Deletion of open reading frames 9, 10 and 11 from the avian adenovirus CELO genome: effect on biodistribution and humoral responses

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In this study, the in vivo effect of the 3-6 kbp deletion of the three open reading frames (ORF) 9, 10 and 11 found at the right end of the CELO genome was examined. Groups of chickens were inoculated oronasally with 10^5 –10^7 p.f.u. per animal of wild-type virus and two recombinant CELO strains (rCELO) expressing luciferase and secreted alkaline phosphatase (SEAP). The tissue biodistribution, assessed by PCR, was similar for both wild-type and recombinant viruses. The infectious viral particle titre was determined by a p.f.u. counting method and the antibody responses to the CELO vector and the SEAP antigen were evaluated by ELISA. Infectious particle titres in tissues from chickens inoculated with the wild-type CELO virus increased up to 6 days post-inoculation, and declined until 11 days while titres in organs from chickens inoculated with the rCELO strain were low and only detectable at 4 days post-inoculation. Moreover, although anti-CELO antibody levels were three times lower in sera from chickens inoculated with rCELO, antibodies directed to the heterologous SEAP antigen were detected. Based on these results, no differences in tropism were observed, but the level of production of viral particles and the humoral responses appeared to decrease. Viruses replicate less efficiently with a deletion performed at the right end of the CELO genome. Nevertheless, the presence of antibodies directed to heterologous antigens makes the CELO virus an advantageous candidate for avian vaccination.

INTRODUCTION

Vaccination in poultry farming is commonly associated with the use of classical vaccines such as whole inactivated viruses or live attenuated viruses. Nevertheless, the emergence of several new antigenic and highly virulent viral strains in broilers, observed for some viruses, such as Infectious bursal disease virus (IBDV) (Brown et al., 1994), has led researchers to develop new kinds of vaccines. Various modalities are under study: subunit vaccines such as protein or naked DNA and recombinant vaccines using viruses or bacteria (Kesik et al., 2004; Lewis & Babiuk, 1999; Lo, 1987).

Members of the family Adenoviridae are non-enveloped, icosahedral viruses, with a linear, 26–45 kbp, double-stranded DNA molecule (Horwitz, 1996; Rekosh et al., 1977; Stewart et al., 1993). As their genome is delivered into the nucleus and can replicate very efficiently, adenoviruses represent advantageous candidates for the expression and delivery of therapeutic genes (Benihoud et al., 1999; Graham, 1990; Hitt et al., 1997; Zhang, 1999). Their genome can be easily modified, and they can be produced at high titres in vitro. Recombinant adenoviruses from various species (human, porcine, bovine, canine and avian species) have therefore been developed as gene delivery vectors for vaccination and gene therapy (Hammond et al., 2000; Klonjkowski et al., 1997; Michou et al., 1999; Paillard, 1997; Rasmussen et al., 1999; Reddy et al., 1999; Sheppard, 1999).

Among fowl adenoviruses (FAdV), the serotype I chicken embryo lethal orphan (CELO) virus or FAdV-1, has been widely studied (Chiocca et al., 1997; Laver et al., 1971; Lehrmann & Cotten, 1999; Michou et al., 1999). There are several advantages of using the CELO virus as a new viral vector for vaccination in poultry farming. It has never been associated with any economic losses or major pathologies in chickens. In fact, CELO virus can be isolated from healthy chickens, and does not cause any evident disease when it is experimentally introduced into chickens (Cowen et al., 1978). Its genome has been completely sequenced and its transcriptional organization has been established (Chiocca et al., 1996; Payet et al., 1998). Moreover, molecular tools have been developed in our laboratory to construct recombinant CELO strains (rCELO) expressing heterologous genes from various pathogens (Francois et al., 2001). We have shown recently that specific pathogen-free
(SPF) chickens vaccinated with a rCELO virus expressing the gene encoding the major capsid protein VP2 of IBDV inserted in the left end of the CELO genome (rCELOa-VP2), were protected from a challenge with the pathogenic virus (Francois et al., 2004). Nevertheless, although the molecular biology of the CELO virus has been well characterized, virus–cell and virus–host interactions are still poorly understood. The mechanisms of CELO infection, target organs and immune responses induced by wild-type (wt) and rCELO strains need to be investigated.

We have previously examined the in vitro replication of the CELO virus and the effect of deletions at various regions of its genome on the in vitro growth of the virus (Francois et al., 2001). Three open reading frames (ORF) 9, 10 and 11 were deleted from the right end of the CELO genome: this 3-6 kbp deletion did not inhibit in vitro virus replication (rCELOd) (Francois et al., 2001; Michou et al., 1999). The aim of this study was to determine whether deletion of these three ORFs had any effects on in vivo virus replication, biodistribution and host immune responses. For this purpose, SPF chickens were inoculated oronasally to mimic the natural route of infection in poultry. We compared the replication and biodistribution of wtCELO virus and rCELOd strains expressing heterologous genes in infected chickens. The humoral responses directed to the various viruses were also analysed.

**METHODS**

**Construction of rCELO vectors.** rCELO viruses were constructed as previously described (Francois et al., 2001). Briefly, the CELO genome was first cloned into plasmid pPolyII by homologous recombination in *Escherichia coli* to generate a viral genome with single restriction sites (i.e. *PacI* and *Ascl* sites) at both ends. With the presence of other unique restriction sites (*Pmel*, *NotI* and *FseI*), the CELO genome can be divided into four fragments named A (*Pmel–Pmatl* 7430 bp), B (*Pmel–NotI* 9956 bp), C (*NotI–FseI* 18298 bp) and D (*FseI–Ascl* 8116 bp) (Fig. 1). A 3-6 kbp deletion, between positions 40065 and 43685 of the CELO genome, was created in the D-terminal region of the CELO genome. This strain has been described previously as CELO AIM70 to be replication competent in vitro (GenBank accession no. U46933) (Michou et al., 1999). Three ORFs were deleted: 9, 10 and 11. The reporter genes encoding the human secreted alkaline phosphatase (SEAP) or luciferase (luc) was inserted into the gap under the control of the immediate-early cytomegalovirus promoter (pCMV). The new rCELO strains were, respectively, called rCELOd-seap and rCELOd-luc (Fig. 1). After modification, the cloned CELO genome was excised from the plasmid and ligated with the cosΔ1 cosmid vector, which was linearized by *PacI* and *Ascl* digestions. The ligation reaction generated concatenated DNA molecules, with cos sites separated by 50 kb from each other, which allowed for packaging into λ bacteriophage heads. The ligation product was then packaged and amplified in *E. coli*, resulting in the cos/CELO DNA cosmid that contained the full-length CELO genome.

**In vitro production of rCELO viruses.** Leghorn male hepatocarcinoma (LMH) cells (ATCC CRL-2117) were directly transfected with the recombinant cos/CELO DNA (Francois et al., 2001) preparation using the Polyfect transfection reagent kit (Qiagen) according to the manufacturer’s recommendations. LMH cells were seeded the day before transfection into six-well plates at 6 × 10⁵ cells per well in 2 ml Williams’ Medium E (Invitrogen) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. Transfection efficiency was assessed by transfecting cells with pcDNA3.1/zeo(+) (Invitrogen) containing the gene encoding the enhanced green fluorescent protein (eGFP). Cell fluorescence was observed 12–18 h post-transfection. After 4–12 days, cytopathic effects were observed depending on the rCELO strain. Cells were lysed by three freeze-thaw cycles and lysate clarified by centrifugation. Supernatants containing viruses were used to infect freshly plated LMH cells. After this second in vitro passage, viral stocks were stored at −80 °C. Before use, DNA restriction endonuclease digestion profiles of viruses were analysed.

**Titration of infectious viral particles.** Viral stocks were titrated using a standard p.f.u. counting method (Francois et al., 2001; Graham & Prevec, 1995). LMH cells were seeded 2 days before infection into six-well plates at 6 × 10⁵ cells per well in 2 ml Williams’ Medium E supplemented as described above. Serial dilutions of viral stocks were prepared in Williams’ Medium E supplemented with 2% FCS, and added to confluent LMH cells. After a 2 h adsorption step at 37 °C, cells were washed in PBS and covered with 2·5 ml Williams’ Medium E supplemented with 10% FCS and 1% non-essential amino acids and 1% sodium pyruvate.

**Fig. 1.** Construction of rCELO vectors. The CELO genome can be divided into four fragments named A to D. A 3-6 kbp deletion was created in fragment D and the genes encoding luciferase and SEAP were inserted in this gap.

**Fig. 2.** Plaques observed on LMH cells infected after 4 days of incubation. Cells were seeded into a six-well plate and were infected with serial dilution of viral stock. After a 2 h incubation at 37 °C, cells were covered with Williams’ Medium E supplemented with 10% FCS and 1% low-melting-point agarose. After 4 days, living cells were stained with 0.05% neutral red solution and plaques were counted. Countable plaques are observed in (b) and each plaque corresponds to one infectious viral particle. No plaques can be seen in the control well (a). The presence of too many infectious viral particles (c) did not allow us to determine the infectious titre.
low-melting-point agarose (SeaPlaque agarose; FMC Bioproducts). Living cells were stained with 0·05 % neutral red solution (Sigma) 4 days after infection, and the plaques were counted (Fig. 2).

**Column purification of viral particles.** wtCELO and rCELOd-seap viral stocks were column-purified using the BD Adeno-X Purification kit according to the manufacturer’s recommendations. Briefly, the virus-containing solution was diluted in the provided dilution buffer and pushed through a purification filter. Adsorbed adenoviral particles were then washed and eluted. Purified particles were quantified by protein content (1 mg ml$^{-1} = 3·4 \times 10^{12}$ virus particles ml$^{-1}$) (Lemay et al., 1980) and by the p.f.u. counting method as described above. It was observed that 1 in 100 virus particles was infectious.

**Virus challenge.** Three in vivo trials were carried out on either 1-day-old or 5-week-old SPF White Leghorn chickens (AFSSA Ploufragan) (Table 1). Chickens were challenged with the wtCELO virus, rCELOd-luc (trial b) or rCELOd-seap (trials a and c). Chickens were inoculated oronasally using 10$^5$ or 10$^3$ p.f.u. of viruses per animal. Fifteen non-inoculated animals were used as negative controls.

In ovo inoculation was performed on 9-day-old embryonated SPF White Leghorn eggs (Ploufragan). Eggs were assigned into four groups of eight eggs. The first group of eggs were inoculated in allantoic fluids with 0·1 ml 10$^5$ p.f.u. of rCELOd-seap or wtCELO column-purified viral particles, the second one with 0·1 ml 10$^3$ p.f.u. of rCELOd-seap or wtCELO column-purified viral particles diluted in LMH cell supernatant, the third one with 0·1 ml 10$^5$ p.f.u. of rCELOd-seap or wtCELO virus clarified lysate. The last group of eggs were inoculated with 0·1 ml LMH cell supernatant. Viral plaques were counted after 3 days of infection.

**PCR analyses of in vivo CELO distribution.** PCR analyses were performed to test the presence of CELO viral DNA extracted from several tissues (i.e. trachea, thymus, liver, spleen, caeca, intestine and bursa of Fabricius) sampled at 1, 4, 6, 10, 20 and 30 days post-inoculation (p.i.) with wtCELO virus and rCELOd-seap. DNA was extracted using the Qiagen DNAeasy tissue kit according to the manufacturer’s recommendations. PCR primers, forward 5′-CGGTGTTCCACACAGC-3′ and reverse 5′-GTTGAAAGGATTGA-TTGAAGTTGTCGTC-3′, were designed from the sequence encoding the hexon capsid protein of CELO and generated a 284 bp product. Amplification reactions were performed in a 50 µl mixture containing 0·2 µM each primer, 0·1 mM each dNTP, 0·3 U AmpliTaq Gold (Applied Biosystem) with the following cycling parameters: 10 min denaturation step at 95 °C followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s with a final elongation step at 72 °C for 10 min. PCR products were analysed on a 2 % agarose gel in 1× TAE (40 mM Tris-acetate, 1 mM EDTA). Samples were considered positive for the presence of CELO genome when a 284 bp product was detected.

**Isolation of viral particles.** Whole organs and tissue samples isolated from experiments b and c were homogenized using a Mixer Mill MM 300 (Qiagen). Briefly, organs were cut into small pieces of 100 mg and dispersed in 1 ml Williams’ Medium E supplemented with 10 % FCS containing two 3 mm diameter beads. Samples were homogenized twice for 2 min at 20 Hz and centrifuged for 5 min at 11 000 g at 4 °C. After two further 2 min cycles at 20 Hz, samples were centrifuged for 10 min at 11 000 g at 4 °C. Supernatants were collected and filtered through a 0·45 µm filter. Samples were stored at −20 °C until titration.

**Measurement of luciferase activity.** Twenty milligrams of each organ collected from chickens at day 1, 4, 6, 8, 11, 15 and 20 p.i. were homogenized in 500 µl lysis buffer supplied by the luciferase assay system (Promega). Freeze–thaw cycles achieved complete cell lysis, and supernatants were stored at −70 °C. Each sample (20 µl) was incubated with 100 µl luciferase reagent buffer, and light emission was measured immediately on a microplate luminometer LB 96P (Berthold) for 5 s, with the signal summed over that time period. Positive results were expressed in relative light units (RLU) compared with negative samples, treated under identical conditions.

**Humoral immune responses induced in serum by wtCELO and rCELOd-seap.** Purified human placental alkaline phosphatase (0·5 µg; SEAP) (Sigma), 10 µg UV-inactivated wtCELO virus or 10 µg total LMH proteins were coated overnight at 4 °C in carbonate buffer (0·05 M NaHCO$_3$, pH 9·6) in 96-well Maxisorp Nunc immunoplates (Rochester). Plates were washed three times with 200 µl 0·05 % Tween 20 in PBS (PBST), and blocked with 3 % bovine serum albumin (BSA) and PBST solution for 1 h at 37 °C to reduce non-specific reactions. After three washes with PBST, 100 µl of serially diluted sera in 1 % BSA and PBST were added to wells. Plates were first incubated for 2 h at 37 °C and then washed three times in PBST. After a 2 h incubation with 1:4000 diluted goat anti-chicken IgG antibody, labelled with horseradish peroxidase, plates were washed three times and 100 µl O-phenylenediamine dihydrochloride substrate (Sigma) in urea buffer was added. Plates were incubated for 15 min at 37 °C before the addition of 50 µl 0·5 M H$_2$SO$_4$ to stop the reaction. Absorbance was measured at 492 nm. Titres were compared with negative sera, treated under identical conditions.

**Statistics.** Statistical analyses were performed using the two sample KS (Kolmogorov–Smirnov) or the Kruskal–Wallis non-parametric tests from the SYSTAT 9 computer software package (SPSS).

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**Table 1. Experimental procedures**

<table>
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<tr>
<th>Experiment</th>
<th>Virus</th>
<th>No. chickens</th>
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<td>wtCELO</td>
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<td></td>
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<tr>
<td></td>
<td>rCELO-seap</td>
<td>15</td>
<td>10$^7$</td>
<td></td>
<td>Oronasal</td>
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RESULTS

Effects of LMH cell supernatant on virus replication

The different viral strains used were produced on LMH cells. In order to quantify the impact of the LMH cell supernatant on the virus replication, the relative efficiency with which wtCELO and rCELO-seap replicate in ovo was compared. This experiment was performed using both clarified viruses prepared directly from infected-LMH-cell lysates and viral particles that had been purified on BD AdenoX columns. The adherent columns do not differentiate between infectious and non-infectious viral particles. Titres of infectious viral particles were determined by plaque assay: approximately 1 in 1000 purified rCELOd-seap or wtCELO particles was capable of generating a plaque (data not shown). Purified infectious viral particles (10^5) supplemented or not with LMH cell supernatant, 10^5 of clarified viruses or with LMH cell supernatant alone were inoculated into allantoic fluids of 9-day-old embryonated eggs. Allantoic fluids were collected 3 days p.i. and viral titre determined by plaque assay. No infectious viral particles were observed in eggs inoculated with LMH cell supernatant (Fig. 3 lane a). However, the results showed that wtCELO and rCELOd-seap replicate in ovo. Moreover, no significant differences in viral titre were observed between the different viral strains or the different treatments.

In vivo genomic detection and biodistribution of wtCELO and rCELOd-seap

Eighty-five chickens were assigned into three groups: the first group was inoculated with the wtCELO virus, the second was inoculated with rCELOd-seap, and in the third group, 15 non-inoculated animals were used as negative controls. Chickens were oronasally inoculated with 10^5 p.f.u. per animal in 0·1 ml PBS of wtCELO virus or rCELOd-seap (Table 1, experiment a). Five chickens from each group were sacrificed at each time point up to 30 days p.i. and tissue samples (i.e. trachea, thymus, liver, spleen, intestine, caeca and bursa of Fabricius) were grossly examined. No obvious macroscopic lesions were observed in these organs. DNA of tissue samples from three of these chickens was extracted and subjected to PCR analyses. Results are expressed as the number of PCR-positive chickens/total tested chickens (Table 2).

Viral DNA was detected 1 and 4 days p.i. in all tested tissues with trachea, caeca and bursa of Fabricius being positive for viral DNA in all samples. Viral DNA could be detected in the thymus, spleens, livers and intestines although less reliably and rapidly declined. At 6 days p.i., the CELO genome was detected in trachea, caeca, bursa of Fabricius and intestine in one chicken of the wtCELO-inoculated group. At 10 and 20 days p.i., viral DNA was only observed in caecal tissues. Viral DNA was no longer detected in any of the tested organs after 20 days p.i. No major differences were observed between the two groups of inoculated chickens. Viral DNA was not detected in any of the tissue samples collected from the non-inoculated group (data not shown).

Table 2. Detection of positive viral PCR products in chickens

<table>
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<tr>
<th>Days p.i.</th>
<th>Trachea</th>
<th>Thymus</th>
<th>Liver</th>
<th>Spleen</th>
<th>Intestine</th>
<th>Caeca</th>
<th>Bursae of F.</th>
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**In vivo replication of wtCELO and rCELOd-seap**

The presence of infectious viral particles was then assessed in all organs, collected in the same experiment described above (Table 1, experiment a), from wtCELO and rCELOd-seap-infected chickens (Fig. 4). At 1 day p.i., no infectious particles were detected in samples from chickens inoculated with either wtCELO virus or rCELOd-seap strain. Infectious viral particles were detected for the first time at 4 days p.i. in the two inoculated groups. The highest titres were obtained in tracheal or caecal tissues from chickens inoculated with wtCELO virus or rCELOd-seap strain. However, the infectious particle load was higher in the wtCELO-inoculated group ($6 \times 10^5$ p.f.u. g$^{-1}$) than in the rCELOd-seap-inoculated group ($2 \times 10^3$ p.f.u. g$^{-1}$).

Viral titres ranging from $2 \times 10^4$ to $4 \cdot 41 \times 10^2$ p.f.u. g$^{-1}$ were detected in liver, spleen, intestine and bursa of Fabricius from the two inoculated groups (Fig. 4).

From 6 days p.i., viral particles were no longer detected in tested organs from the rCELOd-seap-inoculated group, while infectious viral particles were still detected in organs from the wtCELO-inoculated group. In this last group, the level of infectious particles in the tracheae remained similar to those observed at 4 days p.i. ($10^6$ p.f.u. g$^{-1}$), whereas in caecal tissue, infectious particle levels increased slightly from $2 \cdot 2 \times 10^3$ to $4 \cdot 9 \times 10^3$ p.f.u. g$^{-1}$. Values observed in the other organs remained similar to those observed at 4 days p.i. ($1 \cdot 7 \times 10^4$ to $5 \cdot 6 \times 10^5$ p.f.u. g$^{-1}$).

After 6 days p.i., viral particle level rapidly declined in all tissues from both inoculated groups. At 8 days p.i., tracheal and caecal titres decreased to $7 \cdot 57 \times 10^2$ and $1 \cdot 72 \times 10^4$ p.f.u. g$^{-1}$, respectively. Infectious viral particles were still no longer detected in liver except in one chicken ($3 \times 10^1$ p.f.u. g$^{-1}$), whereas bursal titres remained stable at $6 \times 10^2$ p.f.u. g$^{-1}$. At 11 days p.i., viral particles were only detected in caecal tissues, ranging from $2 \cdot 1 \times 10^1$ to $9 \cdot 9 \times 10^1$ p.f.u. g$^{-1}$. No more infectious particles were observed after 11 days p.i. No viral particles were detected in organs from the non-inoculated group at any time points (data not shown).

**Expression of heterologous genes is detected in chickens inoculated with rCELOd-luc**

To test the capacity of CELO recombinants to promote transgene *in vivo*, 35 1-day-old SPF chickens were inoculated with either wtCELO or rCELOd-luc (Table 1, experiment b). The measure of the luciferase activity was assessed in trachea, thymus, liver, spleen, intestine, caeca and bursa of Fabricius at day 1, 4, 6, 8, 10 and 20 p.i. Results...
are shown in Fig. 5. The background level, expressed as RLU, was determined in tissue samples collected from chickens inoculated with wtCELO virus (21 RLU) and from the non-inoculated group (19 RLU) (data not shown).

Luciferase activity could be detected as early as 1 day p.i.; the highest activities were observed in tracheal and caecal samples ($1.26 \times 10^4$ and $1.03 \times 10^3$ RLU, respectively, Fig. 5). A lower activity was also detected in the bursa ($1.35 \times 10^2$ RLU), whereas no significant increase of activity was observed in spleen, liver or intestine. At 4 days p.i., luciferase activity decreased in the trachea while activity increased in caecal and bursal tissues. No changes were observed in spleen, intestine and liver. At 6 days p.i., luciferase activity declined in all tested samples except in the intestine. In the trachea, luciferase activity decreased to $1.92 \times 10^2$ RLU, and the activity in the bursa fell to a background level. However, the luciferase activity measured in caecal samples was less markedly decreased ($1.48 \times 10^3$ RLU). After 8 days p.i., luciferase activity was no longer observed in any of the tested samples (results not shown for 10 and 20 days p.i.).

**Humoral immune responses induced against the CELO virus and heterologous antigens**

One approach to evaluate the suitability of the CELO virus as a gene vector for vaccination is to measure antibody responses against the heterologous protein and the wtCELO vector. Five-week-old chickens were inoculated oronasally with rCELOd-seap and wtCELO. The antibody responses were analysed in sera from each chicken, collected every week for 4 weeks.

As shown in Fig. 6(a), no anti-CELO antibodies were detected during the first week in both inoculated groups. At 2 weeks p.i., IgGs were detected in wtCELO immunized chickens, whereas no anti-CELO antibodies were detected in chickens inoculated with the rCELO strain. At 3 and 4 weeks p.i., antibody titres continued to increase. Serum antibodies to rCELOd-seap appeared at week 3 (Fig. 6a), they were significantly three times lower than the antibody responses observed in the sera of the wtCELO-inoculated group ($P<0.08$). No anti-CELO antibodies were detected in sera from non-inoculated chickens.

The presence of IgGs directed to SEAP was also assessed (Fig. 6b): antibodies were detected 3 weeks p.i. and tended to decrease at the fourth week. No anti-SEAP antibodies were detected in sera from chickens inoculated with wtCELO or in sera from non-inoculated chickens. No response were detected against LMH protein extracts (Fig. 6c).

**DISCUSSION**

In this study, we describe the biodistribution and antibody responses of the avian adenovirus CELO. The potential of a CELO-based vector, with a 3·6 kbp deletion at the right end of the genome (D region), was examined by comparing virus replication and humoral responses in chickens oronasally infected with wtCELO virus and two rCELO strains.

In order to evaluate the impact of LMH cell supernatant on virus replication from viral stocks, in ovo inoculations with the different purified or clarified viral strains were performed. The results showed that the titres detected in allantoic fluids from both wtCELO and rCELOd-seap inoculated eggs were equal after 3 days of infection. These results confirmed that LMH cell supernatant had no effect on virus replication, and allowed the use of clarified cell lysate to inoculate chickens compared to, in a first step, the growth kinetics of the recombinant strain.
PCR performed on tissue samples from inoculated chickens showed that the wt and recombinant virus biodistribution was mainly tracheal, caecal and bursal. Most of the positive PCR results were obtained in these organs. Up to 4 days p.i., PCR products were also detected in spleen and intestine, but the number of positive samples rapidly declined. These results suggest that the virus had a transient occurrence in these organs. Moreover, the use of the rCELO-luc showed the presence of the recombinant viruses in tracheae, caeca and bursae of Fabricius. Luciferase detection also demonstrated that rCELO strains were able to produce a functional heterologous protein.

However, although PCR analysis is a sensitive and qualitative method to assess the presence of the CELO genome, it does not provide any quantitative information. CELO virus titres were therefore determined on organs from inoculated chickens. The results showed that wtCELO virus multiplied in tracheae, caeca and, to a lesser extent, bursae of Fabricius, while few infectious viral particles were observed in intestines and livers. Surprisingly, no viral particles were detected on the first day p.i., with either wtCELO or rCELO strain. The threshold of detection appeared not to be sensitive enough to detect low levels of infectious viral particles.

Nevertheless, titres obtained in tissues from chickens inoculated with the wtCELO virus rapidly increased up to 6 days p.i., and declined until 11 days p.i. while the levels in organs from chickens inoculated with the rCELO strain were very decreased by the 3′-6 kb deletion of the CELO genome and the insertion of a transgene.

Indeed, the recombinant strains were constructed by inserting genes in the gap at the right end of the CELO genome (Francois et al., 2001). Three ORFs were deleted: 9, 10 and 11. Recently, Washietl & Eisenhaber (2003) identified these ORFs as a cluster of genes encoding three putative type-I transmembrane glycoproteins with Ig-like domains. Ig-like domains are known to interact with proteins involved in the recognition processes in the immune system (Williams & Barclay, 1988). The E3 region of the genome of human adenoviruses has been reported to be involved in the escape from the host’s immune responses, and several E3 products have been characterized as Ig-like domains (Deryckere & Burgert, 1996; Windheim & Burgert, 2002). However, no similar E3 region was found in the CELO genome. These ORFs could therefore be significant candidates to substitute the immunosuppressive functions of the CELO virus. Moreover, comparison of the ORF9 sequence and a chicken library showed similarities with an expressed sequence tag (Washietl & Eisenhaber, 2003), suggesting the hypothesis that the CELO virus has acquired an immune receptor from the host and has used it to escape immune mechanism. E3 proteins are involved in the inhibition of presentation of antigenic protein via the major class I histocompatibility complex (MHC-I). It has also been shown that within 1 week human adenoviruses are able to induce MHC-I cytotoxic lymphocytes directed to adenoviral proteins (Yang et al., 1994, 1995). The analyses of viral titres, performed on organs from chickens inoculated with wtCELO virus or rCELO strains, established that the wtCELO virus replicated more efficiently in vivo than the recombinant viruses, while no major differences were observed in the in vitro growth of the virus on L929 cell culture (Francois et al., 2001). This could be explained by the putative immunosuppressive role played by these three ORFs.

The use of PCR-based detection and viral titrations directly from tissues demonstrated the absence of differences in the in vivo targets of the CELO virus and its recombinant strains, as all strains showed a similar viral tropism. Infection tropism is related to the presence of fibres on the virus surface (Nakamura et al., 2003), and no genomic modifications were performed in the genes encoding the fibres of the CELO capsid. One particularity of the CELO virus is the presence of two fibres of different lengths on each penton base (Chiocca et al., 1996). It has already been shown that human adenoviruses serotype 40 and 41 also have two fibres (Favier et al., 2002). These adenoviruses infect gastrointestinal epithelial cells (Brandt et al., 1979; Uhnoo et al., 1984) and, recently, the enteric tropism was related to the particular physico-chemical properties of these fibres (Favier et al., 2004).

Actually, bursa of Fabricius and caecal tonsils are both lymphoid organs but no clinical signs of immunodeficiency were observed in poultry. The results of the CELO virus biodistribution suggest that the main target cells could be epithelial cells. However, in vitro infection of isolated lymphoid cells from caeca or bursa demonstrated that some cells are also permissive to the virus (Le Goff et al., 2003). These cells could correspond to secondary targets or vehicles for viral propagation. Histological studies would confirm this hypothesis.

With the aim to use the CELOd based vector in poultry vaccination, antibody responses against the CELO virus after inoculation with wt virus and rCELOd-seap were examined. Only limited data are currently available concerning the basic immunology of avian adenovirus viral infection and further studies are required in this field. Our results agree with those of previous studies, indicating that the kinetics of appearance of avian adenovirus antibodies are characterized by higher titres 3 or 4 weeks after oral inoculation (Maiti & Sarkar, 1997; Ojkic & Nagy, 2003).

However, the kinetics of appearance of anti-CELO antibodies differed between the wtCELO virus and the recombinant strain. Antibody titres were three times higher in sera from wtCELO-inoculated chickens and were observed 2 weeks p.i. whereas anti-rCELOd antibodies appeared 3 weeks p.i. This difference observed between the two strains was probably related to viral propagation, which was decreased by the deletion performed at the right end of the
CELO genome. Nevertheless, the significant level of anti-SEAP antibodies is a promising element in favour of the use the rCELOd strain as a gene delivery vector despite the fact that virus replication is significantly decreased.

In this study, we examined the biodistribution of the CELO virus and its rCELOd strains. No differences in tropism were observed, but the production level of viral particles and the immune responses appeared to be decreased significantly. Viruses replicate less efficiently with deletion of ORFs 9, 10 and 11 at the right end of the CELO genome. Nevertheless, the production of antibodies against an heterologous antigen was observed. Based on these results, the CELO virus represents an advantageous candidate for avian vaccination. We are now interested in identifying the type of cells that are infected by the CELO virus, to define viral–host interactions and to understand the immune response induced by the CELO-based vectors.

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REFERENCES


