Immunogenicity of modified vaccinia virus Ankara expressing the hemagglutinin stalk domain of pandemic (H1N1) 2009 influenza virus

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ABSTRACT
Background: Vaccination offers protection against influenza, although current vaccines need to be reformulated each year. The development of a broadly protective influenza vaccine would guarantee the induction of heterosubtypic immunity also against emerging influenza viruses of a novel subtype. Vaccine candidates based on the stalk region of the hemagglutinin (HA) have the potential to induce broad and persistent protection against diverse influenza A viruses.

Methods: Modified vaccinia virus Ankara (MVA) expressing a headless HA (hlHA) of A/California/4/09 (CA/09) virus was used as a vaccine to immunize C57BL/6 mice. Specific antibody and cell-mediated immune responses were determined, and challenge experiments were performed by infecting vaccinated mice with CA/09 virus.

Results: Immunization of mice with CA/09-derived hlHA, vectored by MVA, was able to elicit influenza-specific broad cross-reactive antibodies and cell-mediated immune responses, but failed to induce neutralizing antibodies and did not protect mice against virus challenge.

Conclusion: Although highly immunogenic, our vaccine was unable to induce a protective immunity against influenza. A misfolded and unstable conformation of the hlHA molecule may have affected its capacity of inducing neutralizing antiviral, conformational antibodies. Design of stable hlHA-based immunogens and their delivery by recombinant MVA-based vectors has the potential of improving this promising approach for a universal influenza vaccine.

Introduction

Each year, influenza A and B viruses cause seasonal epidemics in the human population worldwide. Currently, available vaccines fully protect against homologous virus infection, but only marginally against heterologous drift virus infection [1,2]. Therefore, influenza vaccines conferring broad and persistent protection against a wide range of influenza viruses, including any emerging pandemic strain, would be highly desirable [3].

Recently, several approaches have emerged as potential solutions for a universal influenza virus vaccine [4,5]. In particular, immunogens that can induce antibodies specific for the highly conserved stalk domain of influenza A virus hemagglutinin (HA) have been described as very promising [6–10]. Antibodies targeting the stalk domain may inhibit major structural rearrangements in HA that are required for the fusion of viral and endosomal membranes, and thus release of the viral contents into the cell [11]. In addition, these antibodies could exert antiviral protection through antibody-dependent cellular cytotoxicity (ADCC) activity in vivo [12]. However, they are generated less abundantly than are antibodies specific for the globular head of the HA during natural infection by influenza viruses [13,14]. As a strategy to improve their induction, the highly conserved disulfide bond (Cys52-Cys277 [H3 numbering]) has been used to construct headless HA (hlHA) immunogens [7], and to generate influenza viruses expressing chimeric HAs that consist of stalk domain and globular head from different influenza virus strains [15,16]. Vaccination regimens based on these chimeric HA structures have been shown to elicit anti-stalk-polyclonal antibody responses that are able to protect mice against challenge with a panel of heterologous and heterotypic viruses [17–20].

Recombinant modified vaccinia virus Ankara (MVA)-based vaccine platform technology for the delivery of foreign antigens has been widely used in pre-clinical and clinical studies to elicit long-term humoral and cell-mediated responses [21]. In the current study, we generated an MVA expressing the hlHA of A/CA/04/09 virus (MVA-hlHA-CA/09), and investigated the immunogenic
properties and ability of this vaccine to protect mice against viral challenge.

**Methods**

**Generation and characterization of MVA-hlHA-CA/09**

The headless deletion of the construct used in this study (hlHA-CA/09) was designed as described previously for hlHA of A/PR8/34 virus [7]. In particular, the nucleotide sequence of the HA gene of A/California/4/09 virus (CA/09, H1N1), encoding a linker peptide of four glycines, was codon-optimized for maximal expression in mammalian cells (GeneScript; Piscataway, NJ, U.S.A). The MVA-hlHA-CA/09 virus was generated through a reliable method to produce marker-free recombinant MVA viruses by swapping green and red fluorescence genes combined with fluorescence-activated cell sorting [22]. Serum-free cultures of chicken embryo fibroblasts (CEF) were utilized for recombinant virus construction, terminal dilution cloning, and virus stock preparation. The MVA wild type (MVA-wt) and the MVA-CA/09 virus, previously generated to encode the full-length HA molecule of CA/09 virus (HA-CA/09) [23], were also included in the study.

The production of CA/09 HA and hlHA by recombinant MVA-infected CEF cells, and their molecular weights was determined by Western blot analysis (SDS-PAGE in both reducing and non-reducing conditions) using an α-H1N1 chicken serum (Serum α-H1N1 3/09; courtesy of Ilaria Capua, IZSV, Legnaro, Italy). Flow cytometric analysis was performed by using a specific α-H1N1 polyclonal antiserum as control and a panel of human monoclonal antibodies specific for stalk conformational epitopes (FB179, FE43, and FG20; courtesy of Antonio Lanzavecchia, IRB, Bellinzona, Switzerland) to determine the surface expression by MVA recombinant viruses [24].

**Immunogenicity and protective efficacy in mice of MVA-hlHA-CA/09 virus**

C57BL/6 mice (6–8 weeks) were obtained from Charles River, Italy, and animal experiments were performed at the Istituto Superiore di Sanità in compliance with institutional guidelines and approved protocols. Groups of mice (6–7 mice per group) were vaccinated intramuscularly (i.m.) with 2 doses of 10⁷ pfu of the MVA-hlHA-CA/09, administered 3 weeks apart. All experiments were conducted after the second immunization.

Cell proliferation was measured by *in vitro* re-stimulation of splenocytes with recombinant HA protein from CA/09 virus or CA/09 virus-loaded and γ-irradiated antigen presenting cells (APC) for 4 days at 37 °C. [³H] thymidine (Amersham Biosciences, UK) (0.5 μCi/well) was added 8 h before harvesting. Antigen-specific IFN-γ-producing cells were determined by using an IFN-γ ELISPOT assay performed *ex vivo* with fresh splenocytes. In the challenge experiments, inflammatory cells were also collected from the mediastinal lymph nodes (MLN) draining the respiratory tract of infected mice, and then directly subjected to the ELISPOT assay [25,26]. Delayed type hypersensitivity (DTH) response was evaluated in mice by recording footpad swelling 1 and 2 days following antigen challenge [27]. Serum samples were collected and tested for the presence of influenza-specific antibodies by ELISA, using plates coated with 0.1 μg per well of purified recombinant HA proteins of H1, H3, and H5 subtypes (eEnzyme® LLC) [7]. The sera were also tested by hemagglutination inhibition (HI) assay, MDCK cell-based microneutralization (MN) assay and plaque reduction assay [15,28].

For challenge experiments, four weeks after the last immunization, mice were anesthetized with Avertin and challenged intranasally (i.n.) with 3 LD₅₀ (50% lethal dose) of CA/09 virus in a 40 μl volume. Mice were then monitored for survival and weight loss for 14 days after infection.

**Results**

The MVA-hlHA-CA/09 virus was generated and the transgene expression of both MVA-hlHA-CA/09 and MVA-HA-CA/09 viruses was monitored by Western blot in infected CEF lysates with a specific α-H1N1 polyclonal antiserum. Figure 1 shows the identity and expected
molecular weight of the influenza proteins (Figure 1). Western blot analysis after SDS-PAGE in non-reducing conditions showed a band of very high molecular weight, not entering the gel (not shown). To test whether the hIHA molecule was transported to the cell surface, cytofluorimetric analysis of CEF infected with MVA recombinant viruses was performed following surface staining of the cells with a panel of monoclonal antibodies specific for conformational epitopes of the HA stalk [24]. The mean fluorescence values measured in recombinant MVA virus-infected cells confirmed the surface expression of the full-length HA whereas no expression was observed on the cell surface from cells expressing the hIHA molecule (Figure 2) or in cells infected with MVA-wt (not shown). The above findings were reproduced using a specific α-H1N1 polyclonal antiserum instead of the monoclonal antibodies as a control (not shown). Similar results were obtained by using transiently transfected HEK 293T cells with plasmids encoding the full-length HA or hIHA, thus suggesting a misfolded conformation of the hIHA protein (data not shown).

To investigate the differential cellular distribution of the full-length HA and its headless derivative, confocal microscopy was performed (courtesy of Andrea Orsi, Dibit, OSR, Milano, Italy) using co-localization markers (calnexin for the ER and concanavalin A for the cell surface), and the α-V5 antibody which recognizes a tag at the C-terminus of both forms of the HA. As shown in Figure 3, the headless derivative is largely retained in the ER, while the full-length localizes independently from calnexin. Conversely, full-length HA co-localizes with concanavalin A, while hIHA does not (data not shown). Confocal microscopy controls of cells infected by MVA-wt showed no background binding of α-V5 antibodies (not shown).

The immunogenic properties of MVA-hIHA-CA/09 virus were investigated by injecting two doses of MVA-hIHA-CA/09, MVA-HA-CA/09 or MVA-wt virus in C57BL/6 mice to first determine the presence of influenza-specific antibodies in serum samples. When tested against recombinant HA protein derived from CA/09 virus, sera from mice vaccinated with MVA-hIHA-CA/09 reacted positively at high titers while sera from mice vaccinated with MVA-wt virus were negative (Figure 4(A)). Because the HA subtypes of influenza viruses are divided in two phylogenetic groups, based on the distinct structures of the stalk region [16], the sera were also tested against recombinant HA proteins derived from A/New Caledonia/20/99 (NC/99, H1N1), A/Japan/305/1957 (Jap/57, H2N2), A/Vietnam/1203/2004 (VN/04, H5N1), and A/Indonesia/5/2005 (IN/05, H5N1) viruses of group 1, and from A/Brisbane/10/2007 (Bris/07, H3N2), as belonging to group 2. In contrast to the background level detected with sera from MVAwt-vaccinated mice, high antibody titers against the heterologous HAs from group 1 were measured in sera from MVA-hIHA-CA/09 vaccinated mice, whereas no reactivity was measured in these sera against the recombinant HA protein of H3 subtype (Bris/07) (Figure 4(A)). Thus, broadly reactive antibodies against group 1 were elicited following vaccination of...

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**Figure 2.** Flow cytometry analysis of CEF infected by MVA-hIHA-CA/09 or by MVA-HA-CA/09 virus using three human monoclonal antibodies specific for stalk conformational epitopes (FB179, FE43, and FG20). Samples were treated with both primary antibody and FITC-labeled anti-human Ig-rabbit antibody (infected cells, red lines, and uninfected cells, black lines). As controls, infected cells were only treated with the second antibody (green lines).
mice with MVA-hlHA-CA/09 virus, and at higher levels than those elicited by MVA-HA-CA/09 virus. However, the absence of a globular head domain of the HA in the hlHA vaccine construct was responsible for lack of reactivity against CA/09 virus in HI assay, and also in MN (Figure 4(B)) and plaque reduction assays (data not shown).

Figure 3. The different cellular distributions of full-length hemagglutinin (HA) (upper panel) and its headless derivative (hlHA) (lower panel) are shown by their co-localization with the ER marker calnexin (cnx). The headless derivative is largely retained in the ER, while the full-length HA localizes independently. The α-V5 antibody recognizes a tag at the C-terminus of both forms of the HA molecule.

Figure 4. Serum antibody titers in mice following immunization with MVA-hlHA-CA/09 virus. Groups of C57BL/6 mice were immunized twice, three weeks apart, with MVA-hlHA-CA/09, MVA-HA-CA/09 or MVA-wt virus. (A) The titers of anti-influenza specific antibodies were determined with use of 50-fold-diluted samples obtained three weeks after the second immunization by titration on ELISA plates coated with recombinant HA proteins of CA/09, NC/99, Jap/87, VN/04, IN/05 or Bris/07 virus. Bars represent means ± standard deviation (SD) for six mice per group. OD 450 = Optical density at 450 nm. (B) Specific antibodies against CA/09 virus were measured in serum pools by HI and MN assays. ND = Not detectable.

Figure 5. Vaccine-induced cellular immune responses in mice. Groups of mice were immunized twice with MVA-hlHA-CA/09 or MVA-wt virus. (A) Seven days later, freshly isolated splenocytes were cultured for 4 days in the presence of the recombinant HA protein from CA/09 virus, unloaded- or CA/09 virus loaded-APC. The proliferative response to Concanavalin A (Con A) was used as a control, and the fold increase in proliferation was calculated by determining the ratio of the stimulated:unstimulated proliferative index. (B) Four weeks later, recombinant HA protein was injected subcutaneously in the footpad and differences in footpad swelling between MVA-hlHA-CA/09- and MVA-wt-vaccinated mice were determined 24 and 48 h after antigen injection by using a caliper. The data are the mean footpad swelling ± SD of three (for 24 h readings) and two (for 48 h readings) independent experiments. *P < 0.01, NS = Not significant, compared with MVA-wt-immunized mice by unpaired Student’s t test.
MVA-wt and then challenged with CA/09 virus (Figure 6(A)). Notably, higher levels of CD8+ T cells specific for the immunodominant epitope (NP366-374) of influenza A virus nucleoprotein were detected in the MLN and spleen of MVA-hlHA-CA/09-vaccinated mice, as compared to those from MVAwt-vaccinated mice, suggesting a role of CD4+ T help at inducing primary effector CD8+ T cells in these mice previously vaccinated with the hlHA-based immunogen. Together, our results provide evidence that MVA-hlHA-CA/09 virus could prime antigen-specific T cell responses that were rapidly recalled following pulmonary challenge with CA/09 virus. Importantly, however, no mice survived in the group immunized with MVA-hlHA-CA/09 or MVA-wt virus and challenged with an infectious dose of influenza virus (Figure 6(B)). As expected, MVA-HA-CA/09 virus could provide complete protection against virus challenge, that correlates with high levels of serum HI and MN neutralizing antibodies induced following vaccination (Figure 4(B)).

Discussion

Here, we show that the stalk region of the hlHA construct, which was obtained by introducing four glycines in place of residues 53 to 276 of HA1 and expressed by MVA-hlHA-CA/09 virus, was able to elicit broad, anti-HA cross-reactive antibodies, and cell-mediated immune responses in C57BL/6 mice. However, it was unable to induce neutralizing antibodies, hence unable to protect mice against virus challenge.

In particular, hlHA-specific antibodies were elicited and capable of binding HA molecules of group 1 in...
ELISA, but they failed to neutralize CA/09 virus infection in vitro, as assessed by MN and plaque reduction assays on MDCK cells. These findings suggest that the hIHA construct still maintains some antigenic determinants that are recognized in the native protein but not those responsible for induction of antibodies neutralizing virus infection, or alternatively these epitopes are less accessible to antibodies when displayed on the virions. Further, lack of in vivo protection against viral challenge suggests that these antibodies were ineffective at inducing Fc-mediated effector mechanisms, such as ADCC and complement-dependent cytotoxicity [12]. Whichever the immunologic reason for lack of protection, a misfolded conformation of the hIHA molecule may have affected the ability to induce a protective immunity against influenza. Indeed, the strategy based on the use of chimeric HAs with ‘exotic’ head domains has been adopted to overcome problems related to stability and/or misfolding of hIHA [15,16]. Recently, further strategies have been described to engineer stable HA stem antigens that were effective in inducing heterosubtypic influenza protection in mice [29,30], thus providing proof-of-concept for design of successful hIHA-based immunogens.

Our results show that this vaccine construct was able to elicit cellular immune responses in C57BL/6 mice. Previous studies by Hessel et al. [31] could detect the induction of CD8+ T cells but not CD4+ T cells and antibodies by a recombinant MVA expressing the hIHA of VN/04 virus in BALB/c mice that, however, did not survive against high challenge virus doses. In the present study, the cell proliferative response to antigen in vitro and DTH response measured in vivo against soluble recombinant HA protein of CA/09 virus, clearly suggest a functional role of CD4+ T cells in these responses. Notably, the priming of CD8+ T cell responses specific to the immunodominant epitope NP 366-374 which occurs earlier in MVA-hIHA-CA/09-vaccinated mice than in MVAwt-vaccinated ones after viral challenge, is likely associated with antigen-specific CD4+ T cell recall responses [32]. Nevertheless, these cell-mediated immune responses elicited by MVA-hIHA-CA/09 failed to protect mice from lethal virus challenge, although they might contribute to dampen viral replication in mice challenged with a sublethal dose of virus. The low, background levels of IFN-γ production from immune splenocytes indicate the inability of CD8+ T cell epitopes in the stalk domain of CA/09 virus to bind H-2b-restricted MHC class I molecules in C57BL/6 mice. This may further contribute to vaccine failure in protecting mice from lethal virus challenge. Thus, our results suggest that a misfolded conformation of the hIHA molecule associated with lack of cell surface expression may predict inefficacy of vaccination and prevent unnecessary efficacy testing of candidate hIHA-based vaccines in animal models.

Overall, design of stable hIHA-based immunogens is highly desirable. Their delivery by recombinant MVA-based vectors and, possibly, in combination with other conserved influenza antigens, would represent a promising strategy for inducing broadly protective immunity to influenza.

**Competing interests**

The authors declare that they have no competing interests.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

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