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Systemically administered DNA and fowlpox recombinants expressing four vaccinia virus genes although immunogenic do not protect mice against the highly pathogenic IHD-J vaccinia strain

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ABSTRACT

The first-generation smallpox vaccine was based on live vaccinia virus (VV) and it successfully eradicated the disease worldwide. Therefore, it was not administered any more after 1980, as smallpox no longer existed as a natural infection. However, emerging threats by terrorist organisations has prompted new programmes for second-generation vaccine development based on attenuated VV strains, which have been shown to cause rare but serious adverse events in immunocompromised patients. Considering the closely related animal poxviruses that might also be used as bioweapons, and the increasing number of unvaccinated young people and AIDS-affected immunocompromised subjects, a safer and more effective smallpox vaccine is still required. New avipoxvirus-based vectors should improve the safety of conventional vaccines, and protect from newly emerging zoonotic orthopoxvirus diseases and from the threat of deliberate release of variola or monkeypox virus in a bioterrorist attack. In this study, DNA and fowlpox recombinants expressing the L1R, A27L, A33R and B5R genes were constructed and evaluated in a pre-clinical trial in mouse, following six prime/boost immunisation regimens, to compare their immunogenicity and protective efficacy against a challenge with the lethal VV IHD-J strain. Although higher numbers of VV-specific IFNγ-producing T lymphocytes were observed in the protected mice, the cytotoxic T-lymphocyte response and the presence of neutralising antibodies did not always correlate with protection. In spite of previous successful results in mice, rabbits and monkeys, where SIV/HIV transgenes were expressed by the fowlpox vector, the immune response elicited by these recombinants was low, and most of the mice were not protected.

1. Introduction

The original vaccinia virus (VV) smallpox vaccine was administered by scarification. Due to the successful eradication of smallpox worldwide, the use of this vaccine was discontinued from 1980, later to be replaced by VV-derived, second-generation, cell-cultured vaccines, such as ACAM2000® (Weltzin et al., 2003). These vaccines showed similar immune responses as Dryvax®, but were associated with health risks and contraindications (Nalca and Zumbrun, 2010; Wiser et al., 2007), as they could spread to immunocompromised, non-vaccinated subjects (Bray, 2003; Centers for Disease Control and Prevention, 2007; Jacobson et al., 2008; Lane and Goldstein, 2003). In the attempt to develop strains with lower reactogenicity and fewer side effects, and to face the potential re-emergence or accidental/deliberate release of orthopoxviruses (OPXV) in the human population (Cardeti et al., 2011; Megid et al., 2012; Vogel et al., 2012; Whitley, 2003), a third generation of attenuated vaccines was developed. Although not as virulent as variola, the monkeypox (MPXV) and cowpox (CPXV) viruses are also a threat to public health, as they cause mortality

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in underdeveloped countries (Reed et al., 2004) and can become a potential bioweapon if adapted to grow and spread in humans (Lewis-Jones, 2004).

The new attenuated vaccines, which include the VV-derived Lister clone LC16m8 (Kenner et al., 2006) and the most advanced modified vaccinia Ankara (MVA; e.g., the IMVAMUNE® (Kennedy and Greenberg, 2009)), have an improved safety profile and induce more rapid responses (Earl et al., 2004). However, their ability to induce long-term immunity is controversial (Earl et al., 2007; Ferrier-Rembert et al., 2008), and they also fail to induce protective immunity in immunocompromised animals (Edghill-Smith et al., 2005a). MVA also induces lower immunogenicity than the traditional smallpox vaccine (Ferrier-Rembert et al., 2008) and shows a limited replication in mammals (Blanchard et al., 1998). A further caveat may be represented by possible recombination events that may rescue endogenous OPXV genes and generate a fully-replicative genotype (Okeke et al., 2009; Verheust et al., 2012). MVA also failed to protect animals with CD4/CD8 combined immunodeficiency (Wiser et al., 2007) and Rhesus macaques infected with simian immunodeficiency virus showing a very low cell count of the immune repertoire (Edghill-Smith et al., 2005a).

Although the VV antigens that protect against smallpox are not completely defined, neutralising antibodies have mainly been raised against the surface proteins of the two OPXV infectious particles: the mature virions (MVs) released after cell lysis and the extracellular virions (EVs), which are wrapped by an additional envelope (Moss, 2011; Pacchioni et al., 2013; Roberts and Smith, 2008; Smith et al., 2002). In particular, the combined use of the L1 and A27 proteins of MV, and the A33 and B5 proteins of EV has conferred better protection than the individual use of MV or EV proteins (Fogg et al., 2004; Hooper et al., 2002). These subunit vaccines are protective against VV intranasal challenge in mice or MPXV intravenous challenge in monkeys (Buchman et al., 2010; Fogg et al., 2007; Hirao et al., 2011; Hooper et al., 2010).

In the present study, four DNA recombinants that express the VV *L1R, A27L, A33R* and *B5R* genes (called the 4DNAmix) were used, followed by four novel fowlpox (FP) recombinants that express the same genes (Pacchioni et al., 2013) (called the 4FPmix). MVA, which also contains the same genes, was used as a control, either alone or in a prime/boost regimen, followed by the 4FPmix. A direct comparison was performed between humoral and cell-mediated responses and the ability to induce protection in the immunised mice. Attenuated avipox viruses have been developed as novel vectors for the construction of recombinant vaccines against several human infectious diseases (Radaelli et al., 1994; Zanotto et al., 2010). These vectors are restricted for replication to avian species, but permissive for entry and transgene expression in most mammalian cells. They are also immunologically non-cross-reactive with vaccinia (Baxby and Paoletti, 1992), and they can escape neutralisation by vector-generated antibodies in smallpox-vaccine-experienced humans.

2. Materials and methods

2.1. Cells

Specific-pathogen-free primary chick embryo fibroblasts (CEF) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated calf serum (Gibco Life Technologies, Grand Island, NY, USA), 5% Tryptose Phosphate Broth (Difco Laboratories, Detroit, MI, USA), 100 U/ml penicillin and 100 mg/ml streptomycin. Green monkey kidney (Vero) cells, and Balb/C mouse fibroblasts (B77 cells) were grown in DMEM supplemented with 10% heat-inactivated calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin.

2.2. Viruses and fowlpox recombinants

The highly pathogenic IHD-J strain of VV was supplied by S. Dales (University of Western Ontario, London, Canada) (Wilton et al., 1986), and was grown in Vero cells, to use as the challenging virus (1×10^6 PFU/mouse) through the airways. IHD-J was amplified in Vero cells, purified on discontinuous sucrose density gradient, and titrated as described previously (Pacchioni et al., 2013). The 4FP recombinants, FP_{L1R}, FP_{A27L}, FP_{A33R} and FP_{B5R}, that express the L1, A27, A33 and B5 proteins of VV, respectively, were generated in our laboratory by *in-vivo* homologous recombination (Pozzi et al., 2009). Gene insertion was performed downstream of the VV H6 early/late promoter (Rosel et al., 1986), inside the 3-β-hydroxysteroid dehydrogenase 5-delta 4 isomerase gene interrupted by a multiple cloning site. Recombinants were grown and amplified in CEF, and purified on discontinuous sucrose gradients. MVA was kindly obtained by A. Siccardi (Dept. of Biology, University of Milan, Italy), and amplified and purified on CEF, as already described (Soprana et al., 2011).

2.3. Plasmids

The expression plasmids $pcDNA3.1_{A27L}$, $pcDNA3_{L1R}$, $pcDNA3_{A33R}$ and $pcDNA3_{B5R}$ were constructed in our laboratory by insertion of the same genes used for the FP recombinants. In particular, pcDNA3.1 (Invitrogen Corp., San Diego, CA, USA) was used for the *A27L* gene, which was inserted into the HindIII/NotI restriction sites, whereas pcDNA3 (Invitrogen Corp.) was used for the *L1R*, *A33R* and *B5R* genes, which were separately inserted into the HindIII/XhoI restriction sites. Both pcDNA3.1 and pcDNA3 contain the human CMV promoter. The mixture of these four recombinants is called the 4DNAmix. $PcDNA3_{gag/pol}$ was used as an irrelevant negative control, and described previously (Zanotto et al., 2010).

2.4. Immunisation protocols

Six groups of eight female Balb/C mice (Charles River Laboratories, Wilmington, MA, USA) were immunised by multiple injections. All of the mice were inoculated three times at 3-week intervals, before challenge. After immunisation, all of the mice remained in good health, without loss of weight. Before each inoculation, the mice were anaesthetised with 300 μl 2.5% 2,2,2-tribromoethanol (Avertin®) (Sigma, St Louis, MO, USA) i.p., and bleeding was performed from the retro-orbital eye plexus. The plasma fraction was aliquoted and frozen at −80 °C. Six different prime/boost immunisation protocols were followed (Fig. 1), using: (i) plasmid 4DNAmix (100 μg of each recombinant/mouse; i.m.) in combination with recombinant FP*gagpol* (10^7 PFU/mouse; s.c.) (Group 1); (ii) 4DNAmix (100 μg of each recombinant/mouse; i.m.) in combination with recombinant 4FPmix (10^7 PFU of each recombinant/mouse; s.c.) (Group 2); (iii) recombinant 4FPmix (10^7 PFU of each recombinant/mouse; s.c.) used for the three inoculations (Group 3); (iv) MVA (10^7 PFU/mouse; s.c.) used for the three inoculations (Group 4); (v) MVA (10^7 PFU/mouse; s.c.) in combination with 4FPmix (10^7 PFU of each recombinant/mouse; s.c.) (Group 5); and (vi) $pcDNA_{gagpol}$ plasmid (100 μg/mouse; i.m.) in combination with FP*gagpol* recombinant (10^7 PFU/mouse; s.c.) (Group 6). All of the mice were maintained in accordance with the Italian national guidelines. Mice were observed for signs of disease and weighed daily, provided food and water *ad libitum*. Every effort was made to minimise suffering and, based on predetermined criteria (loss of more than 30% body weight) moribund animals were euthanized. The approval for this study was granted by the ethical Committee of the University of Milan.