Evaluation of protection against *Chlamydomphila abortus* challenge after DNA immunization with the major outer-membrane protein-encoding gene in pregnant and non-pregnant mice

Céline Héchard,[†] Olivier Grépinet and Annie Rodolakis

Unité de Pathologie Infectieuse et Immunologie, INRA – Centre de Tours, 37380 Nouzilly, France

Correspondence

Olivier Grépinet
grepinet@tours.inra.fr

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The protective effect of DNA vaccination with the gene encoding the major outer-membrane protein (MOMP) of *Chlamydomphila abortus* has been studied in non-pregnant and pregnant mouse models after chlamydial challenge. OF1 outbred mice were vaccinated intramuscularly three times every 3 weeks, mated and challenged with *C. abortus* 2 weeks after the last injection of DNA. In non-pregnant mice, the MOMP DNA vaccine elicited a specific humoral response with predominantly IgG2a antibodies, suggesting a Th1-type immune response. The induced antibodies showed no *in vitro* neutralizing effect on *C. abortus* infectivity. Moreover, immunization with the *momp* gene showed no reduction in the mean splenic bacterial counts of non-pregnant or pregnant mice or in the mean placental bacterial counts of pregnant mice after the *C. abortus* challenge. Nevertheless, the MOMP DNA immunization induced a non-specific and partial protection in fetuses against challenge.

INTRODUCTION

One of the most common causes of considerable loss in breeding is the abortion of goats and sheep induced by the intracellular obligate bacterium *Chlamydomphila abortus* (*Chlamydia psittaci* serotype 1) (Papp & Shewen, 1996). Furthermore, these bacteria present a zoonotic risk to pregnant women, since several cases of human chlamydial abortion have been reported (Buxton, 1986). A live attenuated vaccine is currently used in small ruminants (Rodolakis, 1983). This vaccine is effective and safe but does not allow the detection of infected animals in vaccinated flocks.

The predominant component and immunodominant antigen of the *Chlamydiaceae* surface membrane, the 40 kDa major outer-membrane protein (MOMP), presents four surface-exposed variable domains (VDI–VDIV) flanked by five conserved regions and exhibits an oligomeric form, probably trimeric, that functions as a porin (De Sa, 1996). In a murine model of *C. abortus* infection, protective immunity can be conferred by antibodies (Buzoni-Gatel *et al.*, 1990) and, more particularly, by the passive transfer of specific antibodies to the 110 kDa oligomeric MOMP (De Sa *et al.*, 1995), so MOMP has been considered as the most likely vaccine candidate against *C. abortus* infections. Native MOMP (Pal *et al.*, 1997; Tan *et al.*, 1990), recombinant

MOMP (Tuffrey *et al.*, 1992), synthetic MOMP peptides (Su *et al.*, 1995) and live recombinant vectors (Murdin *et al.*, 1995) expressing *momp* genes from members of the *Chlamydiaceae* have been evaluated in different animal models. Limited immunity and protection were generated in animals when the vaccinal approaches attempted to preserve the conformational structure of MOMP. Nevertheless, most trials were unsuccessful. A recent study showed that protective immunity against a *Chlamydia trachomatis* genital challenge was induced with a vaccine based on the MOMP associated with lipophilic immune response-stimulating complexes (Igiertseme & Murdin, 2000).

DNA vaccination represents an exciting means to induce specific immunity to a single antigen and, consequently, fits in the marker vaccine family (van Oirschot *et al.*, 1996). Indeed, the direct injection of a DNA plasmid encoding an immunogenic protein into animals has resulted in both humoral and cell-mediated specific immune responses (Donnelly *et al.*, 1997). Since DNA vaccination preferentially elicits a Th1-type immune response, it appears to be particularly appropriate for preventing intracellular bacterial infection (Strugnell *et al.*, 1997). This novel strategy of vaccination has been assessed in different animal models and seems suitable for veterinary medicine (Babiuk *et al.*, 2000).

DNA vaccination with the *momp* gene has already been tested in *C. trachomatis* (Pal *et al.*, 1999; Zhang *et al.*, 1997), *Chlamydomphila pneumoniae* (Penttilä *et al.*, 2000) and

[†]Present address: Laboratoire d'Immuno-dermatologie, Centre Médical Universitaire, 1 rue Michel Servet, CH-1211 Genève 4, Switzerland.

Abbreviation: MOMP, major outer-membrane protein.

Chlamydomonas psittaci (Vanrompay *et al.*, 1999). In contrast to that observed in the *C. psittaci* model, no or modest protection was observed in the *C. trachomatis* and *C. pneumoniae* models. A single attempt at DNA vaccination against *C. abortus* in an abortion model is described in the literature (Héchard *et al.*, 2002). In this model, only a non-specific and partial protection, probably due to the CpG motifs of bacterial DNA, was observed in pregnant mice immunized with a plasmid encoding the heat-shock protein DnaK.

The purpose of the present study was to evaluate the protective effect of intramuscular injection of the *C. abortus* AB7 *momp* gene in pregnant and non-pregnant mouse models. Protection was evaluated by chlamydial clearance in spleens, placentas and fetuses and the humoral response was analysed by measuring isotype-specific anti-MOMP antibodies.

METHODS

C. abortus strains. The virulent *C. abortus* strain AB7 isolated from an ovine abortion (Faye *et al.*, 1972) and the vaccinal strain 1B obtained by selection of a temperature-sensitive mutant after nitrosoguanidine mutagenesis of *C. abortus* AB7 (Rodolakis, 1983) were used. Bacteria were propagated in yolk sacs of embryonated specific-pathogen-free chicken eggs inoculated at day 7, purified as described previously (Caldwell *et al.*, 1981) and stored at -80°C .

Cloning of the *momp* gene. To produce the vaccinal vector, the *momp* gene was amplified by PCR and cloned into the vector pcDNA3.1 (Invitrogen) carrying the human cytomegalovirus immediate early promoter and the bovine growth hormone polyadenylation signal. The PCR was performed using chlamydial genomic DNA (80 ng) as template with dNTPs (200 μM each), specific primers (1 μM each) and 1 U *Pfu* DNA polymerase (Promega) in a Perkin Elmer 9600 thermocycler with the following program: 94°C for 5 min, 1 cycle; 30 cycles of 50°C for 45 s, 72°C for 2 min and 94°C for 45 s and 1 cycle of 50°C for 45 s and 72°C for 10 min. Specific primers were determined according to the DNA sequence of *C. abortus* AB7 containing an *EcoRI* site (underlined) and Kozak sequence (bold) in the forward primer (MOMP-FW; 5'-CTC**GAATTC**ACCATGAAAACTCTTGAATCG-3') and an *XhoI* site (underlined) in the reverse primer (MOMP-RV; 5'-CTCCTCGAGTTAGAATCTGAATTGAGCATTC-3'). The resulting fragment was inserted into the vector pcDNA3.1 after linearization by *EcoRI/XhoI* double digestion to generate pcDNA3.1::MOMP. The plasmid pcDNA3.1::MOMP and the pcDNA3.1 control plasmid were purified after an overnight Luria-Bertani culture of recombinant *Escherichia coli* DH5 α using the EndoFree Plasmid Mega kit (Qiagen). Plasmid DNA was dissolved at 1 $\mu\text{g } \mu\text{l}^{-1}$ in endotoxin-free PBS (Sigma). The sequence of the vaccinal vector pcDNA3.1::MOMP was verified by DNA sequencing.

Immunization and challenge. All the studies were carried out with 6-week-old female outbred OF1 Swiss mice (IFFA Credo). Prior to DNA immunization, each mouse was injected with cardiotoxin (Latoxan) into the tibialis anterior muscles of both hind legs to enhance the uptake of plasmid DNA (Davis *et al.*, 1993). Five days later, mice were anaesthetized by an intraperitoneal injection of ketamine and xylazine (respectively 80 and 8 mg kg^{-1} body weight) and immunized with pcDNA3.1::MOMP plasmid by intramuscular injections (50 μg in each tibialis anterior). Mice were boosted in the same way at days 21 and 42. The negative-control mice were immunized intramuscularly with endotoxin-free PBS (virulence control) or pcDNA3.1 plasmid. Positive-

control mice were immunized with one subcutaneous injection of 4×10^4 p.f.u. of the live attenuated 1B vaccine at day 1. Groups of 10 and 20 mice were similarly immunized for evaluation of the protective effect of the DNA vaccine and respectively constituted the non-pregnant and pregnant groups. The five groups of pregnant mice were mated at day 44. Non-pregnant and pregnant mice were challenged at day 58 by an intraperitoneal injection of 4×10^4 p.f.u. *C. abortus* AB7. One group of pregnant mice neither immunized nor challenged was kept as a control for the pregnancy (gestation control).

Antibody response. Blood samples were collected from the retro-orbital sinus of non-pregnant mice for the detection of anti-MOMP-specific antibodies before each DNA injection (days 0, 19, 41), just before the challenge infection (day 56) and at day 63. Blood samples were stored overnight at room temperature and then centrifuged (600 g, 15 min, 4°C) and sera were collected and stored at -20°C . The specificity of anti-MOMP antibodies was checked by ELISA as described previously (Héchard *et al.*, 2002). The recombinant MOMP used as antigen was kindly provided by Dr R. Ashley (University of Edinburgh, UK) (Wyllie *et al.*, 1999). Recombinant MOMP (100 μl), denatured by heating (100°C for 10 min) and diluted in Mg^{2+} -free, Ca^{2+} -free PBS (pH 7.4) at a concentration of 1 $\mu\text{g ml}^{-1}$, was used for coating microtitre plates (Nunc-Immuno Plate Maxisorp Surface) for 16 h at 4°C . Sera used for ELISA were serially diluted in Mg^{2+} -free, Ca^{2+} -free PBS (pH 7.4), 0.05 % Tween 20; 1:200 for the less reactive serum or starting from 1:200 to 1:800 for the more reactive serum.

Determination of antibody isotypes. Determination of antibody isotypes was carried out using the ELISA as described above. Briefly, after incubation with dilutions (1:50) of the pooled sera of pcDNA3.1::MOMP-vaccinated mice collected at day 56 or 63, the isotypes were revealed by adding 100 μl of a 1:1000 dilution of peroxidase-conjugated anti-mouse IgG1 or IgG2a (Nordic Immunological Laboratories). The A_{414} was measured on a microplate reader (Multiskan RC, Thermo Labsystems) after 1 h of incubation with the substrate (ABTS) at room temperature.

In vitro neutralization assay in the presence of the complement.

The neutralization assay was done by the plaque-reduction assay using McCoy cells (Schachter *et al.*, 1974). Pooled sera of PBS-, pcDNA3.1- and pcDNA3.1::MOMP-vaccinated mice were diluted in Mg^{2+} -free, Ca^{2+} -free PBS (pH 7.4) containing 10 % fetal bovine serum and 2.5 % guinea pig serum. Two hundred microlitres of these dilutions (10^{-1} , 10^{-2} and 10^{-3}) were added to an equal volume (200 μl) of an AB7 chlamydial suspension (10^4 p.f.u.), incubated for 2 h at 37°C and then titrated on McCoy cells by the plaque assay method (Rodolakis & Chancerelle, 1977). The results are expressed for each serum as the percentage p.f.u. of the PBS control.

Evaluation of protection. The protective effect of the DNA vaccine was assessed by the evaluation of the number of living offspring per litter at birth or 8 days after birth and the clearance of bacteria in spleens, placentas and fetuses. After challenge, the outcome of pregnant mice was monitored daily. Mice were considered as protected when the number of living offspring per litter 8 days after birth was significantly different ($P < 0.05$) from the number for the virulence control as described previously (Rodolakis, 1983). All the non-pregnant mice and five mice of each group of pregnant mice were euthanized at day 63. The spleens from non-pregnant mice and the uteruses and spleens from pregnant mice were removed aseptically. All the placentas from the same uterine horn were dissected from the fetuses. Placentas from the same uterine horn were pooled as well as the fetuses. Pooled placentas, pooled fetuses and spleens were weighed and frozen at -80°C . Organs were homogenized in PBS with a glass grinder, diluted in PBS-DEAE dextran (0.01 %) and titrated by plaque assay on McCoy cells (Rodolakis & Chancerelle, 1977). The course of infection was evaluated by counting

the number of p.f.u. and expressed as $\log_{10}$ p.f.u. per organ for spleens and $\log_{10}$ p.f.u. per uterine horn for placentas and fetuses.

Statistical tests. Analysis of the results was performed using the software InStat 2.03 for Macintosh. For all protective results, the mean was calculated using a one-way analysis of variance and a comparison of the means was then carried out through a Student–Newman–Keuls multiple comparison test. The minimal statistical significance was judged at $P < 0.05$.

RESULTS

Analysis of the humoral response

The anti-MOMP IgG antibody response was assessed by ELISA (Fig. 1). The anti-MOMP IgG titre reached a maximum in the non-pregnant mice immunized with pcDNA3.1::MOMP after the third DNA injection (day 56) and decreased after challenge (day 63). This decrease in anti-MOMP IgG antibody titre at day 63 was also observed without challenge (data not shown). The substantial variations of the IgG titre in individual mice on the last two sampling days were probably due to the fact that the murine model used in these experiments was an outbred one. No MOMP-specific IgG antibody was identified in sera from non-immunized mice (data not shown) or from mice immunized with pcDNA3.1 (Fig. 1). The specificity of anti-MOMP antibodies was also examined by immunoblotting. Sera collected from immunized mice before any DNA injection (day 0) or before challenge (day 56) and after challenge (day 63) were tested on crude extracts of *C. abortus* AB7 as well as on boiled recombinant MOMP. Since no MOMP-specific antibodies were detected (data not shown), we could conclude that the anti-MOMP antibody titre was probably too low to detect native or recombinant MOMP by immunoblotting.

The isotypes of anti-MOMP antibodies of sera collected at

Fig. 1. Kinetics of the antibody response of DNA-immunized mice. Sera were collected at different times after immunization with pcDNA3.1 (●) or pcDNA3.1::MOMP (■) and tested individually by ELISA for titration of anti-MOMP-specific IgG antibodies. The mean A_{414} ($\pm$ SEM) is shown for each time-point.

days 56 and 63 from pcDNA3.1::MOMP-vaccinated mice were tested by ELISA. The predominant isotype was IgG2a (data not shown). No IgG1 was found.

The ability of the sera to neutralize the infectivity of the virulent AB7 strain of *C. abortus* was assessed by the plaque-reduction test. The sera of 1B-vaccinated mice had a substantial neutralizing effect (90 % at the 10^{-1} dilution; Fig. 2), while the sera of pcDNA3.1- and pcDNA3.1::MOMP-vaccinated mice respectively had a modest neutralizing effect (60 %) and a non-significant neutralizing effect (20 %).

Efficacy of DNA immunization on pregnancy

As expected, the number of live newborn mice observed in the 1B group was not significantly different from the number of live newborns observed in the gestation control group ($P > 0.05$), but it was significantly higher ($P < 0.01$) than the number monitored in the virulence group (Fig. 3). The numbers of living offspring at birth and 8 days after birth for the gestation control and 1B groups were not significantly different. For the virulence group and the pcDNA3.1- and pcDNA3.1::MOMP-vaccinated mice, the number of living offspring at birth was more than at 8 days after birth, but this difference was not significant. The numbers of live newborn in the virulence, pcDNA3.1 and pcDNA3.1::MOMP groups were not significantly different ($P > 0.05$), but were significantly lower ($P < 0.05$) than in the gestation control and 1B groups. Therefore, pcDNA3.1::MOMP-vaccinated mice were not protected against abortion due to *C. abortus* AB7 challenge.

Protection was also evaluated at a placental and fetal level by titration of *C. abortus* in these organs. Chlamydial titres in placentas were higher than in fetuses ($P < 0.001$). As expected, chlamydial titres in the placentas and fetuses of 1B-vaccinated mice were significantly lower ($P < 0.01$) than in the virulence group (Fig. 4). 1B-vaccinated mice were consequently protected against chlamydial infection. Chlamydial titres in the placentas of pcDNA3.1- and pcDNA3.1::MOMP-vaccinated mice were not significantly

Fig. 2. Neutralizing effect of sera from 1B- (●), pcDNA3.1- (■) or pcDNA3.1::MOMP- (▲) vaccinated mice. Mouse sera collected at day 56 were diluted 10^{-1} , 10^{-2} or 10^{-3} and then added to the chlamydial suspension. These solutions had been titrated and the results are expressed relative to the control.

Fig. 3. Effect of DNA vaccination on viable offspring at birth (open bars) or 8 days after birth (filled bars). The gestation control group was neither infected nor immunized and the virulence group was infected but not immunized. Mice were immunized with pcDNA3.1 or pcDNA3.1::MOMP or with the 1B vaccine. After immunization, mice were infected at 12 days of gestation. Results are expressed as mean numbers of living offspring 1 week after birth (+SEM). *, Significantly different from the respective virulence control ($P < 0.05$).

Fig. 4. Chlamydial clearance in placentas (open bars) and fetuses (filled bars) of pregnant mice. Mice were immunized and mated and then challenged with *C. abortus* AB7 and euthanized 5 days after challenge. Quantities of bacteria in placentas and fetuses are expressed as $\log_{10}$ p.f.u. per uterine horn (+SEM). *, Significantly different from the respective virulence control ($P < 0.05$).

different from those of the virulence group ($P > 0.05$). This would indicate that DNA vaccination did not elicit sufficient protection against placental colonization. Nevertheless, the fetuses of pcDNA3.1- and pcDNA3.1::MOMP-vaccinated mice were significantly less infected than those of the virulence group ($P < 0.05$) (Fig. 4). Thus, partial protection was observed in fetuses of 1B-, pcDNA3.1- and pcDNA3.1::MOMP-vaccinated mice.

Clearance of *C. abortus* AB7 from the spleen

The induced protection was evaluated by splenic titration of *C. abortus* on pregnant and non-pregnant mice. As expected,

chlamydial titres in spleens from non-pregnant or pregnant 1B-vaccinated mice were significantly lower ($P < 0.001$ and $P < 0.01$, respectively) than in the virulence group. Thus, 1B-vaccinated mice were protected against chlamydial infection (Fig. 5). Chlamydial titres in the spleens from pcDNA3.1- and pcDNA3.1::MOMP-vaccinated, non-pregnant or pregnant mice were not significantly different from those from mice of the virulence group ($P > 0.05$). These results suggested that DNA vaccination did not induce splenic protection in pregnant or non-pregnant mice.

DISCUSSION

In veterinary medicine, vaccines need to overcome a large number of hurdles, including the cost of production, the ease of delivery, safety and the complete protection of animals against disease. DNA vaccination has recently been tested against many pathogens and is promising in veterinary medicine (Babiuk *et al.*, 2000). The aim of this study was to investigate the protective effect of DNA vaccination with the MOMP-encoding gene against *C. abortus* challenge in a murine model. The mouse model usually employed for the evaluation of *C. abortus* virulence (Rodolakis *et al.*, 1989) as well as vaccine efficiency (Rodolakis, 1983) was the OF1 outbred mouse model of abortion. This model presented various advantages. It was easy to perform, since it consisted of evaluation of the living offspring per mouse. Moreover, the protective effects of vaccines obtained in OF1 outbred mice were correlated with those observed in the ovine model. Finally, this mouse model has been used successfully in the past to evaluate the efficiency of 1B vaccine and is now taken as a reference (Rodolakis, 1983). Nevertheless, this OF1 mouse model presented two major disadvantages. Firstly, as it involves outbred mice, this model is not suitable for

Fig. 5. Chlamydial clearance in spleens of DNA-immunized pregnant (open bars) or non-pregnant (filled bars) mice. Mice were immunized by three intramuscular injections of pcDNA3.1 or pcDNA3.1::MOMP or one injection of the 1B vaccine. Mice were then challenged with *C. abortus* AB7 and euthanized 5 days after challenge. Quantities of bacteria in spleens are expressed as $\log_{10}$ p.f.u. per spleen (+SEM). *, Significantly different from the respective virulence control ($P < 0.05$).

evaluation of the cellular immune response. Secondly, it can be weakly discriminatory, since a small difference in the titre of the *C. abortus* challenge (nearness abortive dose 50 %) can modify the number of living offspring very significantly. Together with the high variability between the results obtained with outbred mice, this could have made the protection observed non-significant. Thus, the comparable mean numbers of living offspring obtained from pcDNA3.1- and pcDNA3.1::MOMP-vaccinated mice drove us to investigate more precisely the protection in other models of infection evaluation, including the splenic, placental and fetus bacterial burden inquiries.

Although DNA immunization with the *momp* gene elicited production of MOMP *in vivo*, since it induced specific anti-MOMP antibodies, it failed to reduce the abortive effect of *C. abortus* challenge. No protection was observed after MOMP DNA immunization in either the placental colonization or splenic colonization model. However, partial reduction of infection was observed in fetuses of pcDNA3.1- and pcDNA3.1::MOMP-vaccinated mice. As placentas were infected, fetuses were probably protected by a barrier effect, but this protective effect was not sufficient to allow the survival of living offspring. This non-specific and partial protection could be explained by the immunostimulating properties of the CpG sequences present in the vaccinal plasmid backbone (Krieg, 2000). These unmethylated CpG motifs are recognized as a danger signal by the vertebrate immune system and trigger a Th1-biased immune response with IgG2a antibody production. This hypothesis is therefore in agreement with the predominant IgG2a isotype of anti-MOMP antibodies found in our experiments. Analysis of cytokine profiles could have confirmed this Th1-like immune response. Buendía *et al.* (1999) reported high levels of interferon- γ and interleukin-12 in the serum of mice infected with the *C. abortus* strains AB7 and 1B, suggesting strongly that effective resolution of the infection involves a Th1-like immune response.

The increased mortality of newborn mice observed in the pcDNA3.1 and pcDNA3.1::MOMP groups 8 days after birth could result from the loss of the nutritious properties of infected placentas, leading to frail living offspring, resulting in death even in the absence of fetal infection. Also, late contamination could be possible when the mice gave birth. Indeed, it has been reported that *C. abortus* was excreted when ewes gave birth and that they could therefore infect newborns as well as other ewes at this time (Rodolakis & Souriau, 1979). This hypothesis could be transposable in the mouse model, since the number of living offspring appeared to be higher at birth than 8 days after birth. We could also propose that the newborn of 1B-vaccinated mice would be protected by the transfer of maternal antibodies via the milk, since the sera of these mice showed neutralizing properties.

In contrast with the pooled sera of 1B-vaccinated mice, the sera of pcDNA3.1- and pcDNA3.1::MOMP-vaccinated mice failed to neutralize chlamydial penetration in cells. As protective anti-MOMP antibodies are known to be directed

against conformational epitopes (De Sa *et al.*, 1995), we could speculate that the MOMP DNA vaccine induced linear epitope-specific antibodies. Probably due to the weak humoral response induced by the DNA vaccination, we failed to detect either conformational or linear MOMP after immunoblotting on whole *C. abortus* extracts. Another observation came from the weak level of anti-MOMP antibodies induced by the MOMP DNA vaccine. Because induction of the immune response is known to be linked directly to protein expression following DNA immunization (Barry & Johnston, 1997), we supposed that MOMP expression *in vivo* was likely to be insufficient to induce a relevant level of anti-MOMP antibodies. This was consistent with the fact that the antibodies of pcDNA3.1::MOMP-vaccinated mice sera failed to detect MOMP by immunoblotting, a less sensitive technique compared with ELISA.

Different methods have been evaluated in an attempt to increase the immune response generated by DNA vaccines (Gurunathan *et al.*, 2000). More particularly, the vaccination protocol could be improved, as has already been done in a *C. trachomatis* model (Dong-Ji *et al.*, 2000), using a DNA prime and a protein boost strategy known to enhance both humoral immune response and protection (Song *et al.*, 2000). In addition, it might be possible to amplify the protective immune response by co-administration of an immunoregulatory cytokine-encoding plasmid with the vaccinal plasmid (Gurunathan *et al.*, 2000). A protein boost and the co-administration of cytokine-encoding plasmids can also be combined in order to achieve protection (Scheerlinck *et al.*, 2001).

In summary, DNA vaccination with a plasmid vector encoding the *momp* gene of *C. abortus* elicited a weak humoral response, with the IgG2a isotype predominant. Despite the antigenic properties of MOMP, DNA vaccination failed to protect pregnant mice from abortion against *C. abortus* challenge. In contrast, fetuses from DNA-vaccinated mice were non-specifically and partially protected. Therefore, it would be of interest to optimize the vaccination protocol and to use genetic adjuvants including cytokines or co-stimulating molecule genes in order to enhance the immune response.

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