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Rabies virus pathogenesis in relationship to intervention with inactivated and attenuated rabies vaccines☆,☆☆

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ABSTRACT

Despite progress in vaccine development in the past century the mechanisms behind immune responses elicited by rabies biologics or via natural infection remain largely unknown. In this study, we compared protection elicited by standard, early, or delayed prophylaxis with a reduced number of vaccine doses using inactivated and live-attenuated vaccines. Two-month-old Syrian hamsters, 4-week-old ICR mice or adult rhesus macaques were inoculated with canine rabies virus variants. Thereafter, prophylaxis was initiated 6 h, 1, 2, 3, 4, 5, 6 or 7 days post-exposure (p.e.). One or several doses of inactivated (HDCV), or reverse genetically attenuated (live), or gamma-irradiated (inactivated)-ERAG333 vaccines were administered intramuscularly. The dynamics of virus spread were measured over time in the rodent models. Rabies virus reached the spinal cord at day 4 and brain at day 6 p.e. All hamsters succumbed in groups in which live ERAG333 was delayed until days 5 and 6 p.e. However, 78%, 44%, 56% and 22% of hamsters survived when one dose of live ERAG333 was administered 6 h, 1, 2, 3, and 4 days p.e., respectively. Similarly, 67% survived when inactivated ERAG333 was administered at 24 h p.e. All hamsters succumbed when standard prophylaxis (the Essen regimen) was delayed until days 3–6, but 67% and 33% of hamsters survived when PEP began 1 or 2 days p.e., respectively. Macaques were protected by one dose of attenuated ERAG333 at 24 h p.e. The highly attenuated (live) and inactivated ERAG333 vaccines elicited potent protective immune responses, even when prophylaxis initiation was delayed. When 2–5 doses of commercial vaccine and HRIG were administered according to the Essen scheme, 89–100% of the animals survived. Reduced vaccine schedules provided efficacious intervention, regardless of the total number of vaccine doses administered.

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1. Introduction

Rabies is essentially fatal once clinical signs appear, but readily preventable if early and adequate post-exposure prophylaxis (PEP) is initiated [1–6]. No single reagent has specific therapeutic effects, but a combination of various biologics with unclear synergistic effects has been suggested and one protocol has been successfully applied for experimental therapy [7,8]. As such, PEP remains the main and sole approach for preventing rabies in humans after exposure [9].

Modern PEP using effective biologics relies upon the neutralization of virus by early administration of passive antibodies (immune globulins of human or equine origin) and further peripheral clearance of virus through induced active immunity [10–13]. The passive administration of immune globulins, providing an immediate supply of virus neutralizing antibodies (VNAs) to bridge the gap until the production of active immunity in response to vaccine administration, is a critical part of PEP, especially for severe exposures [9]. Previous experiments have documented the significance of humoral immune responses in peripheral clearance of rabies viruses (RABV) with only limited involvement of cellular immune responses [14,15]. Besides immediate immune response stimulation, generation of lasting immunological memory is another significant outcome of PEP.

During the past century of rabies PEP, significant changes have been made in the substrate used for vaccine production, the level

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and mode of attenuation or inactivation, and the route of administration of biologics. Additionally, as vaccine immunogenicity, efficacy and safety improved, the number of vaccine doses recommended for PEP was gradually reduced from more than 21 doses to 4 intramuscular (i.m.) doses today [10,17,18]. Regarding future products, subunit, DNA, and live recombinant vaccines have been evaluated experimentally [19–23].

Despite improvement in production of rabies biologics globally and progress in basic understanding of vaccine immunogenicity and efficacy, practical applicability remains restricted by economic and technical limitations. In the 21st century, several countries still produce nerve tissue vaccines as the sole method of biologic intervention following exposure to rabid animals. Moreover, after more than a century of successful vaccination against rabies, molecular mechanisms behind the generation of early humoral and cellular responses following infection or vaccination with inactivated or attenuated vaccines remain poorly understood.

The objective of this study was an experimental comparison of the effectiveness of various inactivated and attenuated (live) rabies vaccines and PEP schedules in a variety of animal models.

2. Materials and methods

2.1. Animals and viruses

Two-month-old female Syrian hamsters (*Mesocricetus auratus*) and 4-week-old female ICR mice (*Mus musculus*) were obtained from commercial suppliers and held for observation for a minimum of 72 h after arrival before use. One-year-old female rhesus macaques (*Macaca mulatta*) were obtained from a commercial supplier and were quarantined for a minimum of 2 months before use.

For comparison, two different street viruses of canine origin were used as a challenge. One was $10^{2.5}$ $MICLD_{50}$/50 μl dog/coyote RABV isolated from the salivary glands of a naturally infected dog in Texas (#323); and the other 10^{2} $MICLD_{50}$/50 μl Mexican dog RABV (MD5951) isolated from the salivary glands of a naturally infected dog in Mexico.

All animal handling and experimental procedures were undertaken in compliance with the Centers for Disease Control and Prevention Institutional Animal Care and Use Committee guidelines.

2.2. Biologics used

For rodent experiments, a volume of 50 μl (and for non-human primates 1 ml) of human diploid cell vaccine (HDCV), Imovax® (sanofi pasteur) or 50 μl of purified chick embryo vaccine (PCEC), Rabavert® (Novartis Vaccines and Diagnostics) with a minimum potency of 2.5 IU/ml was used per administration, according to the experimental design described below. Similarly for rodents only, 50 μl of undiluted human rabies immune globulin (HRIG), HyperRab^{tm} S/D (Talecris Biotherapeutics, 150 IU/ml) or Imogam® Rabies-HT (sanofi pasteur, 150 IU/ml) were administered i.m. into the site of inoculation following initiation of PEP according to the experimental design below.

The ERAG333 (attenuated via the point-mutation-site 333 of the viral glycoprotein (G) gene: Arg_{333} to Glu_{333}) and ERA2G333 (with two G genes with the site 333 mutation) were constructed via reverse genetics, as described previously [24]. The ERAG333 construct has been previously shown to be safe and apathogenic for 3-week-old and adult mice, as well as for other target and non-target species, even when administered via the intracerebral (i.c.) route [24]. Inactivated ERAG333 was prepared via gamma-irradiation with 5×10^6 rads using a Gamma Cell Gamma Irradiator (Gammacell 220 Excel, MDS Nordion, Canada). Control of proper inactivation via isolation of any remaining live virus was attempted in mouse neuroblastoma (MNA) cell culture in three consecutive passages. Fifty microlitres of live-attenuated or inactivated ERAG333 (10^9 $TCID_{50}$/ml) were administered into the right gastrocnemius muscle (hamsters, mice) or 1 ml of ERAG333 (10^9 $TCID_{50}$/ml) into the deltoid muscle (macaques).

2.3. Laboratory methods

2.3.1. Direct fluorescent antibody (DFA) test

The RABV antigens were detected in brain samples using the DFA test [25] with a fluorescein-isothiocyanate (FITC)-conjugated anti-RABV monoclonal antibody (Fujirubio Diagnostics, Inc., Malvern, PA, USA).

2.3.2. Reverse transcription polymerase chain reaction (RT-PCR) and hemi-nested RT-PCR (hnRT-PCR)

The RT-PCR and hnRT-PCR assays were applied to trace the spread of RABV from the site of inoculation to the brain in the hamster model, performed as previously described [26]. Samples from the lumbar and thoracic spinal cord and sections of brain were collected using sterile techniques to prevent cross-contamination. Total RNA was extracted according to the manufacturer's recommendations (TRIzol Reagent Cat No. 15596-026, Invitrogen Corp.). Reverse transcription (RT) was done using a forward primer, 1066fw (forward) (5′ GAG AGA AGA TTC TTC AGG GA 3′, positions 1136–1155) (all primer positions correspond to the RABV strain PV genome, accession number M13215) for 90 min at 42 °C. A primary PCR (RT-PCR) was performed using primer set 1066fw (forward) and 304rv (reverse) (3′ TTG ACA AAG ATC TTG CTC AT 5′, positions 1517–1537). Two additional primer sets were used to detect traces of viral RNA via the hemi-nested (hn)RT-PCR: (a) 1087fw (5′ GAG AAR GAA CTT CAR GA 3′, positions 1087–1104) and 304rv, as well as (b) 504sfw (5′ TCA TGA TGA ATG GAG GT 3′, positions 504–521) and 304rv. Temperature cycles were the same for both primary RT-PCR and hemi-nested PCR as follows: pre-PCR 94 °C 1 min; PCR 40 cycles 94 °C 30 s, 37 °C 30 s, 72 °C 1.5 min; and termination at 72 °C during 7 min. Amplicon size was verified by electrophoresis in 3.5% agarose gels and consequent sequence analysis using standard protocols to confirm viral identity.

2.3.3. Rapid fluorescent focus inhibition test (RFFIT)

The RFFIT was used to measure VNA using CVS-11 as a challenge virus and was performed as described [27].

2.3.4. Statistical analysis

Kaplan–Meier survival curves were calculated in SAS (SAS 9.2. SAS Institute Inc., Cary, NC, USA). The log-rank test was used to test differences between group survival distributions. The null hypothesis of identical survival functions was rejected at $P < 0.05$.

2.4. PEP with inactivated commercial HDCV vs. attenuated (live) ERAG333 or ERA2G333 vaccines in a hamster model

2.4.1. Challenge with Texas dog/coyote RABV

Two-month-old female Syrian hamsters (6 per group) were inoculated into the left gastrocnemius muscle with $10^{2.5}$ $MICLD_{50}$/50 μl of Texas dog/coyote RABV. Animals were divided into groups for treatment with different PEP schedules and biologics. Fifty microlitres of inactivated (HDCV) or attenuated ERAG333 or ERA2G333 vaccines were administered into the left gastrocnemius muscle. The standard "Essen" schedule for PEP (days 0, 3, 7, 14 and 28) was used for the administration of HDCV vaccine (with and without HRIG). For PEP with the attenuated vaccines, only a single dose of vaccine was applied per animal per