Journal of Cellular Biochemistry 58:56–64 (1995)

Molecular Anatomy of an Endodermal Gland: Investigations on Mucus Glycoproteins and Cell Turnover in Brunner's Glands of *Didelphis virginiana* Using Lectins and PCNA Immunoreactivity

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Abstract Brunner's glands are located in the submucosa of the proximal duodenum and are unique to mammalian species. The North American opossum (*Didelphis virginiana*) is generally regarded as a prototype marsupial that closely resembles fossil didelphids which can be placed at the beginning of mammalian evolution. The current investigation provided an opportunity for the analysis of secretory products from these glands in a species thought to be more closely related to earlier evolutionary forms. Extracts of Brunner's glands were subjected to SDS-PAGE and Western blotting. The results indicate the presence of two high molecular weight PAS-positive glycoprotein bands. In addition to these two PAS-positive bands, several other glycoprotein bands were detected in the high molecular weight range that bind several lectins which typically recognize O-linked carbohydrates indicative of mucus type glycoproteins. The same lectins bind to glandular structures in tissue sections. Comparison of lectin binding sites with the pyloric glands of the stomach and duodenal goblet cells indicates that Brunner's glands carbohydrate residues resemble those of the pyloric glands more closely than those of the duodenal goblet cells. The low cell turnover rate in Brunner's glands is in contrast to the rapid turnover rate of goblet cell precursors in the duodenal crypts. The mucus composition and the cell turnover rate correlate well with embryological data and suggest that Brunner's glands of *Didelphis* evolved from an epithelium more closely associated with the stomach than that of the duodenum as the topography of the gland would suggest.

Key words: Brunner's gland, cell proliferation, glycoproteins, lectins, mucin, PCNA

Brunner's glands are located in the submucosa of the proximal duodenum in all mammalian species so far investigated and secrete a mucus rich in carbohydrates. Brunner's glands of the North American opossum (*Didelphis virginiana*) are of importance for several reasons: 1) opossums are placed phylogenetically nearer the beginning of mammalian evolution [Romer, 1971] and Brunner's glands are unique to mammals [Krause, 1987], 2) *Didelphis* has become a popular animal model for gastroenterological studies [Krause and Cutts, 1992], and 3) the functional significance of the secretory products from Brunner's glands is unclear. While it was previously thought that the secretory product of Brunner's glands (which contains bicarbonate and mucus glycoproteins) protected the luminal surface of the duodenum by neutralizing acid-pepsin secretions from the stomach, recent studies using surgical techniques to ablate Brunner's glands indicate that no ulceration in the duodenum results from such a procedure [Krause et al., 1988]. Therefore, the more general effects attributed to mucus glycoproteins such as selective barrier functions and lubrication [Strous and Dekker, 1992] may be of greater significance than neutralizing gastric secretion. Although some biochemical characterization of mucus glycoproteins from Brunner's glands of the rat [Smits et al., 1982a,b] and man [Smits and Kramer, 1984] has been done and histochemical information on Brunner's glands in New World monkeys is available [Posel et al.,

Received July 13, 1994; accepted October 5, 1994.

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1988], no data on Brunner's glands glycoproteins in situ and in isolation are available from marsupials. Brunner's glands of Didelphis are unusual in that they are concentrated in the submucosa at the gastrointestinal junction and can be separated from the remainder of the digestive tract by microdissection without contamination from other mucin-producing cells and glands. Since data on mucus composition combined with lectin histochemistry on Brunner's glands from a species nearer the evolutionary beginnings of mammals, which could be isolated and examined in pure form, is lacking, we decided to undertake the following study. In addition, an estimate of the cell turnover rate in these glands was made utilizing immunohistochemistry with monoclonal antibody PC10. The latter recognizes proliferating cells in processed tissue from a wide variety of species [Møllgård and Schumacher, 1993]. Cell proliferation rates often provide important insights into the dynamics of a tissue.

MATERIALS AND METHODS

Biochemistry

Brunner's glands from 12 individuals were resected from the duodenum, frozen, and held at −20°C until biochemical analysis. The thawed duodenal glands were minced with a razor blade and homogenized in 10 volumes of lysis buffer containing phenylmethylsulfonylfluoride [Schumacher et al., 1993] in a Potter homogenizer. The homogenate was centrifuged at 3,000*g* for 20 min to remove debris. The (glyco)proteins in the supernatant were concentrated by a modified Wessel and Flügge method which has been shown to precipitate high molecular weight glycoproteins [Schumacher et al., 1992]. The pellet was resuspended in the lysis buffer used for SDS-PAGE in a mini protean II chamber (Bio-Rad Hemel, Hempstead Herts., UK) and after the run the gels were stained with Coomassie Brilliant Blue or with the periodic acid Schiff's reaction (PAS). After Western blotting the following peroxidase-labeled lectins or biotinylated lectins with avidin peroxidase (Sigma, Poole, Dorset) were used: HPA, GSA-I, UEA-I, PHA-L, and Con A (for lectins, their abbreviation, and sugar specificity see Table I). The lectin binding sites were visualized by DAB/H_2O_2. For technical details see Schumacher et al. [1993].

TABLE I. Origin of the Lectins Used in This Study, Their Abbreviations, and Their Nominal Monosaccharide Specificity

Lectin	Abbreviation	Sugar specificity
Dolichos biflorus	DBA	N-acetylgalactosamine
Glycine max	SBA	N-acetylgalactosamine
Griffonia simplicifonia-I	GSA-I	N-acetylgalactosamine
Helix pomatia	HPA	N-acetylgalactosamine
Vicia villosa	VVA	N-acetylgalactosamine
Wistaria floribunda	WFA	N-acetylgalactosamine
Arachis hypogaea	PNA	Galactose
Artocarpus integrifolia	JAC	Galactose
Maclura pomifera	MPA	Galactose
Triticum vulgaris	WGA	N-acetaylglucosamine
Canavalia ensiformis	Con A	Mannose
Lotus tetragonolobus	LTA	Fucose
Ulex europaeus-I	UEA-I	Fucose
Maackia amurensis	MAA	N-acetylneuraminic acid
Sambucus nigra	SNA	N-acetylneuraminic acid
Phaseolus vulgaris-L	PHA-L	Complex branched carbohydrates

Histochemistry

The gastroduodenal transition zones containing Brunner's glands from four animals were fixed overnight in 4% neutral buffered formalin and processed into paraffin. Sections seven microns in thickness were cut and adhered to poly-L-lysine–coated slides without heating to avoid destruction of immunoreactivity against PC10 [for details of staining see Møllgård and Schumacher, 1993]. As a general stain for orientation, hematoxylin- and eosin- (HE) stained slides were used. Furthermore the periodic acid Schiff (PAS) reaction as a general staining method for neutral carbohydrate residues was carried out on the slides. The same sections were used for staining with FITC-labeled lectins obtained from

Sigma [for technical details of the staining see Schumacher et al., 1994]. The fluorescent intensity was semiquantitatively evaluated from 0 = no fluorescence labeling to ++++ = very intense labeling. Controls were carried out with the appropriate inhibitory monosaccharides.

RESULTS

Biochemistry

At least 22 separate (glyco)protein bands were consistently stained in several experiments using the combined PAS–Commassie Brilliant Blue gel (Fig. 1b). Using the PAS stain alone, one glycoprotein band was found on top of the stacking and one on top of the running gel (Fig. 1c). Many more glycoprotein bands were detected in the Western blots (Fig. 1d–h). GSA-I (Fig. 1d) stained fewer bands than HPA (Fig. 1e), which stained the band at the beginning of the running gel intensely. UEA-I stained fewer bands than the two former lectins (Fig. 1f). PHA-L did not stain glycoproteins in the higher molecular weight range (Fig. 1g), nor did Con A which stained glycoproteins in the lower molecular weight range particularly well (Fig. 1h).

Histology and Histochemistry

In HE sections, the majority of the cytoplasm of epithelial cells forming Brunner's glands remained unstained, while a narrow rim beneath the plasma membrane and the nucleus were stained (Fig. 2). In contrast, the PAS reaction gave a foamy appearance, with the apical region often being more intensely labeled than the basal portion of the cell (Fig. 3). All cells comprising the secretory epithelia of Brunner's glands reacted consistently, uniformly, and intensely to HPA (Fig. 4), WGA, and UEA-I (Fig. 5) in all animals investigated. The reactivity of these lectins covered almost the entire cell, and a regional distinction as seen with the PAS reaction was not possible. In addition to the above mentioned lectins, the secretory epithelia reacted, but with less intensity, to GSA-I, MPA, and JAC (for complete results see Table II). The ductular epithelium showed a different reaction pattern; often the apical region reacted differently than did the remaining cytoplasm (see, e.g., DBA, VVA, and WFA in Table II). In comparison to the secretory epithelia of Brunner's glands, goblet cells in the duodenum reacted with fewer lectins, while in enterocytes the Golgi region and the brush border region could clearly be delineated by their lectin binding pattern (Fig. 7). Apical, cytoplasmic, and Golgi region lectin binding patterns could be distinguished in the gastric lining epithelium of the pyloric stomach (Fig. 8), whereas the epithelium constituting Brunner's glands reacted uniformly without cytological subdivision. The pyloric glands of the stomach reacted with five lectins that also reacted with Brunner's glands (Table II). Complete inhibition of lectin binding to tissues was achieved by inhibitory sugars in the case of VVA, WFA, PNA, Con A, UEA-I (Fig. 6), MAA, and SNA, whereas for GSA-I, HPA, JAC, and MPA a considerable reduction in fluorescence was achieved. In contrast, almost no inhibition was observed in WGA.

Intense PCNA immunoreactivity was observed in the nuclei of cells at the bases of the duodenal crypts (Fig. 9) and in the neck region of the pyloric glands (Fig. 10). In contrast, immunoreactivity in the nuclei of cells comprising Brunner's glands was absent or very faint; only cells within the connective tissue septae of these glands reacted strongly with this antibody (Fig. 11).

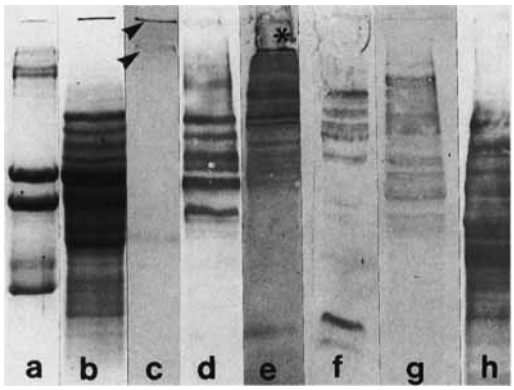


Fig. 1. SDS-PAGE and Western blotting of Brunner's glands extracts. Lanes a–c gels, lanes d–h nitrocellulose strips revealing lectin binding sites. Lane a: High molecular weight standards (in KD) from top to bottom: thyroglobulin 669, ferritin 440, catalase 232, lactate dehydrogenase 140, bovine serum albumin 67. Lane b: Combined Coomassie Brilliant Blue and PAS stained gel. Lane c: PAS stained glycoproteins; note the PAS positive band at the top of the stacking gel and a second one at the beginning of the running gel (arrowheads). Lane d: GSA-I binding. Lane e: HPA binding; note the HPA binding in the stacking gel (asterisk). Lane f: UEA-I binding. Lane g: PHA-L binding. Lane h: Con A binding.

DISCUSSION

This study has demonstrated the presence of high molecular weight PAS positive glycoproteins in extracts of Brunner's glands from a marsupial. The position of these glycoproteins

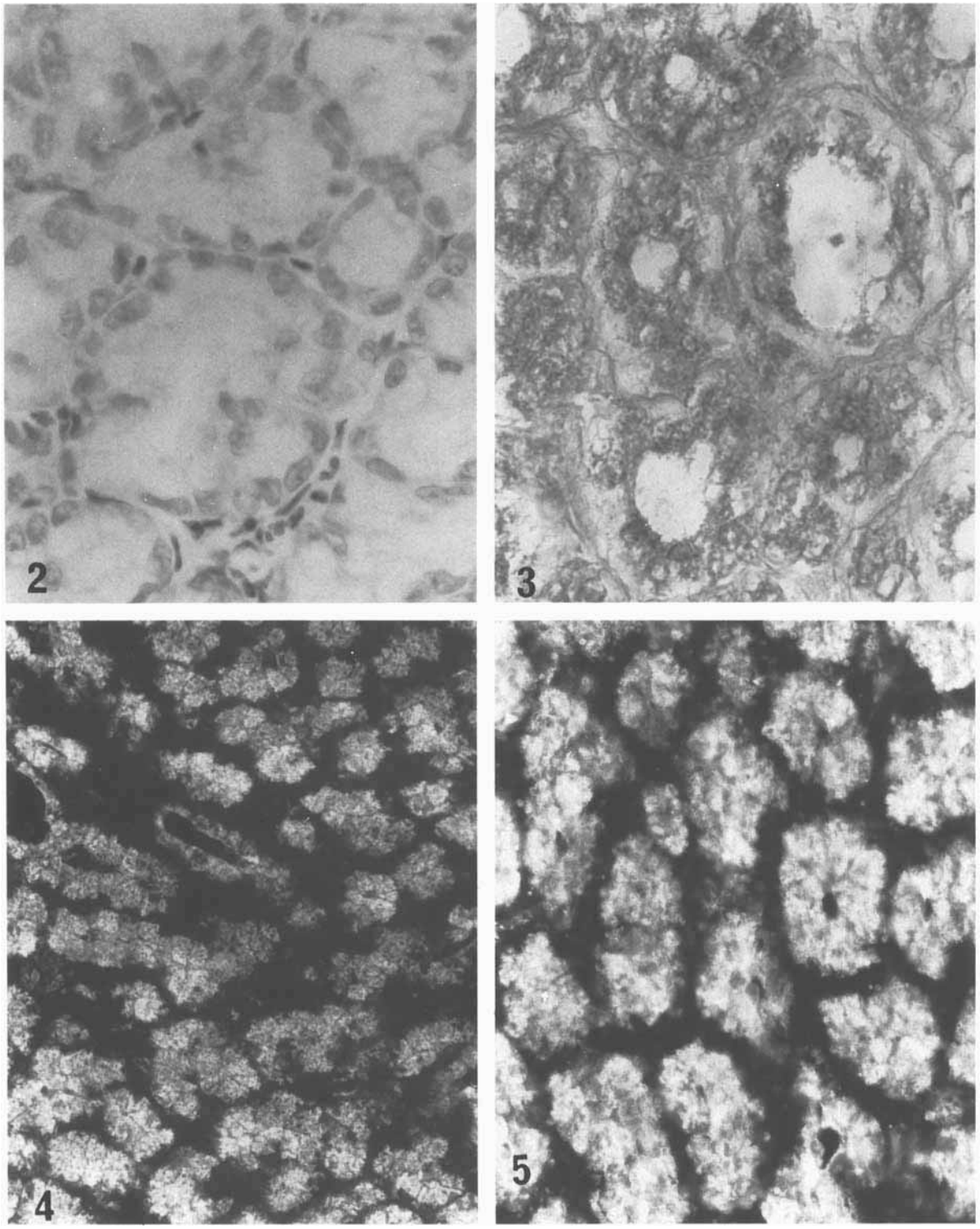


Fig. 2. Hematoxylin- and eosin-(HE) stained section of Brunner's gland. Note that only a rim of cytoplasm and the nuclei are stained which is typical for mucous glands. ×1,200.

Fig. 3. Periodic acid Schiff (PAS) reaction of Brunner's gland without counterstain. Note the foamy appearance of the reaction product in the apical region of the secretory epithelia where the mucinogen granules are concentrated (compare with Figs. 4, 5). ×1,200.

Fig. 4. Intense binding of HPA-FITC to Brunner's gland secretory epithelium. Note the uniform distribution of the fluorescence over the entire cytoplasm of the cells forming the secretory epithelium as compared with Figure 3. ×250.

Fig. 5. A similar lectin binding pattern as demonstrated with HPA-FITC is achieved with UEA-I. ×800.

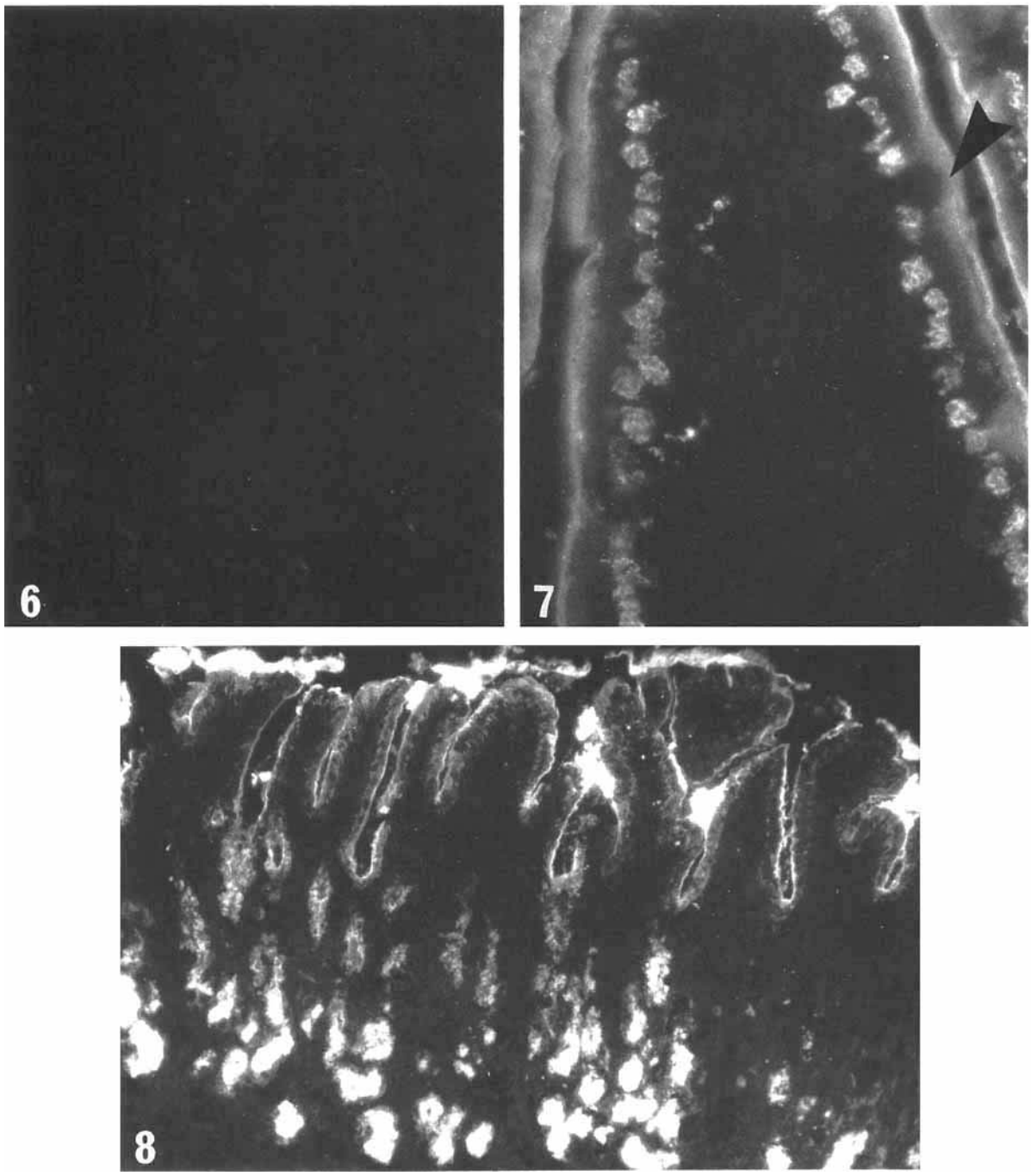


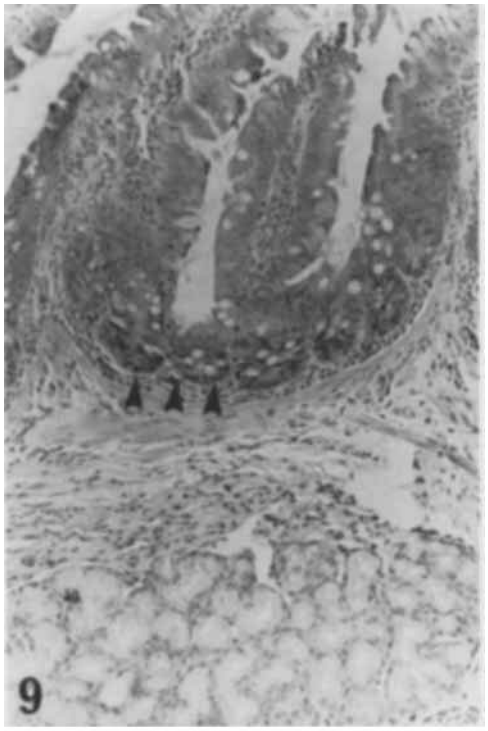
Fig. 7. In contrast to Figure 2, binding of HPA in the duodenum is restricted to the Golgi region and the striated (microvillus) border of the enterocytes. Note the absence of fluorescence in the goblet cell (arrowhead). ×800.

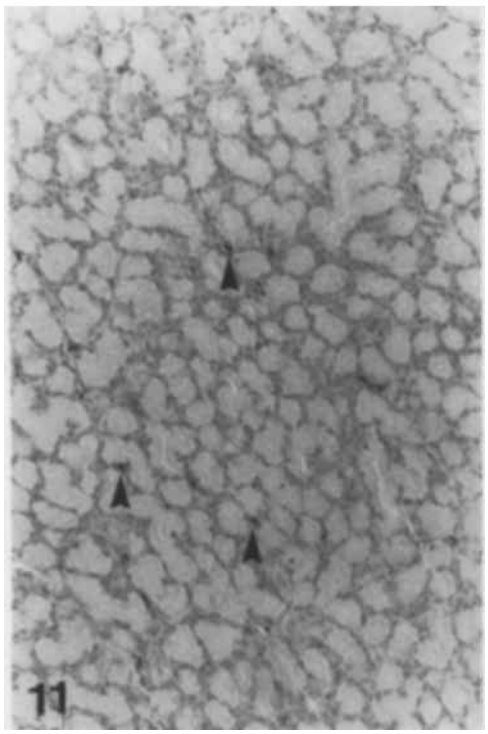
Fig. 8. In the pyloric stomach, HPA labels the glandular cells and the mucus intensely; the surface lining epithelium also is labeled. ×125.

TABLE II. Summary of the Lectin Binding in Brunner's Glands, Duodenum, and Pyloric Stomach*

	Stomach				Duodenum			Brunner's gland		
	Surface epithelium					Enterocyte			Duct	
Lectins	Apex	Cytoplasm	Golgi	Glands	Goblet cells	Brush border	Golgi	Secretory cell	Apex	Cytoplasm
DBA	(+)–++	0–+	0–+	0	0	0	0	0	0–++	0–(+)
SBA	0	0	0	0	0	0	0	0	0	0
GSA-I	0	0	(+)–+	0–+	0	0	0–+	+–++	+–++	0–+
HPA	0	0	(+)–++	0–+++	0	+–++++	+–++	+++	++–+++	0–++
VVA	+–+++	0–++	0–++	0–(+)	0	0	0	0	0–++	0
WFA	+–++	+–(+)	(+)–++	0–+	0	0–+	0	0–(+)	(+)–++	0
PNA	+++	+–++	+–++	+–++	0–++	0–+	0	0	0–+	0
JAC	+–+++	+–++	+–++	+–++	+–+++	+++	0–+	(+)–++	(+)–+++	0–++
MPA	+–+++	0–++	(+)–++	++	0–+	+–++	(+)–+	+–++	+–+++	+–++
WGA	++++	++–+++	++–+++	+++	0–++	+++	0	+++	+++–++++	++–+++
CON A	0–+	0	0	0	0	0–++	0	0	0	0
LTA	0	0	0	0	0	0	0	0	0	0
UEA-I	+++–++++	+–+++	+–+++	++–+++	++–+++	+–++	0	++	++–+++	++
MAA	0–++	0–+	0	0–++	0	0	0	0	0	0–+++
SNA	+–++	0	0	0	0	0	0	0	0	0
PHA-L	0	0	0	0	0	0	0	0	0	0

*The fluorescent intensity was semiquantitatively evaluated from 0 = no fluorescence labeling to ++++ = very intense labeling.





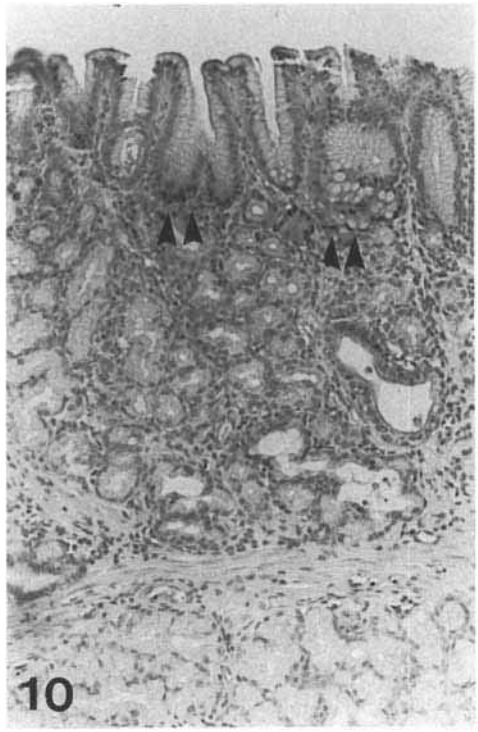


Fig. 9. PCNA immunoreactivity in duodenal crypts where the PCNA immunoreactivity is confined to the nuclei of dividing cells (arrowheads) at the base of the crypt. ×300.

Fig. 10. In the stomach, PCNA immunoreactivity is found primarily in the nuclei of dividing cells in the neck region of the pyloric glands (arrowheads). Note that in the secretory units of the pyloric glands the proliferative activity is much less. ×300.

Fig. 11. In Brunner's glands the PCNA-labeled nuclei (arrowheads) are found mainly in the connective tissue septae. ×300.

in the beginning stacking gel or at the beginning running gel is typical for mucins and was reported for rat duodenal mucin as well [Smits et al., 1982b]. Some of this mucus glycoprotein penetrated into the stacking gel since this region was positive for the lectin HPA in Western blots

demonstrating the higher sensitivity of this method over the PAS stain. The greater sensitivity of the lectins over the PAS reaction also is mirrored in the tissue sections where several lectins including HPA, UEA-I, and WGA react with carbohydrate moities throughout the cytoplasm, whereas the PAS reaction is confined primarily to the apical region. This probably is due to the fact that mucinogen-containing granules are few in the region of the nucleus and endoplasmic reticulum and the number of granules insufficient to be demonstrated by PAS. The higher sensitivity of the lectins on Western blot over PAS SDS-PAGE gels is not particular to this system and has been described in other mucin-containing systems such as the milk-fat-globule membrane [Welsch et al., 1988]. The presence of mucus-type glycoproteins is con-

firmed in tissue sections of the secretory epithelium from Brunner's glands since lectins specific for N-acetylgalactosamine, galactose, N-acetylglucosamine, and fucose, which are specific for O-linked or mucus-type glycoproteins [Strous and Dekker, 1992], are present. The binding pattern of N-acetylgalactoseamine–specific lectins to mucins is complex despite having the same nominal monosaccharide specificity. Our findings indicate that subterminal carbohydrate residues influence the binding behavior of the lectins and can account for this variety. Indeed, the more specific complex saccharides which have higher binding affinities than monosaccharides are quite complex and differ from each other even within the group of N-acetylgalactoseamine–specific lectins [for more refined sugar specificities see Wu et al., 1988]. The problem of specificity with lectin binding to tissue and blot structures also raises the question of specificity of the inhibitory sugars used. For most of the lectins used, a complete or near complete inhibition of binding was observed, suggesting specificity of binding. No inhibition of WGA binding was observed. This could indicate that either WGA binding is nonspecific or that the monosaccharide N-acetyglucosamine inhibitory sugar had a lower affinity than the higher affinity binding sites in the tissue and thus was not able to replace its binding. Since N-acetylglucosamine is such a ubiquitous sugar in mucin-type glycoproteins [Strous and Dekker, 1992] the latter explanation of why no inhibition occurred in the inhibitory experiments is favored.

Mannose and complex carbohydrate-specific lectins did not bind to tissue sections. These two lectins are specific for N-linked glycoconjugates and only very rarely occur in mucins [Strous and Dekker, 1992]. Our findings demonstrate the presence of several glycoprotein bands which stain with lectins specific for O-linked glycoproteins. The correlation of binding patterns of the O-linked–specific lectins such as GSA-I, HPA, and UEA-I in the tissue sections as well as in blots is good. Binding sites for Con A, which binds to immature mannose-containing glycoproteins and for PHA-L (complex type N-linked glycoproteins) was found on blots only and not in tissue sections, again indicating that the blots are the more sensitive tool for the detection of lectin binding sites.

Phylogenetic and developmental studies on the origin of Brunner's glands in prototherian mammals such as the echidna (*Tachyglossus aculeatus*) have demonstrated that in this species they arise as invaginations from a columnar epithelium that lines the stomach of the suckling young [Krause, 1970]. Brunner's glands of *Didelphis virginiana* (a methatherian) arise from a fringe of undifferentiated gastric lining epithelium that extends a short distance beyond the pyloric sphincter into the duodenum and from a narrow band of intestinal epithelium in the proximal most portion of the duodenum [Krause and Leeson, 1969]. If the lectin binding pattern of Brunner's glands is compared to other mucus-producing cells in this region, it is of interest to note that the secretory epithelium of Brunner's glands has three lectin binding patterns (GSA-I, HPA, WFA) in common with the pyloric glands of the stomach which do not react with goblet cells in the duodenum. No exclusive Brunner's glands to goblet cell binding patterns are observed. From the analysis of glycoproteins it appears that epithelial cells of Brunner's glands are more closely related to the pyloric glands of the stomach than to duodenal goblet cells. Such a concept is supported further by the PCNA immunoreactive data which shows that the cellular proliferation pattern in Brunner's glands resembles that of the pyloric glands, both of which are relatively low proliferating compartments. This pattern is quite different from that of precursors of the goblet cells whose turnover rate in the crypts is quite high in comparison.

ACKNOWLEDGMENTS

The skilful technical assistance of Mrs. V. Stauber is gratefully acknowledged.

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