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Identification by Western blots of virulence specific antigens of Chlamydia psittaci isolated from ewes

K Layachi, A Rodolakis *, D Buzoni-Gatel

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Summary — Specific antigens of virulent strains of Chlamydia psittaci isolated from ruminants were characterized by western blotting. The immunoblot analyses were performed with chlamydial elementary bodies from abortive (AB7 strain) or intestinal (iB1 strain) chlamydiae using mouse and rabbit immune sera raised against viable chlamydiae or proteic extracts. Eleven antigens were found to be AB7-specific, but only 4 (96, 90, 88 and 49–50 kDa) were recognized by both mouse and rabbit sera against viable AB7 strains. These could be candidates for specific diagnosis of caprine and ovine abortive chlamydiosis. No iB1-specific antigen recognized by mouse and rabbit anti-sera could be identified.

Chlamydia psittaci / antigen / virulence / ovine abortion

Résumé — Identification par immuno-transfert des antigènes de virulence de Chlamydia psittaci isolée de ruminants. Des antigènes spécifiques des souches virulentes de Chlamydia psittaci ont été caractérisés par immuno-transfert en comparant les réponses obtenues avec des corps élémentaires purifiés de la souche AB7 (souche abortive) et de la souche iB1 (souche intestinale) en présence de sérum polyvalent de souris ou de lapins immunisés avec des Chlamydia vivantes ou des extraits protéiques de ces mêmes souches. Onze antigènes spécifiques de la souche AB7 ont pu être identifiés, mais seulement 4 antigènes de masse moléculaire respective 96, 90, 88 et 49–50 kDa ont été reconnus également par les sérum polyvalent de souris et de lapin immunisés avec des Chlamydia vivantes. Ces antigènes pourraient donc être utilisés pour le diagnostic de la chlamydiose abortive ovine ou caprine. Aucun antigène spécifique de la souche iB1 reconnu par les anti-séums de lapin et de souris n'a pu être identifié par cette technique.

Chlamydia psittaci / antigène / virulence / avortement ovin

* Correspondence and reprints
INTRODUCTION

*Chlamydia psittaci*, a small Gram-negative obligate intracellular bacterium, is a major etiological agent in caprine and ovine abortions. The diagnosis of the disease is usually made by the examination of fixed and stained smears from placentae and by examination of sera for chlamydial antibodies by complement fixation test with genus lipopolysaccharide antigen. However, clinically normal sheep and goats harbor chlamydiae in their intestinal tract and excrete them in their feces (Omori *et al.*, 1957; Kawakami *et al.*, 1958; Dungworth and Cordy, 1962; Storz, 1963; Wilson and Dungworth, 1963). The epidemiological impact of intestinal chlamydial strains in abortive chlamydiosis is not known, but the close antigenic relationships between the 2 chlamydiae disturb the complement fixation test used to diagnose the abortive infection.

With a murine model we showed that most strains isolated from abortion were invasive in mice following subcutaneous inoculation, whereas strains isolated from the feces of healthy sheep were not (Rodolakis *et al.*, 1989). This difference in invasive ability is associated with dissimilar protein patterns in polyacrylamide gel electrophoresis analysis between invasive and non-invasive strains (Buzoni-Gatel *et al.*, 1989).

The aim of this study was to identify specific antigens of invasive chlamydial strains using an immunoblotting technique employing polyclonal sera in order to propose them for a specific diagnosis.

MATERIALS AND METHODS

Strains

*C. psittaci* AB7 strain was originally obtained from Dr P Faye (Faye *et al.*, 1972). Reisolated from an aborted lamb at the Laboratoire de Pathologie Infectieuse et Immunologie (INRA, Nouzilly, France), it was considered as the invasive reference strain. Strain iB1 was isolated from the feces of a healthy Berrichon ewe from a flock with no previous history of abortion (Buzoni-Gatel and Rodolakis, 1983) and was considered as the non-invasive reference strain. The other chlamydial strains examined are listed in table I. Stocks of chlamydia (2nd and 3rd egg

Table I. Chlamydial strains.

<table>
<thead>
<tr>
<th>Strains and associated disease</th>
<th>Invasiveness for mouse</th>
<th>Geographic origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovine abortion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB7</td>
<td>+++</td>
<td>France (Aveyron)</td>
</tr>
<tr>
<td>AB13</td>
<td>+</td>
<td>France (Bouches-du-Rhône)</td>
</tr>
<tr>
<td>S26/3</td>
<td>+</td>
<td>Scotlanda</td>
</tr>
<tr>
<td>A22</td>
<td>+</td>
<td>Scotlanda</td>
</tr>
<tr>
<td>Intestinal (from feces of healthy sheep)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iB1</td>
<td>–</td>
<td>France (Indre-et-Loire)</td>
</tr>
<tr>
<td>iB2</td>
<td>–</td>
<td>France (Indre-et-Loire)</td>
</tr>
<tr>
<td>iB5</td>
<td>–</td>
<td>France (Indre-et-Loire)</td>
</tr>
<tr>
<td>Mo 907</td>
<td>+</td>
<td>USAb</td>
</tr>
</tbody>
</table>

a From ID Aitken, Moredun Research Institute, Edinburgh, Scotland, UK. b From J Storz School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA, USA (Storz, 1963).
passage for AB7 and iBi, respectively) were propagated in the yolk sacs of developing chick embryos as previously described (Rodolakis, 1976), stored at -70 °C and titrated by plaque assay (Banks et al, 1970). For protein analysis the strains were propagated in McCoy cells as previously described (Rodolakis, 1983) and stored in phosphate glutamine sucrose buffer at -70 °C. Chlamydiae were purified on renografin as described by Caldwell et al (1981) using 150 mM Tris–potassium chloride buffer (pH 7.5). The purified chlamydial elementary bodies (EBs) preparation was stored at -70 °C.

**Preparation of proteic extract**

Purified chlamydial EBs were lysed with 4% sodium dodecyl sulphate (SDS) and 3 mM EDTA in phosphate-buffered saline as previously described (Buzoni-Gatel et al, 1989). Proteic concentration was determined by biocinchoninic acid (BCA) protein assay (Pierce, Rockford) (Smith et al, 1985).

**Animals**

Six-week-old 20 g, outbred female OF1 Swiss mice (IFFA Credo; L'Arbresle, France) and female New Zealand White rabbits were used for the production of polyclonal antibodies.

**Preparation of antisera**

Viable chlamydiae or proteic extracts were used to prepare antisera. For production of mouse antibodies, each mouse (20/group) received 3 injections intraperitoneally at 3-week intervals of either $10^5$ PFU viable yolk sac propagated chlamydial strains (AB7 or iB1), or 30 μg per mouse proteic extract emulsified in an equal volume of Freund's complete adjuvant (Difco Labs, Detroit, MI) at first inoculation and in Freund's incomplete adjuvant for the 2 following inoculations. The mice were bled 7 d after the third inoculation, blood was allowed to clot for 2 h at 37 °C and the serum samples were frozen in aliquots at -20 °C. Negative sera were obtained from a control group of 20 uninoculated mice. For production of rabbit antibodies, each rabbit was immunized 3 times at 3-wk intervals either intradermally with $10^7$ PFU of viable yolk sac propagated chlamydial strains AB7 or iB1, or subcutaneously with 500 μg of proteic extract emulsified in an equal volume of Freund's complete adjuvant at the first inoculation and in Freund's incomplete adjuvant for the 2 following inoculations. Control rabbit sera were taken from the same rabbits just before the first immunization.

Serum antibody titers were determined by conventional ELISA using AB7 or iB1 proteic extract (250 ng per well) as coating antigens (Fukushi et al, 1987).

**Separation of chlamydial antigens by SDS-PAGE**

Proteins of purified chlamydial EBs were separated by electrophoresis in 8 to 23% gradient acrylamide gel according to the method of Laemmli (1970). Before electrophoresis the samples were mixed with an equal volume of the solubilizing solution containing 2% SDS, 5% β-mercaptoethanol, 2.5% glycerol and 0.05% bromophenol blue, then boiled for 10 min. Approximately 120 μg of protein was added to the gel. Protein bands were revealed by silver staining (Oakley et al, 1980). Molecular masses were estimated by using low molecular mass markers (Pharmacia, Uppsala, Sweden): phosphorylase 94 kDa, bovine serum albumin 67 kDa, ovalbumin 43 kDa, carbonic anhydrase 30 kDa, soy bean trypsin inhibitor 21 kDa, and lactalbumin 14.4 kDa.

**Immunoblot analysis**

Western blots were performed according to the method of Towbin et al (1979). After transfer, the nitrocellulose membrane (NC) was stained with Ponceau S red to ensure that the chlamydial proteins were transferred successfully. Non-specific reactions on NC were blocked for 1 h at 37 °C with Tris-buffered saline (TBS), 1% skimmed milk and 0.01% Tween 20. The NC sheets were then washed with TBS 0.01% Tween 20 (3 times for 10 min each). These were incubated for 2 h at 37 °C with tested sera.
diluted in TBS and 0.03% skimmed milk and 0.01% Tween 20, washed with TBS and 0.01% Tween 20 (3 times for 10 min each) then incubated with biotinylated sheep antimouse or donkey anti-rabbit F(ab')2 fragments (Amersham Int plc, Amersham, UK) for 2 h at 37 °C. Following a further wash, the antigen-antibody complexes were visualized by the biotin-streptavidin-peroxidase system (Amersham Int plc Amersham, UK).

RESULTS

Serum antibody titers

Immunization of mice or rabbits with viable chlamydiae induced a higher level of antibodies against proteic extracts of AB7 than against those of iB1, even after immunization with viable iB1 (table II), whereas immunization of mice or rabbits with proteic extracts induced a similar level of antibodies against the 2 strains.

The serum dilutions used for immunoblotting are indicated in table II.

**Table II. Serum antibody titers.**

<table>
<thead>
<tr>
<th>Sera</th>
<th>ELISA titers</th>
<th>Dilution used for Western blots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antigens: proteic extract (pe)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AB7</td>
<td>iB1</td>
</tr>
<tr>
<td>From mouse against:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viable AB7 (MvAB7)</td>
<td>1/25 600</td>
<td>1/800</td>
</tr>
<tr>
<td>Viable iB1 (MviB1)</td>
<td>1/3 200</td>
<td>1/800</td>
</tr>
<tr>
<td>pe AB7 (MpeAB7)</td>
<td>1/5 000</td>
<td>1/1 000</td>
</tr>
<tr>
<td>pe iB1 (MpeiB1)</td>
<td>1/1 000</td>
<td>1/1 000</td>
</tr>
<tr>
<td>From rabbit against:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viable AB7 (RvAB7)</td>
<td>1/100 000</td>
<td>1/1 000</td>
</tr>
<tr>
<td>Viable iB1 (RviB1)</td>
<td>1/100 000</td>
<td>1/1 000</td>
</tr>
<tr>
<td>pe AB7 (RpeAB7)</td>
<td>1/100 000</td>
<td>1/100 000</td>
</tr>
<tr>
<td>pe iB1 (RApeiB1)</td>
<td>1/100 000</td>
<td>1/100 000</td>
</tr>
</tbody>
</table>

**Immunoblotting with antibodies against viable chlamydiae (fig 1)**

Mouse antibodies against viable AB7 (MvAB7) recognized 20 bands of AB7 (fig 1A) with apparent molecular masses (MMs) of 27 to 115 kDa. Fourteen bands (28–29, 33, 35, 37, 40, 49–50, 62, 68, 74, 78, 88, 96 and 110 kDa) appeared as specific for AB7 strain, since they reacted only in the homologous system antigens AB7 with MvAB7.

Mouse antibodies against viable iB1 (MviB1) cross-reacted with 7 of AB7 strain bands (fig 1A), 4 of which were also recognized by MvAB7. AB7 or iB1 major outer membrane protein (MOMP) did not react with MviB1. MviB1 recognized 9 bands on iB1 protein extracts (fig 1B), the MMs of which were 30 to 100 kDa; 2 of them (43 and 66 kDa) seemed to be iB1-specific antigens since they reacted only in the homologous system antigens iB1 with MviB1. Only one iB1 band (30 kDa) reacted weakly with MvAB7.

Rabbit antibodies against viable AB7 strain (RvAB7) recognized 24 of AB7 strain
bands (fig 1A) strain and reacted weakly with 8 bands of iB1 strain (fig 1B). As MvAB7 they recognized considerably fewer proteins on iB1 strain than on AB7 strain, but unlike MvAB7 they revealed about as many bands on iB1 strain as rabbit antibodies against viable iB1 strain (RviB1) did on iB1 or AB7 strains. Four bands of iB1 strain (30, 35, 62 and 66 kDa) reacted specifically with RviB1 (they were...
not revealed by rabbit antisera in the heterologous system) but all of them except the 66 kDa band were recognized by mouse antiserum MvAB7 on AB7 strain.

Eleven antigens of AB7 strain reacted specifically with RvAB7; 6 of them (37, 49–50, 54, 78, 88, 90 and 96 kDa) were also found to be specific with MvAB7, and 5 (54, 72, 76, 80 and 82 kDa) reacted only with RvAB7.

**Immunoblotting with antibodies against proteic extracts**

Immunization of mice with proteic extract from AB7 strain (MpeAB7) significantly increased the number of recognized bands from iB1 strain, since MpeAB7 reacted with 10 bands of iB1 strain and 22 of AB7 strain (fig 2). The one band of iB1 strain (30 kDa) recognized by MvAB7 reacted very weakly with MpeAB7 or MpeiB1. Five bands of iB1 strain reacted with MpeiB1 and did not react with MpeAB7, but none of them were specific for iB1 strain. MpeAB7 revealed 2 (60 and 64 kDa) out of 3 proteins of AB7 strain which reacted with MviB1 but not with MvAB7.

We found only 1 specific antigen on iB1 strain, a 66-kDa band which reacted only with MviB1 and 11 specific antigens of AB7 strain, but only one of them (32 kDa) was not previously recognized by MvAB7 or RvAB7.

Rabbit antibodies against proteic extract of AB7 (Rpe AB7) reacted with 30 proteins of AB7 strain (fig 3), 16 of which were recognized by RvAB7. RpeAB7 did not react with 78 to 94 kDa bands as did MpeAB7, but recognized bands of MMs < 30 kDa. RpeiB1 reacted with 17 proteins of iB1 strain, 4 of which were specific to iB1 strain but were not recognized by the other sera, MviB1, RviB1 or MpeiB1.

Fifteen AB7-specific bands were found by rabbit antibodies but only 11 appeared to be truly specific in all systems: MvAB7, RvAB7, MpeAB7, RpeAB7 (table III); 4 of them (MM 22 to 32 kDa) only reacted with sera against proteic extracts. Only 4 (96, 90, 88 and 49–50 kDa) were recognized by both mouse and rabbit sera against viable AB7 strains and could be antigens for specific diagnosis of caprine and ovine chlamydiosis. The one protein (66 kDa) found iB1-specific by MviB1 reacted with RpeAB7.

In order to determine whether the specific antigens revealed on AB7 could be used for diagnosis of abortive chlamydiosis we looked for their evidence in other invasive abortive strains (AB13, S26/3, A22), invasive intestinal strain Mo 907, or non-invasive intestinal strains (iB2, iB5). These strains, used as antigens in immunoblotting (figure 4), confirmed the absence of the specific antigens on non-invasive strains and their presence on invasive strains including the invasive intestinal strains Mo 907.

**DISCUSSION**

AB7 specific antigens were found by immunoblotting with mouse and rabbit sera. As we wanted antigens for diagnosis of chlamydial abortions in ewes and goats, only antigens which reacted with both sera against viable AB7 prepared in mice and rabbits was kept, since the others could be specific to the host species and could be not recognized by ovine or caprine sera. Ovine or caprine sera were not used here because of the antibodies against intestinal chlamydia that almost all sheep and goats have. The one AB7-specific antigen which reacted with all the anti AB7 sera was the 49–50 protein, so this could be the potential antigen for diagnosis of abortive
Chlamydiosis. The other specific antigens recognized by both MvAB7 and RvAB7 but not by Mpe AB7 or Rpe AB7 were 88, 90 and 96 kDa which were previously identified as "virulence" proteins by SDS–PAGE (Buzoni-Gatell et al, 1989) whereas the 76–80 doublet specific of non-invasive strains in SDS–PAGE was not antigenic. No iB1-specific antigen fitting the previous criterion could be found.

Except for the identification of specific antigens, the most notable features of this study were the weak antigenic reactivity of proteic extract from iB1 strain and non-invasive strains, and the small number of common antigens shared among strains.

Fig 2. Immunoblot pattern of mouse sera against proteic extracts of C psittaci strains AB7 (1), or iB1 (2). A = antigen strain AB7, B = antigen strain iB1, C = control serum.
AB7 and iB1 compared to the great similarity of the protein patterns of the 2 strains when analyzed by SDS-PAGE (Buzoni-Gatel et al, 1989). The difference observed between the antibody responses to inoculation with viable strains AB7 or iB1 may reflect: 1), the difference in virulence of the strains. We have previously shown that iB1, as all non-invasive strains, is quickly eliminated in mice contrary to invasive strains such as AB7 (Rodolakis et al, 1989); 2), the difference of presentation of the proteins following antigen processing by an antigen-presenting cell; 3), a difference in sensitivity of the proteins to denaturation by the process used to extract proteins and analyze them by SDS-PAGE.

In order to try to answer these different hypotheses we studied the immunogenicity of proteic extracts of strains AB7 or iB1 in mice and rabbits. This study indicated that some iB1 antigens were denatured by ex-
traction or electrophoresis while those of AB7 were not, as for example the 94 kDa protein which did not react on iB1 whatever the serum, but was revealed on AB7 by MviB1 and RviB1. However the responses of mice and rabbits to immunization with proteic extracts showed that the denaturation of iB1 proteins could not by itself be responsible for those results, since the antibody titers of the anti-proteic extract sera were almost the same whatever the chlamydial strain and the host species. Whereas, with viable chlamydiae the anti-AB7 responses were 8 to 10 times higher than those against iB1 as the differences in multiplication of the 2 strains reflect. These responses clearly indicated that the antigen processing by antigen-presenting cells was not the same according to the host species and/or chlamydial strain. The case of the 90 kDa AB7-specific protein illustrated the

<table>
<thead>
<tr>
<th>Molecular weight (kDa)</th>
<th>MvAB7</th>
<th>RvAB7</th>
<th>MpeAB7</th>
<th>RpeAB7</th>
</tr>
</thead>
<tbody>
<tr>
<td>110</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>96</td>
<td>+</td>
<td>+</td>
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<tr>
<td>90</td>
<td>+</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>88</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>80</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>76</td>
<td>-</td>
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<tr>
<td>49–50</td>
<td>+</td>
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<td>24</td>
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<td>+</td>
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<tr>
<td>22</td>
<td>-</td>
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<td>+</td>
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</table>

MvAB7 = mouse antibodies against viable AB7, RvAB7 = rabbit antibodies against viable AB7, MpeAB7 = mouse antibodies against AB7 proteic extracts, RpeAB7 = rabbit antibodies against AB7 proteic extracts.

**Fig 4.** Immunoblot pattern of rabbit sera against viable *C psittaci* strain AB7 (1) or iB1 (2). A = antigen AB7, B = antigen S26/3, C = antigen Mo907, D = antigen A22, E = antigen iB2, F = antigen iB5, G = antigen AB13.
problem of processing: the 90 kDa protein was not revealed by MpeAB7, but was obviously immunogenic since it was revealed by RpeAB7. When we compared the homologous response after immunization with viable chlamydiae or proteic extracts of AB7 there was > 50% homology in mouse or rabbit, whereas with iB1 there was < 33%.

Our results were in accordance with those previously published on immunoblotting of chlamydial proteic extracts from ovine (Mondesire et al., 1989; Baghian et al., 1990; Cevenini et al., 1991) or bovine (Fukushi and Hirai, 1988) abortive strains: the 58-60 kDa antigens common to AB7 and iB1 could correspond to the 57-62 doublet described by Mondesire et al. (1989) after Sarkosyl extraction, and the 89 kDa bands described by Cevenini et al. (1991) was likely to be the same as the 90 kDa bands we had previously described (Buzoni-Gatel et al., 1989). There was no previously published antigenic characterization of intestinal strains of C. psittaci by immunoblotting; nevertheless, we noticed that the immunoblots observed with iB1 were more similar to those obtained with strains of serotype 2 (Mondesire et al., 1989; Baghian et al., 1990), than to those of abortive strains of C. psittaci. In C. trachomatis, MOMP was readily extracted from intact EBs by SDS (Caldwell et al., 1981), but in C. psittaci (Fukushi and Hirai, 1988; Mondesire et al., 1989; Baghian et al., 1990) or C. pneumoniae (Campbell et al., 1990), Sarkosyl-insoluble fraction was the best way to obtain MOMP. For this reason we only obtained a weak or no antibody response against MOMP, but we were not looking for these antibodies, since MOMP, which harbors genus, species and type epitopes, was not an ideal candidate for a specific diagnosis antigen.

Thus this study showed that it must be possible to find antigens which are specific to virulent chlamydiae; but the very small number of bands revealed on iB1, whatever the polyclonal serum (less than half the number on AB7), indicates that the 2 kinds of strains are more different than SDS-PAGE leads one to suppose: some proteins which seem identical when analyzed by SDS-PAGE must be different. Results from further research on characterization using more discriminating methods such as 2-dimensional gel electrophoresis will clarify the situation, since in 1-dimensional gel electrophoresis more than one protein can give the same band.

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