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

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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-Gatell et al., 1990) and, more particularly, by the passive transfer of specific antibodies to the 110 kDa oligomeric MOMP (De Sa et al., 1995). When the vaccinal approaches attempted to preserve the conformational structure of MOMP, 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 (Igietseme & 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., 1999). 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 Chlamydomphila psittaci (Penttilä et al., 2000) models.
Chlamydia 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 AB8 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 chickens (respectively 80 and 8 mg kg 

anaesthetized by an intraperitoneal injection of ketamine and xylazine into the tibialis anterior muscles of both hind legs to enhance the uptake into the tibialis anterior. Mice were boosted in the same way at days 21 and 42. Positive-control mice were immunized with one subcutaneous injection of 4 × 10⁸ 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 × 10⁷ 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 (400 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 Mg²⁺-free, Ca²⁺-free PBS (pH 7·4) at a concentration of 1 μg 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 Mg²⁺-free, Ca²⁺-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 titres were measured by 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 (Schacter et al., 1974). Pooled sera of PBS-, pcDNA3.1-:MOMP-vaccinated mice were diluted in Mg²⁺-free, Ca²⁺-free PBS (pH 7·4) containing 10 % fetal bovine serum and 2·5 % guinea pig serum. Two hundred microlitres of these dilutions (10⁻⁴, 10⁻³ and 10⁻²) were added to an equal volume (200 μl) of an AB7 chlamydial suspension (10⁵ 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 live 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 live 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 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 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.
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* AB7 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...
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. 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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**REFERENCES**


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.
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