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Evaluation of a novel immunogenic vaccine platform based on a genome replication-deficient Sendai vector

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ABSTRACT

We developed a novel vaccine platform based on a paramyxoviral, genome replication-deficient Sendai virus vector that can express heterologous genes inserted into the genome. To validate the novel approach *in vivo*, we generated a combined vaccine candidate against human respiratory syncytial virus (RSV) and human parainfluenza virus type 3 (PIV3). The present study compares two different methods of displaying heterologous antigens: (i) the RSV fusion (F) protein, encoded as a secretable version in an additional transcription unit, serves as an antigen only after being expressed in infected cells; (ii) PIV3 fusion (F) and hemagglutinin-neuraminidase (HN) genes, replacing Sendai counterparts in the vector genome, are also expressed as structural components on the surface of vaccine particles. The efficacy of this prototype vaccine was assessed in a mouse model after mucosal administration. The vaccine candidate was able to elicit specific mucosal, humoral and T cell-mediated immune responses against RSV and PIV3. However, PIV3 antigen display on the vaccine particles' surface induced higher antibody titers than the RSV antigen, being expressed only after cell infection. Consequently, this construct induced an adequate neutralizing antibody response only to PIV3. Finally, replicating virus particles were not detected in the lungs of immunized mice, confirming the genome stability and replication deficiency of this vaccine vector *in vivo*. Both factors can contribute substantially to the safety profile of vaccine candidates. In conclusion, this replication-deficient Sendai vector represents an efficient platform that can be used for vaccine developments against various viral pathogens.

1. Introduction

Most of our current viral vaccines are made with live-attenuated or inactivated viruses or proteins from pathogens. Among these, live attenuated vaccines have been very powerful for disease control or even eradication, due to their efficacy and long-lasting immunity. However, the safety of such vaccines, or vaccine candidates, is constantly being discussed as they could be associated with genetic instability and residual virulence [1]. A possible reversion of attenuating mutations, as seen with the Sabin polio vaccine [2,3], or finding the right balance of attenuation, which complicates for instance the development of live-attenuated RSV vaccines [4], illustrate the shortcomings of live vaccines. Thus, novel vaccine approaches could help to achieve optimal protection against infectious diseases. Viral vectors have emerged as potent and defined approaches with immunogenic characteristics [5–9] similar to live attenuated vaccines. However, live attenuated vectors still present similar safety concerns. The optimal vaccine vector should therefore combine the following criteria: (i) replication-deficiency & no persistence, (ii) genetic stability, (iii) no interaction with host cell genome, (iv) no pre-existing immunity, and (v) induction of specific humoral and cellular immunity.

To fulfill all these requirements, we tested a recently developed novel viral vector platform for the first time *in vivo* for its applicability as a vaccine vector. The vector is based on a genetically engineered genome replication-deficient Sendai virus (SeV). This vector can still efficiently express genes, including heterologous transgenes, *in vitro*, without spreading in tissue culture [10]. However, as contributing factors to safety of this platform its replication-deficiency and genetic stability still had to be proven *in vivo*.

In addition to these aspects, an efficacious vaccine vector must be capable of efficiently stimulating humoral and cellular immune responses. Due to its replication deficiency, the novel vector showed reduced gene expression compared to

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replication-competent Sendai vectors *in vitro* [10]. In order to evaluate whether the expression of heterologous genes still sufficed to induce substantial immune responses *in vivo*, we designed a prototype vaccine in a special way: the F protein of the respiratory syncytial virus (RSV) was encoded as a soluble antigen being expressed in infected cells; however, the F and HN surface proteins of the parainfluenzavirus type 3 (PIV3) were also encoded in the vector genome and, in addition, present as structural components on the vaccine particle. In this way, two different strategies of displaying antigens by the novel viral vector were compared and analyzed by evaluating specific humoral, local mucosal and cellular immune responses after intranasal immunization of mice.

2. Materials and methods

2.1. Cells and viruses

Vero (ATCC CCL-81), HEp-2 (ATCC CCL-23) and P815 cells (ATCC TIB-64) from the American Type Culture Collection (Rockville, MD, USA) were maintained in Eagle minimal essential medium or RPMI (Invitrogen, Milan, Italy) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Invitrogen), 100 μg/ml streptomycin and 100 U/ml penicillin. The complementing helper cell line "P-HC" is derived from Vero cells expressing SeV phosphoprotein [11]. BSR-T7 cells [12] were kindly provided by Klaus-K. Conzelmann (Munich).

RSV type A (Long strain, ATCC VR-26) and human PIV3 (strain C243, ATCC VR-93) were cultured on HEp-2 cells and Vero cells, respectively, at 37 °C. All recombinant SeV variants, derived from strain D52 (ATCC VR-105), were cultured at 33 °C.

2.2. Genomic vector design

The ORF encoding the ectodomain of RSV F ("sF", amino acids (aa) 1–524) was previously inserted in a transcription cassette between the SeV P and M genes ("rSeV sF") [13]. Plasmids containing the cDNA of PIV3 or SeV F and HN genes, respectively, were used as templates for the construction of chimeric PIV3/SeV F or HN ORFs by an overlapping PCR technique [14]. The sequence-verified chimeric ORFs were inserted consecutively into a triple transcriptional cassette [15] comprising the *San*DI fragment from the SeV genome, including the M gene but without the ORFs for F and HN (details in Supplementary materials and methods). The resulting recombinant SeV genome, following the "rule of six" [16], was designated rc/rdPIRV (replication-competent/-deficient PIV & RSV Vaccine) and was confirmed by restriction analysis and sequencing.

2.3. Virus rescue, propagation and titration

Recombinant viruses were recovered from transfected BSR-T7 cells as described in [11] with slight modifications. FuGENE6 (Roche) was used as transfection reagent at 2.0 μl/μg DNA. Virus was harvested from the supernatant and amplified in Vero cells (replication-competent SeV) or in a helper cell line [11] (replication-deficient SeV vector). To provide a negative control for *in vivo*-experiments, an aliquot of the stock was inactivated by UV-irradiation at 48 mJ/cm^2 (254 nm). Viruses were titrated as previously described [11] and titers were given as cell infectious units per milliliter (ciu/ml). The integrity of the various SeV vectors was confirmed by RT-PCR and sequencing.

2.4. Western blot analysis

Extracts from Vero cells, mock infected or infected with PIV3, RSV or rdPIRV, were collected and separated by SDS-PAGE. After blotting on a nitrocellulose membrane proteins were detected with mouse monoclonal antibodies against PIV3 HN and F proteins (Chemicon, Milan, Italy) and a goat anti-RSV antibody (Meridian Life Science, Saco, ME).

2.5. Animals and immunization

Each experiment was repeated three times to ensure reproducibility of results. Female BALB/c mice at 6–8 weeks of age were purchased from Charles River Laboratories (Milan, Italy). Three groups of four mice were immunized intranasally. Group A received 20 μl (10 μl per nostril) of phosphate buffer (PBS), group B received 20 μl of rdPIRV, group C were given 20 μl of UV-inactivated rdPIRV containing 1.5×10^7 ciu of vaccine, and group D received 20 μl of rcPIRV containing 1.0×10^6 ciu. Each group was inoculated three times, three weeks apart. Ten days after the last immunization, mice were sacrificed for collection of bronchoalveolar lavages (BAL) and nasal washes (NW) as described elsewhere [17]. Blood and spleens were harvested for further analyses. All animal experiments complied with all relevant institutional policies, according to European Parliament directive 2010/63/EU [18].

2.6. ELISA

IgG and IgA antibodies were measured by enzyme-linked immunosorbent assay. For the determination of virus-specific IgG and IgA antibodies, purified virions (1 μg/ml) of inactivated human PIV3 (IBT Systems, Reutlingen, Germany) or RSV type A (Experteam, Venice, Italy) were used as antigens, as previously described [17]. Results were expressed as the mean ± SD of two determinations from three different experiments. Differences were determined by the Mann–Whitney Rank Sum test.

2.7. Neutralization assay

Virus neutralization assay was carried out on Vero cells for PIV3, and Hep-2 cells for RSV, in a 96-well microplate. Briefly, serial two-fold dilutions of immunized mice serum were added to an equal volume of PIV3 or RSV containing 100 $TCID_{50}$ in 50 μl and incubated for 90 min at 37 °C, followed by the addition of 5×10^3 cells to each well. The presence of a cytopathic effect (CPE) was examined four days later. The antibody titer was evaluated as the highest dilution that resulted in a 50% reduction of CPE. The assay was performed twice. An antibody titer of <4 was considered negative.

2.8. Cytokine and cytotoxicity assays

Splenocytes drawn from immunized mice and lymphocytes were collected by Fycoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) gradient. 2×10^5 of unfractionated cells in RPMI1640 plus 10% FCS were cultured in a total volume of 200 μl with 10 μg/ml of purified inactivated virus or 5 μg/ml ConcanavalinA (Sigma, Milan, Italy). Control wells received cell suspension only. After 24 h in culture, cell-free supernatants were harvested for the presence of IL-5 and after 48 h for the presence of IFN-γ. Samples were stored at −80 °C. The assay was performed as previously described [17].

To evaluate virus specific cytotoxicity, splenocytes were stimulated *in vitro* with inactivated PIV3 or RSV before being tested, as described elsewhere [17] and in the Supplementary materials and methods.

2.9. Monitoring of replication-deficiency in vivo

Two groups of BALB/C mice ($n = 4$) were inoculated i.n. with 1×10^5 ciu of rdPIRV or replication-competent virus, both expressing EGFP as a reporter. Three days later, mice were sacrificed and lungs and blood samples were collected. Virus present in