

# *Chlamydia trachomatis*-induced death of human spermatozoa is caused primarily by lipopolysaccharide

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Elementary bodies (EBs) of *Chlamydia trachomatis* serovar E are more toxic to sperm than those from serovar LGV. In this study, lipopolysaccharide (LPS) was prepared from the EBs of both serovars and incubated with human spermatozoa at concentrations that matched the LPS concentration of EBs. The effects of EBs and LPS on sperm motility, viability and acrosomal status were then determined. Sperm motility was measured by computer-assisted sperm analysis and the hypo-osmotic swelling test was used to determine the proportion of dead cells. Acrosomal status was examined using a standard mAb assay. Over a 6 h incubation, LPS from both serovars resulted in a marked reduction in sperm motility (and a concomitant increase in the proportion of dead spermatozoa) in a manner similar to that seen in response to EBs of serovar E. In addition, when sperm were incubated with a range of doses of EBs and LPS, probit analysis revealed that the greater spermicidal effects of EBs from serovar E (when compared with serovar LGV) were not observed when sperm were incubated with LPS from the two serovars. This suggests that the more potent effect of EBs of serovar E cannot be explained entirely by differences in the composition of LPS. Interestingly, *Escherichia coli* LPS was required in doses 500 times more concentrated than chlamydial LPS in order to kill a similar proportion of sperm, suggesting that bacterial LPSs may differ in their spermicidal properties. However, that chlamydial LPS was spermicidal was demonstrated by the use of polymyxin B (a polycationic antibiotic known to neutralize LPS effects), confirming that the effects observed were primarily a result of LPS activity.

Received 3 December 2001

Accepted 1 November 2002

## INTRODUCTION

*Chlamydia trachomatis* is an obligately intracellular bacterium that is responsible for an increasing number of sexually transmitted infections in both males and females (Weström, 1996). The organism has a unique developmental cycle in which it exists in two alternating forms: an extracellular, metabolically inactive, infectious form called the elementary body (EB) and an intracellular, metabolically active, reproducing form called the reticulate body. This developmental cycle is necessary because chlamydiae need to utilize the intracellular machinery of a host cell in order to reproduce. An obvious consequence of this developmental cycle is that, at the site of infection, the reproductive tract will periodically contain significant numbers of highly infectious EBs, and these may be encountered by any gametes that are present in the reproductive tract at that time.

There has been very little research to investigate whether direct exposure to *C. trachomatis* itself may compromise gamete function directly. However, recent work by our group on human spermatozoa has suggested that it may be an important contributor to subfertility in infected men and women. We have shown that, following incubation with EBs from *C. trachomatis* serovar E, the tyrosine phosphorylation of two major sperm epitopes of 80 and 95 kDa is significantly increased (Hosseinzadeh *et al.*, 2000). As a result, we initially hypothesized that *C. trachomatis* serovar E may compromise sperm function by accelerating sperm capacitation, since tyrosine phosphorylation of sperm proteins is closely associated with capacitation *in vitro* (Visconti & Kopf, 1998). However, in a further series of experiments (Hosseinzadeh *et al.*, 2001), we demonstrated that serovar E was causing sperm death, suggesting to us that the chlamydial-induced increase in tyrosine phosphorylation was associated more with cell death than with capacitation.

Abbreviations: EB, elementary body; HOS, hypo-osmotic swelling; LPS, lipopolysaccharide; PMB, polymyxin B.

Interestingly, experiments with *C. trachomatis* serovar LGV did not give the same results. Incubation of human sperma-

tozoa with serovar LGV led only to increased tyrosine phosphorylation of the 80 kDa epitope (Hosseinzadeh *et al.*, 2000) and did not lead to a significant increase in cell death (Hosseinzadeh *et al.*, 2001). This suggests that there are subtle differences between the serovars in terms of their infectivity towards reproductive tissues. This is not surprising, as there are known differences in their clinical presentation. Serovar E is perhaps the most common genital serovar worldwide, whereas serovar LGV is rarely seen outside the tropics and also leads to an infection with a more significant involvement of the lymphatic system (Moulder, 1991; Morré *et al.*, 2000). Moreover, we have also shown that these two serovars differ markedly in the mechanism that they use to infect epithelial cells (Taraktchoglou *et al.*, 2001).

At the present time, we do not know the mechanism by which *C. trachomatis* serovar E may be triggering sperm death. Moreover, it is not known why serovar E has a more marked spermicidal effect, when serovar LGV is thought to lead to the more aggressive clinical infection. In an attempt to investigate these issues, our attention has focussed on the lipopolysaccharide (LPS) component of *C. trachomatis*. This follows the report by Galdiero *et al.* (1994) that LPS purified from *C. trachomatis* (albeit serovar LGV) could lead to a high degree of sperm death during an *in vitro* incubation. LPS is a major component of the chlamydiae, as with all Gram-negative bacteria (Brade *et al.*, 1997). It is also known as endotoxin, which is a known contaminant of *in-vitro* fertilization culture systems that leads to low fertilization rates and impaired embryo development (Snyman & Van der Merwe, 1986; Fishel *et al.*, 1988). As such, we have extended our initial studies to investigate the effect of chlamydial LPS from both serovar E and LGV on the motility, viability and acrosomal status of human spermatozoa *in vitro*.

## METHODS

Semen samples that were identified to be normozoospermic by World Health Organization (WHO) criteria (WHO, 1999) were obtained from donors attending the Andrology Laboratory, Jessop Wing, Royal Hallamshire Hospital (Sheffield, UK). From each sample, a highly motile suspension of spermatozoa was obtained by density centrifugation of a 1 ml aliquot of liquefied semen through a Percoll gradient, as described previously (Hosseinzadeh *et al.*, 2000). This results in a highly motile suspension of spermatozoa with relatively few contaminating leukocytes and, therefore, a low level of reactive oxygen species (ROS) production that may otherwise contribute to sperm damage (Kessopoulou *et al.*, 1992). The final concentration of spermatozoa obtained was adjusted to  $2 \times 10^7$  sperm  $\text{ml}^{-1}$  in Earle's balanced salt solution (EBSS; Sigma) containing 0.3% (w/v) human serum albumin (Sigma) prior to use in the experiments described below.

EBs of *C. trachomatis* serovars E and LGV were prepared from laboratory cultures of McCoy cells that had been maintained as described by Tjiam *et al.* (1984). The culture of serovar E was initially isolated from a clinical source (cervical swab from Department of Genitourinary Medicine, Royal Hallamshire Hospital, Sheffield) and strain LGV1 was kindly provided by M. Ward (University of Southampton, UK). Confirmation of the genotype was conducted by restriction analysis of a nested PCR product of each serovar according to the method of Lan *et al.* (1994). The EBs were then isolated from McCoy cells by density-gradient centrifugation as described by Caldwell

*et al.* (1981) and detailed in our previous report (Hosseinzadeh *et al.*, 2001). A dilution series of EBs was applied to cell monolayers for quantification. Following incubation, monolayers were fixed and stained using the Micro Trak *C. trachomatis* TC confirmation test according to the manufacturer's instructions. Using a fluorescence microscope, the inclusions were counted. The number of inclusion-forming units (i.f.u.) for each dilution was determined and used to calculate an approximate number of i.f.u.  $\text{ml}^{-1}$ .

**Extraction and quantification of chlamydial LPS.** *C. trachomatis* LPS was extracted as described previously by Nurminen *et al.* (1985). Urografin-purified EBs from serovars E and LGV were suspended in 90% aqueous phenol (2.5 ml) and homogenized for 5 min followed by magnetic stirring at 50 °C for 30 min. Four millilitres petroleum ether (boiling point 90–100 °C) and 2.5 ml chloroform were then added to the mixture and stirring was continued for 1 h at room temperature. After centrifugation at 10 000 g for 30 min, the supernatant was freed from organic solvents under reduced pressure and the remaining solution was precipitated with acetone in a –20 °C freezer. The precipitate (LPS) was then washed with cold acetone, dried under vacuum and kept at –20 °C after resuspension in 200  $\mu\text{l}$  2-sucrose phosphate (2-SP) buffer. To confirm the successful extraction of LPS, 20  $\mu\text{l}$  of the LPS sample was then run on a 14% polyacrylamide gel at 100–150 V. Extracted LPS from *Aeromonas caviae* was used to compare with chlamydial LPS on a polyacrylamide gel. After running the gel, it was fixed in 40% methanol plus 10% acetic acid overnight followed by washing five or six times (for 5 min) in the fixative containing 0.7% periodic acid. The gel was then stained using the Bio-Rad silver staining kit.

The *Limulus* amoebocyte lysate (LAL) kit (BioWhittaker) was used to quantify chlamydial LPS. The method was performed as recommended by the manufacturer. A microplate method was used as follows. The microplate was pre-warmed at  $37 \pm 1$  °C in a heating block. Aliquots of 50  $\mu\text{l}$  of samples or standards were dispensed into the appropriate microplate well. Each series of determinations included a blank plus the four endotoxin standards run in duplicate. Reagent additions and incubation times were identical. At time 0, 50  $\mu\text{l}$  LAL was added to the first microplate well. At 10 min, 100  $\mu\text{l}$  substrate solution (pre-warmed to 37 °C) was dispensed into the microplate wells. At 16 min, 100  $\mu\text{l}$  stop reagent was added to stop the reactions. The absorbance of each microplate well was read at 405 nm. A standard curve was drawn and the concentrations of LPS (EU  $\text{ml}^{-1}$ ) were calculated according to the standard curve. The final concentration of LPS was recorded as  $\mu\text{g ml}^{-1}$  of the preparations.

The doses of LPS used in our experiments were calculated to be roughly equivalent to the number of EBs used in the incubations in this and our previous work. Therefore, according to our calculations (not shown), a concentration of  $0.31 \times 10^6$  EBs  $\text{ml}^{-1}$  was equivalent to an LPS dose of 0.05  $\mu\text{g ml}^{-1}$  and so on.

**Time-course incubation of chlamydial-derived LPS.** Semen samples were obtained from six men and prepared as described above to give six 300  $\mu\text{l}$  aliquots. To two aliquots,  $1.75 \times 10^5$  EBs of serovar E or LGV were added in a volume of 20  $\mu\text{l}$ , giving a final concentration of  $0.54 \times 10^6$  EBs  $\text{ml}^{-1}$ . To two further aliquots, LPS extracted from serovar E or LGV (see above) was added in a volume of 4  $\mu\text{l}$ , giving a final concentration of 0.1  $\mu\text{g ml}^{-1}$ . Finally, two control incubations were established. As a positive control, 15  $\mu\text{l}$  of LPS derived from *Escherichia coli* O55:B5 (Sigma) was added to give a final concentration of 50  $\mu\text{g ml}^{-1}$ . As a negative control, 20  $\mu\text{l}$  EBSS was added. Each experimental tube was maintained over a 6 h incubation period in a humid atmosphere at 37 °C in 5% CO<sub>2</sub> in air. After 1, 3 and 6 h incubation, the tubes were gently mixed before removing two 10  $\mu\text{l}$  aliquots for the evaluation of sperm motility characteristics and sperm viability or acrosomal status as described below.

**Dose response to chlamydial LPS.** Semen samples from a further six men were prepared to give twenty-one 300  $\mu\text{l}$  aliquots. To eight aliquots, EBs of *C. trachomatis* serovar E or LGV were added in a volume of 40  $\mu\text{l}$ , giving final concentrations of 0.31, 0.63, 1.25 and 2.50  $\times 10^6$  EBs  $\text{ml}^{-1}$ . To a further eight aliquots, LPS purified from serovar E or LGV was added in a volume of 40  $\mu\text{l}$  to give final concentrations of 0.05, 0.1, 0.2 and 0.4  $\mu\text{g}$  chlamydial LPS  $\text{ml}^{-1}$ . As a positive control, to a further four aliquots, *E. coli* LPS was added in a volume of 40  $\mu\text{l}$  to give final concentrations of 25, 50, 100 and 200  $\mu\text{g}$   $\text{ml}^{-1}$ . A single aliquot of 300  $\mu\text{l}$ , to which 40  $\mu\text{l}$  EBSS was added, served as a negative control for all experiments. All incubations were maintained in a humid atmosphere at 37 °C in 5%  $\text{CO}_2$  in air for 6 h, before removing two 10  $\mu\text{l}$  aliquots for the evaluation of sperm motility characteristics and sperm viability or acrosomal status.

**Dose response to polymyxin B (PMB).** Semen samples from a further six patients were prepared to give five 300  $\mu\text{l}$  aliquots each, to which 40  $\mu\text{l}$  PMB (Sigma) was added to give final concentrations of 50, 100, 200 and 400  $\mu\text{g}$   $\text{ml}^{-1}$ . PMB is a known and potent inhibitor of the biological and toxic effects of bacterial LPS (Duff & Atkins, 1982; Morrison & Jacobs, 1976). To a final aliquot, 40  $\mu\text{l}$  EBSS was added as a negative control. All incubations were maintained in a humid atmosphere at 37 °C in 5%  $\text{CO}_2$  in air for 6 h, before removing two 10  $\mu\text{l}$  aliquots for the evaluation of sperm motility characteristics and sperm viability or acrosomal status.

**Inhibitory effects of PMB.** Semen samples from a further six patients were prepared to give ten 300  $\mu\text{l}$  aliquots each. To four aliquots, EBs of both serovars were added as in the time-course experiments to give a final concentration of 0.54  $\times 10^6$   $\text{ml}^{-1}$ . To one aliquot of E and LGV, PMB was added (as in the dose-response experiments) to give a final concentration of 100  $\mu\text{g}$   $\text{ml}^{-1}$ . To four further aliquots, chlamydial LPS from both serovars was added as in the time-course experiments to give a final concentration of 0.1  $\mu\text{g}$   $\text{ml}^{-1}$ . Similarly, to one aliquot of chlamydial LPS from each serovar, PMB was added at a final concentration of 100  $\mu\text{g}$   $\text{ml}^{-1}$ . As controls, an aliquot containing PMB at a concentration of 100  $\mu\text{g}$   $\text{ml}^{-1}$  and a second aliquot containing medium alone were established. All incubations were maintained in a humid atmosphere at 37 °C in 5%  $\text{CO}_2$  in air for 6 h, before removing two 10  $\mu\text{l}$  aliquots for the evaluation of sperm motility characteristics and sperm viability or acrosomal status.

**Assessment of sperm motility characteristics.** For the assessment of sperm motility, 10  $\mu\text{l}$  of the incubate was transferred into a 20  $\mu\text{m}$  depth Microcell slide (Conception Technologies). The slide was then placed on a heated stage (37 °C) of an Olympus BH-2 microscope fitted with a  $\times 10$  positive-phase objective and a Sony (SPT-M124) CCD camera connected to a video recorder. The videotapes were subsequently analysed using a Hamilton Thorn IVOS Motility analyser (Hamilton Thorn Research) running version X10.8q of the Hamilton Thorn operating system. The set-up parameters of the machine were: frame rate of 50 Hz at 50 frames  $\text{s}^{-1}$ , minimum contrast of 80, minimum size of 2 pixels, lo/hi size gates of 0.30 and 2.99, lo/hi intensity gates of 0.66 and 1.15, magnification factor 4.78.

**Assessment of sperm viability and acrosomal status.** The viability of spermatozoa at each time-point was determined using the hypo-osmotic swelling (HOS) test (WHO, 1999). The HOS test was chosen as it allowed the simultaneous assessment of membrane integrity and acrosomal status to provide a simple method to determine the percentage of live, acrosome-reacted sperm. Briefly, 20  $\mu\text{l}$  of each incubate was transferred to 200  $\mu\text{l}$  HOS solution (containing 0.735 g trisodium citrate and 1.351 g fructose in 100 ml distilled water) and incubated at 37 °C for 30 min. Each incubate was then spotted onto a microscope slide and allowed to air-dry overnight before being fixed in absolute methanol for 45 min the following day. The slides were then stained to evaluate

acrosomal status using the technique described by Moore *et al.* (1987). Each slide was then observed on an Olympus BH-2 microscope fitted with both  $\times 100$  magnification phase-contrast and epifluorescence objectives (UV filter 492 nm). For each incubate or control, 200 sperm were counted to establish the viability of the sample, recording all sperm with straight tails as 'dead'. A further 200 sperm were examined to determine the proportion of viable (coiled-tailed) sperm that were acrosome intact (spermatozoa with entire fluorescence over the acrosomal region).

**Statistical analysis.** Since all of the data generated in these experiments were non-parametric (percentage motility, viability and acrosomal status), the data were analysed by a variety of non-parametric strategies. To determine overall differences between groups, the Mood median test was used with differences between specific groups being evaluated using a Kruskal–Wallis non-parametric analysis of variance and/or Dunn's test. To investigate the relationship between the dose effects of micro-organisms and purified LPS, a probit analysis was performed using version 10 of the Statistical Package for Social Scientists (SPSS Inc.).

## RESULTS

### Time-course incubation of chlamydial-derived LPS

Immediately after density-gradient centrifugation, the sperm preparations had a median sperm motility of 77.0 (72.6–82.1) % with only 13.5 (10.4–17.0) % dead cells and 6.5 (4.4–10.0) % of live sperm having lost their acrosomes [data are medians (95% confidence interval, CI) for  $n = 6$  experiments]. Table 1 shows the results of the subsequent incubation, where aliquots of this preparation were incubated with EBs of serovar E or LGV (0.54  $\times 10^6$   $\text{ml}^{-1}$ ) or LPS extracted from serovar E or LGV (0.1  $\mu\text{g}$   $\text{ml}^{-1}$ ) in addition to LPS derived from *E. coli* (50  $\mu\text{g}$   $\text{ml}^{-1}$ ) and a medium-only control. Over the 6 h incubation period, the characteristics of the control (medium only) incubation did not alter significantly ( $P > 0.05$ ) from that seen immediately after preparation (see above).

However, the characteristics of sperm incubated with EBs or LPS (derived from any source) altered markedly over the 6 h incubation. As expected from our previous work (Hossein-zadeh *et al.*, 2001), the decline in sperm motility seen following incubation with EBs of serovar E was more marked than that following incubation with EBs of LGV (Table 1). Indeed, at all time-points, this was significantly different from the control incubation and from sperm incubated with serovar LGV ( $P < 0.01$ ). Although sperm incubated with serovar LGV also showed a reduction in the percentage of motile sperm, this was not significantly different from the control values at any time-point. In contrast to the obvious differences between the serovars when spermatozoa were incubated with EBs, there was no significant decline in sperm motility following incubation with chlamydial or *E. coli* LPS although, at all time-points, the median sperm motility was numerically lower than the control but with no obvious differences between the sources of LPS.

As with our previous report (Hossein-zadeh *et al.*, 2001), the decline in sperm motility shown in Table 1 was closely

**Table 1.** Levels of motile, dead and acrosome-reacted sperm over a 6 h incubation with EBs and LPS

Sperm were incubated with  $0.54 \times 10^6$  EBs  $\text{ml}^{-1}$  of serovar E or LGV or E- or LGV-derived LPS at a concentration of  $0.1 \mu\text{g ml}^{-1}$ . Also shown are the results of a negative-control incubation where sperm were incubated in medium alone (medium control) and a positive control where sperm were incubated with LPS derived from *E. coli* at a concentration of  $50 \mu\text{g ml}^{-1}$ . Data shown are median percentages (lower–upper 95 % CI) for six experiments.

Incubation	Incubation time (h)		
	1	3	6
<b>Motile spermatozoa</b>			
Medium control	81.5 (73.4–86.3)	79.5 (72.4–84.3)	82.5 (71.7–89.6)
LGV EBs	67.5 (47.1–74.6)	66.5 (48.1–76.3)	63.0 (48.1–73.3)
E EBs	38.0 <sup>b*</sup> (23.5–58.2)	51.5 <sup>b</sup> (35.7–62.7)	41.0 <sup>b</sup> (27.1–58.5)
LGV LPS	57.0 (41.6–69.1)	55.3 (27.0–75.4)	59.5 (32.7–79.9)
E LPS	54.5 (47.2–71.5)	63.0 (43.0–74.4)	56.0 (37.6–72.8)
<i>E. coli</i> LPS	59.5 (42.6–70.8)	70.0 (53.6–79.4)	57.0 (40.5–71.5)
<b>Dead spermatozoa</b>			
Medium control	15.0 (12.0–17.3)	13.5 (11.1–16.6)	15.5 (14.1–18.6)
LGV EBs	23.5 (20.9–25.8)	26.5 (20.2–32.5)	30.5 <sup>a</sup> (21.9–45.4)
E EBs	39.0 <sup>b</sup> (30.5–45.5)	34.5 <sup>b</sup> (28.9–46.1)	38.0 <sup>b</sup> (27.2–53.2)
LGV LPS	26.0 (20.2–35.1)	29.0 (19.9–43.5)	26.0 (14.1–47.5)
E LPS	29.0 (20.6–32.7)	25.0 (19.6–30.0)	28.5 (22.6–36.4)
<i>E. coli</i> LPS	24.5 (21.0–29.7)	25.0 (19.5–32.2)	28.0 (22.9–40.1)
<b>Acrosome-reacted spermatozoa</b>			
Medium control	9.5 (7.8–11.9)	13.5 (9.1–15.6)	16.0 (12.8–20.2)
LGV EBs	14.5 (11.0–17.4)	11.5 (9.5–15.1)	13.5 (9.7–18.0)
E EBs	11.0 (8.2–16.5)	15.0 (9.0–19.0)	14.5 (9.1–17.2)
LGV LPS	8.5 (7.1–12.5)	13.0 (11.0–16.7)	17.5 (12.0–20.3)
E LPS	11.0 (8.4–13.6)	13.0 (8.6–15.8)	16.0 (8.6–20.4)
<i>E. coli</i> LPS	11.5 (9.9–14.4)	14.5 (10.8–16.9)	17.0 (10.3–20.1)

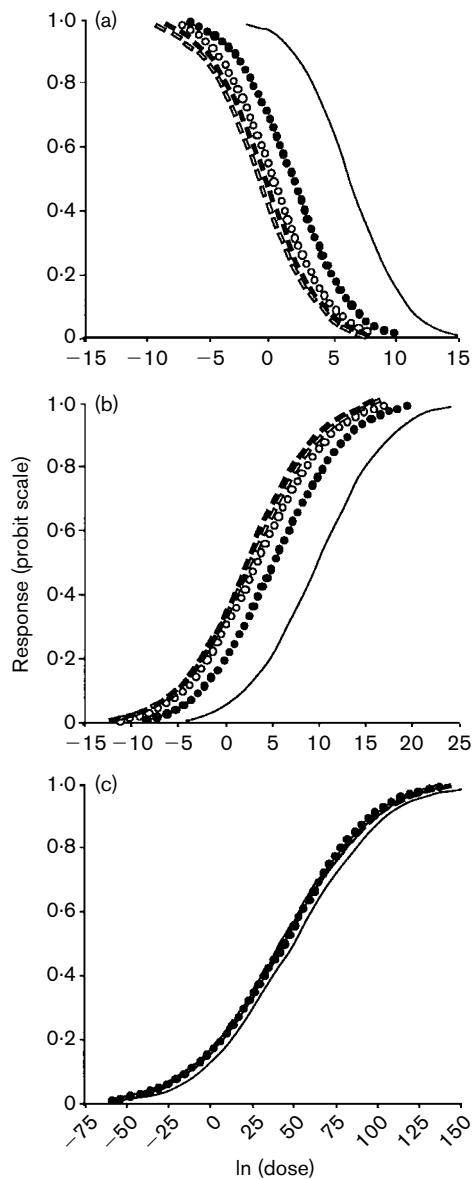
\*Values that are significantly different from the control incubation are indicated by: a,  $P < 0.05$ ; b,  $P < 0.01$ .

paralleled by an increase in the proportion of non-viable sperm (as determined by the HOS test; Table 1). Again, the increase in cell death was not the same between the serovars when spermatozoa were incubated with EBs. As expected, a higher level of cell death occurred at all time-points when spermatozoa were incubated with EBs of serovar E and LGV. This difference was significant for serovar E at all time-points ( $P < 0.01$ ), but only after 6 h incubation for serovar LGV ( $P < 0.05$ ). By contrast, when spermatozoa were incubated with LPS from any source, whilst a higher proportion of non-viable (dead) sperm was observed at all time-points, this was not significantly different from that observed in the control incubation. Moreover, at none of the time-points was a single incubation of LPS significantly different from the others.

The median percentage of acrosome-reacted spermatozoa remained  $< 17.5\%$  in all incubations at all time-points (Table 1) and no statistical differences were observed at any time-point, either between incubation with EBs or between the various preparations of LPS.

### Dose response to chlamydial LPS

Fig. 1 shows the results of a probit analysis on the data obtained for percentage motility, viability and acrosomal status when human sperm were incubated with EBs (from both serovars) over a concentration range of  $0.31$ – $2.50 \times 10^6$  EBs  $\text{ml}^{-1}$  as well as purified LPS from *C. trachomatis* serovars and *E. coli* over respective concentrations of  $0.05$ – $0.4 \mu\text{g}$  and  $25$ – $200 \mu\text{g ml}^{-1}$ . The models show that, although the effects of EBs and LPS on sperm motility (Fig. 1a) and viability (Fig. 1b) were very similar in the type of response observed, the doses involved varied somewhat between the serovars and the source of the LPS. For example, the ln median effective dose ( $\text{ED}_{50}$ ) for sperm motility (with upper and lower 95 % CI) was  $6.32$  ( $5.63$ – $7.45$ ) for *E. coli*,  $1.68$  ( $1.03$ – $2.72$ ) for serovar LGV and  $-0.25$  ( $-0.82$  to  $+0.30$ ) for serovar E, whereas the  $\text{ED}_{50}$  (95 % CI) for LPS from serovar E of  $-0.91$  ( $-1.47$  to  $-0.13$ ) and  $-0.88$  ( $-1.43$  to  $-0.09$ ) for serovar LGV were almost identical to that observed with EBs from serovar E. An almost identical



**Fig. 1.** Probit models generated from the dose-response data obtained following the incubation of human sperm with EBs (dotted lines) and LPS (dashed lines) of *C. trachomatis* serovars E (open dots/dashes) and LGV (filled dots/dashes) and LPS of *E. coli* (solid line). The models show the effect of ln(dose) on (a) sperm motility, (b) viability and (c) acrosomal status following a 6 h incubation period. The variables are presented in the probit scale.

pattern was seen with respect to sperm viability with the ED<sub>50</sub> values (95 % CI) of 9.95 (8.85–11.64) for *E. coli* and 5.21 (4.18–6.79) and 2.94 (2.29–3.91), respectively, for serovars LGV and E. Again, the ED<sub>50</sub> values (95 % CI) for LPS from serovar E of 2.78 (1.85–4.20) and for LGV of 2.67 (1.77–4.07) were almost identical to that observed with EBs from serovar E.

Finally, the ln ED<sub>50</sub> values for acrosomal status (Fig. 1c) were almost identical for all experimental conditions. Moreover,

the predicted range of doses over which a response would be expected was unphysiologically large, from –58 to 149.99 (i.e.  $6.47 \times 10^{-26}$  to  $1.38 \times 10^{65}$  EBs or  $\mu\text{g LPS ml}^{-1}$ ).

### Dose response to PMB

The results of the incubation of sperm over 6 h with doses of PMB between 50 and 400  $\mu\text{g ml}^{-1}$  are shown in Table 2. Briefly, at PMB doses of less than 200  $\mu\text{g ml}^{-1}$ , there was no significant effect upon sperm motility, viability or acrosomal status. Only following a 6 h incubation with 400  $\mu\text{g ml}^{-1}$  was sperm motility significantly reduced when compared with the control ( $P < 0.05$ ). As such, the use of PMB in experiments at a concentration of 100  $\mu\text{g ml}^{-1}$  would not in itself result in a significant change in sperm motility, viability or acrosomal status.

### Inhibitory effects of PMB

In this experiment, the effect of 100  $\mu\text{g PMB ml}^{-1}$  on the EB- and chlamydial LPS-stimulated decline in sperm motility and viability was investigated. Table 3 summarizes these results and indicates that, following a 6 h incubation with EBs of serovar E, sperm motility had declined significantly and the percentage of dead sperm was significantly increased ( $P < 0.001$ ) in comparison with both control incubations (PMB and medium alone). However, in the presence of 100  $\mu\text{g PMB ml}^{-1}$ , the decline in sperm motility and the extent of cell death were markedly reduced. For example, in the presence of PMB, 52.5 (43.7–67.3) % of sperm were motile and 24.0 (18.9–26.7) % of sperm were dead compared with 45.0 (38.7–51.3) % and 32.5 (28.8–35.9) % in the presence of EBs alone [median (95 % CI)]. For each variable, these values were significantly different, confirming that PMB could inhibit the EB-induced decline in sperm motility and the increase in sperm death. Moreover, the median percentages of motile and dead sperm in the presence of PMB were not significantly different ( $P > 0.05$ ) from either the medium-only control or the PMB control. Interestingly, this effect was not seen with EBs of serovar LGV and the percentages of sperm motility and viability in the presence and absence of PMB were almost identical.

When spermatozoa were incubated with chlamydial LPS from both serovars in the presence or absence of PMB, a similar, but not identical, picture emerged. Interestingly, in this experiment, the effect of both E- and LGV-derived LPS was much more marked, leading to a greater decline in sperm motility and a corresponding increase in the proportion of dead sperm, more akin to the incubation with EBs from serovar E. Moreover, similar to the observation in the dose-response experiments, the results obtained with LPS from both serovars were very similar. For example, 42.0 (32.8–56.9) % and 42.5 (32.6–48.4) % of sperm were motile and 33.0 (29.3–35.0) % and 30.5 (26.0–39.0) % of sperm were dead following a 6 h incubation with LPS from serovars LGV and E, respectively. Both of these variables were significantly different from both control incubations (PMB and medium alone). However, following co-incubation of LPS with PMB,

**Table 2.** Effect of PMB dose on sperm motility, viability and acrosomal status following a 6 h incubation period

Data shown are median percentages (lower–upper 95 % CI) for six experiments.

Sperm type	PMB dose ( $\mu\text{g ml}^{-1}$ )				
	0	50	100	200	400
Motile	84.5 (73.9–92.4)	80.5 (71.5–87.1)	78.0 (66.5–84.1)	69.5 (59.5–81.9)	67.0* (56.1–74.6)
Dead	18.5 (11.8–23.2)	20.0 (16.0–24.4)	19.5 (15.4–25.6)	20.5 (16.4–25.6)	25.0 (17.6–30.1)
Acrosome-intact	18.5 (15.2–21.5)	18.0 (13.5–19.8)	19.5 (15.6–22.5)	16.0 (13.1–20.9)	16.0 (14.2–18.5)

\*Significantly different ( $P < 0.05$ ) from control (no PMB).

**Table 3.** Effect of 100  $\mu\text{g PMB ml}^{-1}$  on sperm motility, viability and acrosomal status during a 6 h incubation with EBs and LPS purified from serovars E and LGV

The results of a medium-only control and a PMB-only control are also shown. Data shown are median percentages (lower–upper 95 % CI) for six experiments.

Incubation	Motile	Dead	Acrosome-intact
Medium control	76.0 (69.4–84.6)	19.0 (17.7–21.4)	15.0 (12.5–16.2)
PMB control	73.0 (60.2–82.8)	19.5 (17.8–21.5)	13.0 (12.0–15.6)
LGV EBs	50.5 (41.9–72.5)	26.0 (19.5–32.5)	14.5 (12.2–18.5)
E EBs	45.0 <sup>a*</sup> (38.7–51.3)	32.5 <sup>b,B</sup> (28.8–35.9)	14.5 (11.2–17.1)
LGV EBs+PMB	50.5 (44.3–66.0)	23.5 (19.8–26.9)	15.5 (14.8–16.9)
E EBs+PMB	52.5 (43.7–67.3)	24.0 (18.9–26.7)	14.5 (13.0–17.7)
LGV LPS	42.0 <sup>b,A</sup> (32.8–56.9)	33.0 <sup>b,B</sup> (29.3–35.0)	16.0 (14.5–17.5)
E LPS	42.5 <sup>c,B</sup> (32.6–48.4)	30.5 <sup>a,A</sup> (26.0–39.0)	15.0 (12.7–18.0)
LGV LPS+PMB	54.5 (49.6–69.1)	22.5 (19.0–25.4)	15.5 (13.2–18.8)
E LPS+PMB	55.5 (47.4–73.6)	23.0 (17.5–26.5)	15.0 (13.4–17.0)

\*Values that are significantly different from the medium control (lower case) or PMB control (upper case) incubation are indicated by: a, A,  $P < 0.05$ ; b, B,  $P < 0.01$ ; c, C,  $P < 0.001$ .

the decline in sperm motility and the extent of sperm death were again less marked, with 54.5 (49.6–69.1) % and 55.5 (47.4–73.6) % of sperm remaining motile and 22.5 (19.0–25.4) % and 23.0 (17.5–26.5) % of sperm being dead following incubation with LGV- and E-derived LPS, respectively. In neither case were these values significantly different from the control incubations, confirming that PMB could inhibit the LPS-induced decline in sperm motility and increase in sperm death of both serovars.

Acrosomal status was unaffected by EBs or chlamydial LPS from either serovar ( $P > 0.05$ ) and was unchanged following incubation with PMB.

## DISCUSSION

In our previous reports (Hosseinzadeh *et al.*, 2000, 2001), we have demonstrated that chlamydial EBs can have a direct and detrimental effect on sperm physiology. This was seen primarily via a reduction in sperm motility, which was reflected in a corresponding increase in sperm death. However, at the time of these studies, it was unclear to us how EBs

might be triggering these effects, although a report by Galdiero *et al.* (1994) had suggested to us that the LPS component of chlamydia could be important.

In their report, Galdiero *et al.* (1994) demonstrated that a concentration of LGV-derived LPS of 10  $\mu\text{g ml}^{-1}$  was able to kill 92 $\pm$ 5 % of spermatozoa within 60 min (as determined using the exclusion of eosin as a measure of sperm viability). This is much higher than the maximum dose used in the experiments reported here (0.4  $\mu\text{g LPS ml}^{-1}$ ), although it is possible to determine from the data of Galdiero *et al.* (1994) that, at concentrations as low as 0.1  $\mu\text{g ml}^{-1}$ , more than 60 % of spermatozoa were still classified as dead. This is intriguing, since it suggests that their preparation of LPS was significantly more potent than that we have prepared for this work: after 6 h incubation with LPS from serovars E and LGV, only 27.0 $\pm$ 1.7 % and 27.3 $\pm$ 2.4 % of spermatozoa were dead. The doses of LPS used in our experiments were carefully calculated to be in step with the number of EBs used in the incubations performed in this and our previous work. In turn, the concentration of EBs used had been previously calculated to be within a broad physiological range [see the

discussion of Hosseinzadeh *et al.* (2001) for an explanation]. Using the same arithmetic, we can surmise that an LPS concentration of  $10 \mu\text{g ml}^{-1}$  used by Galdiero *et al.* (1994) would correspond roughly to  $6.3 \times 10^7$  EBs  $\text{ml}^{-1}$ .

More recently, Gorga *et al.* (2001) have described how porins and LPS can increase the level of spontaneous apoptosis in human spermatozoa. In addition, Urata *et al.* (2001) have shown that LPS can induce sperm to generate ROS and that this may be the mechanism by which LPS may affect sperm motility. Curiously, Sikka *et al.* (2001) have reported that LPS has no effect on spermatozoa unless interferon- $\gamma$  is present. This is an interesting observation, and requires further investigation, since the spermicidal activity of LPS has been established for 40 years (Dennis, 1962). However, it does raise the possibility that there may exist significant differences in the spermicidal activity of LPS depending on the source from which it was extracted. In the present study, we have shown that chlamydial-derived LPS is up to 500 times more potent than LPS purified from *E. coli* (see Fig. 1a, b). Brade *et al.* (1997) point out some structural differences between the composition of LPS of *C. trachomatis*, *Chlamydia psittaci* and *E. coli*, but whether this relates to differences in sperm toxicity remains to be established. Sikka *et al.* (2001) did not specify the source of LPS and so we cannot be certain whether this is a contributory factor in their results. However, it is clear that the bacterial source of LPS needs to be taken into consideration in future experiments.

The response of spermatozoa to LPS purified from *C. trachomatis* that we have described in this report is interesting in the context of our previous work, which has shown that serovar E is more effective at killing sperm than LGV (Hosseinzadeh *et al.*, 2001). However, unlike the situation with EBs, when sperm are incubated with LPS, the reduction in sperm motility (Fig. 1a) and the increase in the proportion of dead sperm (Fig. 1b) are almost identical regardless of which serovar the LPS was purified from. This implies that the difference in response between the two serovars cannot be due to an inherent difference in the toxicity of their LPS. Moreover, the reduction in sperm motility (and increase in the proportion of dead sperm) that is observed after incubation with purified LPS is almost identical to that observed following incubation with EBs from LGV (cf. Fig. 1a, b). This suggests that the preparation of EBs from serovar E may possess molecules in addition to LPS that are contributing to sperm death. Another possibility is that differential release of LPS from EBs could be a contributory factor. However, that LPS is primarily responsible for the EB- and LPS-induced cell death is evident from the experiments using PMB. PMB is a polycationic antibiotic that can block many biological and toxic effects of LPS (Duff & Atkins, 1982; Morrison & Jacobs, 1976). In experiments looking at the inhibitory effects of PMB, incubation of sperm with  $100 \mu\text{g ml}^{-1}$  was able to inhibit the spermicidal properties of EBs of serovar E and LPS purified from both serovars (Table 3). Of course, this does not rule out the possibility that there may be additional molecules in the EB preparations of serovar E that are also spermicidal, and this possibility

requires further investigation if we are to understand fully the molecular basis of interactions between sperm and chlamydiae.

## ACKNOWLEDGEMENTS

The authors would like to thank the staff of the Andrology Laboratory at the New Jessop Wing of the Royal Hallamshire Hospital, Sheffield, for their assistance in obtaining semen samples for use in this study. Also, thanks to Harry Moore for access to computer-assisted sperm analysis machines and for the 18:6 mAb used to evaluate acrosomal status.

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