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Spermicidal Activity of Bacterial Lipopolysaccharide Is Only Partly Due to Lipid A

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Abstract

We have previously shown that co-incubation of *Chlamydia trachomatis* lipopolysaccharide (LPS) leads to premature sperm death by an apoptosis-like mechanism. It was always assumed that lipid A is the toxic component of LPS. Here we investigate the possible involvement of 3-deoxy-D-*manno*-octulosonic acid (Kdo), which is an additional component of the LPS in *C. trachomatis*. Highly motile preparations of sperm from normozoospermic patients were incubated for 6 hours with commercial sources of lipid A and Kdo. Conventional lipid A inhibitors, polymyxin B (PMB) and anti-CD14 monoclonal antibody (mAb) were used to test the ability of both lipid A and Kdo to induce an apoptotic-like response in mature sperm. Flow cytometry was used to determine apoptosis by the expression of annexin V. Caspase activity was also measured by fluorometry and by the use of a pan-caspase inhibitor and caspase-3 inhibitor. Both lipid A and Kdo at 50 µg/mL caused significant mortality of sperm. However, although PMB and anti-CD14 mAb were inhibitory to the activity of lipid A on sperm, no such effect was seen against Kdo. In the presence of either lipid A or Kdo, sperm death was caused by an apoptotic-like effect that was caspase mediated. We conclude that Kdo shares its spermicidal properties with lipid A and seems to kill sperm in a similar manner. These results provide an explanation for higher than expected levels of spermicidal activity of LPS that are not caused by lipid A.

Key words: Apoptosis, human sperm, Kdo

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Lipopolysaccharide (LPS) is a heat-stable complex and unique glycolipid that is present in all Gram-negative bacteria. It is the immunodominant antigen of most Gram-negative bacteria and is considered to be intimately associated with the virulence of the pathogen (Volk et al, 1996). LPS consists of 3 structural sections: lipid A, core polysaccharide, and O antigen. Lipid A is responsible for the endotoxin activity of LPS and binds to polymyxin B (Morrison and Jacobs, 1976). The core oligosaccharide region has 2 parts. The inner core is characterized by more unusual sugars, particularly 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) and heptose. The former of these is seen in almost every LPS looked at to date, being α-bound to the carbohydrate backbone of lipid A in every case. The outer core typically consists of common hexose sugars such as glucose, galactose, *N*-acetyl galactosamine, and *N*-acetyl glucosamine and is generally more variable than the inner core. Interestingly, while the O antigen and the majority of the core can be removed in some viable mutants, the Kdo residue, like lipid A, is always required for bacterial viability (Erridge et al, 2002). The interaction between LPS and the effector cell occurs via the CD14 receptor, a phosphatidylinositol-anchored molecule, in the presence of a plasma protein, the LPS binding protein which functions as an opsonin (Tobias et al, 1986). After this stage, cell signaling proceeds via Toll-like receptors and the cell is activated, which in many cases is followed by cytokine release.

Chlamydia trachomatis LPS is known to be particularly spermicidal (Galdiero et al, 1994; Hosseinzadeh et al, 2003) and is composed of a simple structure containing lipid A and Kdo (Rund et al, 1999). Our previous studies showed the toxicity of chlamydial LPS on ejaculated human spermatozoa in vitro, although inhibition by polymyxin B (PMB) was incomplete (Hosseinzadeh et al, 2003). On the basis of these considerations, the purpose of the present study was to verify the probable effect of Kdo in triggering apoptosis-like death of spermatozoa. Although the Kdo we used in the following experiments was synthetic, our aim was to assess any biologic activity of LPS that might be attributed to Kdo rather than lipid A using the specific binding of PMB and inhibitory effect of an anti-CD14 monoclonal antibody (mAb) as well as caspase inhibitors.

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Sperm Preparation

Semen samples were obtained from the andrology laboratory (Jessop Wing, Royal Hallamshire Hospital, Sheffield, United Kingdom) from normozoospermic patients by masturbation. Ethical approval for the use of semen samples in this study was granted by the South Sheffield Research Ethics Committee (project number 02/337). The ejaculates had a high sperm concentration (x60 x 10^6 sperm/mL), with more than 15% ideal morphologic forms according to World Health Organization (1999) criteria. From each ejaculate, fast, progressively motile spermatozoa were purified and washed by a Percoll gradient technique (Moohan and Lindsay, 1995), and the samples which yielded more than 90% motile spermatozoa were used and adjusted to 5 x 10^6/mL in Earle balanced salt

solution (EBSS; Sigma Chemical Co, Poole, United Kingdom) containing 0.3% (w/v) bovine serum albumin.

Assessment of Sperm Viability

Sperm viability was determined using the hypo-osmotic swelling (HOS) test (Jeyendran et al, 1984). Briefly 20 µL of each incubate was transferred to 200 µL of HOS solution (tri-sodium citrate and fructose) and incubated at 37 °C for 30 minutes. 10 µL of each incubate was then spotted onto a microscope slide, and spermatozoa was classified as live or dead according to the method of Jeyendran and coworkers (1984).

Preparation of Control Populations

In the following experiments, 1 mM staurosporine-treated sperm and untreated Percoll-prepared sperm were used as positive and negative apoptosis controls, respectively. A necrosis control was produced by heat treatment of spermatozoa at 56 °C for 1 hour.

Endotoxin Activity of the Kdo Preparation

The absence of endotoxin activity in the Kdo preparation was checked using a limulus amebocyte lysate assay (Cambrex Biosciences, Wokingham, United Kingdom). The method was performed as recommended by the manufacturer.

The Effect of Lipid A and Kdo on Sperm

This experiment was conducted to assess the relative spermicidal activities of the 2 major components of bacterial LPS. Semen samples were obtained from 6 men and prepared as described above to give 4 x 200-µL aliquots of each sample. To 3 aliquots were added 50 µg/mL of lipid A (Sigma), 50 µg/mL of Kdo (Sigma), and a combination of both. To the remaining aliquot, 20 µL of EBSS was added as a control. All were incubated for 6 hours at 37 °C in 5% CO_2 and examined for sperm mortality.

The Effect of PMB and Anti-CD14 mAb on the Spermicidal Activity of Lipid A and Kdo

The LPS inhibitors PMB and anti-CD14 mAb, which work against lipid A, were used in experiments to confirm the ability of lipid A and Kdo to kill spermatozoa. Semen samples were obtained from a further 6 men and prepared as described above to give 9 x 200-µL aliquots of each sample. PMB (Sigma) was used at a final concentration of 100 µg/mL as previously reported (Hosseinzadeh et al, 2003); anti-CD14 mAb (BD PharMingen, Oxford, United Kingdom), which was free of endotoxin and sodium azide, was used at a final concentration of 10 µg/mL. Both were incubated with spermatozoa for 30 minutes prior to adding lipid A and Kdo to the spermatozoa as described above.

Evaluation of the Apoptotic Effect of Kdo and Lipid A on Spermatozoa

Annexin V binding and use of a pan-caspase inhibitor allowed further insights into whether spermicidal activities caused by Kdo and lipid A are due to an apoptotic-like effect. Preparations of 5 x 10^6 sperm/mL from 6 men were incubated with either 50 µg/mL of lipid A or Kdo at 37 °C in 5% CO_2 for 6 hours. In another set of experiments, before the above was added, sperm were incubated with 200 µM of pan-caspase inhibitor (Z-VAD-FMK; Calbiochem, Darmstadt, Germany) for 1 hour at room temperature. To prepare the samples for flow cytometry, the cells were then washed twice with PBS and resuspended in 1x annexin binding buffer (BD PharMingen) at a final concentration of 1 x 10^6 sperm/mL. An aliquot of 100 µL of the solution (1 x 10^5 cells) was then transferred to a 12 x 75-mm polystyrene test tube (Elkay, Basingstoke, United Kingdom) followed by the addition of 5 µL of annexin V-FITC and 50 µg/ml propidium iodide (PI; Sigma). The cells were then mixed by vortexing gently and incubated for 15 minutes at room temperature in the dark. An aliquot of 400 µL of 1x

annexin binding buffer was added to each tube, and the cells were analyzed by flow cytometry in a FACSCalibur (BD Biosciences, Oxford, United Kingdom). A minimum of 10 000 spermatozoa were examined for each test. The sperm population was gated by using forward-angle light scatter. The FITC-labeled annexin V–positive sperm cells were measured in the FL1 channel, and the PI-labeled cells were measured in the FL2 channel of the flow cytometer, as described by Eley and colleagues (2005a).

Quantification of Caspase Activity in Spermatozoa Incubated with Lipid A or Kdo

Confirmatory evidence of an apoptotic-like effect on sperm caused by Kdo and lipid A was obtained by measurement of sperm caspase activities (as described by Weng and coworkers, 2002) and the use of a caspase-3 inhibitor. Sperm lysates were prepared as described by Weng et al (2002). Briefly, 1 x 10^7 sperm (after a 6-hour incubation with either 50 µg/ml lipid A or Kdo) were centrifuged to form a pellet. In another set of experiments, before incubation with lipid A, Kdo, or staurosporine, sperm were incubated with 200 µM caspase-3 inhibitor I (Calbiochem) for 1 hour at room temperature. The pellet was then placed on dry ice for 5 to 10 minutes prior to lysis in 400 µL of 10 µM Tris buffer with 0.5% Triton X-100 (Sigma), pH 7.5, containing general protease inhibitors 2 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 10 mM dithiothreitol (Sigma) for 15 minutes at 4 °C. The lysates were vortexed and sonicated before centrifugation (20 minutes at 4 °C) to remove particulate matter. The supernatant was then removed, and an aliquot of sperm lysate (100 µg, according to the Bradford method [1976]) was diluted with 1 mL of solution containing interleukin 1ß–converting enzyme buffer (50 mM HEPES, 10% sucrose, 0.1% CHAPS [3-([3-chloamidopropyl]dimethylammoniol)-1-propanesulfonate], pH 7.5) and 50 µM of fluorogenic substrate Ac-DEVD-afc (Bachem, Merseyside, United Kingdom) and incubated for 45 minutes at 37 °C. The fluorescent emission (excitation 400 nm and emission 505 nm) was measured in a fluorometer (Perkin Elmer, Beaconsfield, United Kingdom). Background fluorescence was determined using blanks without sperm. Standards containing 1 to 5 µM AFC-(7-amino-4-trifluoromethyl coumarin) were used to determine the amount of fluorochrome released, and apoptotic human neutrophils treated with 1 mmol/L cycloheximide were used as a positive control.

Statistical Analysis

We used the Student's *t* test and 1-way analysis of variance (ANOVA) test as indicated in the figure legends. A *P* value of less than .001 was considered significant.

Analysis was by GraphPad Instat software version 3.0 (GraphPad Software Inc, San Diego, Calif).

Results

The Effect of Lipid A and Kdo on Sperm

Immediately after density gradient centrifugation, only 5.4% of spermatozoa in the control incubation were dead. Over the 6-hour incubation period this value did not change significantly in the control population (*P* > .05). However, the mortality of the spermatozoa that were incubated with lipid A, Kdo, and a combination of both (50 µg/ml) for 6 hours increased up to 23.6%, 20.6%, and 21%, respectively (Figure 1). These values were significantly different from the 6-hour control with a sperm mortality of only 8.8% (*P* < .001). Endotoxin activity in the Kdo preparation was shown to be less than 0.01 ng/mL.

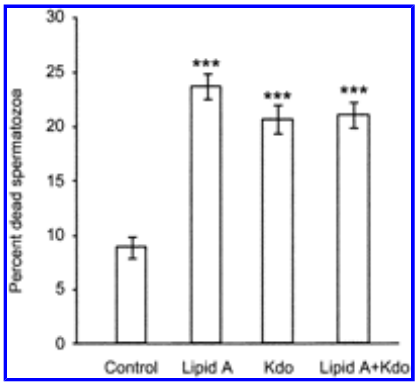

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Figure 1. The effects of lipid A, 3-deoxy-D-*manno*-octulosonic acid (Kdo), and the combination of lipid A and Kdo (all at 50 µg/ml) on human spermatozoa. The parameter measured is sperm mortality. The data shown are the mean of 6 experiments + SEM. *** indicates a significant difference from the control incubation using the Student's *t* test ($P < .001$).

The Effect of PMB and Anti-CD14 mAb on Lipid A and Kdo Acting Against Sperm

After a 6-hour incubation anti-CD14 mAb (8.0%) and PMB (9.8%) had no significant effect on spermatozoa death above that seen in the control population (approximately 10.2%) ($P > .05$). However, the spermicidal activity of lipid A and Kdo was significantly higher than that seen in control incubations ($P > .001$) (Figure 2). Spermatozoa in the presence of a combination of lipid A and anti-CD14 mAb (12.6%) and lipid A and PMB (9.6%) showed significantly fewer dead spermatozoa ($P < .001$). However, anti-CD14 mAb (17.6%) and PMB (17.8%) were not able to reduce the effect of Kdo on sperm ($P > .05$) (Figure 2).

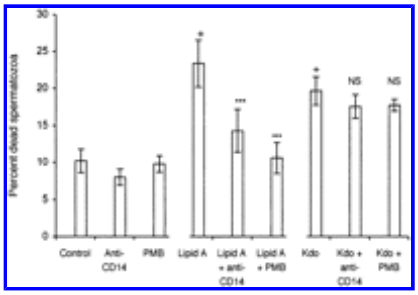

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Figure 2. The effect of anti-CD14 monoclonal antibody (mAb) (10 µg/ml) and polymyxin B (PMB) (100 µg/ml) on lipid A and 3-deoxy-D-*manno*-octulosonic acid (Kdo) (both at 50 µg/ml) acting against human spermatozoa. Spermatozoa were incubated with lipid A or Kdo with and without anti-CD14 mAb and PMB for 6 hours at 37°C. The data shown are the mean of 6 experiments ± SEM. *** indicates significant differences of $P < .001$ for lipid A incubated with anti-CD14 mAb and PMB. Additions to Kdo were not significant (NS) at $P > .05$. + indicates a significant difference of $P < .001$ for additions of lipid A and Kdo when compared with the control incubation using the Student's *t* test.

Evaluation of the Apoptotic-like Effect of Kdo and Lipid A on Spermatozoa

Both lipid A and Kdo induced an apoptotic-like effect in spermatozoa (Figure 3), which was significantly inhibited by the pan-caspase inhibitor Z-VAD-FMK ($P < .001$). This suggested that the lipid A and Kdo-induced apoptotic-like effect might be caspase mediated.

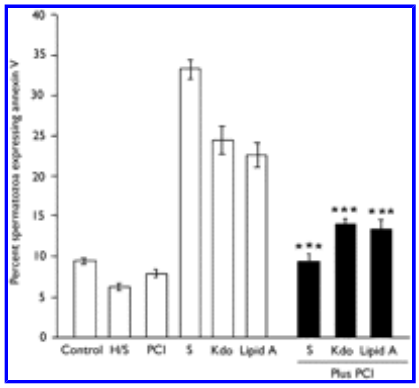

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Figure 3. Percent spermatozoa expressing annexin V in the presence and absence of pan-caspase inhibitor (PCI) when exposed to the following over a 6-hour incubation period: control (no addition), H/S (heat-shock), S (1 mM staurosporine), 50 µg/ml Kdo, and 50 µg/ml lipid A. Data shown are the mean ± SEM of incubations with sperm preparations from 6 men. *** indicates a significant difference ($P < .001$) between incubations with and without PCI using the 1-way analysis of variance test.

Quantification of Caspase Activity in Spermatozoa Stimulated With Lipid A or Kdo

High levels of caspase activity were seen in cycloheximide-treated human neutrophils which were obtained from 6 men and gave a mean level of 129 nM/min mg (results not shown). Spermatozoa treated with staurosporine, lipid A, or Kdo showed significantly higher levels of caspase activity compared with unstimulated controls which were significantly reduced in the presence of caspase-3 inhibitor ($P < .001$), confirming that the apoptotic-like effect in these spermatozoa was caspase mediated (Figure 4).

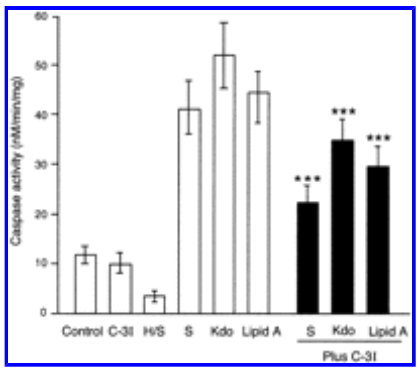

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Figure 4. Caspase activity of spermatozoa in the presence and absence of caspase-3 inhibitor (C3I) when exposed to the following over a 6-hour incubation period: control (no addition), H/S (heat shock), S (1 mM staurosporine), 50 µg/ml Kdo, and 50 µg/ml lipid A. Data shown are the mean ± SEM of incubations with sperm preparations from 6 men. *** indicates a significant difference ($P < .001$) between incubations with and without C3I with the 1-way analysis of variance test.

Discussion

In this study we have shown that Kdo appears to be as spermicidal as lipid A, and this may explain why PMB incompletely inhibits *C. trachomatis* LPS-induced sperm death in ejaculated human spermatozoa (Hosseinzadeh et al, 2003). In *C. trachomatis* LPS, the core region consists of the trisaccharide Kdo$\alpha \rightarrow 8$ Kdo $2 \rightarrow 4$ Kdo, emphasizing the importance of Kdo in this bacterium (Rund et al, 1999).

Although anti-CD14 mAb was able to inhibit the ability of lipid A to kill sperm, this was not seen with Kdo. One possible reason for this is that the α configuration of Kdo (as in the LPS molecule) may be required for CD14 binding (Cavaillon et al, 1996) but that this tautomer was not present in its synthetic form.

Although there is no additive spermicidal effect of lipid A and Kdo, this is not surprising given the findings of dose-response experiments using chlamydial LPS in which significant increases in LPS are required to see marked differences in spermicidal activity (Hosseinzadeh et al, 2003).

On further investigation of the mode of sperm death following exposure to either lipid A or Kdo, it appeared that both induced an apoptotic-like effect (as observed through the expression of annexin V) and that caspases were activated as shown by inhibition with both the pancaspase and caspase-3 inhibitors. These findings support the role of caspases in ejaculated sperm apoptosis as described by Weng and colleagues (2002) and Eley and coworkers (2005a).

While it has been suggested that the exposure of phosphatidylserine may occur in sperm as part of the changes that take place in the plasma membrane as a result of capacitation (de Vries et al, 2003) in a series of previous experiments (Eley et al, 2005a), those were shown to be caspase mediated; therefore, we are confident that the results described here are associated with apoptosis rather than capacitation. Recently our findings have been confirmed by Satta and collaborators (2006) who described *C. trachomatis* producing apoptosis (including expression of annexin V) in human sperm.

The spermicidal activity of Kdo was unexpected. Lack of anti-CD14 inhibition of Kdo to kill sperm suggested that a different method of cell signaling was occurring in comparison to that seen with lipid A. However, the extent and mechanisms of causation of sperm death seemed to be similar.

Recently we have hypothesized (Eley et al, 2005b) that as chlamydial LPS is the principal factor leading to sperm apoptosis following co-incubation with *C. trachomatis*, a new line of inquiry would be to measure the levels of LPS in semen and relate these to parameters of semen quality. However, now that we have shown comparative levels of spermicidal activity for Kdo as well as lipid A, measurement of LPS in semen (which only detects the lipid A portion and not Kdo) would underestimate the total spermicidal activity and is an important factor for consideration in such studies of LPS detection. In possible situations in which the lipid A may be sequestered away from semen, any remaining Kdo present would be undetectable by routine LPS detection methods and yet could be responsible for spermicidal activity, thereby complicating any Gram-negative bacterial association with semen quality.

Because of our surprising findings of the spermicidal activity of synthetic Kdo, they need to be confirmed using Kdo extracted from chlamydial LPS. However, there are difficulties in growing enough chlamydia in tissue culture to purify Kdo from sufficient LPS. We also believe that further work should be performed on Kdo component sugars to investigate those chemical structures that are responsible for sperm death and to assess their spermicide potential.

Acknowledgments

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