Highly attenuated vaccinia virus mutants for the generation of safe recombinant viruses

(human immunodeficiency virus type 1 gag and env genes)

Dolores Rodriguez*, Juan-Ramon Rodriguez*, Jose F. Rodriguez*, David Trauber†, and MARIANO ESTEBAN*‡

Departments of *Biochemistry, Microbiology, and Immunology, and †Medicine, State University of New York Health Science Center at Brooklyn, Brooklyn, NY 11203

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ABSTRACT

An attenuated vaccinia virus mutant with specific genetic lesions has been used to develop a vehicle for safer live recombinant virus vaccines. The mutant virus 48-7 has an 8-MDa deletion starting 2.2 MDa from the left end of the viral genome and point mutations in the gene encoding the 14-kDa fusion protein that determines the plaque-size phenotype of the virus. Using the highly sensitive reporter gene luciferase, we have shown that this mutant can generate recombinant viruses that infect cultured cells and animals with normal vaccinia virus tropism. Insertion of the envelope and gag genes of human immunodeficiency virus type 1 into the attenuated vaccinia mutant resulted in their efficient expression and precursor processing in infected cultured cells. Infection of mice with human immunodeficiency virus–vaccinia recombinant viruses elicited human immunodeficiency virus-specific antibodies. Using mice pretreated with cyclophosphamide as a model for immunosuppression, the reduced virulence of the mutant recombinant virus was clearly evident. These findings demonstrate that the highly attenuated vaccinia virus mutant 48-7 can be used to generate effective and safer vaccines.

Genetically engineered recombinants of vaccinia virus are now used as eukaryotic expression vectors and, because they are potent immunogens, have a potential as vaccines against a broad spectrum of infectious pathogens of animals and human significance (1–3). However, in humans, accidental vaccination with live vaccinia virus of an immunocompromised host can lead to serious and lethal complications, such as systemic vaccinia infection with encephalitis (4, 5). This point was confirmed by the vaccination of a soldier who unknowingly was human immunodeficiency virus (HIV) (6). To diminish the chances of producing such complications, an expert panel from the World Health Organization has recommended the development of attenuated strains of vaccinia virus that carry identifiable genetic markers upon which to base the construction of future recombinant vaccines (7). Attenuated variants of vaccinia virus have been obtained by several methods, including (i) inactivation of the viral thymidine kinase gene (8), (ii) generation of deletions at the left end of the viral genome (9, 10), (iii) mutation of the gene encoding a 14-kDa fusion protein that influences viral plaque size (11), (iv) alterations in size of virion core proteins (12, 13), and (v) insertion of the interleukin 2 gene that greatly decreases virus virulence in athymic nude mice (14, 15).

In this report we describe the effectiveness of recombinant vaccines based on an attenuated vaccinia virus mutant that has specific genetic lesions. Using luciferase as a reporter gene and HIV gag and envelope genes, we have demonstrated the advantage of basing recombinant vaccines on this attenuated variant.

MATERIALS AND METHODS

Construction of Vaccinia Recombinants Expressing Luciferase and HIV Envelope and gag Genes. A 1892-base-pair (bp) BamHI fragment encoding the luciferase gene (16) was inserted into the vaccinia virus expression vector pSC11 and recombinant viruses were selected by marker rescue by following standard protocols (17). Luciferase activity was measured with a spectrophotometer and results were quantitated as described (18). For the construction of HIV–vaccinia recombinants, an Xba I–Xho I DNA fragment containing the coding region of HIV env was created by standard recombinant DNA techniques from a full-length HIV type 1 (HIV-1) DNA genome (19). This fragment extends to the Sp I site 66 bp upstream from the start site of translation of the env gene and 100 bp downstream from the termination codon. The env DNA fragment was blunt-ended by treatment with the Klenow fragment of DNA polymerase I and cloned into the Sma I site of the vaccinia virus insertion vector pSC11. As a result of this cloning strategy, we isolated a plasmid containing HIV env coding sequence under the control of the 7.5-kDa vaccinia virus promoter. A similar strategy was used to construct vaccinia virus insertion vectors containing the intact HIV gag gene. A BssHII–EcoRI DNA fragment containing the coding region of gag was isolated from the full-length HIV-1 DNA. This fragment contains 79 bp upstream from the start site of translation of the gag gene and, from the termination site, it contains 2358 bp of the pol gene. The Luciferase, env, and gag genes were inserted in the correct orientation relative to the vaccinia 7.5-kDa promoter and into the thymidine kinase region of wild-type and mutant viruses by DNA homologous recombination. β-Galactosidase-producing plaques (17) were picked, cloned three times, and amplified in African green monkey kidney cells (BSC-40) grown in culture.

RESULTS

Expression and Tissue Tropism of Attenuated Vaccinia Recombinants. Since attenuated viruses might multiply less efficiently in infected animals than wild-type virus, our first interest was to document the extent of viral gene expression and tissue tropism of attenuated recombinants. This was accomplished by introducing the highly sensitive reporter gene luciferase into vaccinia DNA. Two classes of recombinants were obtained, one derived from wild-type virus and...
Fig. 1. Expression of the firefly luciferase gene by wild-type and mutant recombinants of vaccinia virus. The construction of vaccinia luciferase recombinants and assays of luciferase activity have been described (18). (A) Expression of luciferase in cultured cells. BSC-40 cells were infected with wild-type vaccinia luciferase-recombinant viruses (WRLUC, open symbols) or with mutant 48-7 luciferase-recombinant viruses (MUT7LUC, solid symbols) at 5 plaque-forming units (pfu) per cell with (△, △) or without (○, ●) cytosine arabinonucleoside (ARA-C) at 40 μg/ml. At various times after infection, cells were collected and cell extracts prepared in luciferase buffer (100 mM potassium phosphate/1 mM dithiothreitol) were tested for luciferase activity in the presence of luciferin and ATP (18). (B) Expression of luciferase in organs of infected mice. BALB/c female mice, 6 weeks old (Charles River Breeding Laboratories), were inoculated i.p. with 5 × 10⁵ pfu of recombinant vaccinia viruses and 3 days later tissues were washed in isotonic phosphate-buffered saline (PBS), weighed, homogenized in luciferase buffer containing 1 mM phenylmethylsulfonyl fluoride and leupeptin at 10 μg/ml (5 μl/mg of tissue), and supernatants were assayed for luciferase activity (18). WRLUC, wild-type vaccinia luciferase-recombinant; MUT7LUC, mutant 48-7 luciferase-recombinant.
the other derived from the attenuated vaccinia mutant 48-7. Mutant 48-7 has an 8-MDa deletion starting 2.2 MDa from the left end of the viral genome and two point mutations on the gene encoding the 14-kDa fusion protein that result in high attenuation (refs. 10–12; S. C. Gong, C. F. Lai, and M. E., unpublished results). Vaccinia recombinants containing the luciferase gene were then tested for luciferase activity both in cultured cells and in experimental animals. As shown in Fig. 1A, the levels of luciferase activity were equivalent in monkey cells infected with the luciferase–wild-type recombinant or with the luciferase–mutant 48-7 recombinant. In infected mice luciferase activity was observed in spleen and liver, the known target organs for wild-type vaccinia virus multiplication (20). The levels of luciferase activity found in spleens of mice inoculated with the luciferase–mutant recombinant virus were 30% lower than in mice inoculated with the wild-type recombinant virus. Thus, the mutant recombinant virus efficiently expresses a foreign gene and shows the same tissue tropism as the wild-type virus.

Expression of HIV Envelope and gag Genes by Attenuated Vaccinia Recombinants. To assess that the attenuated vaccinia virus mutant has a vaccine potential, we constructed recombinant vaccinia viruses containing either the HIV-1 env or the gag gene and studied their expression and immunogenicity. Because mature retroviral gag and envelope proteins are derived from precursor molecules by processing (21–23), we first examined processing of env and gag by immunoprecipitation analysis. The results are shown in Fig. 2 A and B. In

Fig. 2. Expression of HIV env and gag by vaccinia (wild-type and mutant) recombinant viruses. Immunoprecipitation of env (A) and gag (B) proteins from lysates of HIV–vaccinia virus-infected cells, as examined by NaDodSO4/PAGE. Hela S1 cells infected (5 pfu per cell) with vaccinia recombinants were labeled with [35S]-methionine (50 μCi/ml; 1 Ci = 37 GBq) from 3 to 18 hr after infection and cell extracts were immunoprecipitated with mouse monoclonal antibodies against gp120 (env) and p24 (gag) (DuPont). Lanes: 1, 3, 5, and 7, total cell extracts; 2, 4, 6, and 8, immunoprecipitates. Uninfected cells (lanes 1 and 2), cells infected with wild-type virus (lanes 3 and 4), and cells infected with HIV–vaccinia recombinants, from wild-type virus (lanes 5 and 6) and from mutant virus (lanes 7 and 8), are shown. (C) Processing of env products in cells infected with HIV–vaccinia env mutant virus (lanes 1–5). Six hours after infection, Hela cells were pulse-labeled with [35S]methionine (100 μCi/ml) for 30 min and chased for various times with a 100-fold excess of unlabeled methionine, and cell extracts were immunoprecipitated with a monoclonal antibody to gp120. Proteins were resolved on a 10% NaDodSO4/polyacrylamide gel. Total cell lysate (lane 1) and immunoprecipitates after a 30-min pulse (lane 2) and after chase periods of 30 min (lane 3), 60 min (lane 4), and 90 min (lane 5) are shown as well as purified HIV-1 (lane 6) and gp120 released from infected cells (lane 7). Released gp120 was measured in culture supernatants from mutant HIV–vaccinia env virus-infected Hela cells at 24 hr after infection after a 50-fold concentration by using an Amicon ultrafiltration cell. Lanes 6 and 7 show a blot reacted with rabbit anti-HIV gp160 serum and developed by immune peroxidase staining.

Fig. 3. Humoral immune response elicited in mice by HIV–vaccinia recombinants. (A) Four BALB/c mice per group were inoculated i.p. (5 × 107 pfu) with purified HIV–vaccinia env mutant recombinant virus, and 21 days later mice were revaccinated i.v. and i.p. with 105 pfu of recombinant virus. After 21 days, mice were given a booster injection i.v. with 100 μg of purified gp160 isolated from HIV–vaccinia-infected cells. Serum was collected from the tail vein 6 days later and tested for specific antibodies to a Western blot by immunoperoxidase staining. Proteins were separated by a minigel (10% polyacrylamide). Lanes: 1, lysates of mutant virus-infected CV-1 cells 24 hr after infection; 2, lysates of HIV–vaccinia env mutant-infected CV1 cells 24 hr after infection; 3, purified HIV–vaccinia env wild-type virus; 4, purified HIV–vaccinia env mutant virus; 5, purified gp160. (B) Mice were primed with purified HIV–vaccinia gag mutant recombinant virus and 21 days later revaccinated as above. Serum was collected 2 weeks after secondary vaccination and tested on a Western blot. Proteins were separated on a blot (10% polyacrylamide). Lanes: 6, purified HIV-1 virus (a reverse transcriptase mutant with unprocessed gag, isolated from the 8E5 human T-cell line); 7, purified gp160; 8, lysates of HIV–vaccinia env wild-type virus-infected Hela cells 36 hr after infection; 9, lysates of wild-type virus-infected Hela cells 36 hr after infection. Protein markers are myosin, β-galactosidase, bovine serum albumin, ovalbumin, and carbonic anhydrase.
lysates of Hela S cells infected with wild-type HIV–vaccinia env virus, monoclonal antibodies against gp120 immunoprecipitates both gp120 and its precursor gp160 (Fig. 2A, lane 6). These polypeptides were also detected in lysates of cells infected with the mutant recombinant virus (lane 8). However, these polypeptides were not detected in lysates of uninfected cells (lane 2) or in cells infected with nonrecombinant virus (lane 4). In lysates of cells infected with HIV–vaccinia gag recombinants (either wild-type or mutant virus), a monoclonal antibody against p24 has specific reactivity with polypeptides p36, p42, p32, and p24 (Fig. 2B, lanes 6 and 8). Next we establish a precursor–product relationship for env by pulse–chase experiments (Fig. 2C). The polypeptide that was most labeled during the pulse period was gp160, which was chased into gp120. The chased gp120 was released extracellularly and was recovered in the cell culture supernatant (lane 7). Two other immunoreactive bands of 70 and 50 kDa (lane 7) were also found and appear to result from a single nick in gp120 (unpublished results). To determine if mutant HIV–vaccinia recombinants elicit specific antibodies against env and gag proteins, mice were vaccinated with the recombinants and serum was tested by Western blot and ELISA. We found that mice vaccinated with mutant HIV–vaccinia env virus have weak antibody response to gp160. However, induction of antibodies to gp160 was markedly increased if vaccinated mice were given a booster injection with purified gp160 (Fig. 3A, lane 5). Mice only vaccinated with mutant HIV–vaccinia gag virus had a strong immune response to gag (Fig. 3B, lane 6). Immune reactivity to env and gag proteins in serum samples was also confirmed by ELISA with HIV-infected cell extracts (Pasteur Institute).

Reduced Virulence of Attenuated HIV–Vaccinia Recombinants in Immunosuppressed Animals. Since it is known that inoculation of immunocompromised individuals with vaccinia virus may lead to generalized vaccinia and encephalitis (4, 5) [and indeed, this has been documented in a vaccinated male seropositive for HIV (6)], it was important to assess the degree of virulence of attenuated HIV–vaccinia recombinants in an immunosuppressed host. Thus, BALB/c mice were first treated with the immunosuppressor drug cyclophosphamide (24) and subsequently infected, and their survival was evaluated daily (Fig. 4). As expected, in normal mice the mortality produced by the vaccinia mutant was about 100 times lower than that induced by wild-type virus (Fig. 4A). Significantly, the differences in mortality between the two viruses were greater with immunosuppressed mice (Fig. 4B). This was also the case with mutant recombinant virus. As shown in Fig. 4C and D, mutant HIV–vaccinia env virus caused markedly decreased mortality in immunosuppressed mice as compared with wild-type recombinant virus.

**DISCUSSION**

These studies demonstrate that a vaccinia virus mutant, 48-7, containing two defined genetic lesions can be used to generate safe recombinant live vaccines. During infection mutant recombinant viruses express significant levels of foreign genes, are good immunogens, and have greatly reduced

![Fig. 4. Reduced virulence of HIV–vaccinia recombinants in immunosuppressed mice. Four BALB/c mice per group were inoculated i.p. (0.2 ml) with cyclophosphamide (Sigma) at 300 mg/kg in PBS, and 5 days later mice were treated with a second dose of the drug but at 150 mg/kg. Twenty-four hours later, untreated and drug-treated mice were inoculated i.p. with various doses of purified parental and recombinant HIV–vaccinia env viruses. Survival was followed daily. (A and B) Results obtained after 1 month with parental viruses in normal (A) and immunosuppressed (B) animals. □, MUT-7; ■, WR. (C and D) Results obtained in immunosuppressed animals with HIV–vaccinia env recombinants. ○, MUT-7 ENV; ■, WR ENV. The surviving mouse (WR ENV) in D died on day 21. The survival pattern shown with mutant recombinant virus was maintained for more than 2 months.](image-url)
virulence in an immunosuppressed host. Expression of the highly sensitive reporter gene luciferase in spleen and liver but not in other organs provided direct evidence that tissue tropism of attenuated recombinant virus is the same as wild-type virus. Cells infected with attenuated HIV–vaccinia viruses were efficient in their ability to process env and gag precursors. Similar processing for HIV-1 env products has been observed with other HIV–vaccinia recombinants derived from wild-type virus (25, 26). However, the processing of HIV-1 gag precursors that we have observed with HIV–vaccinia recombinant has not been observed, and only the unprocessed form of gag p56 has been reported with a wild-type HIV–vaccinia gag recombinant (27). Processing of gag precursors by our vaccinia recombinants is likely to be due to the function of the protease encoded by the 5′ end of the pol gene contained in the BssHII–EcoRI fragment used to introduce the gag sequence as a result of ribosomal “frame-shifting” (28). Of significance in our study is that mice vaccinated with attenuated HIV–vaccinia recombinants develop antibodies to env and gag proteins. The immune response to the gag protein was stronger than to the env protein. However, if after vaccination mice were given a booster injection i.v. with purified gp160, we then observed good antibody response to the env protein (Fig. 3A).

In this study we have established an immunosuppressed mouse model system to evaluate virulence of vaccinia virus recombinants. In this animal–virus system, we observed clear differences in mortality between recombinant viruses that correlated with the viral genetic markers (Fig. 4). In fact, inactivation of wild-type vaccinia thymidine kinase locus that is known to reduce virulence in normal mice (8), has little effect in decreasing virulence in immunosuppressed animals. However, a vaccinia recombinant with a left-end deletion and alterations in the 14-kDa fusion protein encoding gene has about 100 times less virulence in immunosuppressed animals than a wild-type recombinant with a thymidine kinase-negative phenotype. Thus, a potential advantage in generating highly attenuated vaccinia virus recombinants with deletions and point mutations is that these types of recombinants will greatly decrease the risk of virus dissemination after vaccination, particularly in an immunocompromised host.

Considering the characteristics of the highly attenuated vaccinia recombinants that we have generated, these recombinants and derivatives might be useful as immunogens in studies of recognition specificity of immune T cells and in the design of safer vaccines against pathogens of veterinary and human importance.

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