

Glycolipoprotein Cytotoxin from *Leptospira interrogans* serovar *copenhageni*

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Lipopolysaccharide (LPS), glycolipoprotein (GLP) and lipid extract were prepared from *Leptospira interrogans* serovar *copenhageni*. GLP, lipid extract or purified fatty acids from lipid extract produced cytotoxic effects seen as cell enzyme leakage followed by cytotoxic death when tested in mouse fibroblast L929 cells in tissue culture. All extracts also agglutinated mouse erythrocytes but purified LPS was not cytotoxic. Neither GLP nor LPS were pyrogenic but both gelled *Limulus* amoebocyte lysate. Specific anti-GLP IgG neutralized the cytotoxic and haemagglutinating effect of GLP; however, at higher concentrations it enhanced the cytotoxicity of GLP and mediated lysis of the erythrocytes. A high dose of leptospire (i.e. 10^{10} organisms) killed weanling mice causing pathological changes similar to those seen in acute leptospirosis. Similar results were obtained with live, dead, pathogenic and saprophytic leptospire. The results suggest that toxicity is involved in leptospiral infection and that lipid components either of whole leptospire or of a leptospiral GLP may contribute to the pathogenesis of acute leptospirosis.

INTRODUCTION

Clinical and pathological observations suggest that a toxin may play a role in the pathogenesis of leptospirosis (Higuchi, 1930; Faine, 1957*a*; Miller *et al.*, 1974; DeBrito *et al.*, 1979; Chaperon *et al.*, 1979; Sitprija *et al.*, 1980). Undefined toxic substances have been reported in leptospiral culture supernatants (Cinco *et al.*, 1980) or in the plasma of experimentally infected animals (Chaperon *et al.*, 1979; Knight *et al.*, 1973). Biological effects typical of Gram-negative bacterial endotoxins have been demonstrated using extracts from leptospire, but could not be confirmed by others (Arean *et al.*, 1964; Sefer, 1965; Finco & Low, 1967; Johnson, 1976). Preliminary experiments showed that high doses of leptospire (10^{10} organisms) of either virulent or avirulent strains could kill young guinea pigs or weanling mice, resulting in pathological changes similar to those seen in acute leptospirosis and suggesting that the toxic substance might be part of the leptospiral cell. These observations are consistent with those of Faine (1957*b*) and of Miller *et al.* (1970), who found that cytotoxic activity against mouse fibroblasts was retained in whole or in disintegrated leptospire but not in the culture supernatant fluids. Stalheim (1968) found that leptospiral lipids were toxic to guinea pig peritoneal macrophages *in vitro*, although these lipids were not lethal to mice or hamsters.

This report describes the toxic effects of a glycolipoprotein (GLP) extracted from *L. interrogans* serovar *copenhageni* and compares its biological activities with those of leptospiral lipopolysaccharide (LPS).

METHODS

Organisms. Leptospire of strains *Leptospira interrogans* serovars *copenhageni* L45 (Faine & van der Hoeden, 1964) and *pomona* L115 (obtained from the Regional Veterinary Laboratory, Hamilton, Victoria, Australia) and *Leptospira biflexa* serovar *patoc* strains L92 (obtained from H. Dikken, Royal Tropical Institute, Amsterdam, 1974) and L41 (obtained from A. D. Alexander, Walter Reed Army Medical Center, Washington DC, USA, 1965)

Abbreviations: BSA, bovine serum albumin; FCS, foetal calf serum; GLP, glycolipoprotein; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; PBS, phosphate-buffered saline.

were used. Strains L45 and L115 were virulent when injected intraperitoneally into 100 g guinea pigs, which were killed by doses of 10^7 – 10^8 leptospire within 3–5 d. The leptospire were grown in Ellinghausen–McCullough–Johnson–Harris (EMJH) medium for 7 d at 30 °C and harvested by centrifugation at 12000 g, except when used for immunizing rabbits, when they were grown in protein-free medium (Christopher *et al.*, 1982). Formalin-killed leptospire were prepared by adding 1% (v/v) neutral formalin (equivalent to 0.4% v/v, formaldehyde) to leptospiral cultures, incubating for 30 min at room temperature and washing with phosphate-buffered saline (0.137 M-NaCl, 0.007 M- K_2HPO_4 , 0.1 M- KH_2PO_4), pH 7.2 (PBS). Leptospire killed by heat were treated at 56 °C for 3 h.

LPS was extracted from lyophilized leptospire (serovar *copenhageni*) by the method of Westphal & Jann (1965) as described by Vinh *et al.* (1986).

GLP extraction. The procedure was modified from Braun & Wolff (1970). The cell sediment after centrifugation was resuspended in a solution of 0.01 M-Tris/HCl buffer (pH 7.4) containing lysozyme (50 μ g ml⁻¹) (Bacto-Lysozyme, Difco) and kept overnight at 34 °C (Braun & Wolff, 1970). The lysozyme-treated leptospire were centrifuged at 20000 g for 30 min, and the supernatant was collected and treated with 50 μ g ml⁻¹ each of RNAase and DNAase (Sigma) for 3 h at 37 °C, dialysed against 0.01 M-Tris/HCl buffer (pH 7.4) for 24 h at 4 °C and acidified with 1 M-acetic acid to pH 3.7 at 4 °C. The precipitate (GLP) was centrifuged at 37000 g for 30 min, washed twice with 0.1 M-acetic acid and lyophilized.

Treatment of GLP with lipase and proteinase. GLP (2 mg ml⁻¹ in 0.1 M-Tris/HCl buffer, pH 7.4) was treated with either pancreatic lipase (Calbiochem; 100 μ g ml⁻¹), phospholipase C (Calbiochem; 10 μ g ml⁻¹) or proteinase (Calbiochem; 20 μ g ml⁻¹) in the presence of 0.02 M-CaCl₂ for 18 h at 37 °C, dialysed against distilled water and lyophilized. The lyophilized products were dissolved either in PBS (100 μ g ml⁻¹) for haemagglutination tests or in M199 without foetal calf serum (FCS) (250 μ g ml⁻¹) for cytotoxicity assays. The reconstituted solutions were heated at 100 °C for 10 min to destroy enzyme activity before use.

Phospholipase activity. Lecithin agar plates were prepared by incorporating soya bean lecithin (100 μ g ml⁻¹) into 1% (w/v) agar in Tris/HCl buffer (pH 7.4) containing 0.02 M-CaCl₂. Holes of 3 mm diameter were punched in the agar and filled with either GLP or phospholipases A or C (all 100 μ g ml⁻¹) (Sigma). The plates were incubated at 37 °C and the results were read after 3 h. The presence of enzyme activity was indicated by an opaque zone surrounding the holes.

Chemical determination. Protein was measured by the micromethod of Bradford (1976) with Bio-Rad protein assay reagents, using bovine serum albumin fraction V (BSA) (Sigma) to prepare a standard curve.

Total carbohydrate content was measured by the phenol/sulphuric acid method (Dubois *et al.*, 1956), using glucose as standard. Hexosamine was determined by the method of Morgan & Elson (1934), using D-glucosamine hydrochloride as standard. Phosphorus was determined according to Chen *et al.* (1956).

Lipid extraction. Lipids extracted by chloroform/methanol (2:1, v/v) as described by Folch *et al.* (1957) were dried in a rotary evaporator under vacuum followed by exposure to a stream of dry nitrogen. The dried lipid extract was used for cytotoxicity, haemagglutination and complement fixation tests.

Separation of fatty acids by TLC and HPLC. Lipid extracts from whole leptospire were dissolved in hexane, and separated by thin-layer chromatography (TLC) on silica gel (Kieselgel-Merck) with hexane/acetone (2:1, v/v) as solvent. The lipids were visualized by spraying the plates with 5 M-H₂SO₄ and heating at 110 °C for 30 min. The location of the fatty acids corresponded to an *R_f* of 0.6 while phospholipids remained at the origin. The phospholipids were further separated by TLC on silica gel in a solvent of chloroform/acetone/methanol/acetic acid/H₂O (15:6:3:3:1, by vol.). The fatty acids were removed from TLC plates by methanol and dried with a stream of dry nitrogen. Fatty acids were extracted from GLP or LPS by chloroform/methanol (2:1, v/v) and dried with a stream of dry nitrogen. The fatty acids were dissolved in 1 ml tetrahydrofuran (HPLC grade; Waters Associates) and separated at 20 °C by high pressure liquid chromatography (HPLC) (Waters Associates), using a 6000 A pump, a U6K injector, a 3.9 × 30 cm fatty acid analysis column and a differential refractometer detector model 401. The solvent system was tetrahydrofuran/acetonitrile/H₂O (25:35:45, by vol.) and 0.1% (v/v) acetic acid, at a flow rate of 1.2 ml min⁻¹ and ×4 attenuation. The fatty acids were identified by comparison with a profile of the retention times of standard fatty acids.

Preparation of standard fatty acid solution. Each fatty acid standard (5 mg) [caprylic, C_{8:0}; capric, C_{10:0} (Sigma); lauric, C_{12:0}; myristic, C_{14:0}; palmitic, C_{16:0}; stearic, C_{18:0}; oleic, C_{18:1} (National Biochemicals Co.); myristoleic, C_{14:1} and palmitoleic, C_{16:1} (Sigma)] was dissolved in 1 ml tetrahydrofuran. This standard solution was protected from light and stored at -20 °C when not in use. The standard solution (10 μ l) was injected into the HPLC injector and the fatty acid profile recorded.

Rabbit pyrogenicity tests. Either GLP or LPS (500 μ g) was dissolved in 1 ml pyrogen-free water. The GLP solution was sonicated three times for 10 s each at a frequency of 20 kHz with a Branson sonicator. The pyrogenicity test protocol followed the procedure of the British Pharmacopoeia (1980), using three healthy rabbits for each solution. A positive fever test was read as a rise in rectal temperature of at least 0.55 °C within 3 h of intravenous injection.

Limulus amoebocyte lysate assay. The amoebocyte lysate assay for endotoxin was done with the *Limulus* E-Toxate kit (Sigma). GLP resuspended in pyrogen-free water (1 mg ml^{-1}) and sonicated as above, and LPS dissolved in PFW (1 mg ml^{-1}) were diluted in serial 10-fold steps in pyrogen-free water. Lysate (0.1 ml) was added to 0.1 ml of either the LPS or the GLP dilution in a pyrogen-free tube. After mixing, the tubes were incubated at 37°C for 4 h and allowed to stand for 15 h at room temperature. The formation of a gel or starch-like granules in the tube was recorded as positive. An endotoxin reference preparation (Sigma) and pyrogen-free water were used as positive and negative controls respectively.

Crenation of mouse erythrocytes. A suspension of 2% (v/v) packed mouse erythrocytes in PBS was used, to which either LPS or GLP (both $100 \mu\text{g ml}^{-1}$) was added, each in 0.1 ml volumes. The suspensions were incubated at 37°C for 60 min and observed by dark-field and by phase-contrast microscopy.

Preparation of tissue culture. Mouse fibroblast cell line L929 and Vero cells were prepared as described previously (Vinh *et al.*, 1984), except for cytotoxicity experiments using GLP or LPS, where Linbro 96-well plastic tissue culture trays were used instead of Leighton tubes to prepare L-cell monolayers. A 0.2 ml volume of the cell suspension ($1.2 \times 10^5 \text{ cells ml}^{-1}$) in tissue culture medium 199 (M199; Commonwealth Serum Laboratories, Melbourne, Australia) was dispensed into each well. After overnight incubation the cell monolayers were washed twice with M199 without FCS and then used for cytotoxicity assays.

Cytotoxicity assays. In earlier experiments L929 fibroblasts and Vero cells were grown on coverslips in Leighton tubes in M199 and treated with sonicates of leptospires equivalent to concentrations of 10^7 – 10^{10} organisms ml^{-1} .

GLP was suspended in M199 without FCS at 1 mg ml^{-1} and sonicated. LPS was dissolved in M199 without FCS at a concentration of 1 mg ml^{-1} . Lipid extract and individual fatty acids in M199 without FCS were dispersed by sonication. All solutions to be added to the cell monolayers were first pasteurized at 70°C for 20 min and then serially diluted in M199 without FCS. Cytotoxicity was assessed by measuring the release of lactate dehydrogenase (LDH) from damaged L-cells (Mitchell *et al.*, 1980) in the supernatant medium after incubation and centrifuging at 300 g for 5 min. Mouse fibroblast monolayers treated with GLP at 0, 62.5, 125, 250 and $500 \mu\text{g ml}^{-1}$ were incubated for various times up to 24 h after which cytotoxicity was measured by the LDH assay in the supernatant. Cytopathic effects were observed by using an inverted microscope with photographic attachments.

Haemagglutination. Erythrocytes from guinea pigs, humans, mice, rabbits or geese in Alsever's solution were washed twice and resuspended in PBS. GLP, LPS, lipid extract and individual fatty acids were suspended in PBS and treated as above (for cytotoxicity assays) before serial dilution in conical-bottom plastic microtitration trays in $25 \mu\text{l}$ volumes. An equal volume of 1% mouse erythrocyte suspension was added to each well. The tray was incubated at 37°C for 1 h and the cells settled overnight at 4°C before reading the results. Tweens could not be used for solubilizing lipid materials, nor could BSA be added to haemagglutinations because of their interfering effects.

Interference with haemagglutination by leptospiral lipids in the presence of BSA and Tween 80. BSA or Tween 80 (each 1%, w/v, in PBS, pH 7.2) were serially diluted in conical-bottom microtitration plates. Equal volumes ($25 \mu\text{l}$) of lipid (1.5 mg ml^{-1}) and of 1% packed mouse erythrocytes were added to each well, the plates were incubated at 37°C for 1 h and the lowest concentrations of either BSA or Tween that neutralized haemagglutination were recorded.

Preparation of antibodies to GLP and leptospires. GLP (2 mg) was dissolved in PBS and emulsified with an equal volume of Freund's incomplete adjuvant. New Zealand White rabbits were injected intradermally with the emulsion and bled after 3 weeks. Antisera to whole leptospires were prepared in rabbits by intravenous injection of cultures (Faine, 1982). IgG components of antisera to either GLP or whole leptospires or of normal rabbit serum were separated by affinity chromatography using Protein A-Sepharose (Pharmacia). The titre of the IgG from GLP antiserum was determined by enzyme immunoassay and microagglutination test (Adler *et al.*, 1980).

Neutralization tests. For neutralization of haemagglutination by GLP, the protein concentrations in IgG fractions from antisera to either GLP or whole leptospires, or from normal serum, were adjusted to 1 mg ml^{-1} and serially diluted with PBS in conical-bottom microtitration plates in volumes of $25 \mu\text{l}$. A similar volume of GLP solution at concentrations of 10, 25 or $50 \mu\text{g ml}^{-1}$ (w/v) in PBS was added to each well. After incubation for 15 min at 37°C , $50 \mu\text{l}$ of a 1% (v/v) suspension of washed mouse erythrocytes was added to each well and the mixtures incubated at 37°C for 1 h and left overnight at 4°C before the results were recorded.

For neutralization of the GLP cytotoxicity for L-cells, serial dilutions of immune or normal IgG were made in M199 without FCS and added to equal volumes of GLP solution ($250 \mu\text{g ml}^{-1}$) in the same medium. The mixtures were incubated for 15 min at 37°C , after which they were transferred onto the cell monolayers grown in tissue culture trays as described above. The treated cells were incubated in an atmosphere of 5% (v/v) CO_2 in air at 37°C overnight. The medium above the treated monolayers was withdrawn and used to measure LDH activity. The neutralization of cytotoxicity for L-cells caused by whole leptospires was measured by incubating the cell monolayers with 5×10^8 leptospires ml^{-1} in the presence of different concentrations of IgG (immune or normal). LDH activities were determined after 3 d incubation at 37°C .

Complement fixation. Complement fixation by leptospiral extracts was measured in microtitration trays, using 2

haemolytic units of complement, 4 haemolytic units of haemolysin and a final 1% (v/v) suspension of washed sheep erythrocytes in veronal-buffered saline, pH 7.4. Sera, antigen and complement added in 0.25 ml volumes were incubated together at 37 °C for 30 min, and for a further 30 min after addition of sensitized sheep erythrocytes. Normal rabbit sera were used as controls. Antigen stock solutions were prepared by mixing a solution of lipid extract (600 µg ml⁻¹) in ethanol, with an equal volume of an ethanolic solution containing lecithin (250 µg ml⁻¹) and cholesterol (1.5 µg ml⁻¹). The antigen stock solution was stable for at least one month. It was made up freshly for use by rapid 1 in 150 dilution in veronal-buffered saline. It was neither haemolytic nor anticomplementary.

Electron microscopy of L-cell fibroblasts treated with GLP. L-cell fibroblast monolayers grown in plastic Falcon flasks were treated with GLP as described above and the treated cells were processed for transmission electron microscopy (Vinh *et al.*, 1984). Thin sections were stained with uranyl acetate and lead citrate, air dried and examined with a Philips EM300 electron microscope at 60 kV.

Susceptibility of mice to leptospiral infection. Leptospires of virulent *L. interrogans* strains L45 and L115 and saprophytic *L. biflexa* strain L92 were harvested at 7 d, washed twice and resuspended in PBS. Pairs of weanling Balb/c mice (weighing approx. 8 g) were injected intraperitoneally with doses in the range of 1 × 10⁹, 3 × 10⁹, 10¹⁰, 3 × 10¹⁰ and 6 × 10¹⁰ leptospores of each strain in 0.5 ml PBS. Leptospores killed with formalin or by heating at 56 °C were also injected similarly while control mice received PBS alone.

The mice were observed for 7 d. Dead mice were autopsied to observe pathological changes and checked for the presence of leptospores.

RESULTS

Lipid extract from leptospores

The lipid fraction extracted from the virulent strain of serovar *copenhageni* (L45) comprised 11.8% of the total cell dry weight. Phospholipids constituted 80% and free fatty acids 20% of the lipids. Phosphatidylethanolamine accounted for 98% of the phospholipid fraction by TLC. The fatty acids detected in the lipid fraction by HPLC were myristic (14:0), palmitic (16:0), palmitoleic (16:1), stearic (18:0) and oleic (18:1) (Table 1).

Extraction of LPS and GLP complex

LPS extracts (Vinh *et al.*, 1986) comprised approximately 3–5% of the dry weight of the whole leptospores in different preparations. Extraction by Tris/lysozyme (pH 7.4) followed by acetic acid (1 M) precipitation at 4 °C yielded a GLP complex which was 3–5% of the dry weight and poorly soluble in water. The average chemical compositions of LPS and GLP are shown in Table 2. The GLP did not exhibit phospholipase activity.

Table 1. *Fatty acid content of leptospiral lipid extract separated by HPLC*

Results are the means of two determinations ± the range.

Fatty acid	Percentage of total fatty acid detected
14:0	2.9 ± 0.1
16:0	17.5 ± 0.7
16:1	25.0 ± 0.5
18:0	1.0 ± 0.1
18:1	51.5 ± 1.0

Table 2. *Chemical compositions of the glycolipoprotein (GLP) and lipopolysaccharide (LPS) from L. interrogans serovar copenhageni*

Results are the means of two determinations ± the range and are expressed as a percentage of the total dry weight.

Component	GLP	LPS
Protein	20.0 ± 0.5	5.0 ± 0.4
Total carbohydrate	39.0 ± 0.5	65.0 ± 1.2
Lipid	33.3 ± 0.8	25.0 ± 0.5
Amino sugars	ND	4.0 ± 0.5
Phosphorus	ND	0.4 ± 0.0

ND, Not determined.

Substances interfering with cytotoxicity

Early attempts to measure toxic effects in tissue cultures produced unpredictably irregular results. Normal serum, BSA, FCS and Tween 80, all common constituents of either culture media for tissue cells, or of diluents for reagents, neutralized toxicity. The effects of serum and FCS appeared to be due to their content of albumin, which inhibited cytotoxicity at concentrations $> 0.001\%$ (w/v). When serum or FCS was omitted from tissue cultures, reliable, reproducible and sensitive measurements could be made. In experiments where toxicity was measured, the cells were washed free of nutrient medium containing FCS and kept in M199 without serum during exposure to toxins. Control cells, exposed to diluent without toxin for the duration of the experiments and afterwards fed nutrient medium, showed no signs of damage. Erythrocytes used for direct haemagglutination by toxins were suspended in PBS without Tweens or albumin. Tweens could not be used as solubilizing agents for lipids because concentrations as low as 0.01% (w/v) prevented haemagglutination by lipids or GLP, and were toxic to cultured cells.

Cells grown in Leighton tubes treated with sonicates of leptospire developed cytopathic effects within 24–72 h at equivalent concentrations of 10^8 – 10^{10} organisms ml^{-1} . With lower doses, a slower response was seen. Recovery of the cell layer occurred if the sonicate of leptospire was removed after an exposure of up to 4 h.

In later experiments, measurement of LDH leakage at 3–6 h after infection was used as a rapid objective indicator of cell damage.

Biological activities of leptospiral lipids

Lipid extract from leptospire was toxic to mouse fibroblasts (L-cells) and agglutinated erythrocytes, showing a species preference. Mouse erythrocytes were agglutinated at a concentration of $0.53\ \mu\text{g ml}^{-1}$ of lipid extracted with chloroform/methanol. Erythrocytes of other species were either less sensitive (requiring $1.56\ \mu\text{g ml}^{-1}$ for guinea pig erythrocytes), or insensitive (not agglutinated by up to $4.25\ \mu\text{g ml}^{-1}$ for sheep, human, rabbit and goose erythrocytes). Therefore mouse erythrocytes were used in all experiments. Underivatized fatty acids from leptospiral lipid extract were separated by HPLC and were used to test for haemagglutination and cytotoxicity. Since myristic (14:0) and stearic (18:0) acids represented only a small proportion of leptospiral fatty acids, not enough material could be collected to test each individually, and therefore they were not included in these tests.

Preparations of individual fatty acids (palmitic, palmitoleic and oleic) were incubated with mouse erythrocytes. At very low concentrations (3 – $12.5\ \mu\text{g ml}^{-1}$) palmitoleic (16:1) and oleic (18:1) acids agglutinated mouse erythrocytes, whereas similar fatty acids from commercial sources lysed cells at higher concentrations ($> 25\ \mu\text{g ml}^{-1}$) but did not agglutinate these cells. When the same preparations of individual fatty acids were added to L-cell monolayers, the ones that caused haemagglutination also caused damage to the cells as revealed by the leakage of LDH into the medium (Fig. 1) whereas commercial fatty acids had little effect. In addition, the leptospiral phospholipid fraction also agglutinated erythrocytes and caused a cytopathic effect for L-cells at a minimum concentration of $0.1\ \mu\text{g ml}^{-1}$ and $150\ \mu\text{g ml}^{-1}$ respectively.

Biological activities of LPS and GLP

Fever is a feature of leptospirosis. Attempts were therefore made to test the cytotoxic GLP or the LPS for pyrogenicity and/or reaction with *Limulus* lysate.

Pyrogenicity. Neither LPS nor GLP, when injected into rabbits, were significantly pyrogenic. A slight rise ($0.3\ ^\circ\text{C}$) in rabbit body temperatures was recorded at doses of $5\ \mu\text{g kg}^{-1}$ but not less, of either LPS or GLP. A pyrogenic response is regarded as an average temperature rise of $0.55\ ^\circ\text{C}$ or more (British Pharmacopoeia, 1980). Pyrogenic samples tested in the laboratory caused rises in the range 0.4 – $0.8\ ^\circ\text{C}$.

Limulus amoebocyte lysate assay. LPS and GLP induced the gelation of *Limulus* lysate at end-point concentrations of $10\ \text{ng ml}^{-1}$ and $1\ \text{ng ml}^{-1}$ respectively.

Effect on mouse erythrocytes. Both LPS and GLP agglutinated and crenated the erythrocytes (Fig. 2a). Control erythrocytes retained their round appearance (Fig. 2b).

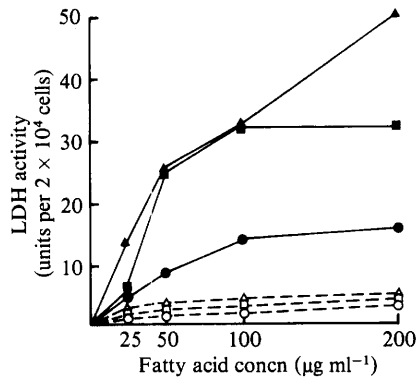


Fig. 1. Lactate dehydrogenase (LDH) activity, as a measure of cytotoxicity, in supernatants of L929 cultured fibroblasts treated with leptospiral or commercially available fatty acids for 24 h. Leptospiral fatty acids: ▲, C_{18:1}; ■, C_{16:1}; ●, C_{16:0}. Commercial fatty acids: △, C_{18:1}; □, C_{16:1}; ○, C_{16:0}.

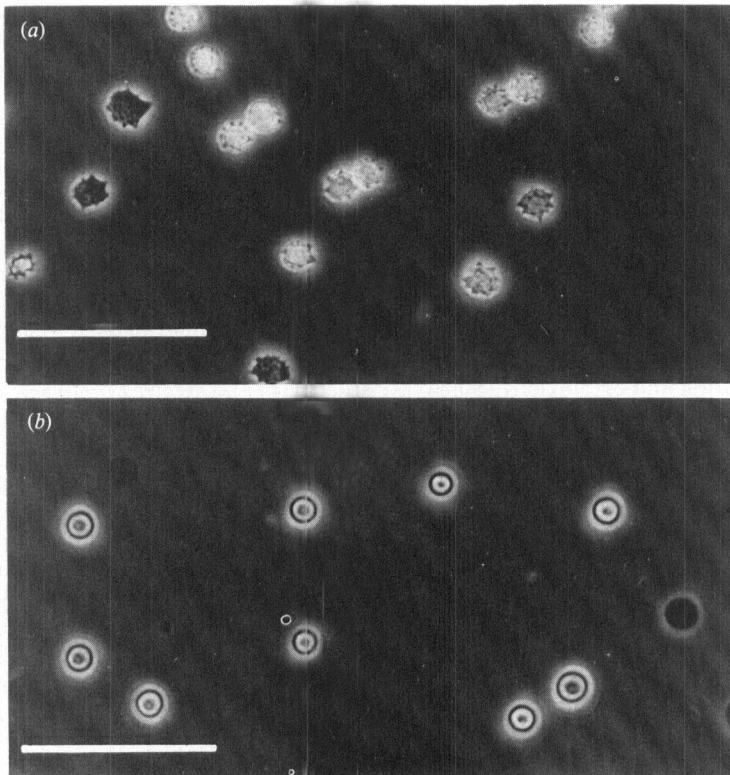


Fig. 2. (a) Effect of 100 µg GLP ml⁻¹ on mouse erythrocytes. A similar result was observed with an equal amount of LPS. (b) Control. Dark-field. Bars, 2 µm.

Cytotoxicity in mouse fibroblasts. The amount of cytotoxicity (Fig. 3) was time and concentration dependent. Cytotoxicity and haemagglutination of GLP were not reduced after heating to 70 °C for 20 min or to 100 °C for 10 min.

Electron micrographs of cells treated with 100 µg GLP ml⁻¹ for 3 h showed that most of the cells were vacuolated and the cell membrane appeared to be damaged (Fig. 4b) compared with the controls (Fig. 4a). Fig. 4(c) shows a severely damaged cell after 24 h of treatment. Similar concentrations of LPS were tested in the same way, but evidence of cytotoxicity was not detected.

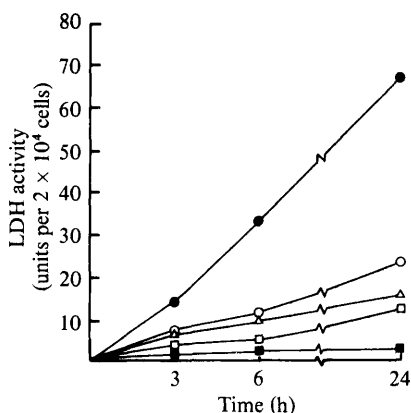


Fig. 3. Lactate dehydrogenase (LDH) activity, as a measure of cytotoxicity, in the supernatants of L929 cultured fibroblasts treated with various concentrations of GLP for times up to 24 h. Concentrations ($\mu\text{g ml}^{-1}$): ●, 500; ○, 250; △, 125; □, 62.5; ■, control, without GLP.

To investigate the component which was responsible for the toxic effect further, the lipids were extracted from GLP, and both lipid and non-lipid fractions were used to treat L-cell monolayers. The cytotoxic effects equivalent to those of $250 \mu\text{g GLP ml}^{-1}$ were reproduced by $70 \mu\text{g ml}^{-1}$ of the lipid fractions, but not by up to $175 \mu\text{g ml}^{-1}$ of the non-lipid moiety (Fig. 5). Haemagglutination and cytotoxicity of GLP were lost after treatment with lipase, but retained after treatment with proteinase or phospholipase C.

Neutralization of the toxic effects of GLP by antibody

Antisera cannot be used for neutralization because the albumin interferes with toxicity. Specific anti-GLP IgG neutralized the haemagglutinating effect of GLP. Concentrations of $3\text{--}25 \mu\text{g IgG ml}^{-1}$ were required to neutralize the agglutination of mouse erythrocytes by $25 \mu\text{g GLP ml}^{-1}$. Higher concentrations of IgG ($> 25 \mu\text{g ml}^{-1}$) induced cell lysis in the presence of GLP, although erythrocytes in controls with IgG alone did not lyse. Haemagglutination by GLP was not neutralized nor was lysis enhanced in controls with IgG from normal rabbit serum. Higher GLP concentrations of $50 \mu\text{g ml}^{-1}$ or more lysed all erythrocytes even in the presence of IgG.

A similar neutralizing effect was observed when IgG against GLP was used to neutralize the cytotoxicity of either GLP or whole leptospire on mouse fibroblasts (Table 3). The neutralizing amounts of IgG ranged from 0.2 to $12 \mu\text{g ml}^{-1}$ for $250 \mu\text{g GLP ml}^{-1}$ and from 6 to $100 \mu\text{g ml}^{-1}$ for 5×10^8 leptospire ml^{-1} . The cytotoxic effect of GLP was enhanced at higher concentrations of IgG, above $12.5 \mu\text{g ml}^{-1}$ for GLP and $100 \mu\text{g ml}^{-1}$ for whole leptospire. IgG from normal rabbit serum did not neutralize the effect of GLP.

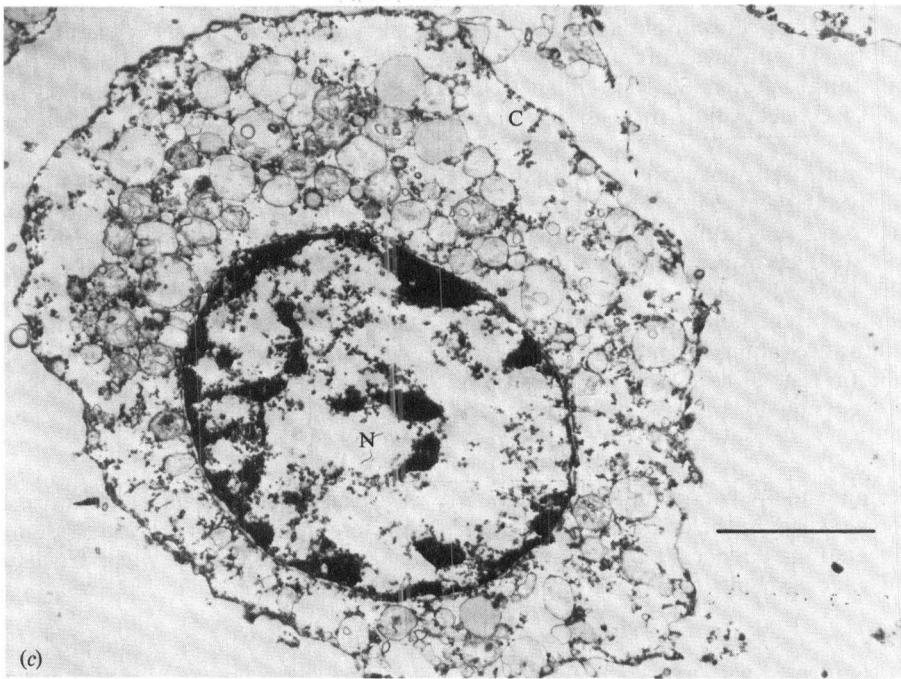
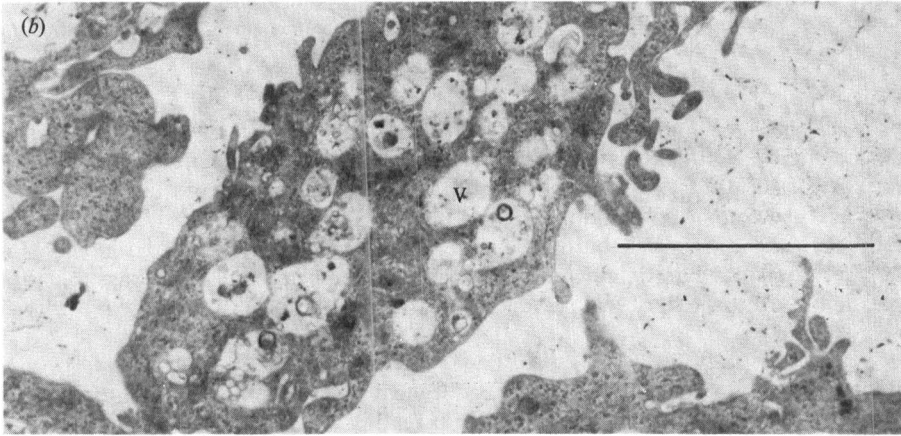
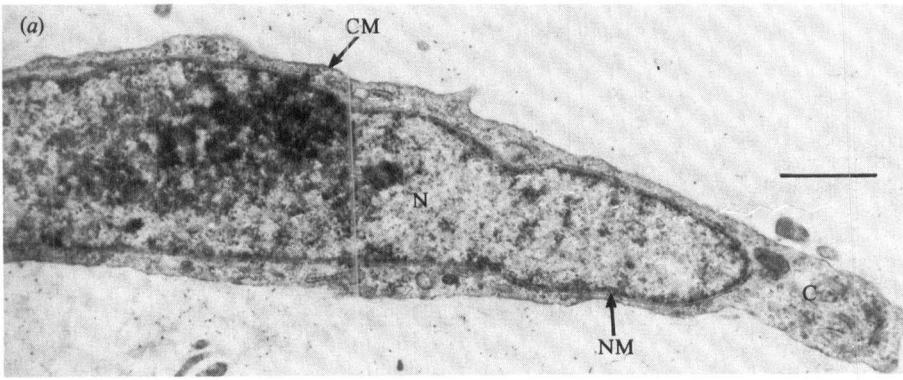
One of the two rabbits immunized with GLP alone produced IgG which reacted with LPS in a gel precipitin test, while IgG of the other rabbit reacted with GLP alone. IgG from both the antisera were equally effective in neutralization tests of GLP toxicity.

Complement fixation

The compound lipid antigen prepared from serovar *pomona* fixed complement to titres of 256 with homologous and heterologous strain *pomona* antiserum and to 64 with antiserum to a *copenhageni* strain, but not at all with antiserum to *L. biflexa* serovar *patoc*, or with normal rabbit serum. The constituents of the antigen were inert when tested alone.

Lethal toxicity in weanling mice

All mice infected with a dose of 10^{10} leptospire or more, regardless of whether these were virulent pathogenic (L45, L115) or saprophytic (L92) strains, died within 12–38 h whereas a



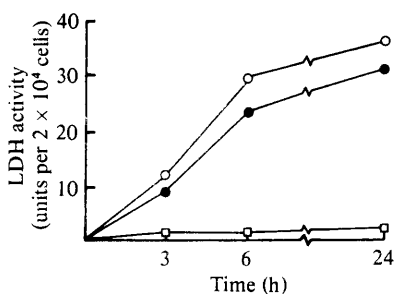


Fig. 5. Comparison of cytotoxicity of GLP and lipid fraction of *L. interrogans* serovar *copenhageni* measured by lactate dehydrogenase (LDH) activity in the supernatants of L929 cultured fibroblasts for times up to 24 h. ●, GLP (250 µg ml⁻¹); ○, lipid fraction (70 µg ml⁻¹); □, non-lipid fraction (175 µg ml⁻¹).

Table 3. Neutralization by anti-GLP IgG of the cytotoxic effect on mouse L-cells by GLP and live leptospires

Results are the means of two determinations ± the range and are expressed as LDH units per 2 × 10⁴ cells.

	Concn (µg ml ⁻¹)	Cytotoxic effect on L-cell monolayers treated by:	
		GLP (250 µg ml ⁻¹)	Live leptospires (5 × 10 ⁸ ml ⁻¹)
Anti-GLP IgG*	0	50.2 ± 1.0	60.7 ± 5.3
	6	23.1 ± 1.6	24.3 ± 5.6
	12.5	20.8 ± 2.1	23.1 ± 7.8
	25	57.2 ± 4.6	NT
	50	77.3 ± 4.8	34.7 ± 4.9
	100	NT	75.5 ± 6.2
	300	NT	131.5 ± 7.7
Normal IgG	100	44.8 ± 1.5	65.7 ± 3.9

NT, Not tested.

* Similar results to those found with 6 µg ml⁻¹ were obtained with concentrations of 0.2–3.0 µg ml⁻¹ in a serial doubling dilutions when tested against 250 µg GLP ml⁻¹. No neutralizing effect was detected with concentrations < 6 µg ml⁻¹ of anti-GLP IgG when tested against live leptospires.

lower dose of 3 × 10⁹ organisms was not lethal. The same results were observed with formalin-killed leptospires but not with heat-killed preparations containing an equivalent number of leptospires.

Autopsies of the dead mice showed scattered haemorrhages in lungs, kidneys and other tissues, and oedema.

DISCUSSION

The results in this paper show that reproducible laboratory experiments depended on recognition and control of interfering factors, that GLP and not LPS is responsible for toxicity in leptospires and that a similarly-acting toxin occurs in both non-pathogenic and pathogenic leptospires, so that pathogenicity and virulence depend on other factors as well as toxicity.

Fig. 4. Electron micrographs of L-cells treated with 100 µg GLP ml⁻¹. (a) Without GLP. Similar appearances were observed with LPS up to 100 µg ml⁻¹. Note intact cell membrane (CM), nuclear membrane (NM), cytoplasm (C) and nucleus (N). (b) After 3 h exposure to GLP. Many vacuoles (V) appear in the cytoplasm. (c) After 24 h exposure to GLP. The cytoplasmic and nuclear membranes have been destroyed, and degeneration of both cytoplasm (C) and nucleus (N) has occurred. Bars, 5 µm.

Interfering substances. Results of cytotoxicity experiments were poorly reproducible until it was recognized that the presence of serum or BSA at concentrations down to 0.001% (w/v) reduced and sometimes abolished the cytotoxicity of lipid fractions or sonicates of leptospire. Reproducible results were obtained only when tissue cells were incubated in media free of serum, BSA or FCS during exposure to toxin, and in neutralization experiments, when a purified albumin-free IgG fraction of antiserum was required.

Albumin is an effective absorbent for fatty acids which may be toxic to microbial and mammalian cells in culture (Davis & Dubos, 1947). Plasma albumin can bind and transport free fatty acids in the body, and can presumably also bind and detoxify leptospiral or other toxic lipids (Kanai & Kondo, 1979), to prevent circulating toxic fatty acids, phospholipids and other leptospiral components from acting on endothelial cells, erythrocytes or other target cells in tissues until the plasma albumin and other lipid transports are saturated. Possible roles for lipoproteins or other plasma proteins or detoxifiers require further investigation.

Tweens, which were similarly inhibitory down to concentrations of 0.01% (w/v) when attempts were made to use them as solubilizers, either lysed or agglutinated erythrocytes in controls or, at lower concentrations, prevented haemagglutination by leptospiral lipids. These effects may be due to either the free or the bound long-chain fatty acids found in Tween preparations, which may compete with the leptospiral lipids for sites of action on target cell membranes.

The interfering substances may account for the unconfirmed and contradictory reports of cytotoxicity studies (Miller *et al.*, 1970; Finn & Jenkin, 1973; Yam *et al.*, 1970), uncharacterized toxins (Finco & Low, 1967; Sefer *et al.*, 1966), toxic proteins in plasma (Chaperon *et al.*, 1979) and toxic lipids (Stalheim, 1967).

Toxicity and possible mode of action. Measurement of toxicity by observation of cytopathic effects using conventional microscopy at 24–72 h after exposure to toxin was relatively slow, coarse and subjective, but measurement of LDH leakage was rapid, sensitive and objective, enabling the demonstration that cell damage at lower concentrations of toxin was reversible and time-dose dependent. Haemagglutination of mouse erythrocytes proved to be a similarly rapid, very sensitive assay for activity and for neutralization.

All lines of evidence (reversible cytopathic effects and LDH leakage at low concentrations of toxin, haemagglutination, crenation of erythrocytes with GLP, enhancement of cytotoxicity by IgG) point to an effect of toxin on cell membranes, although possible mechanisms by which this could occur have not been studied here. Nor has the reason why only fatty acids of leptospiral origin, and not those of similar chain length from other sources, were cytotoxic.

At least one long-chain fatty acid, hexadecenoic acid, from various leptospire has been characterized as an unusual positional isomer (Livermore *et al.*, 1969; Stern *et al.*, 1969). A theoretical basis for the activity of leptospiral fatty acids as toxins, and for mediating the toxicity of GLP and longer fragments, exists in a hypothesis that the leptospiral lipids, whether free or bound in phospholipids, are intercalated into the host cell membrane in place of normally occurring similar fatty acids. Membrane permeability function is then disturbed (Kanai & Kondo, 1979; Karnovsky, 1979), resulting in leakage of cell contents such as LDH, and eventually in cell death seen as a cytopathic effect if not reversed. If the concentration of abnormal fatty acids is low, membrane repair could overcome the physiological disturbance. The hypothesis is supported by the evidence that erythrocytes are crenated by GLP and LPS, and that in neutralization of GLP by specific IgG an antibody excess of IgG enhanced cell leakage in tissue cultured fibroblasts (Table 3) and lysis after haemagglutination of erythrocytes treated with a sub-lytic dose of 25 μg GLP ml^{-1} .

The reason for the relatively selective action on mouse erythrocytes has not been investigated, but is probably connected with the fatty acid composition of the erythrocyte membrane. Leptospiral phospholipases were selective for the cell membranes of various species of animals (Kasarov, 1970), reflecting the interaction of leptospiral enzyme specificity and animal cell membrane lipid composition. Although mouse fibroblast L929 cells were used in our experiments, cell lines from other animal sources were also susceptible.

Neither GLP nor LPS were significantly pyrogenic in rabbits, when using standard protocols

for testing, and leptospiral LPS was not toxic, although LPS preparations were slightly active in clotting *Limulus* lysate and were similar to other Gram-negative bacterial LPS in general chemical composition (Vinh *et al.*, 1986). Leptospiral LPS is in a class of low pyrogenicity, similar to LPS from *Coxiella burnetii* (Amano & Williams, 1984).

Fever is characteristic of leptospirosis, Q fever, the secondary stage of syphilis, relapsing fever and the Jarisch–Herxheimer reaction. Nevertheless there were also other reports (Finco & Low, 1967; Johnson, 1976) as well as previous unpublished results from this laboratory, that LPS from leptospires was not pyrogenic. Fever in *Borrelia recurrentis* infection was not mediated by an endotoxin (Butler *et al.*, 1979) and LPS from other pathogenic spirochaetes, *Treponema pallidum* and *Borrelia hispanica* were also not pyrogenic (Hardy & Levin, 1983).

In contrast to LPS from other Gram-negative bacteria, leptospiral LPS was not toxic. On the other hand, biological effects, including some attributable to LPS in other bacteria, were observed with GLP. Evidence in this paper shows that GLP and LPS are chemically as well as biologically different, although some GLP preparations contained a small amount of LPS. GLP does not have the characteristic electron microscopic structure of leptospiral LPS (Vinh *et al.*, 1986). Furthermore, three different mouse monoclonal antibodies reacted with GLP at concentrations of 5–6 $\mu\text{g ml}^{-1}$ in enzyme immunoassays, but did not react in similar tests with 25–50 $\mu\text{g LPS ml}^{-1}$, which were optimum antigen concentrations in these tests (B. H. Jost, H. E. Farrelly; B. Adler & S. Faine, unpublished observations). The sites of GLP and LPS in leptospires have not been localized.

Role of toxin in pathogenesis and immunity in leptospirosis. It was significant that leptospires did not need to be viable or virulent nor indeed members of the pathogenic species *L. interrogans* in order to be toxic. A strain of *L. biflexa* as well as formalin-killed or sonicated pathogenic leptospires (*L. interrogans*) were toxic in amounts equivalent to the toxic concentrations of whole leptospires. Culture supernatants and heat-killed leptospires were ineffective in comparable doses. Lesions like those of leptospirosis were observed in toxin-treated animals, in the absence of viable leptospires.

The role of toxin in pathogenesis *in vivo* may thus be to produce or facilitate the production of lesions, provided the antibacterial defences of the host animal are incapable of preventing the growth of a sufficient number of leptospires to reach the threshold concentration (Adler & Faine, 1976, 1977; Faine, 1957*b*). In a situation analogous to that existing amongst other Gram-negative bacteria, leptospires of a 'non-pathogenic' species (*L. biflexa*) can exert endotoxin-like activity whether they are alive or not provided sufficient numbers can enter the body. In natural conditions they can enter but cannot reproduce because they are killed before reaching a toxic threshold, so that their lack of pathogenicity depends on the activity of the natural antibody system. In its absence or failure, as in animals artificially inoculated with large numbers of leptospires, they are toxic and lethal. In nature or in the laboratory, virulent pathogenic leptospires, which are not subject to destruction by natural defences (Faine & Carter, 1968; Johnson & Muschel, 1966), are able to survive and grow to threshold toxic levels (Faine, 1957*b*). While avirulent pathogens cannot survive (Faine & Carter, 1968), the lesions produced by different leptospires are essentially similar, even in different animals, so that a single or similar mechanism of production of lesions operates in all serovars.

The toxin described here is a candidate for the first step on the common pathway because its effects are similar in all the strains tested and monoclonal antibodies cross-react serologically between serovars (B. H. Jost, H. E. Farrelly, B. Adler & S. Faine, unpublished observations), although no information is available on cross-neutralization. The further characteristics of capillary endothelial damage, renal ischaemia and tubular necrosis, disseminated intravascular haemolysis and haemorrhages may all result from the effects of the toxin acting similarly to other Gram-negative bacterial endotoxins *in vivo*. Those lesions would be consistent also with the observation that small amounts of antibody were able to enhance toxicity *in vitro*, presumably by interacting with cell membrane bound toxin. If this occurs *in vivo*, the lesions found would correspond with those of a circulating immune complex for which there is already evidence (Lai *et al.*, 1982).

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