contiguous and establish new tight junctional complexes. These migrate along the lateral cell membranes until, eventually, an effective seal is established before the extruding cell detaches into the lumen (Fig. 1). During detachment, enterocytes may pass through a "polyp-like" phase in which the nucleated cell body retains an increasingly slender cytoplasmic attachment to the rest of the epithelium but, finally, even this link is broken. In luminal washouts from these species, nucleated enterocytes showing signs of apoptosis (including cell shrinkage and peripheral condensation of chromatin in the nucleus) are detectable. In human washouts, there is additional evidence of TUNEL-positive reactivity and agarose gel electro-

phoretic DNA laddering (Shibahara et al., 1995).

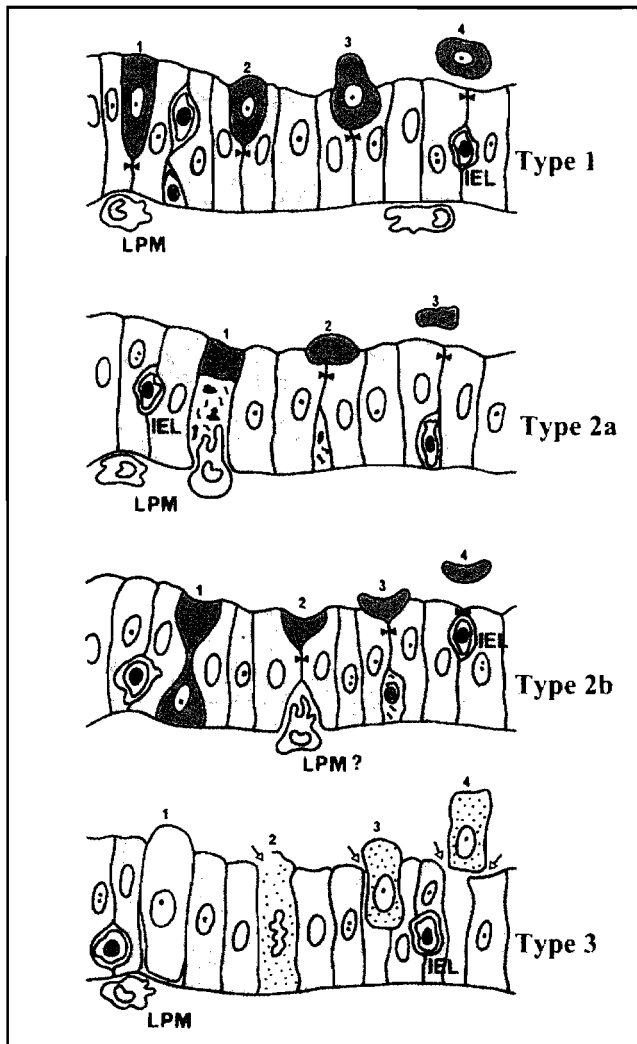
In type 1 loss, IELs and LP macrophages may differ in phenotype and activity from those found in other types of loss. Immunochemical and TEM studies in the rat suggest that IELs are perforin-negative and have short cell processes which do not invade enterocytes deeply. Also, whilst LP macrophages may send substantial pseudopodial processes into basal reaches of the epithelium, they appear not to be so active phagocytically (Iwanaga et al., 1994a,b). These features contrast with those seen during type 2a loss in the guinea pig (see below).

A similar mechanism of whole cell extrusion has been observed *in vitro* in rat colon (Baron and Miller, 1990) suggesting that, within a given species, the extrusion mechanism might be conserved in small and large intestine. Some support for this notion comes from investigations of human intestines (Shibahara et al., 1995; Mahida et al., 1996) but studies on other species are needed in order to test this possibility further.

#### Type 2 loss - extrusion of apical cell fragments

Two variants of this type of loss are now known to exist (Fig. 1). In the first (type 2a), IELs target effete enterocytes and LP macrophages subsequently phagocytose the nucleated basal portions (below tight junction level) creating large basal intercellular spaces below transiently retained anucleate apical fragments (including the tight junctions). The inferior border of this apical fragment displays a plasma membrane. Again, normal enterocytes surrounding the extruding cell establish new tight junctions prior to shedding of the apical fragments. Anucleate apical cell fragments have been observed in luminal washouts from several of the

species in which this process has been observed. These include guinea pig, horse, monkey, reindeer and, probably, cattle (Iwanaga et al., 1992, 1993, 1994a; Han



**Fig. 1.** Types of cell loss seen in small intestine. All types are associated with intraepithelial lymphocytes (IEL) and lamina propria macrophages (LPM). Two types maintain epithelial integrity because new tight junctions (opposed arrowheads) are established before the targetted cell is extruded. In type 1 loss, a sequence of changes (1-4) leads to complete cells being extruded into the lumen. In type 2 loss, only anucleate apical cell fragments are extruded. Two variants are distinguishable by the fate of the nucleated basal cell fragments. Type 2a loss (sequence 1-3) creates large intercellular spaces extending from the apical fragment to the basal lamina and containing debris for phagocytosis by LPM. Type 2b loss involves cell shrinkage and in situ degeneration of nucleated fragments in narrow intercellular spaces between adjacent enterocytes (sequence 1-4). The mechanism of removal of fragments is unclear but may be via LPM. Apoptosis is implicated in type 1 and type 2 loss. Type 3 loss involves changes reminiscent of necrosis. Cell swelling (1) and degradation (2-4) lead to breaks in epithelial continuity (open arrows) and end in spillage of degraded cell remnants into the lumen (2) or subtotal degradation and extrusion of a complete cell remnant (3-4).

et al., 1993a,b; Iwanaga, 1995; Takahashi-Iwanaga et al., 1995; Suzuki et al., 1997; Mayhew et al., 1998; Myklebust and Mayhew, 1998). So far, no consistent evidence of apoptosis has been obtained for the nucleated basal portions but there is certainly rapid phagocytosis of debris by LP macrophages in most species.

In reindeer, enterocyte debris is found in the space created below the apical cell fragment and this is slightly different from the original descriptions of this type of loss in guinea pig (Iwanaga et al., 1993). Near the tips of villi, the latter displays extensive spaces running from the bases of enterocytes up to the level of their tight junctions. This phenomenon was not seen in reindeer and the discrepancy might be related to differences in mode of fixation or some other factor(s) (Myklebust and Mayhew, 1998).

Iwanaga and colleagues (1994a,b) have compared the roles of IELs and LP macrophages in guinea pig (type 2a) and rat (type 1) varieties of extrusion. In contrast to the situation obtaining in the rat, epithelium in the guinea pig is invaded by perforin-positive IELs. Basal portions of targetted enterocytes are interiorised from the large basal intercellular spaces by subepithelial macrophages. In guinea pigs, IELs at the villous tip erode and separate enterocytes by substantial cell processes and seem to be involved in enterocyte apoptosis. Neither this degree of intimate contact between IELs and enterocytes nor clear morphological evidence of lymphocytic enterocytolysis is apparent in the rat. Instead, LP macrophages below the villous tips invade epithelium by projecting thick cell processes into it and attacking enterocytes, probably by mechanical and chemical means (Iwanaga et al., 1994a,b).

A variant of the guinea pig version of apical fragment shedding has been identified by TEM in reindeer and seal (Myklebust and Mayhew, 1998) and involves in situ cell shrinkage (type 2b loss). Initially, enterocytes become more electron-dense and more slender and their nuclei much denser. The typical morphology of the microvillous border is lost. On some enterocytes, microvilli elongate but, on others, they become thicker, clumped and more variable in length. Finally, some enterocytes are denuded of microvilli. In most cases, the apical cell border becomes concave and, therefore, quite distinct from the convex appearance found in type 3 loss (see below). Mitochondria are slightly swollen but, again, their appearance is not the same as that noted in enterocytes undergoing type 3 loss. There is weakening of intercellular contact with adjacent normal cells at the sites of lateral membrane interdigitations. This is partly brought about by adjacent enterocytes extending expanded processes into the extruding enterocytes. However, the latter are invaded also by obtruding thin processes from relatively light cells. These processes contain few organelles beyond free ribosomes and are similar in appearance to the cytoplasm of IELs.

As type 2b loss progresses, enterocytes become



relatively thin and assume hourglass-shaped forms but apical integrity is preserved by maintaining tight junctional complexes with neighbouring cells. These changes produce apical caps resembling inverted umbrellas or mushroom-caps. Often these are homogeneous and electron-dense suggesting that alterations are taking place in the terminal web. Sometimes, apical caps are visibly continuous with an expanded nucleated basal portion via the intermediate isthmus region. At later stages, cells disintegrate leaving apparently isolated pockets of large organelle-bearing cell remnants, or residual membrane whorls, trapped between adjacent enterocytes of normal morphology. Eventually, the apical caps, together perhaps with some subapical regions containing organelle debris, are shed into the lumen without breaking epithelial surface integrity. It is not clear how the intercellular cell debris is removed but the most likely explanation is by the intermediary of macrophages or surrounding enterocytes. This happens in other epithelia in order to remove apoptotic cells (Wyllie et al., 1980) but, as yet, no evidence for apoptosis has been adduced for reindeer or seal (Myklebust and Mayhew, 1998).

The presence of IELs in both seal and reindeer (Myklebust and Mayhew, 1998) is consistent with the mechanism of cell targetting described in guinea pig but the situation in seal differs slightly by virtue of the relative scarcity of mononuclear phagocytes in the epithelium and LP. This may be related to the occurrence of type 2b cell death and the difference in ease with which LP macrophages can remove enterocyte debris from the large basal intercellular spaces created in guinea pig and reindeer during type 2a extrusion. Alternatively, the differences may reflect a different pattern of non-epithelial involvement such as that seen in type 1 versus type 2a loss.

#### *Mechanisms breaching epithelial continuity (type 3 loss)*

Scanning electron microscopy (SEM) of murine small intestine has revealed the presence of scattered holes on the surface of villi where enterocytes, or at least their apical portions, are missing (Potten and Allen, 1977). Taken alone, such observations are not unequivocal evidence for loss of complete cells because enterocyte remnants might exist deep within the holes and at least some of the holes might represent sites of goblet cell secretion. More convincing evidence comes from TEM studies. In the chicken, cell death compromises tight junctional (and, hence, epithelial) integrity and is accompanied by ultrastructural changes similar to events seen in necrosis (Holman, 1975; Watson, 1995b). Comparable alterations have now been confirmed in pig, rat, reindeer and seal small intestine (Mayhew et al., 1998; Myklebust and Mayhew, 1998; Mayhew and Jenkins, unpublished observations). Clearly, such deficiencies could render the epithelium locally leaky since it leads to local breaks in the otherwise tight epithelium. As with type 1 and type 2

cell loss, it is also associated with non-epithelial cells, notably IELs.

In chicken, pig, rat and seal, this type 3 cell loss (Fig. 1) involves alterations in microvilli, the subapical terminal web, mitochondria, rough and smooth endoplasmic reticulum and the nucleus. Early stages are characterised by abnormal microvillous morphology, including a disrupted and vesiculated brush border. The apical cell border is often convex, bulging towards the lumen above the general level of the epithelium. Swelling leads to gradual loss of electron-density and ends with cells lacking microvilli and displaying breakdown of cellular integrity. There are signs of mitochondrial swelling and, ultimately, these organelles disintegrate together with other intracytoplasmic organelles and cell membranes. Nuclei become pleiomorphic and may lose their perinuclear space. Heterochromatin is condensed peripherally but the residual nucleoplasm tends to be less electron-dense overall with scattered electron-dense granules. Eventually, nuclei disintegrate. Formation of chromatin bodies and other ultrastructural features of apoptosis have not been observed. In the final stages, microvilli may disappear and the cell apex (often with a faintly visible terminal web) becomes partially or completely detached. This exposes the cell contents to, or they are spilled into, the lumen leaving holes in the epithelium down to the basal lamina. This mode of cell death is structurally similar to necrosis (Watson, 1995b) and seems to be confined largely to isolated single cells although, occasionally, small clusters of enterocytes are affected. Cells similar in appearance to IELs are sometimes found in intimate contact with enterocytes undergoing this type of cell death and their cell processes are seen to invade surrounding, apparently healthy, enterocytes (Myklebust and Mayhew, 1998). The IELs may be found at the highest levels of the epithelium, i.e. near the disrupted cell surfaces.

In reindeer, swollen dying cells are also found and their contents are lost into the lumen leaving holes in the epithelium. However, in contrast to the pattern described in chicken, pig, rat and seal, there is subtotal degeneration of cells before shedding: swollen enterocytes with swollen spherical nuclei occasionally break tight junctional contacts with adjacent enterocytes before their membranes disintegrate completely. These "cell ghosts" may project well beyond the general epithelial surface as if whole cell remnants are about to be lost. Indeed, nucleated debris of necrotic cells has been observed in the lumen (Myklebust and Mayhew, 1998).

Some of the ultrastructural features of type 3 cell death (vesiculation of microvilli, cell apex convexity, cytoplasmic degradation, involvement of small clusters of cells) are visible also in murine enterocytes but the cells are more electron-dense (see Potten and Allen, 1977). The process may not be confined to villous tips but might occur at sites over the entire villous surface. The authors also noted the likelihood of circadian variation, with cell loss being greatest in the early

morning hours.

Type 3 loss has been observed in immersion- and perfusion-fixed material and its end-stage, viz. release of disrupted cell contents into the lumen, is clearly distinguishable from goblet cell secretion. It could account for at least some of the holes seen in the villous epithelium of rodent intestine by SEM (Potten and Allen, 1977). Given its occurrence in chicken, pig, rat, reindeer, seal and (possibly) mouse, this manner of cell death may be more widespread than previously admitted. Clearly, a more thorough and systematic screening of intestines in other species is required before it can be stated categorically that this necrosis-like form of cell loss does not occur in tandem with other types of enterocyte extrusion.

### Non-epithelial cells

A number of non-epithelial cell types are found in the epithelium and underlying connective tissue and show species and regional differences in incidence and morphology. Within avian and mammalian small intestine, intraepithelial neutrophil polymorphonuclear leucocytes and IELs have been identified (Meader and Landers, 1967; Bjerregaard, 1975). LP harbours a variety of cell types including mononuclear phagocytes (usually mature tissue macrophages), lymphocytes, plasma cells, fibroblasts, eosinophil polymorphonuclear leucocytes and mast cells. However, it is only the lymphocytes which gain access to the epithelium in appreciable numbers. For present purposes, attention is restricted to the LP macrophages and lymphocytes and to these intraepithelial lymphocytes.

#### *Lamina propria mononuclear cells*

LP constitutes an important functional compartment of the intestinal immune system. Within it, the principal cells implicated in enterocyte death and loss are macrophages. These vary with species and region. For example, the incidence, size, granularity and activity of LP macrophages are greater in guinea pig than human, mouse or rat (Sawicki et al., 1977; Iwanaga et al., 1994a,b). The incidence of LP macrophages tends to be highest near villous tips (Holman, 1975; Sawicki et al., 1977; Iwanaga et al., 1992, 1994a,b; Han et al., 1993b; Shibahara et al., 1995; Suzuki et al., 1997) where they have been identified by enzyme histochemistry and immunocytochemistry as having high levels of lysosomal enzyme expression or activity. Time-series studies using Feulgen staining and tritiated thymidine autoradiography or bromodeoxyuridine immunocytochemistry have suggested that LP macrophages phagocytose other cell types and these might include fibroblasts and IELs but the majority are enterocytes (Sawicki et al., 1977; Han et al., 1993b). In contrast to IELs (see below), LP lymphocytes are nearly all T cells of the  $\alpha\beta$  receptor and helper/memory phenotypes (Shanahan, 1994).

In guinea pig small intestine (Han et al., 1993b; Iwanaga et al., 1993), LP macrophages congregate near the tips of villi, stain well for acid phosphatase and cathepsin, and extend cell processes between overlying enterocytes. Elsewhere within the villus, LP macrophages stain less intensely and are not so densely packed. Ultrastructurally, the cells contain numerous lysosomes as well as phagosomes varying widely in size and content (e.g. mitochondria, nuclei). The nuclei appear pyknotic and resemble apoptotic bodies previously identified in enterocytes as well as macrophages (Wyllie et al., 1980). The general appearance of partially digested cell debris suggests an enterocytic rather than fibroblastic or lymphocytic origin. The cytoplasmic fragments lack evidence of microvilli suggesting that, if the debris is enterocytic in origin, it comprises only the nucleated basal portions of cells. This is supported by the appearance of bromodeoxyuridine-stained bodies in LP macrophages 4 days after bromodeoxyuridine administration. Cell loss in guinea pigs is type 1. LP macrophages with similar features overall are observed in horse and monkey (Iwanaga et al., 1992; Han et al., 1993b) but some invade the epithelium completely rather than merely extending processes into it. In similar villous tip positions in hamsters, mice and rats, LP macrophages are not so conspicuous in numbers or phagocytic morphology (Iwanaga et al., 1992; Han et al., 1993b).

In seal LP (Myklebust and Mayhew, 1998), there are rounded cells resembling large granular lymphocytes (containing homogeneously electron-dense granule profiles 200-750 nm in diameter) and mononuclear phagocytes resembling mature tissue macrophages rather than immature monocytes. The large granular lymphocytes are observed commonly whilst LP macrophages are infrequent. However, macrophages do appear to be phagocytically active with intracellular vacuoles containing recognisable cellular debris. They appear not to extend processes into the epithelium and no macrophages have been observed within the epithelium. Overall, their appearance is more reminiscent of that in human, mouse and rat than in guinea pig intestine.

In reindeer, substantial aggregations of mononuclear phagocytes, varying in ultrastructural morphology from relatively immature monocyte-like cells to mature tissue macrophages containing phagolysosomes and sequestered cell remnants, are abundant in the LP and often closely packed together on the same subepithelial plane (Myklebust and Mayhew, 1998). LP macrophages containing ingested material are also seen within the lower reaches of the epithelium. Overall, this pattern is similar to that described in guinea pigs. Granule-rich large lymphocytes (granule profiles 250-750 nm in diameter) are also found in the LP in reindeer and some of these display substantial subepithelial cell processes.

Macrophages produce several cytotoxic factors (interleukins, nitric oxide, oxygen free radicals) as well as tumour necrosis factor- $\alpha$  which is known to induce apoptosis in target cells albeit over a longer timeframe

# Cell loss in small intestine

**Table 2.** Variations along the villus in the fraction of epithelial volume (%; estimated by test point-counting) occupied by intraepithelial lymphocytes in pig, reindeer and seal small intestine.

SPECIES	APICAL HALF	BASAL HALF	MEAN DIFFERENCE	SEM	p LEVEL
Pig	16.0 %	10.6%	5.4%	1.6%	< 0.01
Reindeer	3.8%	3.0%	0.8%	1.0%	NS
Seal	2.2%	1.1%	1.1%	0.7%	NS

p levels obtained from paired Student's t-test; SEM: standard error of difference between means; NS: not significantly difference from null hypothesis

(hours versus minutes) than cytotoxic T cell-mediated killing (Duke, 1991; Ohno et al., 1993; Suda et al., 1993). Therefore, they are not only well-positioned but they are also well-armed for cell targetting. The same is true for IELs.

## Intraepithelial lymphocytes

The intestinal epithelium provides another important compartment of the intestinal immune system and, due to their location, lymphocytes at this site have the closest contact with foreign antigens present in the lumen. Cytokine production by enterocytes and lymphocytes must have a profound influence on the intestinal immune system but the functional implications of this communication are still being elucidated (Elson and Beagley, 1994; Shanahan, 1994; Kagnoff, 1998; Mayer, 1998). The concept is emerging of the enterocyte as a prime player in sampling luminal antigens, processing them and presenting them to lymphocytes.

The epithelium is often rich in lymphocytes, the overwhelming proportion of which are located in the intercellular spaces in basal regions, below the level of the majority of enterocyte nuclei, but they may be found at any level between the basal lamina and subapical region (Meader and Landers, 1967; Bjerregaard, 1975). From pilot stereological estimates of volume fractions (Mayhew, 1991) in top and bottom halves of villous profiles, we have found species- and position-dependent differences (Table 2). The pig displayed 3-10 times greater fractional volumes of lymphocytes than reindeer or seal in both halves of the villous and also demonstrated higher values towards the top half of the villus. Although appearing to contain also higher values in the top half, the differences between villous regions were not significant in reindeer or seal. The functional implications of these findings are not clear and further investigation on larger samples are required.

Few, if any, IELs appear to pass into the lumen. There is evidence that lymphocytes, and other cell types, pass between the LP and epithelium via pores in the basal lamina (McCluggage and Low, 1984; Mahida et al., 1997). They do not form desmosomal or other cell surface specialisations with enterocytes but do extend substantial projections between them. A variable proportion of IELs harbour homogeneously electron-dense intracytoplasmic granules like cytotoxic T lymphocytes. In the latter, granules contain sulphated

mucopolysaccharides, perforins, serine esterases of the granzyme family and fragmentins which induce DNA fragmentation and apoptosis (Guy-Grand et al., 1991; Shi et al., 1992; Iwanaga et al., 1993, 1994a; Sydora et al., 1993; Shanahan, 1994). Fragmentation of target cell DNA via the intermediary of large granular lymphocytes is characteristic for apoptosis and may be perforin-dependent. In marked contrast to peripheral blood and LP lymphocytes, almost all IELs are T cells, the majority having a suppressor/cytotoxic surface phenotype and the remainder a helper phenotype. Another distinction is their expression of the  $\gamma\delta$  form of the T cell receptor (Viney et al., 1990; Suzuki et al., 1997). Such cells are capable of detecting and destroying tumour cells or abnormal enterocytes infected by bacteria or viruses and may monitor such cells continuously in order to identify them and expedite their removal from the epithelium (Janeway et al., 1988; Kagnoff, 1998).

In terms of morphology, IELs often resemble large granular lymphocytes and their granule content is greater in rodent than human intestinal epithelium. However, large granular lymphocytes in rats contain smaller granules of different ultrastructural features to those seen in some other species (Iwanaga et al., 1994a,b; Myklebust and Mayhew, 1998). In fact, large granular lymphocytes comprise a heterogeneous group of cytolytic lymphocytes which includes cytotoxic T cells, natural and lymphokine-activated killer cells (Kaneda, 1989). Their granules contain various cytotoxins including perforin, a peptide which induces cell death by creating ion channels in target membranes (Henkart et al., 1984; Podack et al., 1985). In guinea pigs (Iwanaga et al., 1994a), IELs are known to be perforin-containing. They are dispersed in the epithelium but more numerous towards villous tips. Perforin-containing large granular lymphocytes are rare in LP. In rats, epithelium lacks perforin-containing lymphocytes (Iwanaga et al., 1994a) but they are scattered throughout the LP and more frequently observed near the bases of villi. Further information is needed on the perforin-immunoreactivity of IELs in different mammalian species.

In guinea pig small intestine, numerous lymphocytes are found in the epithelium, especially near villous tips (Han et al., 1993b; Iwanaga et al., 1993). They extend their cell processes between enterocytes. Many contain near-spherical electron-dense granules (profile diameter 300-600 nm) and so resemble large granular lympho-

cytes. Apart from slight differences in granule size, IELs in cattle and monkey small intestine are essentially similar to those seen in the guinea pig (Iwanaga et al., 1992; Suzuki et al., 1997). In the seal, IELs are seen in close proximity to both necrotic and shrinking cells. They tend to have a similar ultrastructure to LP large granular lymphocytes but with fewer intracytoplasmic granules. IELs are found at all levels (basal to subapical) within the epithelium and, sometimes, are surrounded by narrow intercellular spaces containing cell membrane remnants (Myklebust and Mayhew, 1998). In reindeer, as in the seal, enterocytes suffering type 2b loss (shrunk electron-dense cells with expanded apical portions and maintaining tight junction contacts with adjacent enterocytes) are found close to IELs (Myklebust and Mayhew, 1998). Again, these possess relatively poor complements of granules, are detected at all levels within the epithelium and extend processes between and into epithelial cells.

Large electron-dense granules characteristic of those in large granular lymphocytes in other species are demonstrable in guinea pig, monkey, reindeer and seal. In contrast to these species at least, IELs in rat small intestine also harbour smaller (profile diameter 150-200 nm) clear granules whose profiles display rod-like or punctate cores (Kaneda, 1989; Iwanaga et al., 1994a).

### **Apoptosis and necrosis**

Apoptosis and necrosis are different mechanisms of cell death. In necrosis, cells swell; in apoptosis, they shrink. Necrosis is known to be sensitive to radiation, temperature, hypoxia, ischaemia, reactive oxygen metabolites, bacterial and chemical toxins and cytolytic agents (Watson, 1995a,b). This raises questions about whether or not necrosis is part of a targeted cell deletion and what triggers enterocyte loss by this means. The occurrence of necrosis-like enterocyte death in several different species without obvious signs of intestinal pathology suggests that this is a normal process. However, its limitation to scattered single cells or small cell clusters is rather unusual for necrosis which, elsewhere, tends to affect large groups of cells simultaneously and to evoke an inflammatory response (Watson, 1995b). The occasional observation of IELs in close proximity to, or intimate contact with, enterocytes undergoing type 3 loss provides circumstantial evidence that the process is not a chance by-product of targeted cell removal at more distant epithelial sites. However, necrosis may occur in response to noxious influences found locally including, perhaps, from the lumen. Apart from lymphocytes, necrosis may involve the intermediary of polymorphonuclear granulocytes (Parks, 1989; Watson, 1995b) which have also been seen in the LP, but rarely in the epithelium, in seal, reindeer and other species (e.g. see Myklebust and Mayhew, 1998). The possibility cannot be discounted that the type 3 loss described so far (Holman, 1975; Myklebust and Mayhew, 1998; Mayhew et al., 1998) might not be

typical necrosis but a variety of localised programmed cell death with different ultrastructural features to apoptosis.

Apoptosis is a DNA-dependent mechanism of programmed cell death which occurs under physiological and pathological conditions. Various studies have suggested that apoptosis is an important factor in regulating enterocyte number. Assessments of numbers of apoptotic bodies in human, mouse and rat small intestine have been made on paraffin sections stained by haematoxylin-and-eosin and in situ end-labelling as well as on plastic-embedded sections (Hall et al., 1994). Although these studies did not correlate apoptotic incidence or location with mechanism of cell extrusion, they did demonstrate apoptotic figures in the epithelium, particularly near the tips of villi and in subjacent LP. Others have suggested that fragmented DNA typical of apoptosis is found along the entire villous length (Gavrieli et al., 1992) but DNA fragmentation may also occur in necrosis. However, the suggestion of apoptosis being found near villous tips and in LP is reinforced by ultrastructural studies on LP macrophages and cells in luminal washouts in different species.

Studies on mammals have shown that extrusion associated with intact tight junctions may involve enterocyte apoptosis (Han et al., 1993a,b; Iwanaga et al., 1994b; Iwanaga, 1995; Shibahara et al., 1995). There is characteristic chromatin condensation in nuclei of extruded cells found in rat intestinal lumen and evidence of engulfed apoptotic nuclei in LP macrophages in guinea pig (Iwanaga et al., 1994a). Ultrastructural features reminiscent of apoptotic nuclei were not identified in reindeer or seal small intestine (Myklebust and Mayhew, 1998) and further studies are needed on these species.

### **Concluding remarks**

Enterocyte loss in mammalian small intestine proceeds by at least three mechanisms, two of which maintain epithelial integrity whilst the third does not. All are associated with IELs and LP macrophages. Tight junctions are preserved where there is extrusion of whole cells (type 1 loss - hamster, human, mouse, rat) and apical fragment loss (type 2 - guinea pig, horse, monkey, reindeer, seal and, maybe, cattle). With loss of complete cells, the nuclei of enterocytes within the lumen may show signs of apoptosis but there are relatively few active LP macrophages. Loss of isolated apical cell fragments may be accompanied by the creation of substantial subapical spaces from which apoptotic debris is removed by LP macrophages which aggregate in large numbers (type 2a - guinea pig, horse, monkey, reindeer). Alternatively, subapical enterocyte debris may be confined to narrow intercellular clefts (type 2b - reindeer, seal) and interiorised by LP macrophages or other enterocytes. Another type of cell loss (type 3 - chicken, pig, rat, reindeer, seal) is a distinct process which displays ultrastructural features of necrosis and



causes localised breaches of epithelial integrity. It may result in loss of "cell ghosts" or spillage of cell contents from disrupted cells.

Since tight junctional complexes are rate-limiting for paracellular transfer of certain nutrients, the occurrence of cell extrusion mechanisms which are controlled by mononuclear cell targetting and retain tight junction integrity implies some degree of conservation of epithelial permeability. However, the simultaneous presence of localised sets of enterocytes with ultrastructural features of necrotic cells suggests that permeability to macromolecules might be influenced by the occurrence of physical discontinuities in the epithelium. Leakage may not be confined to villous tip extrusion zones as has been thought previously (Walker, 1981).

Finally, recognition of these different forms of extrusion, and their association with apoptotic and necrosis-like forms of cell death, opens up the possibility of new strategies for treating gastrointestinal disorders characterised by changes in cell turnover and intestinal permeability (Watson, 1995a). A number of such diseases are associated with apoptosis of epithelial and other cells. For example, in coeliac disease, a malabsorptive disorder associated with abnormalities of the intestinal mucosa and LP, enterocyte apoptosis (detected by TUNEL-staining of fragmented DNA) is increased greatly and the spatial pattern changes from one in which apoptotic nuclei are seen mainly near villous tips to one in which they are detectable along the crypt-villus axis (Moss et al., 1996). Features return to normality with a gluten-free diet. Another distinctive feature of the disease is a striking increase in the frequency of  $\gamma\delta$  versus  $\alpha\beta$  T cell IELs in small intestine. However, this balance is not restored during remission of the disease (Kagnoff, 1998). Ingestion of anthraquinones in purgative preparations leads to melanosis coli in which there is apoptosis of colonic epithelial cells and subsequent phagocytosis by macrophages. Similar features have been observed in the colon and ileum of a guinea pig model involving oral administration of the anthraquinone, danthron (Walker et al., 1988). Some macrophages ingest apoptotic bodies in the epithelium and transport them through the basal lamina and into the underlying LP. In a neoplasia of the colon, familial adenomatous polyposis, above-normal numbers of apoptotic bodies are phagocytosed by adjacent epithelial cells or cells in early stages of apoptosis are extruded into the lumen (Strater et al., 1995). Antibiotic-associated pseudomembranous colitis is a disease caused by *Clostridium difficile*. Recent studies suggest that the disease may be initiated by toxin A-induction of epithelial cell interleukin-8 and apoptosis after detachment from the basal lamina (Mahida et al., 1996).

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