Paneth Cells and Antibacterial Host Defense in Neonatal Small Intestine

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Paneth cells are specialized epithelia in the small bowel that secrete antimicrobial proteins. Paneth cells are vital to the innate immunity of the small bowel in adult mammals, but their role during neonatal infection of the small bowel is not well established. Dithizone selectively damages Paneth cells, and when dithizone-treated newborn rats are infected enterally with *Escherichia coli*, the numbers of *E. coli* cells in their jejunal and ileal lavage fluid are significantly increased compared to controls. The data support that Paneth cells are necessary for neonatal antibacterial defense.

Antimicrobial proteins and peptides are key effectors of innate immunity at mucosal surfaces in adult animals, but their role in host defense during perinatal intestinal development is not clearly delineated. Paneth cells are specialized epithelia in the crypts of the small bowel that control the intestinal growth of bacterial pathogens through the secretion of antimicrobial proteins and peptides. Paneth cells secrete their antimicrobial-rich granules upon exposure to pathogenic bacteria and bacterial products, and alpha-defensins account for 70% of the secreted bactericidal activity of Paneth cells.

Neonatal necrotizing enterocolitis is a disease mainly of premature human infants, but its pathogenesis is incompletely understood. A causative association between Paneth cells and necrotizing enterocolitis has been proposed because preterm infants inadequately express alpha-defensins in the small bowel. Additionally, Paneth cells in surgical specimens from human infants with necrotizing enterocolitis have a deficiency in lysozyme, a prominent antimicrobial protein in Paneth cell granules. The inability of neonatal Paneth cells to control the growth of bacterial pathogens in the lumen of the small bowel is an attractive hypothesis related to the initiation of necrotizing enterocolitis, but there is no in vivo evidence that Paneth cells provide host defense in the neonatal small bowel.

This investigation postulated that ablating Paneth cells in neonatal rats would reduce the ability of the neonatal small bowel to clear an infection caused by enteroinvasive *Escherichia coli*. To test this hypothesis, dithizone was administered systemically to neonatal rats. Since dithizone selectively damages Paneth cells, and when dithizone-treated newborn rats are infected enterally with *E. coli*, the numbers of *E. coli* cells in their jejunal and ileal lavage fluid are significantly increased compared to controls. The data support that Paneth cells are necessary for neonatal antibacterial defense.

Effect of dithizone on Paneth cells in the noninfected neonatal small bowel. For studies that examined the effects of dithizone, specific-pathogen-free Sprague Dawley rats (Haran, San Diego, CA) were studied between 4 and 5 days of age. The studies described below were approved by the Animal Use Committee of the University of California, Davis.

Paneth cells contain their antibacterial peptides, a Lickert score for neonatal rats was assigned to Paneth cells in adult rats, we quantified the effects of dithizone on the Paneth cells of neonatal rats. After dithizone treatment, newborn rats were infected with an intragastric dose of *Escherichia coli*, and the quantitative clearance of this bacterium from the small bowel lumen was measured.

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applied to Paneth cells that had significantly reduced numbers of poorly staining and pleomorphic eosinophilic granules in their cytoplasm. A score of 2 indicated an intermediate appearance.

After 24 h, there were no signs of illness and survival was 100% in the noninfected rat pups given either i.p. dithizone or control injections. The numbers of Paneth cells identified in 100 crypts 24 h after i.p. injection of saline, Li2CO3 buffer, or dithizone were 27 ± 3, 26 ± 1, and 19 ± 2, respectively (means ± standard errors of the means; P < 0.05). The average numbers of Paneth cells per crypt were, for saline, 0.33 ± 0.03; for Li2CO3 buffer, 0.30 ± 0.02; and for dithizone, 0.22 ± 0.03 (P < 0.05). The granules in Paneth cells of dithizone-treated pups had a mean Lickert score of 1.6, a median score of 1, and a range of 1 to 2, whereas those from pups given i.p. saline or Li2CO3 buffer had average Lickert scores of 2.5 and 2.2, respectively, a median score of 3, and a range of 2 to 3 (Fig. 1). Analysis of Lickert scores for Paneth cells of dithizone-treated pups showed significant abnormalities compared to controls (by the Kruskal Wallis statistic, H = 8.117, P < 0.017, and post hoc analysis for dithizone versus saline or Li2CO3 buffer, each at the P < 0.01 level) (7).

The histological appearance of the crypts and villi, however, did not reveal abnormalities among the three groups, and no inflammatory cell response in any group was apparent. The mitotic activity of intestinal epithelia adjacent to dithizone-exposed Paneth cells was similar to the mitotic indices of epithelia in control pups, suggesting that epithelial cells adjacent to Paneth cells were not injured. This finding is also consistent with the in vitro treatment of the rat intestinal epithelial cell 6 line whose proliferation did not change in high concentrations of dithizone (17). Thus, a model is described here that uses dithizone to chemically ablate and alter the granular characteristics of newborn rat Paneth cells.

In the control neonatal pups, the number of Paneth cells identified per 100 crypts was <10% of that reported for adult rats (16). This low number of Paneth cells may be one reason why neonatal rats are the preferred animal model for studying the pathogenesis of human necrotizing enterocolitis (8). As described in the next series of experiments, the diminished numbers and the altered granules seen in the Paneth cells of dithizone-treated pups may explain why neonatal pups could not effectively clear a burden of pathogenic Escherichia coli from the small bowel.

Effect of dithizone on the antibacterial activity of neonatal small bowel. For studies involving the intestinal clearance of Escherichia coli, dithizone was given i.p. before the infection was induced in 4-day-old rat pups, and at least 3 litters and 24 newborn rats (8 pups per litter) were used for each experimental condition. Six hours after pups were given i.p. dithizone (25 mg/kg or 75 mg/kg) versus 25 mM Li2CO3 buffer or saline as controls, a dispersed suspension of E. coli (1 × 10^{12} CFU/kg of body weight) was administered intraperitoneally. The contrast in the antibacterial activity of Paneth cells in the small bowel of control and dithizone-treated pups is shown in Table 1.
weight) was inoculated into the stomach by using a catheter. Six hours was selected as the time to initiate infection because this is when a maximal reduction in Paneth cells occurs after dithizone administration in adult rats (16). *E. coli* strain Ec5 was used to induce enteral infection because it is an isolate from a human preterm infant and has the following surface determinants associated with virulence, O18: K1:H7 (9). This strain has been used in past reports of enteral infection in neonatal rats and was prepared as described in those studies (6, 18).

Eighteen hours after infection, the number of deaths was recorded and an illness score was assigned in surviving pups (6). After euthanasia, the jejunum and ileum of the surviving pups were aseptically procured for bacteriologic studies. The weight and length of each bowel segment were measured to calculate bowel mass, and each segment was lavaged with sterile saline as previously reported (18). The recovered microorganisms of lavage fluid were measured, and the percent fractional recovery was calculated from the instilled volume. After the lavage procedure, the jejunum and ileum were separately homogenized in 1 ml of sterile saline. Jejunal and ileal lavage effluents and the homogenized jejunum and ileum were serially diluted in sterile saline, and quantitative cultures were performed using 5% sheep blood agar and MacConkey agar plates. After 48 h of incubation, the *E. coli* cells were enumerated. The numbers of *E. coli* cells in intestinal fluid were corrected for fractional recovery of the lavage effluent, and the numbers of *E. coli* cells in the lavage fluid and bowel wall homogenates were then standardized to bowel mass (18).

Figure 2 shows the numbers of *E. coli* cells recovered in the jejunal and ileal lavage fluid of newborn rats given a 75-mg/kg dose of dithizone. *E. coli* cells present in the small bowel fluid of dithizone-treated pups were significantly higher in numbers than those cultured in controls. This difference was not a result of a disparity in recovered lavage fluid, which ranged from 97 to 111% of the instilled lavage volume (*P* was not significant among the groups). Mucosa- and bowel-wall-associated numbers of *E. coli*, determined by cultures of gut wall homogenates, were also significantly higher in the jejuna of dithizone-treated newborn rats versus controls, but the numbers of *E. coli* cells in the ileal segments did not reach statistical significance among the three groups (*P* = 0.06 for dithizone versus controls).

Table 1 shows the death rate and illness scores of newborn rats given 75 mg/kg of i.p. dithizone and infected with enteral *E. coli* compared to the control groups. The higher death rate in dithizone-treated, *E. coli*-infected pups may be related to the postmortem observation that 25% of the ileal specimens had gross necrosis. The devitalized bowel may explain the lower numbers of *E. coli* in the ilea of dithizone-treated pups.

Newborn rats given a lower dose (25 mg/kg) of dithizone before enteral infection with *E. coli* also had a 2- to 3-log increase of *E. coli* in the jejunal and ileal lavage fluids and in jejunal homogenates compared to the control groups (*P* < 0.01); however, their mortality rate (3 of 24 [12.5%]) and their illness scores were lower than those for *E. coli*-infected pups treated with 75 mg/kg of dithizone. None of the *E. coli*-infected pups given 25 mg/kg of dithizone had gross necrosis of the distal ileum.

Previous studies of fetal and neonatal Paneth cells in ani-

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**TABLE 1. Effect of dithizone treatment followed by enteral infection with *E. coli* on the rate of death and illness in neonatal rats**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>E. coli plus:</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Li2CO3</td>
</tr>
<tr>
<td>No. of deaths</td>
<td>2 (8.3%)</td>
<td>2 (8.3%)</td>
</tr>
<tr>
<td>(% of pups)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean and median illness scores</td>
<td>0.3, 0</td>
<td>0.4, 0</td>
</tr>
<tr>
<td>No. of pups and no. of litters studied</td>
<td>24, 3</td>
<td>24, 3</td>
</tr>
</tbody>
</table>
mals and humans have reported their anatomic and biochemical characteristics without a direct linkage to the killing of bacteria (2, 4, 5, 10, 14, 19). The findings reported here show that dithizone-induced alterations in the Paneth cells of newborn rat pups (Fig. 1) are associated with (i) a reduced ability of the small bowel to kill *E. coli* (Fig. 2) and (ii) an increased morbidity and mortality after infectious challenge of the small intestine (Table 1).

This study is consistent with reports showing that Paneth cells are an important antibacterial defense of the adult intestine. Matrilysin-deficient (MAT/−) mice lack processed alpha-defensins in Paneth cells, and when MAT/− mice are infected with intragastric *E. coli*, this bacterium survives in large numbers in the distal small bowel compared to similarly infected wild-type (MAT+/+) mice (20). Further compelling evidence for Paneth cell involvement in innate host defense is the protection of transgenic mice expressing human defensin 5 from gastrointestinal infection caused by *Salmonella enterica* serovar Typhimurium (15). Studying small bowel infections in neonatal mice with genetically engineered abnormalities in Paneth-cell-related antimicrobial proteins would be difficult because of their small size. Dithizone-treated newborn rats with generalized abnormalities in Paneth cells represent a new model for studying the function of their antimicrobial proteins in the neonatal small intestine.

In conclusion, compromised Paneth cell function is detrimental to host defense against *E. coli* infection in the neonatal small intestine. The results of this study, which used dithizone to deplete Paneth cell numbers and decrease their antimicrobial-rich granules, fully support this concept. We suggest that this new model may be useful in future studies of innate intestinal host defense relevant to newborn human infants.

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REFERENCES

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