Vaccine 29 (2011) 3935–3944

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

MHV-68 producing mIFNα1 is severely attenuated *in vivo* and effectively protects mice against challenge with wt MHV-68

Eleonora Aricò[a,*], Domenica M. Monque[a], Giuseppina D'Agostino[a], Federica Moschella[a], Massimo Venditti[a], Ulrich Kalinke[b], Deborah J. Allen[c], Anthony A. Nash[c], Filippo Belardelli[a], Maria Ferrantini[a]

[a] *Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy*
[b] *Institute for Experimental Infection Research, TWINCORE, Centre for Experimental and Clinical Infection Research, Feodor-Lynen-Str. 7, 30625 Hannover, Germany*
[c] *Centre for Infectious Diseases, The Roslin Institute, University of Edinburgh, Edinburgh, UK*

ARTICLE INFO

Article history:
Received 17 January 2011
Received in revised form 3 March 2011
Accepted 25 March 2011
Available online 8 April 2011

Keywords:
Gammaherpesviruses
Type I IFN
Genetically modified viruses
Vaccine

ABSTRACT

Human gammaherpesviruses such as Epstein–Barr virus (EBV) cause lifelong infections and associated diseases, by virtue of their ability to establish latent infection. Many studies performed in the past years in murine herpesvirus 68 (MHV-68) model of infection suggested that the limited immunity generated against isolated viral components by subunit vaccines cannot counteract the multiple immune evasion strategies operated by gammaherpesviruses. Indeed, a significant inhibition of long-term latency establishment could be observed in mice vaccinated with strains of genetically modified MHV-68 defective in reactivation or establishment of latency.

In this study, we focused on the effects of interferon-α (IFN-α) on both the lytic and latent phase of MHV-68 infection, as exerted by the constitutive release of IFN-α1 by a clone of MHV-68 genetically modified to produce this cytokine (MHV-68mIFNα1). Although the MHV-68mIFNα1 recombinant virus exhibited *in vitro* replication features indistinguishable from those of the wild type MHV-68, its pathological properties were severely attenuated *in vivo* in immunocompetent mice and not in mice rendered genetically unresponsive to type I IFN, suggesting that a stronger immune response was primed in the presence of the cytokine. Notably, MHV-68mIFNα1 attenuation did not result in a reduced level of long-term spleen latency establishment. These results prompted us to evaluate the efficacy of MHV-68mIFNα1 in a prophylactic vaccination regimen aimed at inhibiting the symptoms of acute virus infection and the establishment of long-term latency after MHV-68 challenge. Our results show that mice vaccinated with MHV-68mIFNα1, administered as a live-attenuated or partially inactivated (by Psoralen and UV treatment) vaccine, were protected against the challenge with wt MHV-68 from all phases of infection. The ability of MHV-68mIFNα1 to produce IFN-α at the site of the infection, thus efficiently stimulating the immune system in case of virus reactivation from latency, makes this recombinant virus a safer live-attenuated vaccine as compared to the previously reported latency-deficient clones.

1. Introduction

The search for an effective vaccine aimed at reducing the transmission of gammaherpesviruses, such as EBV or HHV8, causing severe diseases in humans, recently drove an increasing interest in studying the murine herpesvirus 68 virus (MHV-68) model of infection. In the last decades, this model has been exploited to understand the physiology of gammaherpesvirus infection and to test the efficacy of vaccination strategies against this class of viruses. The failure of most of the vaccination approaches based on structural proteins, lytic or putative latency-associated antigens in protecting against all phases of MHV-68 infection [1–11], suggested that the limited immunity generated against isolated viral components cannot counteract the multiple immune evasion strategies operated by the virus. It is a general opinion that vaccination against gammaherpesviruses should mainly minimize virus-induced lymphocyte proliferation and reduce the long-term latent viral load, since these are the features more likely responsible for the development of gammaherpesvirus-associated lymphoproliferative diseases [12].

A number of studies utilized DNA recombinant techniques to generate genetically modified MHV-68 viruses, not only to identify the function of viral genes, by assessing the changes in virus replication caused by genome targeted disruptions [13], but also to generate latency defective recombinant MHV-68 clones, and evaluate their efficacy as vaccines in protecting mice against

* Corresponding author. Tel.: +39 06 4990 3004; fax: +39 06 4990 2140.
E-mail address: eleonora.arico@iss.it (E. Aricò).

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doi:10.1016/j.vaccine.2011.03.092

the challenge with the "wild type" (wt) virus [5–8,11]. With one exception [14], the vaccination of mice with live attenuated latency-deficient recombinant MHV-68 gave the best results in terms of protection against the establishment of long-term latency [5–8,11,15]. In general, the advantage of live-attenuated *vs* replication-deficient virus vaccines relies on their high immunogenicity, based on their ability to stimulate both local and systemic response against a broader spectrum of epitopes for cell-mediated and humoral immunity. However, viruses replicating and persisting in a latent state are not considered completely safe, since their long-term persistence might have unpredictable consequences.

In the study presented herein, we generated a recombinant MHV-68 expressing the mouse IFN-α1 coding sequence to evaluate the effects of the constitutive production of this cytokine on MHV-68 replication *in vitro* and *in vivo*. Originally discovered for their antiviral activity, type I IFN (IFN-I) are now recognized to exert a variety of immunomodulatory activities in both human and mouse models [16,17]. Notably, by virtue of its well documented role in dendritic cell (DC) differentiation/maturation and activity, IFN-I are now considered as important cytokines linking the innate and adaptive immune response to infections [18].

As expected on the basis on their antiviral activity, endogenous IFN-I play a significant role in limiting MHV-68 replication at early stages of infection and before the onset of the adaptive immune response [19]. In addition, IFN-I counteract viral latency, both by contributing to the development and maintenance of an anti-MHV-68 innate immune response [20], and by controlling virus reactivation from latency [21]. Notably, like many herpesviruses, MHV-68 evolved several immune evasion strategies to neutralize IFN-I-mediated control of acute and latent infection [22–25]. In the light of all this, we hypothesized that a recombinant MHV-68 producing IFN-α could represent a useful tool for further understanding the role of this cytokine in the different phases of virus infection *in vivo*. Furthermore, we assumed that the secretion of IFN-α by the cells infected with the recombinant virus could neutralize the inhibitory strategies evolved by MHV-68 against IFN-I antiviral activities, thus allowing the immune stimulatory properties of the cytokine to be more effective.

In the study presented herein, we report the effects of the continuous production of IFN-α1 on the course of infection of a clone of MHV-68 genetically modified by the insertion of the mouse IFN-α1 coding gene (MHV-68mIFNα1). Moreover, we describe the efficacy of MHV-68mIFNα1 administered as a live-attenuated or partially inactivated virus (following Psoralen and UV treatment) in protecting mice from the challenge with wt MHV-68.

2. Methods

2.1. Mice

Four to five week-old 129Sv and C57BL/6 mice (H-2^b) were purchased from Charles River Italia (Italy). IFNARI$^{-/-}$ mice have a targeted disruption in an essential chain of the IFN-I receptor gene (IFNARI), and do not respond to IFN-I. A colony of IFNARI$^{-/-}$ 129Sv was established at the Department of Cell Biology and Neurosciences of the Istituto Superiore di Sanità, Rome. IFNARI$^{-/-}$ C57BL/6 mice have been obtained from EMBL, Monterotondo, Italy. Mice were housed in plastic cages and maintained under specific pathogen-free conditions in the animal house of the Istituto Superiore di Sanità.

2.2. Cell lines

IFNARI$^{-/-}$ cells were grown in Dulbecco's medium complemented with 10% FCS, 10% TPB, penicillin/streptomycin (70 μg/ml and 10 μg/ml) and L-glutamine (2 mM). IFNARI$^{-/-}$ cells were derived from embryonic fibroblasts isolated from an IFNARI$^{-/-}$ 129Sv mouse, immortalized *in vitro* by transfecting a SV40 T antigen expressing plasmid. αRec14 cells have been obtained by stably transfecting into a fibroblast cell line originated from IFNARI$^{-/-}$ 129Sv mouse a plasmid encoding the IFNARI coding sequence, to recover a functional IFN-I receptor.

2.3. Virus stocks

MHV-68 was originally obtained from Professor D. Blaskovic [26]. To avoid the possible interference of the antiviral activity of IFN-I produced by recombinant MHV-68, IFNARI$^{-/-}$ cells have been used for the production and titration of the wt and recombinant virus stocks. Working stocks of MHV-68 clone g2.4 [27], MHV-68mIFNα1 and MHV-68-GFP were prepared by infection of IFNARI$^{-/-}$ cells at 0.01 PFU/cell as previously described [28]. Virus stocks were titered by the previously described plaque assay [4] performed on IFNARI$^{-/-}$ cells.

2.4. Construction of recombinant viruses

Two clones of recombinant MHV-68 were produced by homologous recombination in IFNARI$^{-/-}$ cells. To obtain the insertion of the mIFN-α1 gene into the 1400 bp left end region of MHV-68 genome (named EH1,4), comprised between the HindIII and EcoRI restriction site, the IFN-α1 coding sequence was cloned into a plasmid specifically designed to drive homologous recombination in that site. The expression cassette consisted of the CMV-IE promoter and the EGFP-Hygr gene from EGFPHyg (Clontech), followed by EMCV IRES element. The gene encoding mIFN-α1 was cloned downstream from the IRES, followed by the SV40 poly(A) signal. The whole cassette was flanked by two portions of the EH1,4 MHV-68 region, of 0.6 and 0.8 kb, respectively. MHV-68 DNA (5 μg) and 10 μg of linearized plasmid were transfected by electroporation into 2×10^6 IFNARI$^{-/-}$cells by double-pulse setting (high-voltage setting = 600 V, 25 μF, 99 Ω; low-voltage setting = 260 V, 1500 μF, 329 Ω; 0.1-s interpulse delay) on an EasyJect electroporator (EquiBio). Electroporated cells were cultured in a six-well plate. Green fluorescent plaques were picked after 5 days and subjected to four rounds of plaque purification by limiting dilution (in 96-well plates) with hygromycin selection (100 μg/ml) on IFNARI$^{-/-}$ cells. Each round of purification was verified by a PCR to amplify mIFN-α1 or GFP, and by IFN-I titration on cell supernatant. Once a pure population of green fluorescent plaques was obtained, the genomic structure was analyzed. MHV-68 genomic DNA was prepared from purified virions as described previously [27]. PCR was performed on DNA samples to obtain the molecular characterization of the recombinant viruses. The following primers were used: ORF10-For: AAGTCTGCCCCCTCGATTAT (nucleotide position in MHV-68 genome: 22275), ORF10-Rev: TAGAGGGTCTGCCACTCCAT (nucleotide position in MHV-68 genome: 23505), mIFN-α1-Rev: TGAGTCTGAGGCAGGTCACA (nucleotide position in mIFN-α1 gene: 97), GFP-For: CGACCACTACCAGCAGAACA (nucleotide position in GFP gene: 31).

2.5. IFN-I titration

IFN-I was titrated on L929 cells as described previously [29]. IFN-I titers are expressed in International Units (IU). To avoid the possible infection of L929 cells by the virus present in test supernatants, making advantage of IFN-I pH stability, test samples underwent overnight pH 2 acidification by HCl addition, followed by NaOH neutralization before the inoculation on L929 monolayers.