Virus distribution of the attenuated MVA and NYVAC poxvirus strains in mice

Carmen Elena Gómez,† José Luis Nájera,† Elena Domingo-Gil, Laura Ochoa-Callejero, Gloria González-Aseguinolaza, and Mariano Esteban

1Department of Molecular and Cellular Biology, Centro Nacional de Biotecnología, CSIC, Ciudad Universitaria Cantoblanco, 28049 Madrid, Spain
2Division of Hepatology and Gene Therapy, Center for Investigation in Applied Medicine (CIMA), University of Navarra, 31080 Pamplona, Spain

Recombinant vaccinia viruses based on the attenuated NYVAC and MVA strains are promising vaccine candidates against a broad spectrum of diseases. Whilst these vectors are safe and immunogenic in animals and humans, little is known about their comparative behaviour in vivo.

In this investigation, a head-to-head analysis was carried out of virus dissemination in mice inoculated by the mucosal or systemic route with replication-competent (WRluc) and attenuated recombinant (MVAluc and NYVACluc) viruses expressing the luciferase gene. Bioluminescence imaging showed that, in contrast to WRluc, the attenuated recombinants expressed the reporter gene transiently, with MVAluc expression limited to the first 24 h and NYVACluc giving a longer signal, up to 72 h post-infection, for most of the routes assayed. Moreover, luciferase levels in MVAluc-infected tissues peaked earlier than those in tissues infected by NYVACluc. These findings may be of immunological relevance when these vectors are used as recombinant vaccines.

Some of the most promising vaccine candidates being evaluated in clinical trials against AIDS, malaria and cancer are based on poxvirus recombinants. They are valuable tools for the expression of foreign antigens directly inside the cells of the host organism, as would happen in natural infection, and induce potent cellular immune responses against the heterologous product (Bonnet et al., 2000; Zavala et al., 2001). Among poxviruses, highly attenuated strains, such as modified vaccinia virus Ankara (MVA) or NYVAC, are considered the strains of choice for preclinical and clinical vaccine development. MVA was derived from the Ankara strain of vaccinia virus (VACV) by approximately 570 serial passages in primary chick embryo fibroblasts and has genome deletions (30 kbp) that include VACV genes involved in host immune regulation and host range (Mayr et al., 1978; Antoine et al., 1998). NYVAC was derived from the Copenhagen strain of VACV. It was attenuated genetically by the deletion of 18 non-essential genes implicated in host range or virulence (Tartagli et al., 1992a, b). The major advantage of MVA and NYVAC is the safety record and, despite their limited replication in human and most mammalian cell types, both viral strains provide a high level of gene expression and are immunogenic when delivering foreign antigens in animals and humans (Cox et al., 1993; Amara et al., 2001; Hel et al., 2001; Gherardi et al., 2003; Didierlaurent et al., 2004; Gomez et al., 2004, 2007a, b; Webster et al., 2005). Although the capacity of these attenuated vectors to produce levels of recombinant antigens similar to those produced by replication-competent viruses has been demonstrated widely, to date an in vivo comparative analysis of virus dissemination of MVA and NYVAC strains when administered by different routes has not been done.

Molecular imaging offers many unique opportunities to study biological processes in intact organisms. Bioluminescence imaging (BLI) is based on the sensitive detection of visible light produced during enzyme (luciferase)-mediated oxidation of a molecular substrate when the enzyme is expressed in vivo as a molecular reporter (Sadikot & Blackwell, 2005). This technology has been applied in studies to monitor transgene expression, progression of infection, tumour growth and metastasis, transplantation, viral infections and gene therapy (Edinger et al., 1999; Doyle et al., 2004; Ray et al., 2004). This non-invasive technique allows quantification in the same animal of the spatial and temporal progression of the infection, identifying animal-to-animal variations in viral replication and dissemination. In this study, we have followed, by BLI and biochemical analyses, the distribution in mice of MVA and NYVAC vectors, in comparison with the
replication-competent VACV strain Western Reserve (WR), when administered by different routes.

The poxvirus recombinants used in this study expressed the luciferase reporter gene and were derived from MVA
(kindly provided by G. Sutter, Paul-Ehrlich-Institut, Langen, Germany), NYVAC (kindly provided by Sanofi-Pasteur) and WR strains. MVALuc and WRLuc recombinants were described previously (Rodriguez et al., 1988; Ramirez et al., 2000). NYVACLuc was generated in this work according to standard methods by using the same plasmid-transfer vector, pSCLUC, as was used for the generation of WRLuc and MVALuc, which placed the gene under control of the virus p7.5 early/late promoter and the insertion site in the thymidine kinase (TK) locus of the viral genome (Rodriguez et al., 1988).

To visualize dissemination of the different viruses in vivo, female BALB/c mice, 6–8 weeks old (Harlan OLAC), were inoculated by the following routes: intraperitoneal (i.p., 200 µl), intramuscular (i.m., 50 µl), intranasal (i.n., 50 µl), intrarectal (i.r., 50 µl) or intragastric (i.g., 50 µl), with 1 × 10^7 p.f.u. of either MVALuc or NYVACLuc per animal or with 1 × 10^6 p.f.u. of WRLuc diluted in PBS per mouse. In the case of the tail-scariication (t.s.) route, 1 × 10^6 p.f.u. virus per mouse was administered in a total volume of 10 µl. Animals were anaesthetized with 100 µl per 20 g weight of a 1:9 mixture of ketamine-500 (Merial) and 2% xylazine (Bayer) before t.s. and i.m. inoculation, and 100 µl d-luciferin (Xenogen) at a concentration of 30 mg ml^-1 diluted in 150 mM NaCl solution was injected by the i.p. route. The animals were placed in the imaging chamber of the Xenogen IVIS system, which includes a cooled CCD camera. A greyscale photograph of the animals was acquired, followed by a bioluminescent acquisition starting at 10 min after the luciferin injection. Images were collected for 3 min each in the ventral and dorsal positions. Regions of interest (ROIs) were drawn over the positions of greatest signal intensity on the animal, as well as over regions of ‘no’ signal, which were used as background readings. Light intensity was quantified by using photons s^-1 cm^-2 sr^-1. The greyscale photograph and data images from all studies were superimposed by using LivingImage (Xenogen). Luciferase activity is depicted with a pseudocolour scale, using red as the highest and blue as the lowest photon flux. Measurements of BLI were performed daily and the progression of infection was monitored until disappearance of the signal. Serial images were obtained from animals and the mean photon flux was quantified. There was no bioluminescence above background level in mock-infected mice, which were used as a negative control.

First, we determined how the systemic routes, i.p., i.m. and t.s., impacted on luciferase expression, as an index of virus dissemination in the whole animal. In mice inoculated i.p. with either MVALuc or NYVACLuc, light emission was detected in the abdominal region, demonstrating the dissemination of the virus beyond the site of peritoneal infection. This is observed clearly in WRLuc-infected animals, with extensive virus spreading and luciferase expression lasting for longer than 4 days (Fig. 1a). The highest levels of luciferase in animals receiving the attenuated viruses were detected at day 1 post-inoculation (p.i.); however, whereas in MVALuc-infected mice, the signal decreased markedly at day 2 p.i. and no luciferase activity was detected at later times, in NYVACLuc-infected mice, the signal remained detectable until day 3 p.i. This was confirmed by photon-flux quantification performed at the site of inoculation (Fig. 1b). The levels of luciferase increased by about 4 logs above background for WRLuc at the different times assayed, whilst for NYVACLuc and MVALuc, the increments were 74- and 16-fold, respectively, at day 1 p.i., and 15- and 2.5-fold, respectively, at day 2 p.i.

The i.m. route is generally the way used to administer poxvirus and DNA vaccine candidates in clinical trials. This route has been preferred to s.c. or i.d. delivery to minimize the severity of reactions associated with the injection. When we inoculated animals by the i.m. route with the different viruses, we observed that the luciferase signal was mainly restricted to the inoculation site (Fig. 1c). Photon-flux quantification showed that, in contrast to WRLuc-infected mice, the luciferase levels at the site of virus inoculation decreased in a time-dependent manner for MVALuc and NYVACLuc vectors (Fig. 1d). Whilst by day 1 p.i., the two attenuated viruses induced similar levels of luciferase, at day 2 p.i., differences were observed between the vectors. NYVACLuc-induced values were 100-fold higher than background, whereas MVALuc levels were only 4-fold higher. By the t.s. route, the expression of luciferase in animals inoculated with MVALuc or NYVACLuc was very low and was restricted to the site of inoculation, in contrast to mice receiving WRLuc, in which the signal increased gradually after infection, a sign of WR virus spreading in the animal. This was confirmed by photon-flux quantification (Fig. 1e). Next, we examined the expression pattern of MVA Luc and NYVACLuc following i.n., i.r. and i.g. inoculations. By i.n. inoculation, luciferase expression in MVA Luc- and NYVACLuc-infected mice was transient and restricted to the lungs. In contrast, bioluminescence was detected in the nose and the chest of mice receiving WR Luc and increased with time, an indication of virus spreading within the respiratory tract, affecting trachea, lungs and brain (data not shown).

Systemic dissemination of the viruses to abdominal organs was not observed at any time point. Photon-flux quantification revealed that the luciferase-expression levels for the two attenuated vectors were low, and remained longer in NYVACLuc-infected animals (Fig. 1f). Following i.r. inoculation, very low levels of luciferase were observed in animals receiving MVA Luc and NYVACLuc vectors at any time p.i. (Fig. 1g). After i.g. inoculation of MVA Luc and NYVACLuc, luciferase expression was transient, with the highest values observed at day 2 p.i. In NYVACLuc-infected mice, the levels of the reporter gene were higher than for MVA Luc-infected mice and were more sustained (data not shown).
Fig. 1. (a, c) BLI distribution of WRluc, MVA\textsubscript{luc} and NYVAC\textsubscript{luc} in mice inoculated by the i.p. (a) or i.m. (c) route. In the right-hand panel of (a), mock-infected mice (CTRL) are shown. (b, d–g) Quantification of the luciferase signal in the ROI during i.p. (b), i.m. (d), t.s. (e), i.n. (f) and i.r. (g) infections. Mean ± SD values for photon fluxes over time are represented. The solid line represents the background level of bioluminescence.
As revealed by BLI analysis with the luciferase reporter, and by comparison of different virus-inoculation routes in mice, the i.p. and i.m. routes are the most efficient to obtain high levels of heterologous gene expression. In contrast to WRluc, the attenuated MVA\textsubscript{Luc} and NYVAC\textsubscript{Luc} viruses expressed the luciferase gene transiently, demonstrating their restricted replication capacity in vivo, as documented previously for MVA\textsubscript{Luc} (Ramirez et al., 2000). Interestingly, in NYVAC\textsubscript{Luc}-infected mice, the luciferase signal from 24 h p.i. onward was more sustained in the whole animal than that for MVA\textsubscript{Luc}, indicating that the NYVAC\textsubscript{Luc} reporter remains longer within the infected cells.

Whilst the above results revealed differences in levels of bioluminescence from 24 h onward for both MVA and NYVAC vectors when inoculated by systemic routes, it was important to define the kinetics of vector expression shortly after virus infection. To this aim, we quantified the enzyme activity in tissue extracts of mice inoculated i.p., as this is the most effective route for virus dissemination. Gene expression of recombinant viruses in different mouse tissues was monitored by a highly sensitive luciferase assay, described previously (Rodriguez et al., 1988). Different groups of mice received an i.p. inoculation (1 x 10\textsuperscript{7} p.f.u. per animal) of MVA\textsubscript{Luc}, NYVAC\textsubscript{Luc} or WRluc. Peritoneal cells were harvested by mouse peritoneal-cavity lavage with 10 ml sterile PBS, centrifuged at room temperature for 5 min at 1200 r.p.m. and stored at -70 °C. At various times p.i., animals were sacrificed and spleens, draining lymph nodes and ovaries were dissected under sterile conditions and stored at -70 °C. Tissues from individual mice were homogenized in Promega luciferase extraction buffer (300 µl per spleen and 200 µl per ovary, lymph node or peritoneal extract) by using an Ultraturrax T8 mechanical homogenizer (Janke & Kunkel). Luciferase activity was measured in the presence of luciferin and ATP by using a Lumat LB 9501 luminometer (Berthold Technologies).

![Fig. 2. Kinetics of luciferase expression by WR\textsubscript{Luc}, MVA\textsubscript{Luc} and NYVAC\textsubscript{Luc} in different mouse target tissues. (a) BALB/c mice were inoculated i.p. with the different viruses and, at indicated times, the extent of virus gene expression in different tissue extracts was evaluated by luciferase assay. Background levels in control uninfected tissues are shown as dashed lines. Results represent mean values from samples of three animals per time and group with standard deviations covered by each symbol. (b) Virus gene expression in cells from the peritoneal cavity of naïve mice, isolated and infected with 3 p.f.u. of WR\textsubscript{Luc}, MVA\textsubscript{Luc} or NYVAC\textsubscript{Luc} per cell. At the indicated times p.i., the extent of virus gene expression was evaluated by luciferase assay. Results represent mean values of three independent experiments with sd.](image)
according to the manufacturer’s instructions, and was expressed as luciferase reference units (LRU) (mg protein)−1. Protein content in tissue extracts was measured with a BCA Protein Assay kit (Pierce Biotechnology). As shown in Fig. 2, luciferase levels at 4 h p.i. in peritoneal looks, ovaries and lymph nodes from MVAluc-infected mice were 5- to 10-fold higher than those found in tissues from NYVACluc-infected mice, except for the spleen, where the levels were similar between the two viruses. By 6 h p.i., these levels were comparable and decreased with time, falling to background values by 48 h p.i. By this time, the values in WRluc were 2–4 log units higher than for either MVAluc or NYVACluc. We also quantified the virus titres in peritoneal washes, ovaries, lymph nodes and spleen of infected mice. In contrast to tissues from WRluc-infected mice, where infectious virus was observed, there was no infectious virus at 24–48 h p.i. in samples from MVAluc- and NYVACluc-infected mice (data not shown). These results are in agreement with previous findings for MVAluc (Ramirez et al, 2000) and indicate higher efficiency of virus gene expression for MVA versus NYVAC shortly after infection.

To verify the differences observed in vivo between both attenuated recombinants at early times p.i., we isolated cells from the peritoneal cavity of naïve animals and infected them with the different viruses. As shown in Fig. 2(b), expression of luciferase at the different times assayed was about 1 log unit higher in MVAluc- than in NYVACluc-infected cells. This was not due to differences in virus uptake by the cells, but rather to a post-entry event, as similar levels of luciferase were observed between 2 and 4 h p.i. in HeLa cells infected with MVAluc or NYVACluc (3 p.f.u. per cell; data not shown). As MVA and replication-competent VACV exhibited different tropisms for primary human cells (Chahroudi et al, 2005), the differences that we observed between the two attenuated recombinants might be related to the susceptibility of such cells to infection with MVA or NYVAC. Whilst the peritoneal exudates had the highest level of luciferase activity after infection, it remains to be defined whether the same cells present in the peritoneal washout of an uninfected mouse are infected in vivo. As similar levels of binding and infection have been observed in murine splenic B cells exposed to replication-competent VACV or MVA (Chahroudi et al, 2005), our results may also reflect a biological mechanism that distinguishes the post-binding infectious process or the timing of early gene expression of MVA and NYVAC in certain cell types. Further studies should be directed to the identification of the cell types infected by the two attenuated viruses in animals and humans.

The results described here and those described previously on the biology of these two virus strains in cultured cells (Najera et al, 2006), their impact on host genome profiling in HeLa cells (Guerra et al, 2004, 2006) and head-to-head comparisons of the immunogenicity of both vectors expressing human immunodeficiency virus type 1 antigens in mice (Gomez et al., 2007a, b) all suggest that MVA and NYVAC have different behaviours in their ability to replicate and impact on host immune responses. Hence, they should be explored as poxvirus vector vaccines with differential in vitro and in vivo characteristics.

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