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Protection of turkeys against *Chlamydophila psittaci* challenge by parenteral and mucosal inoculations and the effect of turkey interferon-γ on genetic immunization

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SUMMARY

Plasmid DNA (pcDNA1::MOMP A) expressing the major outer membrane protein of an avian *Chlamydophila psittaci* serovar A strain was tested for its ability to induce protective immunity against challenge with the same *C. psittaci* serovar. A combined parenteral (intramuscular injection) and mucosal route (DNA drops administered to the nares) of DNA inoculation was compared to three other, different routes of administration (intramuscular inoculation, DNA drops administered to the nares and aerosol immunization). In addition, the effect of turkey interferon gamma (tIFN-γ) on intramuscular immunization was evaluated by co-expressing pCIneo::tIFN-γ. A significant level of protection was observed in turkeys immunized via the combined parenteral/mucosal route, the intramuscular route or by aerosol. Severe clinical signs and lesions were observed in the non-vaccinated control groups, in 80% of turkeys inoculated with a mixture of pcDNA1::MOMP A and pCIneo::tIFN-γ, and in 60% of turkeys vaccinated with DNA drops administered to the nares. The use of MOMP-based DNA vaccination as a means of preventing severe clinical signs and lesions in a turkey model of *C. psittaci* infection was demonstrated, as was down-regulation of the immune response by co-expression of tIFN-γ.

INTRODUCTION

Chlamydia psittaci, recently reclassified as *Chlamydophila psittaci*[1] is a Gram-negative, obligate intracellular bacterium that causes infections of mucosal epithelial cells and macrophages (Mϕ) of the turkey respiratory tract, which is followed by septicaemia and localization in epithelial cells and Mϕ of various other organs.[2] Chlamydial infections in turkeys present important economic losses, but public health is also a consideration, as poultry workers and veterinary surgeons are at risk of becoming infected with this zoonotic agent. At present, vaccines are non-existent.

Recently, the significance of DNA immunization as a means of preventing severe clinical signs, lesions and bacterial excretion, has been demonstrated in a turkey model of *C. psittaci* infection.[3] In the present work we evaluated how the route of DNA inoculation affects the ability to induce protective immunity. The efficacy of DNA immunization has to reflect both the efficiency of *in vivo* transfection (DNA uptake and expression) and the efficiency with which transfected cells present proteins to the immune system. Muscle is 100–1000 times more permissive than other tissues for the uptake and expression of DNA.[4,5] Tissues, such as the skin and the mucosal linings of the respiratory tract and gut, have associated lymphoid tissues that provide high levels of local immune surveillance. In view of the above, DNA inoculations were administered via several different routes, as follows:

(1) by a route that supports unusually efficient transfection (muscle),
(2) by a route that supports less efficient transfection but delivers DNA to a tissue with high levels of local immune surveillance (DNA drops administered to the nares),
(3) by a route that supports less efficient transfection, but represents a route frequently used for the administration of a vaccine to large poultry groups (spray or aerosol), and
(4) by a combined intramuscular (i.m.)/intranasal (i.n.) route combining the benefits of unusually efficient transfection and efficient antigen presentation at the mucosal surface.

The effect of the different routes of inoculation on DNA vaccination efficiency was evaluated in a turkey *C. psittaci* model.

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In addition, the effect on development of an antigen-specific B- and T-helper cell response was tested by co-inoculation of turkeys with a plasmid vector expressing the major outer membrane protein (pMOMP) of a *C. psittaci* serovar A strain and an additional vector encoding turkey interferon-γ (tIFN-γ). Turkey IFN-γ was chosen for it is known to up-regulate and/or induce expression of major histocompatibility complex (MHC) class I and II molecules on a number of cells, including muscle cells.[6]

MATERIALS AND METHODS

C. psittaci *strain*

In this study, *C. psittaci* strain 84/55, isolated from the lungs of a diseased parakeet, was used. The strain had been characterized previously by using serovar-specific monoclonal antibodies (mAbs) and by restriction-fragment length analysis of the *omp1* gene, and was classified as an avian serovar A and genotype A strain.[7] The strain was grown in Buffalo Green Monkey (BGM) cells as previously described,[8] and the 50% tissue culture infective dose ($TCID_{50}$) was determined on BGM cells, also as previously described.[2]

Vaccine DNA

Plasmid pcDNA1::MOMP A (pMOMP) was constructed as described previously.[3] Plasmids were amplified in *Escherichia coli* MC1061/P3 and purified by use of the Endo Free QIAGEN Tip 2500 plasmid preparation method (QIAGEN GmbH, Hilden, Germany). DNA concentration was determined by measuring the absorbance at 260 nm and confirmed by comparing the intensity of ethidium bromide-stained *Eco*RI restriction endonuclease fragments with standards of known concentration. DNA was stored at −20° in 1 mM Tris (pH 7·8), 0·1 mM EDTA. Expression of MOMP was confirmed by indirect immunofluorescence staining of both DEAE dextran-transfected COS-7 cells and turkey skeletal muscle injected with pMOMP.[9] For each DNA inoculation, 100 μg of pMOMP diluted in saline (0·9% NaCl) was used. pcDNA1 (placebo) was used as a control plasmid.

The plasmid pCIneo::tIFN-γ (pIFN-γ), encoding tIFN-γ, was constructed as described previously.[10] Expression of tIFN-γ was confirmed by using a tIFN-γ bioassay, which measured the ability of supernatants from COS-7 cells (DEAE dextran transfected with pIFN-γ) to induce nitric oxide (NO) production from a chicken Mϕ cell line, as previously described.[10] pCIneo (placebo) was used as a control plasmid.

Vaccination trials

The effect of a combined parenteral (i.m. injection) and mucosal (DNA drops administered to the nares) route of DNA inoculation was compared to i.m. immunization, i.n. immunization (drops), and aerosol immunization, by using the Cirrus™ Nebulizer (Intersurgical, Berkshire, UK). This nebulizer was designed to provide a large volume of particles up to 5 μm (with a mass median diameter of 3·5 μm) to give tracheobronchial deposition in humans. In addition, the effect of tIFN-γ on DNA immunization was evaluated by i.m. administration of a mixture of pMOMP (100 μg) and pIFN-γ (100 μg).

Specific pathogen-free (SPF) turkeys (CNEVA, Ploufragan, France) were divided into 10 groups of five turkeys, reared in negative-pressure isolators. All groups received a primary DNA inoculation at 1 day of age and one booster inoculation 3 weeks later. Turkeys were challenged by aerosol (Cirrus Nebulizer) infection at the age of 5 weeks. The challenge infection consisted of 10^4 $TCID_{50}$ of *C. psittaci* strain 84/55 (avian serovar A strain). The whole vaccination experiment with challenge is presented in Table 1.

Samples

All turkeys were observed daily for clinical signs. Nasal and cloacal swabs were taken every other day from day 6 postchallenge (PC) to day 18 PC. Blood samples were collected for the detection of anti-MOMP-specific antibody titres immediately prior to each DNA inoculation, immediately prior to the experimental infection and at 18 days PC. Blood samples were stored overnight at room temperature, centrifuged (325 ***g***, 10 min, 4°) and afterwards serum was frozen at −20° until tested. When the turkeys were killed 18 days PC, proliferative responses in peripheral blood lymphocytes (PBL) were examined. All killed turkeys were examined for macroscopic lesions. Cryostat tissue sections of the thoracic and abdominal air sacs, the lungs, spleen and liver were examined for the presence of chlamydophila antigen.

C. psittaci *isolation*

Nasal and cloacal swabs were examined for the presence of viable chlamydophilae by isolation in BGM cells, as previously described.[8] Chlamydophilae were identified by using IMAGEN™ direct immunofluorescence staining (Novo Nordisk Diagnostics, Cambridge, UK), as described previously.[8] The number of chlamydophila-positive cells in each turkey was counted in five randomly selected microscopic fields (×500 magnification), and scored in a range from 0 to 3: score 0 indicated that no chlamydophila-positive cells present; scores 1, 2 and 3 were given when a mean of 1–5, 5–10 and >10 chlamydophila-positive cells were identified, respectively.

Table 1. Vaccination trial

Group ($n=5$)	Plasmid	Vaccination route*	Dose (μg)	*C. psittaci* challenge§
1	pMOMP	i.m.†+i.n.‡	100+100	Serovar A
2	pMOMP	i.m.	100	Serovar A
3	pMOMP	i.n.	100	Serovar A
4	pMOMP	Aerosol	100	Serovar A
5	pcDNA1 placebo	i.m.+i.n.	100+100	Serovar A
6	pMOMP	i.m.+i.n.	100+100	No
7	pMOMP+pIFN-γ	i.m.	100+100	Serovar A
8	pcDNA1 placebo+pCIneo placebo	i.m.	100+100	Serovar A
9	pcDNA1 placebo+pIFN-γ	i.m.	100+100	Serovar A
10	pMOMP+pIFN-γi.m.	100+100	No	

*Each group received a primary vaccination at time 0 followed by a booster vaccination 3 weeks later.

†i.m., intramuscular.

‡i.n. intranasal (DNA drops).

§Challenge (10^4 50% tissue culture infective dose [$TCID_{50}$] *Chlamydophila psittaci* 84/55 serovar A) was administered by aerosol at 14 days after the second DNA inoculation.

IFN-γ, interferon-γ.

Cloacal and nasal shedding in all vaccinated groups are presented as mean scores ± standard deviation (SD) in swab cultures of individual turkeys.

Direct immunofluorescence staining

Cryostat tissue sections were examined by the IMAGEN (Novo Nordisk Diagnostics) direct immunofluorescence staining, as previously described. The number of chlamydophila inclusions was counted as described above. The presence of chlamydophila antigen in tissues of all 10 groups are presented as mean scores ± SD in swab cultures of individual turkeys.

Antibody responses

Enzyme-linked immunosorbent assays (ELISAs) were performed on turkey sera following pretreatment with kaolin to remove background activity.[11] Anti-MOMP-specific antibody titres were determined using twofold dilutions of sera (starting at a dilution of 1 : 100) in an indirect ELISA with recombinant MOMP (rMOMP) as antigen. rMOMP was produced in pMOMP-transfected COS-7 cells, as described previously.[9] For the determination of antibody titres, 1 : 4000 and 1 : 2000 dilutions of biotinylated anti-chicken/turkey immunoglobulin G (IgG) (H + L) antibody and peroxidase-conjugated streptavidin, respectively, were used. Anti-MOMP antibody titres were determined as the reciprocal of the highest serum dilution that gave an absorbance reading at 405 nm above the cut-off value. The cut-off value (0·100) was the mean absorbance of seronegative turkeys ± 2 SD. Results are presented as mean anti-MOMP antibody titres per group ± SD in sera of individual turkeys of each group.

Lymphocyte proliferative responses

PBL were isolated from heparinized blood samples obtained by venepuncture (*v. ulnaris*) from each turkey of groups 1–10, at 18 days PC. Lymphocyte proliferative tests were performed as described previously.[3] Briefly, non-adherent cells were grown in duplicate in 96-well tissue culture plates (at a concentration of 6×10^5 cells) in 150 µl of Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 20% heat-inactivated fetal calf serum (FCS; Integro, Zaandam, the Netherlands), 1% non-essential amino acids (Life Technologies, Merelbeke, Belgium), 1% sodium pyruvate (Life Technologies), 1% L-glutamine (Life Technologies), 1% gentamycin (Life Technologies) and 5×10^{-5} M β-mercaptoethanol (β-ME; Life Technologies). For antigen proliferation, 10 µg of recombinant MOMP was added to individual wells. Negative and positive controls included cells stimulated with medium alone or with 10 µg of concanavalin A (Con A), respectively. Cells were incubated at 39·5° in a humidified incubator with 5% CO_2. Con A- or antigen-induced proliferation was measured by incorporation of ^{3}H-thymidine (1 µCi/well) during the last 16 hr of culture, on days 2 and 8, respectively. Cultures were harvested onto glass-fibre filter strips by using a cell harvester (Skatron, Liers, Norway). Filters were placed in 2 ml of Lumasave LSC cocktail (Lumac, Groningen, the Netherlands) and counted in a Beckman β-scintillation counter (Beckman, Gent, Belgium). The stimulation index (SI) was defined as the ratio of counts per minute (c.p.m.) of stimulated cells relative to medium-only cultures.

Statistical analysis

The two-tailed Student's *t*-test was used for all statistical analyses. Results were considered significantly different at a *P*-value of $< 0{\cdot}05$.

RESULTS

Protection against *C. psittaci* challenge

Following vaccination with pMOMP (groups 1–4), pcDNA1 placebo (group 5), pMOMP and pIFN-γ (group 7), pcDNA1 placebo and pCIneo placebo (group 8), or pcDNA1 placebo and pIFN-γ (group 9), SPF turkeys were challenged with an experimental infective dose of the homologous chlamydophila strain. Following challenge, severe clinical signs and lesions were not only observed in the placebo-vaccinated control groups (5, 8 and 9), but also in 80% (four of five) of the pMOMP-vaccinated turkeys simultaneously inoculated with pIFN-γ (group 7), and in 60% (three of five) of the i.n.-vaccinated turkeys (group 3). Turkeys of these five groups displayed depression, anorexia, conjunctivitis, rhinitis, dyspnea, diarrhoea, sinusitis, pneumonia, air-sacculitis, pericarditis and hepatosplenomegaly. Turkeys of groups 1 and 2, vaccinated i.m. + i.n. and i.m., respectively, showed no clinical signs and no macroscopic lesions upon challenge and were similar to non-challenged animals (groups 6 and 10). However, in 40% (two of five) of the aerosol-vaccinated turkeys (group 4) one small greyish area of inflammation was observed unilaterally in the lungs. No other lesions were observed in this vaccinated group.

Results of the nasal and cloacal cultures obtained at 2-day intervals during the 18 days PC are shown in Tables 2 and 3, respectively. A significant level of protection was observed in i.m. + i.n.-, i.m.- and aerosol-vaccinated groups (groups 1, 2 and 4, respectively), as only 20% (one of five) turkeys of these groups showed cloacal shedding and the scores of cloacal shedding were comparable. All turkeys of the challenged placebo-vaccinated groups (5, 8 and 9) and 80% of the challenged pMOMP/pIFN-γ-vaccinated group (group 7) had positive cloacal cultures during the 18 days of observation. In addition, turkeys of the latter four groups shed significantly more chlamydophilae than i.m. + i.n.-, i.m.- and aerosol-vaccinated turkeys (groups 1, 2 and 4, respectively), as shown by the higher mean scores. All non-vaccinated, challenged control turkeys excreted chlamydophila nasally. At 18 days PC, 100% of the control turkeys still excreted chlamydophila nasally, while in all i.m. + i.n.-vaccinated turkeys, and in 80% (four of five) of i.m.- and aerosol-vaccinated turkeys (groups 2 and 4, respectively), nasal excretion was observed no longer than 8 days PC. All turkeys in the sham-immunized, non-challenged control groups remained chlamydophila-negative throughout the experiment.

Immunofluorescence staining of tissues of the placebo-vaccinated turkeys (groups 5, 8 and 9), killed 18 days PC, revealed a high level of chlamydophila replication in both air sacs, and moderate replication in the lungs, liver and spleen. Interestingly, chlamydophila replication in pMOMP/pIFN-γ-vaccinated turkeys (group 7) was comparable to that observed in the placebo-vaccinated groups. Regarding chlamydophila replication, there were no significant differences in protection provided by the i.m. + i.n., i.m., or aerosol routes of DNA

Table 2. Results of nasal cultures of placebo-vaccinated and pcDNA1::MOMP A-vaccinated turkeys after challenge

Group ($n=5$)	Mean score* of *Chlamydophila psittaci* nasal shedding ± SD† for the days postchallenge shown below (values in parenthesis represent the % of positive turkeys in each group)						
	6	8	10	12	14	16	18
1	0·20 ± 0·44 (20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2	0·40 ± 0·54 (40)	0 (0)	0 (0)	0 (0)	0 (0)	0·20 ± 0·44 (20)	0·20 ± 0·44 (20)
3	1·0 ± 0 (100)	0·20 ± 0·44 (20)	0·20 ± 0·44 (20)	0·20 ± 0·44 (20)	0·40 ± 0·54 (40)	0·60 ± 0·89 (60)	0·80 ± 0·22 (80)
4	0·60 ± 0·89 (60)	0 (0)	0 (0)	0 (0)	0 (0)	0·20 ± 0·44 (20)	0·20 ± 0·44 (20)
5	1·40 ± 0·55 (100)	1·40 ± 0·55 (100)	1·40 ± 0·55 (100)	1·60 ± 0·59 (100)	1·80 ± 0·44 (100)	2·0 ± 0 (100)	2·60 ± 0·54 (100)
7	0·60 ± 0·89 (60)	1·20 ± 0·40 (60)	1·20 ± 0·40 (60)	1·40 ± 0·56 (80)	1·80 ± 1·0 (80)	1·80 ± 1·0 (80)	2·0 ± 1·22 (80)
8	1·0 ± 0 (100)	1·40 ± 0·55 (100)	1·20 ± 0·37 (100)	1·80 ± 0·44 (100)	2·0 ± 0 (100)	2·40 ± 0·50 (100)	2·20 ± 0·45 (100)
9	1·40 ± 0·55 (100)	1·40 ± 0·55 (100)	1·60 ± 0·59 (100)	1·80 ± 1·0 (100)	1·80 ± 1·0 (100)	2·0 ± 0 (100)	2·20 ± 0·45 (100)

*Shedding was scored from 0 to 3. Score 0 indicated no viable chlamydophilae present. Scores 1, 2 or 3 were given when a mean of 1–5, 5–10 or >10 inclusion-positive cells, respectively, were present per five randomly selected microscopic fields. No shedding was observed in groups 6 and 10.
†SD, standard deviation.

Table 3. Results of cloacal cultures of placebo-vaccinated and pcDNA1::MOMP A-vaccinated turkeys after challenge

Group ($n=5$)	Mean score* of *Chlamydophila psittaci* cloacal shedding ± SD† for the days postchallenge shown below (values in parenthesis represent the % of positive turkeys in each group)						
	6	8	10	12	14	16	18
1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0·20 ± 0·44 (20)
2	0·20 ± 0·44 (20)	0·20 ± 0·44 (20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3	0·80 ± 1·0 (40)	0·20 ± 0·44 (20)	0·60 ± 0·89 (60)	1·0 ± 0 (100)	1·0 ± 0 (100)	0·80 ± 0·84 (60)	1·0 ± 1·0 (60)
4	0 (0)	0·20 ± 0·44 (20)	0·20 ± 0·44 (20)	0·20 ± 0·44 (20)	0·20 ± 0·44 (20)	0·20 ± 0·44 (20)	0·40 ± 0·89 (20)
5	2·8 ± 0·45 (100)	2·0 ± 0 (100)	2·0 ± 0 (100)	1·80 ± 0·44 (100)	1·0 ± 0 (100)	1·80 ± 0·44 (100)	1·80 ± 0·44 (100)
7	0·80 ± 1·0 (40)	1·20 ± 0·40 (60)	1·80 ± 1·0 (80)	1·80 ± 1·0 (80)	1·40 ± 0·56 (80)	1·80 ± 1·0 (80)	1·80 ± 1·0 (80)
8	2·0 ± 0 (100)	2·0 ± 0 (100)	1·60 ± 0·59 (100)	1·80 ± 0·44 (100)	1·80 ± 0·44 (100)	1·40 ± 0·55 (100)	1·60 ± 0·59 (100)
9	2·20 ± 0·45 (100)	2·0 ± 0 (100)	1·80 ± 0·44 (100)	1·80 ± 0·44 (100)	1·80 ± 0·44 (100)	1·60 ± 0·59 (100)	1·80 ± 0·44 (100)

*Shedding was scored from 0 to 3. Score 0 indicated no viable chlamydophilae present. Scores 1, 2 or 3 were given when a mean of 1–5, 5–10 and >10 inclusion-positive cells, respectively, were present per five randomly selected microscopic fields. No shedding was observed in groups 6 and 10.
†SD, standard deviation.

administration (Table 4). In these vaccinated, challenged animals, a weak replication only was observed in lung tissues from four out of 15 (26·6%) turkeys, in thoracic air sacs from three out of 15 (19·9%) turkeys and in abdominal air sacs from four out of 15 (26·6%) turkeys. Examination of the liver and spleen revealed no chlamydophila replication. Remarkably, aerosol-vaccinated animals showed no chlamydophila replication in the abdominal air sacs.

Antibody responses

Three weeks following the first immunization, low mean anti-MOMP serum antibody titres were observed in i.m.+i.n.-, i.m.-, i.n.-, aerosol- and pMOMP/pIFN-γ-vaccinated turkeys (groups 1–4 and group 7, respectively), as determined by ELISA using homologous recombinant MOMP as antigen (Table 5). Following the second immunization, the highest mean anti-MOMP antibody titres were observed in i.m.+i.n.- (group 1)

Table 4. Chlamydophila replication in tissues of placebo-vaccinated and pcDNA1::MOMP A-vaccinated turkeys, 18 days postchallenge (PC)

Group ($n=5$)	Mean fluorescence score* ± SD for the presence of *Chlamydophila psittaci* in tissues of the groups shown below (values in parenthesis represent the % of positive turkeys in each group)				
	Lung	Thoracic air sac	Abdominal air sac	Liver	Spleen
1	0·60 ± 0·89 (60)	0·20 ± 0·44 (20)	0·40 ± 0·54 (40)	0 (0)	0 (0)
2	0·20 ± 0·44 (20)	0·20 ± 0·44 (20)	0·40 ± 0·54 (40)	0 (0)	0 (0)
3	1·0 ± 0·71 (80)	1·6 ± 0.70 (80)	0·80 ± 0·84 (60)	0·20 ± 0·44 (20)	0·40 ± 0·54 (40)
4	0·60 ± 0·89 (60)	0·20 ± 0·44 (20)	0 (0)	0 (0)	0 (0)
5	1·80 ± 0·44 (100)	1·80 ± 0·44 (100)	2·20 ± 0·45 (100)	1·40 ± 0·55 (100)	1·80 ± 0·44 (100)
7	0·80 ± 0·22 (80)	1·80 ± 1·0 (80)	2·40 ± 0·50 (100)	1·0 ± 0 (100)	1·20 ± 0·37 (100)
8	1·60 ± 0·59 (100)	2·0 ± 0 (100)	2·20 ± 0·45 (100)	1·0 ± 0 (100)	1·40 ± 0·55 (100)
9	1·40 ± 0·55 (100)	2·20 ± 0·45 (100)	2·40 ± 0·50 (100)	1·20 ± 0·37 (100)	1·40 ± 0·55 (100)

*A score of 1 represented an average of 1–5 inclusion-positive cells; 2 an average of 5–10 inclusion-positive cells; and 3 an average of >10 inclusion-positive cells, per five randomly selected.

Table 5. MOMP-specific antibody titres following DNA vaccination and subsequent challenge with the homologous *Chlamydophila psittaci* strain

Time of serum collection	Mean MOMP-specific serum antibody titres ± SD in turkeys in the groups shown below							
	1	2	3	4	5	7	8	9
Prevac*	<	<	<	<	<	<	<	<
21 days PPV	360 ± 89	320 ± 89	360 ± 89	480 ± 165	<	200 ± 122	<	<
14 days PBV	5760 ± 2304	1920 ± 716	500 ± 269	10 880 ± 4293	<	400 ± 346	<	<
18 days PC	2400 ± 1131	2240 ± 876	7040 ± 5258	3400 ± 1315	10 880 ± 4293	8320 ± 4293	10 240 ± 4582	9600 ± 4582

*Prevac, bleed at hatching, before DNA vaccination.
PBV, postbooster vaccination; PC, postchallenge; PPV, postprimary vaccination; <no antibodies detected at the serum dilution used (1 : 100).

and aerosol- (group 4) vaccinated turkeys, suggesting superior priming in these turkeys. Turkeys within these groups showed a fourfold increase in the antibody response, indicating the occurrence of a secondary antibody response. Turkeys (group 3) vaccinated i.n., and pMOMP/pIFN-γ-vaccinated turkeys (group 7), still showed low mean anti-MOMP antibody titres following the second immunization. Following challenge, a fourfold increase in the mean antibody titre was observed only in i.n.-vaccinated turkeys (group 3) and pMOMP/pIFN-γ-vaccinated turkeys (group 7), both less-protected groups. No secondary antibody response could be detected in the best-protected groups, vaccinated i.m.+i.n., i.m. or by aerosol (groups 1, 2 and 4, respectively). In all control turkeys, a primary antibody response was following infection.

Antigen-specific lymphocyte proliferation

Proliferative responses to rMOMP of PBL of vaccinated and non-vaccinated turkeys were determined 18 days PC with the homologous chlamydophila strain. The PBL of pMOMP-immunized turkeys displayed significantly higher proliferative responses (Table 6) than the PBL of placebo-immunized controls. However, the proliferative responses of PBL of pMOMP-immunized animals were significantly down-regulated when the vaccine was co-administered with pIFN-γ.

DISCUSSION

In the present study, the eukaryotic expression vector, pMOMP, encoding the major outer membrane of an avian *C. psittaci* serovar A strain, was used to evaluate how the route of DNA inoculation affects the ability to induce protective immunity in a turkey *C. psittaci* model. The efficacy of combined i.m./i.n., and single i.m., i.n. (DNA drops) and aerosol administration of plasmid DNA in saline was evaluated. In addition, the effect of DNA vaccination in the presence of the tIFN-γ gene was determined.

A significant level of protection was observed in turkeys immunized by the combined i.m.+i.n. route, by the i.m. route or by aerosol. Severe clinical signs and lesions were only observed in the placebo-vaccinated control groups, in four out

Table 6. Proliferative responses of peripheral blood lymphocytes from immunized or non-immunized turkeys to recombinant major outer membrane protein on day 18 postchallenge

Mean stimulation index ± SD for the groups shown below ($n=5$)							
1	2	3	4	5	7	8	9
7·20 ± 2·84	16·0 ± 6·20	7·20 ± 2·84	17·50 ± 2·74	1·50 ± 0·35	5·80 ± 1·23	3·50 ± 0·25	2·50 ± 0·50

of five turkeys inoculated with a mixture of pMOMP and ptIFN-γ, and in three out of five turkeys vaccinated with DNA drops administered to the nares. The high efficacy of i.m. + i.n. vaccination could be explained by the combination of unusually efficient transfection in muscle and the delivery of plasmid to a tissue with high levels of local immune surveillance. However, both the combined i.m. + i.n. and single i.m. immunization methods gave comparably good protection levels although i.m.-immunized turkeys received only half the dose of DNA administered to the i.m. + i.n.-immunized turkeys.

Immunization by the i.n. route was significantly less efficient in protecting experimentally infected turkeys. In 80% of the i.n.-immunized, aerosol-challenged turkeys, chlamydophilae could still replicate in the epithelial cells and Mϕ of the respiratory tract, resulting in positive nasal and cloacal culture for 18 days PC. Consequently, the benefit of i.n. immunization, as performed in our combined i.m./i.n. vaccination method, is questionable. However, the administration of DNA drops to the nares has already been described as a successful mucosal route of vaccination.[12] Fynan *et al.*[12] used 100 µl of DNA in 100 µl of saline to vaccinate Metofane-anaesthetized mice in an avian influenza virus model. It may be that our i.n. vaccination results would have been better if a higher DNA concentration had been used or if the turkeys had been anaesthetized to prevent DNA expulsion by head shaking or sneezing. However, the volume of DNA that can be inoculated in the nares is limited, and anaesthesia removes i.n. vaccination from practical application.

By contrast, aerosol delivery of plasmid DNA to the respiratory tract resulted in significant protection against challenge. This can probably be explained by the fact that the aerosol particle size obtained by the Cirrus Nebulizer resulted in air sac delivery of DNA rather than delivery of DNA to the upper respiratory tract. In our study, air sac delivery of very small DNA particles (<10 µm) probably caused a local inflammatory reaction leading to the attraction of antigen-presenting cells specialized in initiating an immune response at the primary bacterial replication site. Protection upon aerosol challenge was significant as only one out of five aerosol-immunized turkeys gave positive nasal and cloacal cultures at 18 days PC. Only in this turkey were *C. psittaci* still present in the thoracic air sacs at 18 days PC. Considering the effects of evaporation rate on particles, in order to deliver vaccine DNA by inhalation in turkeys, particles in the range of 50–100 µm are probably ideal and should be evaluated starting in 1-day-old turkeys, as turkeys can become infected within the first week of age. Moreover, spray vaccination of 1-day-old chicks produces droplets on the feathers and the eyes, improving vaccination by immunization via the eye, which connects to the Hardarian gland.

Immunization via the i.m./i.n. route, as well as i.m. or aerosol immunization (groups 1, 2 and 4, respectively, in the present study), clearly primed both T and B cells. In these three groups, an indication for the mobilization of B-cell memory in response to primary vaccination was shown by the significantly increased antibody responses following a second DNA administration. That the increased antibody response following the second immunization reflects a booster response, was shown in a previous study for i.m. + i.n.-immunized turkeys.[3] Evidence for the mobilization of T-cell memory in response to challenge was shown by the significantly increased PBL proliferative responses following challenge when compared to the placebo-vaccinated control group 5. The best protection occurred in i.m. + i.n.-, i.m.- and aerosol-vaccinated turkeys (groups 1, 2 and 4, respectively), which upon challenge did not demonstrate secondary antibody responses as the bacteria were probably not able to proliferate to boosting levels. Eighteen days PC, 60%, 40% and 60% of turkeys of groups 1, 2 and 4, respectively, showed weak (low mean scores) chlamydophila replication in the respiratory tract and no replication outside the respiratory tract. The remaining turkeys of these three groups were chlamydophila negative at this time-point and thus seemed to have cleared the infection. Not surprisingly, this contrasts with results of our former study (which used the same *C. psittaci* strain for challenge and where all i.m./i.n.-vaccinated turkeys remained positive for at least 18 days PC), as a 10^4 higher challenge dose was used. Therefore, protection by DNA vaccination was even more efficient when using experimental infective doses that are closer to those of non-experimental situations.

In all turkeys inoculated with a mixture of pMOMP and pIFN-γ, chlamydophilae replicated intensively inside and outside the respiratory tract, resulting in 80% positive cloacal and nasal cultures from postinfection day 12 to the end of the observation period. Both the T-cell response and the antibody response were reduced in the presence of tIFN-γ. Similar findings were described by Xiang & Ertl, who tested the effect of co-inoculation of inbred mice with the rabies virus G-protein-expressing plasmid and a plasmid encoding mouse IFN-γ.[13] A potential explanation for the reduced immune response might be the negative effect of tIFN-γ on the viral (cytomegalovirus) promoter driving expression of the *C. psittaci omp1* gene. However, Xiang *et al.*[14] found no such negative effect when testing the *in vitro* influence of tIFN-γ on the expression of rabies virus G-protein in cells stably transfected with pSG5rab.gp, expressing the rabies virus G-protein under control of the SV40 early promoter. In the present study, the *in vitro* effect of tIFN-γ on the CMV promoter (driving expression of MOMP) was not evaluated.

Initiation of cell-mediated immune responses following plasmid DNA immunization requires the presentation of

antigen by professional antigen-presenting cells (APCs). How APCs acquire antigen is less clear. Professional APCs could acquire antigen through direct transfection and expression within the APC, or via the uptake of exogenous antigen synthesized within other transfected cell types. The presentation by APCs of endogenously synthesized antigen and exogenously acquired antigen is not mutually exclusive, and both mechanisms may be operative following *in vivo* gene delivery. Although the relative contribution of each mechanism to prime is unclear, it is not improbable that antigen release from transfected non-APCs could potentiate or prolong the resulting immune response, especially when these transfected cells are targeted by the first wave of antigen-specific cytotoxic T cells. Evidence for immune-mediated destruction of muscle fibres following direct gene transfer with antigen-expressing plasmid DNA has indeed been found and supports this hypothesis.[15] In view of the above, tIFN-γ-induced up-regulation of MHC I determinants on muscle cells might have resulted in a more efficient and rapid clearance of transfected MOMP-expressing muscle cells, which might consequently have given a shortened and reduced release of recombinant MOMP. The latter might explain the weak immune responses observed in pMOMP/ptIFN-γ-immunized animals as less endogenous antigen became available to the APCs.

In conclusion, the use of MOMP-based DNA vaccination as a means of preventing severe clinical signs, lesions and bacterial replication and excretion in a turkey model of *C. psittaci* infection was demonstrated, as well as down-regulation of the immune response, by co-expression of tIFN-γ during vaccination.

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