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Microvillus Membrane Differentiation: Quantitative Difference in Cholera Toxin Binding to the Intestinal Surface of Newborn and Adult Rabbits

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Summary

Microvillus membranes (MVM) were isolated from newborn and adult New Zealand rabbit small intestine. The isolation procedure provided a mean enrichment of 25 ± 4 for sucrose activity in adult preparations and of 27 ± 3 for lactase activity in newborn preparations. These purified MVM were incubated with increasing concentrations of ^{125}I -labeled cholera toxin (CT). ^{125}I -CT binding to adult MVM reached saturation at 6.4×10^{-9} M; in contrast ^{125}I -CT binding to newborn MVM did not reach saturation but instead continued to increase with increasing ^{125}I -CT concentrations. Scatchard plot analysis of adult data supported the existence of a single binding site ($K_d = 1.2 \pm 0.2 \times 10^{-9}$ M); analysis of newborn data, however, suggested the existence of additional binding sites, as ^{125}I -CT binding to newborn MVM was inhibited by preincubation with unlabeled CT. These results show that CT binding to both preparations is quantitatively different and is higher in newborn preparations. This difference may be accounted for by the existence of additional binding sites in newborn MVM preparations in contrast to the presence of only the unique receptor previously reported in adult MVM preparations.

Abbreviations

MVM, microvillus membrane

CT, cholera toxin

Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

In many species, the small intestines undergo important modifications during postnatal development such as structural changes of the epithelial layer (6) or changes in MVM enzymatic activities (25). These modifications are likely to induce, as a

result, changes in MVM surface properties that may be reflected by events such as the "closure" of macromolecular transport through the small intestine (24). Comparison of MVM surface properties between newborn and adult are not yet available. Indirect approaches, such as those of Extler and Branstrator (9), have been used to study the lectin-binding capacity of small intestine mucosa during postnatal development; however, examination of histologic preparations under light microscopic conditions does not allow for a more precise conclusion about MVM-binding properties to be made.

In order to study MVM surface property changes with differentiation, we designed an *in vitro* system using MVM vesicles purified from newborn and adult rabbit small intestine, and tested these vesicles for binding characteristics using a molecular probe with an extensively studied microvillus receptor.

MATERIALS AND METHODS

Microvillus membrane isolation was performed according to a modification (3) of the Ca^{2+} precipitation method (27). Fasted adult ($n = 8$; more than 8 weeks old) and newborn ($n = 50$; 12 to 24 h of life) New Zealand rabbits (Margaret's Home Farm, Greenfield, MA) were sacrificed and the small intestines were removed and washed with cold saline. The following steps were carried out at 4°C . Scrapings of the whole adult mucosa or 4–5-cm pieces of opened newborn small intestines were homogenized in 300 mM mannitol, 12 mM Tris buffer at pH 7.4, diluted 1:6, and filtered on a $40\text{-}\mu\text{m}$ nylon mesh (Tetko, Elmsford, NY). CaCl_2 was added to a final concentration of 10 mM. After 15 min on ice, homogenates were centrifuged at $2500 \times g$ for 15 min on a Beckman J21B centrifuge with a JS7.5 rotor. Supernatants were saved and centrifuged with a JA20 rotor at $28,000 \times g$ for 30 min, then suspended in 50 mM mannitol, 1 mM Hepes buffer at pH 7.4, and centrifuged again. The pellets (crude preparation) were resuspended in 1 mM Hepes buffer and applied on the top of 10 to 60% sucrose gradients (Sigma Co., St. Louis, MO) which were centrifuged at $64,000 \times g$ for 25 min on a Beckman L5-65 ultracentrifuge with a SW 28 rotor. Bands were removed from the top, diluted 1:10, centrifuged at $100,000 \times g$ for 60 min on a 50 Ti rotor. Pellets (final preparation) were

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resuspended in distilled water. Proteins were assayed according to Lowry *et al.* (18); disaccharidase activities were according to Dahlqvist (8); organic phosphorus measurements were performed according to McClare (21), and used as an indirect assessment of membrane phospholipids, assuming that their mean molecular weight was 750; electron micrographs were done on final preparations either fresh or after Epon embedding according to Luft (19).

Kinetic studies were performed using purified CT obtained from Schwartzman Co. (Orangeburg, NY). After reconstitution in 0.25 M sodium phosphate buffer, and filtration on Sephadex G-75 (Pharmacia), the toxin was labeled, using Bolton-Hunter reagent (2) and separated from free label by an additional gel filtration. Cholera toxin concentration was measured by spectrophotometry, assuming $A_{1\text{cm}}^{1\%} = 11.42$ (16). The mean specific activity was 200 mCi/ μmol . Experiments were carried out as described by Cuatrecasas (7). A previous report (7) and preliminary experiments (30) showed that ^{125}I -CT binding to MVM was complete within 10 min and irreversible. Samples of adult or newborn preparations (50 μg of membrane protein) were incubated for 20 min at 37°C with 0.08, 0.32, 0.64, 0.96, 1.28, 1.9 and 2.56 pmol of freshly prepared ^{125}I -CT in a 0.2-ml final volume. Samples were diluted 1:10 with cold buffer and immediately poured on EGWP 25 Millipore filters (Millipore, Bedford, MA) which were washed, dried, and counted in a Beckman Gamma 7000 gamma counter. For each experiment, at each concentration, controls were done in which microvillus membranes were replaced by the same volume of buffer; these controls

were processed as described for samples and counted to determine the nonspecific binding of ^{125}I -CT to filters. Inhibition studies were done by preincubating samples from either preparation, at 37°C with unlabeled CT for 10 min. ^{125}I -CT was then added and each sample was incubated for another 15 min at 37°C; in this time interval, no exchange between labeled and unlabeled CT was observed (7). Student's *t* test was used to compare newborn and adult data; values are given as mean $\pm$ SD.

RESULTS

Purity of microvillus membrane preparation. Purification of microvillus membranes was monitored by assays of sucrase activity in adult preparations and lactase in newborn ones. The adult final preparation yielded $1.8 \pm 0.3\%$ of the homogenate protein amount, with a mean enrichment factor of 25 ± 4 for sucrase activity. The newborn final preparation yielded $2.1 \pm 0.2\%$ of the homogenate protein amount, with a mean enrichment factor of 27 ± 3 for lactase activity. Newborn microvillus membranes appeared to be lighter than adult ones on velocity gradients (density of 1.102 versus 1.150), the protein to phospholipid ratio was 6.35 ± 0.26 in adult final preparations as opposed to 2.7 ± 0.5 in newborn preparations ($p < 0.001$). Electron micrographs showed that membranes were present in the form of closed vesicles which displayed "knob-like" formation on their external side in adult preparation; vesicles from newborn preparations however did not demonstrate such an asymmetry (Fig. 1).

Fig. 1. A, electron micrographs of adult final MVM preparation after Epon embedding. B, negative staining of newborn final MVM preparation.

¹²⁵I-CT binding to adult and newborn MVM. ¹²⁵I-CT binding reached a steady state in 10 min at all the concentrations studied. Binding to adult MVM reached a plateau at ¹²⁵I-CT concentrations of 6.4×10^{-9} M (Fig. 2). Scatchard analysis (30) of these data provided a straight line ($r = 0.98$; $p < 0.001$) with a $K_d = 1.2 \pm 0.2 \times 10^{-9}$ M and a binding site concentration of 1.1×10^6 M (2.6×10^9 binding sites/ μ g of MVM protein). In contrast, saturation of binding to newborn MVM was observed at concentrations up to five times the CT concentration at which ¹²⁵I-CT binding to adult MVM reached saturation (Fig. 2). ¹²⁵I-CT binding to newborn MVM increased linearly with increasing CT concentration. Scatchard analysis of these data provided a curve suggesting that either nonspecific binding or additional receptor sites were present (Fig. 3). To test specificity of ¹²⁵I-CT binding to these membrane preparations, inhibition studies were performed. ¹²⁵I-CT binding to adult MVM was inhibited by preincubation with 37.5×10^{-9} M unlabeled CT; ¹²⁵I-CT binding to newborn MVM was also inhibited by preincubation with unlabeled CT, higher concentrations of unlabeled CT causing greater inhibition (Fig. 4).

DISCUSSION

Enrichment in sucrase and lactase activities obtained in the microvillus membrane preparations are in close agreement

Fig. 2. Binding of ¹²⁵I-CT to adult (●) and newborn (○) membranes. Each point is the mean of at least eight experiments; CT binding to each preparation is significantly different beyond 0.96 pmol.

Fig. 3. Scatchard plot analysis of ¹²⁵I-CT binding to newborn and adult MVM. Scatchard plot of adult data is consistent with a single class of receptor sites with $K_d = 1.2 \pm 0.2 \times 10^{-9}$ M. Analysis of newborn data suggests that additional binding might either be due to nonspecific binding or to additional receptor sites.

Fig. 4. Inhibition of ¹²⁵I-CT binding to newborn MVM by preincubation with 0.8 (○), 0.96 (●), and 1.8 (▲) pmol of unlabeled CT.

with reports by Schmitz *et al.* (27) and other workers (11). Unlike adult small intestine, which gave comparable results using either fresh or frozen preparations, newborn MVM purification gave better results with fresh material. Physical and chemical properties of the two preparations seem to differ markedly, as suggested by differences in apparent velocity on differential centrifugations already noted by Galand and Forstner (11), and by difference in the protein to phospholipid ratios; the observed value in adult MVM is close to what has been found by Hauser *et al.* (14) and the difference between newborn and adult preparations is in close agreement with the results obtained by Glickman and Bonhours (12) with crypt and villus cells. The morphology of vesicles in our adult MVM preparations supports the fact that most of them are "right-side out" (13, 17, 20); although it is already known that vesicles isolated from infant rats are largely oriented right-side out (29), it is not as certain in our newborn preparations. However, since it has been shown that glycoproteins and glycolipids are located on the external surface of the cell (5, 10, 15), the presence of "inside out" newborn vesicles could only result in an underestimation of cholera toxin binding to newborn MVM.

Binding of ¹²⁵I-CT to adult microvillus membranes is saturable and inhibited by unlabeled CT. Scatchard analysis yields results suggesting the existence of only one type of receptor; both K_d ($1.2 \pm 0.2 \times 10^{-9}$ M) and receptor concentration (2.6×10^9 receptors/ 10^{-6} g of MVM protein) are in close agreement with results obtained in other systems (14). In contrast, binding of ¹²⁵I-CT to newborn MVM is higher than to adult ones and this difference might be underestimated if a portion of vesicles were oriented as inside out in the MVM preparation purified from newborns. This difference in binding capacity could be explained either by nonspecific binding or by additional binding sites; however, inhibition of ¹²⁵I-CT binding to newborn MVM by preincubation with unlabeled CT suggests that the difference is accounted for by the existence of additional CT receptor sites. Scatchard analysis of newborn data is then consistent with the presence of several types of receptors with decreasing binding affinity. Although monosialoganglioside GM_1 is thought to be a specific receptor for CT, binding to MVM glycoproteins (23, personal observation) has been observed as well as inhibition of CT binding to GM_1 by other glycosylated molecules (7). It was recently shown (28) that interaction between CT and GM_1 only involves the nonreducing end of the glycosylated chain, meaning that CT probes for a family or families of glycosidic side chains present on membrane lipids and/or membrane glycoproteins.

The present results then suggest that CT is able to recognize glycosylated molecules present in the newborn MVM but absent in adult MVM. Such a difference could be due to: 1) complete disappearance of MVM binding molecules with differentiation or 2) disappearance of the binding capacity by partial structural modification of the binding molecules due to a change in glycosylation of membrane components in the fully mature enterocytes. Our data (4) as well as results from Miki *et al.* (22) and from Auricchio *et al.* (1) do suggest that changes in glycosylation of membrane components occur with age-related differentiation.

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Immune Response to Respiratory Syncytial Virus: Prevention of Syncytia Formation by Human Serum during *in Vitro* Infection

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Summary

Human serum specimens containing respiratory syncytial virus (RSV)-specific neutralizing antibody were found to prevent the formation of syncytia when applied to HEP-2 tissue culture monolayers which had been infected with RSV 12 h previously. This was evidenced by the demonstration of RSV-infected cells

without any syncytia formation in the monolayers treated with RSV antibody-positive serum. On the other hand, widespread syncytia formation was observed with antibody-negative control serum. The inhibitory effects of RSV antibody progressively declined when applied beyond 12 h after infection. Protection of the monolayer against syncytia formation occurred only in the presence of antibody and was quickly lost after the serum was removed. The titer of antisyncytial antibody correlated with the titer of neutralization antibody.

Abbreviations

RSV, respiratory syncytial virus
CPE, cytopathologic effects

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