Vaccine xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

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

Generation of a novel live rabies vaccine strain with a high level of safety by introducing attenuating mutations in the nucleoprotein and glycoprotein

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ARTICLE INFO

Article history:
Received 25 February 2016
Received in revised form 10 August 2017
Accepted 20 August 2017
Available online xxxx

Keywords:
Rabies
Attenuated live vaccine
Gene manipulation
Glycoprotein
Nucleoprotein

ABSTRACT

The current live rabies vaccine SAG2 is attenuated by only one mutation (Arg-to-Glu) at position 333 in the glycoprotein (G333). This fact generates a potential risk of the emergence of a pathogenic revertant by a back mutation at this position during viral propagation in the body. To circumvent this risk, it is desirable to generate a live vaccine strain highly and stably attenuated by multiple mutations. However, the information on attenuating mutations other than that at G333 is very limited. We previously reported that amino acids at positions 273 and 394 in the nucleoprotein (N273/394) (Leu and His, respectively) of fixed rabies virus Ni-CE are responsible for the attenuated phenotype by enhancing interferon (IFN)/chemokine gene expressions in infected neural cells. In this study, we found that amino acid substitutions at N273/394 (Phe-to-Leu and Tyr-to-His, respectively) attenuated the pathogenicity of the oral live vaccine ERA, which has a virulent-type Arg at G333. Then we generated ERA-N273/394-G333 attenuated by the combination of the above attenuating mutations at G333 and N273/394, and checked its safety. Similar to the ERA-G333, which is attenuated by only the mutation at G333, ERA-N273/394-G333 did not cause any symptoms in adult mice after intracerebral inoculation, indicating a low level of residual pathogenicity of ERA-N273/394-G333. Further examination revealed that infection with ERA-N273/394-G333 induces *IFN-β* and *CXCL10* mRNA expressions more strongly than ERA-G333 infection in a neuroblastoma cell line. Importantly, we found that the ERA-N273/394-G333 stain has a lower risk for emergence of a pathogenic revertant than does the ERA-G333. These results indicate that ERA-N273/394-G333 has a potential to be a promising candidate for a live rabies vaccine strain with a high level of safety.

1. Introduction

Rabies is a viral zoonotic disease characterized by severe neurological symptoms with a case-mortality rate of almost 100%. Although rabies is a vaccine-preventable disease, more than 55,000 people, mostly in developing countries, die from rabies every year [1]. In developing countries, dogs are the major reservoir/transmitter of rabies, while in developed countries, wild animals play an important role in maintenance of the infection cycle. Since more than 99% of human rabies cases are transmitted by dogs, mass vaccination of dogs is an effective measure to control rabies [2,3].

Live vaccines have some advantages such as a higher level of immunogenicity and lower production cost than inactivated vaccines [4]. Flexibility of administration is also another advantage: live rabies vaccines can be administered not only parenterally

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http://dx.doi.org/10.1016/j.vaccine.2017.08.050
0264-410X/

but also orally. Indeed, attenuated rabies virus (RABV) strains contained in baits have been used as oral vaccines for immunization in wild animals and have successfully contributed to control of rabies in these animals [5–8]. This oral vaccination strategy is attractive for mass vaccination of dogs in developing countries, where cultural practices allow unowned, free-roaming dogs, which are hard to access for traditional vaccination strategies [9,10]. However, it is concerned that the attenuated virus contained in the live vaccine causes rabies by its residual pathogenicity or pathogenic reversion [11–14]. Hence, there exists an urgent need to establish a highly and stably attenuated RABV strain that is applicable to a live vaccine for dogs, which share habitats with humans, in developing countries. To develop such live vaccine strains, it is necessary to establish the molecular basis of attenuation of RABV.

Previous studies demonstrated that RABV glycoprotein (G protein) plays important roles in the pathogenicity: the strains with an Arg or Lys at position 333 in the G protein (G333) are virulent, whereas mutants with other amino acids at G333 are avirulent [15–17]. Based on these findings, a current live vaccine, SAG2, which is attenuated by an Arg-to-Glu mutation at G333, has been established [18]. SAG2 has been used as an oral bait vaccine for wild animals, and the efficacy has been experimentally confirmed in several animals including dogs [19–22]. Meanwhile, the possibility that SAG2 reverts to the virulent phenotype cannot be denied, since only a single amino acid change (Glu-to-Arg or Glu-to-Lys mutation at G333) results in pathogenic reversion [18]. Furthermore, Faber et al. [23] reported that an attenuated strain with Glu at G333 reverted to the virulent phenotype by an Asn-to-Lys mutation at position 194 in the G protein (G194), even though Glu at G333 was retained. To circumvent these potential risks, a live vaccine strain attenuated by multiple mutations not only in the G protein but also in other viral proteins would be required for development of live vaccines with a high level of safety. However, information about attenuating mutations other than that at G333 has remained limited.

We previously reported that the RABV strain Ni-CE is highly attenuated by novel mutations, including Phe-to-Leu and Tyr-to-His mutations at positions 273 and 394, respectively, in the nucleoprotein (N protein) (N273/394) [24,25]. Subsequently, by using a gene manipulation system, we established an Ni-CE mutant with Glu at G333, Ni-CE(G333), as a live vaccine candidate strain attenuated by multiple mutations and confirmed its high level of safety [26]. However, the potential of Ni-CE(G333) as an oral vaccine strain has remained to be examined. Meanwhile, all RABV strains that have been applied for oral vaccines so far belong to a specific lineage composed of the SAD strain and its derivative strains including SAG2, SAD-Bern, SAD-B19 and ERA. This indicates the possibility that these strains will provide more suitable platforms for development of a novel oral vaccine. From this aspect, it would be interesting to know whether introduction of the Ni-CE-type mutations at N273/394 into these oral vaccine strains attenuates their pathogenicity.

It was previously demonstrated that a live vaccine strain ERA induces protective immunity in dogs after intramuscular and oral immunization [27,28]. However, this strain shows relatively high pathogenicity, since it possesses a virulent-type Arg at G333 [29], indicating a need of further attenuation of this strain before applying for a live oral vaccine for dogs. In this study, to generate a vaccine strain highly and stably attenuated by multiple mutations, we established an ERA mutant (ERA-N273/394-G333) by introducing the Ni-CE-type mutations at N273/394 in combination with the Arg-to-Glu mutation at G333. Then we investigated the attenuated phenotype and its stability of ERA-N273/394-G333 by comparing with those of ERA-G333, which is attenuated by a single mutation at G333.

2. Materials and methods

2.1. Cells

Mouse neuroblastoma NA cells, human neuroblastoma SYM-I cells (kindly provided by Kawai) [30] and Vero cells (ATCC number CCL-81) were maintained in Eagle's minimal essential medium supplemented with 10% fetal calf serum.

2.2. Viruses

To generate ERA-N273/394, ERA-G333 and ERA-N273/394-G333, carrying mutation(s) in the N and/or G proteins shown in Figs. 1A and 2A, we introduced nucleotide substitutions into the corresponding positions of the full-length genome plasmid of

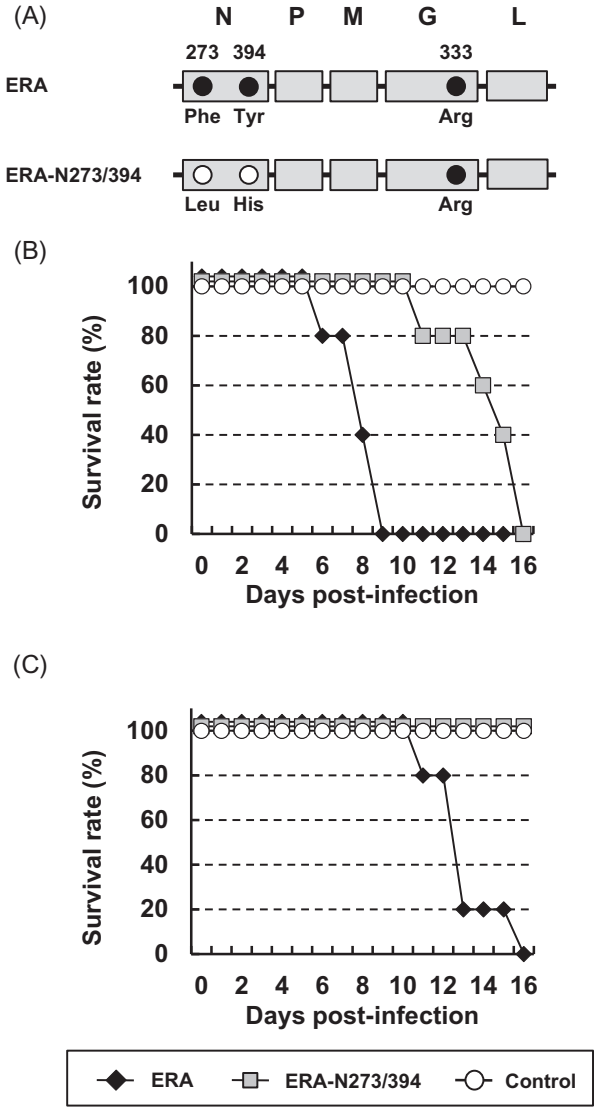

Fig. 1. Pathogenicities of ERA and ERA-N273/394. (A) Schematic diagrams of genomes of ERA and ERA-N273/394. Boxes represent open reading frames derived from ERA. The filled and open circles in the genome represent pathogenic and attenuating amino acids, respectively. (B) Survival curves of mice intracerebrally inoculated with each strain. Groups of five adult mice were inoculated intracerebrally with 10^5 FFU of each strain and observed for 16 days to record their mortalities daily. Mice injected with a diluent served as controls. Representative data from two independent experiments are shown. (C) Survival curves of mice intramuscularly inoculated with each strain. Groups of five adult mice were inoculated intramuscularly with 10^6 FFU of each strain and observed for 16 days.

Please cite this article in press as: Nakagawa K et al. Generation of a novel live rabies vaccine strain with a high level of safety by introducing attenuating mutations in the nucleoprotein and glycoprotein. Vaccine (2017), http://dx.doi.org/10.1016/j.vaccine.2017.08.050