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DEVELOPMENT OF THE GASTROINTESTINAL MUCOSAL BARRIER

EVIDENCE FOR STRUCTURAL DIFFERENCES IN MICROVILLUS MEMBRANES FROM NEWBORN AND ADULT RABBITS

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Electron spin resonance (ESR) and spin label methods with 5-doxylstearic acid as a probe were used to investigate the structure of microvillus membrane from the small intestine of adult and newborn rabbits. The spin label in microvillus membrane of newborns appeared to be in a more disordered environment than spin label in microvillus membrane of adult animals in the temperature range from 4 to 56°C. In addition, a temperature transition at 39.6 ± 0.3°C was observed in the temperature dependence of the hyperfine splitting parameter for microvillus membrane from adult animals whereas a linear temperature dependence of the hyperfine splitting parameter was found for microvillus membrane from newborns. Cholera toxin was used as an external stimulus to test for the structural response in these two membrane preparations. Cholera toxin at 6 pM caused a decrease in the hyperfine splitting parameter at temperatures below 40°C and a shift in the temperature break from 39.6°C to 30.7°C in microvillus membrane from adults. Using microvillus membrane from newborns, the temperature dependence of the hyperfine splitting parameter remained linear with a cholera toxin stimulus and the disordering effect of cholera toxin was only observed below 30°C. These studies suggested that microvillus membrane from newborns were inherently more disordered than microvillus membrane from adult animals and that this difference in membrane organization might in part account for the increased attachment and penetration of macromolecules noted during the perinatal period.

Introduction

The microvillus surface of the small intestine represents an important barrier to the penetration of foreign substances such as proteins, toxins, bacteria and viruses present in the external environment. In previous studies from this laboratory performed with everted gut sacs in vitro [1,2] and with mesenteric lymph fistulas in vivo [3], it was noted that a trace protein-enzyme, horseradish peroxidase, was taken up by pinocytosis into enterocytes and subsequently transported to the extracellular space of the lamina propria and from there into lymph. In subsequent physiologic experiments, radiolabelled bovine serum albumin (^{125}I-BSA) was noted in the circulation 4 h after the oral feeding of bovine serum albumin to adult rats [4]. These combined experiments suggested that macromolecules could be transported intact across the small intestine into the systemic circula-

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tion. In recent studies, we have reported that bovine serum albumin was transported in larger quantities across the small intestine of newborn and young rabbits compared to adult rabbits after oral ingestion of the protein [5]. This apparent increase in macromolecular transport in newborn and young animals suggests that the mucosal barrier to foreign materials may be incomplete in the newborn period. Since attachment of macromolecules to the intestinal surface (adsorption) represents an important initial step in the pinocytosis of antigens and the structure of microvillus membrane may influence its interaction with macromolecules, the present study was designed to investigate if any structural differences could be demonstrated in microvillus membrane isolated from the small intestine of newborns compared to adult rabbits.

Electron spin resonance (ESR) with various kinds of spin labels has been widely used as an important technique for probing membrane structure [6,7]. In this study, we used the spin label 5-doxylstearic acid to compare the structure of microvillus membrane isolated from the small intestine of newborn and adult rabbits. In addition, cholera toxin, a bacterial enterotoxin and macromolecule which depends on interaction with the microvillus membrane for its action and which has a different binding characteristic in microvillus membrane from newborn compared to adult animals [8], was used as an external membrane stimulus to test the structural response in these animals.

Methods

Microvillus membrane preparation

Membranes were prepared from small intestine of female New Zealand white rabbits (Margaret's Home Farm, Greenfield, MA) weighing about 3 kg and from small intestine of 12–24-h-old newborn rabbits removed from mother before suckling. Microvillus membrane were prepared by a slight modification of the method of Schmitz, et al. [9]. All steps in the preparation were carried out at 4°C. Mucosal scrapings from the small intestine were homogenized in buffer containing 500 mM mannitol and 12 mM Tris-HCl at pH 7.4 for 15 min. The homogenate was diluted with buffer to 6 times of its original volume before filtering through a 40 μm nylon mesh (Tetko®, Elmsford, NY) to exclude large particulate matter. Crystalline $CaCl_2$ was added to the filtrate to make a final concentration of 10 mM. After mixing for 45 min, the filtrate was centrifuged at $3000 \times g$ for 15 min to clear cellular debris and other organelles. The supernatant was saved and recentrifuged at $28\,000 \times g$ for 30 min to collect crude microvillus membrane. The cruce microvillus membrane was resuspended in 100 mM mannitol, 10 mM Tris-HCl (pH 7.4) and centrifuged under the same conditions. Resuspended pellets were further purified on a 10% to 60% glycerol gradient containing 5 mM $MgCl_2$ maintained at $64\,000 \times g$ for 15 min. A band containing purified microvillus membrane noted on the upper half of the gradient was collected. The final membrane preparation was maintained in a 100 mM mannitol, 10 mM Tris-HCl (pH 7.4) buffer. Purity of membrane preparations was assessed by determining the specific activity of marker enzymes, sucrase (adult) and lactase (newborn). The final ratio of specific activities of sucrase from the purified membrane preparation compared to the initial homogenates ranged from 13 to 20. The enrichment of lactase ranged from 20 to 30. Membrane protein concentration was determined by the method of Lowry et al. [10]. Membrane lipids were extracted according to the method of Folch et al. [11]. Total phospholipids, cholesterol and hexose were determined from the lipid extract by the methods of McClare [12], Rudel and Morris [13] and Dubois et al. [14], respectively.

Measurement of membrane structure using the membrane probe

The spin label method was used to monitor structural differences between microvillus membrane from newborn and adult animals and to determine the effect of cholera toxin on these membranes. The microvillus membrane labeled with 5-doxylstearic acid (Syva Co., Palo Alto, CA) used in these experiments was prepared as follows: 5-doxylstearic acid was dissolved in absolute methanol at 6.5 mM concentration and stored at −20°C. 10–20 μl of spin label stock solution was dried under a stream of nitrogen gas in a test tube. 200–500 μl of freshly prepared or frozen microvillus membrane stored at −20°C in a 100 mM

mannitol, 10 mM Tris-HCl (pH 7.4) buffer was added to the test tube. The solution was vortexed for 1 min to ensure the dispersion of spin labels into the microvillus membrane. The molar ratio of spin label to membrane phospholipid was 1%. Electron spin resonance spectra were recorded on a Varian E-9 spectrophotometer equipped with a variable temperature control and a digital thermometer (model 72, Yellow Spring Instrument Co., Yellow Spring, OH). An external calibrated thermometer probe was used to monitor temperatures of the samples.

The order parameters of the spin labeled microvillus membrane was quantitated by first measuring the hyperfine splitting parameters $2T'_{\parallel}$ (parallel) and $2T'_{\perp}$ (perpendicular) expressed in Gauss (G) units and shown in Fig. 1. These data correspond to the separation of the outer and inner spectral extrema, respectively. $2T'_{\parallel}$ and $2T'_{\perp}$ were used to calculate the order parameter S' according to the method of Hubbel and McConnell [15]. Temperature was used as an external probe to induce changes in membrane structure and to examine its effect on the hyperfine splitting parameter. Membrane preparations were cooled to either 4 or -2°C and the ESR spectra were recorded at 1 or 2°C increments up to 56°C; the ESR spectra were then repeated at lowered temperatures to determine whether the process was reversible. To detect thermotropic transition, an experimental curve comparing the hyperfine splitting parameter $2T'_{\parallel}$ with temperature was prepared and analyzed in terms of linear components by applying expression lines to predesignated sections of the curve using the method of least squares. The existence of a break point in the thermotropic transition dependent curves suggested a sudden change in the spin label environment within the membrane.

Binding of cholera toxin to microvillus membrane

The effect of cholera toxin on microvillus membrane structure was also examined using spin labeled ESR techniques. The binding of cholera toxin to microvillus membrane was investigated according to the method of Bresson et al. [8]. 100 μl of cholera toxin (Schwartz-Mann, Orangeburgh, NJ) (1 mg/ml, in 0.001 M Tris-HCl, 0.25 M NaCl, pH 7.5 buffer), was mixed with 0.1 ml of either adult or newborn microvillus membrane (3 mg/ml protein concentration), labeled with 5-doxylstearic acid (100 mM mannitol, 10 mM Tris-HCl, pH 7.4 buffer). The final concentration of cholera toxin used in experiments was 6 pM. The mixture was incubated at room temperature for 30 min before transfer to a quartz tube for spectral measurements. Hyperfine splitting parameters for each membrane preparation with and without exposure to cholera toxin at temperature ranges from 4°C to 56°C were recorded and compared.

Results

Protein and lipid composition of microvillus membranes

Total protein in microvillus membrane was determined by the method of Lowry et al. [10] using bovine serum albumin as a standard. Lipids were extracted from the known amount of microvillus membrane; phospholipid, cholesterol and hexose were in turn determined from the extracted lipid. The results are shown in Table I. Microvillus membrane from newborn rabbit contained a significantly higher amount of phospholipid, cholesterol and hexose (glycolipid) than that of microvillus membrane from adult animals, and consequently, a higher lipid to protein ratio (Table II). However the molar ratio of phospholipid to cholesterol was similar in these two microvillus membrane preparations.

Microvillus membrane in newborn and adult animals

A comparison of representative ESR spectra of 5-doxylstearic acid labeled microvillus membrane

TABLE I

PHOSPHOLIPID, CHOLESTEROL AND HEXOSE CONTENT OF MICROVILLUS MEMBRANE (MVM) OF RABBIT SMALL INTESTINE

Phospholipid, cholesterol and hexose were determined as described in Materilas and Methods. The results are expressed (in μg/mg protein) as the mean ± S.E., the number of preparations analysed is given in parentheses. Statistical analysis was done by Student's *t*-test.

MVM	Phospholipid	Cholesterol	Hexose
Adult	131 ± 18(5)	77 ± 7(5)	86 ± 3(3)
Newborn	216 ± 23(4)	134 ± 11(6)	142 ± 7(4)
P	< 0.02	< 0.01	< 0.02

TABLE II

PERCENTAGE OF MICROVILLUS MEMBRANE COMPOSITION OF ADULT AND NEWBORN RAT SMALL INTESTINE

Data are taken from Table I. It was assumed that microvillus membrane neutral lipid was 70% cholesterol [16]. Total glycolipids were calculated from the hexose content on the assumption that mixed glycolipids have a molecular weight of 846 [17]. Prot, protein; PL, phospholipid; NL, neutral lipid; GL, glycolipid; Prot/L, protein/lipid ratio (w/w).

MVM	Prot	PL	N	GL	Prot/L
Adult	60.8	8.0	6.7	24.6	1.55
Newborn	48.2	10.4	9.2	32.2	0.93

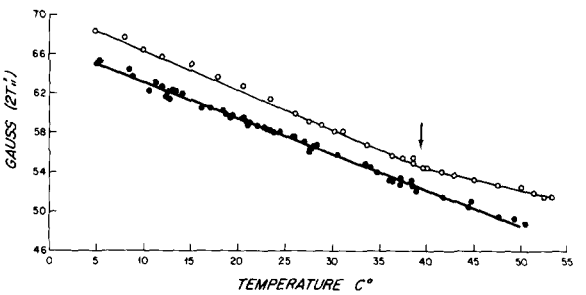

Fig. 2. A representative curve for the temperature dependence of the hyperfine splitting parameter of 5-doxylstearic acid labeled adult (○) and newborn (●) microvillus membrane. The transition temperature for adult microvillus membrane is 39.6 ±0.3°C, whereas there is no transition temperature for newborn microvillus membrane.

from adult and newborn rabbits at 23°C are shown in Fig. 1. These data indicate that the label undergoes rapid, anisotropic motion about its long molecular axis in a fairly restricted environment [15], i.e. flexing motions of the probe are relatively restricted. At the same temperature, the spin probe of microvillus membrane from newborn rabbits experienced a less ordered environment than that of the spin probe of microvillus membrane from adult rabbits, as suggested by a lower $2T'_{\parallel}$ and a smaller S' in newborn microvillus membrane (Fig. 2 and Table III).

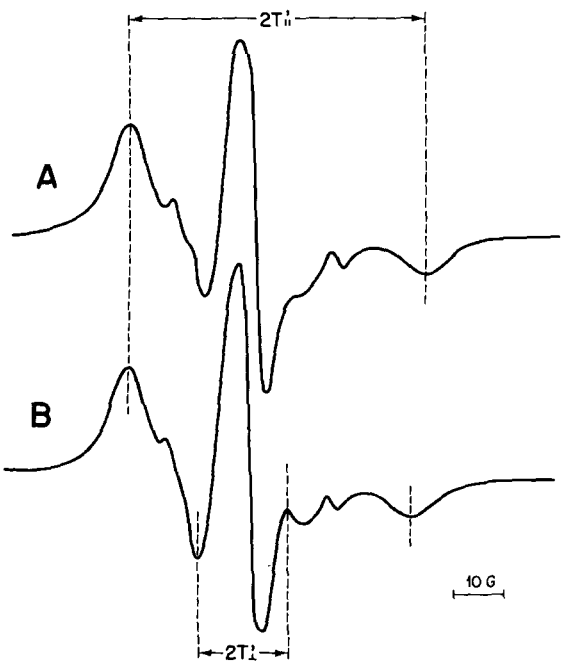

Fig. 1. A comparison of the typical ESR spectra of 5-doxylstearic acid labeled adult (A) and newborn (B) microvillus membrane at 23°C.

Temperature dependence of the hyperfine splitting parameter

It has been shown extensively in the literature that the characterization of temperature dependence of spin labeled membranes provided a useful approach to the study of lipid-protein or lipid-lipid interactions. For example, in bacteria and in mouse fibroblasts, a close correlation have been observed between the 'characteristic temperature' of membrane lipids and the capacity for sugar transport [18]. In pure phospholipids vesicles, the solubility of the spin label, 2,2,6,6-tetramethyl peperidine-1-oxyl (Tempo) in the hydrophobic re-

TABLE III

COMPARISON OF THE ORDER PARAMETER S' FROM ADULT AND NEWBORN MICROVILLUS MEMBRANES LABELED WITH 5-DOXYLSTEARIC ACID

The results are expressed as the mean ± S.E., the number of experiments is given in parentheses.

Temp. (°C)	S'		
	Adult	Newborn	P
20	0.749 ± 0.014(5)	0.707 ± 0.010(4)	< 0.01
30	0.699 ± 0.011(5)	0.660 ± 0.005(4)	< 0.01
40	0.648 ± 0.009(5)	0.589 ± 0.009(4)	< 0.001
50	0.599 ± 0.008(4)	0.535 ± 0.008(4)	< 0.001

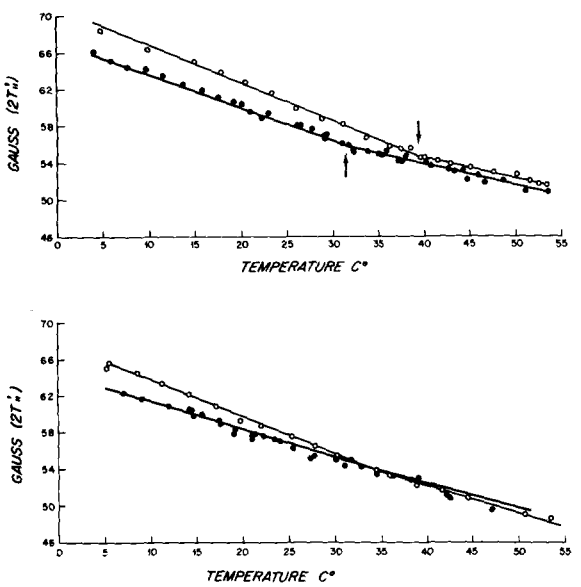

Fig. 3. A representative curve showing the effect of cholera toxin on temperature-dependence of the hyperfine splitting parameter in 5-doxylstearic acid labeled microvillus membranes. Upper panel represents adult microvillus membrane with (●) and without (○) cholera toxin at 6 pM. The transition temperature for microvillus membrane with 6 pM of cholera toxin is 30.7 ± 0.2°C. Lower panel represents newborn microvillus membrane with (●) and without (○) cholera toxin at 6 pM.

gion of the lipid exhibits abrupt changes in slope at characteristic temperatures corresponding to the onset and completion of lateral phase separation [19]. In this report, temperature-induced structural change was evaluated; it was found that the hyperfine splitting parameters for microvillus membrane decreased with increasing temperatures in both newborn and adult microvillus membrane (Fig. 2). However, major differences were observed in the $2T'_{\parallel}$ response between these two membrane preparations. At any given temperature, the hyperfine splitting parameter ($2T'_{\parallel}$) of microvillus membrane from newborn was significantly lower than that of microvillus membrane from adult. A linear dependence of the hyperfine splitting parameter on temperature from 4 to 56°C was found in microvillus membrane from newborn rabbits with a slope of −0.36 (G/K) whereas a single break point at 39.6 ± 0.3°C was seen in adult microvillus membrane. A slope of −0.37 (G/K) was observed from 4 to 39°C and a lesser slope of −0.22 (G/K) was found from 40 to 50°C in the temperature-dependent curve of adult microvillus membrane.

The hyperfine splitting parameters $2T'_{\parallel}$ and $2T'_{\perp}$ measured from 5-doxylstearic acid labeled microvillus membrane were also used to calculate its order parameters S' [15]. Order parameter reflects an amplitude of anisotropic motion expressed as the mean angular deviation of a spin labeled fatty acid long chain from its average orientation in the lipid bilayer. Table III compares order parameters (S') of microvillus membrane from adult and newborn rabbits. S' values of microvillus membrane from adult are significantly higher than those from newborn from 20 to 50°C, indicating a more restricted environment of the label in adult microvillus membrane.

Effect of cholera toxin on the membrane structure

It has been shown previously in this laboratory and others [20,21] that cholera toxin exhibits a

TABLE IV

EFFECT OF CHOLERA TOXIN (CT) ON THE ORDER PARAMETER S' OF ADULT AND NEWBORN MICROVILLUS MEMBRANE (MVM) LABELED WITH 5-DOXYLSTEARIC ACID

The results are expressed as the mean ± S.E., the number of experiments is given in parentheses.

Temp. (°C)	S'					
	Adult MVM			Newborn MVM		
	without CT	with CT	P	without CT	with CT	P
20	0.749 ± 0.014(5)	0.725 ± 0.002(4)	< 0.05	0.707 ± 0.010(3)	0.689 ± 0.013(4)	< 0.05
30	0.699 ± 0.011(5)	0.679 ± 0.004(4)	< 0.05	0.660 ± 0.005(3)	0.646 ± 0.004(4)	< 0.05
40	0.648 ± 0.009(5)	0.633 ± 0.009(4)	< 0.05	0.601 ± 0.009(3)	0.603 ± 0.021(4)	> 0.05
50	0.599 ± 0.008(4)	0.585 ± 0.007(4)	> 0.05			

high affinity for microvillus membrane with primary binding sites being the ganglioside G_{M1}. However, recent studies by Bresson et al. [8] have suggested that additional glycoprotein binding sites are present in newborn microvillus membrane which may be unique to those newborn membranes. Having made this observation, cholera toxin was chosen as an external membrane stimulus to determine if cholera toxin binding induced a different structural response between newborn and adult microvillus membrane.

The effect of cholera toxin on microvillus membrane structure was examined by adding cholera toxin to 5-doxylstearic acid labeled membranes over a wide temperature range. Results are shown in Fig. 3. Cholera toxin at 6 pM was noted to decrease the hyperfine splitting parameter at temperatures below 40°C and to shift the temperature break from 39.6°C to 30.7°C in adult membranes. Above 40°C, cholera toxin did not change hyperfine splitting parameters significantly. In newborn microvillus membrane, the temperature dependence for hyperfine splitting remained linear at 6 pM cholera toxin and the disordering effect of cholera toxin was only observed below 30°C. S' values calculated from microvillus membrane with and without cholera toxin are shown in Table IV. After the addition of cholera toxin to microvillus membrane, S' values decreased in adult microvillus membrane from 20 to 40°C, as well as decreased in newborn microvillus membrane from 20 to 30°C, essentially in agreement with the results from the hyperfine splitting parameter ($2T'_{\parallel}$) alone. These data strongly suggest that cholera toxin has a disordering effect on microvillus membrane.

Discussion

The microvillus surface of the small intestine represents a highly differentiated and metabolically active compartment which contributes to the end states of digestion and active transport of nutrients as well as providing an important barrier to the penetration of toxic substances present in the external environment. We have previously reported that macromolecules are transported intracellularly by enterocytes as metabolically-active or antigenic molecules via a pinocytotic process involving adsorption to and invagination of the microvillus membrane surface [2]. We have also shown that the uptake of macromolecules was much greater in newborn than in adult animals and a cessation of excessive macromolecular uptake during the newborn period can be influenced by nutrition (natural versus artificial feeding) [22]. Since the development of the microvillus membrane structure may represent an important factor in the control of macromolecular transport during the newborn period, the present study was designed to determine if any structural differences could be demonstrated in newborn and adult microvillus membranes that might account for the increased uptake of macromolecules in newborns.

Using 5-doxylstearic acid as a standard spin label probe for isolated microvillus membranes, we were able to reproduce a spectra similar to that previously reported for 5-doxylstearic acid labeled model membranes [6,7,15] and other biological membranes [18,23,24] (Fig. 1). Both $2T'_{\parallel}$ and S' have previously been noted to be highly sensitive to the changes in the order of the lipid bilayer of membranes [6,7,15]. In analysis of microvillus membrane from adult animals (Fig. 2 and Table I) both $2T'_{\parallel}$ and S' were significantly higher than corresponding values of newborn microvillus membrane in a temperature range of 4 to 56°C. These results strongly suggested that the spin probe in newborn microvillus membrane was maintained in a much more disordered environment than the probe in adult microvillus membrane.

Although not completely understood, it has been shown that the composition of a membrane could greatly influence its structure [25–27]. The results from compositional analysis of these two membrane preparations suggested that there were significantly more phospholipids, cholesterol and hexose (glycolipids) in microvillus membrane from newborn rabbits (Table I) and consequently, a higher lipid to protein ratio (Table II). This result is consistent with our previous report that newborn microvillus membrane was lighter than adult microvillus membrane in sucrose density gradient ultracentrifugation [8]. The same phenomenon had been reported by other investigators in rats [28]. Based on these observations, it is possible that a greater lipid to protein ratio noted in microvillus membrane from newborns compared to adult animals may account for the more disordered en-

vironment observed in these studies.

To further define structural differences between microvillus membrane from immature and mature animals, temperature dependence of the hyperfine splitting parameter $2T'_{\parallel}$ for 5-doxylstearic acid labelled membranes was examined. In newborn microvillus membrane, $2T'_{\parallel}$ was inversely related to temperature in a linear manner from 4 to 56°C; whereas there was a break at 39.6 ± 0.3°C in the temperature dependence curve for adult microvillus membrane. (Fig. 2). Brasitus et al. [29] using calorimetry observed a thermotropic transition in the temperature range from 23 to 39°C for microvillus membrane from adult rat small intestine. Such breaks in the temperature dependence for the spin label parameter may represent a lipid phase transition from a gel to liquid crystalline state [19], lateral lipid phase separation [18] or the interaction between the boundary lipid phase associated with membrane protein and the bulk lipid phase [23]. Although the exact meaning of the temperature break in microvillus membrane is not completely understood, a lower $2T'_{\parallel}$ and a lack of temperature break from newborn microvillus membrane from 4 to 56°C strongly suggests that this membrane already exist in a more disordered environment.

The first event in the action of cholera toxin on a cell is a rapid high affinity binding to receptors on the cell surface; this is followed by a characteristic lag period of 10 to 60 min before the effect of cholera toxin on adenylate cyclase can be demonstrated. It has been well documented that the B subunit of cholera toxin is the effector in binding and the A subunit is responsible for activation of adenylate cyclase activity [30–32]. In this report, cholera toxin was shown to disorder the adult microvillus membrane at a physiologic temperature (39.4°C) and cause a shift in transition temperature from 39.6 to 30.7°C (Fig. 3). These results suggest a disordering effect of cholera toxin on adult microvillus membrane as a result of binding. The same disordering effect was only observed with newborn microvillus membrane at temperature below 30°C. However, at 39°C, cholera toxin does not change the order of newborn microvillus membrane. As previously reported, cholera toxin binds differentially to newborn and adult microvillus membrane at higher concentrations, but at lower concentrations (6 pM) their binding curves appear similar. The differences observed in membrane disordering by cholera toxin at physiologic temperatures in newborn and adult microvillus membrane cannot be explained in terms of binding characteristic of cholera toxin, and more likely was related to events after binding to microvillus membrane. The biologic action of cholera toxin appears to require penetration of the toxin-receptor complex from the microvillus surface of enterocytes and diffusion to the basolateral membrane where subsequent direct interaction with and modulation of adenylate cyclase occurs [34]. Fisherman et al. [35] in their study of the lipid moiety of choleragen action, showed that the lipid-protein portion of the ganglioside receptor which anchors the ganglioside to the cell surface can modulate the toxin-membrane interactions necessary for activation of adenylate cyclase. Their data implied that the fluidity of the lipid bilayer could directly influence the physiological manifestations of cholera toxin actions. A more disordered microvillus membrane such as exists in newborns could allow for a more efficient penetration and diffusion from the microvillus surface by the receptor-toxin complex, and subsequently allow more A subunits to gain access to the adenylate cyclase complex. In the more ordered adult microvillus membrane, disordering of the lipid bilayer must occur before penetration of A subunit can take place. This observation may account for the differential effect of cholera toxin in these two groups of animals.

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