LOCALIZED LANTHANUM STAINING OF THE INTESTINAL BRUSH BORDER

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The use of lanthanum salts has made possible the staining of extracellular regions which are not electron opaque when conventional methods of fixation and staining for electron microscopy are employed (3, 6, 10). The present work describes the staining of the intestinal epithelium by this means. Intercellular regions in the intestinal wall generally stain intensely, as has been described previously in other tissues (3, 10), but the free surface of the epithelium is distinctive in that the stain is taken up here in localized regions. It is the purpose of this report to describe the character of these regions and their fine structure as shown by enzymatic breakdown.

MATERIALS AND METHODS

Experimental material consisted of C57/10 mice which were fed on Rockland Mouse Breeder Diet (Teklad Inc., Monmouth, Ill.). Animals were etherized, and the proximal loop of the duodenum was removed, split open, and cut into 1–2-mm fragments while immersed in cold fixative. Tissue was fixed for 2 hr in 2.5% glutaraldehyde in cacodylate buffer (pH 7.2) to which 1% $La(NO_3)_3 \cdot 6\ H_2O$ had been added. It was then rinsed for 1 hr in buffer with lanthanum nitrate, fixed for ½ hr in 1% osmium tetroxide in cacodylate buffer (pH 7.2), then dehydrated in alcohol and propylene oxide, and embedded in Araldite 502 (9). This procedure was also varied by omitting the postfixation with osmium tetroxide, by omitting the addition of lanthanum nitrate to the buffer rinse, or both. Lanthanum staining for prolonged periods of up to 8 hr did not appear to increase the intensity or extent of localized staining in the brush border, nor did rinsing in buffer alone for up to 12 hr appear to decrease it. Two other staining methods were tried. Following Revel and Karnovsky (10), a solution of lanthanum nitrate was brought to a pH of 7.6–7.8 with 0.01 N NaOH and then added to both the glutaraldehyde and the osmium tetroxide fixatives to a final concentration of 1%. This method gave a localized but coarse precipitate in the brush border and was therefore abandoned. Following Lesseps' method (6), the free cell-surface stained uniformly, as was described by him for other cell surfaces, but showed no indications of a localized effect. Fresh or fixed tissue was treated with a number of chemical agents in an effort to characterize regions of intense staining in more detail. Fresh tissue fragments were treated for 30 min. or less, at 37°C on a shaker, in the following media: Ca- and Mg-free Tyrode's (pH 7.2) with 0.2 g/l EDTA (disodium salt, Fischer Scientific Company, Pittsburgh, Pa.; or tetrasodium salt, Matheson Co., Inc., East Rutherford, N. J.); Ca- and Mg-free Tyrode's (pH 7.2) with 1 mg/ml pronase (Calbiochem, Los Angeles, Cal.; B grade); Ca- and Mg-free Tyrode's with 0.5% trypsin (2X crystal; Nutritional Biochemicals Corp., Cleveland, Ohio); Tyrode's with (pH 7.2) 0.01 or 0.001 mg/ml phospholipase C (Worthington Corporation, Harrison, N. J.; PHLC7CA); Hanks' solution (pH 7.2) with 1 mg/ml α-amylase (Worthington AA); and Hanks' solution (pH 7.2) alone.

Tissue which had been fixed for 15 min in 2.5% glutaraldehyde in the cold, then washed in four to five 10-min changes of Hanks' solution in the cold, was treated with EDTA, trypsin (Tryptar, Armour Pharmaceutical Co., Kankakee, Ill.; 25,000 NF U/ml), phospholipase C, α-amylase, and Hanks' solution alone as described above.

Embedded tissue was sectioned on a Porter-Blum MT-2 ultramicrotome and viewed with no further staining in a Hitachi HU-11A electron microscope.

RESULTS

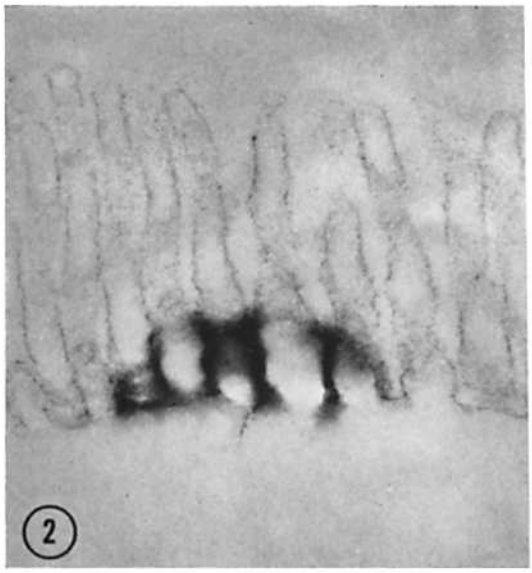
As described by Revel and Karnovsky (10), lanthanum penetrates the interstices between cells but does not as a rule penetrate the cells themselves. In the intestinal epithelium, staining between cells is generally limited to their basal regions and rarely appears between cells towards

their free surface. The only cells into which lanthanum frequently penetrates are the goblet cells. The character of the staining of the free sufrace is illustrated in Fig. 1. At relatively regular intervals, intense patches of stain appear in the brush border, and in addition a fine layer of stain covers the entire surface of the microvilli. A thin section of tissue which was not postfixed in osmium tetroxide shows no structure within the cells and hence shows lanthanum deposition only (Fig. 2). Each microvillus is lightly outlined, and the intensely staining region characteristically forms a mound-like contour when the surface is cut in cross-section. Densely staining regions occur over cell boundaries. Since postfixation in osmium tetroxide permits one to recognize the tissue structure in thin sections, this procedure was used most frequently.

Patches of intense lanthanum staining may appear strikingly symmetrical, but a comparison of Figs. 1, 2, and 3 shows that the patches may also vary considerably in size. The stained region may extend well beyond the tips of the microvilli (Fig. 3). Lanthanum staining over cell boundaries may also be absent (see Fig. 1) or confined to a very small region just above the tight junction. In some sections, through the villi intensely lanthanum-stained patches are reduced in size or are infrequent, but nevertheless this staining is characteristic of the duodenum and has been found in every block sampled in the villus region. Where three cells are juxtaposed, staining is particularly prominent (Fig. 3). Where sections are cut tangential to the surface (Fig. 4), it is also apparent that staining follows cell boundaries; in such a section one can see that the stain fills the region between microvilli solidly. No stain was found associated with the free cell surface in the crypts in a sample of 20 or more blocks studies.

Since different levels of the intestine show bifferent absorptive capacities (11), it seemed worthwhile to compare staining characteristics of the duodenum with those of the jejunum and ileum. Some patches of intense lanthanum staining were found at those levels also, but it was not feasible to make a quantitative comparison. The duodena of newborn mice showed the characteristic staining, as did that of mouse embryos just before term. In the duodena of the three newborn mice which were examined, the lanthanum-staining mound never extended up to or beyond the tips of the microvilli, and in those of mouse embryos the staining was even more confined. Thus the developmental sequence of this staining feature paral-

FIGURE 1 Intestinal brush border with localized lanthanum staining. Arrow indicates cell boundary where ntense lanthanum staining is missing. OsO_4 postfixation. × 4,500.



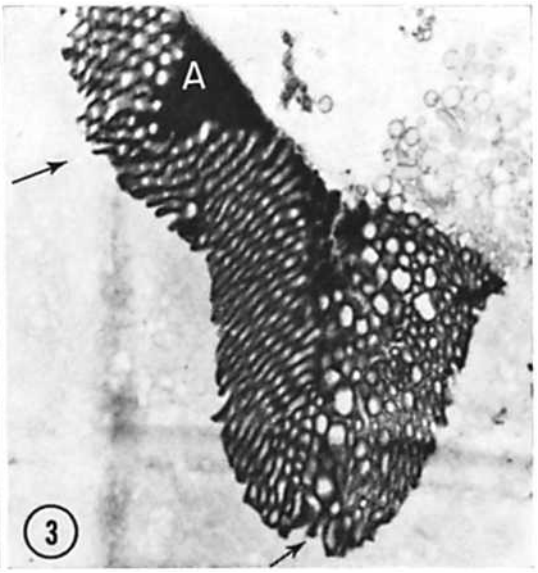


FIGURE 2 Patch of lanthanum staining in brush border, showing variation in size of patches. Glutaraldehyde fixation only. × 45,000.

FIGURE 3 Lanthanum staining where three cells are apposed. Arrows indicate cell boundaries. *A*, a lanthanum-staining material which has accumulated above microvilli. × 11,000.

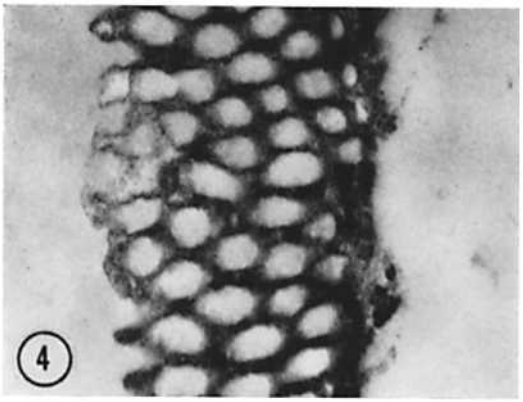


FIGURE 4 Tangential section of surface of brush border in fixed tissue after treatment with α-amylase, showing that lanthanum staining follows cell boundaries and that the stain fills the regions between microvilli. × 16,000.

lels in time that of a number of biochemical and morphological characteristics (7, 8).

Finally, the tissue was treated with several chemical agents. EDTA was chosen since it has been suggested that lanthanum may act by replacing calcium (3). Fresh tissue treated with EDTA for as long as ½ hr showed no patches of intense lanthanum staining at cell boundaries. This treatment, however, caused considerable distortion (2). Therefore the experiment was repeated with fixed tissue. In this case, patches of lanthanum staining were never found with their characteristic conformation, but rather in smaller, dispersed fragments showing a fibrous substructure (Fig. 7). In control experiments, tissue incubated at 37°C in Hanks' solution showed no change.

Attempts at enzymatic degradation were made. Treatment of fresh tissue with pronase or with trypsin (Figs. 5 and 6) resulted in the elimination of characteristic lanthanum-staining patches and in the appearance of masses of lanthanum-staining fibrils between the microvilli or just above the surface of the tissue near cell boundaries. Trypsin digestion of fixed tissue with Armour Tryptar resulted in a more complete breakdown of lanthanum-staining material. The brush border was largely free of any material showing intense lanthanum uptake, though occasionally some small scattered amorphous material remained.

Treatment of fresh tissue with phospholipase C at the lowest concentration used produced tissue disruption. Cells tended to be partially separated

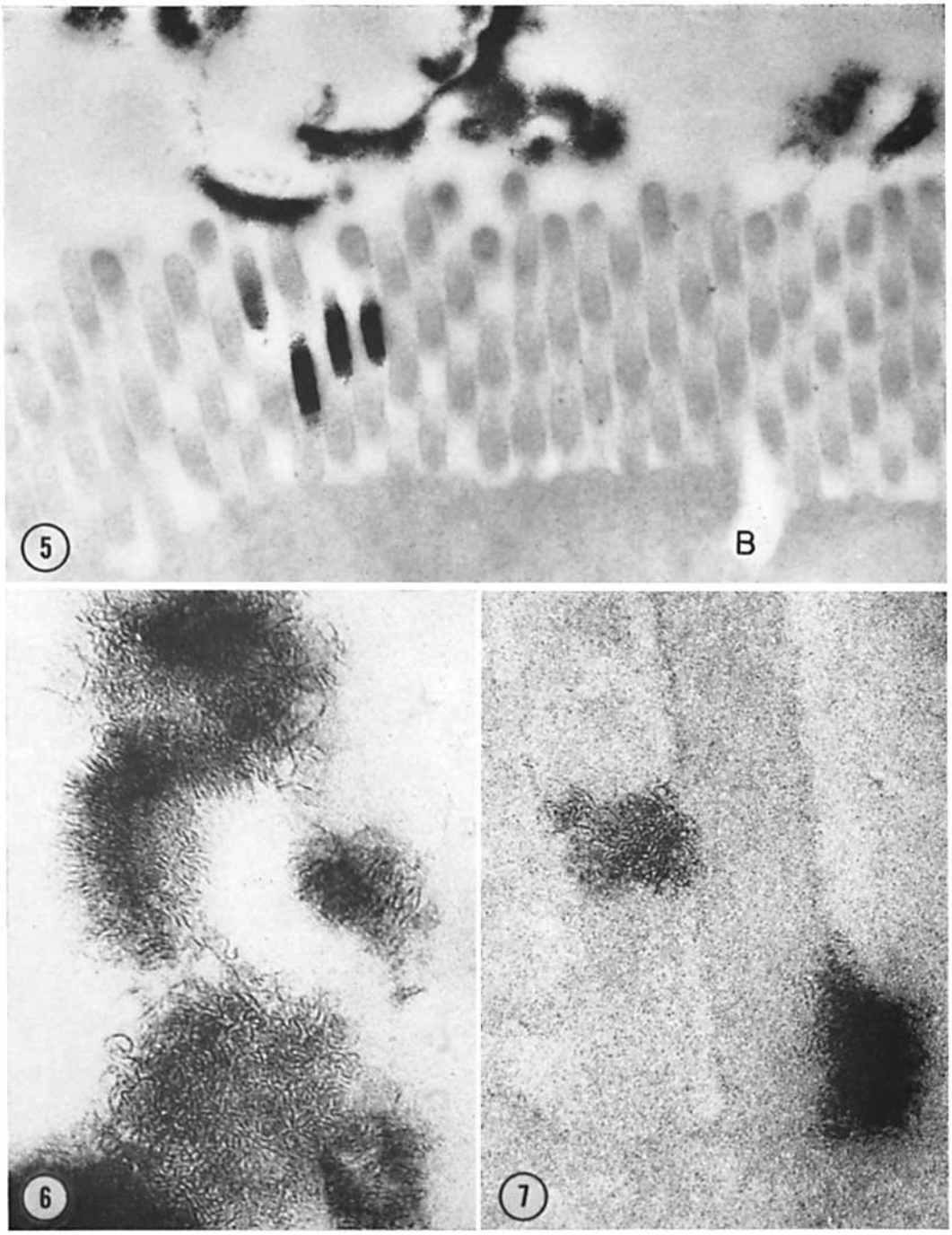


FIGURE 5 Brush border after trypsin treatment of fresh tissue. Note the elimination of characteristic lanthanum-staining patches but the appearance of lanthanum-staining fibrils between microvilli or lying above the surface of the tissue near cell boundaries. *B*, cell boundary. OsO_4 postfixation. × 36,000.

FIGURE 6 Same as Fig. 5. × 160,000.

FIGURE 7 Brush border after EDTA treatment of fixed tissue. Note that the lanthanum-staining patches are in the form of small, dispersed fragments showing a fibrous substructure. × 160,000.

and vacuolated, and the brush border was often free of lanthanum-staining material. No characteristic lanthanum-staining patches were observed. Some fibrous material was found. Fixed tissue treated with phospholipase C, however, gave no indication of enzymatic breakdown and appeared to be entirely normal. In experiments with α-amylase, no apparent disruption of lanthanum-staining patches was present in either fresh or fixed tissue (Fig. 4).

The lanthanum-staining fibrils which appeared after the different treatments described above were all similar. A highly electron-opaque, tortuous strand of rougly 25 A in width was conspicuous.

DISCUSSION

It seems reasonable to accept the lanthanum-staining fibrils as normal tissue components, for two reasons. First, if this amount of material had been forced out from within or between the cells during fixation, one might expect the lanthanum to be able to pass into the cells. Lanthanum does stain the interior of ruptured cells (10), and it stains goblet cells which may rupture during secretion. Second, there is a precedence for finding a fibrous extracellular material associated with the brush border (1, 4, 5). The source of the lanthanum-staining material is unclear, however. There are no morphological clues to suggest that the material arises from the cells which it overlies as in the case of the surface coat (4, 5), nor does any evidence exclude the possiblity that lanthanum-staining material arises elsewhere, for example, from the goblet cells. It has been suggested that lanthanum may replace calcium (3), that it may stain by nonspecific trapping (10), and that it may stain complex molecules (6). With the staining method used here, the morphological appearance of the tissue, as well as its response after enzyme treatment, was quite different from that obtained by Lesseps (6), so that the two cases are not comparable. This method of staining is also different from lanthanum staining as used by Revel and Karnovsky (10) since the stain did not leach out when rinsed for prolonged periods. The fact that treatment with EDTA reduced or abolished the staining in the brush border is consistent with the suggestion of Doggenweiler and Frenk (3) that lanthanum replaces calcium. However, the suggestion of Lesseps (6) that lanthanum may stain complex molecules is certainly not excluded. The results of enzyme treatment also give no indication of function of the material, though they do suggest that the material has a protein component. An attractive notion that could apply to the extracellular material described here is that mucopolysaccharide on the cell surface, or "glycocalyx", may act as an ion trap (1). However, since this material is invariably associated with juxtaposed cell boundaries, it might also function in cell adhesion.

SUMMARY

An extracellular material in the intestinal brush border is described which is associated with limited regions of the free surface near cell boundaries. This material strains when La $(NO_3)_3$ is added to the glutaraldehyde fixative. The material breaks down when treated with EDTA, trypsin, or pronase, revealing its fibrillar structure. The fibrils are long, tortuous, densely staining, and roughly 25 A in diameter.

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