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Insusceptibility of fetal intestinal mucosa and fetal cells to *Clostridium difficile* toxins

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ABSTRACT Infants are often carrying toxigenic *Clostridium difficile* in large numbers in the gastrointestinal tract without evidence of ill effects. We cultivate human amnion (epitheloid cells) and chorion (fibroblasts) *in vitro* and determine their susceptibility to *C difficile* toxin A and B three times weekly for a period of 3 weeks. On continued toxin exposure, these cells were found to be relatively insensitive to toxins during the first week of cultivation. When toxin exposure was limited to 1 h, the period of insensitivity was prolonged further. As cell cultures grew older, both types of cells gradually gained susceptibility to toxins. On subculture, neither amnion nor chorion exhibited early insensitivity to toxins, as did serially passed human lung fibroblasts (MRC-5). Fetal intestinal mucosal tissue did not absorb toxin A or B when exposed, while adult mucosal tissue did so readily. Meconium and cord serum showed no neutralizing activity. Our study suggests that resistance of infantile intestine to *C difficile* toxins is probably related to the nature of intestinal cells rather than intraluminal factors.

KEY WORDS *Clostridium difficile*; toxins; antitoxins; intestinal mucosa; amnion; chorion; fetal cells; cultured cells; meconium

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It is paradoxical that *Clostridium difficile* can be both harmless inhabitant in the gastrointestinal tract of infants and causative agent of antimicrobial agent-associated diarrhea and colitis in adults[1-3]. Infants are frequently colonized by toxigenic organisms in numbers comparable to that found in patients with pseudomembranous colitis, but the incidence of the disease is very low[4,5]. The reasons are not clear. It is possible that the intestinal mucosa is protected from toxin by substances that interfere with toxin attachment, or the mucosa cells fail to pick up toxin because of lack of receptors. It is also possible the fetal cells are resistant to toxins. To investigate these possibilities, we compare toxin binding capacity of fetal intestinal mucosa with that of adult tissue, examine the effect of muconium and fetal serum on toxin activity, and finally investigate the effect of age of fetal cell cultures on susceptibilty to *C difficile* toxins. Our results showed that fetal intestinal mucosa did not pick up toxins as well as adult cells and that fetal cells were relatively resistant to the action of *C difficile* toxins.

MATERIALS AND METHODS

Preparation of tissue culture Human placentae were obtained sterilly from cesarean section. Fetal membranes were stripped from placental tissue, washed repeatedly with Earl's balanced salt solution

and cut into small pieces. Both amnion and chorion were prepared at the same time, but in separate containers. 50 ml of trypsin was added, and the containers were placed in a shaker with a rotary motion, 120/min, at 37℃. After 30 min, the membranes were transferred to another set of containers containing 100 ml for fresh trypsin. After 4 h of trypsinization, the containers were vigorously shaken by hand. The membrane pieces were discarded and the cells were harvested by centrifugation at 1000 rpm for 10 min. The cells were resuspended in minimal Eagle's medium (MEM) containing 10% fetal calf serum. Final cell concentration of 100 000 cells per ml were distributed to 96 well tissue culture plates and incubated at 37℃ under 5% CO_2. Medium changes were done once weekly with MEM containing 3% fetal calf serum. MRC-5 cells (human lung fibroblast) were purchased from the Flow Laboratories, McLean, Virginia. The flask culture of MRC-5 cells was trypsinized, suspended in MEM containing 10% fetal calf serum, and distributed to 96-well tissue culture plates.

Morphologically, amnion cells appear to be epithelial cells, while chorion and MRC-5, fibroblasts.

Preparation of human intestinal mucosal tissue Fetal intestine was obtained from two second trimester abortuses. Segments of intestine were dissected from omental attachment, rinsed with Earle's balanced salt solution, and opened to expose intralumenal mucosal surface. After several rinses with Earl's solution, the mucosal tissue was scraped off from the underlying submucosal layers with a sterile microscopical slide. The harvested tissue was suspended in Earle's solution, washed twice, and resuspended to make a 10% suspension in NEM. Microscopic examination showed villi segments of varying sizes, as well as clumps of epithelial cells. Adult colon was obtained from a patient with carcinoma. About one inch of the normal colon was used for mucosal preparation. The intestinal villi were harvested in the same manner. All mucosal tissues were metabolically active as indicated by pH changes of the medium throughout incubation.

Toxin and antitoxin Methods for purification of toxin A and B, and for preparation of antitoxins A and B have been described[6]. Sordelli antitoxin was obtained from the Office of Biologics, Food and Drug Administration, Bethesda, MD. All toxins and antitoxins were stored at 2-8℃.

Collection of meconium and paired cord and mothers' blood Meconium was collected from three 1-2 day-old infants. Cord bloods were collected at the time of delivery. Mothers' blood from three corresponding infants were also saved. While serums were gathered in pairs, the meconiums were randomly sampled.

Toxin neutralization To determine the relative potency of different antitoxins, checker board titrations were carried out for each pair: toxin A and antitoxin A, toxin B and antitoxin B, toxin A, B or AB with sordellii antitoxin. Both toxin and antitoxin were diluted initially 1:10 and titrations were done in a 96-well MRC-5 cell culture plate. Serial 5-fold dilutions of toxin were made on horizontal plan, and the same 5-fold dilutions of antitoxin on vertical plan. The plates were incubated in a 5% CO_2 incubator and examined microscopically daily for 3 d. The end point was read as the highest dilution showing no evidence of cytopathic effects.

To determine the effect of incubation time on toxin neutralization, equal volumes of toxin dilution containing 100 TCD-50 (50% tissue culture dose) and serial 2-fold dilutions of antitoxins were mixed and incubated at room temperature. At 15 min intervals, aliquots from each dilution were

inoculated to MRG-5 cell cultures. Daily examination microscopically was done for 3 d. The end point was recorded as the highest serum dilutions showing no evidence of cytopathic effects.

Effect of age of cell cultures on susceptibility to toxins Primary cell cultures of human amnion and chorion and serially passed culture of human lung fibroblast (MRC-5) were prepared. Serial dilutions of toxin A or B were added to the cell cultures three times weekly for a period of 3.5 wk. For each toxin dilution, 4 wells of cell culture were inoculated. The plates were incubated at 37℃ for 1 h, at which time half of the inoculated wells (2 sets) were neutralized with sordellii antitoxin (1:10). Daily observation for cytopathic effects was made and the end point was recorded on d 3.

Subcultures of both amnion and chorion cells were inoculated with serial dilutions of toxin A or B on d 2 and 5, without using sordellii antitoxin.

RESULTS

Neutralization to toxin A and B with antitoxins Fig 1 shows that sordellii antitoxin was as effective in neutralizing toxin A (AS) as the specific antitoxin (AA).

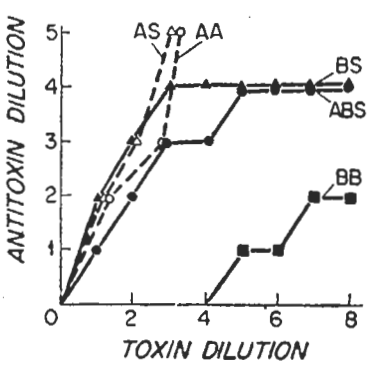

Fig 1. Neutralization of *C difficile* toxins A and B (first letter) with 3 different antitoxins (A, B, & Sordellii, last letter). ABS, both toxins with sordellii antitoxin. Numbers represent number of log 5.

Against toxin B, sordellii antitoxin (BS) was more potent than the specific antitoxin (BB). Against both toxin A and B, sordellii antitoxin (ABS) showed similar activity as against toxin B alone.

The effect of incubation time on neutralization of toxin A and B is shown in Fig 2. With specific antitoxins (AA, BB),

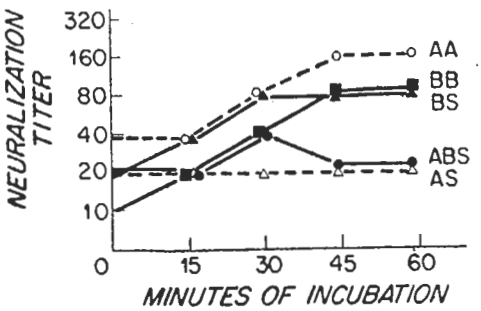

Fig 2. Effect of incubation time on neutralization of toxin A or B (first letter) with antitoxins (A, B, S or Sordellii last letter). ABS refers to both toxins with sordelii antitoxin.

the degree of neutralization increased with time of incubation until 45 min, when a plateau was reached. Sordelli antitoxin behaved similarly when tested against toxin B (BS). On the other hand, there was a change in neutralizing activity when incubated with toxin A (AS) or combined A and B (ABS).

Effect of age of cell cultures on susceptibility to toxins. Fig 3 shows that

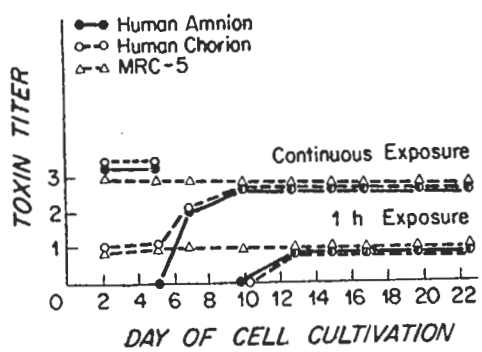

Fig 3. Effect of age of cell cultures on susceptibility to toxin A. Numbers 1-3 represent number of log 5. Top lines represent subcultures of amnion and chorion cells on continuous toxin exposure.

both amnion and chorion cell cultures were relatively insensitive to toxin A during the first week of cultivation. Amnion cells began to show cytopathic changes after d 7. By d 10, full susceptibility developed. The degree of susceptibility of chorion cells to toxin A was low during the first week, but increased steadily thereafter. MRC-5 cells, on the other hand, did not exhibit initial resistance. When toxin exposure was limited to 1 h, both amnion and chorion cells showed no evidence of cytotoxicity until d 13 of cultivation. Again, MRG-5 did not exhibit initial period of resistance. Similarly, subcultures of amnion and chorion cells showed no lag period of cytotoxicity (represented by 2 top lines in Fig 3). These cells, like MRC-5 cells, exhibited the same levels of susceptibility throughout cultivation.

Fig 4 shows the effect of age of cell

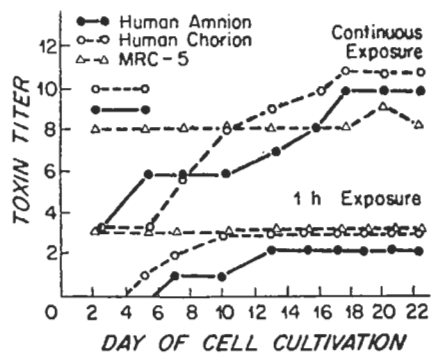

Fig 4. Effect of age of cell cultures on susceptibility to toxin B which is measured as number of log 5. Top 2 lines (left upper corner) represent subcultures of amnion and chorion cells on continuous toxin exposure.

culture susceptibility to toxin B. On continued toxin exposure, both amnion and chorion were relatively insensitive during the first week of cultivation, gradually developed their susceptibility, and reached the maximum on d 18. When toxin exposure was limited to 1 h, a lag period of 5 and 7 d respectively, was observed. Subcultures of both amnion and chorion cells show full susceptibility to toxin B. MRC-5 cells, likewise, did not exhibit initial resistance.

Toxin uptake by intestinal mucosa Suspension of fetal intestinal mucosa in medium containing either toxin A or B did not result in reduction of toxin titer after 60 and 120 min exposure. On the other hand, toxin uptake by colonic mucosa from adults was demonstrated (Tab 1). There was a 5-25 fold reduction of toxin titers in the medium containing adult mucosal tissue.

Tab 1. Toxin uptake by colonic mucosa from adults

Time of exposure	60		120 min	
Tissue suspension*	Yes	No	Yes	No
Toxin A(titer in medium)**	25	625	125	625
	5	25	5	25
Toxin B (titer in medium)	5	125	25	125
	5	25	5	25

* 10% suspension of colonic villi in Eagle's minimal essential medium.

** Reciprocal of 5-fold serial dilutions.

Absence of neutralizing activity of meconium and sera Neither mother nor infant's sera showed neutralizing activation against toxin A or B. None of the meconium from 6 infants neutralized toxin activity.

DISCUSSION

Although there are differences in sensitivity of different types of cell cultures to *C difficile* toxins, it is not known why the differences exist. Reports have suggested that cytotoxicity is correlated with changes in the cytoskeletal system. For instance, toxin treated cells showed marked clumping and derangement of microtubules[7]. Fibronectin and microfilaments of the toxin treated cells greatly diminished in quantity or disappeared entirely[8,9]. Fibronectin levels in the serum are

significantly lower in infants[10]. The levels in the amniotic fluid are alto low, only one-third of adult levels[11]. Whether the initial insensitivity of human amnion and chorion cells to toxins is related to the deficiency state of fibronectin remains to be studied. It is also not clear whether the cytoskeletal system is undeveloped in infancy.

A striking difference in toxin uptake between fetal and adult intestinal mucosa has been demonstrated. It is likely that the degree of toxin binding can be measured more precisely by labeling toxins. The failure to demonstrate toxin-inhibitory substances in the muconium and serum suggests that toxins are not inactivated by the intraluminal contents. Our observations indicate that the lack of harmful effects of *C difficile* toxins in infants is related to the nature of the intestinal mucosa and not to the intraluminal factors.

In performing neutralization test for diagnosis of *C difficile* diarrhea or colitis, sordellii antitoxin is preferred because of its activity on both toxin A and B and its prompt action[12]. With specific monovalent antitoxins, it is necessary to preincubate toxin-antitoxin mixture prior to tissue culture inoculation.

REFERENCES

1 Start PL, Adrian L, Parsonage BD. Colonization of large bowel of *Clostridium difficile* in health infants. Quantitative study. *Infect Immun* 1982; 35 : 895

2 Hoist E, Helin I, March PA. Recovery of *Clostridium difficile* from Children. *Scand J Infect Dis* 1981; 13 : 41

3 Fekety R, Kyung-Hee K, Batts DH, Browne RA, Cudmore A, Silva J, Toshniwal R, Wilson KH. Studies on the epidemiology of antibiotic-associated *Clostridium difficile* colitis. *Am J Clin Nutr* 1980; 33 : 2527

4 Viscidi RP, Bartlett JG. Antibiotic-associated pseudomembranous colitis in children. *Pediatrics* 1981; 67 : 381

5 Sutphen JL, Grand RJ, Flores A, Chang TW, Bartlett JG. Chronic diarrhea associated with *Clostridium difficile* in children. *Am J Dis Child* 1983; 137 : 275

6 Sullivan NM, Pellett S, Wilkins TD. Purification and characterization of toxin A and B of *Clostridium difficile*. *Infect Immun* 1983; 35 : 1032

7 Chang TW, Lin PS, Gorbach SL, Bartlett JG. Ultrastructural changes of cultured human amnion cells by *Clostridium difficile* toxin. *Ibid* 1979; 23 : 795

8 Ahlgren T, Florin I, Jarstrand C, Thelestam M. Loss of surface fibronectin from human lung fibroblasts exposed to cytotoxin from *Clostridium difficile*. *Ibid* 1983; 39 : 1470

9 Thelestam M, Bronnegard M. Interaction of cytopathogenic toxin from *Clostridium difficile* with cells in tissue culture. *Scand J Infect Dis (Suppl)* 1980; 22 : 16

10 McCaffety MH, Lepow M, Sabe TM, Cho E, Neuwissen H, White J, Zuckerbrod SF. Normal fibronectin levels as a function of age in the pediatric population. *Pediatr Res* 1983; 17 : 482

11 Crouch E, Holbrook BK, Hoehn H, Bornstein P. Amniotic fluid fibronectin and the synthesis of fibronectin by amniotic fluid cells in culture. *Ann NY Acad Sci* 1978; 312 : 410

12 Chang TW, Gorbach SL, Bartlett JG. Neutralization of *Clostridium difficile* toxin by *Clostridium sordellii* antitoxin. *Infect Immun* 1978; 22 : 418

胎儿肠粘膜和胎儿细胞对难辨梭状芽孢杆菌毒素的不易感性

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提要　体外培养人羊膜上皮细胞和绒毛膜纤维母细胞，每周测其对难辨梭状芽孢杆菌毒素A，B的易感性，共3 wk。结果细胞连续接触毒素1 wk对毒素不敏感，接触时间在1 h之内，不敏感时间延长。延长培养时间，两种细胞都逐渐增加对毒素的敏感性。胎儿肠粘膜不吸收毒素，成人的则容易吸收，胎粪和脐血无中和作用，提示胎儿肠道对难辨梭状芽孢杆菌毒素的抵抗力与肠细胞有关。

关键词　难辨梭状芽孢杆菌；毒素；抗毒素；肠粘膜；羊膜；绒毛膜；胎儿细胞；培养细胞；胎粪

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