Papers

Chlamydia pneumoniae in vitro and in vivo: a critical evaluation of in situ detection methods

A Meijer, P J M Roholl, S K Gielis-Proper, Y F Meulenberg, J M Ossewaarde

Abstract

Aims—**There is a considerable discrepancy between data from the detection of *Chlamydia pneumoniae* in atherosclerotic lesions obtained by means of immunocytochemistry and data obtained using the polymerase chain reaction. This study evaluated methods for the in situ detection and assessment of the viability of *C pneumoniae* bacteria.**

Methods—***Chlamydia pneumoniae* membrane protein, heat shock protein 60, and lipopolysaccharide were detected by immunocytochemistry, and genomic DNA and 16S rRNA by in situ hybridisation in paraffin wax embedded sections of cultured HEp2 cells infected with *C pneumoniae* and of lungs from mice infected intranasally with *C pneumoniae*.**

Results—**Inclusions reactive for all three antigens, DNA, and 16S rRNA were seen in infected HEp2 cells, in all positive bronchus and alveolar epithelial cells, and in some of the positive infiltrate cells in the lungs of mice up to seven days after infection. In all alveolar macrophages and in the infiltrate cells positive for antigens only, the staining pattern was granularly dispersed throughout the cytoplasm up to seven days after infection. At 21 days after infection, only this granular staining pattern was seen for antigens in infiltrate cells and macrophages in the alveoli and bronchus associated lymphoid tissue. At this time point, DNA or 16S rRNA were detected sporadically, but always as inclusion-like staining.**

Conclusions—**Because antigens with an inclusion-like staining were detected only together with DNA and 16S rRNA, this type of staining pattern suggested the presence of viable bacteria. Thus, the granular staining pattern of antigens in the absence of staining for DNA and 16S is most likely caused by non-viable bacteria. In conclusion, these methods are suitable for the in situ detection of *C pneumoniae* and the assessment of its viability.**

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Keywords: *Chlamydia pneumoniae*; immunocytochemistry; in situ hybridisation; animal model

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Chlamydia pneumoniae, an important respiratory pathogen and the third recognised species of the Chlamydiaceae family, has been associated with atherosclerotic vascular diseases.[1] Initially, this association was observed in seroepidemiological studies.[2] Subsequently, *C pneumoniae* has been demonstrated in atherosclerotic lesions of all major arteries by several techniques.[3] However, discrepancies were noted between the results of culture, immunocytochemistry (ICC), electron microscopy, the polymerase chain reaction (PCR), and in situ hybridisation (ISH).[3–7] These discrepancies could be explained by the poor sensitivity of *C pneumoniae* culture methods, sample error, PCR inhibitors, or different avidities of the antibodies used in ICC. Although the detection of *C pneumoniae* DNA by PCR is important in the study of the role of *C pneumoniae* in the pathogenesis of atherosclerosis, this technique does not provide information on the location or viability of the microorganism in the diseased tissue. To comply with the molecular guidelines of Fredricks and Relman[8] for establishing microbial disease causation, confirmation of the presence of *C pneumoniae* DNA in vessel walls by PCR should be obtained by in situ techniques. So far, ISH for the detection of *C pneumoniae* DNA in vessel walls has been reported three times, showing variable results.[4 7 9] These studies found that one coronary artery specimen from only one of eight patients with specimens positive in PCR and/or ICC,[4] none of the abdominal aortic aneurysm specimens of 19 patients positive in ICC but negative in PCR,[7] and five of eight abdominal aortic aneurysm specimens positive in ICC[9] were positive in ISH for *C pneumoniae*. To date, no systematic evaluation of in situ techniques for the detection of *C pneumoniae* has been reported. Here, we report an evaluation of ICC for the in situ detection of various antigens of *C pneumoniae*, and of ISH for the in situ detection of DNA and 16S rRNA of *C pneumoniae* by using well defined specimens from in vitro and in vivo experiments.

Methods

CHLAMYDIA PNEUMONIAE STRAINS AND INOCULUM PREPARATION
Chlamydia pneumoniae strains TW-183 (Washington Research Foundation (WRF), Seattle,

Washington, USA) and AR-39 (WRF) were propagated in HEp2 cells (code CCL23; American Type Culture Collection, Manassas, Virginia, USA), as described previously.[10] Briefly, one day old monolayers in six well microtitre plates were inoculated with *C pneumoniae* by means of centrifugation (one hour, 25°C, 4800 ×*g*). The monolayers were incubated for 72 hours at 35°C and 5% CO_2 in Iscove's modified Dulbecco's medium (Gibco BRL, Life Technologies, Breda, The Netherlands) supplemented with antibiotics (100 μg/ml vancomycin, 100 μg/ml streptomycin, and 25 E/ml nystatin), cycloheximide (1 μg/ml), and 10% fetal calf serum (chlamydia culture medium). The cells were scraped off the microtitre plates and suspended in culture medium (Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum and antibiotics, 100 μg/ml vancomycin, 100 μg/ml streptomycin, and 25 E/ml nystatin). Then, the bacteria were released from the cells by sonication. Cellular debris was removed by centrifugation for five minutes at 500 ×*g*. The suspensions were aliquoted and stored at −80°C. One vial was thawed to determine the number of inclusion forming units (IFU) by titration.

IN VITRO EXPERIMENTS
One day old monolayers of HEp2 cells in six well microtitre plates were either infected at a multiplicity of infection (MOI) of 0.1 IFU with *C pneumoniae* strain TW-183 or mock infected with chlamydia culture medium as described above. Cells infected with *C pneumoniae* were harvested by trypsinisation at five, 13, 21, 29, 46, and 72 hours after infection, washed once with culture medium, and once with phosphate buffered saline (PBS), then fixed for 24 hours in 10% buffered formalin. Mock infected cells were harvested at 72 hours after infection only. The fixed cells were washed once with PBS, dehydrated, and embedded in paraffin wax.

IN VIVO EXPERIMENTS
All in vivo experiments were approved by the institutional animal care and animal experiments committee. Four to six week old female C57B6/JIco mice were randomised before infection. Each group was held in a separate incubation box and the animals were given access to food and water ad libitum. Infections were carried out under light ether anaesthesia to induce hyperventilation. One day before infection with *C pneumoniae* three control mice were mock infected intranasally with supernatant from a mock infected HEp2 cell culture. On the day of infection with *C pneumoniae* the control mice were sacrificed. Six mice were infected intranasally with 4.3×10^8 IFU and four mice with 1×10^6 IFU of *C pneumoniae* strain AR-39 in 50 μl culture medium. Of the six mice infected with 4.3×10^8 IFU of *C pneumoniae* three were sacrificed two days after infection, and three were sacrificed seven days after infection. The mice infected with 1×10^6 IFU of *C pneumoniae* were sacrificed 21 days after infection. Lungs were removed, intratracheally filled with 10% buffered forma-lin, fixed for 24 hours in 10% buffered formalin, and embedded in paraffin wax.

HISTOLOGY AND IMMUNOCYTOCHEMISTRY
One 4 μm paraffin wax section each of HEp2 cells infected with *C pneumoniae*, mock infected HEp2 cells, the lungs of mice infected with *C pneumoniae*, and the lungs of mock infected mice was stained with haematoxylin and eosin for histological examination.

Consecutive 4 μm paraffin wax sections were used to detect chlamydial antigens by ICC using an indirect immunoperoxidase method, as described previously.[7] One *C pneumoniae* species specific antimembrane protein monoclonal antibody RR-402[11] (WRF), three chlamydia genus specific antilipopolysaccharide (LPS) monoclonal antibodies (two (16.3B6 and 16.1D10) produced and characterised in our laboratory[12] and CF-2[13] (WRF)), and one chlamydia genus specific anti-heat shock protein 60 (hsp60) monoclonal antibody A57-B9[14] (Affinity Bioreagents, SanverTECH, Breda, The Netherlands) were used. Irrelevant primary monoclonal antibodies of the same isotype were used as negative control antibodies.

IN SITU HYBRIDISATION
The detection of *C pneumoniae* DNA by in situ DNA hybridisation (ISDH) on 4 μm paraffin wax sections adjacent to those stained by ICC was carried out with a digoxigenin (DIG) labelled *C pneumoniae* major outer membrane protein (MOMP) gene fragment as a probe and with a DIG labelled minute virus of mouse (MVM) genome fragment as a negative control probe, as described previously.[7]

The detection of *C pneumoniae* 16S rRNA by in situ RNA hybridisation (ISRH) in 4 μm paraffin wax sections adjacent to those stained with ISDH or ICC was carried out with an antisense 3' and 5' DIG labelled oligonucleotide probe specific for the order Chlamydiales 16S rRNA (5'-ATGTA(T/C)TACTAACCC TTCCGCCACTA-3'), including recently discovered chlamydia-like sequences.[15] A sense 3' and 5' DIG labelled oligonucleotide probe (5'-TAGTGGCGGAAGGGTTAGTA(G/A)TAC AT-3') was used to assess the specificity of the antisense probe for rRNA. A nonsense 3' and 5' DIG labelled oligonucleotide probe composed of the same nucleotides as the antisense probe but in a different sequence (5'-ATCCTAC GCTACTAAGTCTCTCATCA-3') was used as a negative control probe. After dewaxing and proteinase K treatment (2.5 μg/ml) of the sections as described for ISDH,[7] the slides were immersed for five minutes at room temperature in 0.2% glycine in PBS followed by five minutes in 2× saline sodium phosphate EDTA buffer (SSPE; 1× SSPE is 10 mmol/litre $NaPO_4$ containing 180 mmol/litre NaCl and 1 mmol/litre Na_2EDTA) at 95°C. Next, the slides were immersed in prewarmed hybridisation mixture (2× SSPE containing 100 μg/ml denatured herring sperm DNA and 5.1 pmol/ml probe) and incubated for 45 minutes at 55°C. After two washes with 2× SSPE for 10 minutes at

55°C the hybridised probes were detected as described for ISDH.[7]

DNAse (Boehringer Mannheim; 1 U/μl in 0.1 mmol/litre NaAc (pH 5.0) containing 5 mmol/litre $MgSO_4$ for one hour at 37°C) or RNAse A (Invitrogen BV, Leek, The Netherlands; 100 μg/ml in 2× SSC containing 10 mmol/litre $MgCl_2$ for one hour at 37°C) pretreatment of the sections was used to confirm the specificity of the hybridisation reactions.

Results

IN VITRO EXPERIMENTS

Mock infected cells did not react with the monoclonal antibodies, and cells infected with *C pneumoniae* did not react with the irrelevant monoclonal antibodies. No hybridisation signal was seen in mock infected cells with the MOMP probe, the antisense 16S rRNA probe, or the sense 16S rRNA probe. In addition, no hybridisation signal was observed at any point in time after infection in cells infected with *C pneumoniae* with the MVM probe, the nonsense probe, or the sense 16S rRNA probe.

By five hours after infection, *C pneumoniae* LPS was detected with monoclonal antibody 16.1D10. At 13 hours post infection *C pneumoniae* DNA was detected with the MOMP probe, and LPS was detected using the monoclonal antibodies 16.1D10 and 16.3B6. From 21 hours post infection onwards, *C pneumoniae* membrane protein (fig 1A–C); LPS with monoclonal antibodies 16.1D10, 16.3B6 (fig 1D–F), and CF-2; and DNA and 16S rRNA (fig 1J–L) were detected. The staining pattern for DNA was similar to that for 16S rRNA, as shown in fig 1. Hsp60 was detected at 29 hours after infection for the first time (fig 1G–I).

Up to 29 hours after infection the observed inclusions were very small (fig 1A, B, D, E, H, J, and K). Their size increased at least 10 times at 46 hours after infection. At this point in time, more positive cells became visible in agreement with an MOI of 0.1 (fig 1C, F, I, and L). At completion of the developmental cycle at 72 hours after infection, the size of the inclusions was again two to three times larger.

The immunoreactivity of the antimembrane protein and of the anti-hsp60 monoclonal antibodies was similar in size and pattern, and the staining was restricted to clearly recognisable inclusions (fig 1C and I). In addition to inclusions, all three anti-LPS monoclonal antibodies also stained the membranes of many cells without visible inclusions, and numerous vesicle-like structures (fig 1F). This type of staining was seen from 29 hours post infection onwards with monoclonal antibody 16.1D10, and from 46 hours post infection onwards with the other two anti-LPS monoclonal antibodies.

The staining pattern for *C pneumoniae* DNA and 16S rRNA was restricted to inclusions. At 46 hours after infection the inclusions stained with the MOMP DNA probe and the antisense 16S rRNA probe (fig 1L) were at least twice as large as those stained with the antimembrane protein (fig 1C) and anti-hsp60 (fig 1I) mono-

clonal antibodies, but similar to those stained with the anti-LPS monoclonal antibodies (fig 1F).

After DNAse pretreatment of sections of cells infected with *C pneumoniae*, no reaction was observed with the MOMP probe, whereas the inclusions were clearly stained with the antisense 16S rRNA probe. After RNAse A pretreatment of these sections, no staining was observed with the antisense 16S rRNA probe, whereas the inclusions were clearly stained with the MOMP probe.

IN VIVO EXPERIMENTS

Histologically, the lungs of mock infected mice were normal. Also, no reaction was seen with any of the monoclonal antibodies and probes. Sections of the lungs of mice infected with *C pneumoniae* did not stain with the irrelevant antibodies, the MVM probe, the nonsense probe, or the sense 16S rRNA probe.

At two days post infection, the lung pathology of the mice was characterised by focal interstitial infiltrates of predominantly polymorphonuclear cells and hyperplasia of bronchus epithelial cells. At seven days post infection, an interstitial pneumonia was observed with focal infiltrates of macrophages only. In addition, polymorphonuclear cells were seen in the alveoli and alveolar macrophages showed vacuolisation. At 21 days post infection, small multifocal periarterial and peribronchial infiltrates of macrophages and lymphocytes were observed. These infiltrates were also located focally in the subpleura. Sometimes, focal infiltrates—predominantly of lymphocytes—were seen in the alveoli. Polymorphonuclear cells were not noted at this time point. Bronchus epithelial cells showed sporadic vacuolisation and blebbing.

Table 1 summarises the cellular distribution pattern of *C pneumoniae* components in the lungs. At two days post infection, clearly recognisable inclusions positive with all the monoclonal antibodies and the probes were observed in bronchus epithelial cells (fig 2A–C) and in alveolar epithelial cells (fig 2A and E). In addition, blebs derived from bronchus epithelial cells stained positive for membrane protein and LPS. Many alveolar macrophages showed a vacuolised and granular immunostaining pattern (fig 2D and F), but only some of them showed a hybridisation reaction with the two probes. Viable bacteria were also present in infiltrate cells, as suggested by positive DNA and 16S rRNA (fig 2E) hybridisation reactions. These cells were also immunoreactive for LPS (fig 2F), and to a lesser extent for membrane protein, but not for hsp60. At seven days post infection, a lower number of epithelial cells was stained for the various *C pneumoniae* components. However, a higher number of macrophages in alveoli and in the infiltrates was positive for all three antigens, especially for LPS. In addition to blebs derived from bronchus epithelium, frequently the apical membrane of bronchus epithelial cells was stained for LPS. The cytoplasmic staining pattern of the different cell types was similar to that seen at two days post infection. In addition, macrophages

in bronchus associated lymphoid tissue (BALT) sporadically stained granularly for all three antigens. At 21 days post infection, no staining for any of the *C pneumoniae* antigens was observed in epithelial cells, and only a few alveolar macrophages (fig 2I) and infiltrate cells were positive for one or more of the *C pneumoniae* antigens. However, several macrophages in BALT stained strongly for membrane protein (fig 2G), LPS, and hsp60 (fig 2H). Staining was seen as dots scattered throughout the cytoplasm of positive cells. However, in all the sections examined, viable bacteria were observed only sporadically in epithelial and infiltrate cells, and in macrophages in BALT, as suggested by a positive

ISDH, although no 16S rRNA hybridisation reactivity was seen.

Discussion

We have shown that, in the early stages of the developmental cycle of *C pneumoniae* in cultured cells, the most sensitive techniques for the in situ detection of *C pneumoniae* were ICC for chlamydial LPS and ISDH. In the later stages of the developmental cycle the sensitivities of ICC for membrane protein, LPS, and hsp60; and ISDH and ISRH for 16S rRNA were similar. The detection of the antigens in sections of the lungs of mice infected with *C pneumoniae* showed considerable heterogeneity, depending on the type of cell that was
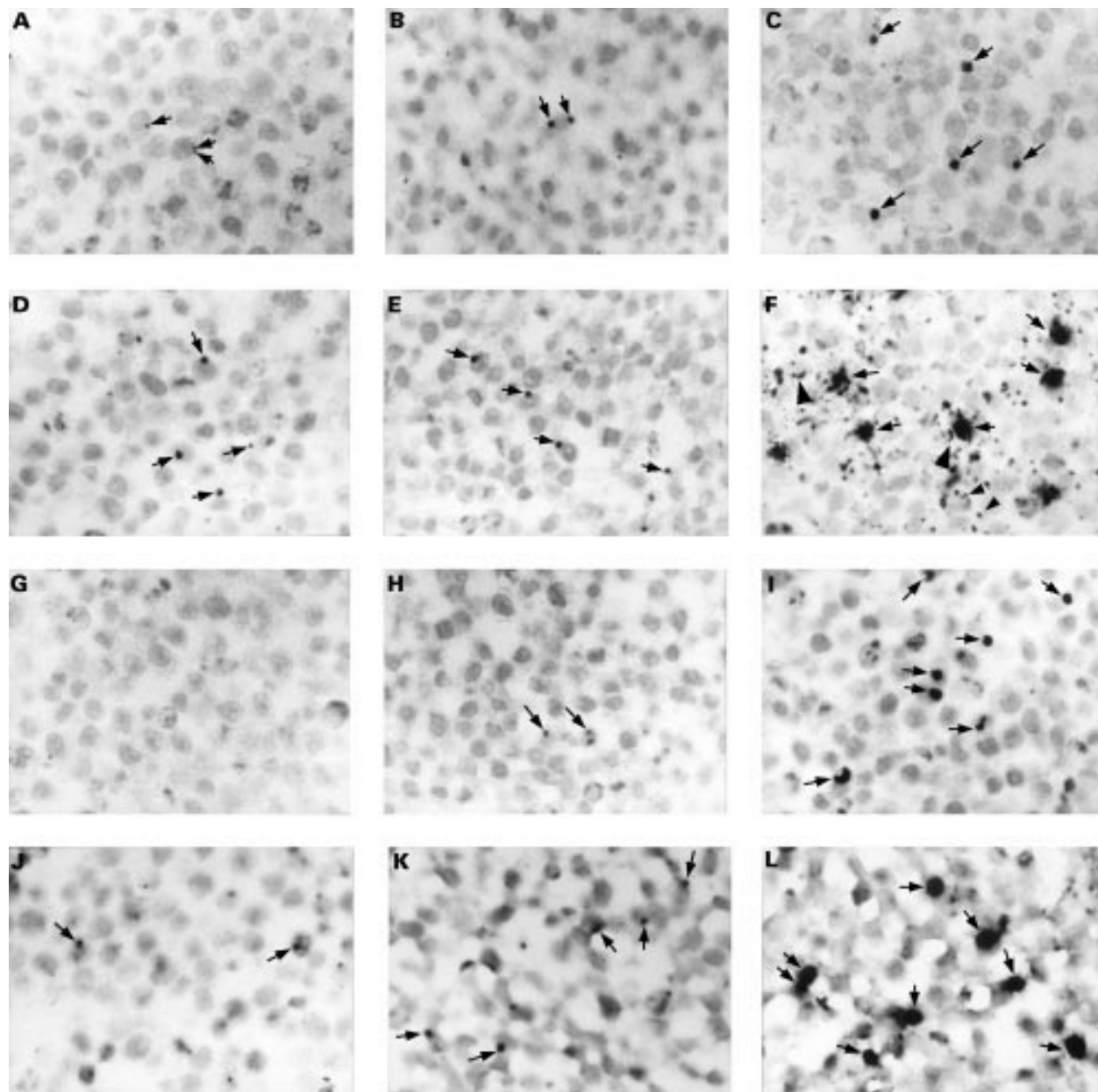

Figure 1   The detection of Chlamydia pneumoniae in paraffin wax embedded sections of formalin fixed HEp2 cells infected with a multiplicity of infection of 0.1 inclusion forming units of C pneumoniae. Cells were fixed at 21 (A, D, G, and J), 29 (B, E, H, and K), and 46 (C, F, I, and L) hours after infection. Sections were immunostained for C pneumoniae membrane protein (A–C), LPS (D–F; with monoclonal antibody 16.3B6), or hsp60 (G–I), or hybridised for 16S rRNA (J–L). In the early stages of the developmental cycle of C pneumoniae, up to 29 hours post infection, only very small inclusions were seen (A, B, D, E, H, J, and K; arrows). No immunostaining was observed for hsp60 at 21 hours after infection (G). Inclusions were up to 10 times larger at a later stage of the developmental cycle (C, F, I, and L; arrows). Membranes of non-infected cells (F; large arrowheads) and vesicle-like structures (F; small arrowheads) were also immunostained for LPS. Counterstaining, nuclear fast red. Original magnification, ×125 (A–L).

infected and on the point in time after infection. Overall, viable bacteria, as suggested by a positive 16S rRNA hybridisation reaction, were seen in the early stages of infection in bronchus and alveolar epithelial cells, in infiltrate cells, and in alveolar macrophages. In the later stages of infection antigens were seen mainly in alveolar macrophages and macrophages in BALT. Furthermore, in the early stages of infection, clearly defined inclusions could be demonstrated by immunostaining, whereas in the later stages the immunostaining was dispersed throughout the cytoplasm.

A possible explanation for the differences in the detection of *C pneumoniae* antigens in the early stages of the infection of cultured cells could be the amount of antigen present, because the synthesis and maturation of proteins and lipopolysaccharides are temporally regulated.[16] By the later stages of infection, when larger numbers of bacteria had produced mature proteins and lipopolysaccharides, all monoclonal antibodies performed equally well. The staining of the membranes of many cells without visible inclusions and numerous vesicle-like structures with the anti-LPS monoclonal antibodies is probably caused by the shedding of LPS. This observation is in agreement with previously reported results for the shedding of *Chlamydia trachomatis* LPS.[17] Our results of the detection of *C pneumoniae* hsp60 in cultured cells with monoclonal antibody A57-B9 are consistent with the results obtained for *C trachomatis* with the same antibody.[14]

We found that the sensitivity of ISRH was slightly less than that of ISDH, detecting *C pneumoniae* 21 and 13 hours after infection for the first time, respectively. This point in time corresponds with the beginning of the replication of the bacterium after the conversion of the infectious but metabolically inactive elementary body into the non-infectious but metabolically active reticulate body.[16] However, the number of copies of rRNA present in one bacterium is much higher than that of genomic DNA. Thus, it is probable that the difference in sensitivity is related to the number of DIG labels for each probe. The sensitivity of ISRH for the detection of *C pneumoniae* can be increased by using a larger probe or by the incorporation of more labels.[18–22] However, the 16S rRNA antisense probe was designed to detect all members of the order Chlamydiales, including the chlamydia-like organisms,[15] imposing restrictions on the length of this probe. In addition, an RNA directed probe was chosen because the detection of 16S rRNA provides information on the metabolic activity of the bacterium. Chlamydial elementary bodies contain mainly 4S rRNA, whereas the reticulate bodies consist predominantly of 21S, 16S, and 4S rRNA, in much higher quantities.[16]

The lung pathology of the mice infected with *C pneumoniae* was similar to that reported previously.[23 24] In both of these earlier studies, the lung pathology was also characterised by patchy interstitial pneumonitis, with predominantly polymorphonuclear cell infiltration in the early stages of infection and mononuclear cell infiltration in the later stages.

Clearly recognisable inclusions were seen only in the early stages of infection in bronchus and alveolar epithelial cells that were positive for *C pneumoniae* membrane protein, LPS, hsp60, DNA, and 16S rRNA, suggesting active replication. This finding is in agreement with the frequently observed inclusions in bronchus epithelial cells in the early stages of the infection by electron microscopy, as reported previously.[25] However, in the later stages of infection no clearly recognisable inclusions were seen in any type of cell positive for *C pneumoniae* antigens, and hybridisation was positive only very sporadically, suggesting only limited replication of *C pneumoniae*. In addition, the staining pattern for the antigens was granularly dispersed throughout the cytoplasm. These observations suggest the persistence and/or accumulation of antigens in cells, but not the persistence of viable bacteria. Because most of the positive cells at the later stages of infection were macrophages, the accumulation of the phagocytosed remnants of infected cells is likely. However, *C pneumoniae*

Table 1 *Cellular distribution pattern of Chlamydia pneumoniae components at different times after infection in the lungs of mice infected intranasally with C pneumoniae*

|			*C pneumoniae antigens*			
Type of cells	*Days post infection*	*C pneumoniae DNA and 16S rRNA*	*Membrane protein*	*Lipopoly-saccharide*	*Heat shock protein 60*	*Staining pattern*
Bronchus and alveolar epithelium	2	++	++	++	+	I
	7	ND	+	+	+	I
	21	±	–	–	–	NA
Alveolar macrophages	2	+	++	++	++	G
	7	ND	+	+++	++	G
	21	–	+	+	±	G
Infiltrate cells	2	++	±	++	–	I and G
	7	ND	++	++	+	I and G
	21	±	+/–	–	+	G
Macrophages in bronchus associated lymphoid tissue	2	–	–	–	–	NA
	7	ND	±	±	±	G
	21	±	+	+	+	G

The results are the combined results of three mice at two days, three mice at seven days, and four mice at 21 days after infection. All mice at the same time point after infection showed a similar staining pattern.
Chlamydia pneumoniae DNA and 16S rRNA were determined by in situ hybridisation; the staining pattern was always inclusion-like.
Chlamydia pneumoniae antigens were determined by immunocytochemistry.
Number of positive cells graded: –, none; ±, <5/section; +, 5–25/section; ++, 26–100/section; +++, >100/section; ND, not done.
Staining patern: G, granular; I, inclusion-like; NA, not applicable.
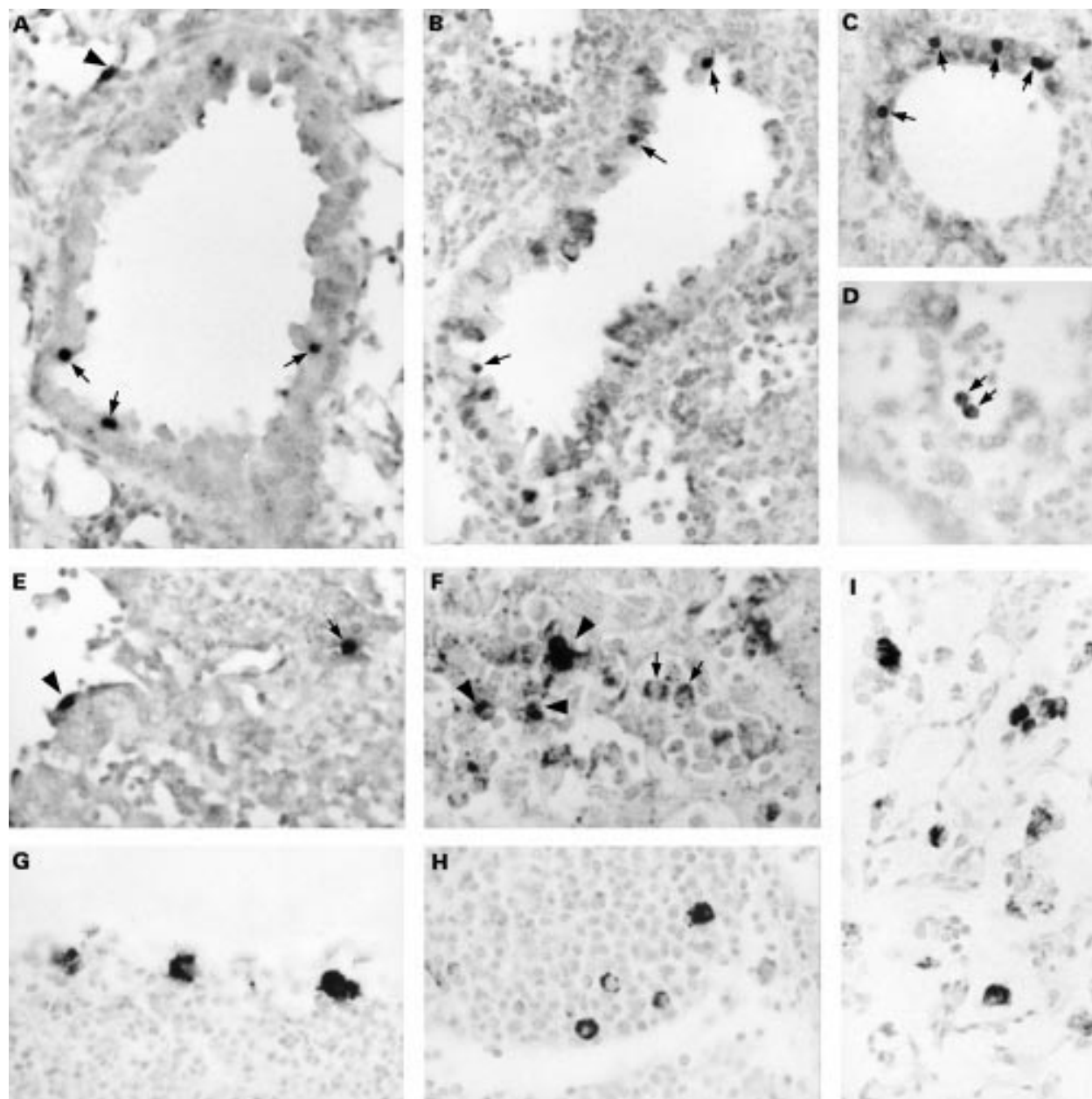
Figure 2 The detection of Chlamydia pneumoniae in paraffin wax embedded sections of formalin fixed lungs of mice intranasally infected with C pneumoniae. Results are from mice sacrificed at two (A–F) and 21 (G–I) days after infection. Sections were immunostained for membrane protein (G), lipopolysaccharide (B, F, and I; with monoclonal antibody 16.3B6), or hsp60 (C, D, and H), or hybridised for DNA (A) or 16S rRNA (E). In the early stages of infection, clearly recognisable inclusions were observed in bronchus epithelial cells (A–C; arrows) and in alveolar epithelial cells (A and E; arrowhead). Alveolar macrophages showed a vacuolised immunostaining pattern (D and F; arrows). Infiltrate cells were positive by immunostaining (F; arrowheads) and by in situ hybridisation (E; arrow). In the later stages of infection, macrophages in bronchus associated lymphoid tissue (G and H) and alveolar macrophages (I) showed a granular immunostaining pattern dispersed throughout their cytoplasm. Counterstaining, nuclear fast red. Original magnification, ×125 (A–I).

can also replicate in these cells, as indicated by a positive hybridisation reaction in some infiltrate cells and BALT macrophages, in agreement with the replication of *C pneumoniae* in cultured macrophages.[26] Furthermore, the appearance of antigen positive cells in BALT in the later stages of infection is consistent with the function of macrophages: to present antigens phagocytosed elsewhere in the lung to lymphocytes in BALT to induce an immune reaction.[27] The granular immunoreactivity pattern of the antigens highly resembled the staining pattern seen previously in human abdominal aortic aneurysm specimens that were

negative by ISDH also.[7] We conclude that this pattern of staining of antigens and the negative reactions in ISDH and ISRH suggest the presence of non-viable *C pneumoniae*.

In contrast to the presence of membrane protein and LPS in epithelial cells in the early stages of infection, hsp60 was present in very low amounts. Because the presence of DNA and 16S rRNA indicated replication, hsp60 values were probably close to the detection limit. Previously, in vitro studies with *C trachomatis* showed that hsp60 is expressed constitutively under normal culture conditions and the addition of interferon γ results in its upregula-

tion.[28] However, it is not clear if these results can be extrapolated to in vivo conditions. Hsp60 was present more abundantly in macrophages in the later stages of infection than in the early stages. This can most likely be explained by the accumulation of hsp60 as a result of the phagocytosis of remnants of infected cells, because these macrophages were negative in ISDH and ISRH. Because of the absence of evidence for the presence of viable bacteria in these macrophages, the upregulation of hsp60 expression as a result of local factors is less likely.

In conclusion, ICC for the detection of membrane protein, hsp60, and LPS; and ISDH and ISRH for 16S rRNA are suitable techniques to demonstrate the presence of *C pneumoniae* in formalin fixed tissues. In addition, infection and antigen persistence can be discriminated from each other by a combination of ICC, ISDH, and ISRH. Previously, we demonstrated that cells that stain positively for *C pneumoniae* antigens in abdominal aortic aneurysm specimens are negative in ISDH.[7] Our present results from the in vivo experiments support our hypothesis that *C pneumoniae* antigens persist in these cells, rather than viable bacteria.

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