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Maturation of Intestinal Defenses Against Peroral Infection with Group B Coxsackievirus in Mice

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The intestinal tract of adult mice provides effective protection against peroral infection with group B coxsackievirus. This protective function consists of at least two separate components. One is a barrier effect that prevents virus from passing through the mucosal side of the gut into the circulation. It becomes clearly evident at 18 days of life and is present thereafter. The other is a clearance mechanism that acts to eliminate virus from the enteric tract after infection has occurred. This is first demonstrable at about 14 to 18 days and also persists. The appearance of these protective functions coincides with the known development of enzymatic and morphological changes in the gut associated with the transition from suckling to weanling.

A previous report from our laboratory has shown that newborn mice are equally susceptible to group B coxsackievirus by the peroral (p.o.) and intraperitoneal (i.p.) routes (9). Although adult mice are also susceptible to infection by both these routes, the intestinal tract provides some degree of protection against p.o. infection for this host (10). This protection consists of at least two separate components: (i) a barrier effect that prevents passage of virus from the intestinal lumen through the mucosal side of the gut into the circulation; and (ii) a clearance mechanism that eliminates virus from the enteric tract after infection has occurred. The present report traces the development of these systems with age.

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MATERIALS AND METHODS

Virus. The virus used in these studies (strain GR) was isolated in monkey kidney tissue culture from a patient with aseptic meningitis. It was identified as a coxsackievirus B5 by neutralization tests with specific rabbit antiserum in monkey kidney tissue. After four passages in HeLa cell cultures, a virus pool was prepared as follows. Confluent HeLa cell cultures grown in 32-ounce (0.946-liter) tissue culture bottles containing 50 ml of medium were used. The medium was removed and the cell sheet was inoculated with 5 to 6 ml of a virus pool (titer, 10^7 plaque-forming units [PFU]/ml). After incubation for 1 to 2 h at 37 C, fresh medium was added and the culture was incubated again. At about 24 h, when most of the cells were lysed, the fluid and cell debris were harvested and centrifuged at 2,100 × *g*, and the supernatant was removed. Distilled water (5 ml) was added to the sedimented cell pellet, which was then subjected to three cycles of freezing and thawing with intermittent mixing. This suspension was also centrifuged, and the two supernatants were then combined. The virus pool was stored in 3-ml aliquots at −70 C until needed.

Culture medium. Eagle minimal essential medium supplemented with 10% fetal calf serum and containing 50 U of penicillin/ml, 50 μg of streptomycin/ml, and 50 μg of amphotericin/ml was used as the medium for HeLa cell cultures. This mixture also served as the virus diluent.

Animals. All experiments were performed with CD-1 mice obtained from Charles River Breeding Laboratories, Wilmington, Mass. Animals of the following ages were used: 1, 4, 10, 14, 18, 22, 26, and 33 days. Mice were maintained at 21 C in individual disposable cages (Lab-Line Instruments, Melrose Park, Ill.) and given Purina Lab Chow and water ad lib. Animals less than 18 days old (i.e., suckling) were kept with their mothers during these experiments. Before infection, mothers and mice 18 days and older were tested for the presence of neutralizing antibodies to coxsackievirus B5, using a standard plaque reduction technique (11).

A minimum of 12 mice was employed for each age and route of inoculation examined.

Mode of infection. Parenteral infection for mice 14 days old and younger was induced by the i.p. injection of a 0.1 ml of viral inoculum through a 30-gauge, 1.5-inch (ca. 3.81-cm) needle, which was

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inserted subcutaneously into the anteromedial surface of the thigh and guided into the abdominal cavity. Parenteral infection in the older mice was induced by an i.p. injection of 0.5 ml of viral inoculum. The 1-day-old mice received 10^5 PFU of virus; all others were given 10^8 to 10^9 PFU of virus.

Peroral infection was induced in mice 14 days old and younger through a sterile 3.5-cm polyethylene tube (P. E. 10, Intramedic, Clay-Adams, Parsipanny, N.J.) which was guided gently into the stomach with the aid of the swallowing reflex. For older mice a 5-cm, P.E. 60 polyethylene tube was used. Inoculum volumes and virus doses were similar to those used for parenteral infection.

Experiments in which p.o. and i.p. routes of infection were compared were done on the same day under identical conditions.

Preparation of tissues for viral assay. Blood obtained by either decapitation or cardiac puncture was collected into 1-dram screw-capped vials containing 40 U of heparin/ml of blood (Panheprin, 20,000 U.S.P. units/ml, Abbott Laboratories, North Chicago, Ill.) and quick-frozen in an ethanol-dry ice bath. The gastrointestinal tract was dissected free of fat and blood vessels and cut open along the longitudinal axis (from the stomach distally to the rectum). Gut and stomach contents were removed by thorough agitation in ice-cold phosphate-buffered saline followed by an additional fresh buffer rinse. This procedure was repeated four times using a fresh 15-ml volume of phosphate-buffered saline in a separate container for each agitation and rinse. All specimens were stored at −70 C until needed.

Viral quantitation. Tissues were weighed and ground with sterile alundum in twice-distilled water containing 200 U of penicillin/ml and 200 μg of streptomycin/ml to make a 10% tissue suspension. After two cycles of freezing and thawing, the samples were centrifuged for 10 min at 1,000 × g, and the supernatants were used for viral titration. All quantitations of virus were carried out by plaquing on HeLa cells by means of a standard technique (11).

Experimental procedure. At 1 and 3 days after infection, six mice from each age category through 18 days were sacrificed. Analogous tissues were then divided into two pools, and each pool was titrated separately. For older mice, two animals from each group were similarly sacrificed and titrations for virus were done on each. Titrations were repeated on aliquots of some specimens as a check on the validity of the results.

RESULTS

The intestinal virus barrier is defined here as the capacity of the intestinal tract to prevent penetration of virus into the host circulation. In this study it is expressed as the blood virus titer ($\log_{10}$ PFU/g) at 1 day after i.p. infection minus the blood virus titer at 1 day after p.o. infection.

In mice infected perorally at 1, 4, 10, and 14 days of age, respectively, virus titers in the blood were greater or in the same general range as in mice infected parenterally (Table 1). In animals infected at 18 days and thereafter, however, a significant quantity of perorally administered virus was prevented from reaching the host circulation, e.g., the concentration of virus in the blood after p.o. infection at 18 days was only 1% of that after i.p. infection. With larger viral inocula, moreover, this barrier appeared to be even more effective.

We have used the term "intestinal virus clearance" to denote the capacity of the intestine to reduce virus titers in this tissue after p.o. infection. A quantitative estimate of this capacity may be made by determining the difference between the virus inoculum and virus titers per gram of intestinal tissue at 1 and 3 days after infection. Such data for mice infected perorally at 1 through 10 days of age showed no virus clearance by the gut (Table 2). Virus titers in these mice were, instead, generally higher than the inoculum, indicating that viral replication was occurring. In the 1-day-old mice, viral titers in gut tissue increased by over 1,000-fold. By contrast, mice perorally infected at 14 to 18 days of age and later showed significant drops in virus titer by 1 day after infection, and these became more marked by day 3. These findings indicate that a clearance mechanism is present beginning at 14 days, and that it represents an active process because it continues to act over the 3-day period of observation.

Virus clearance by the gut has alternatively been measured in these studies by comparing virus titers per gram of intestinal tissue at 1 day after i.p. infection with titers after p.o.

TABLE 1. *Virus titers in blood after i.p. and p.o. infection with coxsackievirus B*

Age (days) at infection	Inoculum ($\log_{10}$ PFU/animal)	Blood[a]		Intestinal virus barrier[b] (i.p. minus p.o.)
		i.p.	p.o.	
1	5.0	3.5	5.1	−1.6
4	8.0	5.2	5.1	0.1
10	8.0	6.7	6.2	0.5
14	8.0	5.6	5.6	0.0
18	8.0	5.5	3.5	2.0
22	8.0	5.5	3.0	2.5
26	8.0	5.1	3.4	1.7
33	9.4	8.0	3.5	4.5

[a] $\log_{10}$ PFU virus per gram blood, obtained 1 day after infection.

[b] Intestinal virus barrier is defined as the blood virus titer ($\log_{10}$ PFU/g) after i.p. infection minus the titer after p.o. infection.

infection. Such data (Table 3) again show viral replication rather than clearance in intestinal tissue of animals through 10 days of age. As in Table 2, a clearance mechanism first became apparent in mice infected at 14 to 18 days of age and persisted thereafter.

Table 3 also presents viral titers per gram of intestinal tissue at 3 days after i.p. infection. These data permit a comparison of events in this tissue at 3 days after infection by both routes. Viral titers at 3 days after i.p. infection were generally higher than or at the same levels as the viral inoculum. These titers, moreover, were significantly higher than at 1 day after i.p. infection. This indicates that i.p. infection with coxsackievirus is associated with virus replication in intestinal tissue.

Mice infected perorally at 1 through 10 days of age had viral titers in intestinal tissue at 3 days after infection that were in the same range as the i.p.-infected animals, indicating that viral replication had occurred. By contrast, a significant decrease in viral titers was observed in animals infected perorally at age 14 days and older. This decrease probably represents a combined effect of the intestinal barrier, clearance activity, and possibly reduced viral replication. At 3 days virus titers in gut tissue of i.p.-infected animals had generally attained levels 100,000 times or more greater than those in p.o.-infected animals.

TABLE 2. *Virus titers in intestinal tissue following peroral infection and comparison with virus inoculum (coxsackievirus B)*

Age (days) at infection	Inoculum (log_{10} PFU/animal)	Intestine[a]		Intestinal virus clearance[b]	
		Day 1	Day 3	Day 1	Day 3
1	5.0	6.4	8.5	−1.4	−3.5
4	8.0	7.1	7.9	0.9	0.1
10	8.0	8.3	9.7	−0.3	−1.7
14	8.0	6.6	5.3	1.4	2.7
18	8.0	5.1	3.2	2.9	4.8
22	8.0	5.1	3.4	2.9	4.6
26	8.0	3.6	3.1	4.4	4.9
33	9.4	4.5	1.6	4.5	7.4

[a] Log_{10} PFU of virus/g of tissue at 1 and 3 days after infection.

[b] Intestinal virus clearance is defined as the virus inoculum (log_{10} PFU) minus virus titer in intestine (log_{10} PFU/g of tissue) at 1 and 3 days after peroral infection.

TABLE 3. *Coxsackievirus B titers in intestinal tissue after i.p. and p.o. infection*

Age (days) at infection	Intestine[a]				Intestinal virus clearance[b]	
	i.p. (day 1)	p.o. (day 1)	p.o. (day 3)	i.p. (day 3)	Day 1	Day 3
1	5.5	6.4	8.5	8.6	−0.9	−3.0
4	6.2	7.1	7.9	8.1	−0.9	−1.7
10	8.4	8.3	9.7	9.6	0.1	−1.3
14	7.0	6.6	5.3	8.2	0.4	1.7
18	6.4	5.1	3.2	8.2	1.3	3.2
22	7.1	5.1	3.4	9.1	2.0	3.7
26	7.0	3.6	3.1	8.8	3.4	3.9
33	8.0	4.5	1.6	7.3	3.5	6.4

[a] Log_{10} PFU of virus/g of tissue.

[b] Intestinal virus clearance, virus titers in intestinal tissue log_{10} PFU at day 1 after i.p. infection minus titers in this tissue at days 1 and 3 after p.o. infection.

DISCUSSION

The digestive tract of the newborn host shows considerably less differentiation than that of the more mature host. This has been reported for various species including chicks (12), rats (2), mice (7), rabbits, and humans (3). The high susceptibility of newborn mice to oral infection with group B coxsackievirus may be related to the state of morphological and functional development of the gut in this host. Lecce found that the intestinal epithelium of neonatal mice was capable of nonselectively absorbing a wide variety of albumins, including egg, porcine, rabbit, bovine, and human albumin (7). He found that gamma globulin was also absorbed into the gut epithelium and circulation of this host for up to 17 days after birth. In mice, at 16 to 18 days of life a "closure" of the intestinal epithelium to absorption of antibody and albumins occurs. This coincides with a change in the morphology of the microvilli from a short, broad configuration, to the mature, narrow, elongated form (15, 16). O'Connor reported a drop in mitotic activity in the small intestine of mice at the end of the 2nd week and at the start of the 3rd week after birth (15). These morphological changes are accompanied by a variety of functional developments. These include an increase in activity of hydrolytic enzymes, especially alkaline phosphatases (13, 14), and an increase in the adhesive strength of the intestinal epithelial cells (1).

The age at which barrier and clearance mechanisms become apparent in the suckling mouse intestinal tract coincides quite closely with the period of morphological and functional changes in this organ. Although the capacity to clear virus appears to precede the appearance of the virus barrier function, it is uncertain whether these two defense mechanisms are associated with different tissue components or

whether they represent functions of the same cells which become apparent at somewhat different times during development.

A major characteristic of the coxsackieviruses is their marked virulence for newborn mice and their relative avirulence for older mice. Several studies have indicated that this age-specific susceptibility is related to the greater abundance and distribution of cells with specific receptor sites in newborn as compared with older mice (4–6). Such receptors have been demonstrated in various organs of this host, although the gut has not been directly examined (6). Enteroviral receptors have been demonstrated, however, in the gut of other susceptible species, including human fetuses and rhesus monkeys (4). In addition, fluorescent antibody studies in 7-day-old mice perorally infected with group B coxsackieviruses have shown viral antigen in the intestinal mucosa (8), indicating that such receptors are present in the gut of suckling mice.

The occurrence of coxsackievirus receptors in very young mice and their decrease with age in at least some tissues (6) suggest that development of barrier and clearance functions may be due to a decrease in viral receptors in the gut mucosa of the older host. Opposed to this hypothesis, however, are results of previous studies in this laboratory which show that virus given perorally to both newborn and adult mice may be found in relatively high titers in washed gut tissue at 2 h after infection, although the titers drop sharply thereafter in the adult mice, where the clearance mechanism is active (9, 10). The occurrence of high virus titers in enteric tissue shortly after infection indicates that there is no paucity of viral receptors on the mucosal surface of the gut in adult mice. The subsequent drop in virus titers would suggest either that the mucosal cells in older mice adsorb virus less firmly (i.e., more reversibly) than in the young host, that virus penetrates but fails to replicate as readily in such cells, or alternately that virus is more effectively inactivated after attachment. The relative importance of these mechanisms remains to be explored.

The possibility that antibodies or interferon may play a role in gut barrier function appears unlikely. Experiments in this laboratory have shown that by 2 h after i.p. infection of adult mice with 3×10^9 PFU of virus, the blood contained 10^8 PFU of virus per g; 2 h after p.o. infection with the same viral dose, however, only 10^2 PFU of virus per g was found in the blood (7). The apparent presence of a barrier function within 2 h after p.o. infection in mice not previously exposed to group B coxsackievirus and its absence in mice infected i.p. would indicate that neither antibodies nor interferon is involved. It is possible, however, that these factors may play some role in virus clearance.

The transition in mice from suckling to weanling is associated with loss of maternal antibodies and possibly other defensive factors against infection that are acquired through the mother's milk. Since weaned mice now derive their food from multiple sources, the risks of enteric infection and disease are increased. It is noteworthy, therefore, that the developmental processes in the gut that prepare this host for weaning are associated with the appearance of new protective mechanisms against enteric infection.

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