Technical method

Direct immunoperoxidase method for demonstrating *Chlamydia psittaci* in tissue sections

J FINLAYSON, D BUXTON, IE ANDERSON, KM DONALD *Animal Diseases Research Association, Moredun Institute, Edinburgh*

The modified Ziehl-Neelsen¹ and the methylene blue² staining methods combined with darkground illumination are useful techniques for viewing chlamydial organisms in smears and cell monolayers, but they have the disadvantage of being non-specific. Immunological methods offer greater precision and both immunofluorescence and immunoperoxidase techniques have been described for use with chlamydia infected tissue culture cell monolayers.^{3,4} We describe a method for specifically demonstrating *Chlamydia psittaci* in histopathological sections and in monolayers of infected cells.

Material and methods

ANTISERUM TO *C PSITTACI*

Serum samples from a group of sheep experimentally infected with *C psittaci* (strain A22-EAE) were examined by the complement fixation test.⁵ Eight samples giving a strongly positive (4+) complement fixation reaction at a dilution of >1/512 were pooled. The IgG fraction was separated and conjugated with horseradish peroxidase.⁶ The resultant conjugate was designated SaCPO.

CONTROL MEASURES

To adsorb out *C psittaci* antibody 2 g of a placental cotyledon from a sheep experimentally infected with the A22-EAE strain of *C psittaci* and which contained the organism was homogenised in 3 ml of 0.3 M phosphate buffered saline (PBS), sonicated, and centrifuged. The supernatant from this preparation was used to make a 1/20 dilution of SaCPO and incubated for 1 h at room temperature before being centrifuged. This supernatant fluid was designated SaCPO/PL. A similar preparation was made from a non-infected cotyledon and designated control

SaCPO/PL. SaCPO was adsorbed similarly either with a homogenate of chick embryo membranes infected with A22-EAE strain of *C psittaci* (SaCPO/CE) or with non-infected chick embryo (control SaCPO/CE). A group specific monoclonal antibody to *C psittaci* and *C trachomatis* was used as positive control antiserum.

TISSUE SECTIONS

Placental tissues were collected at 120 days' gestation from a group of sheep experimentally infected with A22-EAE strain of *C psittaci* 30 days earlier. Thin slices of placenta were fixed with modified Bouin's solution (190 ml of saturated aqueous picric acid, 10 ml of 40% formaldehyde, and 5 ml of glacial acetic acid) for 4 h at room temperature, then dehydrated through graded alcohols, cleared in xylene, and embedded in paraffin wax. Sections 4 µm thick were cut and dried overnight at 37°C.

PREPARATION OF COVERSLEIPS

C psittaci (A22-EAE) and *C trachomatis* (LGV-L2) were cultured separately in BHK 21 cell monolayers grown on 16 mm diameter glass coverslips in universal bottles. The cells were treated for three days with 80 µg/ml of 5-iodo-2'-deoxyuridine before being inoculated with a suspension of either *C psittaci* (A22-EAE) or *C trachomatis* (LGV-L2) diluted in BHK maintenance medium (BHK 21 Glasgow medium supplemented with 2% adult bovine serum, 2.95 mg of tryptose phosphate broth per ml, 200 µg of streptomycin per ml, and 50 units of mycostatin per ml) such that the inoculum would infect 25-50% of the cells. The cultures were then centrifuged at 200 g for 30 min at 20°C and incubated at 37°C. After three days the coverslips were rinsed twice in PBS, fixed in 2% paraformaldehyde in PBS for 10 min, and rinsed again.

DIRECT METHOD FOR PARAFFIN SECTIONS

Sections were dewaxed in xylene, rinsed in 100% ethanol, and placed in 1% H₂O₂ in methanol for 30 min to block endogenous peroxidase activity. They were then washed in 0.01 M PBS containing 2% egg albumen (pH 7.5) for 30 min to reduce non-specific background staining. Slides were gently agitated when rinsing. After the slides had been removed from the rinsing fluid, the appropriate antiserum was layered on to the sections, which were placed in a humidity chamber and left for 90 min. Sections were then rinsed with PBS containing 2% egg albumen for 15 min followed by two 10 min changes of 0.05 M Tris buffer, pH 7.6. The substrate 3,3'-diaminobenzidine (4 mg in 10 ml of Tris buffer containing 0.01% H₂O₂) was then applied to the sections for 10 min and washed off in running tap

Fig. 1 Intracytoplasmic inclusions (arrows) stained with *Chlamydia psittaci* antiserum conjugated with horseradish peroxidase in epithelial cells lining a gravid ovine uterus. $\times 500$.

water. The slides were then placed in 5% cobalt chloride for 10 min to enhance the intensity of the reaction product and, after a further wash in water, they were counterstained with haematoxylin, dehydrated, cleared, and mounted in DPX. In order to test the specificity of SaCPO, cell monolayers on coverslips infected with A22-EAE and LGV-L2 groups of chlamydia were stained by the same immunoperoxidase method.

IMMUNO-ULTRASTRUCTURAL DEMONSTRATION OF CHLAMYDIA

Cells infected with *C psittaci* (A22-EAE) were detached from coverslips by agitation in the presence of glass beads and after two washes in PBS they were divided into two aliquots, fixed in paraformaldehyde for 10 min, and rinsed in PBS. SaCPO was added to one aliquot only and incubated for 90 min at room temperature. After rinsing with PBS both

Fig. 2 Cultured BHK cell containing a *Chlamydia psittaci* (A22-EAE) cytoplasmic inclusion stained with specific antiserum conjugated with horseradish peroxidase showing initial bodies (IB), intermediate forms (IF), and elementary bodies (EB). $\times 9000$.

Fig. 3 Direct immunoperoxidase method using horseradish peroxidase conjugated *Chlamydia psittaci* antiserum showing specific reaction product adherent to the outer membrane of chlamydial organisms within a cytoplasmic inclusion (arrow). $\times 37\,500$.

aliquots were treated with 3'3-diaminobenzidine for 10 min, washed in PBS, and fixed in 3% glutaraldehyde in 0.01 M phosphate buffer for 2 h. The cells were rinsed and centrifuged to form a pellet. Osmium tetroxide (1%) was layered on to each pellet and left for 1 h, after which each was rinsed with PBS, dehydrated, and embedded in Araldite.

Results and discussion

In the experimentally infected ovine placental tissues examined, chlamydial inclusions were found in uterine epithelial cells and in fetal trophoblast cells. When treated with SaCPO a dense reaction product made these inclusions prominent (Fig. 1). In addition, reaction product was found in the cytoplasm of adjacent inflammatory cells. Similar results were obtained with sections treated with control SaCPO/PL, control SaCPO/CE, and with the monoclonal antibody. On serial sections treated with either SaCPO/PL, SaCPO/CE, or 3'3-diaminobenzidine alone, reaction product was absent. Thus since staining can be prevented by reacting the conjugated antiserum with chlamydial antigen either from ovine placental tissue or chick embryo cultures before its application to the slide we conclude that SaCPO specifically stains *C psittaci* in these tissues. Subsequent experience showed that this direct immunoperoxidase method could be performed on formal fixed, paraffin embedded sections with results similar to those described using modified Bouin's fixation.

Examination of the coverslips infected with *C psittaci* or *C trachomatis* and subsequently stained by this immunoperoxidase method showed that the antiserum reacted equally well with both. Thus in common with the monoclonal antibody it appears to be detecting group specific antigen(s).

Ultrastructural examination of chlamydial inclusions showed reaction product adhering to the outer membrane of all three stages: initial bodies, intermediate forms, and elementary bodies (Figs. 2 and 3).

Thus the direct immunoperoxidase method described has the advantage that it is quick and simple to use, it can be performed on routinely fixed paraffin embedded sections, and it provides a permanent preparation which can be viewed with the light microscope.

We thank Mr J Redmond for preparation of the conjugate, Mr B Easter for the photographs, and Boots-Celltech Diagnostic Limited for kindly donating the monoclonal antibody.

References

- Stamp JT, McEwen AD, Watt JAA, Nisbett DI. Enzootic abortion in ewes. *Vet Rec* 1950; **62**:251-4.
- Johnson FWA, Chancerelle LYJ, Hobson D. An improved method for demonstrating the growth of *Chlamydiae* in tissue culture. *Med Lab Sci* 1978; **35**:67-74.
- Richmond SJ, Stirling P. Localisation of chlamydial group antigen in McCoy cell monolayers infected with *Chlamydia trachomatis* or *Chlamydia psittaci*. *Infect Immun* 1981; **34**:561-70.
- Chasey D, Davies P, Dawson M. Immunoperoxidase detection of *Chlamydia ovis* in experimentally-infected cell culture. *Br Vet J* 1981; **137**:634.
- Stamp JT, Watt AA, Cockburn RB. Enzootic abortion in ewes. *J Comp Pathol* 1950; **62**:93-101.
- Wilson B, Nakane PK. Recent developments in the peroxidase method of conjugating horseradish peroxidase (HRPO) to antibodies. In: Knapp W, Holubar K, Wick G, eds. *Immunofluorescence and related staining techniques*. Elsevier/North-Holland: Biomedical Press, 1978:215.

Requests for reprints to: Mrs J Finlayson, Animal Diseases Research Association, Moredun Institute, 408 Gilmerton Road, Edinburgh EH17 7JH, Scotland.