jfms

Modified vaccinia virus Ankara as a vaccine against feline coronavirus: immunogenicity and efficacy

Matthias Hebben[a]*, Véronique Duquesne[a], Joëlle Cronier[a], Bernard Rossi[b], André Aubert[a]

[a]*Laboratoires VIRBAC, Recherche et Développement Biologie, BP 447, 06515 Carros cedex, France*
[b]*Institut National de la Santé et de la Recherche Médicale, Unité 364, Laboratoire d'Immunologie cellulaire et moléculaire, Faculté de Médecine Pasteur, Nice, France*

Summary Feline infectious peritonitis virus (FIPV) is a coronavirus that induces a fatal systemic disease mediated by an inappropriate immune response. Most previous vaccination attempts against FIPV were unsuccessful because IgG antibodies against the surface protein enhance the infection. However, two studies have shown that poxvirus vectors (vaccinia WR and canarypox) expressing only the FIPV membrane (M) protein can elicit a partially protective immunity which is supposed to be cell-mediated (Virology 181 (1991) 327; International patent WO 97/20054 (1997)). In our study, we report the construction of another poxvirus, the modified vaccinia virus Ankara (MVA), as an expression vector for the FIPV M protein. In this vector, the M gene has been inserted downstream a strong early/late promoter, whereas the two previously described poxviruses expressed the M protein during their early stage only. The immunogenicity of the recombinant MVA-M was evaluated in the murine model which revealed an effect of the vector on the Th1/Th2 balance. The vaccine was then tested in cats to evaluate its efficacy in an FIPV 79-1146 challenge. Vaccinated kittens developed FIPV-specific antibodies after immunization, however, none of them was protected against FIPV. Our results suggest a crucial role for the type of poxviral promoter that must be used to induce an effective immune response against FIPV.

Introduction

Feline coronaviruses (FCoVs) cause either a mild enteritic disease or a fatal peritonitis in cats. They are thus classified in two groups depending on their pathogenicity: feline enteritic coronaviruses (FECVs) and feline infectious peritonitis viruses (FIPVs) (see for review: Olsen, 1993). Several vaccination trials against the lethal FIPV strains have been attempted but they were often unsuccessful due to a problem of antibody-dependent enhancement (ADE) of the disease that leads to early death. In contrast, cats naturally resistant to FIPV present a strong cell-mediated immunity (CMI) and a weak antibody response (Pedersen, 1987). Therefore, an efficient vaccine should preferentially induce a CMI, also called Th1-type response, characterized by CD4+ helper T-lymphocytes secreting interleukin 2 (IL-2) and Interferon gamma (IFN-γ) (Th1 cells) and by CD8+ cytotoxic T-lymphocytes (CTLs).

---

* Corresponding author. Tel.: +33-4-92-08-79-28; fax: +33-4-92-08-71-99

  E-mail address: mhebben@virbac.fr (M. Hebben).

The FIPV virion contains three major structural proteins: the 200 kDa spike glycoprotein (S), the 30 kDa Membrane protein (M) and the 45 kDa nucleoprotein (N). The S protein has been shown to be responsible of ADE and thus does not induce a protective immune response (Vennema et al., 1990). In contrast, the M protein, but not the N protein, is partially protective when expressed by poxvirus vectors like vaccinia or canarypox viruses (Paoletti and Gettig, 1997; Vennema et al., 1991). Such protection occurred in the absence of neutralizing antibodies, and was thought to be due to CMI.

These canarypox and vaccinia-based vaccines expressed the M protein only during the early stage of their viral cycle. This is because canarypox does not achieve its late stage in mammalian cells, and because the M gene was inserted downstream the early promoter P7.5 in vaccinia.

In this study, we report the use of another poxvirus vector, the modified vaccinia virus Ankara (MVA), which recently showed very promising results as a vaccine against simian–human immunodeficiency virus (Amara et al., 2002). MVA is an attenuated strain of the vaccinia virus Ankara, that has lost the ability to replicate in mammalian cells (Sutter and Moss, 1992). Thus MVA is safe but remains a strong expression vector because it can still express foreign antigens during both the early and late stages of its life cycle. Herein, we used this feature to construct a recombinant MVA expressing the M protein (MVA-M) during both stages in order to synthesize larger amounts of antigen compared to the canarypox-M and vaccinia-M vaccines.

The immunogenicity of this enhanced expression vector was tested in vivo. A preliminary trial in the murine model showed an effect of the MVA on to the Th1/Th2 balance. These data were taken in account to design a cat trial, in which animals were challenged with the virulent FIPV strain 79-1146 (Pedersen et al., 1984) to evaluate the efficacy of the MVA-M vaccine.

Materials and methods

Viruses and cells

MVA (kindly provided by B. Moss, NIH) and recombinant MVA are cultured on primary chicken embryo fibroblasts (CEF), in Eagle minimum essential medium (MEM, Gibco Brl) supplemented with 10% fetal calf serum (FCS). For vaccination studies, both viruses are purified from infected cell lysates by centrifugation at 35,000 g for 1 hour and 30 minutes (Beckman L8-M, rotor 45Ti) through a 36% sucrose cushion.

FIPV strain 79-1146 (kindly given by N. Pedersen) is propagated on *Felis catus* whole fetus-4 (Fcwf-4) cells in Dulbecco's modified Eagle medium (DMEM, Gibco Brl) with 10% FCS. Virus-containing culture supernatant was used for cat challenge after titration. For ELISA antigen and splenocytes stimulation, cell culture supernatants were inactivated with β-propionolactone and centrifuged at 100,000 g. Pelleted virus was resuspended in phosphate buffered saline (PBS) and stored at −80 °C.

M gene cloning into MVA transfer plasmid

The VR-M plasmid containing the M gene cDNA from FIPV 79-1146 was kindly given by the Veterinary University of Utrecht (Vennema et al., 1991). The M gene was isolated by digesting VR-M with *Bam*HI and *Hinc*II, and was then inserted into *Bam*HI and *Hinc*II treated pLW-9 plasmid (obtained from the NIH) (Wyatt et al., 1996). PLW-9 contains the modified early/late promoter from the H5R gene of vaccinia virus (PH5) and two flanks targeting the recombination into the deletion III of the MVA genome.

Generation of recombinant viruses

Recombination was performed as described elsewhere with some modifications (Wyatt et al., 1996): briefly, a monolayer of nearly confluent CEF were infected by MVA at a multiplicity of 0.5 plaque-forming units (pfu) per cell and then transfected for 7 hours by pLW9-M (5 μg per 35 mm well) using DOTAP (Roche). Cells were harvested 48 hours later, lysed by freezing–thawing and sonication. Recombinant viruses expressing the M protein were detected by immunostaining infected CEF with the ascite of an FIPV infected cat. Specific antibodies were revealed by peroxidase conjugated anti-cat IgG (Jackson) and 3-amino-9-ethyl carbazol as substrate. After six successive plaque purifications, stocks were prepared on CEF.

Titration of the MVA-M stocks were performed by double staining both with the ascite of the FIPV-infected cat and with rabbit anti-vaccinia antibodies (Jackson).

Protein expression analysis

CEF were infected by MVA-M or wild type MVA at a multiplicity of 3 pfu/cell. Twenty-four hours later, the supernatants were centrifuged at 100,000 g for 2 hours (Beckman Optima TL centrifuge, rotor TLA 100.2) in order to harvest the cell debris containing the M protein. The pellets were loaded on a NuPAGE 4–12% gel (Novex) and then analyzed by Western

blot using two monoclonal antibodies specific to the FIPV 79-1146 M-protein (precisely the B5D10H5 and B15C12C7 antibodies which were made in our laboratory), followed by an alkaline phosphatase-conjugated anti-mouse IgG (Southern Biotechnology Associates). Nitro-blue-tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Sigma) were used as phosphatase substrate.

Animals and immunizations

Mice

All the studies were carried out on 7-week-old female BALB/c mice (IFFA CREDO, France). Animals were specific pathogen free (SPF).

Groups of five mice were vaccinated subcutaneously with 200 µl of vaccine containing 10^8 pfu of purified MVA or MVA-M in phosphate buffered saline (PBS).

Blood samples from retro-orbital plexus and spleens were collected either two or four weeks after the last immunization.

Cats

Fifteen specific pathogen free European female kittens were purchased from Harlan US. Animals were aged eight to nine weeks at the beginning of the experiment. Two groups of five cats were immunized subcutaneously with one injection (at week 0 of the experiment) or two injections (week 0 and week 3) of 10^8 pfu of recombinant MVA-M in 1 ml of PBS. Five control cats were non-vaccinated.

Cat challenge protocol

Six weeks after the beginning of the study, all the cats were inoculated oronasally with 220 $TCID_{50}$ of FIPV 79-1146 in 1 ml of DMEM 10% FCS.

Blood samples were collected from animals anaesthetized with Tiletamine and Zolazepam (Zoletil® 50, Virbac), at the vaccination and challenge times, in order to analyze the FIPV-specific serology.

Clinical signs (anorexia, icterus, anemia, prostration and peritoneal swelling) were monitored daily and hematological parameters were analyzed weekly.

Animals were sacrificed six weeks after the challenge day or when they were deemed moribund.

Determination of the antibody response

Antigen-specific IgG antibodies were quantified by enzyme-linked immunosorbent assay (ELISA).

Briefly, microtiter plates (Dynex Immulon 2) were coated with 100 µl of purified FIPV 79-1146 at a concentration of 5 µg/ml in carbonate buffer, and incubated overnight at 4 °C. Wells were washed with PBS 0.2% Tween 20 and saturated with 1% BSA during 1 hour at 37 °C. After washing, three-fold serial dilutions of cat sera or mouse sera were incubated in the wells for 1 hour at 37 °C. FIPV-specific murine IgG, IgG1 and IgG2a were then detected with appropriate peroxidase-conjugated goat anti-mouse antibodies (Southern Biotechnology Associates) diluted 1:5000 in TNE 0.1% BSA, for 1 hour at 37 °C. For detection of feline IgG, a peroxidase-conjugated sheep anti-cat IgG (Cappel, Organon Teknika) was diluted 1:3000. The plates were then washed five times, and bound peroxidase activity was revealed with 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) for 10 minutes at room temperature. Absorbance was measured at 405 nm. Antibody titers were defined as the higher serum dilution having an absorbance greater than three times the blank value.

Analysis of murine cytokine mRNA

Spleens of mice from a same group were pooled and crushed with a syringe. Mononuclear white cells were isolated by centrifugation on Ficoll at 620 g for 20 minutes (Jouan GR-422). Recovered splenocytes were then stimulated for 48 hours in RPMI medium complemented with 5% FCS, 50 IU/ml penicillin, 50 µg/ml streptomycin (Gibco) and 10 µg/ml of inactivated FIPV, or 2 µg/ml of concanavalin A (Sigma) as positive control. Then total RNA from 10^7 splenocytes was extracted with RNeasy midi kit (Qiagen) according to the manufacturer's procedure and was treated with RNase-free DNase I. The mRNAs were then reverse transcribed to cDNA with the Superscript pre-amplification system (Gibco Brl) and an oligo-dT as primer. Five microliters of cDNA were used per reaction of the Cytokine Th1/Th2 Multiplex PCR assay (Biosource, Mouse Th1/Th2 set 2) according to the manufacturer's protocol. PCR products were separated on a 2% agarose gel.

Results

Construction and characterization of recombinant MVA-M

The FIPV M gene was inserted into the deletion III of the MVA genome by homologous recombination.
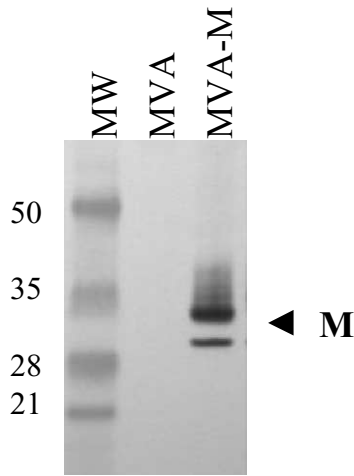
Figure 1 Expression of the FIPV M protein from cells infected with the recombinant MVA-M. CEF were infected with MVA-M or MVA during 24 hours and culture supernatants were analyzed by Western blot using two monoclonal antibodies specific for the FIPV 79-1146 M protein. The molecular weights are given in kDa on the left and the double band corresponding to the glycosylated and unglycosylated forms of the M protein is indicated by an arrow on the right.

The recombinant MVA-M is stable and replicates at high titer in CEF.

Expression of the M protein in CEF infected by MVA-M was visualized by Western Blot using two appropriate monoclonal antibodies. A double band of approximately 30 kDa can be detected in MVA-M-infected CEF culture supernatants but not in the MVA control (Fig. 1). These two bands correspond to the glycosylated and unglycosylated forms of the M protein, since a tunicamycin treatment resulted in the disappearance of the upper form (data not shown).

Antibody and cytokine responses in mice immunized with MVA-M

As a preliminary study, the immunogenicity of MVA-M was evaluated in the murine model. Mice were subcutaneously vaccinated with one or two doses of 10^8 pfu of MVA-M. In addition, we wanted to check if the anti-vector immunity could inhibit the vaccine uptake. Therefore, in one group, naive mice were pre-immunized with 10^8 pfu of wild type MVA (wtMVA) and were then vaccinated with 10^8 pfu of MVA-M, two or four weeks later. Production of the different IgG isotypes was compared to that of naive mice inoculated once or twice with 10^8 pfu of MVA-M.

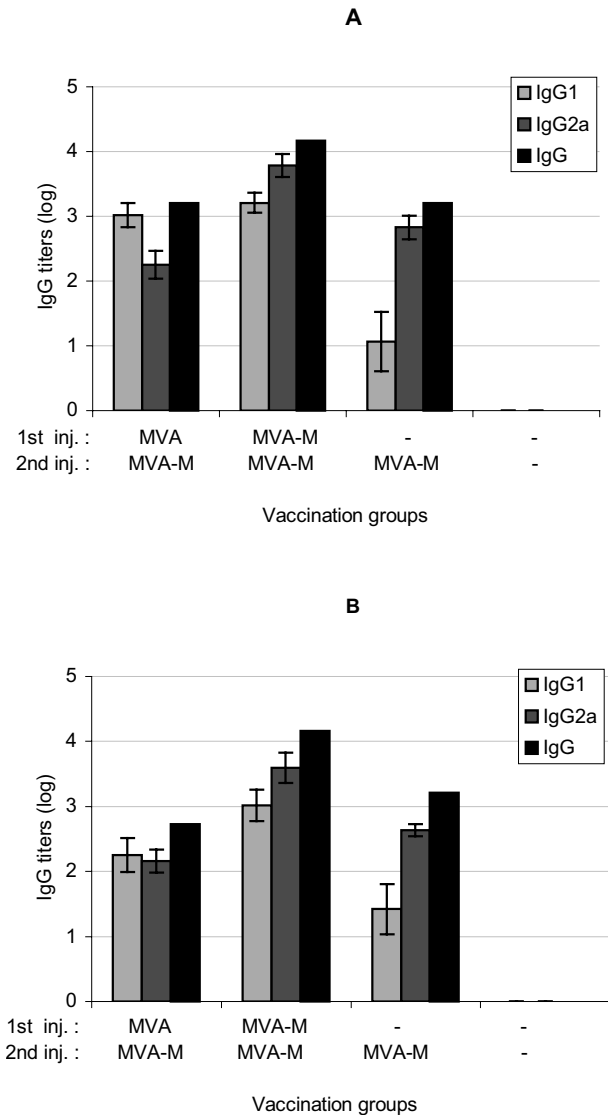All mice immunized with the MVA-M contruct developed a strong FIPV-specific humoral response

Figure 2 Humoral responses after MVA-M inoculation into naive or MVA-immunized mice. Animals received 10^8 pfu of MVA or MVA-M or were non-inoculated. Two weeks later (A) or 4 weeks later (B), they all received 10^8 pfu of MVA-M. Injections were administered subcutaneously. Sera were collected two weeks after the last immunization. IgG1 and IgG2a titers correspond to the mean of the five mice of each group, total IgG titers correspond to the values of pooled sera.

(titers greater than 1000). The pool of sera from mice pre-immunized with wtMVA and the pool of sera from naive mice that received only one injection of MVA-M gave the same IgG titer, indicating that pre-immunity against the vector was not able to inhibit the M protein expression by the recombinant virus (Fig. 2A, B).

However, the analysis of the IgG subclasses showed important differences among the three groups. Naive mice immunized once with MVA-M
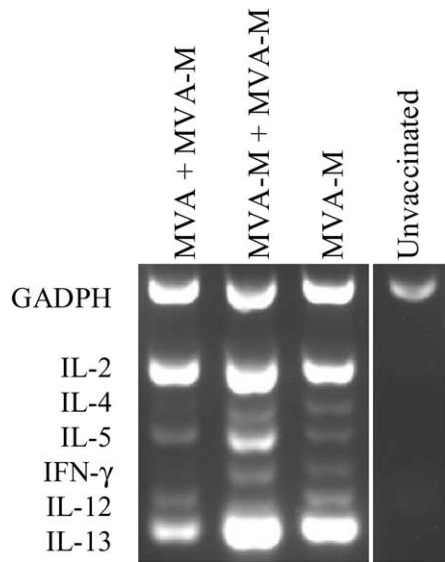
Figure 3 Cytokine profiles after MVA-M inoculation into naive or MVA-immunized mice. The results correspond to the mice from Figure 2A. Splenocytes were isolated two weeks after the last immunization and stimulated for 48 hours with inactivated FIPV 79-1146. Total RNA was reverse transcribed and assessed in a multiplex PCR assay for Th1/Th2 cytokines (Biosource). The detected cytokines are indicated on the left and the vaccination groups on the top. Th1-type cytokines are IL-2, IL-12 and IFN-γ, Th2-type cytokines are IL-4, IL-5 and IL-13. The GADPH gene is amplified as an internal control.

developed a low IgG1 response and high IgG2a titers suggesting a predominant activation of Th1 lymphocytes. Mice immunized twice with the same virus produced significantly higher titers of both IgG1 and IgG2a (Newman—Keuls multiple-comparison test). Surprisingly, mice pre-immunized with wtMVA produced predominantly FIPV-specific IgG1 after inoculation of 10^8 pfu of MVA-M, equivalently to a double injection, but a weaker IgG2a level (significant difference according to the Newman-Keuls multiple-comparison test). This inversion of the IgG1/IgG2a ratio was observed when the two injections were at either two or four week intervals (Fig. 2A, B).

To confirm these observations, the cytokine mRNA pattern was analyzed in a qualitative multiplex RT—PCR assay in order to determine the Th1/Th2 profile. The experiment was carried out on total RNA extract from splenocytes in vitro re-stimulated with FIPV. The results showed the presence of IL-2, IL-12 and IFN-γ (Th1 type cytokines) but also IL-4, IL-5 and IL-13 (Th2 type cytokines) mRNA in spleen cells of naive mice vaccinated once or twice with MVA-M (Fig. 3). These data indicate a mixed Th1 and Th2 response in

these animals which correlates with the production of both IgG2a and IgG1 antibodies, respectively. Furthermore, the bands corresponding to IL-4, IL-5 and IL-13 were more intense for the animals receiving two injections of MVA-M, giving a good correlation with their high IgG1 titers.

Mice pre-immunized with wtMVA showed the same cytokine pattern (presence of IL-2, IL-12, IL-5, IL-13 and a slight IL-4 band) except for IFN-γ which was surprisingly undetectable, revealing an inhibition of the Th1 lymphocytes.

In conclusion, the two groups presenting high levels of FIPV-specific IgG1 have raised either a strong Th2 response demonstrated by the cytokines IL-4, IL-5 and IL-13 (after two MVA-M injections) or a moderate Th2 response accompanied by an inhibition of the IFN-γ-secreting Th1 cells (after wtMVA and MVA-M immunizations). In the latter case, the lack of IFN-γ was correlated with the low IgG2a titers.

Antibody response in cats before and after challenge

The MVA-M vaccine was then tested in the feline model in order to validate the protective effect of the M protein and to investigate the efficacy of this new vector. Cats were immunized following a one-step or two-step schedule with 10^8 pfu per animal and were subsequently oronasally challenged with 220 TCID$_{50}$ of FIPV 79-1146. This challenge dose was defined as the FIPV titer inducing 100% of FIP clinical signs in unvaccinated kittens, during a preliminary trial (unpublished data). Cats were bled at vaccination and challenge times and one week following the FIPV challenge for coronavirus serology monitoring (Fig. 4).

After a single MVA-M inoculation at week 0, all the vaccinated animals developed FIPV-specific IgG. This humoral response was very homogenous within the vaccinated groups and was boosted after the second injection, reaching titers up to 1:1000.

One week after the FIPV challenge, cats vaccinated once or twice with MVA-M showed an increase of their coronavirus-specific IgG, while control cats started to develop a specific humoral response.

Outcome of the FIPV 79-1146 challenge

One week post-infection, all the kittens showed a severe drop in their lymphocytes count (data not shown). This lymphopenia persisted during the six weeks following the challenge. There was no significant difference between the two vaccinated
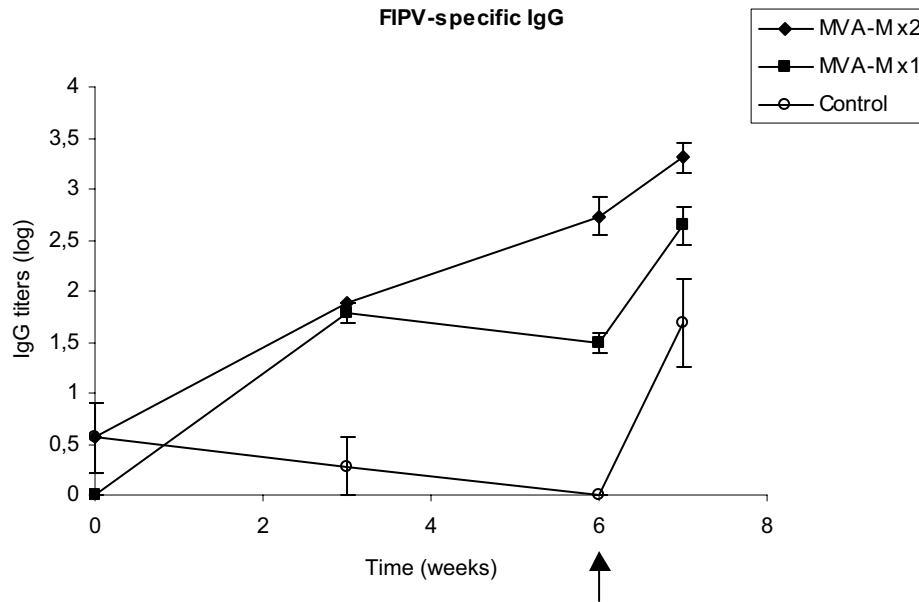
FIPV-specific IgG

Figure 4 FIPV-specific IgG response in cats immunized once (MVA-M ×1, at week 0) or twice (MVA-M ×2, at weeks 0 and 3) with 10^8 pfu of MVA-M and challenged with FIPV 79-1146 (black arrow, at week 6). Titers correspond to the mean of each group. Control cats were unvaccinated.
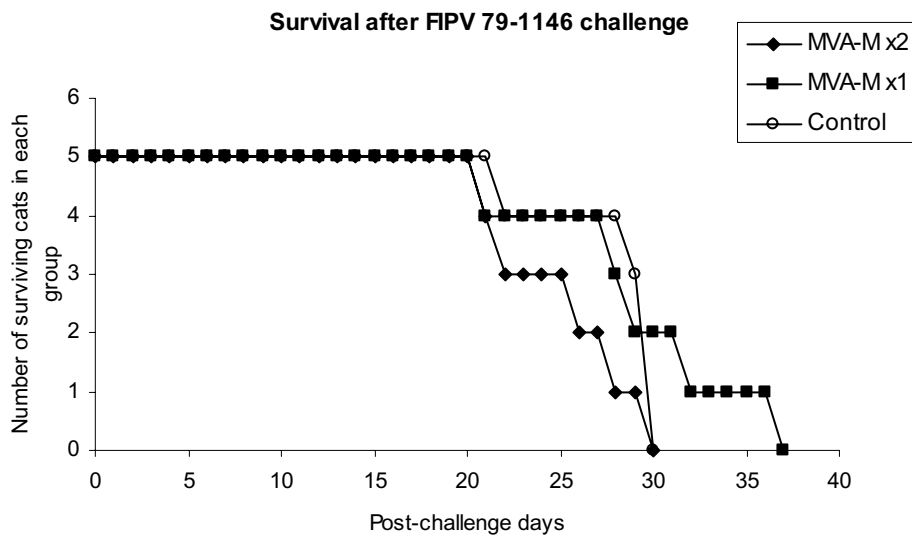
Survival after FIPV 79-1146 challenge

Figure 5 Representation of the mortality after FIPV challenge in groups of cats immunized once or twice with MVA-M (MVA-M ×1 and MVA-M ×2 respectively) or unvaccinated (control).

groups and the control group. Similarly, all the cats, vaccinated or not, presented a strong anemia after the first week of infection. Every cat from each group presented FIP symptoms from the 2nd or the 3rd week post-infection, leading to death, or euthanasia, on weeks 5 and 6, as shown by the mortality curves (Fig. 5). There was no correlation between the serological status and the evolution of the disease.

Discussion

The aim of this study was to evaluate the poxvirus MVA as a vaccine against FIPV. First, the M gene was inserted into the MVA genome by homologous recombination, downstream the early/late promoter PH5. The MVA-M was then injected into mice and cats in order to evaluate its immunogenicity and its efficacy.

Mice experiment

After either one or two injections of 10^8 pfu, we observed that the MVA-M vector induced a strong FIPV-specific IgG response. This result indicates that the MVA-M expresses the FIPV M protein in vivo and is immunogenic.

One of the drawbacks in using a recombinant virus is the immune response directed against the vector itself, that may preclude expression of the gene of interest. To gain information on this point, a group of mice was first primed with MVA, before being vaccinated with MVA-M. We observed that MVA-immune mice developed, after the MVA-M boost, FIPV-specific antibody titers equal to naive mice after a single MVA-M injection. These data support the idea that the vector is not neutralized. This is in accordance with what was reported with a recombinant MVA expressing the HIV-1 Env gene (Ramirez et al., 2000b). Surprisingly, this mode of vaccination modified the T helper status of the response, since MVA-primed mice clearly showed a Th2-type response against FIPV, as evidenced by the predominant anti-M IgG1 antibodies and by IL-4, IL-5 and IL-13 mRNA expression in splenocytes. But the most striking effect was that IFN-γ was no longer detectable by RT–PCR in wtMVA-primed mice, whereas this cytokine was expressed after vaccination of naive mice. These results confirm those of Ramirez et al. who reported a decrease of IFN-γ-secreting CD8+ T cells in mice primed with MVA and boosted with MVAenv (Ramirez et al., 2000b). Thus, these observations indicate that MVA is able to induce a Th2 environment by itself, hence predisposing the immune system to raise a Th2 response against co-expressed antigens.

Our experiments also indicate the onset of an immune memory as evidenced by the increase of Th2 lymphocytes four weeks after the prime. This observation suggests that MVA not only creates a transient Th2 environment, but also generates memory Th2 cells against its own antigens, that can act as helper signals during a subsequent immunization.

It has been already reported that increasing doses of MVA can elicit Th2 cells differentiation (Ramirez et al., 2000a). Here, we show that iterative injections of MVA-M and especially pre-existing immunity against poxvirus can also modulate the immune response.

This phenomenon has no clear explanation. It is well known that poxviruses possess factors, like virokines, to escape the immune system (Kotwal, 2000). These factors may contribute to the re-orientation of the immune response. However,

MVA has lost the majority of these sequences except a receptor for IL-1β and a soluble receptor for IL-18 (Antoine et al., 1998; Smith et al., 2000). The latter could influence or decrease activation of Th1 cells since IL-18 acts in synergy with IL-12 to promote IFN-γ secretion (Okamura et al., 1998). This interference could provide one explanation for the emergence of a Th2 environment and especially for inhibition of IFN-γ expression consequently to a poxvirus prime, despite the induction of IL-12 (Fig. 3). This type of hypothesis has to be confirmed in further experiments including IL-18 receptor-deleted MVA.

This finding may have important implications, especially for vaccination against FIPV. Indeed, FIPV infection may be constrained by a strong cell-mediated immunity, whereas the Th2-type immunity seems to be correlated with the pathology.

Cat experiment

As the murine model is not informative on the protective effect of an FIPV vaccine, the MVA-M was then evaluated in a cat trial. Two protocols were deduced from the mice experiment: on one hand, a single injection inducing a Th1 response seemed to be promising to control FIPV. On the other hand, a two-step immunization eliciting a mixed Th1/Th2 immunity should be effective since a similar vaccination schedule was partially protective with a replicative vaccinia virus (Western Reserve strain) (Vennema et al., 1991) and with the non-replicative canarypox virus (Paoletti and Gettig, 1997).

MVA has been shown to be a more potent expression vector than vaccinia WR in terms of immune response induction (Ramirez et al., 2000a), probably because it is replication-defective in mammals and it has lost several of its virulence factors. Furthermore, MVA can still produce the antigen of interest during both early and late stages of the poxvirus cycle (like vaccinia), unlike canarypox which only expresses its early genes in mammals (Sutter and Moss, 1992). Therefore, we expected that the protective effect of the M protein could be enhanced in the MVA context, compared to vaccinia and canarypox vectors.

Cats were immunized once or twice with 10^8 pfu of the recombinant MVA-M. All the animals developed coronavirus-specific antibodies, demonstrating the effectiveness of the vector. However, despite the induction of a relatively high IgG response (titers of 1:1000 for animals vaccinated twice), none of the vaccinated cats survived after the lethal FIPV challenge.

In the murine model, we observed that the number of MVA-M injections influences the Th1/Th2 balance. However, in this cat trial, in terms of protection, no difference was observed between the two vaccinated groups.

In similar assays, vaccinia virus WR expressing the same antigen and canarypox-M were partially protective against the same FIPV 79-1146 strain. The lack of efficacy of our MVA-M might be explained by the difference of promoters between the constructions. In vaccinia WR and canarypox, the M gene expression was restricted to the early stage of the viral cycle. In MVA, the M gene is controlled by the modified PH5 promoter, which enables a strong expression during both early and late stages of the viral cycle. Early expression of an antigen by a poxvirus is known to induce a cell-mediated immunity, whereas late expression favors the humoral immunity (Bronte et al., 1997). Thus the late expression of the M protein by MVA-M explains the high FIPV-specific IgG response. These antibodies may have contributed to the pathology because FIPV is an immune-mediated disease. Although the M-specific antibodies do not enhance the virus entry into macrophages, they might have been involved in the formation of immune complexes, for example.

In conclusion, this study demonstrates that this protocol of immunization with this MVA-M construct is not protective against a FIPV challenge in kittens, probably because of the strong induction of FIPV-specific antibodies. The type of poxviral promoter seems to have a crucial importance to minimize the humoral response while eliciting an efficient cell-mediated immunity. This type of immunity may be enhanced by a DNA vaccine prime as seen in other studies (Ramshaw and Ramsay, 2000). Furthermore the M protein is apparently not sufficient to induce a protective immunity by itself, so it should be associated with other FIPV antigens that still have to be determined.

References

Amara, R.R., Villinger, F., Staprans, S.I., Altman, J.D., Montefiori, D.C., Kozyr, N.L., Xu, Y., Wyatt, L.S., Earl, P.L., Herndon, J.G., McClure, H.M., Moss, B., Robinson, H.L., 2002. Different patterns of immune responses but similar control of a Simian-Human Immunodeficiency Virus 89.6P mucosal challenge by Modified Vaccinia Virus Ankara (MVA) and DNA/MVA vaccines. Journal of Virology 76(15), 7625–7631.

Antoine, G., Scheiflinger, F., Dorner, F., Falkner, F.G., 1998. The complete genomic sequence of the Modified Vaccinia Ankara strain: comparison with other Orthopoxviruses. Virology 244, 365–396.

Bronte, V., Carroll, M.W., Goletz, T.J., Wang, M., Overwijk, W.W., Marincola, F., Rosenberg, S.A., Moss, B., Restifo, N.P., 1997. Antigen expression by dendritic cells correlates with the therapeutic effectiveness of a model recombinant poxvirus tumor vaccine. Proceedings of the National Academy of Science U S A 94(7), 3183–3188.

Kotwal, G.J., 2000. Poxviral mimicry of complement and chemokine system components: what's the end game? Immunology Today 21(5), 242–248.

Okamura, H., Tsutsui, H., Kashiwamura, S., Yoshimoto, T., Nakanishi, K., 1998. Interleukin-18: a novel cytokine that augments both innate and acquired immunity. Advances in Immunology 70, 281–312.

Olsen, C.W., 1993. A review of feline infectious peritonitis virus: molecular biology, immunopathogenesis, clinical aspects, and vaccination. Veterinary Microbiology 36, 1–37.

Paoletti, E., Gettig, R., 1997. Recombinant poxvirus-feline infectious peritonitis virus, compositions thereof and methods for making and using them. International patent, IPN: WO 97/20054.

Pedersen, N.C., 1987. Virologic and immunologic aspects of feline infectious peritonitis virus infection. Advances in Experimental Medical Biology 218, 529–550.

Pedersen, N.C., Evermann, J.F., McKeirnan, A.J., Ott, R.L., 1984. Pathogenicity studies of feline coronavirus isolates 79-1146 and 79-1683. American Journal of Veterinary Research 45(12), 2580–2585.

Ramirez, J.C., Gherardi, M.M., Esteban, M., 2000a. Biology of attenuated modified vaccinia virus Ankara recombinant vector in mice: virus fate and activation of B- and T-cell immune responses in comparison with the Western Reserve strain and advantages as a vaccine. Journal of Virology 74(2), 923–933.

Ramirez, J.C., Gherardi, M.M., Rodriguez, D., Esteban, M., 2000b. Attenuated modified vaccinia virus Ankara can be used as an immunizing agent under conditions of preexisting immunity to the vector. Journal of Virology 74(16), 7651–7655.

Ramshaw, I.A., Ramsay, A.J., 2000. The prime-boost strategy: exciting prospects for improved vaccination. Immunology Today 21(4), 163–165.

Smith, V.P., Bryant, N.A., Alcami, A., 2000. Ectromelia, vaccinia and cowpox viruses encode secreted interleukin-18-binding proteins. Journal of General Virology 81(5), 1223–1230.

Sutter, G., Moss, B., 1992. Nonreplicating vaccinia vector efficiently expresses recombinant genes. Proceedings of the National Academy of Science U S A 89, 10847–10851.

Vennema, H., de Groot, R.J., Harbour, D.A., Dalderup, M., Gruffydd-Jones, T., Horzinek, M.C., Spaan, W.J.M., 1990. Early death after feline infectious peritonitis virus challenge due to a recombinant vaccinia virus immunization. Journal of Virology 64(3), 1407–1409.

Vennema, H., de Groot, R.J., Harbour, D.A., Horzinek, M.C., Spaan, W.J.M., 1991. Primary structure of the membrane and nucleocapsid protein genes of feline infectious peritonitis virus and immunogenicity of recombinant vaccinia viruses in kittens. Virology 181, 327–335.

Wyatt, L.S., Shors, S.T., Murphy, B.R., Moss, B., 1996. Development of a replication-deficient recombinant vaccinia virus vaccine effective against parainfluenza virus 3 infection in an animal model. Vaccine 14(15), 1451–1458.