Properties of Monoclonal Antibodies to the Genus-specific Antigen of *Chlamydia* and Their Use for Antigen Detection by Reverse Passive Haemagglutination

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The chlamydial genus-specific antigen was extracted with phenol/chloroform/petroleum ether (PCP) from preparations of *Chlamydia trachomatis* and *C. psittaci*, and quantities measured using an assay for lipopolysaccharide (LPS). The LPS from *C. trachomatis* contained 2.2% (w/w) of ketodeoxyoctanoic acid. Five IgG monoclonal antibodies reacted in an ELISA with LPS from both species, the antigen being periodate-sensitive and heat-resistant, confirming that all antibodies were against the genus-specific antigen. All the antibodies bound to the PCP extract of *C. trachomatis* on an immunoblot, at a position corresponding to the periodate-Schiff-stained bands of both *C. trachomatis* extract and *Salmonella* Re-LPS. When linked to trypsin-treated sheep erythrocytes and used in reverse passive haemagglutination tests, all antibodies gave indicator cells capable of detecting chlamydial LPS or crude preparations of chlamydiae grown in McCoy cells, the sensitivity varying with the antibody used. The antibodies varied in IgG subclass (either IgG_{2a} or IgG₃), and in ability to precipitate in immunodiffusion tests. Two antibodies cross-reacted with one strain of *Acinetobacter* in ELISA and with *Salmonella* Re-LPS in both ELISA and immunodiffusion tests. The other three did not react in ELISA with *Acinetobacter* strains or *Salmonella* Re-LPS, and none of the five reacted with LPS of *E. coli* or *Pseudomonas morsprunorum*.

INTRODUCTION

The genus-specific antigen of *Chlamydia* is a glycolipid containing ketodeoxyoctanoic acid (KDO) (Dhir *et al.*, 1971, 1972). Recently, Nurminen *et al.* (1983), using polyclonal antisera, have shown that this antigen bears a close structural and immunological similarity to LPS of the Re-chemotype (Re-LPS) from certain 'deep-rough' mutants of *Salmonella* species, and they accordingly described the genus-specific antigen as the LPS of *Chlamydia*. Caldwell & Hitchcock (1984), using animal sera, confirmed the findings of Nurminen *et al.* (1983) of complete cross-reactions between chlamydial and *Salmonella* Re-LPS, whilst their monoclonal anti-chlamydial LPS antibody did not cross-react with Re-LPS or LPS of several other bacteria when tested by the immunoblotting technique. This antibody was therefore thought to react with a determinant specific to chlamydial LPS. Cross-reactions between the chlamydial genus-specific antigen and strains of *Acinetobacter* have also been reported (Brade & Brunner, 1979) but not studied with monoclonal antibodies.

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Abbreviations: DOC, sodium deoxycholate; KDO, 2-keto-3-deoxyoctonate; PCP, phenol/chloroform/petroleum ether; RPH, reverse passive haemagglutination; SRBC, sheep red blood cells.

Monoclonal antibodies to the chlamydial genus-specific antigen have been described by Stephens *et al.* (1982) and Thornley *et al.* (1983). Our study of the production and characteristics of such monoclonal antibodies, and their possible eventual application in diagnostic methods (Thornley *et al.*, 1983) has been extended to include four additional monoclonal antibodies, characterization of their reactions with chlamydial antigens, a study of cross-reactions with *Salmonella* Re-LPS and *Acinetobacter* strains, and further quantitative characterization of the chlamydial genus-specific antigen. The sensitivity of the monoclonal antibodies when coupled to trypsin-treated sheep red blood cells and then used to detect chlamydial antigen by reverse passive haemagglutination (RPH) has also been studied.

METHODS

Growth of chlamydiae and extraction of the genus-specific antigen. *C. trachomatis* strains 48/80H and SA₂(f) were cultured in McCoy cells (Evans & Taylor-Robinson, 1979); SA₂(f) was additionally cultured in egg yolk sacs (Schachter & Dawson, 1978). Elementary bodies from egg yolk cultures were purified by density centrifugations and enzyme treatment, followed by extraction with 1% (w/v) sodium deoxycholate (DOC) to yield the genus-specific antigen (Thornley *et al.*, 1983).

C. trachomatis strain SA₂(f) grown in McCoy cells (a gift from Drs M. Macnaughton and D. Sherwood, Boots-Celltech Diagnostics, Slough, UK) was harvested in distilled water, inactivated by heating at 50 °C for 1 h, then centrifuged at 20000 *g* for 1 h at 4 °C. The pellet was resuspended in distilled water, treated in an ultrasonic generator (MSE, 100 W) for 30 s at full power and centrifuged at 500 *g* for 10 min. The supernatant was retained and freeze-dried before extraction.

C. psittaci, ovine enzootic abortion (OEA) strain A22, grown in egg yolk sacs, harvested in PBS, pH 7.3 and inactivated with formalin (0.2%, v/v) was a gift from Dr F. Shand, Wellcome Research Laboratories, Beckenham, UK). PBS contained per litre: 8 g NaCl; 0.2 g KCl; 1.15 g Na₂HPO₄; 0.2 g KH₂PO₄. A suspension of 50 infected egg yolks in 200 ml PBS was dispersed in an Atomix (MSE) used at full power for 5 min, and then centrifuged at 20000 *g* for 1 h at 4 °C. The supernatant, together with a layer of 'egg lipid' on its surface, was discarded and the pellet resuspended in 200 ml PBS. The dispersion and centrifugation were repeated three more times, and the final pellet was resuspended in 150 ml PBS and dispersed in the Atomix. Phospholipase C (Sigma, P-7633; 0.1 mg ml⁻¹) was added and the mixture incubated at 37 °C for 90 min, followed by the addition of protease (Sigma, P5130 0.5 mg ml⁻¹) and further incubation for 90 min at 37 °C. The preparation was then disrupted and centrifuged twice as before. The final pellet was dispersed in 50 ml PBS and freeze-dried.

The genus-specific antigen (LPS) was extracted from freeze-dried preparations using phenol/chloroform/petroleum ether (PCP) according to the method of Galanos *et al.* (1969) with the following modifications. Freeze-dried material was sonicated with 2 ml of the extraction mixture (5 min, full power, MSE ultrasonic generator, 14 mm diameter probe). The mixture was centrifuged at 5000 *g* for 15 min and the supernatant retained. Extraction of the pellet was repeated twice and the supernatants combined. Chloroform and petroleum ether were removed by rotary evaporation at 40 °C; distilled water (10 ml) was added to the remaining phenol and the mixture dialysed exhaustively against distilled water at room temperature. The extract from egg yolks contained insoluble 'lipid' material which formed a layer on the surface of the supernatant after centrifugation at 5000 *g* for 10 min. This layer was removed and the step repeated twice. Extracts from both egg yolk sac and McCoy cells were further treated with a chloroform/methanol mixture, to reduce contamination of the extract by nucleic acid and protein, as follows.

Three volumes of chloroform/methanol (2:1) were added to the aqueous extract with shaking. The chloroform phase (containing most of the antigen) was retained. An equal volume of distilled water was added and the chloroform removed by rotary evaporation at 40 °C. The antigen was solubilized by ultrasonication (2 min, full power). This extraction step was repeated.

Preparations from other organisms. Extracts from strains of *Acinetobacter calcoaceticus* (NCTC 7844, 10303 and 10305) were prepared by heating the bacteria at 100 °C for 4 h in 0.9% (w/v) NaCl (Brade & Brunner, 1979) as described previously (Thornley *et al.*, 1983).

Deep-rough LPS (Re-LPS) from *Salmonella minnesota* mR595 was a gift from Dr M. Nurminen, National Public Health Institute, Helsinki, Finland; rough LPS from *Escherichia coli* ATCC 12408 (Knox *et al.*, 1967) was a gift from Dr Elizabeth Work. Lipopolysaccharides were prepared from strains of *Pseudomonas morsprunorum* by phenol/water extraction (Westphal & Jann, 1965).

Preparation of the monoclonal antibodies. The methods used for the monoclonal CF2/47 were described in detail by Thornley *et al.* (1983). Briefly, Balb/c mice were given a series of injections with viable *C. trachomatis* strain SA₂(f) elementary bodies, grown in McCoy cells, followed by a series of injections with rifampicin, and a boost with chlamydiae inactivated by ⁶⁰Co irradiation. After an interval, a second boost with inactivated chlamydiae was followed 3 or 4 d later by fusion of spleen cells from one mouse with cells of the mouse myeloma cell line

NSO/U (Clark & Milstein, 1981), a gift from Dr C. Milstein. Mice for the CF2, CF4 and CF6 fusions were prepared in the same way, except that the intervals from the first injection to the fusion were 96, 46 and 181 d, respectively. The methods for fusion, selection and growth of the hybridoma cells were as described by Waldmann & Milstein (1982). Supernatant fluids were screened for the presence of antibody by the ELISA method and for IgG antibody by the protein A-ELISA method, in both cases using the DOC extract of genus-specific antigen from *C. trachomatis* strain SA₂(f) as antigen.

The antibody class was confirmed by SDS-PAGE following ¹⁴C incorporation (Clark & Milstein, 1981) and the sub-class by Ouchterlony analysis (Miles reagents). Ascitic fluids were prepared and IgG fractions separated on protein A-Sepharose as described previously (Thornley *et al.*, 1983).

ELISA. This was as described by Thornley *et al.* (1983), except that antigens in either distilled water or 0.5% (w/v) DOC in PBS were dried onto the wells of microtitre plates and fixed for 15 min at room temperature with 50% (v/v) ethanol instead of 20% (v/v) acetone. All washes and dilutions of antibodies were made with PBS containing 0.055% (w/v) Tween 20 (Koch-Light). Bound antibodies were detected with a peroxidase-conjugated anti-mouse immunoglobulin serum (Dako).

A 'protein A-ELISA' method was used to detect IgG antibodies (Crowther & Abu Elzein, 1980). Details were as described above, except that the test antibodies were diluted in PBS/Tween containing 0.5% (v/v) foetal calf serum (Sera-lab, Crawley Down, UK) to prevent non-specific binding of the conjugate. The detection reagent was peroxidase-conjugated protein A (Sigma).

Double immunodiffusion. This was done according to the method of Ouchterlony using 0.6% (w/v) agarose in PBS, placed in 3 ml portions on 5 × 5 cm glass plates. Wells 2.5 mm in diameter and 5 mm apart contained 10 µl of reagents. Antigens were diluted in distilled water containing 0.2% (w/v) SDS and placed in a central well. Ascitic fluid preparations (undiluted) of the monoclonal antibodies were added to the surrounding wells. After 18 h at 4 °C, plates were examined by dark ground illumination, washed and stained with Coomassie blue.

Chemical methods. Chlamydial LPS was oxidized with sodium periodate (0.05 M) in distilled water (0.5 ml) at room temperature for 2 h in the dark, and was terminated by the addition of 10 µl ethanediol. Modified LPS was diluted and used as antigen in ELISA.

Concentrations of chlamydial LPS were estimated using the carbocyanine dye spectral shift assay of Janda & Work (1971). Re-LPS from *S. minnesota* was used as the standard. KDO was detected using thiobarbituric acid (Osborne, 1963).

SDS-PAGE. Slab gel electrophoresis was done according to the method of Laemmli (1970) using a final acrylamide concentration in the separating gel of 12% (w/v) and gels of dimensions 16 × 14 × 0.1 cm. Electrophoresis (20 mA, constant current) was continued until the dye front had migrated 10 cm into the separating gel. LPS was visualized in the gels using either the periodate-Schiff stain or the silver stain of Tsai & Frasch (1982).

Immunoblotting (Western blotting). After separation on the SDS-polyacrylamide slab gels, LPS was electrophoretically transferred (blotted) from the gels onto nitrocellulose sheets according to the method of Towbin *et al.* (1979), in a Bio-Rad Trans-Blot electrophoretic transfer cell. The nitrocellulose sheet was placed nearest to the anode and electrophoresis was carried out at 40 V, 130 mA for 18 h. IgG preparations of monoclonal antibodies, purified from supernatants of tissue cultures, were used for antigen detection. The nitrocellulose sheet was incubated with shaking in 50 ml of 0.01 M-Tris/HCl, 0.154 M-NaCl (TBS) buffer, pH 7.4, containing bovine haemoglobin Type II (Sigma; 30 g l⁻¹) for 4 h at room temperature. The nitrocellulose was then divided into strips and these were incubated separately either with a mixture of all five monoclonal antibodies, each at a concentration of 1 µg ml⁻¹ in 10 ml TBS buffer with haemoglobin, or, to investigate the reaction of the antigen with single monoclonal antibodies, with 50 µg of a single monoclonal antibody in 10 ml TBS with haemoglobin. Incubation was for 18 h at 4 °C with shaking. Normal mouse IgG (50 µg in 10 ml) was used as a control.

Strips were washed in 10 ml TBS, followed by 10 ml TBS containing 5 µl Nonidet P40 detergent and finally by one wash of 10 ml TBS. Each wash was with shaking for 20 min. The strips were then incubated for 4 h at room temperature with a rabbit anti-mouse immunoglobulin (Sigma) diluted 1 in 300 in TBS/haemoglobin then washed as described above. Finally they were incubated for 18 h at 4 °C with sheep anti-rabbit immunoglobulin conjugated to horse radish peroxidase (a gift from Dr Peter Jackson, Department of Clinical Biochemistry, University of Cambridge, UK) diluted 1 in 50 in TBS/haemoglobin. The strips were washed as described above and developed with a solution (0.2 g l⁻¹) at 3,3'-diaminobenzidine tetrahydrochloride in TBS containing 200 µl hydrogen peroxide (100 vol.). The reaction was stopped by repeated washing with distilled water.

Reverse passive haemagglutination (RPH). Sheep red blood cells were treated as described by Scott *et al.* (1981), with either trypsin (Difco grade 1:250) or chymotrypsin from bovine pancreas (Sigma C-4129) at a final concentration of 0.5 mg ml⁻¹. IgG preparations of the monoclonal antibodies, or normal mouse IgG, at concentrations of 0.25 or 0.5 mg ml⁻¹ in 0.15 M-NaCl were linked to the enzyme-treated sheep red blood cells by chromic chloride, as described by Scott *et al.* (1981). Coupled cells prepared in this way were either used fresh (for up to 2 weeks after preparation) or were stabilized with glutaraldehyde (Cranage *et al.*, 1983).

For RPH tests, antigens were diluted in PBS, pH 7.3, containing 0.005% (w/v) Tween 20 (Koch-Light). Portions of 25 µl of each dilution were added to U-shaped wells of polystyrene microtitre plates (Sterilin). One drop (about 30 µl) of a 1% (v/v) suspension of fresh or glutaraldehyde-treated coupled red cells in PBS was added to each well and the plates were agitated for 30 s on a micro-mixer (Triyo-Bussan). The haemagglutination patterns were read after 90 min.

PCP extracts of chlamydial LPS were diluted and tested directly, but crude preparations of McCoy cell-grown *C. trachomatis* strains were pre-treated by heating at 100 °C for 5 min in PBS containing 0.1% (w/v) SDS and 20 mM-EDTA, pH 8.2, before testing.

RESULTS

Production of monoclonal antibodies

With the mouse inoculation schedule described, the monoclonal antibodies to the chlamydial group-specific antigen were predominantly IgM. However, there were indications that longer intervals between priming and fusion resulted in a higher proportion of IgG-producing clones. In the CF6 fusion, both types of ELISA were used at an early stage in the testing of cell lines. Selection was made of lines giving a positive protein A-ELISA and presumed to be IgG producers, and some which were negative with protein A but positive with the normal ELISA. The cell lines eventually isolated proved to be four IgG producers (three IgG_{2a} and one IgG₃) and four IgM producers, and all had been correctly indicated by the protein A-ELISA.

The antibodies studied in detail and described here were all IgG, which gave high readings in ELISA and included CF2/47, some properties of which have already been reported (Thornley *et al.*, 1983).

Properties of LPS extracts

The genus-specific antigen prepared by extraction with PCP allowed the application of tests which could not be used on a DOC extract.

PCP extracts from both *C. trachomatis* and *C. psittaci* produced spectral shifts in the carbocyanine dye assay for LPS (Janda & Work, 1971) giving a dye adsorption maximum of 460 nm, as did *Salmonella* Re-LPS, which was used as the standard. The assay was used for detection and estimation of small quantities of LPS. The amounts of LPS obtained from freeze-dried preparations of egg-grown *C. psittaci*, OEA strain and tissue-culture grown *C. trachomatis* SA₂(f) were 0.08% (w/w) and 1–2.4% (w/w), respectively.

The LPS contained KDO, as indicated by the production of an absorption spectrum in the thiobarbituric acid assay identical with that of authentic KDO. LPS from tissue culture grown *C. trachomatis* contained 2.2% (w/w) KDO.

ELISA

Extracts obtained with PCP from both *C. trachomatis* and *C. psittaci* were used as antigen at 5 and 1 µg ml⁻¹ in ELISA tests, and gave binding of monoclonal antibodies which was comparable to, or higher than, that shown by a DOC extract, previously used as an arbitrary standard for comparison of experiments but containing an unknown amount of LPS (Table 1). The PCP extract of *C. psittaci* from egg-grown material gave higher readings at the lower concentration, probably because of the dilution of lipid material, which prevented adsorption of the antigen to the ELISA plates. The monoclonal antibodies CF6/J12, CF6/L13 and CF6/L5 gave higher readings than CF2/47 or CF4/B2 with all chlamydial LPS preparations.

When the PCP extract of *C. trachomatis* had been treated with periodate, binding of all monoclonals was much reduced, whereas heating at 100 °C for 1 h gave no change in subsequent reactions. These facts, together with the binding of all monoclonals to extracts of both *C. trachomatis* and *C. psittaci* LPS, confirmed that their reaction was with the genus-specific antigen.

The sensitivity of the ELISA as a means of detecting chlamydial antigen was not fully investigated, but in one test, using as antibody a 1:10 dilution of a tissue culture supernatant of CF6/J12, a PCP extract of *C. trachomatis* LPS was detectable in a dilution containing 30 ng ml⁻¹ but not at 3 ng ml⁻¹.

Table 1. *ELISA assay of binding of monoclonal antibodies to chlamydial and other LPS preparations*

LPS preparations were in distilled water, apart from *C. trachomatis* DOC extract (used at 1:10 dilution in 0.5% w/v DOC). Monoclonal antibodies were tested in tissue culture supernatants, diluted 1:10 in PBS with 0.05% Tween 20, and compared with the tissue culture medium [Iscoe's Modified Dulbecco's medium (Gibco) + 5% v/v foetal calf serum (Sera-lab)] also diluted 1:10. Controls (not shown) for 0.5% DOC and distilled water dried on wells gave readings ≤ 0.02 for all monoclonals. A preparation of *Pseudomonas morsprunorum* LPS, tested at 5 and 1 $\mu\text{g ml}^{-1}$, gave negative results (not shown) with all monoclonals. Results of a typical experiment are shown; all observations were repeated three or more times.

Monoclonal antibody	Concn ($\mu\text{g ml}^{-1}$)	A_{492} with LPS from:									
		<i>C. trachomatis</i> (PCP extract)		<i>C. psittaci</i> (PCP extract)		<i>C. trachomatis</i> (DOC extract)	<i>Salmonella</i> Re-LPS		<i>E. coli</i> LPS		
		5	1	5	1		5	1	5	1	
		...									
CF2/47		1.18	0.63	0.82	1.18	0.86	0.19	0.14	0.01	0.03	
CF4/B2		1.09	0.48	0.74	1.14	0.48	0.08	0.09	0.03	0.02	
CF6/J12		1.78	1.06	1.14	1.95	1.72	0.02	0.02	0.02	0.00	
CF6/L13		1.98	1.48	1.25	1.88	1.72	0.02	0.02	0.01	0.04	
CF6/L5		> 2	1.17	1.50	1.98	1.78	0.02	0.02	0.02	0.06	
Tissue culture medium control		0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.02	0.01	

SDS-PAGE and immunoblotting

LPS preparations from *C. trachomatis*, *C. psittaci* and Re-LPS from *Salmonella* each revealed one fast-migrating band after electrophoresis through 12% (w/v) SDS-polyacrylamide and staining with periodate-Schiff. Each LPS had the same electrophoretic mobility, migrating just a little more slowly than the dye front.

Immunoblotting revealed a single band corresponding to that detected on the gel with periodate-Schiff. Blots of *C. trachomatis* and *C. psittaci* LPS were detected using a mixture of monoclonals as the primary antibody; both yielded identical bands. Blots of *C. trachomatis* LPS were detected with each monoclonal antibody individually, all five monoclonals giving identical bands, but with varying intensity. Blots so obtained were most intense with CF6/L13 and CF6/J12, and least intense with CF2/47 and CF4/B2.

Immunodiffusion

Ascitic fluids containing the monoclonal antibodies CF2/47 and CF4/B2 reacted with *C. trachomatis* and *C. psittaci* LPS in double immunodiffusion to form single precipitin lines. Precipitin lines fused to show a reaction of complete identity between the two monoclonals. Monoclonal antibodies CF6/J12 and CF6/L5 did not cause precipitation.

Reverse passive haemagglutination (RPH)

PBS containing Tween 20 at 0.005% (w/v) was routinely used to dilute the antigen for RPH tests with either fresh or glutaraldehyde-stabilized cells in suspension in PBS. This enabled cells which exhibited some degree of autoagglutination in PBS alone to be used.

A PCP extract of *C. psittaci* LPS was titrated against indicator cells linked to each of four different monoclonal antibodies and the resulting values for LPS concentration at the end-points (Table 2) showed a wide range in sensitivity. Of the two most sensitive indicators, trypsin-treated sheep red blood cells (SRBC) coupled to CF6/J12 could be used in the absence of Tween 20 in the diluent, whilst CF6/L13 coupled to chymotrypsin-treated SRBC exhibited autoagglutination in PBS alone, but resulted in very sensitive cells when diluted with

Table 2. *Sensitivities of indicator cells linked to IgG of various monoclonal antibodies in RPH of an extract of C. psittaci*

Results are given with the range of values in parentheses.

Monoclonal antibody coupled to SRBC*	Chlamydial LPS concn at end-point of titration (ng ml ⁻¹)	
	Fresh cells	Stabilized cells
CF2/47	6.2 (3.1-12.5)	200 (100-200)
CF4/B2	25 (12.5-50)	2000 †
CF6/J12	1.6 (0.8-1.6)	6.2 (3.1-9.2)
CF6/L13	0.4 (0.2-0.4)	3.1 (1.6-3.1)

* Chymotrypsin-treated SRBC were coupled to CF6/L13, and trypsin-treated SRBC to all other antibodies.

† One observation only. All other values were obtained with two or more preparations of antibody-coupled SRBC. Stabilized cells coupled to CF6/J12 and CF6/L13 were prepared in three different experiments, using SRBC from two different sheep, and tested after storage for periods from 1 to 98 d.

PBS/Tween. When fresh, these latter cells detected 0.4 ng LPS ml⁻¹ at the titration end-point which was equivalent to 10 pg antigen per well.

Glutaraldehyde stabilization of indicator cells coupled to CF6/J12 or CF6/L13 usually resulted in the reduction of sensitivity by a factor of 4 or 8, as seen in Table 2, giving stabilized cells which detected chlamydial LPS at concentrations of 3 or 6 ng ml⁻¹. Such cells retained unchanged activity for at least 3 months.

Crude preparations of *C. trachomatis* grown in McCoy cells gave very low titres when tested by RPH without pre-treatment. Heating at 100 °C for 5 min in PBS/SDS/EDTA, pH 8.2, yielded higher titres than those obtained after ultrasonic vibration, as used by Thornley *et al.* (1983). Results obtained with stabilized cells for the heated preparations are related to the numbers of inclusion-forming units (i.f.u.) initially present (Table 3). Considerable variation between two preparations of *C. trachomatis* strain 48/80H was found. In tests with indicator cells plus CF6/J12, antigen concentrations between 9 and 469 i.f.u. per well were detected at the end-points and between 2 and 59 i.f.u. per well in tests with cells plus CF6/L13.

Cross-reactions

The monoclonal antibodies CF2/47 and CF4/B2 cross-reacted in ELISA with *Salmonella* Re-LPS, although binding to Re-LPS was less than to chlamydial LPS when both antigens were tested at the same concentrations (Table 1). Antibodies CF6/J12, CF6/L13 and CF6/L5 did not react with Re-LPS in any ELISA tests.

Antibodies CF2/47 and CF4/B2 gave reactions in ELISA with both cell suspensions and extracts of *Acinetobacter calcoaceticus* NCTC 10303, and, additionally, CF2/47 reacted with NCTC 7844. No other antibodies listed in Table 1 gave any binding to these strains, and none reacted with *Acinetobacter* NCTC 10305.

Cross-reactions were also observed in immunodiffusion tests between monoclonal antibodies CF2/47 and CF4/B2 and *Salmonella* Re-LPS, where single precipitation lines were observed. No cross-reactions were observed in ELISA or immunodiffusion tests with *E. coli* LPS, and none were found in ELISA with *Pseudomonas morsprunorum* LPS.

In RPH tests, *Salmonella* Re-LPS at concentrations of 1 µg ml⁻¹ or more caused some non-specific agglutination of freshly prepared indicator cells; at 0.5 µg ml⁻¹ or less, all tests were negative. Glutaraldehyde-stabilized cells gave no non-specific reactions with Re-LPS at 5 µg ml⁻¹, and all tests, including those with indicator cells linked to CF2/47 and CF4/B2, were negative.

Extracts of *Acinetobacter* strains NCTC 7844, 10303 and 10305, tested neat and diluted gave negative results in RPH with the stabilized indicator cells listed in Table 2, and with freshly prepared cells linked to CF2/47 and CF4/B2.

Table 3. Sensitivity of stabilized indicator cells in RPH to detect *C. trachomatis* antigens present in crude McCoy cell preparations with known concentrations of inclusion-forming units (i.f.u)

The crude preparations of *C. trachomatis* were heated at 100 °C for 5 min in PBS/SDS/EDTA before titration. Two sets of stabilized indicator cells of both types, previously stored for 15 and 64 d, were used and gave identical results. Results of a typical experiment are shown.

<i>C. trachomatis</i> preparation		Indicator cells	RPH titre	Antigen concn at end-point of titration	
Strain	I.f.u. ml ⁻¹			I.f.u. ml ⁻¹	I.f.u. per well
48/80H	7.5 × 10 ⁶	CF6/J12	400	18750	469
		CF6/L13	3200	2343	59
48/80H	3.0 × 10 ⁵	CF6/J12	800	375	9
		CF6/L13	3200-6400	47-94	1.2-2.3
SA ₂ (f)	6.5 × 10 ⁵	CF6/J12	800	812	20
		CF6/L13	6400	102	2.5

Table 4. Summary of properties of five monoclonal antibodies

Monoclonal antibody	IgG sub-class	ELISA reactions*				Immunodiffusion			RPH		
		Chlamydial LPS (both spp.)	<i>Salmonella</i> Re-LPS	<i>Acinetobacter</i> NCTC 10303†	Immunoblot <i>C. trachomatis</i> LPS	Chlamydial LPS (both spp.)	<i>Salmonella</i> Re-LPS	<i>Salmonella</i> Chlamydial antigens	<i>Salmonella</i> Re-LPS (0.5 µg ml ⁻¹)	Chlamydial antigens	<i>Acinetobacter</i> extracts
CF2/47	IgG ₃	+	+	+	+	+	+	+	-	+	-
CF4/B2	IgG ₃	+	+	+	+	+	+	+	-	+	-
CF6/J12	IgG _{2a}	+	-	-	+	-	-	+	-	+	-
CF6/L5	IgG _{2a}	+	-	-	+	-	-	+	-	+	NT
CF6/L13	IgG _{2a}	+	-	-	+	NT	NT	+	NT	+	-

NT, Not tested.

* ELISA with the following antigens gave negative results with all monoclonal antibodies: *E. coli* LPS, *Pseudomonas morsprunorum* LPS, *Acinetobacter* NCTC 10305 cell suspension and extract.

† Results for cell suspension and extract of *Acinetobacter* NCTC 10303.

DISCUSSION

The genus-specific antigen of *Chlamydia* spp. has been extracted with PCP from both tissue-culture grown *C. trachomatis* as described by Nurminen *et al.* (1983), and from egg-grown *C. psittaci*. Chlamydial LPS could be estimated in small quantities in these extracts, by the carbocyanine dye spectral shift assay which has not previously been applied to chlamydial material. *Salmonella* Re-LPS, shown to resemble chlamydial LPS in several properties (Nurminen *et al.*, 1983), was thought to be an appropriate standard, and both caused shifts in the absorption maximum of the dye to 460 nm.

In general, properties of the extracted genus-specific antigen were as previously reported. It was soluble in chloroform/methanol and diethyl ether, was of limited solubility in water, and contained KDO or a closely related compound. The KDO represented 2.2% (w/w) of the LPS in an extract from tissue-culture grown *C. trachomatis*, a rather lower figure than those of approximately 5% (Nurminen *et al.*, 1983) or 8.8% (Caldwell & Hitchcock, 1984) reported previously. SDS-PAGE confirmed that the chlamydial LPS was the same or similar in molecular size to *Salmonella* Re-LPS as was reported by Nurminen *et al.* (1983).

The properties of the monoclonal antibodies are summarized in Table 4. All reacted with chlamydial LPS from both species, the antigenic determinant(s) being heat-resistant and periodate-sensitive, confirming that the reaction was with the genus-specific antigen. The binding could be demonstrated by immunoblotting, as previously shown for polyclonal sera by Nurminen *et al.* (1984) and by Caldwell & Hitchcock (1984) for their monoclonal antibody. The antibodies differed in their ability to give a precipitin line in immunodiffusion tests (Table 4). When linked to trypsin-treated sheep erythrocytes, all would detect chlamydial antigens in RPH tests.

The pattern of cross-reactions shown in Table 4 indicates the existence of at least two antigenic determinants on the chlamydial LPS. Monoclonal antibodies CF6/J12, CF6/L5 and CF6/L13 showed no cross-reactions with *Salmonella* Re-LPS, *E. coli* or *Pseudomonas* LPS, nor with three strains of *Acinetobacter*, indicating that at least one determinant of chlamydial LPS is not shared with these other organisms. The monoclonal anti-chlamydial LPS described by Caldwell & Hitchcock (1984) also failed to react with *Salmonella* Re-LPS, but its reaction with *Acinetobacter* was not described.

The cross-reaction between chlamydial LPS, Re-LPS and *Acinetobacter* NCTC 10303 shown by monoclonal antibodies CF2/47 and CF4/B2 indicates the existence of a determinant common to all three organisms. This has presumably been involved in the cross-reaction revealed by human and animal sera between chlamydiae and *Acinetobacter* (Brade & Brunner, 1979) and between chlamydial LPS and Re-LPS (Nurminen *et al.*, 1983; Caldwell & Hitchcock, 1984).

Nurminen *et al.* (1984) found that the cross-reacting component of an *Acinetobacter* strain was located in a part of its LPS. Since LPS was not measured in our *Acinetobacter* extracts, it is not known how much significance should be given to the negative results obtained with strain NCTC 10303.

The absence of cross-reactions in RPH tests of *Acinetobacter* extracts with cells coupled to CF2/47 and CF4/B2 confirmed previous tests with CF2/47 (Thornley *et al.*, 1983) but was surprising since both monoclonal antibodies reacted with *Acinetobacter* NCTC 10303 in ELISA tests.

RPH proved to be a sensitive method for the detection of chlamydial antigen, as in other systems, e. g. the measurement of IgE (Scott *et al.*, 1982). The modification of adding a very low concentration of Tween 20 to the diluent allowed the use of slightly clumped cells, and this apparently increased the sensitivity. The provision of stabilized cells means that this method might be useful in tests for chlamydial antigens in human and veterinary samples.

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REFERENCES

- BRADY, H. & BRUNNER, H. (1979). Serological cross-reactions between *Acinetobacter calcoaceticus* and chlamydiae. *Journal of Clinical Microbiology* **10**, 819–822.
- CALDWELL, H. D. & HITCHCOCK, P. J. (1984). Monoclonal antibody against a genus-specific antigen of *Chlamydia* species: location of the epitope on chlamydial lipopolysaccharide. *Infection and Immunity* **44**, 306–314.
- CLARK, M. R. & MILSTEIN, C. (1981). Expression of spleen cell immunoglobulin phenotype in hybrids with myeloma cell lines. *Somatic Cell Genetics* **7**, 657–666.
- CRANAGE, M. P., GURNER, B. W. & COOMBS, R. R. A. (1983). Glutaraldehyde stabilization of antibody-linked erythrocytes for use in reverse passive and related haemagglutination assays. *Journal of Immunological Methods* **64**, 7–16.
- CROWTHER, J. R. & ABU ELZEIN, E. M. E. (1980). Detection of antibodies against foot and mouth disease virus using purified *Staphylococcus A* protein conjugated with alkaline phosphatase. *Journal of Immunological Methods* **34**, 261–267.
- DHIR, S. P., KENNY, G. E. & GRAYSTON, J. T. (1971). Characterization of the group antigen of *Chlamydia trachomatis*. *Infection and Immunity* **4**, 725–730.
- DHIR, S. P., HAKOMORI, S., KENNY, G. E. & GRAYSTON, J. T. (1972). Immunochemical studies on chlamydial group antigen (presence of a 2-keto-3-deoxycarbohydrate as immunodominant group). *Journal of Immunology* **109**, 116–122.
- EVANS, R. T. & TAYLOR-ROBINSON, D. (1979). Comparison of various McCoy cell treatment procedures used for detection of *Chlamydia trachomatis*. *Journal of Clinical Microbiology* **10**, 198–201.
- GALANOS, C., LUDERITZ, O. & WESTPHAL, O. (1969). A new method for the extraction of R lipopolysaccharide. *European Journal of Biochemistry* **9**, 245–249.
- JANDA, J. & WORK, E. (1971). A colorimetric estimation of lipopolysaccharide. *FEBS Letters* **16**, 343–345.
- KNOX, K. W., CULLEN, J. & WORK, E. (1967). An extracellular lipopolysaccharide-phospholipid-protein complex produced by *Escherichia coli* grown under lysine-limiting conditions. *Biochemical Journal* **103**, 192.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- NURMINEN, M., LEINONEN, M., SAIKKU, P. & MÄKELÄ, P. H. (1983). The genus specific antigen of *Chlamydia*: resemblance to the lipopolysaccharide of enteric bacteria. *Science* **220**, 1279–1281.
- NURMINEN, M., WAHLSTRÖM, E., KLEEMOLA, M., LEINONEN, M., SAIKKU, P. & MÄKELÄ, P. (1984). Immunologically related ketodeoxyoctonate-containing structures in *Chlamydia trachomatis*, Re mutants of *Salmonella* species, and *Acinetobacter calcoaceticus* var. *anitratus*. *Infection and Immunity* **44**, 609–613.
- OSBORN, M. J. (1963). Studies on the Gram-negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctonate in the LPS of *Salmonella typhimurium*. *Proceedings of the National Academy of Sciences of the United States of America* **50**, 499–506.
- SCHACHTER, J. & DAWSON, C. R. (1978). *Human Chlamydial Infections*, 1st edn, pp. 210–212. Littleton, Mass., USA: PSG Publishing Co.
- SCOTT, M. L., THORNLEY, M. J., COOMBS, R. R. A. & BRADWELL, A. R. (1981). Measurement of human serum IgE and IgA by reverse passive antiglobulin haemagglutination. *International Archives of Allergy and Applied Immunology* **64**, 222–229.
- SCOTT, M. L., MERRET, T. G., ISHIZAKA, K., THORNLEY, M. J. & COOMBS, R. R. A. (1982). Comparison of reverse passive haemagglutination with double antibody radioimmunoassay for estimation of total human serum IgE. *Clinical and Experimental Immunology* **48**, 417–422.
- STEPHENS, R. S., TAM, M. R., KUO, C. C. & NOWINSKI, R. C. (1982). Monoclonal antibodies to *Chlamydia trachomatis*: antibody specificities and antigen characterization. *Journal of Immunology* **128**, 1083–1089.
- THORNLEY, M. J., LUSHER, M., SCOTT, M. L., COOMBS, R. R. A., EVANS, R. T., THOMAS, B. J. & TAYLOR-ROBINSON, D. (1983). Characterization of a monoclonal antibody to the group antigen of *Chlamydia* spp. and its use for antigen detection by reverse passive haemagglutination and indirect immunofluorescence. *FEMS Microbiology Letters* **17**, 45–49.
- TOWBIN, H., STAHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 4350–4354.
- TSAI, C. M. & FRASCH, C. E. (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Analytical Biochemistry* **119**, 115–119.
- WALDMANN, H. & MILSTEIN, C. (1982). Monoclonal antibodies. In *Clinical Aspects of Immunology*, 4th edn, pp. 476–503. Edited by P. J. Lachmann & D. K. Peters. Oxford: Blackwell.
- WESTPHAL, O. & JANN, K. (1965). Bacterial lipopolysaccharides extraction with phenol-water and further application of the procedure. *Methods in Carbohydrate Chemistry* **5**, 83–91.