LESIONS PRODUCED BY *CLOSTRIDIUM BUTYRICUM* STRAIN CB 1002 IN LIGATED INTESTINAL LOOPS IN GUINEA PIGS

M. R. POPOFF AND P. RAVISSE*

*Unité des Anaérobies and *Unité d'Histopathologie, Institut Pasteur, 25, rue du Docteur Roux, 75724 Paris Cedex 15, France*

SUMMARY. Heated spores (80°C, 10 min) of *Clostridium butyricum* strain CB 1002 isolated from a fatal case of necrotising enterocolitis in a human neonate were inoculated into ligated intestinal loops prepared in young conventional guinea pigs. Necropsy findings 18 h later included congestion, patchy haemorrhage of the intestinal mucosa and bacteraemia. No abnormalities were observed in control loops given inocula of inactivated spores (heated at 100°C for 10 min) or TYG 6 medium. The results suggest that vascular lesions are produced by *C. butyricum* in the intestine of young conventional guinea pigs.

INTRODUCTION

Bacterial proliferation in the intestinal tract is associated with the pathogenesis of neonatal necrotising enterocolitis (NNE) in infants (Brown and Sweet, 1982). Clostridia are frequently isolated from the faeces of these patients (Kosloske *et al.*, 1978; Kliegman, 1979; Kosloske and Ulrich, 1980), and *Clostridium butyricum* has been identified in several outbreaks of NNE (Howard *et al.*, 1977; Laverdière *et al.*, 1978; Smith *et al.*, 1980; Sturm *et al.*, 1980; Popoff and Sebald, 1981). To investigate the pathogenicity of *C. butyricum* we examined its effects in ligated intestinal loops in guinea pigs.

MATERIAL AND METHODS

Bacterial strain and growth medium. *C. butyricum* strain CB 1002 (Magot *et al.*, 1983) was isolated from stools of a neonate aged 3 weeks that died of NNE (Popoff and Sebald, 1981).

Two growth media were used: TYG contained trypticase (Difco, Paris, France) 3%, yeast extract (Difco) 2%, and glucose 0.5%; TYG 6 contained glucose 6%. Spore stocks were prepared on D medium (Labbe, 1981) incubated anaerobically for 24 h at 37°C and stored at 4°C.

Surgical and experimental procedures. The ligated intestinal loop technique (LILT) described previously in rabbits (Duncan *et al.*, 1968), was used. Young male Hartley conventional guinea pigs, weighing 100–150 g, were starved for 24 h and anaesthetised by intramuscular injection of ketamine (Imalgene 500, Institut Merieux, Lyon, France; 200 mg/kg). The abdomen was shaved and an incision was made in the midline; one 10-cm loop was prepared in the ileum with no. 4-0 silk (Moria-Dugast, Paris, France) 20 cm proximal to the caecum of each guinea pig. Spore suspensions were heated at 80°C for 10 min, diluted in 2 ml of freshly prepared TYG 6 and injected through a 30 G $\frac{1}{2}$ -in needle into the lumen of the loop. TYG 6 alone or spores inactivated

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by heating at 100°C for 10 min were inoculated into control loops. The abdomen was closed with no. 4-0 silk. The animals were killed 18 h later and a necropsy was performed immediately.

Bacterial examination. Immediately after death, 0.5 ml of heart blood was removed into 7 ml of TYG medium and incubated in anaerobic conditions at 37°C. The bacteria were identified after 24 h by the methods of Brefort and Sebald (1977).

A selective medium was used to count *C. butyricum* in the contents of loops; it consisted of

TABLE
Results of ligated-intestinal-loop experiments with C. butyricum CB1002 in guinea pigs

Number of spores	Treatment of spores	Number of animals	Bacterial counts (cfu/ml of intestinal loop content)	Severity of intestinal lesions
<i>Bacteraemic animals</i>				
2×10^8	80°C for 10 min	$\left\{ \begin{array}{l} 1 \\ 1 \\ 1 \\ 1 \\ 1 \end{array} \right.$	5×10^8	acute
2×10^8			5×10^8	moderate
2×10^6			2×10^8	moderate
2×10^7			9×10^7	acute
2×10^6			6×10^7	moderate
<i>Non-bacteraemic animals</i>				
2×10^7	80°C for 10 min	$\left\{ \begin{array}{l} 1 \\ 1 \\ 1 \\ 1 \\ 1 \end{array} \right.$	7×10^8	moderate
2×10^8			2×10^8	moderate
2×10^6			2×10^8	moderate
2×10^7			8×10^7	acute
2×10^6			6×10^7	moderate
2×10^8	100°C for 10 min	5	0	...
TYG 6 medium alone	...	5	0	...

FIG. 1.—Macroscopic appearance of ligated intestinal loops: (a and b) inocula of *C. butyricum* CB 1002 spores, heated at 80°C for 10 min; (c) inoculum of TYG 6 alone. The intestinal loops were opened longitudinally and observed by transillumination ($\times 2$); (a) shows extensive haemorrhage and congestion of the intestinal mucosa.

FIG. 1 (cont.). Macroscopic appearance of ligated intestinal loops: (b) shows patchy haemorrhage and milder congestion and (c) is a control loop.

basal medium (Cummins and Johnson, 1971), trimethoprim (Sigma, Paris, France) 16 $\mu\text{g}/\text{ml}$, D-cycloserine (Sigma) 10 $\mu\text{g}/\text{ml}$ and polymyxin B sulphate 20 $\mu\text{g}/\text{ml}$. Serial ten-fold dilutions of intestinal loop contents were made in fluid basal medium, and plates were inoculated with 0.1 ml of each dilution and incubated at 37°C for 48 h. All manipulations and incubations were done in an anaerobic chamber (La Calhène, Paris, France).

Morphological and histological examinations. The loop was excised, placed in a petri dish and its length was measured. A longitudinal incision was made and the volume of the contents was measured. A sample was fixed in Bouin solution, embedded in paraffin, serially sectioned and stained with haematoxylin and eosin. After gentle washing of the loop with phosphate buffered saline (NaCl 0.8%; Na_2HPO_4 2.7%; NaH_2PO_4 0.34%; pH 7.2) to remove the luminal debris, the loop was spread out and the lesions compared with those in control loops.

FIG. 2.—Histopathological changes in ligated intestinal loop after inoculation of *C. butyricum* CB 1002 spores heated at 80°C for 10 min (Hematoxylin and eosin): (a) shows haemorrhage in the mucosa and submucosa, and inflammatory cell infiltrate in the lamina propria ($\times 100$); (b) shows congestion of the lamina propria ($\times 250$).

RESULTS

Morphological changes in intestinal loops

Intestinal loops in 10 guinea pigs inoculated with spores of *C. butyricum* CB 1002 heated at 80°C for 10 min showed severe to moderate changes (table). In severely affected loops, haemorrhagic fluid (0.5–0.6 ml/cm) had accumulated and there was severe congestion and extensive haemorrhages of the mucosa (fig. 1a). Moderate

FIG. 2 (cont.). Histopathological changes in ligated intestinal loops: (c) shows microabscesses in the mucosa ($\times 250$); and (d) shows inflammatory-cell infiltrate in the villi (lymphocytes, macrophages, histiocytes and a few polymorphonuclear neutrophils ($\times 250$)).

lesions comprised a smaller accumulation of fluid (0.1–0.3 ml/cm), patchy haemorrhage and petechiae of the mucosa (fig. 1b). The mesenteric vessels were dilated in every case.

No obvious abnormalities were seen when spores of *C. butyricum* CB 1002 inactivated by heating at 100°C for 10 min or TYG 6 alone were inoculated into control loops (fig. 1c).

Bacterial counts

The counts of *C. butyricum* CB 1002 in intestinal loops are shown in the table. The organisms were also recovered from the heart blood of 5 out of 10 animals.

Histological observations

By light microscopy, the severe intestinal lesions were characterised by congestion and small areas of haemorrhage in the mucosa; the lamina propria and submucosa were infiltrated with lymphocytes, macrophages, histiocytes and a few polymorphonuclear neutrophils, and in one case micro-abscesses were present in the mucosa. These changes are shown in figs 2a–2d. Lysis of epithelial cells was observed in some areas, but it was mild and the intestinal villi were not disrupted. Staining of sections by Gram's method demonstrated gram-positive rods in the intestinal lumen; small numbers were also present in the crypts, but not in the lamina propria or in the mucosa. Less severe changes were seen in the loops with moderate macroscopic changes. In the control loops, infiltration of a small number of neutrophils was present in the lamina propria and the submucosa.

DISCUSSION

The results of this study show that *C. butyricum* strain CB 1002 caused intestinal damage and bacteraemia after inoculation into ligated loops in guinea pigs. The lesions consisted of vascular alterations (congestion and patchy haemorrhages in the intestinal mucosa) and inflammatory infiltration of the intestinal wall. Necrotic lesions were absent or mild, and may have been attributable to early autolysis. Vascular modifications, haemorrhages in the mucosa and submucosa, and extensive mucosal necrosis occur in natural disease (Pedersen *et al.*, 1976; Laverdière *et al.*, 1978) and the vascular modifications may be an early stage of NNE (Swanson and Landing, 1980); this might be demonstrated in intestinal loops left for longer than 18 h which is unacceptable with this technique.

We used conventional guinea pigs in these experiments because we were unable to obtain germ-free animals. Although the intestinal bacteria may have interacted with *C. butyricum*, we did not observe significant vascular alterations in the control intestinal loops.

Little is known about the pathogenicity of *C. butyricum*. Enteritis was produced in neonatal germ-free rats after oral inoculation of *C. butyricum* (Lawrence *et al.*, 1982). The pathological findings were mainly confined to the distal ileum, and ranged from loss of villi to patchy haemorrhage of the gut wall and necrosis. However, the authors failed to reproduce these results (Lawrence and Bates, 1983). Host factors may account for the different responses between the human neonate and experimental animals. Moreover, it has been shown that susceptibility to NNE is closely related to the functional maturation of the gastrointestinal tract (Wilson *et al.*, 1982 and 1983). Such events could explain the inconsistent results of experimental infections in laboratory animals.

Our experimental model shows that *C. butyricum* strain CB 1002 can induce vascular intestinal damage, but the other lesions of NNE (extensive necrosis and pneumatosis) were not observed. Further work is in progress to develop a more suitable animal model for NNE.

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