INFECTION AND IMMUNITY, Dec. 1995, p. 4953–4956 Vol. 63, No. 12
0019-9567/95/$04.00+0

Sodium Deoxycholate Facilitates Systemic Absorption of Verotoxin 2e from Pig Intestine

THOMAS E. WADDELL* AND CARLTON L. GYLES

Department of Veterinary Microbiology and Immunology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Received 22 June 1995/Returned for modification 1 August 1995/Accepted 24 September 1995

Injection of verotoxin 2e together with sodium deoxycholate, which increases intestinal permeability to macromolecules, into the intestine of pigs resulted in fluid accumulation, intestinal damage, and signs and lesions of edema disease. Intragastric administration of verotoxin 2e to newborn piglets, who normally absorb protein nonspecifically, resulted in systemic verotoxemia. These results suggest that development of natural edema disease requires a state of increased intestinal permeability.

Edema disease (ED) in pigs, which occurs during the postweaning period, is characterized by vascular necrosis, neurological signs, and edema formation in the gastrointestinal and central nervous systems (5). The clinical signs and pathologic lesions of the disease are due to vasotoxic effects of verotoxin 2e (VT2e) produced by *Escherichia coli*, which colonizes the small intestine of the pig. These *E. coli* cells are noninvasive and colonize without causing visible damage to the intestinal mucosa (3, 21, 28). Furthermore, intraintestinal inoculation of postweaning pigs with very large amounts of VT2e does not reproduce ED (12a) despite the low 50% lethal dose of VT2e of 3 ng/kg of body weight by the intravenous route (19). These observations suggest that absorption of VT2e requires an increase in the permeability of the intestinal gut barrier to macromolecules.

This study tested the hypothesis that intraintestinal challenge of pigs with VT2e under conditions of increased intestinal permeability would allow the development of systemic effects of VT2e. VT2e was injected with sodium deoxycholate (DOC) into the intestine of postweaning pigs. DOC was used because it and other dihydroxy unconjugated bile salts have been reported to arise during bacterial overgrowth of the small intestine and to disrupt the intestinal gut barrier, allowing small increases in the nonspecific transmucosal passage of intact macromolecules (24). VT2e was also administered intragastrically to newborn piglets, who can absorb macromolecules in an intact form (2, 34).

(Portions of this study were originally presented at the 93rd Annual Meeting of the American Society for Microbiology, Atlanta, Georgia, 16 to 20 May 1993.)

VT2e was purified from *E. coli* TB1(pCG6) (17) by fast protein liquid chromatography-cation-exchange chromatography (Mono S, Pharmacia, Baie d'Urfe, Quebec, Canada) employing a linear gradient of 100 to 500 mM NaCl–10 mM NaH_2PO_4 (pH 6.0). Purified toxin displayed a specific activity greater than 1.2×10^9 50% cytotoxic doses/mg in a Vero cell cytotoxicity assay (17).

Under general anesthesia, 10-cm ligated loops were formed in the colon, lower ileum, and mid-jejunum of 5- to 6-week-old weaned female Yorkshire-Landrace pigs (Arkell Swine Unit, University of Guelph) and injected with 3 ml of phosphate-buffered saline (PBS) containing 0 to 40 μg of VT2e and 0 to 5 mM DOC (Sigma, St. Louis, Mo.). After 24 h, the ligated loops were examined for the presence of fluid and local intestinal damage. Three pigs were used to determine the effects of VT2e and DOC in various regions of the pig intestine, and four pigs were used to determine the dose response within the lower ileum.

Forty micrograms of VT2e in the presence of 5 mM DOC, but not 5 mM DOC alone, induced accumulation of mucoid and occasionally slightly red fluid and intestinal damage in ligated loops of ileum, but not jejunum or colon (Table 1). The synergy between VT2e and DOC in causing fluid accumulation and tissue damage in ligated loops in the lower ileum was investigated by observation of the effect of a range of VT2e doses with 5 mM DOC and a range of DOC concentrations with 40 μg of VT2e (Fig. 1 and 2). The minimum dose of VT2e which induced fluid accumulation in the presence of 5 mM DOC was 40 μg per loop, and the minimum concentration of DOC which induced fluid accumulation in the presence of 40 μg of VT2e was 2.5 mM. VT2e alone (40 μg per loop) was very weakly enterotoxic in ligated loops of ileum.

In the ileum, 40 μg of VT2e in the presence of 5 or 2.5 mM DOC resulted in severe tissue damage (Fig. 3), which included submucosal edema and hemorrhage, microthrombus formation and loss of villus architecture, formation of apoptotic bodies in the lamina propria of the lower portion of the villi, and release of fibrin, enterocytes, and leukocytes to the intestinal lumen. The intestinal damage observed in response to 8 μg of VT2e in the presence of 5 mM DOC occurred without fluid accumulation and was more focal and less severe than that which occurred in response to 40 μg of VT2e in the presence of 5 or 2.5 mM DOC. Five millimolar DOC did not affect the integrity of the intestinal epithelium, consistent with effects reported in the literature (12).

The mechanism of induction of fluid accumulation by VT2e and DOC together may be different from that of VTs alone in the rabbit small intestine. In the rabbit, VTs bind specifically to and are cytotoxic toward globotriaosyl ceramide-containing apical absorptive enterocytes (13), and fluid accumulation in response to treatment with Shiga toxin or VT1 is believed to be due to inhibition of Na^+ reabsorption by toxin-damaged apical absorptive enterocytes (13, 14). The fluid which accumulated in ligated loops of pig ileum after treatment with VT2e and DOC in combination was likely an exudate arising from submucosal edema and mucosal damage induced by local action of VT2e on the intestinal microvasculature.

* Corresponding author. Present address: Health of Animals Laboratory, Agriculture and Agri-Food Canada, 110 Stone Road West, Guelph, Ontario, Canada N1G 3W4. Phone: (519) 822-3300. Fax: (519) 822-2280. Electronic mail address: waddellt@em.agr.ca.

TABLE 1. Effect of VT2e plus DOC on fluid accumulation and mucosal damage in ligated intestinal loops in three regions of the pig gut[a]

Location of ligated loop	Effect of:			
	VT2e + DOC		DOC	
	Fluid accumulation[b]	Intestinal damage[c]	Fluid accumulation[b]	Intestinal damage[c]
Jejunum	0	−	0	−
Ileum	1.6 ± 0.9	+	0	−
Colon	0	−	0	−

[a] Pairs of ligated loops in the mid-jejunum, lower ileum, and mid-colon were injected with 40 μg of VT2e in 3 ml of PBS containing 5 mM DOC (VT2e + DOC) or with 3 ml of PBS containing 5 mM DOC (DOC). The loops were examined 24 h later.

[b] Expressed as volume (milliliters) per length (centimeters) ± standard error of the mean.

[c] Assessed by examination of hematoxylin-and-eosin-stained paraffin sections. +, submucosal edema and hemorrhage, microthrombus formation, and release of fibrin, enterocytes, and leukocytes to the intestinal lumen; −, no intestinal damage.

VTs alone, including VT2e, have been reported not to cause fluid accumulation in ligated loops of pig small intestine (10, 18); however, only fluid accumulation greater than 0.5 ml/cm was considered positive. In the present study, large amounts of VT2e caused very weak fluid responses in the lower ileum. The VT2e receptor glycolipids globotriaosyl ceramide and globotetraosyl ceramide have been detected in the pig small intestine mucosal scrapings (25); however, their location is unknown. Pig erythrocytes contain both globotetraosyl ceramide and globotriaosyl ceramide (7) and would be present in mucosal scrapings. The glycolipids may also be present in enterocytes or other cells in the lamina propria of the villi. The presence of glycolipids in apical absorptive enterocytes could account for the weak enterotoxic response observed. Alternatively, it is possible that endogenous deconjugated bile salts which arise in ligated loops could effect changes to the intestinal barrier to the toxin.

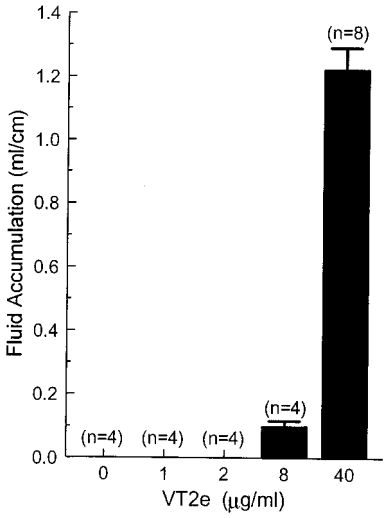

FIG. 1. Effect of various dosages of VT2e in the presence of 5 mM DOC on fluid accumulation in ligated ileal loops. Ligated loops were injected with VT2e in 3 ml of PBS containing 5 mM DOC. The data are expressed as volume (milliliters) per length (centimeters) ± standard error of the mean. *n*, number of loops per treatment.

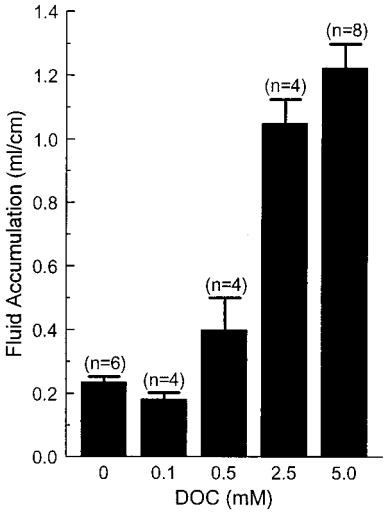

FIG. 2. Effect of various concentrations of DOC in the presence of 40 μg of VT2e on fluid accumulation in ligated ileal loops. Ligated loops were injected with 40 μg of VT2e in 3 ml of PBS containing DOC. The data are expressed as volume (milliliters) per length (centimeters) ± standard error of the mean. *n*, number of loops per treatment.

To test the hypothesis that VT2e administered intraintestinally under conditions of increased mucosal permeability would allow the development of ED in weaned pigs, the lower ileum (approximately 1 m from the ileocecal junction) of two pigs was inoculated with 400 μg of VT2e in the presence of 50 ml of PBS containing 5 mM DOC, and the pigs were observed for clinical signs of ED. The concentration of VT2e administered (8 μg/ml) was less than that which induced fluid accumulation and severe intestinal damage in ligated loops of ileum (13.3 μg/ml). Control pigs consisted of two pigs who received 400 μg of VT2e in 50 ml of PBS and two pigs who received 50 ml of PBS containing 5 mM DOC. Pigs in which VT2e together with DOC was injected into unligated ileum showed no signs of systemic toxemia during the first 24 h postinoculation. However, they began to show clinical signs of ED during the second 24 h (edema of the eyelid and face, depression, altered squeal, incoordination, fine muscle tremors, dyspnea, and convulsions) and became very ill and were euthanized 48 h after inoculation. At postmortem, edema of the mesentery of the spiral colon, small intestine, and associated lymph nodes, face, eyelid, larynx, and brain was observed in the pigs. Hemorrhage of the cerebellum was observed in one pig. Tissues of these pigs contained microvascular lesions consistent with those reported in natural and experimental ED (not shown) (16, 19). The ileum and mesenteric lymph nodes posterior to, but not anterior to, the site of injection were edematous and hemorrhagic (not shown). The control pigs remained completely healthy.

In another experiment, newborn Yorkshire-Landrace piglets less than 6 h old were administered 100 μg (six piglets), 20 μg (six piglets), or 0 μg (three piglets) of VT2e in 5 ml of 5% sodium bicarbonate intragastrically or 1 μg of VT2e in PBS intravenously (three piglets). The piglets had free access to colostrum and were returned to the sow immediately after the administration of toxin. The neonatal piglets who received VT2e intragastrically remained healthy for 24 h, but then they subsequently developed signs of verotoxemia (inappetence, edema of the eyelid, profound edema of the neck, incoordination, dyspnea, fine muscle tremors, and paralysis). The piglets who received 100 μg of VT2e experienced signs of toxemia

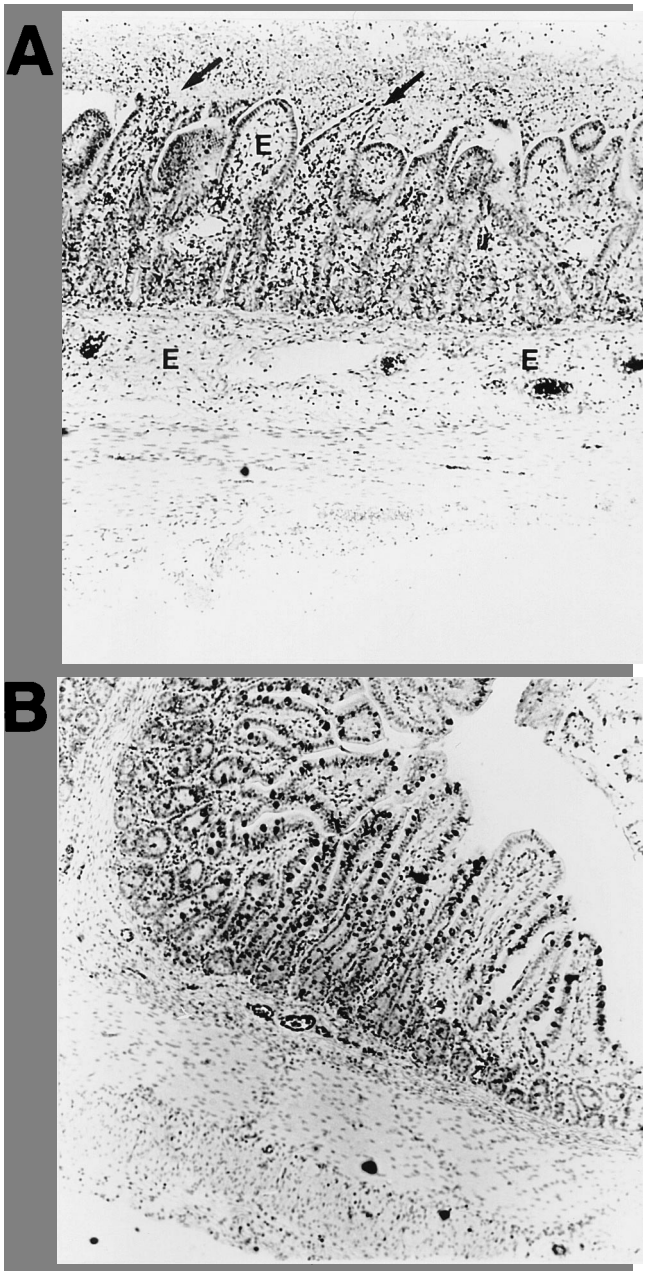

FIG. 3. Effect of 40 μg of VT2e in PBS containing 5 mM DOC (A) and of PBS containing 5 mM DOC (B) on the intestinal mucosa of ligated ileal loops after 24 h. (A) Note edema of villi and submucosa (E) and release of fibrin, enterocytes, and leukocytes to the intestinal lumen (arrows). (B) Note intact epithelium. Staining was with hematoxylin and eosin. Magnification, ×100.

earlier and died or were euthanized in extremis earlier (mean time to death, 37 h) compared with the piglets who received 20 μg of toxin (mean time to death, 48 h). All piglets who received 1 μg of VT2e intravenously developed severe signs of toxemia and died or were euthanized in extremis (mean time to death, 55 h). The piglets who were administered bicarbonate alone remained completely healthy. Edema and vascular lesions similar to those observed in postweaning pigs administered VT2e together with DOC intraintestinally were observed in the tissues of neonatal piglets administered VT2e intragastrically (not shown).

The hypothesis that VT2e is absorbed during increased intestinal permeability was supported by the demonstration that systemic effects of intraintestinal VT2e required administration of an agent which increases intestinal macromolecular permeability (1) or the protein-permeable intestine of newborn piglets. These observations suggest that during natural ED, intestinal permeability may be increased. Numerous factors may increase the permeability of the intestine. These include viruses (15), bacteria (6, 30), bacterial products such as deconjugated dihydroxy bile salts (1, 8, 31), or toxins (9, 32). During postweaning diarrhea and ED, there are marked changes in the intestinal flora of pigs (20). Possibly, the altered intestinal flora in the weaned pig may cause an accumulation of dihydroxy bile salts in the ileum, leading to increased permeability to macromolecules. Also, ED strains of *E. coli* generally produce α-hemolysin and may make one or a number of enterotoxins (11, 27), but neither α-hemolysin nor enterotoxins appear to be necessary for bacterial colonization or systemic absorption of VT2e (3, 28, 29). It is possible that ED strains of *E. coli* produce another unknown toxin which directly increases the permeability of the pig intestinal mucosa to macromolecular antigens, including VT2e.

High-protein feed at weaning promotes the development of ED by enhancing intestinal colonization with *E. coli* (4, 28). Dietary factors may also alter intestinal macromolecular permeability by enhancing colonization by certain bacteria and viruses or by inducing intestinal hypersensitivity (26, 35). In pigs with ED, infiltration of eosinophils in the intestine, evidence of hypersensitivity, has been observed (16, 23).

The amount of macromolecule absorbed from the intestine nonspecifically is extremely small and is proportional to the concentration of the solute (33). During ED, VT2e at the mucosal surface adjacent to adhering bacteria may persist at a high concentration and lead to nonspecific absorption of toxin. In vitro experiments indicate that the biological effects of heat-labile toxin produced by *E. coli* adherent to Y-1 cells were enhanced approximately 40-fold compared with those of toxin produced by nonadherent bacteria (22). VT2e produced by adherent bacteria may be similarly more effective in inducing ED than luminal toxin.

Since macromolecules are not readily absorbed from the normal postweaning pig intestine, increased intestinal permeability due to changes in the intestinal microflora or diet may play a role in the development of natural ED. Currently, we are not able to determine if the permeability of the intestine is altered during disease, since it is difficult to reproduce experimentally and a ready source of pigs which are susceptible to intestinal colonization with ED-causing strains of *E. coli* is not available in Canada or the United States. More information regarding the absorption of VT2e by pigs may be gained by studies with pigs naturally infected with ED-causing strains of *E. coli*.

We acknowledge with gratitude the support of this work by a Natural Sciences and Engineering Research grant.

REFERENCES

1. **Argenzio, R. A., and S. C. Whipp.** 1983. Comparison of the effects of disodium ethylenediaminetetraacetate, theophyline, and deoxycholate on colonic ion transport and permeability. Am. J. Vet. Res. **44:**1480–1487.
2. **Asplund, J. M., R. H. Grummer, and P. H. Phillips.** 1962. Absorption of colostral gamma-globulins and insulin by the newborn pig. J. Anim. Sci. **21:**412–413.
3. **Bertschinger, H., and J. Pohlenz.** 1983. Bacterial colonization and morphology of the intestine in porcine *Escherichia coli* enterotoxemia (edema disease). Vet. Pathol. **20:**99–110.
4. **Bertschinger, H. U., H. Jucker, H. P. Pfirter, and J. Pohlenz.** 1983. Role of nutrition in the pathogenesis of porcine *Escherichia coli* enterotoxaemia. Ann. Rech. Vet. **14:**469–472.

5. **Bertschinger, H. U., and N. O. Nielsen.** 1992. Edema disease, p. 498–509. *In* A. D. Leman, B. E. Straw, W. L. Mengeling, D. J. Taylor, and S. D'Allaire (ed.), Diseases of swine, 7th ed. Iowa State University Press, Ames.
6. **Boedeker, C. E.** 1994. Adherent bacteria: breaching the mucosal barrier? Gastroenterology **106:**255–257.
7. **Boyd, B., G. Tyrrell, M. Maloney, C. Gyles, J. Brunton, and C. Lingwood.** 1993. Alteration of the glycolipid binding specificity of the pig edema toxin from globotetraosyl to globotriaosyl ceramide alters *in vivo* tissue targeting and results in a verotoxin 1-like disease in pigs. J. Exp. Med. **177:** 1745–1753.
8. **Fagundes-Neto, U., S. Teichberg, M. A. Bayne, B. Morton, and F. Lifshitz.** 1981. Bile salt-enhanced rat jejunal absorption of a macromolecular tracer. Lab. Invest. **44:**18–26.
9. **Fasano, A., B. Baudry, D. W. Pumplin, S. S. Wasserman, B. D. Tall, J. M. Ketley, and J. B. Kaper.** 1992. *Vibrio cholerae* produces a second enterotoxin, which affects intestinal tight junctions. Proc. Natl. Acad. Sci. USA **88:**5242–5246.
10. **Gannon, V., and C. Gyles.** 1990. Characteristics of the Shiga-like toxin produced by *Escherichia coli* associated with porcine edema disease. Vet. Microbiol. **24:**89–100.
11. **Gannon, V. P. J., C. L. Gyles, and R. W. Friendship.** 1988. Characteristics of verotoxigenic *Escherichia coli* from pigs. Can. J. Vet. Res. **52:**331–337.
12. **Gullikson, G. W., W. S. Cline, V. Lorenzsonn, L. Benz, W. A. Olsen, and P. Bass.** 1977. Effects of anionic surfactants on hamster small intestinal membrane structure and function: relationship to surface activity. Gastroenterology **73:**501–511.

12a. **Gyles, C. L.** Unpublished results.

13. **Kandel, G., A. Donohue-Rolfe, M. Donowitz, and G. T. Keusch.** 1989. Pathogenesis of shigella diarrhoea. XVI. Selective targeting of Shiga toxin to villus cells of rabbit jejunum explains the effect of the toxin on intestinal electrolyte transport. J. Clin. Invest. **84:**1509–1517.
14. **Keenan, K. P., D. D. Sharpnack, H. Collins, S. B. Formal, and A. D. O'Brien.** 1986. Morphologic evaluation of the effects of shiga toxin and *E. coli* shiga-like toxin on the rabbit intestine. Am. J. Pathol. **125:**69–80.
15. **Keljo, D. J., D. G. Butler, and J. R. Hamilton.** 1985. Altered jejunal permeability to macromolecules during viral enteritis in the piglet. Gastroenterology **88:**998–1004.
16. **Kurtz, H. J., M. E. Bergeland, and D. M. Barnes.** 1969. Pathologic changes in edema disease of swine. Am. J. Vet. Res. **30:**791–806.
17. **MacLeod, D. L., and C. L. Gyles.** 1990. Purification and characterization of an *Escherichia coli* Shiga-like toxin II variant. Infect. Immun. **58:**1232–1239.
18. **MacLeod, D. L., C. L. Gyles, A. Valdivieso-Garcia, and R. C. Clarke.** 1991. Physicochemical and biological properties of purified *Escherichia coli* Shiga-like toxin II variant. Infect. Immun. **59:**1300–1306.
19. **MacLeod, D. L., C. L. Gyles, and B. P. Wilcock.** 1991. Reproduction of edema disease of swine with purified Shiga-like toxin-II variant. Vet. Pathol. **28:**66–73.
20. **McAllister, J. S., H. J. Kurtz, and E. C. Short, Jr.** 1979. Changes in the intestinal flora of young pigs with postweaning diarrhoea or edema disease. J. Anim. Sci. **49:**868–879.
21. **Methyiapun, S., J. F. L. Pohlenz, and H. U. Bertschinger.** 1984. Ultrastructure of the intestinal mucosa in pigs experimentally inoculated with an edema disease-producing strain of *Escherichia coli* (O139:K12:H1). Vet. Pathol. **21:**516–520.
22. **Ofek, I., D. Zafriri, J. Goldhar, and B. I. Eisenstein.** 1990. Inability of toxin inhibitors to neutralize enhanced toxicity caused by bacteria adherent to tissue culture cells. Infect. Immun. **58:**3737–3742.
23. **Ohshima, K.-I., and S. Miura.** 1961. Pathological study on an occurrence of swine edema disease in Iwate prefecture in 1959. Jpn. J. Vet. Sci. **23:**111–121.
24. **Palmer, R. H.** 1979. Bile acid heterogeneity and gastrointestinal epithelium. J. Lab. Clin. Med. **94:**655–660. (Editorial.)
25. **Samuel, J. E., L. P. Perera, S. Ward, A. D. O'Brien, V. Ginsberg, and H. C. Krivan.** 1990. Comparison of the glycolipid receptor specificities of Shiga-like toxin type II and Shiga-like toxin type II variants. Infect. Immun. **58:** 611–618.
26. **Sanderson, I. R., and W. A. Walker.** 1993. Uptake and transport of macromolecules by the intestine: possible role in clinical disorders (an update). Gastroenterology **104:**622–639.
27. **Smith, H. W., P. Green, and Z. Parsell.** 1983. Vero cell toxin in *Escherichia coli* and related bacteria: transfer by phage and conjugation and toxic action in laboratory animals, chickens and pigs. J. Gen. Microbiol. **129:**3121–3137.
28. **Smith, H. W., and S. Halls.** 1968. The production of oedema disease and diarrhea in weaned pigs by oral administration of *Escherichia coli*: factors that influence the course of experimental disease. J. Med. Microbiol. **1:**45–59.
29. **Smith, H. W., and M. A. Linggood.** 1971. Observations on the pathogenic properties of the K88, HLY and ENT plasmids of *Escherichia coli* with particular reference to porcine diarrhoea. J. Med. Microbiol. **4:**467–485.
30. **Spitz, J., G. Hecht, M. Taveras, E. Aoys, and J. Alverdy.** 1994. The effect of dexamethasone administration on rat intestinal permeability: the role of bacterial adherence. Gastroenterology **106:**35–41.
31. **Teichberg, S., E. McGarvey, M. A. Bayne, and F. Lifshitz.** 1983. Altered jejunal macromolecular barrier induced by α-dihydroxy deconjugated bile salts. Am. J. Physiol. **245:**G122–G132.
32. **Triadafilopoulos, G., C. Pothoulakis, M. O'Brien, and J. T. LaMont.** 1987. Differential effects of *Clostridium difficile* toxins A and B on rabbit ileum. Gastroenterology **93:**273–279.
33. **Walker, W. A., and K. J. Isselbacher.** 1974. Uptake and transport of macromolecules by the intestine: possible role in clinical disorders. Gastroenterology **67:**531–550.
34. **Westrom, B. R., J. Svendsen, B. G. Ohlsson, C. Tagesson, and B. W. Karlsson.** 1984. Intestinal transmission of macromolecules (BSA and FITC-labelled dextrans) in the neonatal pig: influence of age of piglet and molecular weight of markers. Biol. Neonate **46:**20–26.
35. **Wilson, A. D., C. R. Stokes, and F. J. Bourne.** 1989. Effect of age on absorption and immune responses to weaning or introduction of novel dietary antigens in pigs. Res. Vet. Sci. **46:**180–186.

Editor: A. O'Brien