The effect of campylobacter lipopolysaccharide on fetal development in the mouse

A. M. O'SULLIVAN, C. J. DORÉ*, S. BOYLE†, C. R. COLD and A. P. JOHNSON‡

*Divisions of Comparative Medicine, *Medical Statistics, †Histopathology and ‡Sexually Transmitted Diseases, Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ*

Summary. Purified lipopolysaccharide (LPS) obtained from isolates of *Campylobacter fetus* ss. *fetus* and *Campylobacter jejuni* impaired fetal development when administered to mice on day 13 of pregnancy. Strikingly more fetal resorption was produced by *C. jejuni* LPS than by similar amounts of *C. fetus* ss. *fetus* LPS. Three of the four *Campylobacter* strains examined produced LPS that had no effect on maternal health, but LPS from one *C. jejuni* strain killed all of the mice to which it was administered.

Introduction

Organisms of the genus *Campylobacter* are associated with various infections in man and animals. The principal disease in man is enterocolitis, but cases of meningitis, abortion, arthritis and other systemic infections have also been reported. Infertility and abortion are the main features of the infection in ruminants (Skirrow, 1984).

Little is known about the mechanisms by which campylobacters cause disease. Earlier work in this laboratory showed that both live and heat-killed campylobacters impaired fetal development when injected into the 13-day pregnant mouse. It was suggested that endotoxin-like substances played a role in pathogenesis (O'Sullivan *et al.*, 1988). The present report describes the effect of purified lipopolysaccharide—the endotoxin moiety (Smith, 1978)—extracted from *C. fetus* ss. *fetus* (henceforth referred to as *C. fetus*) and *C. jejuni* on the development of the mouse embryo.

Materials and methods

Campylobacter strains

The two *C. fetus* strains were kindly supplied by Dr M. B. Skirrow, Worcester Royal Infirmary and the two *C. jejuni* strains by Dr D. Seal, Northwick Park Hospital. These four human isolates were subcultured four times in this laboratory before use. Stock cultures were stored at -70°C . The *C. fetus* strains comprised no. 23907, isolated from an ovarian abscess, and no. 613, from a wound. The *C. jejuni* strains comprised no. 42734, isolated from the watery faeces of a 77-year-old female patient, and no.

40630, from the blood-stained faeces of a 26-year-old male patient. The campylobacters were cultured on horse-blood agar plates (Gibco) and incubated at 37°C for 48 h in a micro-aerophilic environment produced by means of a "Campypack" (Oxoid). Before harvesting, each plate was checked for contaminants.

Lipopolysaccharides (LPS)

LPS was extracted from campylobacters by the method of Johnson and Perry (1976). Briefly, the bacteria were scraped from the surface of blood-agar plates with a loop and suspended in phenol 1% in pyrogen-free water. The bacterial suspensions were then placed in 50-ml tubes (Falcon, Becton Dickinson) and centrifuged at 8000 rpm at 4°C for 15 min. The supernates were discarded and the pellets washed in ethanol 95% and lyophilised. The lyophilised cells were then suspended in 50 ml of 50 mM sodium phosphate buffer. After ultrasonic disintegration the cells were treated with ethylenediaminetetra-acetic acid (EDTA; BDH Chemicals Ltd), with lysozyme to digest cell-wall material, and with DNAase and RNAase to remove nucleic acids. Protein was then removed by the addition of phenol at 70°C and, after extensive dialysis, the LPS in the aqueous phase was collected by ultra-centrifugation.

The presence of protein contaminants ($<1.0\text{ }\mu\text{g/ml}$) in the LPS was estimated by the Bio-Rad Protein Assay (Bio-Rad Laboratories Ltd) and the presence of nucleic acids ($<0.1\text{ }\mu\text{g/ml}$) by electrophoresis on agarose gels followed by staining with ethidium bromide (Maniatis *et al.*, 1982). DNA at a concentration of $0.1\text{ }\mu\text{g/ml}$, kindly supplied by Dr S. Rastan, was used as a standard. The Limulus Amoebocyte Assay (Sigma) was used for the detection of endotoxic activity. The LPS pellet was then lyophilised. For use it was suspended in pyrogen-free distilled water.

The LPS extracts of the four campylobacter strains were highly reactive (firm gel) in the Limulus clotting

assay. The LPS, weighed before it was dissolved in pyrogen-free water, gave a Limulus activity equivalent of 3.04×10^0 Endotoxin Units (EU)/ml and 3.04×10^{-1} EU/ml for *C. fetus* strains 23907 and 613 respectively and 3.04×10^3 EU/ml and 3.04×10^1 EU/ml of LPS for *C. jejuni* strains 42734 and 40630 respectively. *Escherichia coli* LPS, at the same w/v, used for comparison, gave an activity equivalent of 3.04×10^4 EU/ml.

Mice

Female mice of an outbred strain (TO/Crc) aged 10 weeks were obtained from the specific pathogen-free breeding unit at this Centre. The first day of pregnancy was taken to be that on which a vaginal plug was observed. Groups of mice were given LPS intravenously in 0.2-ml doses containing 0.147, 0.294, 0.588 and 1.176 mg. The control groups received 0.2 ml of sterile pyrogen-free water. A further control group received 0.147 mg of *E. coli* LPS (O127:B8; Difco Laboratories) in 0.2 ml of pyrogen-free water.

Statistical methods

Probit analysis (Ross, 1980) was used to compare the dose-response relationship of the two *C. fetus* strains; a logarithmic scale was used for dose. The Mann-Whitney U and Fisher's exact tests (Armitage, 1971) were used for other comparisons as indicated in the text.

Histology

Fetal and placental tissues as well as sections from maternal liver, spleen, lung and kidneys were prepared

for histological examination and stained with haematoxylin and eosin.

Results

Response of pregnant mice to campylobacter LPS

As shown in the table, administration of LPS from both *C. fetus* and *C. jejuni* resulted in impaired fetal development.

To examine possible species and strain differences in the ability of LPS to cause fetal resorption, the dose (RD50) that would produce 50% resorptions was estimated by probit analysis. The RD50 for the LPS from *C. fetus* strain 23907 was 0.30 mg/mouse (95% confidence interval 0.23–0.40); for *C. fetus* strain 613 it was 0.45 mg/mouse (95% confidence interval 0.32–0.61). There was no more than a suggestion of a difference in the dose-response relationship between curves of the two strains ($p=0.08$).

At all doses investigated, *C. jejuni* LPS produced resorption of all of the mouse fetuses. The RD50 must therefore have been <0.147 mg/mouse, the lowest dose studied. Furthermore, LPS from *C. jejuni* strain 42734 killed all of five mice when given in a dose of 1.176 mg. No deaths occurred in any other groups ($p=0.02$; Fisher's exact test). The deaths occurred 24–48 h after administration of LPS; no diarrhoea was observed, but vaginal bleeding was evident.

These experiments demonstrated a significant difference in the ability of LPS from *C. fetus* and *C.*

Table. Effects of campylobacter LPS on the development of the 13-day mouse fetus

Campylobacter strain	Mean percentage fetal resorption (range) in mice treated with LPS in a dose (mg/mouse) of				Mean percentage fetal resorption in mice treated with	
	0.147	0.294	0.588	1.176	pyrogen-free water	<i>E. coli</i> LPS 0.147 mg
<i>C. fetus</i> 23907	18 (0–40) n=4	34 (0–100) n=4	98 (90–100) n=4	95 (75–100) n=5	0 (0–0) n=5	100 (100–100) n=5
<i>C. fetus</i> 613	0 (0–0) n=3	13 (0–38) n=3	64 (0–100) n=4	100 (100–100) n=4	2 (0–11) n=6	100 (100–100) n=6
<i>C. jejuni</i> 42734	100 (100–100) n=4	100 (100–100) n=5	100 (100–100) n=5	100 (100–100) n=5	7 (0–33) n=5	100 (100–100) n=4
<i>C. jejuni</i> 40630	100 (100–100) n=5	100 (100–100) n=4	100 (100–100) n=5	100 (100–100) n=5	4 (0–14) n=4	100 (100–100) n=5

n = Number of mice per group.

jejuni to impair fetal development ($p=0.001$ by the Mann-Whitney U test at LPS dose 0.147 mg).

Histological examination

The pathological changes observed in the fetal and maternal tissues of animals inoculated with

endotoxin from *E. coli*, *C. fetus* or *C. jejuni* were similar. Embryonic tissues were degenerate, cells lining the vascular spaces of the placenta were swollen, and there was focal necrosis (figure). Patchy fibrin deposition in vascular spaces and focal calcification were also evident in the placentas.

Figure. Transverse section of the fetal (F)-placental (P) junctions from mice on day 18 of pregnancy; $\times 102.5$. C. Normal fetal-placental junction (Top). T. Fetal-placental junction from a mouse treated on day 13 with *C. jejuni* LPS (Bottom).

Maternal livers showed focal necrosis and inflammation. The spleens appeared congested and showed occasional macrophages containing haemosiderin. The lungs, which were also congested, showed focal haemorrhages. No pathological changes were seen in the kidneys.

Discussion

Recent studies (O'Sullivan *et al.*, 1988) demonstrated that the inoculation of 13-day pregnant mice with live or heat-killed strains of *Campylobacter* led to impaired fetal development, the findings resembling those observed in mice treated with purified *E. coli* endotoxin (Coid, 1976). It was suggested, therefore, that endotoxin contributed, at least in part, to the pathogenesis of infection.

The present experiments demonstrate that LPS extracted from campylobacters also causes resorption of the 13-day mouse fetus and the pathological changes are indeed similar to those seen after inoculation with purified *E. coli* LPS or with live or heat-killed campylobacters. This observation strengthens the view that endotoxin is one of the active components responsible for impaired fetal development. A similar conclusion was reached by Osborne and Smibert (1964), who injected campylobacter organisms into pregnant heifers, and by Osborne (1965), who showed that the pathological changes were similar to those produced by *E. coli* endotoxin. The LPS of the Enterobacteriaceae has been well characterised (Lüderitz *et al.*, 1982; Hitchcock and Brown, 1983) and studies by Perez-

Perez *et al.* (1986) and Naess and Hofstad (1984) have shown that the LPS of *Campylobacter* spp. is similar. Moreover, electronmicroscopic studies in this laboratory on campylobacter LPS have shown the same morphological structure as in LPS isolated from other gram-negative bacteria (Shands, 1971).

The response of pregnant mice to the campylobacter LPS varied both between species and between strains. It was interesting to note that there was a correlation between the results obtained *in vivo* and *in vitro*; the extract that gave the highest activity in the Limulus test was the most toxic in mice in that it not only caused resorption of all embryos but was lethal for the pregnant mice in higher doses. Campylobacter LPS was much less potent than that of *E. coli*. Fumarola *et al.* (1982) observed that *E. coli* endotoxin was more potent than *C. jejuni* or *C. coli* heat-killed suspensions, in the dermal Shwartzman reaction as well as in the Limulus assay. Studies by Perez-Perez and Blaser (1985) showed that *C. jejuni* strains possessed a rough type of LPS with few or no polysaccharide side chains, whereas *C. fetus* strains possessed a smooth type of LPS. These differences suggest that the toxicity of the LPS is related to its structure.

It is concluded that LPS is, at least in part, responsible for bringing about the pathological changes caused by organisms of the genus *Campylobacter*.

We thank Dr S. Rastan and Mrs H. Davies for helpful advice and comments during the preparation of this paper and Miss J. MacKenzie for the photography.

REFERENCES

- Armitage P 1971 Statistical methods in medical research. Blackwell Scientific, Oxford.
- Coid C R 1976 Bacterial endotoxin and impaired fetal development. *Experientia* **32**:735-736.
- Fumarola D, Miragliotta G, Jirillo E 1982 Endotoxin-like activity associated with heat-killed organisms of the genus *Campylobacter*. In: Newell D G (ed) *Campylobacter*: epidemiology, pathogenesis and biochemistry. MTP Press, Lancaster, pp 185-187.
- Hitchcock P J, Brown T M 1983 Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *Journal of Bacteriology* **154**:269-277.
- Johnson K G, Perry M B 1976 Improved techniques for the preparation of bacterial lipopolysaccharides. *Canadian Journal of Microbiology* **22**:29-34.
- Lüderitz O, Freudenberg M A, Galanos C, Lehmann V, Rietschel E T, Shaw D H 1982 Lipopolysaccharides of gram-negative bacteria. *Current Topics in Membranes and Transport* **17**:79-151.
- Maniatis T, Fritsch E F, Sambrook J 1982 Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Naess V, Hofstad T 1984 Chemical composition and biological activity of lipopolysaccharides prepared from type strains of *Campylobacter jejuni* and *Campylobacter coli*. *Acta Pathologica Microbiologica et Immunologica Scandinavica, Section B* **92**:217-222.
- Osborne J C 1965 Pathological responses in animals after *Vibrio fetus* toxin shock. *American Journal of Veterinary Research* **26**:1056-1067.
- Osborne J C, Smibert R M 1964 *Vibrio fetus* toxin. 1. Hypersensitivity and abortifacient action. *The Cornell Veterinarian* **54**:561-572.
- O'Sullivan A M, Doré C, Coid C R 1988 Campylobacters and impaired fetal development in mice. *Journal of Medical Microbiology* **25**:7-12.
- Perez-Perez G I, Blaser M J 1985 Lipopolysaccharide characteristics of pathogenic campylobacters. *Infection and Immunity* **47**:353-359.
- Perez-Perez G I, Hopkins J A, Blaser M J 1986 Lipopolysaccharide structures in Enterobacteriaceae, *Pseudomonas aeruginosa* and *Vibrio cholerae* are immunologically related to *Campylobacter* spp. *Infection and Immunity* **51**:204-208.

- Ross G J S 1980 MLP—Maximum Likelihood Program. Rothamsted Experimental Station, Harpenden.
- Shands J W 1971 The physical structure of bacterial lipopolysaccharides. In: Weinbaum G *et al.* (eds) vol. 6, Bacterial endotoxins. Microbial toxins, Academic Press, London, pp 127–144.
- Skirrow M B 1984 *Campylobacter* infections of man. In: Easmon C S F, Jeljaszewicz J (eds) Medical microbiology, vol. 4. Academic Press, London, pp 105–141.
- Smith H 1978 The determinants of microbial pathogenicity. In: Norris J R, Richmond M H (eds) Essays in microbiology, chapter 13. John Wiley and Sons, Chichester, pp 13/1–13/32.